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MINIREVIEWS

Opportunities for treatment of the hepatitis C virus-infected patient with chronic kidney disease

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Abstract

The prevalence of hepatitis C virus (HCV) infection amongst patients with chronic kidney disease (CKD) and end-stage renal disease exceeds that of the general population. In addition to predisposing to the development of cirrhosis and hepatocellular carcinoma, infection with HCV has been associated with extra-hepatic complications including CKD, proteinuria, glomerulonephritis, cryoglobulinemia, increased cardiovascular risk, insulin resistance, and lymphoma. With these associated morbidities, infection with HCV is not unexpectedly accompanied by an increase in mortality in the general population as well as in patients with kidney disease. Advances in the understanding of the HCV genome have resulted in the development of direct-acting antiviral agents that can achieve much higher sustained virologic response rates than previous interferon-based protocols. The direct acting antivirals have either primarily hepatic or renal metabolism and excretion pathways. This information is particularly relevant when considering treatment in patients with reduced kidney function. In this context, some of these agents are not recommended for use in patients with a glomerular filtration rate < 30 mL/min per 1.73 m^2. There are now Food and Drug Administration approved direct acting antiviral agents for the treatment of patients with kidney disease and reduced function. These agents have been demonstrated to be effective with sustained viral response rates comparable to the general population with good safety profiles. A disease that was only recently considered to be very challenging to treat in patients with kidney dysfunction is now curable with these medications.

Key words: Hepatitis C virus; Chronic kidney disease; Direct acting antiviral agents; Kidney transplantation

Core tip: Advances in the understanding of the molecular

biology of hepatitis C virus (HCV) have ushered in a new era in treatment. Recent studies have shifted the focus to the more difficult-to-treat cohorts of patients. The presence of chronic kidney disease and end stage renal disease were exclusion criteria for the pivotal clinical direct-acting antiviral agents trials, creating a group of patients with a large unmet medical need. This review will update the reader on the use of the direct acting antiviral agents in the HCV-infected patient with kidney disease. Recommendations for the timing of therapy, choice of agents and management of the kidney transplant candidate will be presented.

Ladino M, Pedraza F, Roth D. Opportunities for treatment of the hepatitis C virus-infected patient with chronic kidney disease. *World J Hepatol* 2017; 9(19): 833-839 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i19/833.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i19.833

INTRODUCTION

Hepatitis C virus (HCV) infection is a recognized public health concern with global implications that affects approximately 170 million individuals worldwide[1-4]. Infection with HCV is associated with an increased morbidity and mortality secondary to hepatic injury and associated complications[4]. The infection, however, can also affect other organs with significant extrahepatic manifestations (Figure 1). Most noteworthy of these include insulin resistance, cryoglobulinemic vasculitis, sicca syndrome, neurocognitive dysfunction, B-cell non-Hodgkin lymphoma and an increase in cardiovascular adverse events[5-11]. On note, patients with HCV infection also have an increased incidence of proteinuria and chronic kidney disease (CKD)[5], often in the setting of essential mixed cryoglobulinemia or "idiopathic" membranoproliferative glomerulonephritis[5,9,12]. Furthermore, it has also been well established that patients with end stage renal disease (ESRD) have an even higher prevalence of HCV infection that is likely a consequence of greater blood product exposure and patient-to-patient transmission of disease within the dialysis clinics due to breakdowns in universal precautions[12,13].

This review will summarize the most recent data and treatment options recommended for HCV-infected patients with kidney disease. A population of patients that for years had extremely limited options for therapy can now be successfully and safely treated for eradication of HCV.

HCV AND THE KIDNEY

HCV-related glomerulonephritis with or without cryoglobulinemia

The HCV has an unusual tropism for B lymphocytes through linkage of envelope protein 2 and the CD81 molecule on the B cell. B cell activation can result in expansion of malignant cell lines or the production of unique antibodies that are of the IgM isotype and possess rheumatoid factor like activity[14-16]. As a consequence of these events, clinical syndromes including mixed cryoglobulinemia, lymphoproliferative disorders and glomerulonephritis with distinct histological patterns including membranous or membranoproliferative glomerulonephritis can be seen[5,6,17,18]. Of note, co-infected HIV/HCV patients have an increased mortality and an overall worse prognosis[19,20].

The glomerular diseases commonly associated with HCV infection are a consequence of the formation of circulating immune complexes that become trapped in the glomerular basement membrane. The clinical expression of this process can occur through type 2 mixed cryoglobulinemia with resulting type 1 membranoproliferative glomerulonephritis (GN), mesangial proliferative and focal proliferative GN, IgA nephropathy, membranous GN and polyarteritis nodosa[6,14,18]. Typically, the patient that develops cryoglobulinemia has been infected with HCV for many years. These patients may present with a skin rash (palpable purpura), polyneuropathy, multi-organ vasculitis, hypertension and the nephritic syndrome[14].

Suppression of viral replication is necessary to interrupt immune-complex production and subsequent injury to the kidney. The VASCUVALDIC study described the use of sofosbuvir and ribavirin in 24 patients with HCV-vasculitis syndrome and cryoglobulinemia. Patients were treated with direct-acting antiviral agents (DAAs) for 24 wk and achieved a sustained viral response at week 12 (SVR_{12}) of 74% with minimal side effects[21]. The less common presentation of an active vasculitic syndrome as part of the cryoglobulinemic syndrome requires a more aggressive treatment strategy targeted at the ongoing endothelial inflammatory process. Options include high dose corticosteroids, rituximab and therapeutic plasma exchange in addition to appropriate DAA therapy to eradicate viral replication[21-24].

Hepatitis C and CKD

HCV infection is highly prevalent in CKD patients[5] and HCV-infected patients have an increased risk for the development of CKD and proteinuria[5,25,26]. Furthermore, emerging data suggests that the rate of CKD progression to ESRD is greater when compared to non-infected patients[26-31]. In this context, HCV-infected patients with CKD stages Ⅰ (GFR > 90 mL/min per 1.73 m^2), Ⅱ (GFR 60-89 mL/min per 1.73 m^2) and Ⅲa (GFR 45-59 mL/min per 1.73 m^2) should be considered for DAA therapy with the goal to slow the progression of CKD. HCV-infected patients with CKD stages Ⅲb (GFR 30-44 mL/min per 1.73 m^2), Ⅳ (GFR 15-29 mL/min per 1.73 m^2) and Ⅴ (GFR < 15 mL/min per 1.73 m^2) will require a more individualized approach depending on the renal replacement therapy options being considered. The major decision point in this context is whether treatment should

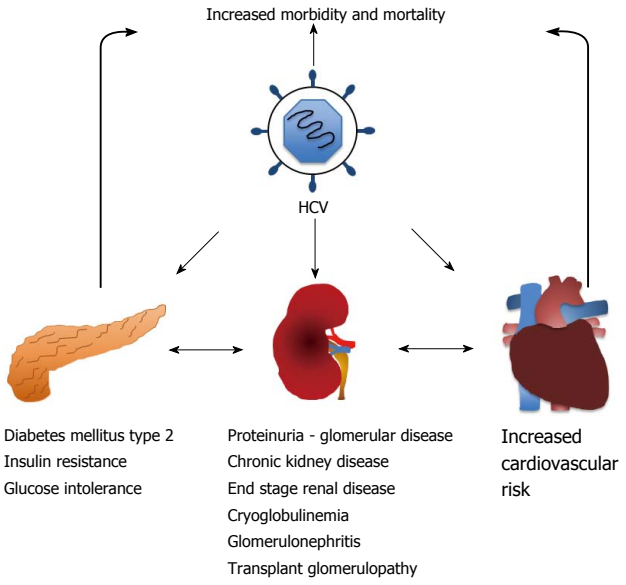

Figure 1 Extrahepatic manifestations of hepatitis C virus. HCV: Hepatitis C virus.

be recommended before or after kidney transplantation. Patients with a living kidney donor should be treated to achieve a SVR prior to transplantation. For the patient that is going to receive a deceased donor kidney the options may include delaying antiviral treatment in order to receive a kidney from an anti-HCV positive donor with the initiation of DAA treatment post transplantation. Alternatively, the patient could be treated pre-transplant and then transplanted with a kidney from an anti-HCV negative donor. Since not all centers currently accept kidneys from anti-HCV positive donors, this option is not available for all patients. Initial reports have demonstrated that accepting a kidney from a positive donor is associated with substantially shortened waiting time on the deceased donor waiting list in the United States[32-34]. Recent studies have demonstrated the safety and efficacy of DAAs in the kidney transplant recipient, with sustained viral response rates equal to that obtained in the general population with minimal side effects[35-37].

HCV in the ESRD patient

It is estimated that 5%-10% of the United States dialysis population is infected with HCV[38]. Many studies have demonstrated that HCV infection is associated with an increased risk of mortality and worse clinical outcomes in ESRD patients[39-43]. In a meta-analysis of ESRD patients, Fabrizi *et al*[41] found that HCV infection was associated with a relative risk of mortality of 1.35 (95%CI: 1.25-1.47). The increased morbidity and mortality associated with HCV infection emphasizes the systemic impact of this disease which can manifest with multiple extrahepatic manifestations and complications[5,40]. In this context, an increased cardiovascular risk attributable to HCV infection has been demonstrated in the ESRD patient[40]. In a recent update from the Dialysis Options and Practice Patterns Study data, it was concluded that HCV infection in ESRD patients was associated with an increased risk of death and hospitalization, anemia and worse quality of life scores for physical function, pain, vitality and mental health[44]. Relevant to any discussion on the associated risks accompanying HCV infection is whether successful treatment delivers a positive impact on outcomes. In this context, Hsu *et al*[45] reported that IFN-based therapy increased survival in HCV-infected ESRD patients. In another report, ESRD patients receiving IFN plus ribavirin obtained improved renal and cardiovascular outcomes compared to those who were untreated[46]. Prospective studies in ESRD patients will be necessary to determine if viral eradication alters the long-term outcome of this challenging population of patients with multiple co-morbidities.

HCV and kidney transplantation

Kidney transplantation is associated with an increase in long-term survival for ESRD patients with HCV infection[47,48]. This was clearly demonstrated in a longitudinal cohort study in which there was a decreased risk of death post-transplantation for the HCV-infected kidney transplant recipients when compared to those remaining on the waiting list[49]. This survival benefit was largely the result of a decrease in cardiovascular events within the first-year post-transplant[50].

HCV infection has been linked to several extra-hepatic manifestations that combine to increase morbidity and mortality after kidney transplantation[51]. It has been well established that HCV is the primary cause of liver disease in kidney allograft recipients[52] and these patients express an increased risk of insulin resistance and diabetes mellitus[53-58]. Furthermore, HCV-infected kidney recipients have a higher probability of developing transplant glomerulopathy[59] and recurrent membranoproliferative glomerulonephritis secondary to immune-complex injury to the renal allograft[60,61].

DIRECT ACTING ANTIVIRAL TREATMENT OPTIONS IN PATIENTS WITH CKD AND POST KIDNEY TRANSPLANT

The availability of DAAs with high SVR rates and favorable adverse event profiles allowed for the study of these drugs in patients with kidney disease, a group that had been excluded from of all the large pivotal trials. Emerging data are now demonstrating an excellent safety and efficacy profile in this patient population (Tables 1 and 2). The HCV-TARGET is a real-world study that collects data on the use of sofosbuvir-based regimens in HCV-infected patients. A total of 73 patients with a GFR $\leqslant$ 45 mL/min per 1.73 m^2 (n = 18 with GFR $\leqslant$ 30 mL/min per 1.73 m^2 and n = 5 on hemodialysis) were included in the analysis[62]. The SVR rate was 83% in patients with GFR $\leqslant$ 45 mL/min per 1.73 m^2 which was similar to patients with GFR > mL/min per 1.73 m^2, however patients with a GFR $\leqslant$ 45 mL/min per 1.73 m^2 had higher rates

Table 1 Direct acting antiviral agents: Dose and use in chronic kidney disease Ⅳ, Ⅴ, end stage renal disease and kidney transplant patients

Medication dose	Use in CKD stage Ⅳ, Ⅴ and ESRD	Use in kidney transplant patients - interactions with Immunosuppressant
Sofosbuvir/Simeprivir 400 mg daily/150 mg daily	CKD IV - GFR 15-29 mL/min: Not recommended CKD V - GFR < 15 mL/min: Not recommended ESRD (dialysis): Not recommended	Decrease in TAC levels with Simeprivir Increase levels of both CyA and Simeprivir Increase or decrease levels of SRL with Simeprivir No changes in TAC, CyA and SRL with Sofosbuvir
Sofosbuvir/Velpatasvir 400 mg/100 mg daily	CKD IV - GFR 15-29 mL/min: Not recommended CKD V - GFR < 15 mL/min: Not recommended ESRD (dialysis): Not recommended	Increase in TAC levels with Velpatasvir No changes in CyA levels with Velpatasvir Increase in SRL levels with Velpatasvir No changes in TAC, CyA and SRL with Sofosbuvir
Sofosbuvir/Daclastavir 400 mg daily/60 mg daily	CKD IV - GFR 15-29 mL/min: Not recommended CKD V - GFR < 15 mL/min: Not recommended ESRD (dialysis): Not recommended	No changes in TAC levels with Daclastavir No changes in CyA levels with Daclastavir Increase in SRL levels with Daclastavir No changes in TAC, CyA and SRL with Sofosbuvir
Sofosbuvir/Ledipasvir 400 mg/90 mg daily	CKD IV - GFR 15-29 mL/min: Not recommended CKD V - GFR < 15 mL/min: Not recommended ESRD (dialysis): Not recommended	No changes in TAC levels with Ledipasvir No changes in CyA levels with Ledipasvir No changes in SRL levels with Ledipasvir No changes in TAC, CyA and SRL with Sofosbuvir
Ombitasvir/Paritaprevir/ritonavir/Dasabuvir 12.5 mg/75 mg/50 mg × 2 tabs/250 mg × 2 tabs	CKD IV - GFR 15-29 mL/min: Dose adjustment not required CKD V - GFR < 15 mL/min: Dose adjustment not required ESRD (dialysis): Dose adjustment not required. Dialysis population studied. Minimal adverse events in patients with advanced CKD and ESRD on hemodialysis	Increase in TAC levels (ritonavir) Increase in CyA levels (ritonavir) Increase in SRL levels (ritonavir) No changes in TAC, CyA and SRL with Ombitasvir/Paritaprevir/Dasabuvir
Grazoprevir/Elbasvir 100 mg/50 mg daily	CKD IV - GFR 15-29 mL/min: Dose adjustment not required CKD V - GFR < 15 mL/min: Dose adjustment not required ESRD (dialysis): Dose adjustment not required. Dialysis population studied. Minimal adverse events in patients with advanced CKD and ESRD on hemodialysis	Increase in TAC levels with Grazoprevir Use of both CyA and Grazoprevir increase levels of Grazoprevir, contraindicated to use together Increase in SRL levels with Grazoprevir

GFR: Glomerular filtration rate; CKD: Chronic kidney disease; ESRD: End stage renal disease; TAC: Tacrolimus; CyA: Cyclosporine; SRL: Sirolimus.

of anemia, worsening kidney function and increased adverse events irrespective of the use of ribavirin[62]. Two open label treatment studies with simeprevir and dose-adjusted sofosbuvir exhibited high rates of SVR with a low incidence of adverse events in patients with advanced CKD and ESRD[63,64]. The RUBY-I trial evaluated the 3D regimen [ombitasvir (OBV)/paritaprevir (PTV)/ritonavir (r) plus dasabuvir (DSV)] in patients with advanced CKD (stages 4/5) and on dialysis. SVR rates were 90% for patients with HCV genotype (GT) 1 with minimal side effects except for the patients with genotype 1a who received ribavirin as part of the protocol[65]. This group had more anemia events and required erythropoietin dose adjustments. Grazoprevir and elbasvir were studied in HCV-infected GT 1 patients with advanced CKD and ESRD in the C-SURFER trial. Sustained viral response rates of 99% were reported with a minimal adverse events profile[66]. The RUBY-I Cohort 2 study included patients with stage F4 fibrosis and GT 1a who were treated for 24 wk with the 3D regimen plus ribavirin. SVR_{24} rates of 89% were reported for this cohort with minimal side effects[67]. The RUBY-Ⅱ study evaluated the use of the 3D regimen in CKD 4 and 5 patients with HCV GT 1a (n = 13) infection without the addition of ribavirin. Genotype 4 patients received OBV/PTV/r without DSV (n = 5). Modified intention to treat (mITT) SVR_{12} rates of 100% were obtained in both groups[68]. Finally, a recent report described the use of glecaprevir (NS3/4A inhibitor) and pibrentasvir (NS5A inhibitor) in patients with advance kidney disease and HCV genotype 1-6 infection (n = 104). In this trial, patients with a GFR < 30 mL/min per 1.73 m^2 (n = 13 with GFR 15-29 mL/min per 1.73 m^2, n = 6 with stage 5 CKD and n = 85 on hemodialysis) obtained a 98% ITT SVR_{12} with no serious adverse events[69] and no viral relapses.

IFN-based protocols have not been recommended after kidney transplantation due to an unacceptably high incidence of rejection events. In contrast, DAA use in kidney transplant recipients has been shown to be safe and effective with minimal side effects[34-37]. Caution to avoid drug-drug interactions related to different drug metabolism/interactions (Table 1) is necessary in addition to high vigilance to maintain therapeutic calcineurin inhibitor levels as HCV viremia is suppressed[34,37].

The availability of DAA agents has dramatically changed the way HCV-infected patients with CKD and ESRD can be managed. While providing outstanding results, these excellent outcomes raise new questions as to which patients should be treated and when is the best time to initiate therapy. Further studies will be

Table 2 Direct acting antiviral agent options for patients with kidney disease

HCV/kidney disease consideration	Complications and observations from HCV infection	DAA options	Other DAA options/notes
HCV related acute glomerulonephritis with or without cryoglobulinemia	HCV has tropism for B-cells with subsequent: Mixed cryoglobulinemia Glomerulonephritis with distinct histological patterns: Membranous nephropathy Membranoproliferative GN	Sofosbuvir 400 mg/d combined with Simeprivir 150 mg/d Daclastavir 60 mg/d Velpatasvir 100 mg/d Ledipasvir 90 mg/d	Can use: Grazoprevir 100 mg/elbasvir 50 mg/d Ombitasvir 12.5 mg/paritaprevir 75 mg/ritonavir 50 mg × 2 tabs/dasabuvir 250 mg × 2 tabs
The HCV-infected patient with stage 1-3a chronic kidney disease (GFR > 45 mL/min)	Increased risk for CKD development Increased rate of CKD progression to ESRD Higher mortality rate	Sofosbuvir 400 mg/d combined with Simeprivir 150 mg/d Daclastavir 60 mg/d Velpatasvir 100 mg/d Ledipasvir 90 mg/d	Can use Grazoprevir 100 mg/elbasvir 50 mg/d Ombitasvir 12.5 mg/paritaprevir 75 mg/ritonavir 50 mg × 2 tabs/dasabuvir 250 mg × 2 tabs
The patient with advanced stage 3 and stage 4/5 chronic kidney disease (GFR < 45 mL/min)	Receiving an anti-HCV positive allograft decreases waiting times for a deceased donor kidney	Sofosbuvir 400 mg/d combined with Simeprivir 150 mg/d Daclastavir 60 mg/d Velpatasvir 100 mg/d Ledipasvir 90 mg/d	Sofosbuvir not recommended with GFR < 30 mL/min Can use Grazoprevir 100 mg/Elbasvir 50 mg/d Ombitasvir 12.5 mg/Paritaprevir 75 mg/ritonavir 50 mg × 2 tabs/dasabuvir 250 mg × 2 tabs
The ESRD patient on dialysis	Increased risk of mortality and poor clinical outcomes in ESRD patients Increased cardiovascular risk	Grazoprevir 100 mg/Elbasvir 50 mg/d Ombitasvir 12.5 mg/Paritaprevir 75 mg/ritonavir 50 mg × 2 tabs/dasabuvir 250 mg × 2 tabs	Grazoprevir/elbasvir, ombitasvir/paritaprevir/ritonavir/dasabuvir, Dialysis population studied Minimal adverse events in patients with advanced CKD and ESRD on hemodialysis
The kidney transplant recipient with eGFR > 30 mL/min	DAA use after kidney transplant is safe and well tolerated with SVR > 97%	Sofosbuvir 400 mg/d combined with Simeprivir 150 mg/d Daclastavir 60 mg/d Velpatasvir 100 mg/d Ledipasvir 90 mg/d	Can use Grazoprevir 100 mg/elbasvir 50 mg/d (caution with cyclosporin) Ombitasvir 12.5 mg/paritaprevir 75 mg/ritonavir 50 mg × 2 tabs/dasabuvir 250 mg × 2 tabs

DAA: Direct-acting antiviral agent; GFR: Glomerular filtration rate; CKD: Chronic kidney disease; ESRD: End stage renal disease; HCV: Hepatitis C virus; DAA: Direct-acting antiviral; SVR: Sustained viral response.

necessary to answer these important questions.

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MINIREVIEWS

Imaging guided percutaneous interventions in hepatic dome lesions: Tips and tricks

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Abstract

Percutaneous hepatic interventions are generally safe given the fact that liver closely abuts the abdominal wall and hence it is easily accessible. However, the superior portion of liver, adjacent to the diaphragm, commonly referred as the "hepatic dome", presents unique challenges for interventionists. Percutaneous access to the hepatic dome may be restricted by anatomical factors and special considerations may be required to avoid injury to the surrounding organs. The purpose of this review article is to discuss certain specific maneuvers and techniques that can enhance the success and safety of interventions in the hepatic dome.

Key words: Hepatic dome; Radiofrequency ablation; Hepatocellular carcinoma; Percutaneous intervention

Core tip: Percutaneous interventions for lesions in the hepatic dome can be technically challenging. This review article discusses various maneuvers and techniques to safely access and treat lesions in this region.

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INTRODUCTION

Image guided hepatic interventions are integral to management of infective and neoplastic liver lesions[1-5]. A gamut of hepatic interventions including abscess drainage, thermal ablation, biopsy of focal liver lesions

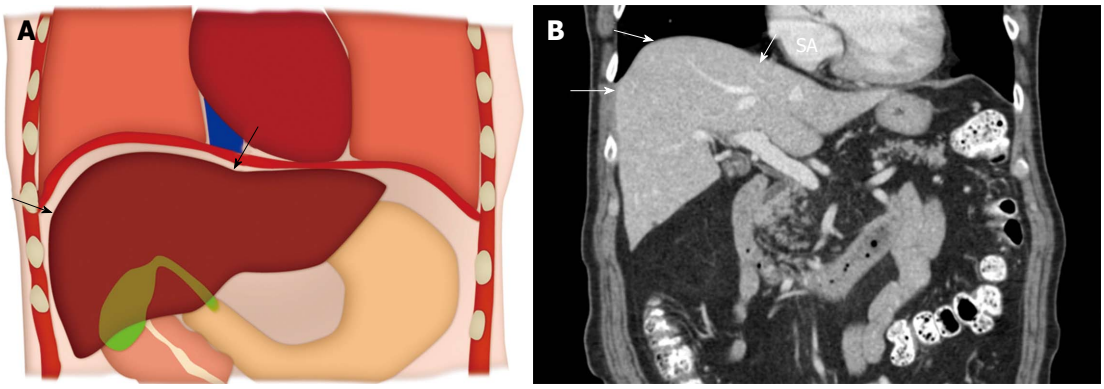

Figure 1 Anatomy of the hepatic dome. A: Colored schematic diagram; B: Coronal reformatted computed tomography image demonstrating the anatomy of the hepatic dome (arrows).

have significantly improved the morbidity and mortality associated with hepatic surgeries[1,2,4-6]. They offer several advantages over other invasive procedures in the liver such as laparascopy/laparotomy including absence of a laparotomy scar, shorter hospital stay, avoidance of general anesthesia and lower risk of complications, morbidity and mortality[2,4-6]. Liver lesions are generally easily accessible for percutaneous procedures, however access to certain regions may be challenging such as the hepatic dome. Certain interventional procedures in the hepatic dome, particularly thermal ablative procedures including radiofrequency ablation (RFA) can be associated with complications related to diaphragmatic and/or pleural injury[6]. Therefore, it is important to adhere to certain guiding principles of safety when performing percutaneous interventions in the hepatic dome. The purpose of this article is to review the anatomy, challenges, technical considerations and various different adjunctive maneuvers to safely access and treat lesions in the hepatic dome.

HEPATIC DOME: ANATOMIC CONSIDERATIONS AND TECHNICAL CHALLENGES

The term hepatic dome in general refers to the liver parenchyma close to the diaphragm and roughly accounts for nearly one-third of the liver volume. For most of its part, the hepatic dome is related on the anterior, lateral and posterior aspects to diaphragm, lung parenchyma with the accompanying pleura and thoracic cage (Figure 1). On the medial aspect, the hepatic dome is related to the cardia and inferior vena cava (IVC) anteriorly and the vertebral column posteriorly. Given the intricate anatomic relations, there is potential risk of severe pain during thermal ablative procedures due to diaphragmatic irritation that can limit complete treatment and can increase need for deeper sedation/anesthesia[7-12]. Percutaneous catheter drainage of hepatic dome abscesses can be difficult due to the need for transgression of the sterile pleural space and/or the lung which increases risk of pleural space contamination and the resulting pleural fluid collections and/or empyemas can often be challenging to treat[1].

Other technical challenge encountered during percutaneous intervention of hepatic dome lesions is accessibility and localization. The technical difficulty is particularly amplified in patients receiving conscious sedation or general anesthesia as the liver becomes increasingly subcostal in position due to shallow respirations brought on by sedation[13].

HEPATIC DOME INTERVENTIONS: TIPS AND TRICKS

Imaging modality

Ultrasound can be useful in approaching lesions of hepatic dome as various angles can be used, given non-axial nature of ultrasound imaging. However ultrasound guidance can be challenging for deeply seated lesions. Computed tomography (CT) provides a 3-dimensional orientation of the needle/catheter and the target during navigation and allows performance of several additional maneuvers as discussed later in the review article. CT permits fluoroscopic capabilities and allows access to the hepatic dome through the transpleural/transpulmonic route. Particularly in patients undergoing ablation, the role of CT encompasses planning, positioning of needles, ablation monitoring, verification of completion and post-ablation assessment. Disadvantages of CT include inability to visualize certain lesions thereby necessitating administration of intravenous contrast and exposure to ionizing radiation. C-arm cone beam CT (CBCT) application may be useful in hepatic dome interventions. Respiratory gating application in CBCT can minimize motion mis-registration during navigation in thoracic and hepatic dome tumors[14]. Ablations of the liver with CBCT are often performed after administration of intra-arterial or intravenous contrast and obtaining an intra-arterial access might

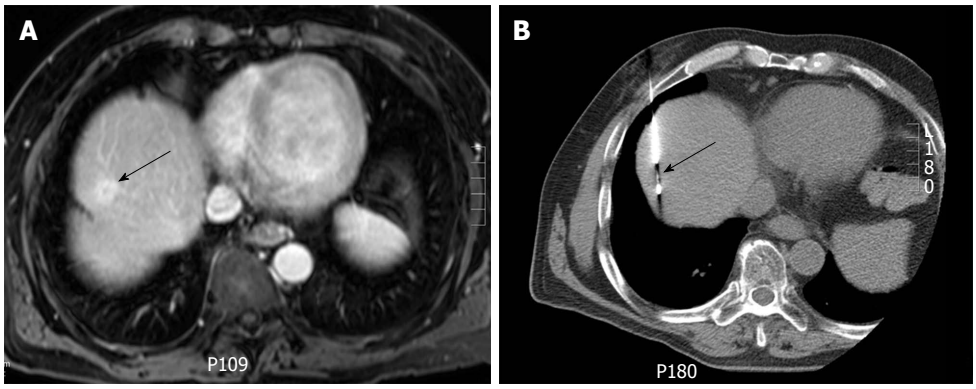

Figure 2 Computed tomography guided biopsy of a liver dome lesion in a 61-year-old man. A: Axial post gadolinium T1-weighted magnetic resonance image shows a 2 cm lesion (arrow) in the hepatic dome. On pre-procedural computed tomography, the tumor was not well seen and contrast could not be administered due to iodine allergy; B: Needle placement for biopsy was done based on use of anatomic landmarks (arrow) (configuration of inferior vena cava, cardiac margin and aorta) *via* a transpulmonary approach. Histopathology: Hepatocellular carcinoma.

be logistically difficult at certain centers[15,16]. Real time magnetic resonance imaging (MRI) guidance for biopsy of hepatic dome lesion has been described by Lu *et al*[3] to be beneficial in targeting lesions best depicted on MRI. MRI guidance has its own caveats such as need for specialized equipment (including open magnet configuration), limited availability and expertise.

Lesion localization

Precise lesion localization within the hepatic dome is crucial, particularly during biopsies and ablations to maximize diagnostic yield and achieve complete tumor destruction respectively. Accurate definition and localization of lesions on CT can be accomplished by either use of anatomic landmarks, contrast administration or additional techniques[17].

Extrapolation based on anatomic landmarks: Tumors seen on pre-procedural MR scans may not be well visualized on preliminary CT images or ultrasound during the procedure. In such circumstances, a comprehensive review of the imaging modality best depicting the lesion helps to identify the orientation of the lesion relative to adjacent landmarks such as blood vessels, bones, vascular or parenchymal calcification. Extrapolation of lesion relationship to hepatic veins, cardiac margin, aorta and IVC can be particularly helpful while performing interventions in hepatic dome. Sainani *et al*[17] reported that this strategy is highly accurate (98%) for percutaneous biopsy in liver and can obviate the need of intravenous contrast during the procedure (Figure 2).

Contrast administration: Administration of intravenous contrast can be used to guide exact placement of biopsy needle or RF electrode and is generally done after the guiding needle has been placed in to the presumed lesion location[11]. The relationship of the guiding needle and the lesion then facilitates the accurate placement of the biopsy needle or the RF electrode. It is important to ensure that the patient's serum urea and creatinine are within normal limits prior to administering contrast to avoid the risk of contrast induced nephropathy[18].

Other techniques: Several techniques have been described for targeting poorly visible hepatic lesions during interventional procedures[19-22]. Image fusion techniques combining real time ultrasonography with preprocedural CT/MRI images can be used effectively to enhance detectability for focal hepatic lesions with poor sonographic visibility[19,20]. A variety of tracking methods are available for image fusion such as image-based, optical, and electromagnetic tracking (most frequently used)[19]. The image fusion techniques however have limitations related to mis-registration because while the reference images (CT, MRI) are often obtained in a static breath-holding state, real-time ultrasound is affected by tissue deformation due to patient's respiration and movements. Most commercially available image fusion systems lack compensating mechanism for patient respiration and movement[23,24]. Hookwire and Suture localization under CT guidance followed by microwave ablation under ultrasound guidance for a sonographically invisible lesion has also been described by Kanazawa *et al*[21]. Such an approach however is cumbersome as it requires two different procedures for an ablation that can be entirely performed under CT guidance. Prior use of lipiodol might be helpful in the visualisation of hepatocellular carcinomas treated with lipoidal-transarterial chemoembolization (TACE)[22].

Optimizing access route

Selection of the approach and proper patient positioning are two important considerations for optimizing access to localization of lesions in the hepatic dome.

Percutaneous approach: The lesions of the hepatic dome can be accessed either by subcostal, intercostal or epipericardial fat pad approach. The choice of percutaneous approach is often based on operator

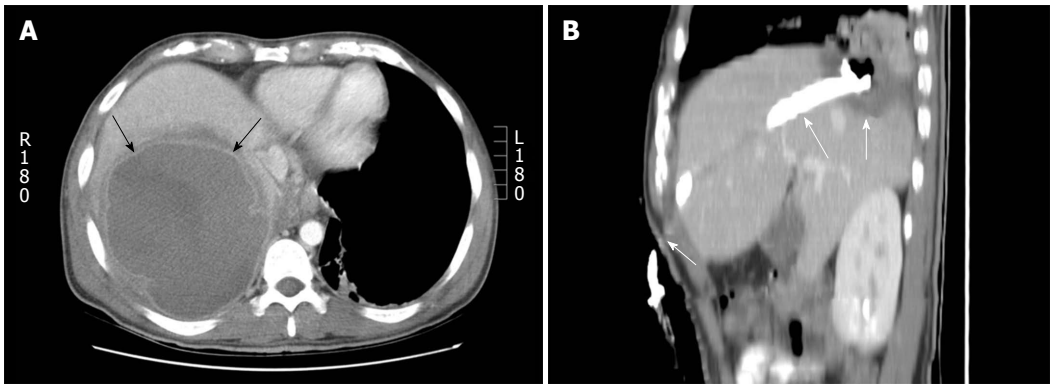

Figure 3 Percutaneous drainage of hepatic dome abscess in a 36-year-old man. A: Axial contrast computed tomography shows the large hepatic dome abscess (arrows). Pleural transgression carried an increased risk of pleural complications; B: Percutaneous catheter drainage using a subcostal approach (arrows) allowed successful abscess treatment while avoiding pleural transgression.

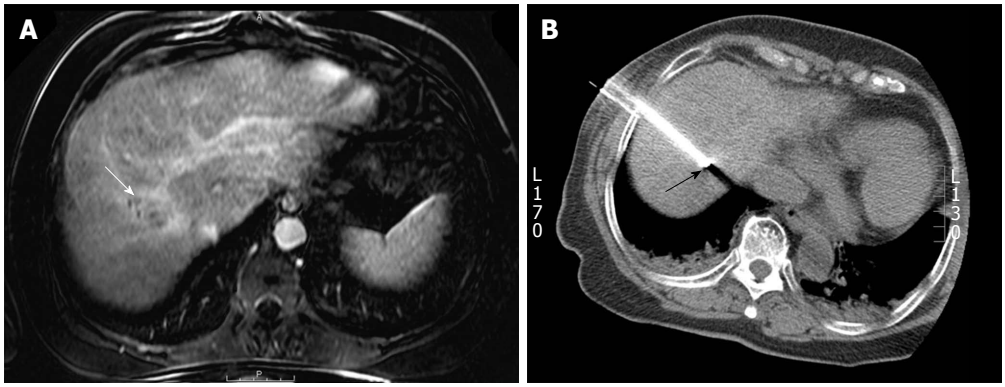

Figure 4 Percutaneous radiofrequency ablation of a hepatic dome hepatocellular carcinoma in a 54-year-old man. A: Axial post gadolinium T1-weighted image in the portal venous phase demonstrates a 3.4 cm hepatocellular carcinoma in the hepatic dome (arrow); B: During the radiofrequency ablation procedure, the patient was placed in the oblique position and using a lateral intercostal approach the tumor was accessed for a successful ablation (arrow).

experience and the access route to the regional anatomy of each individual patient is determined by reviewing prior imaging or preliminary scans. Subcostal approach is most preferred whenever feasible as this usually avoids transgression of lung and pleura (Figure 3). This approach is particularly beneficial in drainage of hepatic abscesses especially while using ultrasound guidance and can be facilitated by placing the patient in decubitus position. However this approach may not always be feasible and an intercostal route is frequently required (Figure 4) which may necessitate pleural or pulmonic transgression[1]. Pulmonary transgression can be avoided by creation of artificial pleural effusion or pneumothorax. Although pulmonary transgression may be unavoidable, for example, in patients with pleural adhesions[25]. In cases where pleural or pulmonary transgression is unavoidable it is important to limit the number of punctures to minimize the risk of pneumothorax. Furthermore, the interventionalist and patient must be prepared for the possibility of a pneumothorax and be aware of the management of such a complication. The technique of pulmonary transgression is of limited value in patients with severe emphysema or coagulopathy[25,26].

Brennan *et al*[27] described a novel epipericardial fat pad approach for safe access to hepatic dome lesions. The epipericardial fat pad is a variable sized structure located in the anterior mediastinum, outside the fibrous pericardium[27]. The authors recommend that an epipericardial fat pad exceeding 1 cm in thickness, may provide a safe window for percutaneous image guided RFA using CT fluoroscopy (Figure 5)[27].

Patient positioning: Optimal patient positioning not only determines a safe percutaneous path to the lesion but also ensures patient comfort and minimizes motion. An ideal position is one which allows the least complicated access to the hepatic dome. Supine position is the most common position employed and is generally the most comfortable one. It allows the use of anterior and lateral approach to access the dome (Figure 6). Oblique patient position can also be employed to improve the safety of a percutaneous path to the hepatic dome (Figure 4). Oblique patient position is usually employed when using a lateral approach to the hepatic dome. Lateral decubitus position can also be used when accessing the hepatic dome using a lateral approach. A lateral decubitus position is beneficial in

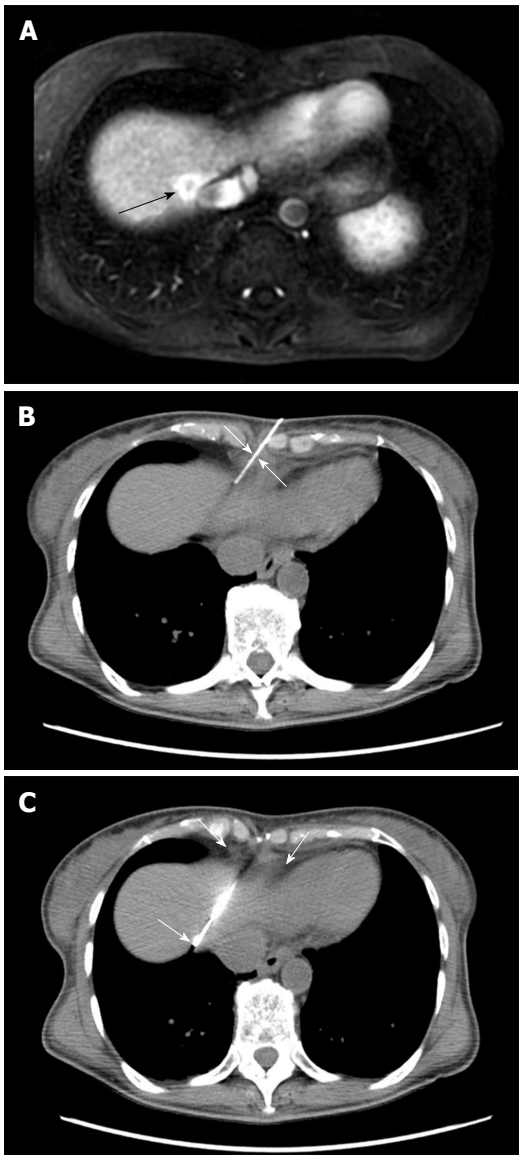

Figure 5 Computed tomography guided biopsy of a liver lesion adjacent to the inferior vena cava in a 56-year-old woman with breast cancer. A: Axial post gadolinium image T1 weighted magnetic resonance image demonstrates an enhancing lesion adjacent to the inferior vena cava (arrow); B and C: Intraprocedural computed tomography images demonstrate placement of the biopsy needle into the lesion through the epipericardial fat pad (arrows). Biopsy: Breast cancer metastases.

obese patients and women with large amount of breast tissue where an anterior approach is not feasible.

Adjunctive techniques

Non-target organ injury is the most feared complication during ablative procedures of hepatic dome. Several maneuvers could be performed in order to minimize collateral damage such as CT gantry angulation, creation of artificial ascites/pleural effusions and artificial pneumothorax.

Gantry angulation: Angulation of the CT gantry is a useful approach when the presence of overlying structures precludes a safe path to the hepatic dome (Figure 7). Angling the gantry allows optimum needle track visualization and permits a lower site of entry relative to lesion location, helping avoid transgression of pleura and lungs and thus reducing the risk of pneumothorax and pleural contamination. In this technique, the CT gantry is tilted towards the patient feet to achieve a caudo-cephalad beam direction. After preliminary scanning and identification of a safe path to the lesion, the needle shaft and hub are aligned to the CT gantry with the help of the localizing light. The needle is then advanced by constantly monitoring the needle tip position at frequent intervals while maintaining the angle of the needle. Gantry angulation technique is frequently performed for hepatic dome lesions and allows a subcostal approach to lesions high in the subphrenic location[28].

Hydro dissection/artificial ascites: Creation of artificial ascites or hydrodissection is an effective techniques for safe percutaneous ablation of hepatic dome lesions[7,8,29,30]. Hydrodissection involves injection of fluid into the peritoneal space around the liver to create separation of hepatic dome from the diaphragm, thereby preventing damage to the diaphragm and pleura during thermal ablation. Additionally, this technique diminishes post procedure pain resulting from diaphragmatic irritation, and reduces the need for general anesthesia for pain control allowing use of conscious sedation[30]. Hydrodissection can be performed using ultrasound or CT guidance and we most commonly use a 14-20 G Chiba needle or a 5 French vascular catheter/sheath for instilling fluid (Figure 8)[7,8,29]. For hepatic dome interventions, the puncture sites for creation of artificial ascites are typically at the level of left subphrenic space. Prolonged procedures such as ablations necessitate continued hydrodissection throughout the primary procedure to maintain sustained separation of the lesion from the diaphragm. Five percent dextrose water (D5W) is preferred over normal saline for hydrodissection since it provides significantly better electrical isolation, reduces unwanted heat dissipation to the adjacent organs and is least likely to cause volume shifts due to its iso-osmolar nature[7,8,29,31]. While no definitive amount of separation has been universally agreed upon, at least 5 mm separation between the diaphragmatic margin and liver capsule is recommended to minimize organ damage. The instilled fluid usually resorbs spontaneously within a week and does not decrease the therapeutic efficacy of RFA[29]. Despite its benefits, occasionally the fluid dissipates away from the intended site and hydrodissection is not effective in the presence of peritoneal adhesions due to prior treatments such as surgical resection, TACE or thermal ablation. Additionally, lesions located in the bare area of liver cannot be separated by hydrodissection as this area is surrounded by peritoneal ligaments. Omentum interposed during hepatic surgeries can also

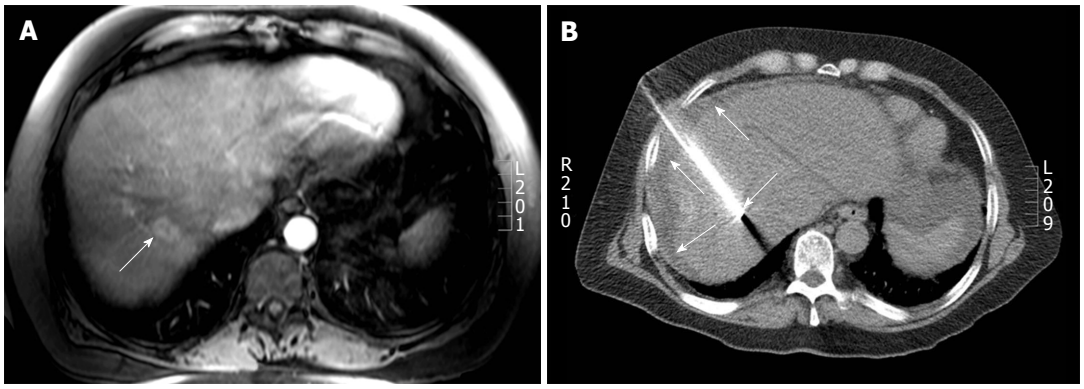

Figure 6 Computed tomography guided radiofrequency ablation in a 56-year-old lady with colorectal liver metastases. A: Axial post gadolinium T1 weighted magnetic resonance image shows a 2.7 cm (arrow) hepatic dome metastases; B: The radiofrequency ablation was performed with the patient in supine position and needle placement through the anterolateral intercostal approach. Hydrodissection was performed in this patient (arrows).

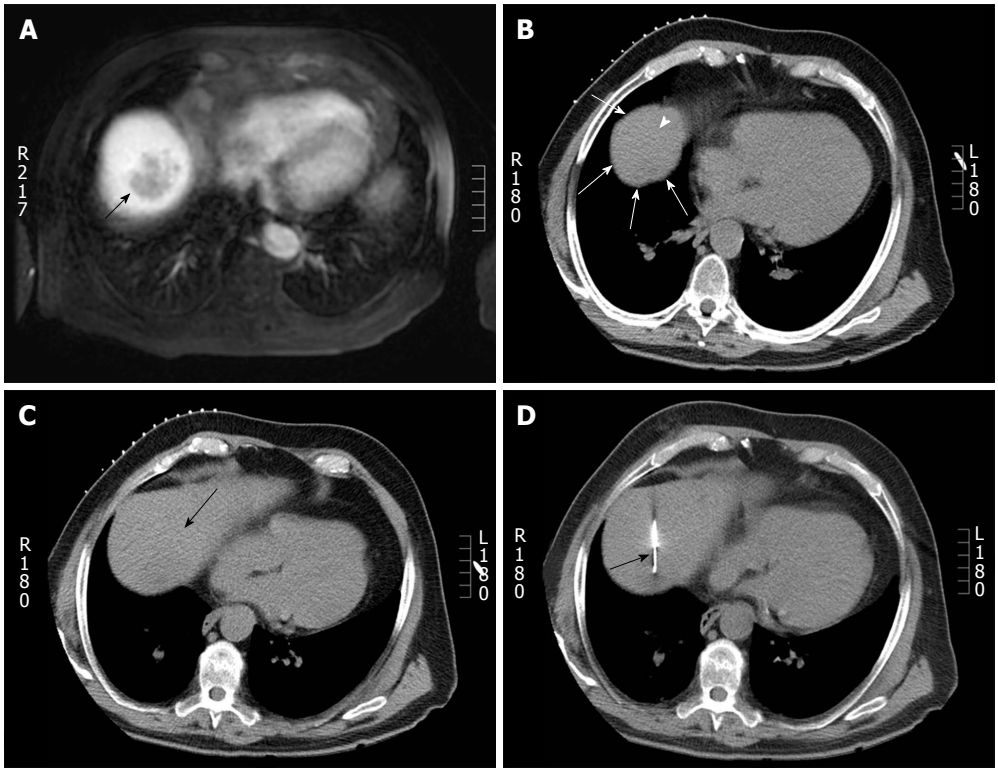

Figure 7 Computed tomography guided biopsy in a 65-year-old man. A: Axial post gadolinium magnetic resonance imaging shows a 4 cm hepatic dome lesion; B: On preprocedural CT, the lesion in the high dome is surrounded by lung (arrow head) on all sides. Pulmonic transgression was not possible as the patient had severe emphysema; C: The CT gantry was angulated in the craniocaudal direction (20 degrees) which created a safe path to the tumor from the anterior aspect (arrow); D: Axial intraprocedural CT image shows biopsy needle within the lesion (arrow). Biopsy: Hepatocellular carcinoma. CT: Computed tomography.

impede successful induction of artificial ascites[7].

Artificial pleural effusion: Bare area of liver (not lined by peritoneum) is in direct contact with the diaphragm. The fluid form an artificial ascites cannot dissect this region from the diaphragm. Similar situation arises when intra-peritoneal adhesions limit separation of the diaphragm from the liver. Artificial pleural effusion using saline is a valuable adjunctive technique in such situations. It also creates a safe percutaneous path or good sonographic window when using ultrasound for image guidance[32-36].

Iatrogenic pneumothorax: An iatrogenic pneumothorax can be created when other approaches fail[37,38]. In this technique, an 18 gauge epidural needle is appropriately positioned in the pleural space and around 50 mL of air, obtained through a micro-porous filter, is injected into the pleural space. Subsequently, serial boluses of 200, 400, 600 and 800 mL are injected to separate the lung from the pleura (Figure 9). Following completion of the interventional procedure, the intrapleural air is aspirated through the catheter into the syringe and expelled through the stopcock[37]. The patient is usually admitted overnight

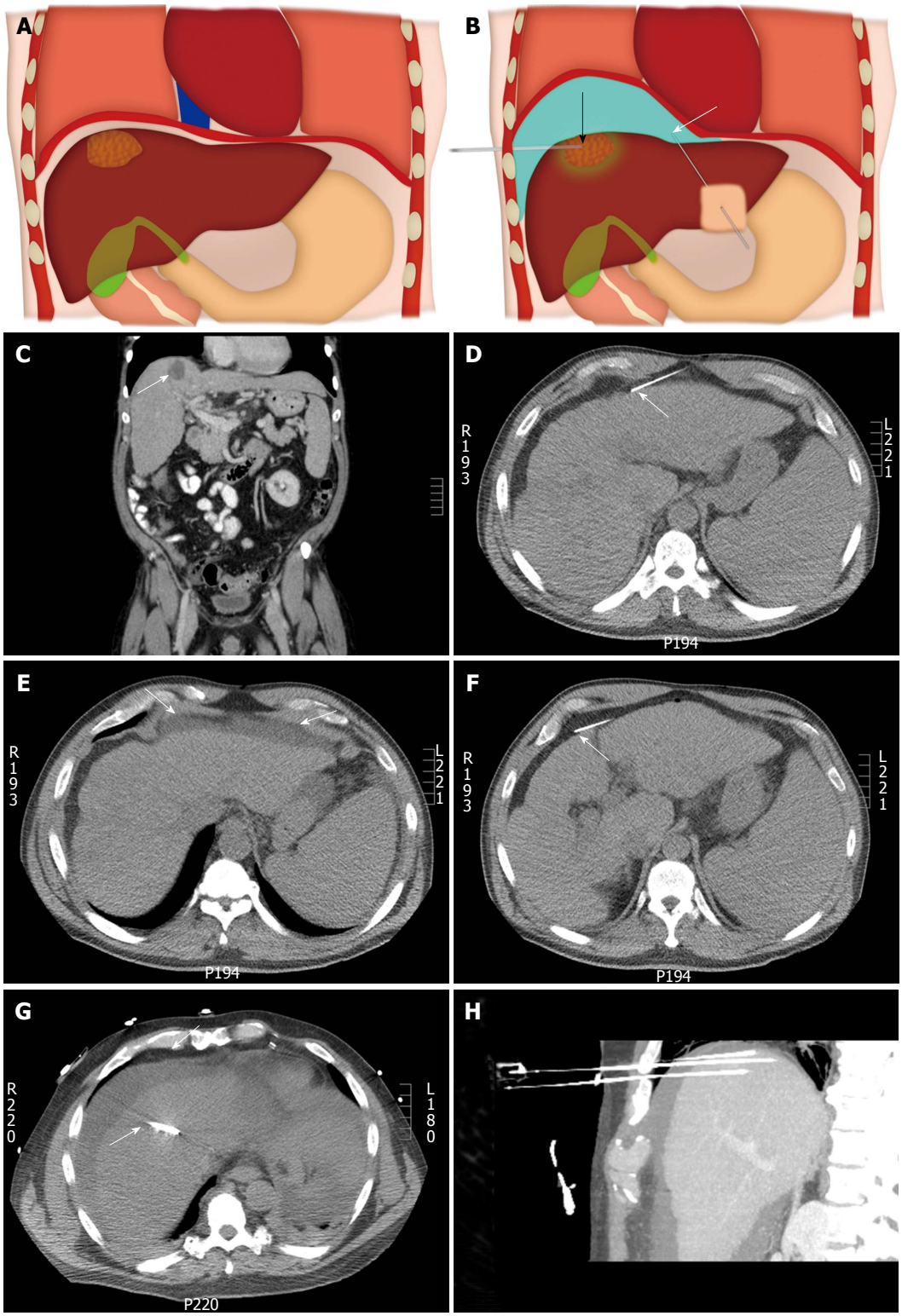

Figure 8 Illustration of the upper abdomen demonstrating the hydro-dissection technique. A: Coronal colored image shows a hepatic dome lesion very close to the diaphragm; B: Coronal colored image after hydro-dissection (shown in blue color) shows the separation of the dome of liver from the diaphragm which improves percutaneous access to the lesion and limits diaphragmatic injury. White arrow shows the needle for hydodissection and black arrow shows the needle into the lesion. An example of a hepatic dome lesion (C) (arrow) where hydrodissection was attempted by needle placed anteriorly (D) (arrow); E: Axial computed tomography showed accumulation of fluid within the properitoneal fat (arrows); F: The needle was repositioned with the tip of the needle into the peritoneal cavity (arrow); G: Successful hydrodissection achieved using instillation of 500 cc of D5W through the needle (arrow); H: The fluid was used to create a safe path for radiofrequency ablation of the hepatic dome hepatocellular carcinoma. Sagittal Maximum intensity projection image demonstrating the artificial ascites and electrodes in position in the hepatic dome lesion.

for observation and serial radiographs are obtained to monitor the resolution of the pneumothorax. Pneumothorax tends to accumulate in non-dependent locations and hence patient positioning is of critical

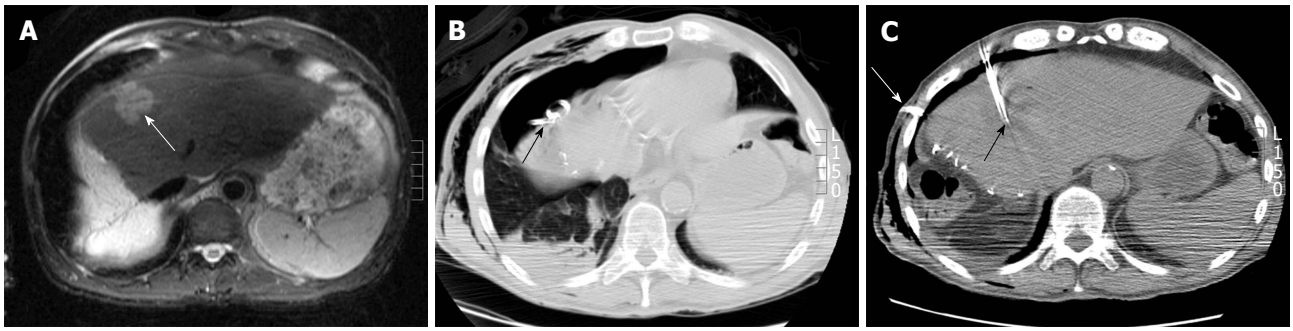

Figure 9 Artificial pneumothorax for radiofrequency ablation of hepatic dome hepatocellular carcinoma in a 69-year-old man. A: Axial T2WI magnetic resonance imaging shows A 4 cm lesion (arrow) in the hepatic dome; B: Artificial pneumothorax was created after instillation of intrapleural air. A chest tube was placed for drainage (arrow); C: Intra-procedural computed tomography shows radiofrequency electrode within the lesion for a successful ablation (black arrow).

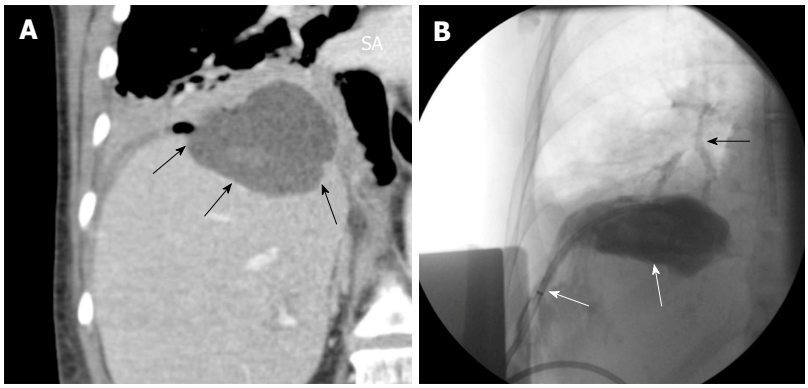

Figure 10 Hepatic abscess complicating a hepatic dome metastases ablation. A: Coronal reformatted image shows a abscess in the dome of liver (arrows); B: Percutaneous drainage was performed and drain injection shows communication with the bronchi (black arrow).

importance. For example, if anterior approach is adopted, the patient should be placed in supine position to direct the pneumothorax anteriorly[37].

Other techniques: Investigators have tried using different barriers for diaphragmatic protection such as intraabdominal carbon dioxide insufflations or angiographic balloon interposition, although the experience with their use is limited[10,39]. Raman *et al*[10] studied the use of intraperitoneal carbon dioxide insufflations for diaphragmatic protection during hepatic RFA ablations in porcine model and proved its efficacy in limiting diaphragmatic injury during superficial hepatic RFA. Knuttinen *et al*[39] interposed an angiographic balloon catheter during RFA ablation of the hepatic dome in a porcine model and demonstrated that balloon interposition is an effective technique for diaphragmatic protection. Balloon interposition has been reported to be superior to hydro-dissection or carbon dioxide insufflation, as the balloon remains stable during the procedure, while fluid and gas have a tendency to dissipate, however evidence in this regard is limited[39]. Electrode "retraction/torquing" technique is another maneuver with the use of expandable RFA probes in kidney, liver and lung tumors[40]. In this technique, the expandable electrode is retracted or torqued to displace the organ after the electrode is in position and fully expanded. This technique may be ineffective in isolation as only a few millimetres of displacement is achieved but could be used as an adjunct to other techniques.

COMPLICATIONS

Most dreaded complications during hepatic dome interventions include diaphragmatic and lung injury, pleural effusion, pneumothorax and empyema. Specific maneuvers like CT-guided transpulmonary needle insertion for liver tumors may lead to pneumothorax, lung hemorrhage and hemothorax, pleural effusion, diaphragmatic injury, tumor seeding in the pleura and/or lung parenchyma, lung abscess and systemic air embolism[26]. Serious complications such as massive pulmonary hemorrhage and systemic air embolism may result from transpulmonary RF needle insertion[41-43]. Diaphargmatic injury can lead to severe pain due to irritation, diaphragmatic palsy and/or perforation[7-10,30,31,39,44]. Diaphragmatic injury can also lead to fistulization of hepatic dome processes into the thorax (Figure 10). Injury to the lung and pleura can result in pneumothorax, pleural effusion and empyema which often need chest tube drainage[9,45]. The reported incidence of major diaphragmatic complications is low and has been reported to be more frequent with deployable radiofrequency electrodes and multiple treatments[29,46-49]. Post-ablation local

tumor progression may be slightly higher for peri-diaphragmatic tumors as compared to central tumors as these tumors are ablated more cautiously because of concern for collateral damage[50].

CONCLUSION

Image guided interventions in the hepatic dome often pose unique challenges to interventional radiologists. Interventionists should use their anatomic expertise along with the wide range of available imaging and interventional techniques to safely access and successfully manage hepatic dome lesions.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Improved Hepascore in hepatitis C predicts reversal in risk of adverse outcome

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Author contributions: Jeffrey AW, Huang Y and Jeffrey GP were responsible for study design, data collection and analysis and manuscript preparation; de Boer WB, Adams LA, MacQuillan G, Speers D and Joseph J were involved in data interpretation and manuscript preparation.

Institutional review board statement: The study was reviewed and approved by the Sir Charles Gairdner Hospital Human Ethics Research Committee.

Informed consent statement: This manuscript did not require informed consent as the presented data is anonymized and risk of identification is low. This retrospective study complies with the NHMRC National Statement on Ethical Conduct in Human Research.

Conflict-of-interest statement: All the Authors have no conflict of interest related to the manuscript.

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Abstract

AIM

To establish if serial Hepascore tests (referred to as delta Hepascore) in those with chronic hepatitis C (CHC) correlate with the increase and/or decrease in risk of liver related complications.

METHODS

Three hundred and forty-six CHC patients who had two Hepascore tests performed were studied. During 1944 patient years follow-up 28 (8.1%) reached an endpoint. The Hepascore is a serum test that provides clinically useful data regarding the stage of liver fibrosis and

subsequent clinical outcomes in chronic liver disease.

RESULTS
Patients with a baseline Hepascore > 0.75 had a significantly increased rate of reaching a composite endpoint consisting of hepatocellular carcinoma, liver death, and/or decompensation (P < 0.001). In those with an initial Hepascore > 0.75, a subsequent improved Hepascore showed a significantly decreased risk for the composite endpoint (P = 0.004). There were no negative outcomes in those with a stable or improved delta Hepascore. The minimum time between tests that was found to give a statically significant result was in those greater than one year (P = 0.03).

CONCLUSION
In conclusion, Hepascore is an accurate predictor of liver related mortality and liver related morbidity in CHC patients. Of note, we have found that there is a decreased risk of mortality and morbidity in CHC patients when the patient has an improving delta Hepascore. Repeat Hepascore tests, when performed at a minimum one-year interval, may be of value in routine clinical practice to predict liver related clinical outcomes and to guide patient management.

Key words: Chronic; Prognosis; Direct acting antivirals; Serum; Hepatitis C

Core tip: The growing burden of hepatitis C is well recognized. The use of serum fibrosis markers such as Hepascore to monitor change in clinical risk in hepatitis C has a significant potential benefit to optimise the management in these patients. However, there is no information on the value of serial serum fibrosis tests and their improvement over time in determining changes in liver related clinical outcomes. We have found that there is a decreased risk of mortality and morbidity in chronic hepatitis C patients when the patient has an improving delta Hepascore, and serial tests may be of use in clinical practice.

Jeffrey AW, Huang Y, de Boer WB, Adams LA, MacQuillan G, Speers D, Joseph J, Jeffrey GP. Improved Hepascore in hepatitis C predicts reversal in risk of adverse outcome. *World J Hepatol* 2017; 9(19): 850-856 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i19/850.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i19.850

INTRODUCTION

The use of direct acting antivirals (DAA) therapy in chronic hepatitis C (CHC) treatment has resulted in up to 99% eradication of hepatitis C virus (HCV) in patients receiving treatment, depending on the genotype and type of DAA used[1,2]. The increased efficacy and minimal side effects of newer DAA's means that many more patients will access therapy, if financially able. To this end, in March 2016 the Pharmaceutical Benefits Scheme (PBS) in Australia listed sofosbuvir, ledipasvir/sofosbuvir and daclatasvir for the treatment with CHC (4) which will provide access to treatment for all Australians. It is estimated that there will be a 93% reduction in advanced liver disease cases due to the new DAA therapies compared to current regimens or no treatment[3]. HCV eradication has been shown to reduce liver fibrosis and liver related complications but the time required for this reversal is not known[2,4,5]. In addition, other co-factors such as NAFLD and alcohol use may be present and prevent or impair reversal of hepatic fibrosis. Therefore the problem remains that CHC patients with significant or advanced liver fibrosis at the time of successful HCV eradication may require long term monitoring for liver related complications for an uncertain period of time[6].

Fibrosis severity is currently measured non-invasively using serum fibrosis markers or transient elastography (Fibroscan®). The histopathological staging of fibrosis using liver biopsy has historically been the best predictor of liver related mortality and liver related morbidity associated with CHC[7]. However liver biopsy is now rarely used to stage CHC patients due to the risk of serious complications and issues with sampling error[8]. Several non-invasive serum fibrosis markers have been developed and are currently used as non-invasive alternatives to liver biopsy. Recent advances have now demonstrated that some serum fibrosis markers are able to directly predict adverse liver related outcomes rather than just provide a surrogate marker of liver fibrosis[9]. Hepascore is one of these markers, and it is used to predict liver related complications in patients with CHC. Hepascore has also been shown to be comparable to liver biopsy[10-12]. The Hepascore result itself ranges from 0 to 1.0 with a lower value indicating less severe or absent liver fibrosis and consequently better liver related clinical outcomes[10]. Measurement of the change in severity of liver fibrosis over time is also a strong prognostic tool in CHC[7]. The use of non-invasive serum fibrosis markers to monitor regression/progression of fibrosis in CHC has a significant potential benefit to optimise the clinical management in these patients. However, there is no information on the value of serial serum fibrosis tests and their change over time in determining changes in liver related clinical outcomes.

This aim of this study is to establish if serial Hepascore tests (referred to as delta Hepascore) in those with CHC correlate with the increase and/or decrease in risk of liver related complications.

MATERIALS AND METHODS

Cohort

Hepatitis C patients who presented to Sir Charles Gairdner Hospital (SCGH) based in Western Australia

from 1992 to 2012 and who also had two Hepascore tests performed were studied. We defined our inclusion criteria as all patients with hepatitis C, both treated and untreated. We also included patients regardless of if they achieved a sustained virological response (SVR). Our exclusion criteria consisted those with co-existing hepatitis B infection, human immunodeficiency virus as well as any other liver diseases. We also excluded patients who had received a previous liver transplantation. We received ethics approval for this study from the Department of Health Ethics Committee and the SCGH Ethics Committee.

Data collection

Baseline and second Hepascore test dates and results were collected for each patient. The WA based Data Linkage System, called WADLS was used to collect long term patient morbidity and mortality figures[13]. This is a wide scale population based linkage system that has been used extensively in the past and validated in previous population and cohort studies[14,15]. The WADLS contains records of cancer registrations as well as in-patient hospital morbidity and death records of the Western Australian population, from 1966 to the present. For this study, the events collected were all-cause mortality, liver related mortality, liver related morbidity and cancer registration. The WADLS database has previously been used as part of published and validated studies on liver fibrosis assessment and use of other non-invasive markers including Hepascore[10,11].

The primary endpoint for this study was liver related death (LRD) or liver transplantation. Secondary endpoints included onset of hepatocellular carcinoma (HCC) and liver decompensation (LD) of all causes. A composite endpoint included all of these endpoints but patients were only included once. The follow-up time used for the analysis of the baseline Hepascore test was from the time of the test until a primary or secondary endpoint or the conclusion of the study. The follow-up time used for the analysis of delta Hepascore was from the time of the second Hepascore test until an end point or end of study was reached. Delta Hepascore was calculated as the second Hepascore minus the baseline Hepascore. Patients who reached an endpoint before the second Hepascore test were excluded from delta Hepascore analysis. Hepascore is a serum marker that incorporates gamma glutamyl transpeptidase, hyaluronic acid and alpha 2 macroglobulin.

Statistical analysis

Statistics were undertaken using the SPSS Statistics software package and Kaplan-Meier survival analysis. Multivariate cox regression was used to assess the prognostic significance of an initial Hepascore, second Hepascore, and delta Hepascore to predict LRD, HCC or LD. Significance was defined as $P < 0.05$. Patients were placed into groups based on the baseline Hepascore value (0-0.25, 0.26-0.5, 0.51-0.75, 0.76-1.0) and the delta Hepascore (delta < -0.1, -0.1 $\leqslant$ delta $\leqslant$ 0.1, delta > 0.1) for the analysis. Survival probabilities for using baseline Hepascore values and delta Hepascore values were then calculated using Kaplan-Meier curves with significance calculated using the log rank test.

Area under Receiver Operating Characteristic (AUROC) curves were calculated to assess the capacity of baseline Hepascore and delta Hepascore values to predict liver related outcomes. The optimal time interval between Hepascore tests was assessed by Kaplan-Meier analysis according to the time between tests: < 1 year and $\geqslant$ 1 year.

RESULTS

A total of 346 patients met the inclusion criteria and were followed for a mean of 5.5 years, during which 28 (8.1%) had a LRD, developed LD and/or HCC (Table 1). The mean age of the cohort was 53.6 years and 220 (63.6%) were male. Of the total cohort, 8 (2.3%) had a LRD, 15 (4.6%) developed LD and 16 (4.3%) developed HCC. The mean baseline and second Hepascore values were 0.48 (SD ± 0.34) and 0.57 (SD ± 0.34) respectively and the mean delta Hepascore was 0.09 (SD ± 0.23). The time between Hepascore tests ranged from 0.03 and 12.5 years, with a mean of 3.3 and the mean follow-up time after the second Hepascore was 2.4 years. Multivariate cox regression showed that baseline Hepascore and delta Hepascore were independently predictive of reaching a composite clinical endpoint (LRD, HCC or LD), with *P* values of 0.02 and 0.013 respectively (Table 2).

Patients were grouped into 4 categories according to their baseline Hepascore (0-0.25, 0.26-0.5, 0.51-0.75 and 0.76-1.0). One hundred and twenty-nine (37%) had a Hepascore $\leqslant$ 0.25, 73 (21%) had a Hepascore from 0.26 to 0.5, 43 (12%) had a Hepascore from 0.51 to 0.75 and 100 (29%) had a Hepascore > 0.75. Kaplan-Meier survival curve analysis found that those patients with a baseline Hepascore > 0.75 had a significantly increased rate of LRD ($n \leqslant 0.001$), HCC ($n \leqslant 0.001$), LD ($n \leqslant 0.001$) and composite endpoint ($P < 0.001$) (Table 3 and Figure 1). Hazard ratios could not be calculated because of the lack of adverse liver related outcomes in the other three lower value Hepascore groups.

Patients with a baseline Hepascore > 0.75 were then analysed using the delta Hepascore value. The delta Hepascore values were divided into those with an improved Hepascore (delta < -0.1), a stable Hepascore (-0.1 $\leqslant$ delta $\leqslant$ 0.1) and a worsened Hepascore (delta > 0.1). Survival curve analysis found that in those with an improved Hepascore there was a significantly decreased risk of LRD, LD and a composite endpoint ($P = 0.048$, $P = 0.001$, $P = 0.004$ respectively) as shown in Figure 2. Twelve (17%) patients with a stable or worsened Hepascore reached a composite end point in contrast with those patients

Table 1 Patient characteristics and outcomes

Characteristic	All patients		Patients with first Hepascore > 0.75		All patients		Patients with first Hepascore > 0.75	
	Number	Percent	Number	Percent	mean	Range	mean	Range
Number	346	-	100	-	-	-	-	-
Gender (male)	220	63.6	76	76	-	-	-	-
SVR	38	11.0	16	16	-	-	-	-
Composite endpoint	28	8.1	21	21	-	-	-	-
LRD	8	2.3	8	8	-	-	-	-
LD	16	4.6	12	12	-	-	-	-
HCC	15	4.3	12	12	-	-	-	-
Result					-	-	-	-
Bilirubin (μmol/L)[1]	-	-	-	-	9.0	1.0-200	12	2.3-200
GGT (U/L)[1]	-	-	-	-	55.0	8.0-1005	93.5	17-713
HA (μg/L)[1]	-	-	-	-	30.3	1.0-1211	124.5	16-1211
A2M (μg/mL)[1]	-	-	-	-	2.5	0.6-6	3.6	1.5-6.0
Age (yr)	-	-	-	-	53.6	30-80	58.3	36-80
Baseline Hepascore	-	-	-	-	0.48	0.02-1.0	0.93	0.77-1.0
Second Hepascore	-	-	-	-	0.57	0.04-1.0	0.87	0.13-1.0
Delta Hepascore	-	-	-	-	0.09	-0.80-0.94	-0.06	-0.8-0.23
Time between baseline and second Hepascore (yr)	-	-	-	-	3.3	0.03-12.5	2.8	0.03-10.3
Follow-up after second Hepascore (yr)	-	-	-	-	2.2	0.01-7.3	1.9	0.01-5.7

[1]Serum markers used in Hepascore calculation. GGT: Gamma glutamyl transpeptidase; HA: Hyaluronic acid; A2M: Alpha 2 macroglobulin.

Table 2 Predictors of composite clinical endpoint (liver related death, hepatocellular carcinoma, liver decompensation) using Multivariate Cox Regression

Variable	Follow-up from the baseline Hepascore		Follow-up from the second Hepascore	
	P	Hazard ratio (95%CI)	*P*	Hazard ratio (95%CI)
Baseline Hepascore	< 0.001	5.85 (2.25-15.18)	0.020	12.86 (1.49-111.17)
Second Hepascore	-	-	0.891	3288.82 (0.0-4.6E + 53)
Delta Hepascore	-	-	0.013	4.77 (1.35-16.45)

Table 3 Predictors of survival using Kaplan-Meier survival curves

Test	End point	*P* value (log rank)	Cohort size
Baseline Hepascore alone	Composite Endpoint	< 0.001	346
	LRD	< 0.001	352
	LD	< 0.001	348
	HCC	< 0.001	350
Delta Hepascore	Composite Endpoint	0.004	96
	LRD	0.048	105
	LD	0.001	101
	HCC	0.178	100

LRD: Liver related death; LD: Liver decompensation; HCC: Hepatocellular carcinoma.

Table 4 Predictors of survival Using Area under Receiver Operating Characteristic

Test	End point	AUROC
Baseline Hepascore alone	Composite endpoint	0.80
	LRD	0.89
	LD	0.75
	HCC	0.87
Baseline Hepascore > 0.75 and Delta Hepascore	Composite endpoint	0.84
	LRD	0.95
	LD	0.77
	HCC	0.93

AUROC: Area under Receiver Operating Characteristic; LRD: Liver related death; LD: Liver decompensation; HCC: Hepatocellular carcinoma.

who had an improved Hepascore, who had no negative outcomes. Comparison between those patients with a stable Hepascore and those with a worse Hepascore was not possible as 19.5% of patients had a baseline Hepascore value > 0.9 (the maximum Hepascore value is limited to 1.0). Thirty-eight (11%) patients had anti-viral treatment and reached a SVR. Of those achieving SVR only 4 patients reached an endpoint. Excluding these patients from the analysis made no difference to the results.

AUROC analysis was performed using the baseline Hepascore alone and with a combination of the baseline Hepascore and delta Hepascore (Table 4). There was a marked improvement in the AUROC for the combined baseline and delta Hepascore values compared to baseline Hepascore values alone with an AUROC for LRD of 0.95 and 0.89, for LD of 0.77 and 0.75 and for HCC of 0.93 and 0.87, respectively (Table 4).

Sub-group analysis was then completed to determine the minimum time required between Hepascore tests to determine delta Hepascore. Survival curve

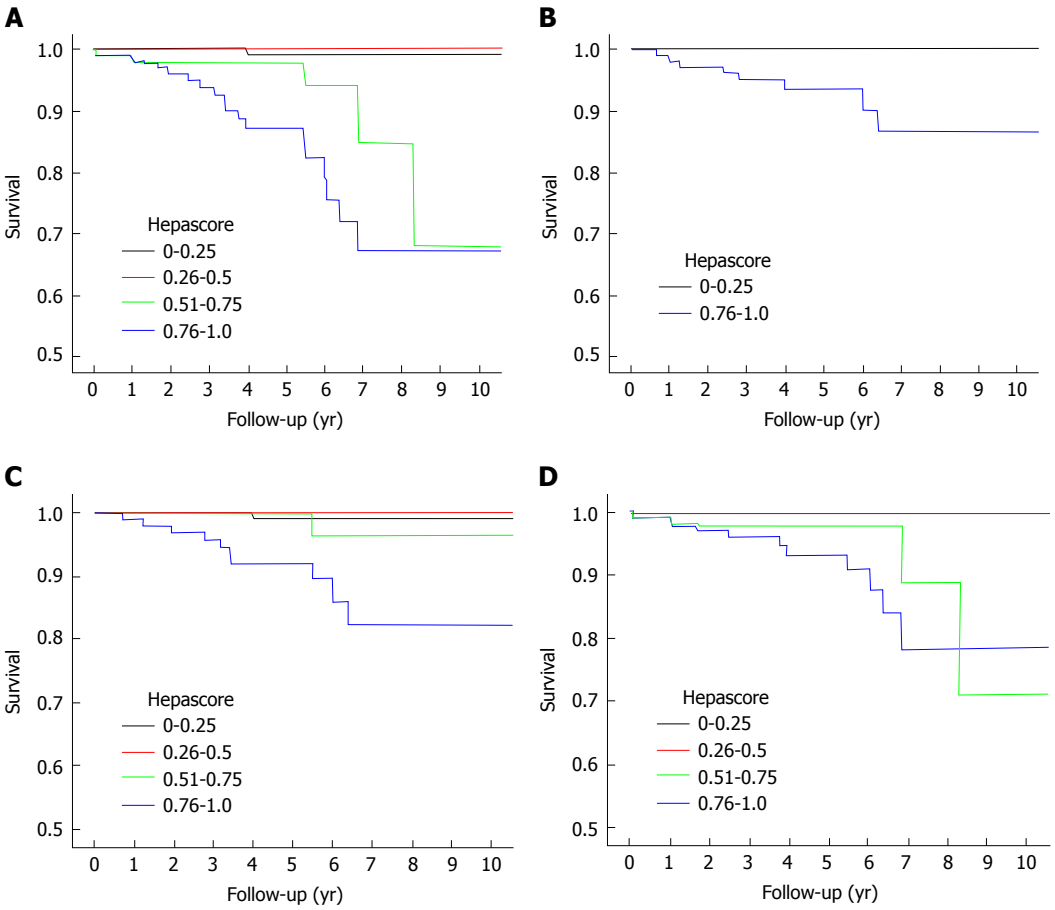

Figure 1 Kaplan-Meier curves specifying survival for liver related death, liver decompensation, hepatocellular carcinoma and a composite end point as a function of baseline Hepascore in the whole cohort. A: Time to composite end point using baseline Hepascore ($P < 0.001$); B: Time to LRD according to Hepascore ($P < 0.001$); C: Time to LD according to Hepascore ($P < 0.001$); D: Time to HCC according to Hepascore ($P < 0.001$). LRD: Liver related death; LD: Liver decompensation; HCC: Hepatocellular carcinoma.

analysis found that in those patients with a baseline Hepascore > 0.75, delta Hepascore is only predictive of a composite endpoint if the time between Hepascore tests is more than one year ($P = 0.03$) (Figure 3).

DISCUSSION

No previous studies have reported the use of repeated non-invasive serum fibrosis markers to predict improved liver related clinical outcomes. In this well documented cohort of CHC patients with a long follow-up period, 8.1% had an adverse liver related outcome after a mean of 5.5 years of follow-up. Cox regression found that a high (> 0.75) baseline Hepascore value was independently associated with increased rates of adverse liver related outcomes ($P < 0.001$), consistent with previous reports[11,12]. Importantly the delta Hepascore was also independently associated with predicting a composite clinical endpoint (LRD, HCC, LD) ($P = 0.004$). The AUROC for predicting the composite end point using the initial Hepascore and delta Hepascore was 0.84, which was increased compared to the AUROC using Hepascore alone (0.80).

Patients with an initial Hepascore value greater than 0.75 had an increased risk of developing an adverse liver related end point and this equated to a 5-year risk of 10% and a 10-year risk of 35%. CHC patients with an initial Hepascore less than or equal to 0.75 had a negligible (%) risk for developing these complications over 10 years. Further analysis found that in patients with a baseline Hepascore greater than 0.75 and who had a subsequent improvement in their second Hepascore of more than 0.1 (delta < -0.1), no adverse liver related end points occurred after a mean of 2.5 years. In contrast, those CHC patients with an initial Hepascore greater than 0.75 and who had a stable or worsened delta Hepascore there was an increased risk of experiencing an adverse liver related outcome. Hepascore has a range of values from 0 to 1.0, therefore only those patients with a baseline Hepascore below 0.9 could have an increased delta Hepascore (delta > 0.1) on subsequent testing. This limited the value of sub-group analysis comparing worsening (delta > 0.1) or stable ($-0.1 \leqslant$ delta $\leqslant 0.1$) delta Hepascore values in those with an initial Hepascore greater than 0.75.

The minimum time interval between Hepascore tests that resulted in useful clinical information was one year. Only when the Hepascore test interval was one year or more was there a significant association

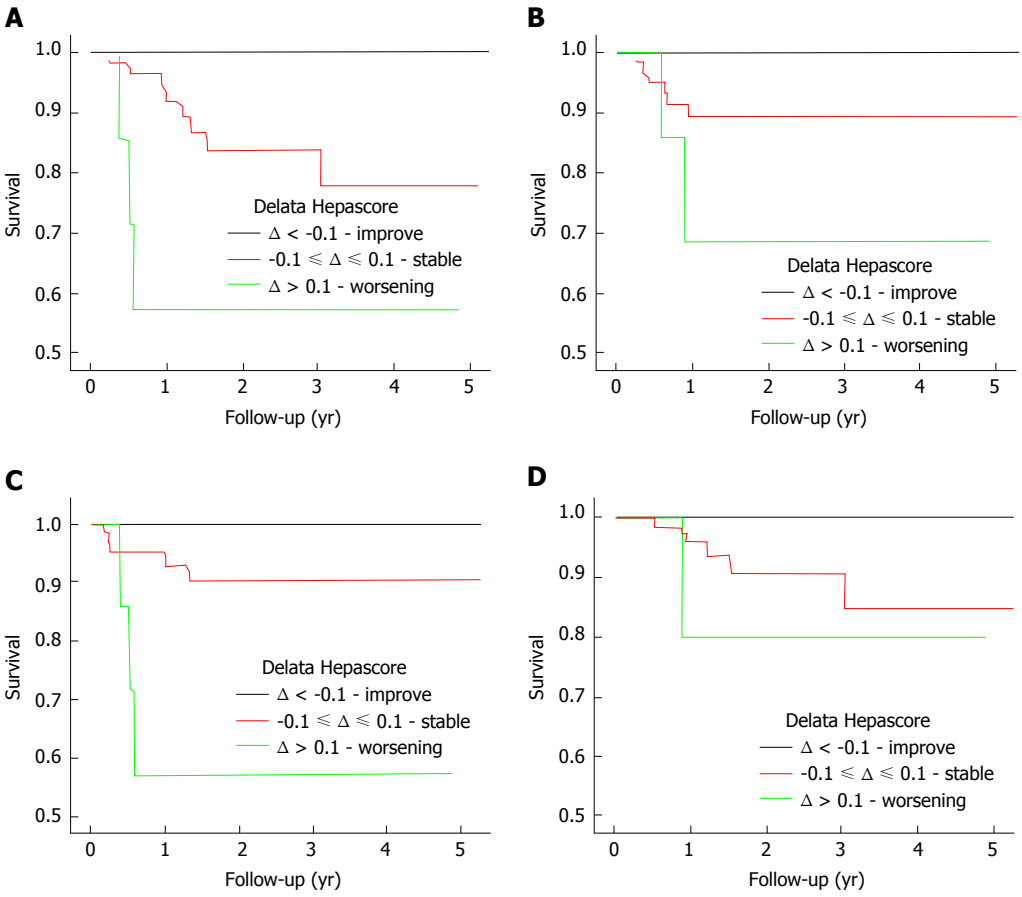

Figure 2 Kaplan-Meier curves specifying survival for liver related death, liver decompensation, hepatocellular carcinoma and a composite end point as a function of Delta Hepascore in the cohort with a baseline Hepascore > 0.75. A: Composite end point according to delta Hepascore, with a baseline Hepascore result of > 0.75 (P = 0.004); B: LRD according to delta Hepascore, with a baseline Hepascore result of > 0.75 (P = 0.048); C: LD according to delta Hepascore, with a baseline Hepascore result of > 0.75 (P = 0.001); D: HCC according to delta Hepascore, with a baseline Hepascore result of > 0.75 (P = 0.178). LRD: Liver related death; LD: Liver decompensation; HCC: Hepatocellular carcinoma.

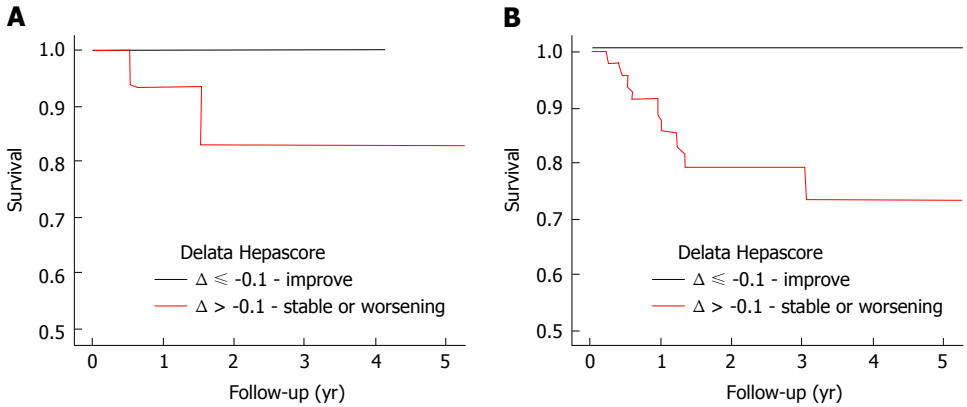

Figure 3 Kaplan-Meier curves specifying survival for a composite end point as a function of Delta Hepascore calculated at varying time intervals between tests. A: Time between tests - 0 to 12 mo (P = 0.347); B: Time between tests from - 1 year onwards (P = 0.03).

between delta Hepascore and the risk of adverse liver related outcomes (P = 0.03). Our findings show that there is a reduced risk of negative outcome in CHC patients who have an initial Hepascore over 0.75, but have an improved delta Hepascore, and will potentially allow a change in clinical management whereby the need for surveillance for varices and hepatocellular cancer may be reduced.

This study has some limitations. Firstly, due to the retrospective nature of this study, the second Hepascore test was not performed after a fixed time period. This time period was sufficient to demonstrate variation in delta Hepascore, however a fixed follow-up period could be established for future research.

Secondly, the data linkage system, which has allowed the collection of comprehensive data from a central source did not include information on alcohol consumption, diet and exercise. However, we believe that this data would not impact on the results of this study.

In conclusion, Hepascore is an accurate predictor of liver-related mortality and morbidity in CHC patients. Of note, we have found that there is a decreased risk of mortality and morbidity in CHC patients when the patient has an improving delta Hepascore. Repeat Hepascore tests, when performed at a minimum one-year interval, may be of value in routine clinical practice to predict liver related clinical outcomes and to guide patient management.

COMMENTS

Background

Several non-invasive serum fibrosis markers have been developed and are currently used as non-invasive alternatives to liver biopsy. Hepascore is one such marker that is able to predict severity of fibrosis, comparable to liver biopsy. Recent advances have now demonstrated that serum fibrosis markers such as Hepascore are able to directly predict adverse liver related outcomes rather than just provide a surrogate marker of liver fibrosis. Hepascore can also be used to monitor regression/progression of fibrosis in chronic hepatitis C (CHC).

Research frontiers

The use of Hepascore to monitor regression/progression of fibrosis in CHC has a significant potential benefit to optimise the clinical management in these patients. However, there is no information on the value of serial serum fibrosis tests and their change over time in determining changes in liver related clinical outcomes.

Innovations and breakthroughs

Hepascore was found to an accurate predictor of liver-related mortality and morbidity in CHC patients. Of note, the authors have found that there is a decreased risk of mortality and morbidity in CHC patients when the patient has an improving delta Hepascore. Repeat Hepascore tests, when performed at a minimum one-year interval, are of value in routine clinical practice to predict liver related clinical outcomes and to guide patient management.

Applications

Repeat Hepascore tests, when performed at a minimum one-year interval, may be of value in routine clinical practice to predict liver related clinical outcomes and to guide patient management.

Terminology

SVR: Sustained viral response. SVR is specific to hepatitis C and is the absence of HCV RNA for 24 wk after the cessation of treatment.

Peer-review

Current study was from the group who originally described Hepascore as a non-invasive marker of fibrosis in patients with chronic hepatitis C. In the current study, the authors use "baseline" Hepascore as a prognostic indicator. In addition, the authors also found the change in Hepascore over time ("Delta Hepascore") was also a predictor of liver related events or death.

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ORIGINAL ARTICLE

Prospective Study

Is hepatic steatosis associated with left ventricular mass index increase in the general population?

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Author contributions: Piontek K and Schmidt CO contributed equally to this work; Völzke H designed the research; Schmidt CO analysed the data; Piontek K wrote the paper; Baumeister SE, Lerch MM, Mayerle J, Dörr M and Felix SB contributed to writing the paper; all authors read and approved the final manuscript.

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Informed consent statement: All study participants provided informed consent prior to study enrollment.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

Data sharing statement: Statistical code is available from the corresponding author. The data set can be requested from the Institute for Community Medicine, University Medicine Greifswald, 17489 Greifswald, Germany, under consideration of local data protection formalities.

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Abstract

AIM

To investigate the association between hepatic steatosis and change in left ventricular mass index (LVMI) over five years, and examine whether systolic and diastolic blood pressures are mediators of the association between hepatic steatosis and LVMI using a general population sample.

METHODS

We analyzed data from the Study of Health in Pomerania. The study population comprised 1298

individuals aged 45 to 81 years. Hepatic steatosis was defined as the presence of a hyperechogenic pattern of the liver together with elevated serum alanine transferase levels. Left ventricular mass was determined echocardiographically and indexed to height$^{2.7}$. Path analyses were conducted to differentiate direct and indirect paths from hepatic steatosis to LVMI encompassing systolic and diastolic blood pressure as potential mediating variables.

RESULTS

Hepatic steatosis was a significant predictor for all measured echocardiographic characteristics at baseline. Path analyses revealed that the association of hepatic steatosis with LVMI change after five years was negligibly small (β = -0.12, s.e. = 0.21, P = 0.55). Systolic blood pressure at baseline was inversely associated with LVMI change (β = -0.09, s.e. = 0.03, P < 0.01), while no association between diastolic blood pressure at baseline and LVMI change was evident (β = 0.03, s.e. = 0.05, P = 0.56). The effect of the indirect path from hepatic steatosis to LVMI *via* systolic baseline blood pressure was small (β = -0.20, s.e. = 0.10, P = 0.07). No indirect effect was observed for the path *via* diastolic baseline blood pressure (β = 0.03, s.e. = 0.06, P = 0.60). Similar associations were observed in the subgroup of individuals not receiving beta-blockers, calcium channel blockers, or drugs acting on the renin-angiotensin system.

CONCLUSION

Baseline associations between hepatic steatosis and LVMI do not extend to associations with LVMI change after five years. More studies are needed to study the longitudinal effects of hepatic steatosis on LVMI.

Key words: Hepatic steatosis; Left ventricular mass index; Blood pressure; General Population; Study of Health in Pomerania

Core tip: Data regarding the association between hepatic steatosis and left ventricular remodeling are limited and previous studies revealed conflicting results. In the present study, hepatic steatosis as defined by liver hyperechogenicity and increased alanine transferase levels was a significant predictor for all measured echocardiographic characteristics at baseline. In contrast, hepatic steatosis was not a predictor of relevance for left ventricular mass index (LVMI) change. Systolic and diastolic blood pressures did not mediate the association between hepatic steatosis and LVMI.

Piontek K, Schmidt CO, Baumeister SE, Lerch MM, Mayerle J, Dörr M, Felix SB, Völzke H. Is hepatic steatosis associated with left ventricular mass index increase in the general population? *World J Hepatol* 2017; 9(19): 857-866 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i19/857.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i19.857

INTRODUCTION

Hepatic steatosis is highly prevalent in Western countries and regarded as the hepatic manifestation of the metabolic syndrome[1]. Results from previous studies indicate that the metabolic syndrome and its components such as overweight and hypertension are associated with an increase in left ventricular mass (LVM)[2,3]. Data on the association between hepatic steatosis and LVM are limited; only four cross-sectional studies of small sample size exist addressing this relationship. The first study investigated the effect of hepatic steatosis on left ventricular geometry and function in normotensive, nondiabetic patients and demonstrated that patients with hepatic steatosis had mildly altered left ventricular geometry and early signs of left ventricular diastolic dysfunction compared to controls[1]. The second study analyzed the relationship between left ventricular morphology, metabolic parameters and hepatic steatosis in patients with hypertension and revealed that individuals with hepatic steatosis had a similar prevalence of left ventricular hypertrophy (LVH) compared to individuals without hepatic steatosis[4]. The third study using data from hypertensive, diabetic patients revealed that the frequency of LVH was higher in individuals with hepatic steatosis compared to individuals without hepatic steatosis. This study further showed that individuals with hepatic steatosis yielded 6-fold higher odds ratios for LVH than individuals without hepatic steatosis[5]. The fourth study was of case-control design and demonstrated that hepatic steatosis was significantly associated with left ventricular dysfunction in diabetic patients[6]. Due to the design of the aforementioned studies, inferences about effect directions between hepatic steatosis and left ventricular remodelling cannot be made. In particular, there is no differentiation between direct paths from hepatic steatosis to LVM progression or indirect effects *via* mediators. However, the evaluation of potential mediators is important for a better understanding of the mechanisms underlying a putative association between hepatic steatosis and LVM. We hypothesize that blood pressure is a potential key mediator on the path from hepatic steatosis to LVM as LVH is known to be the major cardiac sequel of hypertension[7,8]. Thus, blood pressure should be adequately considered in studies aimed to investigate the association between hepatic steatosis and LVM.

To our knowledge, there is no previous research providing data on the association between hepatic steatosis and left ventricular mass index (LVMI) encompassing the following criteria: (1) using a general population sample; (2) using longitudinal data to improve inferences on the direction of effects; and (3)

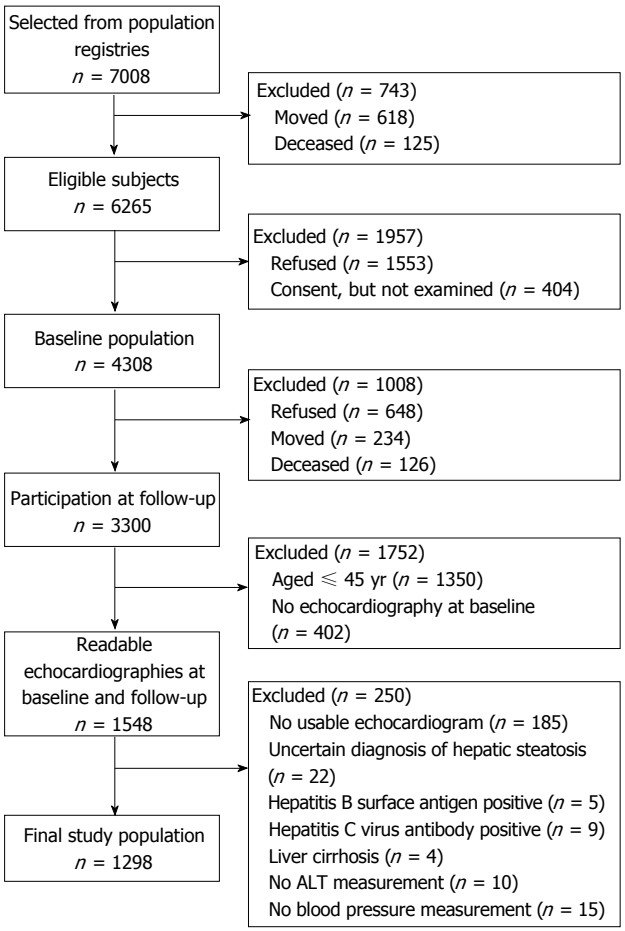

Figure 1 Flow-chart according to sample recruitment. ALT: Alanine transferase.

using methods to differentiate between direct and indirect pathways of hepatic steatosis on LVMI *via* blood pressure. The two major aims of the present study were, first, to investigate the association between hepatic steatosis and LVMI in a general population sample with prospective 5-year follow-up examination and, second, to analyze the mediating role of systolic and diastolic blood pressure on the pathway from hepatic steatosis to LVMI.

MATERIALS AND METHODS

Setting and study population

The Study of Health in Pomerania (SHIP) is a population-based cohort study conducted in West Pomerania, the northeastern area of Germany[9]. The sample recruitment procedure is displayed in Figure 1. At baseline, a sample of 7008 individuals aged 20 to 79 years was drawn from population registries. Only individuals with German citizenship and main residency in the study area were included. The net sample (without migrated or deceased persons) comprised 6265 eligible individuals. Each individual received a maximum of three postal invitation letters. In case of non-response, letters were followed by a phone call or by home visits. The SHIP population finally comprised 4308 participants (response 68.8%). Baseline examinations were conducted between 1997 and 2001. Between 2002 and 2006, all participants were re-invited for an examination follow-up, in which 3300 individuals (83.5% of eligible persons) took part[10]. Follow-up examinations were conducted on average 5.3 years after baseline (median: 5.0, 25th percentile: 5.0, 75th percentile: 5.3). All participants gave informed written consent. The study protocol was consistent with the principles of the Declaration of Helsinki and approved by the Ethics Committee of the University of Greifswald. The study was monitored by a review board of independent scientists.

Among the 3300 participants with follow-up data, only those aged 45 years and older underwent echocardiographic examination at baseline (n = 1950). Of these, 1548 participants received a second echocardiography at follow-up. Readable echocardiograms from both examinations were available for 1538 individuals. Among these, 185 echocardiograms were not evaluable, 22 individuals had an uncertain diagnosis of hepatic steatosis, five were tested positive for hepatitis B surface antigen, nine were tested positive for anti-hepatitis C virus antibody, and four had a self-reported history of liver cirrhosis. Furthermore, ten participants had missing data on serum alanine transferase (ALT), and 15 participants lacked blood pressure measurements. Exclusion of these participants resulted in a final study population of 1298 individuals for the present analyses.

Measurements

Baseline assessments included data on demographics, behavioural risk factors, the individual's medical history and medication as well as data from somatometric, sonographic, echocardiographic and laboratory examinations.

Data on demographics, behavioral risk factors such as physical activity, alcohol consumption, and smoking status were collected using computer-assisted personal interviews. The following demographic variables were assessed: Gender, age and school educational attainment (in years of schooling completed). Individuals who participated in physical training during summer or winter for at least one hour a week were classified as being physically active. Alcohol consumption was assessed using a beverage-specific quantity-frequency measure: Number of days with alcohol consumption (beer, wine, spirits), and the quantity of alcohol consumed on such a day over the last month. Average daily consumption (in grams of pure ethanol) was calculated by multiplying frequency and amount, using beverage specific standard ethanol contents[11]. According to smoking habits, individuals were categorized into current, former, and never-smokers. Data on diabetes mellitus were obtained by self-reported physician's diagnosis of the disease.

The somatometric measures included body weight and height as well as waist circumference (WC). Height

and weight were measured for the calculation of the body mass index [BMI, weight (kg)/height^2 (m^2)]. WC was measured to the nearest 0.1 cm using an inelastic tape midway between the lower rib margin and the iliac crest in the horizontal plane, with the subject standing comfortably with weight distributed evenly on both feet.

Systolic and diastolic blood pressure were measured between 8 am and 7 pm three times after an initial five minute rest period at the right arm of seated individuals using a digital blood pressure monitor (HEM-705CP, Omron Corporation, Tokyo, Japan). Each reading was followed by a further rest period of three minutes. One of two differently sized cuffs was applied according to the circumference of the participant's arm. The mean of the second and third measurement was calculated and used for the present analyses. Pulse pressure was defined as the difference between mean systolic and diastolic pressures. Hypertension was defined as systolic blood pressure $\geqslant$ 140 mmHg or diastolic blood pressure $\geqslant$ 90 mmHg or use of antihypertensive medication.

For the laboratory examinations, non-fasting blood samples were drawn from the cubital vein in the supine position. The laboratory takes part quarterly in the official national German external proficiency testing programs. In addition, internal quality controls were analyzed daily. Hepatitis B surface antigen and anti-hepatitis C virus antibodies were determined by enzyme-linked immunosorbent assays (AxSym HBSAG and AxSym HCV, Abbot, Abbot Park, IL, United States). Serum ALT levels were measured photometrically (Hitachi 704; Roche, Mannheim, Germany) and expressed as μmol/L × s, which corresponds to (μmol/L × s) × 60 = IU/L.

Sonographic examinations were performed by physicians using a 5 MHz transducer and a high resolution instrument (Vingmed VST Gateway, Santa Clara, CA, United States). The sonographers were unaware of the participants' clinical and laboratory characteristics. In SHIP, ultrasound examinations and readings underlie strict quality standards[12]. Hepatic steatosis was defined as the presence of a hyperechogenic liver pattern, with evident density differences between hepatic and renal parenchyma[13-15] together with increased serum ALT levels (> 75th percentile)[16].

Two-dimensional and M-mode echocardiography was performed by trained physicians using a Vingmed CFM 800A system (GE Medical Systems, Waukesha, WI, United States). All data and measurements were stored digitally. M-mode images of the left ventricle were recorded at the papillary level. Left ventricular dimensions [interventricular septum thickness (IVS), posterior wall thickness (LVPW), and left ventricular end-diastolic diameter (LVDD)] were measured off-line using the leading edge convention. LVMI was calculated as follows: LVMI = 0.80 × {1.04 × [(LVDD + IVS + LVPW)^3 − LVDD^3]} + 0.60/height$^{2.7}$[17,18]. LVH was defined as a LVMI of > 48 g/m$^{2.7}$ in men and > 44 g/m$^{2.7}$ in women[19]. Comparisons of intra-reader, intra-observer, inter-reader, and inter-observer LVMI measurements revealed Spearman coefficients of > 0.85 and differences in mean (± 2 SD) of < 5% (< 25%).

Statistical analysis

The study population was divided into two groups based on the presence or absence of liver hyperechogenicity and increased ALT levels at baseline: Category 1 comprised individuals without hyperechogenic liver pattern and without increased serum ALT levels and individuals fulfilling only one of the named criteria. Category 2 comprised individuals with hepatic steatosis as defined by both liver hyperechogenicity and increased serum ALT levels.

Using analyses of variance and χ^2-statistics, differences in baseline characteristics between individuals with and without hepatic steatosis regarding demographics, behavioural risk factors, and clinical characteristics were analyzed. Changes in echocardiographic parameters and blood pressure are depicted using absolute numbers and percentages. Bivariate correlations were calculated based on Pearson correlation coefficients.

We conducted path analyses to evaluate direct effects of hepatic steatosis on LVMI and the indirect effects *via* systolic and diastolic blood pressure. Standardized regression coefficients for systolic and diastolic blood pressure as well as LVMI are presented in the figures. The χ^2-value, comparative fit index (CFI), and the root mean square error (RMSEA) are provided as indicators of model fit. CFI is an incremental fit index comparing the fit of the model of interest with the independence model with values ranging from zero to one. RMSEA is a descriptive approximate estimation of the overall fit of the model in the population. Values have a lower bound of zero. A CFI > 0.96 and a RMSEA < 0.05 are commonly regarded as indicative of a satisfactory model fit[20,21]. Parameter estimates were obtained based on a robust weighted least square approach (WLSMV), which is suitable to handle categorical and non-normal data[21]. Age and sex were considered as independent predictors for all variables in the models. In addition, baseline body weight was included. LVMI was not regressed on body weight since body weight is part of the calculation of LVMI. The time of the day of blood pressure measurement was controlled for all indicators of blood pressure.

To evaluate possible bias due to missing data, we applied statistical inverse probability weights accounting for known individual characteristics of the study participants related to missing data on the echocardiographic examination at follow-up. These inverse probability weights were derived from logistic regression analyses with age, sex, body weight, waist circumference, alcohol intake, smoking, and a summative comorbidity index as predictors.

We repeated our analyses in the subgroup of indivi-

Table 1 General and echocardiographic characteristics of the study population with and without hepatic steatosis at baseline *n* (%)

	No/one criterion for hepatic steatosis *n* = 1106	US$^+$ and ALT$^+$ *n* = 192	*P*-value
Age (yr), M (SD)	59.6 (8.8)	57.2 (7.8)	$P < 0.01$
Male gender	442 (40.0)	139 (72.4)	$P < 0.001$
School education			n.s.
< 10 yr	570 (51.5)	102 (53.1)	
10 yr	358 (32.4)	67 (34.9)	
> 10 yr	178 (16.1)	23 (12.0)	
Waist circumference (cm), M (SD)	89.0 (11.5)	100.8 (10.9)	$P < 0.001$
Body weight (kg), M (SD)	75.6 (12.8)	88.6 (13.9)	$P < 0.001$
BMI, (kg/m^2), M (SD)	27.4 (4.3)	30.5 (4.6)	$P < 0.001$
Smoking			$P < 0.001$
Never-smoker	516 (46.6)	57 (29.7)	
Ex-smoker	382 (34.5)	99 (51.7)	
Current smoker	208 (18.8)	36 (18.8)	
Alcohol consumption (g/d), M (SD)	9.1 (14.5)	15.6 (19.5)	$P < 0.001$
Diabetes mellitus	100 (9.0)	26 (13.5)	$P < 0.001$
Systolic blood pressure (mmHg), M (SD)	139.3 (20.2)	148.5 (17.4)	$P < 0.001$
Diastolic blood pressure (mmHg), M (SD)	84.7 (10.8)	89.9 (10.4)	$P < 0.001$
Pulse pressure (mmHg), M (SD)	54.6 (14.7)	58.6 (13.4)	$P < 0.01$
Hypertension	660 (59.7)	163 (84.9)	$P < 0.001$
Intake of drugs with ATC07	239 (21.6)	39 (20.3)	n.s.
Intake of drugs with ATC08	140 (12.7)	28 (14.6)	n.s.
Intake of drugs with ATC09	198 (17.9)	58 (30.2)	$P < 0.001$
IVS, M (SD)	9.7 (2.2)	10.9 (2.5)	$P < 0.001$
LVEDD, M (SD)	50.9 (5.6)	52.4 (5.9)	$P < 0.01$
PWD, M (SD)	9.6 (1.9)	10.4 (2.0)	$P < 0.001$
LVM (g), M (SD)	181.8 (53.5)	215.8 (61.3)	$P < 0.001$
LVMI (g/m$^{2.7}$), M (SD)	46.2 (13.3)	51.0 (13.7)	$P < 0.001$
LVH	499 (45.1)	114 (59.4)	$P < 0.001$

Pearson χ^2 and ANOVAs were used for bivariate comparisons. Data are given as numbers and percentages or means (standard deviation). US: Ultrasound; ALT: Alanine aminotransferase; ATC: Anatomical-therapeutic code; IVS: Interventricular septum thickness; LVEDD: Left ventricular end-diastolic diameter; PWD: Posterior wall thickness; LVMI: Left ventricular mass index; LVH: Left ventricular hypertrophy; n.s.: Non-significant.

duals not receiving medication with possible influence on LVM [beta-blockers, anatomical-therapeutical (ATC) codes C07; calcium channel blockers, ATC codes C08; and drugs acting on the renin-angiotensin system, ATC codes C09] as sensitivity analysis.

P values were estimated for two-sided tests. A value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using STATA 10.2 (Stata Corporation, College Station, TX, United States) to conduct descriptive statistics. MPLUS 5.1 (Muthén and Muthén, Los Angeles, CA, United States) was used for path analyses. Data analyses were performed by Carsten O Schmidt who is an expert in the field of biomedical statistics.

RESULTS

Sample characteristics

At baseline, 1106 (85.1%) individuals fulfilled no or one criterion for hepatic steatosis, while 192 (14.9%) individuals had hepatic steatosis as defined by the combined presence of hyperechogenic liver pattern and increased serum ALT levels. LVH was present in 48.3% of the study population. The mean LVMI was 49.8 g/m$^{2.7}$ (SD = 14.7). General characteristics of the study population at baseline are presented in Table 1.

Baseline associations

Compared to individuals fulfilling no or one criterion for hepatic steatosis, individuals with hepatic steatosis were more often male, had lower educational attainment, a higher WC, a higher body weight, a higher BMI, were less often never-smokers and reported a higher average daily alcohol consumption. Moreover, individuals with hepatic steatosis reported more often diabetes mellitus, had higher systolic and diastolic blood pressure, higher pulse pressure and were more often hypertensive compared to individuals fulfilling no or one criterion for hepatic steatosis. Individuals with hepatic steatosis reported more often the intake of drugs acting on the renin-angiotensin system compared to the reference group. Regarding echocardiographic characteristics, individuals with hepatic steatosis showed a higher interventricular septum thickness, a higher posterior wall thickness, a higher left ventricular end-diastolic diameter, a higher left ventricular mass, a higher left ventricular mass index and more often left ventricular hypertrophy than the reference group.

Echocardiographic characteristics and blood pressure at baseline and follow-up

There was an increase in echocardiographic parameters from baseline to follow-up with higher values

Table 2 Echocardiographic characteristics and blood pressure at baseline and follow-up in the study population with and without hepatic steatosis

	Baseline M (SD)	Follow-up M (SD)	*P*-value
IVS, M (SD)			
No/one criterion for hepatic steatosis	9.7 (2.2)	11.2 (2.7)	$P < 0.001$
US$^+$ and ALT$^+$	10.9 (2.5)	12.0 (2.9)	$P < 0.001$
LVEDD, M (SD)			
No/one criterion for hepatic steatosis	50.9 (5.6)	48.8 (5.5)	$P < 0.001$
US$^+$ and ALT$^+$	52.4 (5.9)	50.6 (5.3)	$P < 0.001$
PWD, M (SD)			
No/one criterion for hepatic steatosis	9.6 (1.9)	9.9 (1.9)	$P < 0.001$
US$^+$ and ALT$^+$	10.4 (2.0)	10.9 (2.1)	$P < 0.01$
LVM (g), M (SD)			
No/one criterion for hepatic steatosis	181.8 (53.5)	192.2 (56.8)	$P < 0.001$
US$^+$ and ALT$^+$	215.8 (61.3)	226.1 (62.4)	$P < 0.01$
LVMI (g/m$^{2.7}$), M(SD)			
No/one criterion for hepatic steatosis	46.2 (13.3)	49.2 (14.6)	$P < 0.001$
US$^+$ and ALT$^+$	51.0 (13.7)	53.7 (14.4)	$P < 0.01$
SBP (mmHg), M (SD)			
No/one criterion for hepatic steatosis	139.3 (20.2)	136.3 (19.2)	$P < 0.001$
US$^+$ and ALT$^+$	148.5 (17.4)	142.8 (19.0)	$P < 0.001$
DBP (mmHg), M (SD)			
No/one criterion for hepatic steatosis	84.7 (10.8)	81.2 (10.3)	$P < 0.001$
US$^+$ and ALT$^+$	89.9 (10.4)	85.0 (11.1)	$P < 0.001$
LVH			
No/one criterion for hepatic steatosis	499 (45.1)	597 (54.0)	$P < 0.001$
US$^+$ and ALT$^+$	114 (59.4)	128 (66.7)	$P < 0.001$
Hypertension			
No/one criterion for hepatic steatosis	660 (59.7)	686 (62.0)	$P < 0.001$
US$^+$ and ALT$^+$	163 (84.9)	154 (80.2)	$P < 0.001$

IVS: Interventricular septum thickness; LVEDD: Left ventricular end diastolicdiameter; PWD: Posterior wall thickness; LVM: Left ventricular mass; LVMI: Left ventricular mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; LVH: Left ventricular hypertrophy; US: Ultrasound; ALT: Alanine aminotransferase.

in individuals with hepatic steatosis compared to individuals fulfilling no or one criterion (Table 2). Blood pressure decreased from baseline to follow-up in both groups, while the proportion of hypertensive individuals slightly increased in the reference group and decreased in individuals with hepatic steatosis.

Cross-sectional correlations between hepatic steatosis, blood pressure and LVMI

Hepatic steatosis was significantly correlated with all variables in the path models, but effect sizes were small (standardized coefficients ranging from 0.11 to 0.17, Table 3). Baseline measures of LVMI and blood pressure were most closely related to their respective counterparts at follow-up. Systolic blood pressure was consistently more closely associated to LVMI than diastolic blood pressure.

Prediction of LVMI change

Figure 2 depicts the results of path analyses in the whole study population with systolic and diastolic blood pressure as potential mediators. The model fit was very good. Analyses revealed a very small, non-significant direct effect of baseline hepatic steatosis on LVMI change (β = -0.12, s.e. = 0.21, P = 0.55) and a negligible indirect effect *via* diastolic blood pressure (β = 0.03, s.e. = 0.06, P = 0.60, respectively). The moderate indirect effect *via* systolic blood pressure was borderline significant (β = -0.20, s.e. = 0.10, P = 0.07). Systolic blood pressure at baseline was inversely associated with LVMI change (β = -0.09, s.e. = 0.03, $P < 0.01$), while no association between diastolic blood pressure at baseline and LVMI change was evident (β = 0.03, s.e. = 0.05, P = 0.56).

Repeating our analyses after excluding individuals not receiving beta-blockers, calcium channel blockers or drugs acting on the renin-angiotensin system revealed similar results (Figure 3).

We further repeated our analyses after excluding 30 individuals with high risk drinking according to the recommendations of the World Health Organization (consumption levels of 40 g/d in women and > 60 g/d in men). Analyses revealed almost identical results (direct effect of baseline hepatic steatosis on LVMI change: β = -0.13, s.e. = 0.21, P = 0.54).

DISCUSSION

To the best of our knowledge, the present study is the first to investigate the association between hepatic steatosis and change in LVMI and the mediating role of systolic and diastolic blood pressure in this association using data from a prospective population-based cohort. While we observed relevant baseline

Table 3 Bivariate Pearson correlations

	Sex	Age	FLD	$LVMI_{t0}$	$LVMI_{t1}$	SBP_{t0}	SBP_{t1}	DBP_{t0}
Sex								
Age	-0.04							
FLD	-0.23[b]	-0.10[b]						
$LVMI_{t0}$	-0.12[b]	0.30[b]	0.13[b]					
$LVMI_{t1}$	-0.08[b]	0.26[b]	0.11[b]	0.62[b]				
SBP_{t0}	-0.21[b]	0.24[b]	0.16[b]	0.36[b]	0.24[b]			
SBP_{t1}	-0.13[b]	0.18[b]	0.12[b]	0.22[b]	0.22[b]	0.49[b]		
DBP_{t0}	-0.20[b]	-0.14[b]	0.17[b]	0.19[b]	0.11[b]	0.71[b]	0.32[b]	
DBP_{t1}	-0.15[b]	-0.28[b]	0.13[b]	0.04	0.05	0.28[b]	0.65[b]	0.52[b]

[b]$P < 0.01$. FLD: Fatty liver disease; $LVMI_{t0}$: Left ventricular mass index at baseline; $LVMI_{t1}$: Left ventricular mass index at follow-up; SBP_{t0}: Systolic blood pressure at baseline; SBP_{t1}: Systolic blood pressure at follow-up; DBP_{t0}: Diastolic blood pressure at baseline; DBP_{t1}: Diastolic blood pressure at follow-up.

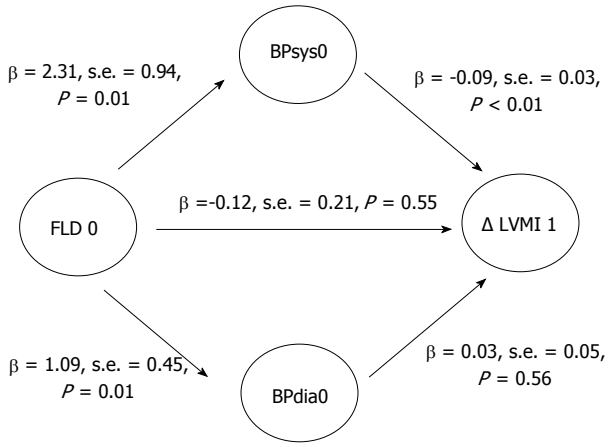

Figure 2 Path model for the effects of hepatic steatosis *via* systolic and diastolic blood pressure on left ventricular mass index in the whole study population (*n* = 1298). $\chi^2 = 3.2$, *df* = 3, *P* = 0.36; RMSEA < 0.01; CFI > 0.99. Indirect Effect *via* $BPsys_0$: β = -0.20; s.e. = 0.10; *P* = 0.07; Indirect Effect *via* $BPdia_0$: β = 0.03; s.e. = 0.06; *P* = 0.60. FLD: Fatty liver disease; LVMI: Left ventricular mass index; BPsys: Systolic blood pressure; BPdia: Diastolic blood pressure; RMSEA: Root mean square error; CFI: Comparative fit index; s.e.: Standard error.

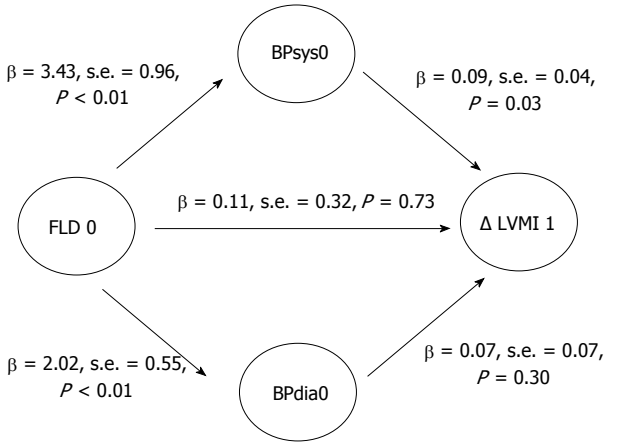

Figure 3 Path model for the effects of hepatic steatosis *via* systolic and diastolic blood pressure on left ventricular mass index in the subgroup of individuals without medication (*n* = 811). $\chi^2 = 1.9$, *df* = 3, *P* = 0.60; RMSEA < 0.01; CFI > 0.99. Indirect Effect *via* BPsys0: β = -0.30; s.e. = 0.17; *P* = 0.07; Indirect Effect *via* BPdia0: β = 0.15; s.e. = 0.14; *P* = 0.30. FLD: Fatty liver disease; LVMI: Left ventricular mass index; BPsys: Systolic blood pressure; BPdia: Diastolic blood pressure; RMSEA: Root mean square error; CFI: Comparative fit index; s.e.: Standard error.

associations between hepatic steatosis, blood pressure and LVMI, these associations were not relevant in the prediction of LVMI change. Our analyses suggest that hepatic steatosis is no predictor of relevance for LVMI change over time.

Previously, only four studies addressed the association between hepatic steatosis and left ventricular morphology[1,4-6]. These studies were of cross-sectional design, used data from small and inhomogeneous samples of patients and yielded conflicting results. The findings of the present study are in good agreement with results from the case-control study by Goland *et al*[1] demonstrating normotensive patients with hepatic steatosis to have larger intraventricular septum and posterior wall thickness and larger LVM than controls. In our study, LVM at baseline was 181.8 g in individuals fulfilling no or one criterion for hepatic steatosis and 215.8 g in individuals with hepatic steatosis. LVH was present in 45.1% of the individuals fulfilling no or only one criterion for hepatic steatosis and in 59.4% of the individuals with hepatic steatosis. Larger differences were found in the study by Mantovani *et al*[5] analyzing data from hypertensive, diabetic patients with hepatic steatosis. In that study, 82% of the patients with hepatic steatosis had LVH, while the proportion was 18% in patients without hepatic steatosis. Furthermore, patients with hepatic steatosis yielded 6-fold higher odds ratios for LVH compared to patients without hepatic steatosis. In contrast to the cross-sectional findings of our study, Bonapace *et al*[6] demonstrated no significant differences between patients with hepatic steatosis and patients without hepatic steatosis regarding left ventricular mass. Fallo *et al*[4] reported a comparable prevalence of LVH in patients with and without hepatic steatosis. However, that study was performed in hypertensive inpatients, in which a high prevalence of both FLD and LVH has been reported[4,22,23]. Therefore, the reported results cannot be directly compared with results from a general population sample.

Regarding longitudinal associations, we only found negligible direct effects of baseline hepatic steatosis

on LVMI change. We hypothesized that blood pressure is a mediating factor involved in the pathway from hepatic steatosis to LVMI as blood pressure has been found to be a major risk factor for left ventricular remodelling[24,25]. Yet, we failed to demonstrate indirect effects from hepatic steatosis on LVMI change *via* systolic and diastolic blood pressure. Interestingly, we observed an inverse association between systolic blood pressure at baseline and change in LVMI after five years. This finding is in contrast to previous studies revealing that both systolic and diastolic blood pressure are important correlates of LVM, whereas systolic blood pressure has been found to be more closely related to LVM than diastolic blood pressure[26]. Our data showed a drop in systolic and diastolic blood pressure from baseline to follow-up in the study sample, whereas this drop was more pronounced in individuals with hepatic steatosis than in individuals fulfilling no or one criterion for hepatic steatosis. We suppose that information on high blood pressure given by study physicians after baseline examination may have led to lifestyle modification or a rise in health consciousness in the study participants including the intake of blood pressure-lowering medication resulting in lower blood pressure at follow-up examination.

Regarding pharmacological interventions, treatment with antihypertensive drugs is indicated in the management of patients with cardiac hypertrophy, whereas the validity of data regarding the effects of antihypertensive medication on LVH regression is limited due to methodological weaknesses of existing studies[27]. Drugs acting on the renin-angiotensin system, beta blockers, and calcium channel blockers have been shown to diminish left ventricular mass with different efficacy[28]. In the present study population, 20.3% of the individuals with hepatic steatosis reported the intake of beta blockers, 14.6% the intake of calcium channel blockers and 20.3% the intake of drugs acting on the renin-angiotensin system. In addition to blood pressure lowering effects, these drugs may lead to LVMI regression[29]. It might be assumed that the observed decrease in blood pressure in the present sample was attended by LVMI regression covering a potentially present association between hepatic steatosis and LVMI. Repeating our analyses after excluding individuals taking beta blockers, calcium channel blockers, and drugs acting on the renin-angiotensin system confirmed our results in general. This finding indicates that the use of the respective medication did not have an influence on the association between hepatic steatosis and LVMI in the entire population as these drugs may prevent further increase of LVM or support regression of LVH[30,31].

Besides pharmacological treatment, lifestyle modification including weight loss and a reduction of alcohol and salt intake may contribute to LVH regression[29]. The role of physical activity remains controversial. It has been demonstrated that regular physical activity is associated with lower blood pressure and reduced cardiac remodeling, while exercise can also lead to the development of LVH[32]. In hypertensive individuals, exercise may have a positive effect on cardiac remodelling with regression or prevention of LVH[32].

With respect to alcohol consumption, analyses after excluding participants with high risk drinking did not change the results of our study. We therefore assume that alcohol consumption had no major role in the association between hepatic steatosis and LVMI. However, it needs to be considered that the number of individuals with high risk drinking was low and drinking above recommended levels is a risk factor for both hepatic steatosis and changes in cardiac structure.

In the present general population sample, both hepatic steatosis and LVH were highly prevalent stressing the public health relevance of these disease conditions in the general population.

Our study has several strengths, but also potential limitations that should be considered. Major strengths encompass the population-based longitudinal design, the large sample size and the high prevalence of hepatic steatosis and LVH in the study region[13,33]. Further strengths encompass the ultrasound and laboratory methods to detect hepatic steatosis and the strict quality management by standardized protocols and certified staff[9]. Limitations may arise from the inability to perform liver biopsy due to ethical concerns although known as the gold standard in the diagnosis of hepatic steatosis. Regarding methodological issues, path analyses allow for a useful differentiation of direct and indirect effects and therefore improve the interpretation of relationships among multiple variables. Limitations comprise potential selection bias due to selective drop out and initial non-response. However, previous analyses do not suggest a major effect on the outcomes under study[10,34]. More measurement points covering a larger time interval might be needed to improve our inferences on direct and indirect effects. Limitations may further arise from the inability to perform liver biopsy due to ethical concerns although known as the gold standard in the diagnosis of hepatic steatosis.

We conclude that hepatic steatosis as defined by liver hyperechogenity and increased ALT levels was not a predictor of relevance for LVMI change after five years in the present population-based cohort of individuals aged 45 to 81 years. Nevertheless, both hepatic steatosis and LVH were highly prevalent in the present indicating the importance of both disease conditions in the general population and the necessity for risk factor reduction to avoid subsequent morbidity and mortality.

COMMENTS

Background

Hepatic steatosis is highly prevalent in Western countries and regarded as the hepatic manifestation of the metabolic syndrome. The metabolic syndrome and its components such as overweight and hypertension are associated with

an increase in left ventricular mass (LVM). Data on the association between hepatic steatosis and LVM are limited; only four cross-sectional studies of small sample size exist addressing this relationship. Due to the design of the aforementioned studies, inferences about effect directions between hepatic steatosis and left ventricular remodelling cannot be made. In particular, there is no differentiation between direct paths from hepatic steatosis to LVM progression or indirect effects *via* mediators.

Research frontiers

There is no previous research providing data on the association between hepatic steatosis and left ventricular mass index (LVMI) encompassing the following criteria: (1) using a general population sample; (2) using longitudinal data to improve inferences on the direction of effects; and (3) using methods to differentiate between direct and indirect pathways of hepatic steatosis on LVMI *via* blood pressure.

Innovations and breakthroughs

The present study is the first to investigate the association between hepatic steatosis and change in LVMI and the mediating role of systolic and diastolic blood pressure in this association using data from a prospective population-based cohort.

Applications

The authors conclude that hepatic steatosis as defined by liver hyperechogenity and increased ALT levels was not a predictor of relevance for LVMI change after five years in the present population-based cohort of individuals aged 45 to 81 years. Nevertheless, both hepatic steatosis and LVH were highly prevalent in the present study population indicating the importance of both disease conditions in the general population and the necessity for risk factor reduction to avoid subsequent morbidity and mortality.

Peer-review

This is an interesting and well-written manuscript. This study investigated the association between hepatic steatosis and change in LVMI over 5 years in a study population of 1298 individuals aged 45 to 81 years. Hepatic steatosis was demonstrated to be a significant predictor for all measured echocardiographic characteristics at baseline but not for LVMI change.

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World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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REVIEW

Value of quality of life analysis in liver cancer: A clinician's perspective

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Abstract

Health related quality of life (HRQOL) is increasingly recognized as an important clinical parameter and research endpoint in patients with hepatocellular carcinoma (HCC). HRQOL in HCC patients is multifaceted and affected by medical factor which encompasses HCC and its complications, oncological and palliative treatment for HCC, underlying liver disease, as well as the psychological, social or spiritual reaction to the disease. Many patients presented late with advanced disease and limited survival, plagued with multiple symptoms, rendering QOL a very important aspect in their general well being. Various instruments have been developed and validated to measure and report HRQOL in HCC patients, these included general HRQOL instruments, *e.g.*, Short form (SF)-36, SF-12, EuroQoL-5D, World Health Organization Quality of Life Assessment 100 (WHOQOL-100), World Health Organization Quality of Life Assessment abbreviated version; general cancer HRQOL instruments, *e.g.*, the European Organisation for Research and Treatment of Cancer (EORTC) QLQ-C30, Functional Assessment of Cancer Therapy (FACT)-General, Spitzer Quality of Life Index; and liver-cancer specific HRQOL instruments, *e.g.*, EORTC QLQ-HCC18, FACT-Hepatobiliary (FACT-Hep), FACT-Hep Symptom Index, Trial Outcome Index. Important utilization of HRQOL in HCC patients included description of symptomatology and HRQOL of patients, treatment endpoint in clinical trial, prognostication of survival, benchmarking of palliative care service and health care valuation. In this review, difficulties regarding the use of HRQOL data in research and clinical practice, including choosing a suitable instrument, problems of missing data, data interpretation, analysis and presentation are examined. Potential solutions are also discussed.

Key words: Hepatocellular carcinoma; Health related quality of life; Palliative care; Prognosis; Survival; The European Organisation for Research and Treatment of Cancer QLQ-C30; QLQ-HCC18; Index score; Functional Assessment of Cancer Therapy; EQ-5D; Spitzer; Short form 36; FHSI-8; World Health Organization Quality of Life Assessment

Core tip: Health related quality of life (HRQOL) is an important clinical parameter and research endpoint in hepatocellular carcinoma (HCC) patients. Instruments discussed are short form (SF)-36, SF-12, EQ-5D, World Health Organization Quality of Life Assessment (WHOQOL) 100, WHOQOL-BREF, the European Organisation for Research and Treatment of Cancer (EORTC) QLQ-C30, Functional Assessment of Cancer Therapy (FACT)-G, Spitzer QoL index, EORTC QLQ-HCC18, FACT-Hep, FHSI-8, TOI. Important utilization of HRQOL included measurement and monitoring of HRQOL, treatment endpoint in clinical trial, prognostication of survival, benchmarking of palliative care service and health care valuation. Various difficulties in using HRQOL data in research and clinical practice, including choosing a suitable instrument, missing data, data interpretation, analysis and presentation are explained. Potential solutions are also discussed.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common and aggressive cancer that arises usually in a cirrhotic liver. Etiological pattern differs between Caucasians (mostly alcoholic liver disease and hepatitis C viral infection) and Asians (predominantly chronic hepatitis B)[1,2]. HCC carries high morbidity and mortality, since many patients present only when symptomatic. Patients with early disease are typically asymptomatic and their diseases are usually detected by regular HCC screening or incidental finding during investigation for other diseases[3]. Advanced disease at presentation is common and patients suffer from symptoms resulting from large space occupying lesion(s) in the liver or associated hepatic dysfunction/failure.

Early diseases are potentially curable by complete surgical extirpation[4,5]. Local tumor ablation, for example radiofrequency ablation (RFA), is a reasonable alternative to partial hepatectomy for small HCC[6,7]. Liver transplantation is considered if the disease falls within the Milan criteria but the anticipated residual liver function is not adequate[8]. Liver directed therapies, such as transarterial chemoembolisation (TACE) and selective internal radiation therapy (SIRT), are palliative treatment for patients with higher tumor burden that is confined to the liver[9-11]. For patients with advanced disease palliative treatment with systemic targeted agents, namely sorafenib and regorafenib, were demonstrated to improve their overall survival (OS)[12-14]. However, in the two phase Ⅲ trials of first-line sorafenib in advanced HCC patients, the improvement in median OS was modest at 2-3 mo[12,13] when compared to placebo. Similar magnitude of benefit was observed in the second-line setting using regorafenib when compared to placebo[14].

In most clinical trials on patients with advanced HCC, the endpoints of interest are disease-free survival (DFS), progression-free survival (PFS) and OS. However in this poor prognostic group, treatment is mainly palliative and the survival benefit is modest. Hence, apart from survival improvement, health related quality of life (HRQOL) becomes very relevant. Thus, increasing number of phase Ⅲ HCC trials have adopted QOL as additional study endpoints. HRQOL therefore has become an important monitoring parameter and treatment goal in clinical research and practice.

HRQOL in HCC patients is a complicated and multidimensional issue that involves medical, psychological, social and spiritual factors. Apart from symptoms arising from HCC and its complications, underlying liver disease and oncological treatment are intertwined with other factors including palliative care service, social and spiritual support, individual's coping skill, patients' function and general well being as well as cultural background, educational level and health literacy.

Therefore HRQOL intrinsically is a multifaceted and complex assessment of human life. Assessment of HRQOL should be comprehensive. Various instruments have been developed to measure and report HRQOL in these patients, they also serve as a means to communicate and reflect on patient's overall well being.

HRQOL INSTRUMENTS UTILIZED TO ASSESS HCC PATIENTS

HRQOL assessment using general tools

HRQOL in HCC patients could be measured using general cancer QOL instruments, *e.g.*, the European Organization for Research and Treatment of Cancer QLQ-C30[15], Functional Assessment of Cancer Therapy - General[16], Spitzer Quality of Life Index[17]; as well as general disease QOL instruments, *e.g.*, Short Form 36[18], short form (SF) 12[19], World Health Organization Quality of Life Assessment 100[20], World Health Organization Quality of Life Assessment abbreviated version[21], EuroQoL-5D[22,23]. These are described in Table 1.

HRQOL assessment using liver-cancer specific tools

Since HCC patients commonly have symptoms related to concomitant underlying liver disease in addition to the tumor(s) within the liver, liver-cancer specific HRQOL instruments have been developed to address symptoms in relation to the malignancy as well as chronic liver disease. These include the European Organization for Research and Treatment of Cancer QLQ-HCC18[24], Functional Assessment of Cancer Therapy-Hepatobiliary[25], Functional Assessment of

Table 1 Health related quality of life instruments commonly used in hepatocellular carcinoma studies

General instruments	
European Organization for Research and Treatment of Cancer QLQ-C30	EORTC QLQ-C30 is a general cancer instrument containing multiple items, measured in multiple-point Likert scales, that reflect the multidimensionality of HRQOL construct[15]. It includes five functional domains (physical, role, cognitive, emotional and social), three symptom domains (fatigue, pain, nausea/vomiting), and a global health and QOL domain. Six single items assess common symptoms in cancer patients (dyspnea, appetite loss, sleep disturbance, constipation and diarrhea) and financial problem. All scales and domains are transformed to scores ranging from 0 to 100. A lower score for a functional or global QOL scale reflects a relatively poorer functioning level or global QOL, a higher score for a symptom/problem scale reflects a more disturbing symptom/problem
Functional Assessment of Cancer Therapy - General	The FACT-G questionnaire is a commonly used tool for HRQOL assessment in general cancer patients[16]. It consists of 27 items for assessment of symptoms and four domains of HRQOL: (1) physical well being (PWB) containing seven items with a subscale score ranging from 0 to 28 points; (2) socio-family well being (SFWB) containing seven items with a subscale score of 0-28 points; (3) emotional well being (EWB) containing six items with a subscale score of 0-24 points; and (4) functional well being (FWB) containing seven items with a subscale score of 0-28 points. Patients were asked to score each item according to how true each statement was to them during the past week on a 5-point ordinal scale, from 0 indicating "not at all" to 4 indicating "very much". The FACT-G total score is the summation of the four subscales (PWB, FWB, SFWB and EWB) scores and can range from 0 to 108. Higher scores reflect better HRQOL
Spitzer Quality of Life Index (Spitzer QoL index)	Spitzer QoL index is a general cancer HRQOL measurement[17]. A score of 0 (worst QOL) to 10 (best QoL) was calculated after the patient answered five items of the questionnaire in the areas of activity, daily life, health perceptions, social support and behavior. Each item is rated on a 3-point Likert scale
Short form 36	SF-36 is a general disease questionnaire to measure the following 8 domains of health: General health, bodily pain, social functioning, role-physical, physical functioning, vitality, role-emotional and mental health[18]. The raw scores of each subscale are converted to scores that range from 0 to 100, with higher scores indicating higher levels of functioning or well being. Scores representing overall physical functioning and mental functioning were calculated from the subscales and are grouped as the physical component summary scale and mental component summary scale
Short form 12	SF-12 is a shortened version of SF-36. It contains a 12-item generic measure of health status developed from SF-36[19]. It also yields scores for eight domains: Physical functioning, role physical, bodily pain, general health, vitality, social functioning, role emotional, and mental health. It likewise provides overall summaries of the physical and mental components
World Health Organization Quality of Life Assessment 100	The WHOQOL-100 questionnaire comprises of 100 items grouped into 25 facets[20]. One of the facets measures overall quality of life/health. The remaining 24 facets are organized in 6 domains: (1) physical health; (2) psychological health; (3) level of independence; (4) social relationships; (5) environment; and (6) spirituality/religion/personal beliefs. Each facet includes four items, rated on a 5-point Likert scale, with higher scores indicating more positive evaluations. Domain and facet raw scores can also be transformed onto a 0 to 100 scale. Higher scores denote higher HRQOL
World Health Organization Quality of Life Assessment abbreviated version	The original 6-domain structure of WHOQOL-100 was subsequently reduced into 4 comprehensive domains by the WHOQOL Group, comprising: (1) physical health (merging the level of independence domain); (2) psychological health (merging the spirituality/religion/personal beliefs domain); (3) social relationships; and (4) environment[21]. It contains a total of 26 questions. Attributes incorporated within the physical health domain of the WHOQOL-BREF include: activities of daily living, dependence on medicines or medical aids, energy and fatigue, mobility, pain and discomfort, sleep and rest and work capacity. Attributes incorporated within the psychological health domain are: body image and appearance, negative and positive feelings, self-esteem, spirituality, religion and personal beliefs, thinking, learning, memory and concentration. Measurements of social health domain include personal relationships, social support and sexual activity. Features incorporated in the environmental health domain are: Financial resources, freedom, physical safety and security, health and social care, home environment, opportunities for acquiring the new information and skills, participation in and opportunities for recreation, physical environment and transportation. Higher scores denote higher HRQOL
EuroQoL-5D	EQ-5D is a general disease instrument for describing and valuing HRQOL developed by the EuroQoL Group[22,23]. The questionnaire consists of 2 sections: The EQ-5D descriptive system and the EQ visual analogue scale (EQ VAS). The descriptive system contains one question in each of the 5 dimensions (mobility, self-care, usual activities, pain/discomfort and anxiety/depression). In the 3-point Likert version (EQ-5D-3L), each question has three levels of response: No problems, some problems or extreme problems. A specific value (weight) is attached to each response of each question according to that country's specific value sets. Studies have been conducted to elicit preferences from general population samples to derive these value sets. A summary score is calculated by deducting all values of the 5 responses from the full mark of 1. A summary score of 1 represents perfect health, 0 represents death, below 0 represents a state being worse than dead. This summary score could be used for quality adjusted life-year (QALY) calculations. Thus EQ-5D is an important tool for economic valuation. The EQ VAS lets the respondent place an "x" on a vertical VAS to reflect his/her self rated health. The endpoints are labeled "best imaginable health state" at 100 and "worst imaginable health state" at 0

Liver-cancer specific instruments	
European Organization for Research and Treatment of Cancer QLQ-HCC18	EORTC QLQ-HCC18 includes eighteen multiple item scales organized into six domains (fatigue, body image, jaundice, nutrition, pain and fever) and two items (abdominal swelling and sex life)[24]. All scales are grouped and transformed to score ranging from 0 to 100. A lower score represents a less severe symptom/problem. EORTC QLQ-HCC18 is used together with EORTC QLQ-C30
Functional Assessment of Cancer Therapy-Hepatobiliary	The FACT-Hep questionnaire is a 45-item instrument for measuring HRQOL in patients with hepatobiliary cancers (liver, bile duct and pancreas)[25]. FACT-Hep is used together with FACT-G. It consists of the 27 items (PWB, FWB, SFWB and EWB domains) in FACT-G together with an 18-item disease-specific hepatobiliary cancer subscale (HepCS) which address specific symptoms of hepatobiliary carcinoma, such as back/stomach pain, gastrointestinal symptoms, anorexia, weight loss, jaundice, as well as side-effects of treatment. An aggregate HepCS score could be obtained. The FACT-G and HepCS scores are summed to form the FACT-Hep total score. Higher scores on all scales of the FACT-Hep reflect better HRQOL or fewer symptoms
Functional Assessment of Cancer Therapy-Hepatobiliary Symptom Index	FHSI-8 is a subset of FACT-Hep. It includes eight items from the FACT-Hep that measure specific symptoms of patient priority concern and side effects of hepatobiliary carcinoma[26]. Higher scores on all items of the FHSI-8 reflect fewer symptoms
Trial Outcome Index	TOI is also a subset of FACT-Hep. It consists of the summation of the PWB, FWB and HepCS subscales[25]. Higher scores reflect better HRQOL and fewer symptoms

EORTC QLQ-C30: European Organization for Research and Treatment of Cancer QLQ-C30; FACT-G: Functional Assessment of Cancer Therapy - General; QoL: Quality of Life; SF-36: Short form 36; SF-12: Short form 12; WHOQOL-100: World Health Organization Quality of Life Assessment 100; FACT-Hep: Functional Assessment of Cancer Therapy-Hepatobiliary; FHSI-8: Functional Assessment of Cancer Therapy - Hepatobiliary Symptom Index; TOI: Trial Outcome Index; HCC: Hepatocellular carcinoma; HRQOL: Health related quality of life.

Cancer Therapy-Hepatobiliary Symptom Index[26] and Trial Outcome Index[25]. Liver specific tools are used together with their general counterparts. See Table 1 for description of each instrument.

Validation of HRQOL instruments

All the above instruments were validated, many were widely validated in patients of different languages and cultural backgrounds[15-17,19-21,25-30].

Validation of an HRQOL instrument encompasses reliability and validity analyses. Internal consistency reliability determines if there is satisfactory correlation between items within the same multi-item scale. Test-retest reliability assesses if there is good correlation between measurements of the same patient at 2 closely separated time points when major QOL discrepancy is not expected. Convergent validity tests for adequate correlation between conceptually related scales within the same instrument or a different validated instrument. Discriminant validity evaluates the ability to differentiate between patients of different clinical statuses. Responsiveness to change looks for significant change in score corresponding to patient's improvement or deterioration in condition with time. Good convergence and discrimination are required for scaling success to support the hypothesized scale structure. These are the essential statistical analyses to validate QOL instruments.

UTILIZATION OF HRQOL INSTRUMENTS

HRQOL assessments have been conducted in HCC patients in different settings, and these are listed in Table 2.

To describe symptomatology and HRQOL of HCC patients

Baseline QOL at HCC diagnosis: HRQOL instruments were frequently used in HCC studies to assess baseline symptomatology and QOL of patients at presentation (Table 2). For instance, a case-control study compared baseline HRQOL of HCC patients at diagnosis with that of normal population[31]. HCC patients had significantly worse physical domain QOL but better environmental QOL of WHOQOL-BREF compared to healthy controls. Another case-control study reported bodily pain, role limitation-physical and physical component summary of SF-36 were significantly worse in HCC patients compared to matched cirrhotic control[32]. Similarly, another report found significantly worse physical, functional, emotional, social-family well-being and overall QOL of FACT-Hep in HCC patients when compared to general population; it also found significantly worse functional well-being and overall QOL in HCC patients when compared to controls with chronic liver disease[33].

Observational studies with QOL assessment during treatment: Many case series on HCC patients underdoing surgical resection, liver transplantation, local ablation, SIRT or transarterial chemoembolisation (TACE) for HCC also reported patients' QOL.

HCC patients after curative intent treatment, for example partial hepatectomy, typically had transient deterioration in QOL followed by improvement of QOL. For long term survivors, their QOL could be comparable to that of control cirrhotic patients but worse than that of general population[34-37]. Patients with recurrent disease after curative treatment had deterioration in QOL[34].

In a prospective cohort study, 388 patients with solitary HCC of $\leqslant$ 3 cm were treated with either surgical resection or percutaneous RFA, there was no difference in DFS or OS between the 2 groups. However, FACT-Hep total scores at 3, 6, 12, 24, 36 mo post treatment were significantly better in percutaneous RFA group compared to resection group[38].

Table 2 Clinical studies in hepatocellular carcinoma that involved health related quality of life assessment

Ref.	Year	Study type	*n*	HCC status	Intervention(s)	HRQOL instruments used	HRQOL assessment time point(s)	Remarks
Poon *et al*[34]	2001	Cohort	76	Resectable and unresectable	Resection (66) *vs* TACE (10)	FACT-G	Baseline, 3, 6, 7, 12, 18 and 24 mo	Observational study with QOL assessment during treatment
Brans *et al*[40]	2002	Cohort	26	Unresectable	SIRT (14) *vs* TACE (14)	EORTC QLQ-C30	Baseline, 1 and 3 mo	Observational study with QOL assessment during treatment
Bianchi *et al*[32]	2002	Case-control	101	Any stage	NA	SF-36	Baseline	To describe symptomatology and/or HRQOL of HCC patients -HRQOL of HCC patients compared to 202 matched cirrhotic patients
Chow *et al*[59]	2002	Phase Ⅲ trial	329	Unresectable	Tamoxifen 120 mg/d (121) *vs* tamoxifen 60 mg/d (76) *vs* placebo (132)	Global QOL domain of EORTC QLQ-C30	Baseline, then every 1 mo	Phase III trial with HRQOL endpoint
Steel *et al*[46]	2004	Cohort	28	Allocated to SIRT or TACE	SIRT (14) *vs* TACE (14)	FACT-Hep, HepCS, TOI, FHSI8	Baseline, 3, 6 and 12 mo	Observational study with QOL assessment during treatment. Included in[97]
Poon *et al*[47]	2004	Randomized phase Ⅱ trial	88	Allocated to TACE	Branched chained amino acid *vs* control	FACT-G	Baseline, 3, 6, 9 and 12 mo	Phase Ⅱ trial with HRQOL endpoint
Steel *et al*[84]	2005	Cohort	82	Any stage	Various treatments	FACT-Hep, HepCS, TOI, FHSI8	Baseline, 3 and 6 mo	To describe symptomatology and/or HRQOL of HCC patients -Compared HRQOL between patients and proxy-raters. Included in[97]
Steel *et al*[96]	2005	Case-control	21	TNM stage Ⅲ or Ⅳ	NA	FACT-Hep, Sexual History Questionnaire	Baseline	To describe symptomatology and/or HRQOL of HCC patients - Included 23 patients with chronic liver disease
Barbare *et al*[58]	2005	Phase Ⅲ trial	420	Not eligible for resection or local treatment	Tamoxifen (210) *vs* control (210)	Spitzer QoL index	Baseline, then every 3 mo	Phase Ⅲ trial with HRQOL endpoint
Kirchhoff *et al*[48]	2005	Randomized phase Ⅱ trial	70	Eligible for TACE	TACE with microspheres (35) *vs* TACE (35)	Global QOL of EORTC QLQ-C30	Baseline, then every 6 mo	Phase Ⅱ trial with HRQOL endpoint
Steel *et al*[97]	2006	Combined analysis of 3 studies	157	Mixed patient populations from 3 studies	Various treatments	FACT-Hep, HepCS, TOI, FHSI8	Baseline, 3 and 6 mo	Observational study with QOL assessment during treatment - evaluates minimally important difference in HRQOL
Eid *et al*[36]	2006	Cohort	7	Allocated to hepatic ablation or resection	Hepatic ablation (3) *vs* resection (4)	EORTC QLQ-C30, FACT-Hep, FHSI8, Profile of Mood States (POMS)	Baseline, postoperative visit, 1.5, 3 and 6 mo	Observational study with QOL assessment during treatment. Study included other liver tumor types (33 patients)
Yeo *et al*[65]	2006	Combined analysis of 2 phase Ⅲ trials	233	Unresectable or metastatic	Chemotherapy, hormonal therapy	EORTC QLQ-C30	Baseline	As prognostic tools for overall survival - baseline HRQOL was prognostic of overall survival in advanced HCC
Wang *et al*[98]	2006	Cohort	83	Non-metastatic, 3 nodules or less	TACE + RFA (43) *vs* TACE (40)	FACT-G	Baseline, 3 mo	Observational study with QOL assessment during treatment
Cebon *et al*[49]	2006	Phase Ⅰ/Ⅱ trial	63	Not eligible for standard therapies	Octreotide long acting release	FACT-Hep, patient disease and treatment assessment form (Pt DATA form), patient benefit form	Baseline, then every 1 mo	Phase Ⅰ/Ⅱ trial with HRQOL endpoint

Llovet *et al*[12]	2006	Phase Ⅲ trial	602	Not eligible for local treatment or had disease progression after surgery or local treatment	Sorafenib (299) *vs* placebo (303)	FHSI-8	Baseline then every 3 wk	Phase Ⅲ trial with HRQOL endpoint
Lee *et al*[31]	2007	Case control	161	Any stage	Surgical, TACE, percutaneous ethanol injection, supportive care	EORTC QLQ-C30, WHOQOL-BREF	Cross sectional one-time assessment	To describe symptomatology and/or HRQOL of HCC patients - compared with national matched healthy controls
Kondo *et al*[37]	2007	Case-control	97	Non-metastatic, 3 nodules or less	Percutaneous ablation	SF-36	Baseline	To describe symptomatology and/or HRQOL of HCC patients - HRQOL compared to 97 matched chronic liver disease controls, and normal population values
Steel *et al*[33]	2007	Case-control	83	Any stage	NA	FACT-Hep	Baseline	To describe symptomatology and/or HRQOL of HCC patients - HRQOL compared to 51 matched chronic liver disease controls, and 138 controls from general population
Martin *et al*[35]	2007	Cohort	4	Resectable	Resection	EORTC QLQ-C30, FACT-Hep, FHSI-8	Baseline, discharge, postoperative visit, 1.5, 3, 6 and 12 mo	Observational study with QOL assessment during treatment. Included 28 patients with other liver tumors
Becker *et al*[50]	2007	Randomized phase Ⅱ trial	120	Not eligible for resection or local treatment	Octreotide (61) *vs* placebo (59)	EORTC QLQ-C30	Baseline, 1, 3 mo, then every 3 mo	Phase Ⅱ trial with HRQOL endpoint
Dimitroulopoulos *et al*[51]	2007	Randomized phase Ⅱ trial	127	Advanced stage. Somatostatin receptor overexpression for randomisation	Octreotide (31) *vs* placebo (30) observation (66)	EORTC QLQ-C30	Baseline then every 1 mo	Phase Ⅱ trial with HRQOL endpoint
Sun *et al*[99]	2008	Cohort	22	Mainly advanced disease	Various treatments	FACT-Hep, Functional assessment of chronic illness therapy spirituality subscale (FACIT-Sp-12)	Baseline, 1, 2 and 3 mo	Observational study with QOL assessment during treatment. Included 23 patients with pancreatic cancer
Méndez Romero *et al*[52]	2008	Phase Ⅰ / Ⅱ trial	9	Not eligible for other local treatments	SBRT	EORTC QLQ-C30 EQ-5D VAS	Baseline, 1, 3 and 6 mo	Observational study with QOL assessment during treatment. Included 19 patients with liver metastases. Phase Ⅰ / Ⅱ trial with HRQOL endpoint
Bonnetain *et al*[66]	2008	Combined analysis of 2 phase Ⅲ trials[59,101]	538	Not eligible for resection, transplantation or percutaneous ablation	Tamoxifen *vs* supportive care; TACE + tamoxifen *vs* tamoxifen	Spitzer QoL index	Baseline	As prognostic tools for overall survival - baseline HRQOL was prognostic of overall survival in advanced HCC
Doffoël *et al*[100]	2008	Phase Ⅲ trial	138	Eligible for TACE	TACE + tamoxifen (70) *vs* tamoxifen (68)	Spitzer QoL index	Baseline, then every 2 mo during treatment, every 3 mo after treatment	Phase Ⅲ trial with HRQOL endpoint
Barbare *et al*[60]	2009	Phase Ⅲ trial	272	Not eligible for curative treatment	Octreotide (135) *vs* placebo (137)	EORTC QLQ-C30	Baseline, then every 1 mo during treatment, every 3 mo after treatment	Phase Ⅲ trial with HRQOL endpoint
Cheng *et al*[13]	2009	Phase Ⅲ trial	271	Unresectable or metastatic, no prior systemic therapy	Sorafenib (150) *vs* placebo (76)	FHSI-8. Physical well being domain of FACT-Hep	Baseline then every 3 wk	Phase Ⅲ trial with HRQOL endpoint
Wible *et al*[44]	2010	Cohort	73	Allocated to TACE	TACE	SF-36	Baseline, 4, 8 and 12 mo	Observational study with QOL assessment during treatment

Dollinger *et al*[101]	2010	Phase Ⅲ trial	135	Locally advanced or metastatic	Thymostimulin (67) *vs* placebo (68)	FACT = Hep	Baseline then every 3 mo	Phase Ⅲ trial with HRQOL endpoint
Chow *et al*[61]	2011	Phase Ⅲ trial	204	Advanced disease, not eligible for standard therapies	Megestrol acetate (195) *vs* placebo (69)	EORTC QLQ-C30	Baseline, then every 1 mo during treatment, then every 3 mo after treatment completed	Phase Ⅲ trial with HRQOL endpoint
Shun *et al*[102]	2012	Cohort	89	Allocated to TACE	TACE	SF-12, Symptom Distress Scale, Hospital Anxiety and Depression Scale	3 d before discharge, 1 and 2 mo	Observational study with QOL assessment during treatment
Qiao *et al*[103]	2012	Observational	140	Any stage	NANANAdadsdfsaNA	FACT-epHHep	Baseline	To describe symptomatology and/or HRQOL of HCC patients - HRQOL worsens with advancing stage
Eltawil *et al*[45]	2012	Cohort	48	Allocated to TACE	TACE	WHOQOL-BREF	Baseline then every 3-4 mo	Observational study with QOL assessment during treatment
Fan *et al*[104]	2012	Cross sectional	286	Any stage		EORTC QLQ-C30, EORTC QLQ-HCC18	Baseline	To describe symptomatology and/or HRQOL of HCC patients - HRQOL compared with population norms. Correlation between HRQOL and coping and illness perception
Diouf *et al*[67]	2013	Reanalysis of a phase Ⅲ trial[61]	215	Not eligible for curative treatment, baseline HRQOL data available	Octreotide *vs* placebo	EORTC QLQ-C30	Baseline	As prognostic tools for overall survival - baseline HRQOL was prognostic of overall survival in advanced HCC. HRQOL data may improve existing staging systems
Soliman *et al*[53]	2013	Phase Ⅱ trial	21	Not eligible for or refractory to standard therapies, symptomatic	Liver radiotherapy	EORTC QLQ-C30, FACT-Hep, HepCS, TOI, FACT-G	Baseline, 1, 3 and 6 mo	Phase II trial with HRQOL endpoint. Included 20 patients with liver metastasis
Salem *et al*[41]	2013	Cohort	56	Allocated to SIRT or TACE	SIRT (29), TACE (27)	FACT-Hep	Baseline, 2 and 4 wk	Observational study with QOL assessment during treatment
Brunocilla *et al*[105]	2013	Cohort	36	Allocated to sorafenib	Sorafenib	FACT-Hep, FHSI-8, FACT-G	Baseline, 1 wk, 1 and 2 mo	Observational study with QOL assessment during treatment
Johnson *et al*[62]	2013	Phase Ⅲ trial	1150	Not eligible for resection or local treatment, no prior systemic treatment	Brivanib (577) *vs* sorafenib (578)	Physical function and role function of EORTC QLQ-C30	Baseline then every 6 wk	Phase Ⅲ trial with HRQOL endpoint
Meyer *et al*[63]	2013	Phase Ⅱ / Ⅲ trial	86	Unresectable, non-metastatic	TACE *vs* TAE	EORTC QLQ-C30, EORTC QLQ-HCC18	Baseline, 1.5, 3 and 6 mo	Phase Ⅱ trial with HRQOL endpoint
Mise *et al*[106]	2014	Cohort	69	Allocated to resection	Resection	SF-36	Baseline then every 3 mo	Observational study with QOL assessment during treatment
Huang *et al*[38]	2014	Cohort	388	Solitary HCC ⩽ 3 cm	Resection, radiofrequency ablation	FACT-Hep, HepCS, TOI, FACT-G	Baseline, 3, 6, 12, 24 and 36 mo	Observational study with QOL assessment during treatment
Zhu *et al*[64]	2014	Phase Ⅲ trial	564	Progressive disease during or after sorafenib	Everolimus (362) *vs* placebo (184)	Global QOL and physical function of EORTC QLQ-C30	Baseline, then multiple reassessments	Phase Ⅲ trial with HRQOL endpoint
Palmieri *et al*[107]	2015	Case control	24	Any stage	NA	SF-36	Baseline	To describe symptomatology and/or HRQOL of HCC patients - evaluates relationship between psychological profile and HRQOL in HCC. Included 22 cirrhotic patients without HCC, 20 control subjects
Chie *et al*[108]	2015	Cohort	171	Allocated to respective treatments	Surgery (53), ablation (53), TACE (65)	EORTC QLQ-C30, EORTC QLQ-HCC18	Baseline, then 4-6 wk for post-ablation/post-TACE, 12-15 wk post-operation	Observational study with QOL assessment during treatment

Heits *et al*[109]	2015	Cross sectional	173	Allocated to liver transplanation	liver transplantation	EORTC QLQ-C30	At one variable time point post-transplantion	To describe symptomatology and/or HRQOL of HCC patients
Xie *et al*[110]	2015	Cohort	102	Allocated to resection or TACE	resection (58), TACE (44)	SF-36	Baseline, 1, 3, 6, 12 and 24 mo	Observational study with QOL assessment during treatment
Xing *et al*[111]	2015	Cohort	118	Allocated to TACE	TACE with doxorubicin eluted beads	SF-36	Baseline, 1-3, 6 and 12 mo	Observational study with QOL assessment during treatment
Kolligs *et al*[54]	2015	Randomized phase II trial	28	Allocated to SIRT or TACE	SIRT (13), TACE (15)	FACT-Hep	Baseline, then every 6 wk	Phase II trial with HRQOL endpoint
Klein *et al*[42]	2015	Combined analysis of prior phase I/II trials	98	Allocated to SBRT	SBRT	EORTC QL-C30, FACT-Hep	Baseline, 1, 3, 6 and 12 mo	Phase I/II trial with HRQOL endpoint
Kensinger *et al*[48]	2016	Case-control	139	Allocated to priority liver transplantation	Liver transplantation	SF-36	Baseline, post transplantation	Observational study with QOL assessment during treatment - included 362 subjects without HCC
Lei *et al*[39]	2016	Cohort	205	Allocated to resection or transplantation	Liver transplantation (110), resection (95)	SF-36	Baseline, then every 1-2 mo for the first 6 mo, then every 2-3 mo for the next 6 mo, then every 6 mo	Observational study with QOL assessment during treatment
Yang *et al*[112]	2016	Cohort	17	Portal vein thrombosis	TACE and transarterial ethanol ablation	EORTC QLQ-C30	Baseline then every 1 mo	Observational study with QOL assessment during treatment
Anota *et al*[55]	2016	Phase I trial	21	Not eligible for curative treatment	TACE with idaurubicin eluted beads	EORTC QLQ-C30	Baseline, 15, 30 and 60 d	Phase I trial with HRQOL endpoint
Chie *et al*[88]	2016	Case-control	227	Any stage	Various treatments	EORTC QLQ-C30, EORTC QLQ-HCC18	Baseline, post-treatment	Observational study with QOL assessment during treatment - Compared HRQOL between Asian and European HCC patients
Lv *et al*[56]	2016	Randomized phase II trial	120	Allocated to TACE	COX2 inhibitor (60) *vs* placebo (60)	Locally developed questionnaire	Baseline, 24 and 48 h	Phase II trial with HRQOL endpoint
Koeberle *et al*[57]	2016	Randomized phase II trial	106	Unresectable or metastatic	Sorafenib + everolimus (60) *vs* sorafenib (46)	FACT-HepCS, EQ-VAS	Baseline, then every 2 wk until week 12	Phase II trial with HRQOL endpoint
Shomura *et al*[113]	2016	Cohort	54	TNM stage IV	Sorafenib	SF-36	Baseline, then every 3 mo	Observational study with QOL assessment during treatment
Bruix *et al*[14]	2016	Phase III trial	573	Progressive disease during sorafenib	Regorafenib (379) *vs* placebo (193)	FACT-Hep, TOI, FACT-G, EQ-5D, EQ-VAS	Baseline, then multiple reassessments	Phase III trial with HRQOL endpoint
Li *et al*[69]	2017	Cohort	472	Any stage	Various treatments	EORTC QLQ-C30, EORTC QLQ-HCC18, C30 index score, HCC18 index score	Baseline	As prognostic tools for overall survival - baseline HRQOL was prognostic of overall survival in advanced HCC. QOL derived scoring system resembles a staging system

EORTC: European Organisation for Research and Treatment of Cancer; EQ-5D: EuroQoL-5D; FACT-G: Functional Assessment of Cancer Therapy - General; FACT-Hep: Functional Assessment of Cancer Therapy - Hepatobiliary; FHSI-8: Functional Assessment of Cancer Therapy - Hepatobiliary Symptom Index; HCC: Hepatocellular carcinoma; HepCS: Hepatobiliary cancer subscale; HRQOL: Health related quality of life; *n*: Sample size; NA: Not applicable; RFA: Radiofrequency ablation; SBRT: Stereotactic body radiation therapy; SF-12: Short Form 12; SF-36: Short Form 36; SIRT: Selective internal radiation therapy; Spitzer QoL Index: Spitzer Quality of Life Index; TACE: Transarterial chemoembolization; TOI: Trial Outcome Index; VAS: Visual analogue scale; WHOQOL-BREF: World Health Organization Quality of Life Assessment abbreviated version.

A surgical series compared post operative QOL using SF-36 between liver transplantation (*n* = 95) and resection (*n* = 110) in HCC patients fulfilling Milan's criteria. It reported no significant difference in all domains, physical component summary scale and mental component summary scale between these 2 cohorts. However it did not correlate with survival outcomes[39].

Patients received palliative locoregional therapies, *e.g.*, TACE, SIRT, stereotactic body radiation therapy (SBRT) commonly reported early deterioration of HRQOL, which could be attributable to treatment toxicity[40-43].

A case series reported HRQOL (SF-36) of HCC patients who received TACE[44]. Overall patients' mental component summary scale improved at 4 mo after TACE. For patients received more than 2 cycles of TACE, their mental component summary scale improved after the initial 2 cycles of TACE, and their bodily pain score also improved. Another TACE series observed deterioration of physical health domain of WHOQOL-BREF that coincided with HCC progression[45]. A cohort study using FACT-Hep reported better functional well-being and overall QOL in HCC patients after treatment with SIRT when compared to TACE[46].

As clinical trials endpoint

HRQOL has been increasingly used as secondary endpoint in HCC clinical trials. Phase Ⅰ/Ⅱ trials put emphasis on treatment tolerability or toxicity, and thus QOL impact is a logical endpoint of interest. Quite a number of phase Ⅰ/Ⅱ HCC trials have QOL as secondary endpoints[47-57] (Table 2).

QOL analysis in phase Ⅰ/Ⅱ clinical trials: A phase Ⅰ/Ⅱ trial assessed the use of octreotide in 63 untreatable HCC patients[49]. Grade 3/4 toxicities were uncommon and responses were rare. QOL assessment using FACT-Hep was performed at baseline and every 1 mo afterwards. There was no significant change in reassessment QOL compared to baseline.

A combined analysis of 3 phase Ⅰ/Ⅱ trials of SBRT addressed the QOL of 98 HCC, 86 liver metastasis and 21 intrahepatic cholangiocarcinoma patients[42]. EORTC QLQ-C30 and FACT-Hep were used for QOL assessment, which was scheduled at baseline, 1, 3, 6 and 12 mo. Overall the QOL deteriorated at 1 mo after SBRT, then recovered at 3 mo. Patients with liver metastasis had significantly better QOL at 1 and 6 mo than patients with primary liver cancer.

A randomized phase Ⅱ trial evaluated TACE with microspheres *vs* TACE in 70 HCC patients[48]. G4 toxicities were rare in both arms. Global QOL domain of EORTC QLQ-C30 was used for QOL monitoring, which was measured at baseline and every 3 mo afterwards. There was no significant difference in QOL in both arms.

QOL analysis in phase Ⅲ clinical trials: Although phase Ⅲ trials focus on evaluation of treatment efficacy, there is an increasing trend for these phase Ⅲ clinical trials to incorporate HRQOL as a study endpoint. Effective treatment could improve QOL, whereas treatment-related toxicity, disease progression with ineffective treatment could worsen QOL. Thus it is important to investigate whether a treatment could provide a net QOL benefit. Capturing HRQOL data in clinical trials could provide valuable information to guide clinicians in treatment decision. Commonly used tools included EORTC QLQ-C30, EORTC QLQ-HCC18, Spitzer QoL index, FACT-G, FACT-Hep, FHSI-8[12-14,58-64] (Table 2). Some trials defined *a priori* 1-2 scales of interest within an HRQOL instrument as study endpoint, *e.g.*, global QOL or physical functioning domain of EORTC QLQ-C30[59,60,64].

A phase Ⅲ trial comparing first-line tamoxifen *vs* best supportive care alone in advanced HCC patients found no significant difference in OS in both arms. HRQOL, measured using Spitzer QoL index, decreased in both groups of patients with time[58].

A phase Ⅲ trial compared first-line megestrol acetate *vs* placebo in advanced HCC patients[61]. There was no significant impact on OS with megestrol acetate. However, patients received megestrol acetate had significantly better scores in EORTC QLQ-C30 appetite loss, nausea/vomiting and emotional functioning scales compared to placebo. Such prospective randomized HRQOL data might provide rationale in using megestrol acetate for palliative symptom relief in advanced HCC patients.

The SHARP study and the phase Ⅲ trial reported by Cheng *et al*[13] were pivotal trials demonstrating PFS and OS benefits of first-line sorafenib in advanced HCC patients compared to placebo[12]. Drug related serious adverse events were more frequent in sorafenib arm than placebo arm in both studies. Both trials employed deterioration in FHSI-8 score as one of the definitions of symptomatic progression. In both trials, median time to symptomatic progression was not significantly different between sorafenib and placebo arms.

The phase Ⅲ BRISK-FL study randomized 1150 advanced HCC patients to first-line brivanib or sorafenib[62]. There was no significant difference in OS, time to tumor progression or response rate between the 2 arms. The overall incidence of serious adverse events was 56% for brivanib arm and 48% for sorafenib arm. The study used EORTC QLQ-C30 physical and role functioning domains as HRQOL endpoint. There was no significant difference in HRQOL at baseline between the 2 arms. The mean scores for physical and role functions declined at 12 wk in both brivanib and sorafenib patients, but the deterioration was significantly worse in brivanib arm. The objective of non-inferiority in OS was not met for brivanib. Should the onjective be met, the available QOL could potentially be a key in guiding clinicians on the use of a more tolerable agent (in this case sorafenib) which has less impairment in QOL.

From these first-line trials on tyrosine kinase inhibitors, it appear that the toxicity profile of brivanib was worse than sorafenib, while that of sorafenib was worse than placebo. The deterioration in QOL may be due to treatment-related toxicities, which can be offset by improvement in QOL due to disease control by a more effective treatment. This postulation could

Table 3 Algorithm of C30 and HCC18 index scores

QOL Index scores for survival prognostication	
C30 index score	∑ [(100-Physical functioning), (100-Role functioning), (100-Emotional functioning), (100-Cognitive functioning), (100-Social functioning), (100-global QOL), scores of Fatigue, Nausea/vomiting, Pain, Dyspnoea, Insomnia, Appetite loss, Constipation, Diarrhea, Financial Difficulty]/15
HCC18 index score	∑(scores of Fatigue, Body Image, Jaundice, Nutrition, Pain, Fever, Sex life, Abdominal distension)/8

QOL: Quality of life.

theoretically be explored in a meta-analysis of these studies, however, the usage of different HRQOL instruments across studies precluded such an attempt.

In the EVOLVE-1 trial, HCC patients who failed sorafenib were treated with everolimus or placebo[64]. Disease control rate was significantly better in the everolimus arm, but there was no significant difference in PFS or OS between the 2 arms. On the other hand, the time to definitive deterioration in EORTC QLQ-C30 physical functioning was significantly shorter in the everolimus arm. This might be related to the significantly increased incidence in grade 3/4 adverse events in the everolimus arm compared to the placebo arm. This study again exemplified the importance in inclusion of HRQOL assessment in clinical trial because the intervention itself could have negative effect on QOL.

The phase Ⅲ RESORCE trial evaluated second-line regorafenib *vs* placebo in advanced HCC patients with prior sorafenib. Compared to placebo arm, patients randomized to regorafenib had significantly longer OS and PFS (using modified Response Evaluation Criteria in Solid Tumors for HCC), and reported more drug related adverse events. HRQOL was assessed using FACT-G, FACT-Hep, TOI, EQ-5D and EQ-VAS. The FACT-Hep total score and TOI were significantly lower in regorafenib arm than placebo arm, while FACT-G, EQ-5D and EQ-VAS were not significantly different[14]. Cost-effectiveness analysis of this expensive intervention is essential in parts of the world where medical resources are particularly limited, the use of EQ-5D will allow such analysis to be conducted.

As prognostic tools for overall survival

One interesting use of HRQOL data in HCC patients is prognostication for OS. Three studies showed that in advanced HCC patients, baseline HRQOL at diagnosis was prognostic for OS[65-67]. Our group reported the prognostic significance of EORTC QLQ-C30 in advanced HCC patients, where worse scores in appetite loss, physical function and role function domains were independent risk factors for shorter OS[65]. In another study using EORTC QLQ-C30, better baseline role function score was found to be a significant prognostic factor for longer OS in advanced HCC patients[67]. Baseline Spitzer QoL index was also reported to be prognostic of survival in 538 advanced HCC patients, where higher baseline Spitzer QoL index score was associated with longer OS[66]. However, a study recruiting HCC patients of all stages reported FACT-G was not prognostic of overall survival[68].

Our group subsequently evaluated the prognostic value of baseline EORTC QLQ-C30 and QLQ-HCC18 in a cohort of newly diagnosed HCC patients including all stages and found both were significant prognostic factors for OS irrespective of stage of disease[69]. Better scores in QLQ-C30 pain, QLQ-C30 physical functioning, QLQ-HCC18 pain, QLQ-HCC18 fatigue scales at diagnosis were significant independent prognostic factors for longer OS. In order to enhance the user-friendliness of these instruments, two summative scoring systems, the C30 index score and HCC18 index score, were derived. See Table 3 for the formulae.

Both of these scores were found to be highly significant factors for OS and their prognostic values resemble that of a staging system.

For C30 index score of 0-20, 21-40, 41-60, 61-100, the median OS were 16.4, 7.3, 3.1, 1.8 mo respectively ($P < 0.0001$). For HCC18 index score of 0-20, 21-40, 41-60, 61-100, the median OS were 16.4, 6.0, 2.8, 1.8 mo respectively ($P < 0.0001$).

Attempts have been made to enhance existing staging systems with HRQOL data[66,67]. Addition of EORTC QLQ-C30 data has been shown to improve the performance of the Cancer of the Liver Italian Program (CLIP)[70,71], the Barcelona Clinic Liver Cancer system[72], the Groupe d'Étude et de Traitement du Carcinome Hépatocellulaire system[73]. Spitzer QoL index could improve the prognostic value of CLIP[66].

Valuation of health care service

Cost-effectiveness studies analyze the cost per outcome (effectiveness) of health care interventions, and compare this with reference to the country's willingness to pay threshold. In cancer setting, this outcome is commonly QALY. HRQOL measurement allows valuation of HRQOL specific to the population. When this is combined with time, QALY could be calculated[74]. A popular instrument for this purpose is EQ-5D.

Certain treatments for HCC, such as liver transplantation and tyrosine kinase inhibitors, carry significant economic burden due to high utility and cost, particularly in areas with endemic hepatitis B viral infection. Cost-effectiveness analysis is therefore important to assist societal economic consideration by policy makers in health care service. A number of cost-effectiveness analyses in HCC have been carried out in this regard[75-80].

Palliative care service benchmark

HRQOL is an important benchmark for palliative care service and clinical trial[81]. Palliative care in cancer setting aims to improve QOL of cancer patients. It involves prevention, early identification and relief of sufferings (physical, psychological, social and spiritual) of cancer patients during the whole course of their illnesses. Therefore effective palliative care could be reflected in improvement in QOL.

Palliative care trials commonly recruit patients with a wide range of malignant diseases, including HCC. A prospective study conducted in Germany assessed the change in HRQOL using EORTC QLQ-C30 in cancer patients admitted to a hospital unit or palliative home care service where palliative treatment was given for symptoms relief[82]. Of all the patients who received palliative service for 7 d, 57% had a better rating in symptom domains and 42% had a better rating in functional domains when compared to their rating before receiving the service.

DIFFICULTIES IN UTILIZATION OF HRQOL IN CLINICAL TRIAL AND PRACTICE

Prospective study design

Although retrospective analysis of QOL can be conducted, HRQOL data have to be prospectively collected to be usable. Unless an institute has routine HRQOL assessment for all patients, a retrospective study is impossible to have HRQOL as a parameter.

Choosing a suitable tool

Choosing a suitable HRQOL instrument for a study could be challenging. Although the majority of the mentioned instruments were extensively validated, which instrument prevails over another is largely unknown. The aim of a study and the characteristics of individual HRQOL instruments should be considered. If the symptom aspect of HRQOL was of interest, one may favor an instrument housing more liver-cancer related symptoms, for example, EORTC QLQ-C30 plus QLQ-HCC18, or FACT-Hep. One should also take into account the instrument's responsiveness to change with clinical condition in order to accurately capture significant HRQOL deterioration or improvement in subsequent reassessment time points. If follow-up cost-effectiveness analysis of an intervention is anticipated, the study needs to include an instrument with QOL valuation ability, for example, EQ-5D.

Missing data

Missing data is common in HRQOL studies, and inadequate reporting and handling of missing data are also common[83]. Analysis of incomplete data could give biased results. Therefore missing data should be prevented, identified and handled appropriately.

Prevention of missing data should be planned before a study begins. As opposed to survival data that could be captured even when patients have succumbed, follow-up QOL assessment relies mainly on active participation of patients. They need to have adequate physical and cognitive function and motivation to answer relevant questionnaires. This could be demanding to patients with deteriorated clinical status. This proves particularly challenging in clinical trial involving advanced HCC patients because their PFS generally is short and the clinical downhill course can be rapid. More frequent HRQOL reassessment may maximize the capture of HRQOL data before significant clinical deterioration occurs. Proxy (treating clinicians or patients' care-giver) filled questionnaires could be a reasonable substitute[84] but still creates significant bias because HRQOL is a personal and subjective measurement. Computerized questionnaire during follow up visit could be programmed to forbid submission of incomplete questionnaire. Patients may forget to return reassessment questionnaires by mail if such system is utilized. Some studies employed reminder system to reduce this non-compliance.

When missing data occurred, it is essential to identify the mechanism of missing data and tackle it accordingly. There are 3 mechanisms of missing data: (1) missing completely at random (MCAR): MCAR is said to occur if the reason of missing data is unrelated to any variable of the study. For example, an on-site hand-held device for HRQOL assessment broke down for a certain period of time; (2) missing at random (MAR): If the reason of missing data was related to non-QOL data, MAR is present. For example, elderly patients are more prone to forget returning the reassessment questionnaire by mail than younger patients; and (3) missing not at random (MNAR): MNAR is assumed when the reason of missing data is related to the QOL data. For example, severely ill patients with the worse QOL may feel too weak to complete reassessment questionnaires.

MCAR and MAR are categorized as ignorable missingness. Whereas MNAR is categorized as non-ignorable missingness, because the observed (available) QOL data are typically biased. Therefore it is important to investigate the mechanism of missing data in order to employ specific method of handling. Various statistical methods have been established to investigate the mechanism of missing data[85]. Nevertheless, confirmation of the underlying mechanism may not be possible. Once assumption of the mechanism is made, appropriate method to deal with missing data follows[86].

The following are the methods to handle missing data: (1) complete case analysis: Patients with missing data are excluded from the analysis; (2) single imputation: Single imputation replaces a missing value by a single value and analysis is carried out as if all data are observed. The replacement value could be the mean or mode of observed data, last observed value carried forward, baseline observed value carried forward, or predicted value from a regression equation based on information from observed data. Single imputation

may have a higher risk of biasing the analysis because the uncertainty of imputed values was not addressed; (3) multiple imputation: Multiple imputation generates multiple copies of the original dataset by replacing missing values using a specified regression model. Analysis is then performed for each dataset and the results are pooled into one estimate with standard error taking into account the uncertainty of the imputation process; and (4) statistical models: Mixed models and generalized estimating equations could be used to allow for missing data without imputation, making assumptions about their relationships with the observed data.

Option (1) will only be unbiased in case of MCAR or MAR. For MNAR, options (2-4) are more appropriate. Sensitivity analysis is then carried out. It involves separate analysis of every dataset generated by various imputation methods and comparison of the results. Sensitivity analysis reflects whether an analysis is robust (insignificant distortion of conclusion) after handling of missing data[87]. These are the key steps to minimize the detrimental effect of missing data on the results of QOL studies.

Population related difference in HRQOL

HRQOL changes significantly across different diseases, cultures and ethnicities. For example, in Chinese culture people take endurance as a merit, they often minimize the verbalization or expression of discomfort, thus symptoms scales might underestimate their symptomatology. Oriental culture tends not to discuss sex issue openly, therefore missing data rate in the sexual problem scale could be particularly high. Different languages and dialects could also affect patient's interpretation of the intended questions. Therefore HRQOL instruments need validation in different countries, since HRQOL data from one country may not be applicable to another.

This is evident in a study that compared HRQOL between Asian and European HCC patients[88]. It reported significantly better scores in emotional functioning and insomnia (based on EORTC QLQ-C30) and sexual interest (based on EORTC QLQ-HCC18) in Asian when compared to European patients, after adjusting for demographic and clinical variables.

Data interpretation

Most HRQOL instruments consist of a collection of scores in various domains. How can one define a domain score being significantly good or bad? How can one define a clinically significant change in a domain score? Attempts have been made to evaluate minimally important differences in HRQOL measurements by comparing the scores among different patient groups stratified according to various clinical anchors, for example, stage of disease, performance status, *etc*[89-92]. This permits meaningful interpretation of HRQOL data. Studies sometimes employed these findings to define their HRQOL endpoints. However caution has to be exercised as these cutoffs or thresholds might be population- or disease-specific and might not be applicable to all.

Data analysis

Raw HRQOL ordinal data are commonly used as continuous variables in data analysis. Analysis is usually in the form of comparison of mean domain score between 2 patient groups or 2 time points within the same group. The situation is complicated by the fact that when all domain scores are included in a multivariate analysis model, the numerous raw HRQOL data could cause excessive multiple comparisons and instability of model[93,94].

Studies using limited number of domains within an HRQOL instrument may have avoided such problem, but may sacrifice potentially significant HRQOL variables.

Diouf *et al*[67] dichotomized all EORTC QLQ-C30 scale scores using 50 as an empirical cut-off for analysis. This may prevent overfitting and multi-collinearity and allows clinicians to understand HRQOL data in a simpler manner. As these cut-offs were supposed to be population-specific, another analysis was performed and reported the real cut-off for various scales[95].

Another way of HRQOL data analysis while avoiding multi-collinearity, yet without sacrificing any QOL data, is to use 1 score to represent all scales in the whole instrument. As discussed earlier, by transforming the EORTC QLQ-C30 into C30 index score, and EORTC QLQ-HCC18 into HCC18 index score for data analysis, our group has shown that these index scores were the most significant independent factors for OS among all the individual HRQOL variables, whether continuous or dichotomized[69].

Different studies used different HRQOL instruments. QOL data, unlike survival data or response assessment, are not unified to allow cross trial communication. Cross study comparison of HRQOL result is not usually possible. Performing meta-analysis on HRQOL studies is therefore difficult.

Limitation for use in clinical practice

Measurement of HRQOL in clinical practice is desirable. QOL changes over time in HCC patients when their diseases improve or progress, or when treatment complications arise. Deterioration in QOL reflects the need for palliative care intervention. However routine capturing of QOL data is difficult. Filling in the instruments, calculating all domain and total scores could be cumbersome in the clinical setting. Difficulty in interpretation of a collection of numerical scores also deters a clinician from welcoming it. Modern handheld device might help patients to self-administer the questionnaires during waiting time, it can help generate all domain and total scores automatically, as well as support interpretation of individual score according to published local reference values.

CONCLUSION

Quality of life could be as important as survival in HCC patients because majority of them have advanced disease and limited survival. QOL measurement provides valuable information in clinical practice and research. Future research into utilization in clinical trials as well as routine clinical practice are warranted.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Impact of comorbidity on waiting list and post-transplant outcomes in patients undergoing liver retransplantation

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Abstract

AIM

To determine the impact of Charlson comorbidity index (CCI) on waiting list (WL) and post liver retransplantation (LRT) survival.

METHODS

Comparative study of all adult patients assessed for primary liver transplant (PLT) (n = 1090) and patients assessed for LRT (n = 150), 2000-2007 at our centre. Demographic, clinical and laboratory variables were recorded.

RESULTS

Median age for all patients was 53 years and 66% were men. Median model for end stage liver disease (MELD) score was 15. Median follow-up was 7-years. For retransplant patients, 84 (56%) had $\geqslant$ 1 comorbidity. The most common comorbidity was renal impairment in 66 (44.3%). WL mortality was higher in patients with $\geqslant$ 1 comorbidity (76% *vs* 53%, P = 0.044). CCI (OR = 2.688, 95%CI: 1.222-5.912, P = 0.014) was independently associated with WL mortality. Patients with MELD score $\geqslant$ 18 had inferior WL survival (Log-Rank 6.469, P = 0.011). On multivariate analysis,

CCI (OR = 2.823, 95%CI: 1.563-5101, P = 0.001), MELD score ⩾ 18 (OR 2.506, 95%CI: 1.044-6.018, P = 0.04), and requirement for organ support prior to LRT (P < 0.05) were associated with reduced post-LRT survival. Donor/graft parameters were not associated with survival (P = NS). Post-LRT mortality progressively increased according to the number of transplanted grafts (Log-Rank 18.455, P < 0.001). Post-LRT patient survival at 1-, 3- and 5-years were significantly inferior to those of PLT at 88% *vs* 73%, P < 0.001, 81% *vs* 71%, P = 0.018 and 69% *vs* 55%, P = 0.006, respectively.

CONCLUSION

Comorbidity increases WL and post-LRT mortality. Patients with MELD ⩾ 18 have increased WL mortality. Patients with comorbidity or MELD ⩾ 18 may benefit from earlier LRT. LRT for ⩾ 3 grafts may not represent appropriate use of donated grafts.

Key words: Hepatic; Organ; Outcome; Diabetes; Renal

Core tip: The prevalence and impact of comorbidity on waiting list (WL) and post-transplant survival is unknown in patients who had liver retransplantation. This study identified comorbidity(ies) were common (56%) in this cohort, most with renal impairment. WL mortality was higher in patients with ⩾ 1 comorbidity and model for end stage liver disease (MELD) score ⩾ 18. Post-transplant survival was inferior in patients with ⩾ 1 comorbidity, MELD score ⩾ 18 and in patients who required organ support prior to retransplantation. Comorbidity increases WL and post-transplant mortality. Patients with comorbidity or MELD ⩾ 18 may benefit from earlier retransplantation.

Al-Freah MAB, Moran C, Foxton MR, Agarwal K, Wendon JA, Heaton ND, Heneghan MA. Impact of comorbidity on waiting list and post-transplant outcomes in patients undergoing liver retransplantation. *World J Hepatol* 2017; 9(20): 884-895 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i20/884.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i20.884

INTRODUCTION

Liver retransplantation (LRT) represents the only viable option for survival for some patients who develop graft failure following primary liver transplant (PLT). Published reports on cohorts of patients who underwent LRT indicate inferior post-transplant survival in these patients[1-5]. There has been an increase in the number of patients awaiting PLT which was not associated with increase in donated organs[6]. Although transplant programmes have tried to compensate for this increase in demand by more liberal use of marginal grafts, there is evidence that death on the waiting list (WL) for patients listed for PLT remains high[7]. Therefore, the combination of increased WL mortality with increasing demand for PLT coupled with the known inferior outcomes of LRT; raises concerns and generates ethical debate in the transplant community on the use of scarce resource of donated organs for LRT[8].

This debate has motivated researchers to identify predictors of survival following LRT to improve the selection of patients who might benefit most from LRT. Model for end-stage liver disease (MELD) score > 25, recipient age, creatinine level, bilirubin level, indication for retransplantation, the urgency for LRT, coma episodes, haemoglobin (Hb) level and the number of fresh frozen plasma units transfused were identified as factors associated with reduced post-LRT survival in a number of studies[3,5,9,10]. Death or graft loss was shown to increase gradually following LRT according to the timing of LRT with marked increase in risk between 4-38 d following LRT[11-13]. Inferior survival was also observed according to increasing number of transplanted graft[13].

Comorbidity as defined by the Charlson comorbidity index (CCI) was found to adversely affect post-transplant survival in patients who underwent PLT[14]. Thuluvath *et al*[7] analysed the data of the scientific registry of transplant recipients (SRTR) in the United States from 1999 to 2008. The prevalence of comorbidity such as diabetes mellitus (DM), renal impairment (RI) and obesity was found to have steadily increased in candidates listed for liver transplantation over the ten year period[7]. However, the prevalence and impact of comorbidity on WL and post-transplant survival in patients listed for LRT have not been studied previously.

The aims of this study were three fold, firstly, to identify the prevalence of comorbidity according to CCI in patients listed for LRT, secondly, to study the impact of comorbidity on WL and post-LRT mortality, and finally, to identify other factors associated with reduced WL and post-LRT survival.

MATERIALS AND METHODS

Patients and design

This is a retrospective study of all patients referred to the liver unit at King's for LRT assessment between January 2000 and December 2007. There were 151 assessments for LRT on 137 patients over the 8 year period. One patient was excluded because of incomplete information. Data analysis was performed on 150 LRT assessments. We utilized a cohort of patients who underwent PLT over the same time period for comparison of outcomes of PLT and LRT (n = 1332). Patients assessed for acute liver failure (n = 175), familial amyloid polyneuropathy (43) and 24 with incomplete information were excluded. We analysed data on 1090 patients with end stage liver disease (ESLD) who were assessed for PLT.

Data

All patients assessed for liver transplantation at our centre had their clinical, laboratory, radiological and histological data as well as the outcome of transplant assessment entered at the time of liver transplant assessment into a prospective electronic database. This database was analysed in addition to electronic patient records and clinical notes to record demographic, clinical and laboratory variables of this cohort. Prognostic scores such as MELD and United Kingdom model for end- stage liver disease (UKELD) scores were calculated at the time of assessment and at the time of transplantation. MELD was calculated according to the UNOS adjustment[15]. The UKELD score was calculated according to Barber *et al*[16]. Donor and graft variables were collected and donor risk index was calculated according to Feng *et al*[17]. Patient survival was recorded according to their survival status in our hospital information system and further confirmed using the National Health System electronic portal. This is a United Kingdom wide national database, where patient survival status is updated according to the generation of death certificates in the United Kingdom.

Definitions of outcome measures

WL outcome was defined for this study by death on WL or delisting because of significant deterioration or hepatocellular carcinoma (HCC) progression beyond Milan criteria whilst awaiting LT. To study the influence of comorbidity and other variables on listing outcome, we used the transplant free survival (defined as time from listing to death, time to delisting or time to transplant) to eliminate the artificial impact of transplantation on survival of this cohort. Post-transplant patient survival was defined as time from transplantation to death, and if alive, censored on 01/11/2011. Graft survival was defined as time from transplantation to retransplantation or death, and if alive censored on 01/11/2011. Patients who were lost to follow-up were censored as being alive at the date of their last follow-up. Post-LRT patient survival was defined as time from second or subsequent transplant to death, and if alive, censored on 01/11/2011. Post-LRT graft survival was defined as time from second or subsequent transplant to further retransplantation or death, and if alive censored on 01/11/2011. One-year post transplant patient survival was defined as time from LRT to death, and if alive, censored at 12 mo following transplantation. One-year post transplant graft survival was defined as time from transplantation to retransplantation or death, and if alive censored at 12 mo following transplantation. Marginal grafts were defined as graft with Donor Risk Index > 1.8[7]. Cut off values for MELD score of 18 and 25 were chosen according to Rosen *et al*[18] and Edwards and Harper[19].

Comorbidities

Nine comorbidities were prospectively defined according to Volk *et al*[14]. These included congestive heart failure, coronary artery disease, DM, peripheral vascular disease, cerebro-vascular disease, chronic pulmonary disease, connective tissue disease, RI and malignancy. DM was defined as a chronic hyperglycaemia requiring medication use at any time during the month preceding transplant assessment. RI was defined as serum creatinine of $\geqslant$ 1.5 mg/dL ($\geqslant$ 132 μmol/L) on transplant assessment, being on renal replacement therapy or history of renal transplantation. Congestive heart failure was defined as documented decrease in left ventricular function on echocardiogram or left ventricle angiogram; or increased pulmonary artery pressure of $\geqslant$ 25 mmHg on echocardiogram or on invasive pulmonary artery pressure study, including patients with porto-pulmonary hypertension. Coronary artery disease was defined as documented history of myocardial infarction or abnormal coronary angiography. All patients underwent a functional cardiac assessment of ischemia either with Bruce protocol exercise tolerance test or cardio-pulmonary exercise test. Those with positive functional test but negative coronary angiogram were not considered as having coronary artery disease. Peripheral vascular disease was defined as documented history of peripheral ischaemia on angiography, abnormal ankle-brachial index or history of vascular bypass surgery. Cerebrovascular disease was defined as a history of stroke with residual neurological deficit. Chronic pulmonary disease was defined as chronic pulmonary disease requiring medication, a forced expiratory volume of < 1.5 L or history of intubation for respiratory failure. Connective tissue disease was defined as a rheumatologist diagnosis of rheumatoid arthritis, systemic lupus erythematosis, scleroderma or spondyloarthropathies excluding patients with arthralgia without evidence of inflammatory arthritis or those with osteoarthritis. Malignancy was defined as documented history of any malignancy excluding HCC or non melanoma skin cancers. To calculate the CCI, each comorbidity was assigned 1 point when present and was assigned 0 points when absent. The CCI was calculated as the sum of points of all 9 comorbidities. CCI was calculated at the time of assessment and at the time of transplantation.

Statistical analysis

Continuous variables were presented as median (range) and analysed using non-parametric methods (Mann Whitney-*U* or Kruskall Wallis test) for non-normally distributed data as appropriate. Categorical variables were presented as numbers (percentages) and analysed using χ^2 test or Fisher's exact test as appropriate. Cox proportional hazard analysis was used to identify factors associated with listing and transplant outcomes. Factors associated with outcome (*P*-value < 0.05) were entered into multivariate analysis. Collinearity diagnostics were used to determine whether variables

Table 1 Baseline characteristics for patients who had primary liver transplantation and those who had liver retransplantation

Variables	PLT (n = 1090)	LRT (n = 150)	P value
Demographic			
Age	54 (18-82)	46 (18-72)	< 0.001
Gender (male, %)	736 (67.5)	80 (53.3)	0.001
Etiology			
ALD (%)	345 (31.7)	18 (12.0)	< 0.001
Viral (%)	303 (27.8)	50 (33.3)	0.159
Cholestatic and autoimmune (%)	227 (20.8)	34 (22.7)	0.604
Clinical			
Na, mmol/L	135 (116-151)	138 (118-150)	< 0.001
Creatinine, mg/dL	1.0 (0.4-6.8)	1.3 (0.7-8.3)	< 0.001
Bilirubin, mg/dL	2.7 (0.2-68.4)	4.7 (0.4-56.3)	< 0.001
INR	1.3 (0.8-5.0)	1.2 (0.8-13.0)	0.078
MELD	14 (6-40)	20 (6-40)	< 0.001
UKELD	55 (43-77)	56 (44-79)	0.041
Ascites (%)	669 (62.9)	38 (25.5)	< 0.001
Encephalopathy (%)	350 (33.0)	52 (34.9)	0.637

ALD: Alcohol-related liver disease; INR: International normalised ratio; MELD: Model for end-stage liver disease; UKELD: United Kingdom end-stage liver disease model; PLT: Primary liver transplantation; LRT: Liver retransplantation.

entered into a model were collinear. MELD, UKELD, Child-Turcotte-Pugh (CTP), renal impairment and sodium level showed high collinearity (variance inflation factor - VIF > 5). Once MELD and UKELD were removed of the model, CTP, sodium level and renal impairment and all other individual comorbidities showed no collinearity (VIF < 3). Kaplan-Meier analysis was performed to assess survival outcomes. Statistical analyses were performed with SPSS software (SPSS® 17.0 for Windows ®SPSS Inc, Chicago, IL, United States).

RESULTS

Patient characteristics

One hundred and fifty assessments for LRT were examined and compared to a control group of 1090 patients assessed for PLT. Median follow-up was 7 years (3-12). There were 124 assessments for a second transplant, 21 assessments for a third transplant, 3 assessments for a fourth transplant, and 1 assessment each for a fifth and sixth transplant out of 150 LRT assessments. Out of these150 assessments for LRT, six were not listed for LRT (two because of early referral, 1 because of alcohol abuse, 1 declined re-listing, 1 with complete porto-mesenteric thrombosis and 1 died during the assessment process. Only121 patients received LRT of the144 listed patients. Twenty three patients were delisted for the following reasons: 12 died awaiting a graft, 6 had significant clinical improvement and 5 were delisted because of significant clinical deterioration, whilst on WL. Information regarding mechanical ventilation, renal replacement therapy, vasopressor support and location of patient [home, hospital or intensive care unit (ICU)] prior to LRT was available on 113 patients. Thirty two patients (28%) received renal replacement therapy, 21 (19%) received mechanical ventilation and 20 (18%) received vasopressor support prior to LRT. Forty four patients (36%) were transplanted from the hospital ward, 40 (33%) were transplanted from ICU and 28 (23%) were transplanted from home.

Table 1 summarises baseline characteristics according to PLT and LRT. LRT patients were significantly younger and were less likely to have ascites. However, this group were more likely to have higher median serum sodium levels (Na), creatinine values, bilirubin levels, MELD and UKELD scores (P < 0.05). There were no significant differences in proportion of patients with encephalopathy or median INR level between groups (P = NS).

Indications for LRT

The most common indication for LRT was vascular complications (thrombotic and non-thrombotic infarction of the graft) in 49 (33%) followed by graft rejection in 40 (27%), disease recurrence in 35 (23%), early graft dysfunction in 18 (12%) and 8 for other indications (5%). There were 30 patients (20%) who had biliary complications; however, only 3 (10%) patients developed graft failure secondary to biliary complications. Biliary strictures following PLT (anastomotic, hilar, papillary stenosis) were managed endoscopically, except for 2 patients who required per cutaneous biliary interventions. Eventually, 8 patients (27%) underwent biliary reconstruction for definitive management of post-transplant biliary complications. Thirty seven (78%) of patients with vascular-related complications had hepatic artery thrombosis, 9 (18%) had non thrombotic graft infarction, 1 (2%) had veno-occlusive disease and 1 (2%) had portal vein thrombosis resulting in graft infarction.

Comorbidities

There were 84 patients (56%) who had ⩾ 1 comorbidity as defined by CCI. The most common comorbidity was RI in 66 (44.3%), followed by DM in 25 (16.8),

Table 2 Factors associated with waiting list mortality in liver retransplantation patients on univariate and multivariate Cox proportional hazard analysis

Variable	Univariate			Multivariate		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age > 60 yr	2.959	0.550-3.896	0.048	3.102	1.015-9.484	0.047
DM	1.587	0.499-5.042	0.434			
Renal impairment	4.771	1.496-15.217	0.008	3.802	1.147-12.603	0.029
CCI continuous	3.121	1.589-6.130	0.001	2.688	1.222-5.912	0.014
CCI dichotomous	6.528	1.472-28.962	0.014	5.475	1.177-25.464	0.030
Hb, g/dL	0.755	0.545-1.047	0.092			
Platelet count, $\times 10^9$/mL	0.994	0.986-1.001	0.090			
Bilirubin, mg/dL	1.012	0.979-1.045	0.481			
Creatinine, mg/dL	3.200	1.888-5.421	< 0.001	2.691	1.261-5.740	0.010
INR	1.489	1.055-2.102	0.024	1.406	0.967-2.044	0.075
Encephalopathy	2.049	0.620-6.770	0.239			
Ascites	2.781	1.006-7.682	0.049			
MELD	1.154	1.067-1.248	< 0.001	2.691	1.261-5.740	0.01
MELD ⩾ 18	3.827	1.190-12.315	0.024	4.369	1.255-15.215	0.021
Na, mmol/L	0.945	0.870-1.027	0.180			
UKELD	1.121	1.029-1.220	0.009	1.117	1.037-1.204	0.003

DM: Diabetes mellitus; CCI: Charlson comorbidity index; Hb: Haemoglobin; INR: International normalised ratio; MELD: Model for end-stage liver disease; Na: Serum sodium; UKELD: United Kingdom end-stage liver disease model.

chronic pulmonary disease in 2 (1.3%) and 1 patient (0.7%) for each of cerebrovascular disease, connective tissue disease and history of previous malignancy. None of the patients had coronary artery disease, congestive heart failure or peripheral vascular disease according to CCI definitions. There was higher percentage of patients who died on the WL or delisted with ⩾ 1 comorbidity compared to those without any comorbidity (76% *vs* 53%, $P = 0.044$). There was a higher percentage of patients with ⩾ 1 comorbidity in those assessed for LRT compared to those assessed for PLT (56% *vs* 43%, $P = 0.002$). The CCI (HR = 2.688, 95%CI: 1.222-5.912, $P = 0.014$) and the presence of any comorbidity (HR = 5.475, 95%CI: 1.177-25.646, $P = 0.030$) were independently associated with WL mortality on Cox proportional hazard analysis (Table 2). Only DM and RI as individual comorbidities were included in the Cox model because of the infrequency of other comorbidities in this cohort. RI (HR = 3.802, 95%CI: 1.147-12.603, $P = 0.029$) was independently associated with WL mortality. WL mortality in patients with any comorbidity was higher compared to those without comorbidities as shown in Figure 1.

With regards to post-transplant outcomes, the CCI (HR = 2.823, 95%CI: 1.563-5101, $P = 0.001$) and the presence of comorbidity (HR = 2.870, 95%CI: 1.306-6.307, $P = 0.009$) were independently associated with 12-mo patient and graft survival post-LRT on Cox proportional hazard analysis (Table 3).

WL mortality

Sixteen out of 144 patients (11%) died awaiting a graft. Eight had disease recurrence (of which 5 had HCV recurrence), 3 had vascular complications, 4 had graft rejection and 1 had other indication for LRT. None of the patients with early graft dysfunction died awaiting a graft. WL mortality for PLT was significantly higher compared to LRT (24% *vs* 11%, $P < 0.001$). However, median waiting time was significantly shorter for LRT compared to PLT (16 d, range: 0-1118 d *vs* 100 d, range: 1-922, $P < 0.001$).

Table 2 summarises variables associated with WL mortality on univariate and multivariate analysis. Only age > 60 years and the presence of ascites were included as fixed variables in the multivariate model to prevent interaction of variables with similar clinical relevance (such as creatinine, MELD, RI, comorbidity). Factors which were independently associated with WL mortality were age > 60 years, RI, creatinine level, the presence of comorbidity, CCI, MELD score and UKELD score. Figure 2 illustrates significantly increased 12 mo WL mortality in patients with MELD score ⩾ 18 on Kaplan Meier survival analysis (Log-Rank: 6.741, $P = 0.009$). Similar findings observed with MELD cut-off value of 25 (Log-Rank: 8.195, $P = 0.004$). WL mortality was not increased when comparing patients listed for their second graft ($n = 118$) to those listed for their third or more grafts ($n = 26$) (Log-Rank: 0.156, $P = 0.693$).

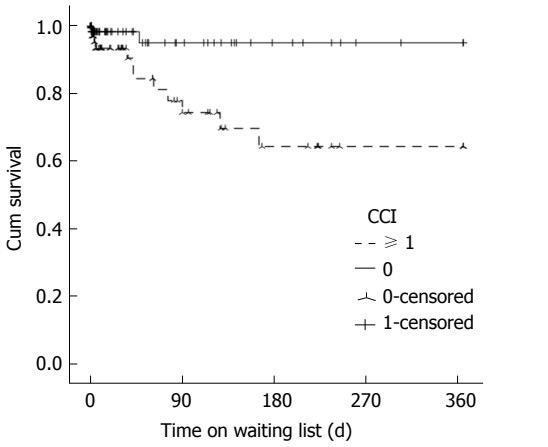
Post-transplant outcomes

The 1-, 3- and 5-year post-transplant patient and graft survival were significantly lower for patients who had LRT compared to those who had PLT. Figure 3 summarise these findings. In retransplanted patients, patient and graft survival were significantly different according to the number of grafts transplanted as analysed by Kaplan Meier survival method (Figure 4). The difference is mainly attributed to the inferior post-transplant survival of patients who had ⩾ 3 transplants. There was no significant difference in patient or graft survival between patients who had

Table 3 Univariate and multivariate analysis of factors associated with 1-year post-transplant patient and graft survival of retransplanted patients on Cox proportional hazard analysis

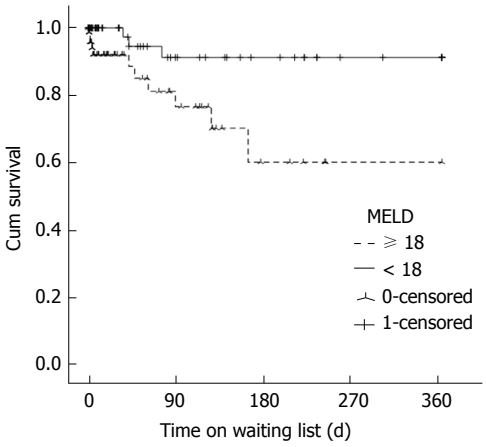
Variable	Patient survival			Graft survival		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Univariate analysis						
Age	0.796	0.973-1.021	0.997	0.992	0.969-1.016	0.515
Early graft dysfunction	2.143	0.919-4.998	0.078	1.788	0.776-4.123	0.173
DM	2.242	0.961-5.228	0.062	2.004	0.869-4.618	0.103
Renal impairment	4.385	2.133-9.017	< 0.001	3.494	1.759-6.941	< 0.001
CCI continuous	3.344	1.949-5.738	< 0.001	2.755	1.638-4.633	< 0.001
CCI dichotomous	3.56	1.691-7.493	0.001	2.751	1.377-5.494	0.004
Pre-LRT mechanical ventilation	3.044	1.461-6.342	0.003	2.456	1.210-4.983	0.013
Pre-LRT vasopressor support	4.714	2.239-9.928	< 0.001	3.618	1.758-7.443	< 0.001
Pre-LRT renal replacement therapy	4.233	2.029-8.829	< 0.001	3.271	1.630-6.562	0.001
Transplant from ICU	2.744	1.318-5.712	0.007	2.101	1.049-4.206	0.036
MELD score ⩾ 18	4.714	2.239-9.928	0.009	3.105	1.399-6.890	0.005
Encephalopathy at LRT	2.593	1.213-5.544	0.014	2.28	1.121-4.639	0.023
Hb, g/dL	0.791	0.629-0.994	0.044	0.792	0.636-0.985	0.037
ABO mismatch	2.37	1.015-5.532	0.046	2.338	1.053-5.190	0.037
Cold ischemia time (h)	1.113	0.987-1.255	0.082	1.081	0.962-1.216	0.191
DRI	0.68	0.236-1.963	0.476	0.693	0.250-1.918	0.476
DRI > 1.8	1.736	0.772-3.902	0.180	1.67	0.747-3.737	0.212
Multivariate analysis						
Renal impairment	3.215	1.147-12.603	0.005	2.543	1.160-5.573	0.020
CCI Continuous	2.823	1.563-5.101	0.001	2.350	1.331-4.148	0.003
CCI Dichotomous	2.87	1.306-6.307	0.009	2.223	1.067-4.633	0.033
Pre-LRT mechanical ventilation	2.52	1.126-5.637	0.024	2.099	0.968-4.552	0.060
Pre-LRT vasopressor support	4.004	1.554-10.314	0.004	3.023	1.216-7.514	0.017
Pre-LRT renal replacement therapy	2.691	1.261-5.740	0.01	2.441	1.107-5.383	0.027
Transplant from ICU	1.859	0.794-4.354	0.153	1.437	0.640-3.230	0.380
MELD score ⩾ 18	2.506	1.044-6.018	0.04	2.512	1.098-5.743	0.029
Encephalopathy at LRT	1.922	0.856-4.315	0.113	1.626	0.752-3.515	0.217
Hb, g/dL at LRT	0.883	0.694-1.125	0.314	0.883	0.698-1.116	0.297
ABO mismatch	1.795	0.739-4363	0.197	1.827	0.791-4.220	0.158

DM: Diabetes mellitus; LRT: Liver retransplant; ICU: Intensive care unit; CCI: Charlson comorbidity index; Hb: Haemoglobin; INR: International normalised ratio; MELD: Model for end-stage liver disease; DRI: Donor risk index.



No. at risk					
CCI = 0	63	60	60	60	60
CCI ⩾ 1	81	60	53	53	53

Figure 1 One-year waiting list survival according to the presence or absence of comorbidity. Log-Rank = 6.798, *P* = 0.009. CCI: Charlson comorbidity index.



No. at risk					
CCI < 18	63	58	58	58	58
CCI ⩾ 18	81	62	49	49	49

Figure 2 Waiting list survivals according to Model for end-stage liver disease score at listing (cut-off value of 18). Log-Rank = 6.741, *P* = 0.009. CCI: Charlson comorbidity index; MELD: Model for end stage liver disease.

PLT and patients who had a second transplant, Log-Rank = 1.741, *P* = 0.187 and Log-Rank = 2.225, *P* = 0.136, respectively. Patients who received ⩾ 3 grafts had significantly decreased 5-year survival of 40% compared to 72% in those who received 1 graft and 64% in patients who received 2 previous grafts (Log-Rank test: 13.737, *P* < 0.001).

With regards to the time interval between liver

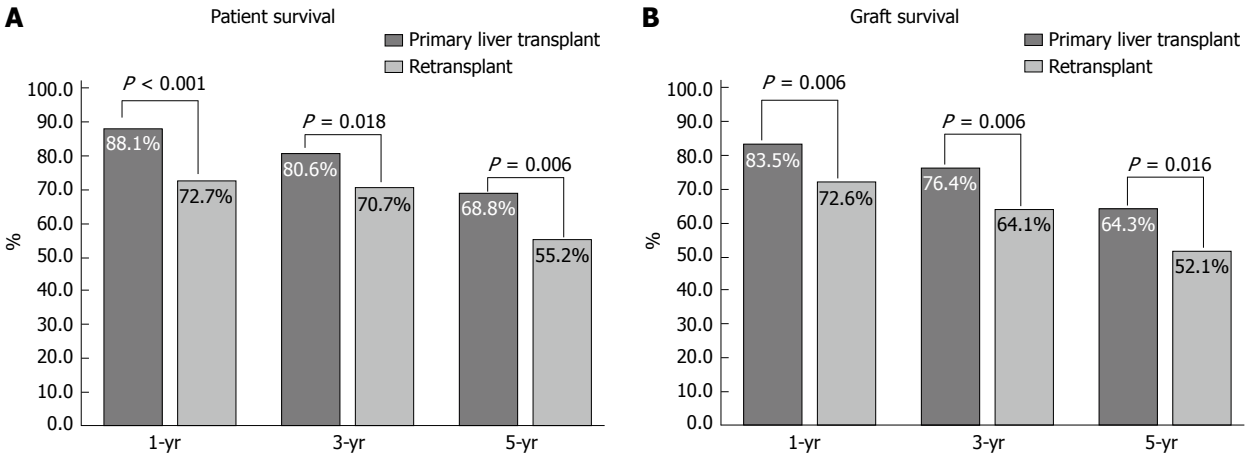
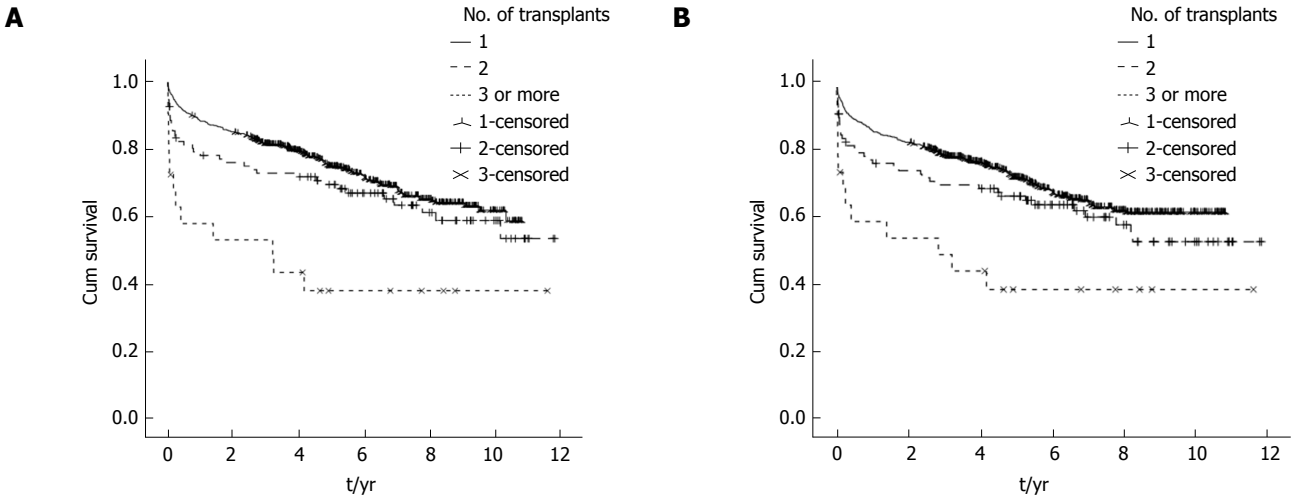


Figure 3 Post-transplant survival at 1, 3 and 5 years. A: Post-transplant patient survival for PLT and LRT; B: Post-transplant graft survival for PLT and LRT. LRT: Liver retransplantation; PLT: Primary liver transplantation.



No. at risk						
1	598	511	481	450	433	429
2	99	76	72	68	65	64
3 or more	22	12	10	9	9	9

No. at risk						
1	598	488	456	423	408	407
2	99	73	68	64	60	59
3 or more	22	12	10	9	9	9

Figure 4 Post-transplant survival according to the number of transplants. A: Kaplan Meier survial analysis of post-transplant patient survival according to the number of transplants. Log-Rank = 18.455, $P < 0.001$; B: Kaplan Meier survial analysis of post-transplant graft survival according to the number of transplants. Log-Rank = 13.737, $P = 0.001$.

transplantation and repeat transplant, patients who were transplanted between day 8-30 had the worse 1-year post transplant survival followed by those transplanted within the first 7 d. Patients who were transplanted > 30 d had significantly improved 1-year post-LRT survival (Log-Rank test: 6.952, $P = 0.031$). Table 4 summarises prognostic variables and indications for LRT according to time interval between transplants. Age, MELD at transplantation and the presence of comorbidity were not significantly different between the groups. Majority of patients who had LRT within 7 d of index transplant had early graft dysfunction. Majority of patients who had LRT 8-30 d of index transplant had vascular complications.

We performed Cox proportional hazard analysis to identify factors associated with post-transplant patient and graft survival in retransplanted patients. CCI (OR = 2.048, 95%CI: 1.294-3.241, $P = 0.002$), the presence of any comorbidity (HR = 1.920, 95%CI: 1.092-3.373, $P = 0.023$) and requirement for RRT (HR = 1.890, 95%CI: 1.044-3.424, $P = 0.036$) were associated with post-LRT patient survival on univariate analysis. Liver prognostic models (MELD, UKELD), donor or graft variables were not associated with patient survival in retransplanted patients. With regards to graft survival, only vasopressor support prior to LRT was associated with increased graft loss on univariate analysis (HR = 1.974, 95%CI: 1.033-3.744, $P = 0.04$).

Table 3 summarises variables associated with 1-year post-LRT patient and graft survival on Cox proportional hazard analysis. Only the presence of encephalopathy at LRT, Hb level at LRT and ABO

Table 4 Comparison of prognostic variables according to time interval of liver retransplantation

Time between transplants (d)	0-7 (n = 19)	8-30 (n = 16)	> 30 (n = 86)	P value
Age	54 (18-67)	44 (20-63)	43 (19-70)	0.086
Transplant MELD	22 (10-40)	17 (8-36)	17 (6-31)	0.104
CCI ⩾ 1, n (%)	10 (71)	11 (61)	44 (49)	0.095
Indication, n (%)				
Early graft dysfunction	14 (74)	2 (13)	2 (2)	< 0.001
Graft rejection	0 (0)	1 (6)	30 (35)	< 0.001
Vascular	5 (26)	13 (81)	23 (27)	< 0.001
Disease recurrence	0 (0)	0 (0)	24 (28)	0.001
Other indications	0 (0)	0 (0)	7 (8)	0.106

CCI: Charlson comorbidity index; MELD: Model for end stage liver disease.

mismatch were chosen as fixed variables in the model to avoid cross interaction between variables of similar clinical significance. The CCI, RI, pre-LRT mechanical ventilation, requirement for renal replacement therapy, vasopressor support and listing MELD ⩾ 18 were independently associated with 1-year patient survival. Similar findings were found for graft survival except for mechanical ventilation which was not associated with outcome.

Graft quality

Median donor age was 44 years (10-76), 55 (45.5%) of donors were males. Donor - recipient gender mismatch was seen in 45 cases (37.2%). Donor ethnicity was Caucasian in 117 (96.7%). Donor cause of death was trauma related in 20 donors (16.5%). Median donor height was 170 cm (147-196), median donor weight was 70 kg (28-110) and BMI was 24 kg/m^2 (16-34). Only 4 patients received split organs and 1 patient received a graft donated after cardiac death (DCD). Blood group mismatch was seen in 17 cases (14%). Median cold ischemia time was 10.55 h (0.92-19.53) and median DRI was 1.511 (1.0-2.8). There were 24 patients (20.2%) who received marginal grafts (DRI > 1.8). The DRI, or components of DRI in isolation, and DRI > 1.8 were not associated with 12-mo or long-term post-transplant patient or graft survival on Cox proportional hazard analysis.

DISCUSSION

The CCI was originally developed and validated as a tool to predict hospital outcome in general medical patients[20]. Composed of medical conditions with varying assigned weights, versions of CCI were found to predict outcomes in multiple clinical settings[21-26]. In this study, we reported on 150 episodes of assessment for LRT from a single centre. We demonstrated that comorbidity as defined by CCI is common (56%) in patients assessed for LRT, and higher than that reported for PLT (40%)[14]. This high prevalence of comorbidity is mainly attributed to the high prevalence of renal impairment (44%) in this cohort. It is difficult to estimate the rate of renal dysfunction in LRT patients from previously published studies[3,4,12,18]. RI was seen in 33% of candidates listed for PLT according to the data of the SRTR[7].

Renal dysfunction is a well recognised complication in patients with ESLD, critical illness and in PLT[27-30]. Renal impairment is known to have detrimental impact on survival of patients with ESLD[31,32]. Therefore, the increased prevalence of RI in our cohort can be explained by the fact that patients listed for LRT have more severe liver dysfunction, reflected by higher MELD scores compared to PLT patients and also by the large proportion of patients who were transplanted from ICU (33%) reflecting the severity of their illness. Furthermore, standard immunosuppression agents with Calcineurin inhibitors such as Ciclosporin or Tacrolimus which is routinely used following liver transplantation to prevent rejection are known to cause or at least contribute to renal impairment following liver transplantation[33]. Other comorbidities, apart from DM, were rare which may be explained by the relatively young median age of patients listed for LRT compared to PLT. The younger age of LRT patients compared to PLT is consistent with previous reports[18,34].

This is the first study to demonstrate the impact of comorbidity on WL mortality in LRT patients. The presence of any comorbidity defined by the CCI was independently associated with a greater than 5 times the risk of death on the wait list. Furthermore, this study has shown that the presence of any comorbidity was associated with twice the risk of post-LRT patient death. Similarly, comorbidity was associated with a three-fold increased risk of patient death and two fold increased risk of graft loss within 12 mo post-LRT. The only study to date which investigated the effect of comorbidity on post liver transplant outcome showed that the presence of any comorbidity was associated with 21% increase in patient death following PLT[14]. Comorbidity was also found to predict post-transplant outcome in patients who received renal and allogeneic stem cell transplantation[35-38].

We have demonstrated in this study that the median MELD score for patients assessed for LRT was significantly higher compared to PLT patients. We have also shown that the increase in MELD among

LRT candidates was attributable to the high median bilirubin and creatinine levels but not to an increase in INR which is consistent with UNOS data (Table 1)[34]. We have also shown that the already established models to assess the severity of hepatic impairment (MELD and UKELD) were independently associated with WL mortality. Furthermore, MELD score at a cut-off as low as 18 was associated with WL mortality which was increased by more than 4 fold. This suggests that patients listed for LRT with MELD score ⩾ 18 may benefit from prioritization on WL and earlier transplantation to improve LRT outcome.

Our data showed increased WL mortality in LRT patients with MELD score of 18 or higher. In a report from The University of Nebraska, Watt *et al*[39] showed that MELD score was predictive of WL mortality in 63 patients listed for a second transplant. WL mortality was also shown to increase with increasing MELD scores, especially at the lower range of MELD[34]. None of the other previously reported studies examined the performance of MELD in predicting WL mortality in LRT patients. Instead, these reports focused on factors predictive of post-LRT outcomes[2,3,5,11,18,40,41]. Surprisingly, WL mortality was lower for LRT patients (11% *vs* 24%, $P < 0.001$), discordant to previous reports[34]. This can be explained by the fact that patients listed for LRT had significantly shorter median waiting time (16 d *vs* 100 d, $P < 0.001$) which may indicate an informal prioritization mechanism for patients listed for LRT in our hospital. Our report also suggests that UKELD score retains its predictive capacity of WL mortality in patients listed for LRT with a 12% rise in WL mortality with every point increase in the UKELD score. Another important finding of the current study is that recipient age > 60 years was independently associated with death on the WL in LRT patients, consistent with previous studies that identified advanced recipient age as a risk factor for WL mortality in patients listed for PLT[28,42,43].

We have shown that 1-, 3- and 5-year patient and graft survival were inferior in patients who underwent retransplantation, consistent with previously published reports[5,13]. This inferior post-transplant survival in our cohort is mainly attributed to the poor post-LRT survival in patients who received ⩾ 3 grafts. Patients who had a second graft had slightly lower patient survival compared to PLT. Although these findings contrast with the outcome of patients who underwent retransplantation 1984-2001 at The University of California Los Angeles, improved survival of patients who had a second transplant in our cohort may be explained by both a different era of transplantation, advances made in immunosupression and local patient selection processes[13]. Our findings also suggest that a second liver transplant may represent an acceptable use of donated organs in selected patients. However, if we take into consideration the rule of 50% survival benefit at 5 years post-transplant, according to our findings a third or subsequent grafts may not represent an appropriate use of donated organs, except in rare instances[6].

Published reports suggested that the time interval between PLT and LRT has an influence on post-transplant outcome. Reports from 2 transplant programs indicated that LRT 4-30 d or 8-30 d following first transplant carries a worse post-transplant survival[11,13,40]. Our data showed inferior survival in patients who were transplanted within 30 d from previous liver transplantation, irrespective of whether LRT occurred in the first 7 d or between 8-30 d. In our cohort, the most common indication for LRT within the first 7 d following a previous transplant was early graft dysfunction whilst vascular complications (thrombotic and non-thrombotic graft infarction) were the primary indication in patients who had LRT 8-30 d following a previous transplant. This increased post-LRT mortality in patients who receive early LRT may be explained by severity of illness, intense immunosuppression, hence increased risk of infections[2,44]. Our findings are consistent with those of Rosen *et al*[18] who reported significantly inferior long term survival in patients who had LRT for PNF and vascular complications. In both United States and the United Kingdom, in recognition of the severity of illness and the high mortality associated with PNF and early HAT without LRT, an urgent priority for LRT is given[45,46].

Regarding post-LRT survival, we demonstrated that the CCI, RI, MELD score ⩾ 18 and requirement for organ support were independent factors associated with 1-year post-LRT patient and graft survival consistent with the reported literature in which MEDL, or individual components of MELD, were associated with post-LRT outcome[2,3,9,10,12,40,41]. Similarly, requirement for mechanical ventilation and renal replacement therapy were found to negatively impact on post-LRT outcome in agreement with the reported literature[2,5,12,40]. Interestingly, we identified pre-LRT vasopressor support as the only factor associated with long term graft outcome. Vasopressor use was also an independent factor associated with 12 mo post-transplant patient and graft survival. This finding has never been reported in previous studies. The requirement for vasopressors may therefore reflect the severity of recipient illness with hemodynamic instability and it may indirectly suggest the negative impact of graft ischemia on patient and graft survival.

Despite our detailed analysis of donor and graft variables, we found no association between graft quality and post-LRT outcomes. This is likely to reflect our local donor-recipient matching practices demonstrated by the limited use of marginal grafts in this cohort and a low median donor age of 44 years which is well within the confines of non-extended criteria donor parameters. Few studies analyzed the impact of graft and donor factors on post-LRT survival. Whilst Pfitzmann *et al*[5] found no correlation between graft survival and donor variables, others identified donor age, ethnicity and warm ischemia time as factors

independently associated with inferior outcome[5,10,40].

Limitations of this study were that it represents a single centre experience; therefore, applicability of the findings on other cohorts may be limited. Secondly, data on immunosuppression were not included in our analysis, although standard immunosuppression was used in all cases except for patients with eGFR < 50 mL/min, a renal sparing regimen of low dose Tacrolimus and interleukin-2 (IL-2) blocker and prednisolone was used preferentially. Indeed, the choice of immunosuppression not only can influence post-transplant outcomes in patients underwent PLT, it can influence the rate of complications related to immunosuppression such as RI which we found as an important factor associated with inferior patient and graft survival[33,47,48]. Thirdly, we used a version of the CCI tested in liver transplant cohort[14]. Therefore, the impact of other comorbidities on post-transplant survival such as inflammatory bowel disease, peptic ulcer disease, valvular heart disease or obesity, which were found to affect patient survival, remains unknown given they were not incorporated in the model[20,49]. Lastly, although the definitions of individual comorbidities were consistent with previous reports, clinical applicability of these definitions maybe limited.

In conclusion, our data indicates that the presence of comorbidity in liver retransplant candidates increases mortality on the WL and following LRT. The severity of recipient liver disease was associated with WL mortality. MELD score was able to discriminate between survival and death whilst on the WL at a lower cut-off value of 18 which suggests that patients undergoing LRT should be transplanted at lower MELD scores. Post-transplant mortality progressively increased according to the number of transplanted grafts; however, the greatest adverse impact was seen after transplanting ⩾ 3 grafts with only 40% 5-year survival seen in this group. Graft and donor variables were not found to influence patient or graft survival in this study which may reflect centre-related donor-recipient matching.

COMMENTS

Background

The prevalence and impact of comorbidities on waiting list (WL) and post-transplant survival in patients undergoing liver retransplantation (LRT) is not known. This study evaluates the impact of comorbidity on the above parameters.

Research frontiers

Model for end-stage liver disease (MELD) score > 25, recipient age, creatinine level, bilirubin level, indication for retransplantation, the urgency for LRT, coma episodes, haemoglobin level and the number of fresh frozen plasma units transfused were identified as factors associated with reduced post-LRT survival in a number of studies.

Innovations and breakthroughs

Comorbidity in liver retransplant patients increases mortality on the WL and following LRT. MELD score of ⩾ 18 was associated with increased risk of death on WL and within 12 mo following retransplantation. Post-transplant mortality progressively increased according to the number of transplanted grafts.

Applications

Patients undergoing LRT should be transplanted at lower MELD scores. Assessment of comorbidity in LRT candidates can provide important prognostic information. A third or subsequent grafts may not represent an appropriate use of donated organs, except in rare instances.

Terminology

ALD: Alcohol-related liver disease; CCI: Charlson comorbidity index; CI: Confidence interval; CIT: Cold ischemia time; DBD: Donation after brain death; DCD: Donation after cardiac death; DM: Diabetes mellitus; DRI: Donor risk index; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus infection; ICU: Intensive care unit; IL-2: Interleukin-2; INR: International normalized ratio; LRT: Liver retransplantation; MELD: Model for end-stage liver disease; Na: Sodium; NHS: National Health Services; OR: Odds ratio; PLT: Primary liver transplantation; RI: Renal impairment; SRTR: Scientific Registry of Transplant Recipients; UCLA: University of California Los Angeles; UKELD: United Kingdom End-stage Liver Disease; UNOS: United Network of Organ Sharing; WL: Waiting list.

Peer-review

Very well written paper.

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ORIGINAL ARTICLE

Prospective Study

Bacterial infections post-living-donor liver transplantation in Egyptian hepatitis C virus-cirrhotic patients: A single-center study

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Abstract

AIM

To determine risk factors, causative organisms and

antimicrobial resistance of bacterial infections following living-donor liver transplantation (LDLT) in cirrhotic patients.

METHODS

This prospective study included 45 patients with hepatitis C virus-related end-stage liver disease who underwent LDLT at Ain Shams Center for Organ Transplant, Cairo, Egypt from January 2014 to November 2015. Patients were followed-up for the first 3 mo after LDLT for detection of bacterial infections. All patients were examined for the possible risk factors suggestive of acquiring infection pre-, intra- and post-operatively. Positive cultures based on clinical suspicion and patterns of antimicrobial resistance were identified.

RESULTS

Thirty-three patients (73.3%) suffered from bacterial infections; 21 of them had a single infection episode, and 12 had repeated infection episodes. Bile was the most common site for both single and repeated episodes of infection (28.6% and 27.8%, respectively). The most common isolated organisms were gram-negative bacteria. *Acinetobacter baumannii* was the most common organism isolated from both single and repeated infection episodes (19% and 33.3%, respectively), followed by *Escherichia coli* for repeated infections (11.1%), and *Pseudomonas aeruginosa* for single infections (19%). Levofloxacin showed high sensitivity against repeated infection episodes (P = 0.03). *Klebsiella*, *Acinetobacter* and *Pseudomonas* were multi-drug resistant (MDR). Pre-transplant hepatocellular carcinoma (HCC) and duration of drain insertion (in days) were independent risk factors for the occurrence of repeated infection episodes (P = 0.024).

CONCLUSION

MDR gram-negative bacterial infections are common post-LDLT. Pre-transplant HCC and duration of drain insertion were independent risk factors for the occurrence of repeated infection episodes.

Key words: Living-donor liver transplantation; Bacterial infection; Multi-drug resistance; Hepatitis C virus; Liver cirrhosis

Core tip: We evaluated 45 patients with hepatitis C virus-related end-stage liver disease for the occurrence of bacterial infections during the first 3 mo post-living-donor liver transplantation. Thirty-three patients (73.3%) suffered from bacterial infections; 21 of them had a single infection episode, and 12 had repeated infection episodes. Bile was the most common site for both single and repeated episodes of infection (28.6% and 27.8%, respectively). Multi-drug resistant gram-negative bacteria, especially *Klebsiella*, *Acinetobacter* and *Pseudomonas*, were the most commonly isolated bacteria. Pre-transplant hepatocellular carcinoma and duration of drain insertion were independent risk factors for occurrence of repeated infection episodes.

Montasser MF, Abdelkader NA, Abdelhakam SM, Dabbous H, Montasser IF, Massoud YM, Abdelmoaty W, Saleh SA, Bahaa M, Said H, El-Meteini M. Bacterial infections post-living-donor liver transplantation in Egyptian hepatitis C virus-cirrhotic patients: A single-center study. *World J Hepatol* 2017; 9(20): 896-904 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i20/896.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i20.896

INTRODUCTION

Infection following living-donor liver transplantation (LDLT) is a serious problem with a high mortality rate reaching 50%. Many factors were associated with high risks of acquiring infection following LDLT, including the difficulty of surgery, the poor patient's condition, and the immunosuppressive drugs[1].

Nearly 80% of recipients develop one infection episode during the first year, predominantly during the first three months post-transplant. Bacterial infections account for 50%-75% of infections post-LDLT and commonly occur in the first month post-transplant[2].

Patients may become infected with antimicrobial-resistant bacteria, especially methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecalis*, *Clostridium difficile*, and gram-negative bacteria[3]. Currently, multidrug-resistant (MDR) organisms are the most common cause of nosocomial infections in liver transplant recipients[1].

Multiple organism infection is common as well as concurrent infections caused by different infectious agents[4]. Infections are usually difficult to diagnose because the usual manifestations of infection, such as fever and leukocytosis, may be absent and because of the need to exclude an acute rejection episode[5].

The aim of the present study was to determine risk factors, causative organisms and antimicrobial resistance patterns of bacterial infections following LDLT in Egyptian cirrhotic patients.

MATERIALS AND METHODS

Forty-five adult patients with hepatitis C virus (HCV)-related end-stage liver disease (ESLD) who were eligible for and underwent LDLT at Ain Shams Center for Organ Transplant, Cairo, Egypt, during the period from January 2014 to November 2015, were included in the current prospective study. They were followed-up for the first 3 mo post-LDLT for detection of bacterial infections.

Patients with other etiologies for ESLD (hepatitis B virus, primary biliary cirrhosis, and others) and patients with pre-operative infections, infections within 48 h after transplantation or early post-operative death

were excluded.

Each patient provided an informed written consent prior to enrollment. The study protocol was accepted by the Research Ethical Committee of the Faculty of Medicine-Ain Shams University. This was in accordance to the ethical guidelines of the 1975 Declaration of Helsinki.

Immunosuppressive drugs

Immediately following liver transplantation (LT), we used triple-therapy of immunosuppressive drugs which was comprised of a steroid, a calcineurin inhibitor: Cyclosporine or tacrolimus, and mycophenolate mofetil. In patients with renal dysfunction, immunosuppression with monoclonal antibodies to T-cells was used. In patients with hepatocellular carcinoma (HCC), tacrolimus monotherapy was used to decrease the incidence of HCC recurrence.

Antimicrobial prophylaxis

Piperacillin/tazobactam 4.5 mg/d was used post-operatively for 5 d. A polymerase chain reaction (PCR) assay for cytomegalovirus (CMV) was done every two weeks until patient's discharge. Ganciclovir for prevention of CMV disease was used if the PCR assay was positive.

Checked parameters

All patients were checked for the following parameters: (1) pre-operatively: Demographic data, other co-morbidities, presence of HCC, any bridging techniques, Child and MELD scores, CBC with differential cell count, liver profile, C-reactive protein, serum ferritin, documented or suspected SBP and third generation cephalosporin administration, renal impairment, and positive cultures; (2) intra-operatively: Total operative period, cold and warm ischemia time, amount of transfused blood or blood products and type of biliary anastomosis; and (3) post-operatively: Intensive care unit stay, ventilator duration, duration of central venous line and catheter insertion, duration of abdominal drain placement, dialysis post-transplant and immunosuppressive drugs.

Case identification

Post-operative infection was defined as any positive culture, based on clinical suspicion, within 3 mo following LDLT, according to the Centers for Disease Control and Prevention's definition of a nosocomial infection and as described in liver transplant recipients[6,7]. The diagnosis of wound infection was established by the presence of redness/induration and the presence of pus on exploration and/or positive wound culture. The diagnosis of urinary tract infection was based upon the following criteria: The patient has at least one of the following symptoms or signs with no other identified cause: fever (> 38 ℃), dysuria, frequency, urgency, suprapubic or costovertebral angle pain or tenderness, as well as a positive urine culture, that is, $\geqslant 10^5$ CFU/mL of urine with no more than 2 species of microorganisms. The diagnosis of pneumonia was based upon the presence of pulmonary infiltrates together with clinical symptoms indicating lower respiratory tract infection, the identification of a relevant etiologic microorganism, and the absence of another possible diagnosis during the follow-up. Bloodstream infection was diagnosed when microorganisms were isolated from one blood culture. Ascitic fluid cultures were performed for all patients with manifestations of bacterial peritonitis or who were suspected of having bacterial peritonitis. Samples were collected before the start of any anti-microbial treatment. Bile samples were withdrawn for those suspected of having a biliary tract infection. In cases of suspected sepsis-induced cholestasis, cultures from blood, the biliary tube, abdominal drains, urine, and sputum were collected, and culture based-treatment was started accordingly.

The term multidrug-resistant (MDR) was used to refer to pathogens resistant to three or more classes of the following antibiotics: extended-spectrum penicillins, 3rd generation cephalosporins, quinolones, carbapenems, and aminoglycosides[8].

Recruited patients were divided into two groups. Group 1 included patients who had a single episode of post-operative bacterial infection, and Group 2 included those patients who had more than one episode of a bacterial infection.

Statistical analysis

Statistical analyses were performed using IBM© SPSS© Statistics version 22 (IBM© Corp., Armonk, NY, United States). Continuous numerical variables were shown as the mean and standard deviation, and differences between groups were compared using the unpaired *t* test. Discrete numerical variables were shown as the median and interquartile range, and the Mann-Whitney test was used to compare intergroup differences. Categorical data were shown as ratios or as the number and percentage, and differences between groups were compared using Pearson's χ^2 test or Fisher's exact test. Variables shown to be significantly associated with the occurrence of repeated infection episodes by univariate analysis were entered in multivariate binary logistic regression analysis to identify independent predictors of this outcome. Time-to-event analysis was done using the Kaplan-Meier method, and the log-rank test was used to compare individual Kaplan-Meier curves. A *P*-value < 0.05 was considered significant.

The statistical methods for this study were performed by Sameh M. Hakim, Diploma of Medical Biostatistics, Faculty of Medicine of Ain Shams University, Cairo, Egypt.

RESULTS

The present study enrolled forty-five adult patients

Table 1 Comparison between patients who developed post-living-donor liver transplantation single episode and those who developed repeated episodes of infection regarding pre-operative parameters *n* (%)

	Single episode of infection (*n* = 21)	Repeated episodes of infection (*n* = 12)	*P* value
Recipient's age (yr, mean ± SD)	51.2 ± 8.3	52.08 ± 8.7	0.767
Donor's age (yr, mean ± SD)	26.9 ± 6.3	32.3 ± 6.1	0.021
Recipient's gender (male/female)	19/2	12/0	0.523
Donor's gender (male/female)	19/2	10/2	0.610
Hepatocellular carcinoma	6 (28.6)	8 (66.7)	0.033
History of bridging procedures[1]	4 (19.0)	6 (50.0)	0.114
History of SBP	10 (47.6)	1 (8.3)	0.052
History of paracentesis	11 (52.4)	4 (33.3)	0.290
Diabetes mellitus	8 (38.1)	5 (41.7)	1.000
Child-Pugh class (B/C)	10/11	5/7	0.741
MELD score (median, interquartile range)	14 (12-16)	16 (15-18)	0.136
Thrombocytopenia[2]	20 (95.2)	12 (100.0)	1.000
Leucopenia[3]	9 (42.9)	5 (41.7)	0.947
Renal impairment	2 (9.5)	1 (8.3)	1.000
High serum ferritin[4]	13 (61.9)	5 (41.7)	0.261
High C-reactive protein[5]	14 (66.7)	11 (91.7)	0.206

[1]Bridging procedures included: Radiofrequency ablation, trans-arterial chemo-embolization and micro-wave ablation; [2]Thrombocytopenia: Platelets less than 150000/mm^3; [3]Leucopenia: WBCs less than 4000/mm^3; [4]High serum ferritin: More than 333 ng; [5]High C-reactive protein: More than 0.5 mg/L. SBP: Spontaneous bacterial peritonitis.

with HCV-related ESLD, and each patient was followed-up for 3 mo post-LDLT for the occurrence of bacterial infections. Thirty-three patients (73.3%) suffered from bacterial infections post-transplant and fulfilled the inclusion criteria. They were further subdivided into two groups. Group 1 included 21 patients who developed a single episode of infection (19 males and 2 females), and Group 2 included 12 patients (all of them were males) who developed recurrent episodes of infection (total number of attacks = 36) throughout the follow-up period.

Table 1 shows the comparison between patients who developed a single episode of infection post-LDLT and those who developed repeated episodes of infection regarding pre-operative parameters. The presence of pre-transplant hepatocellular carcinoma (HCC) showed a statistically significant increased risk of developing repeated episodes of infection post-LDLT (P = 0.033).

There was no significant difference between patients who developed a single episode and those who developed repeated episodes of infection regarding the operative details (P > 0.05) (Table 2).

Table 3 shows that the duration of drain insertion revealed a statistically significant increased risk for the development of repeated episodes of infection (P = 0.002).

Table 4 shows that bile was found to be the most common site for both single and repeated episodes of infection (28.6% and 27.8%, respectively), followed by the bloodstream for repeated infection episodes (22.2%) and drains for a single infection episode (23.8%).

The most common isolated organisms were gram-negative bacteria for both single and repeated episodes of infections. *Acinetobacter baumannii* was found solely to be the most common organism isolated from both single and repeated infection episodes (19% and 33.3%, respectively), followed by *Escherichia coli (E. coli)* for repeated infections (11.1%), and *Pseudomonas aeruginosa* for a single infection (19%). Additionally, *Acinetobacter baumannii* was found in combination with other organisms in three cultures.

Table 5 shows the antimicrobial sensitivity pattern in patients who suffered from single *vs* repeated episodes of infection. The sensitivity of levofloxacin was found to be statistically significant against repeated episodes of infection (P = 0.03). Repeated episodes of infection showed 100% resistance to penicillins. Single episodes of infection were 100% resistant to ciprofloxacin and co-trimoxazole. Both single and repeated episodes of infections were 100% resistant to cefotaxime and aztreonam.

Regarding the pattern of resistance of isolated organisms to the major antibiotic groups, most of the isolated gram-negative organisms were found to be resistant to several groups of antibiotics; especially *Klebsiella* species, *Acinetobacter baumannii* and *Pseudomonas aeruginosa,* all of which were proven to be MDR.

The detailed antibiotic-resistance pattern was as follows: For *Klebsiella* species, 100% of the isolates showed resistance to each of the quinolones and aminoglycosides, 87.5% showed resistance to cephalosporins, 80% to carbapenems, and 25% showed resistance to piperacillin-tazobactam. For *Acinetobacter baumannii*, 100% of the isolates showed resistance to aminoglycosides, 60% to carbapenems, 46.5% to quinolones, 42% to cephalosporins, and 33.3% showed resistance to piperacillin-tazobactam. For *Pseudomonas aeruginosa*, 100% of the isolates showed resistance to quinolones, and 83.3% showed

Table 2 Comparison between patients who developed single episode and those who developed repeated episodes of infection regarding operative details

	Single episode of infection (n = 21)	Repeated episodes of infection (n = 12)	P value
CIT (min), mean ± SD	43.6 ± 17.3	50.8 ± 17.7	0.259
WIT (min), mean ± SD	45.7 ± 13.4	50.8 ± 12.4	0.288
Recipient's operative time (h), mean ± SD	10.3 ± 1.1	10.5 ± 1.5	0.704
Packed red cell transfusion (U), (median, interquartile range)	2 (2-4)	3 (2-6)	0.493

CIT: Cold ischemia time; WIT: Warm ischemia time.

Table 3 Length of intensive care unit stay, length of exposure to invasive procedures, and time to occurrence of infection in patients who suffered from single *vs* repeated episodes of infection

	Single episode of infection (n = 21)	Repeated episodes of infection (n = 12)	P value
Length of ICU stay (d)	6 (5-7)	7 (5-7)	0.969
Days on mechanical ventilator	1 (1-1)	1 (1-1)	0.176
Days with CVC	6 (5-7)	6 (5-7)	0.770
Days with urinary catheter	6 (5-7)	7 (6-8)	0.467
Days with drains	17 (15-20)	25 (21-30)	0.002
Time-to-infection (d)	14 (12-17)	9 (6-19)	0.189

Data are presented as median (interquartile range). ICU: Intensive care unit; CVC: Central venous catheter.

Table 4 Site of infection and implicated organisms in patients who suffered from single *vs* repeated episodes of infection n (%)

	Single episode (n = 21)	Repeated episodes (n = 36)[1]	P value
Site of organism isolation			0.896
Bile	6 (28.6)	10 (27.8)	
Wound	1 (4.8)	2 (5.6)	
Sputum	3 (14.3)	7 (19.4)	
Drains	5 (23.8)	7 (19.4)	
Blood	3 (14.3)	8 (22.2)	
Urine	2 (9.5)	2 (5.6)	
Ascitic fluid	1 (4.8)	0 (0.0)	
Oragnism isolated			0.456
Goagulase (-) Staph. aureus	3 (14.3)	1 (2.8)	
Staph. aureus	0 (0.0)	1 (2.8)	
MRSA	3 (14.3)	1 (2.8)	
E. coli	2 (9.5)	4 (11.1)	
Klebsiella species	2 (9.5)	3 (8.3)	
Pseudomonas aeruginosa	4 (19.0)	3 (8.3)	
Acinetobacter baumannii	4 (19.0)	12 (33.3)	
Proteus	0 (0.0)	2 (5.6)	
Enterobacteriaceae	1 (4.8)	1 (2.8)	
Enterococci	1 (4.8)	2 (5.6)	
Bacillus species	0 (0.0)	2 (5.6)	
Pseudomonas + Acinetobacter	1 (4.8)	0 (0.0)	
Pseudomonas + Klebsiella	0 (0.0)	2 (5.6)	
Acinetobacter + Klebsiella	0 (0.0)	1 (2.8)	
Acinetobacter + coagulase (-) Staph. aureus	0 (0.0)	1 (2.8)	

[1]Represents the total number of attacks occurred among the 12 patients who developed repeated episodes of infections. MRSA: Methicillin-resistant *Staphylococcus aureus*.

resistance to cephalosporins. Meanwhile, 100% of them were sensitive to aminoglycosides, piperacillin-tazobactam and carbapenems. For *E. coli,* 70% of the isolates showed resistance to cephalosporins, 50% to quinolones, and 25% showed resistance to aminoglycosides. Moreover, 100% of them were sensitive to piperacillin-tazobactam and carbapenems.

Table 6 and Figure 1 show that the two variables identified by multivariate analysis as independent risk factors for the occurrence of repeated episodes of infection were HCC and the duration of drain insertion (in days) (P = 0.024 and odds ratio = 25.44 and 1.38, respectively).

The median time-to-infection was 14 d in the single infection episode group and 8.5 d in the repeated infection episodes group, with no significant difference observed between groups (P = 0.647) (Table 7 and Figure 2).

DISCUSSION

Infectious complications have become the most common sources of mortality and morbidity following LT. Multiple organism infection is common. The occurrence of infection following LT is due to the dysfunction of the patient's defensive mechanisms, as a result of liver cirrhosis and the use of immunosuppressant drugs[4].

The current study included 45 patients with HCV-related ESLD who were eligible for and underwent LDLT at Ain Shams Center for Organ Transplant, Cairo, Egypt during the period from January 2014 to November 2015. They were followed-up for the first 3 mo post-LDLT for the detection of bacterial infections.

In the current study, 73.3% of included patients developed a nosocomial bacterial infection in the first 3 mo post-LDLT. This finding is in agreement with previous reports, which denoted a high incidence of bacterial infections post-LDLT ranging from 50% to 75%[1,2].

Table 5 Antimicrobial sensitivity in patients who suffered from single *vs* repeated episodes of infection *n* (%)

Antimicrobial		All episodes of infection (*n* = 57)	Single episode of infection (*n* = 21)	Repeated pisodes of infection (*n* = 36)	*P* value
Levofloxacin	S	11 (52.4)	2 (22.2)	9 (75.0)	0.030
	R	10 (47.6)	7 (77.8)	3 (25.0)	
Ciprofloxacin	S	5 (38.5)	0 (0.0)	5 (45.5)	0.487
	R	8 (61.5)	2 (100.0)	6 (54.5)	
Co-trimoxazole	S	1 (7.1)	0 (0.0)	1 (10.0)	1.000
	R	13 (92.9)	4 (100.0)	9 (90.0)	
Penicillin	S	1 (11.1)	1 (14.3)	0 (0.0)	1.000
	R	8 (88.9)	6 (85.7)	2 (100.0)	
Doxycycline	S	14 (77.8)	5 (100.0)	9 (69.2)	0.278
	R	4 (22.2)	0 (0.0)	4 (30.8)	
Vancomycin	S	8 (88.9)	4 (100.0)	4 (80.0)	1.000
	R	1 (11.1)	0 (0.0)	1 (20.0)	
Piperacillin-tazobactam	S	8 (72.7)	3 (75.0)	5 (71.4)	1.000
	R	3 (27.3)	1 (25.0)	2 (28.6)	
Aminoglycosides	S	9 (75.0)	1 (50.0)	8 (80.0)	0.455
	R	3 (25.0)	1 (50.0)	2 (20.0)	
Imipenem	S	20 (69.0)	8 (88.9)	12 (60.0)	0.201
	R	9 (31.0)	1 (11.1)	8 (40.0)	
Ceftrioxone	S	7 (38.9)	1 (16.7)	6 (50.0)	0.316
	R	11 (61.1)	5 (83.3)	6 (50.0)	
Cefotaxime	R	8 (100.0)	7 (100.0)	1 (100.0)	-
Aztreonam	R	6 (100.0)	1 (100.0)	5 (100.0)	-

S: Sensitive; R: Resistant.

Table 6 Multivariate binary logistic regression model for prediction of the occurrence of repeated episodes of infection

	Regression coefficient	SE	Odds ratio	95%CI	*P* value
Donor's age (yr)	0.05	0.08	1.05	0.90-1.23	0.552
Hepatocellular carcinoma (HCC = 1, no HCC = 0)	3.24	1.43	25.44	1.53-422.21	0.024
Duration of drain insertion (d)	0.32	0.14	1.38	1.04-1.83	0.024
Constant	-10.28				
Model diagnostics					
-2 Log Likelihood test			*P* value, < 0.001		
Hosmer and Lemeshow test			*P* value, 0.369		
Correct classification rate			87.88%		
ROC curve analysis					
AUC			0.935 (95%CI: 0.791-0.991; *P* value < 0.0001)		
Sensitivity, %			91.7 (95%CI: 61.5-99.8)		
Specificity, %			81.0 (95%CI: 58.1-94.6)		
PPV, %			73.3 (95%CI: 43.8-92.7)		
NPV, %			94.4 (95%CI: 72.7-99.9)		

HCC: Hepatocellular carcinoma; AUC: Area under ROC curve; ROC: Receiver-operating characteristic; PPV: Positive predictive value; NPV: Negative predictive value.

In the current study, the presence of pre-transplant HCC was an independent risk factor for the occurrence of repeated episodes of bacterial infection in the recipients during the early post-transplant period. HCC patients are more susceptible to infection due to poor long-term nutrition, poor physical condition and weak immune system[1].

In the present study, the duration of time for abdominal drain placement was considered an independent risk factor for the development of repeated episodes of bacterial infection as confirmed by the multivariate binary logistic regression model. Patients with prolonged drain insertion time had an increased risk of developing recurrent episodes of infection compared to patients who had less drain insertion time.

Results in our study revealed that the major sites of bacterial infections in patients who experienced a single infection episode were as follows: Bile (28.6%), followed by the drains (23.8%), sputum (14.3%), bloodstream infections (14.3%), urine (9.5%) and lastly wound and ascitic fluid infection (4.8% each). These results were in accordance with another Egyptian multicenter study performed by Mukhtar *et al*[1]. In contrast, Kim *et al*[9] and Iida *et al*[10] revealed that the most dominant bacterial infection was bacteremia, which was catheter-related. El-Araby *et al*[11] showed that the main sites of infection were the chest (24.4%), followed by the bile duct or cholangitis (17.1%), and

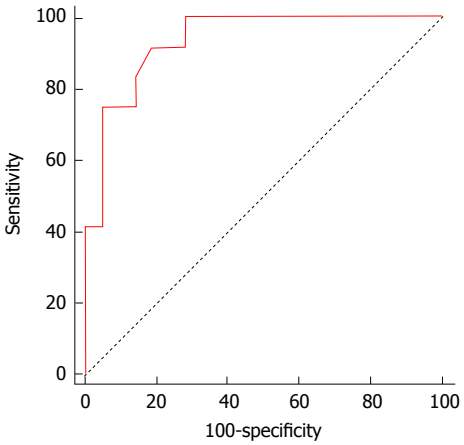

Figure 1 Receiver-operating characteristic curve derived from the multivariate binary logistic regression model for prediction of the occurrence of repeated episodes of infection. AUC = 0.935 (95%CI: 79.1%-99.1%; $P < 0.0001$); sensitivity: 91.7% (95%CI: 61.5%-99.8%); specificity: 81.0% (95%CI: 58.1%-94.6%); PPV: 73.3% (95%CI: 43.8%-92.7%); NPV: 94.4% (95%CI: 72.7%-99.9%). AUC: Area under ROC curve; PPV: Positive predictive value; NPV: Negative predictive value.

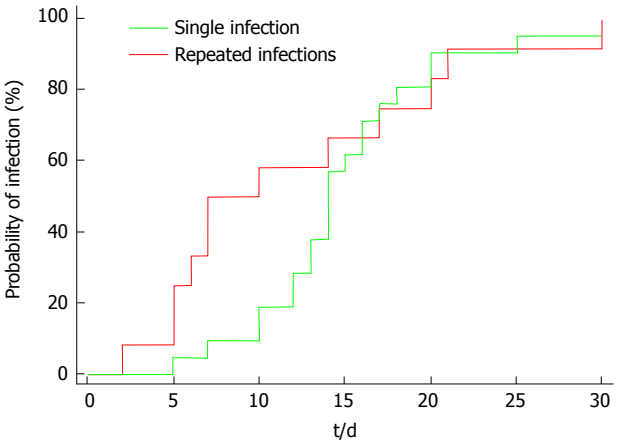

Figure 2 Kaplan-Meier curves for the time-to-infection in patients who suffered from single and repeated episodes of infection. HR = 1.16 (95%CI: 56%-92.4%; $P = 0.647$).

lastly the bloodstream (12.2%). However, Kawecki *et al*[12] revealed that the urinary tract was the main site of infection after LDLT. The discrepancies between the major sites of post-transplant infection between the different centers are most likely related to the variability of the hygienic measures, infection control programs, as well as the peri-, intra- and post-operative disparities.

In the current study, the most common isolated organisms were the gram-negative bacteria for both single and repeated episodes of infections, and these results were consistent with El-Araby *et al*[11] and Linares *et al*[13]. Shi *et al*[14] reported the same results and explained that the prevalence of gram-negative bacteria may be because these bacteria are inhabitants of the digestive tract. In the current study, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were found to be the most common organisms in the

Table 7 Results of the Kaplan-Meier analysis for time to infection in patients who suffered from single and repeated infection episodes

	Single infection episode (n = 21)	Repeated infection episodes (n = 12)
Median time to infection (d)	14 (95%CI: 13-16)	8.5 (95%CI: 6-17)
Hazard ratio	1.16 (95%CI: 56-2.40)	
Log-rank test	P value = 0.647	

single infection episode group (19% each), followed by methicillin resistant *Staphylococcus aureus (MRSA)* and coagulase-negative *Staphylococcus aureus* (14.3% each), and *Klebsiella* species and *E. coli* (9.5% each). These results were in accordance with Zhong *et al*[15]. However, Sganga *et al*[5] and Iida *et al*[10] concluded that *Pseudomonas aeruginosa* was the most common isolated organism.

At present, MDR organisms are the most common causes of nosocomial infections in post-LDLT patients. Zhong *et al*[15] found that MDR gram-negative bacilli were isolated in 56% of patients with gram-negative infection, which was in accordance with Shi *et al*[14], who stated that the three most common pathogens of MDR gram-negative bacilli were *Acinetobacter baumannii*, *E. coli* and *Klebsiella* species. This finding is not fully consistent with a previous report by Pappas *et al*[16] who found that the four most common MDR gram-negative bacilli were *E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Acinetobacter baumannii*. The difference in the findings between the studies was related to differences in patients' underlying diseases and nosocomial infections.

Our results are consistent to some extent with that of Mukhtar *et al*[1] in their retrospective multicenter Egyptian study on bacterial infections post-LDLT. The authors reported that *Pseudomonas aeruginosa* was the most commonly isolated species (26%), followed by *Klebsiella* (19%), *E. coli* (16%), *Acinetobacter baumannii* (8%), and *MRSA* (7.7%). In their study, 75% of the gram-negative bacteria were MDR, including 90% of *Acinetobacter baumannii* isolates, 76% of *Pseudomonas aeruginosa* isolates, 57% of *Klebsiella* species isolates, and 53% of *E. coli* isolates.

Our study revealed that most of the gram-negative organisms were found to be resistant to several groups of antibiotics, especially *Klebsiella* species, *Acinetobacter baumannii* and *Pseudomonas aeruginosa,* which proved to be MDR. In the study of Zhong *et al*[15], *Acinetobacter baumannii* displayed resistance to all antibiotic groups, including β-lactams, quinolones, and aminoglycosides and even showed high resistance to carbapenems, including 100% resistance to meropenem and imipenem. *E. coli* was found to be sensitive to aminoglycosides, carbapenems and piperacillin-tazobactam but showed a pattern of resistance to cephalosporins. Among all the antibiotics used in the current cohort, levofloxacin was found to be of

statistical significance regarding its sensitivity in the treatment of repeated episodes of infection.

It is worth mentioning that all infection episodes in our study occurred in the first month post-operative and by applying Kaplan-Meier analysis for time-to-infection. The median time-to-infection was 14 d in the single infection episode group and 8.5 d in the repeated infection episodes group. Similarly, previous studies have reported that the majority of bacterial infections occurred during the first month following LT[1,17].

In conclusion, MDR gram-negative bacterial infections are common post-LDLT. Pre-transplant HCC and duration of drain insertion are independent risk factors for the occurrence of repeated infection episodes.

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COMMENTS

Background

Bacterial infections are common following living-donor liver transplantation (LDLT), especially multiple-organism infections. The occurrence of infection following liver transplantation is due to the dysfunction of the patient's defensive mechanisms, as a result of liver cirrhosis and the use of immunosuppressant drugs.

Research frontiers

The authors assessed 45 patients with hepatitis C virus-related end-stage liver disease for the occurrence of bacterial infections during the first 3 mo post-LDLT. Thirty-three patients (73.3%) suffered from bacterial infections; 21 patients experienced a single episode of infection, and 12 patients experienced repeated episodes of infection. Bile was the most common site for both single and repeated episodes of infection (28.6% and 27.8%, respectively). Multi-drug resistant (MDR) gram-negative bacteria, especially *Klebsiella*, *Acinetobacter* and *Pseudomonas*, were the most commonly isolated bacteria. Pre-transplant hepatocellular carcinoma and duration of drain insertion were independent risk factors for the occurrence of repeated infection episodes.

Innovations and breakthroughs

This study is a single-center Egyptian study that addresses risk factors, causative organisms and antimicrobial resistance of bacterial infections following LDLT in cirrhotic patients.

Applications

The findings in this study may help in determining the proper antimicrobial prophylaxis for cirrhotic patients pre-LDLT.

Terminology

MDR was used to refer to pathogens resistant to three or more classes of the following antibiotics: Extended-spectrum penicillins, 3rd generation cephalosporins, quinolones, carbapenems, and aminoglycosides.

Peer-review

Acceptance of this manuscript for publication is recommended.

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LETTERS TO THE EDITOR

Changing landscape of hepatitis C virus-positive donors

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Abstract

With the introduction of the new highly effective antiviral therapies, there has been a dramatic increase in the use of the hepatitis C virus (HCV)-positive livers in HCV-positive recipients. In the majority of studies, HCV positivity was defined as a donor testing HCV Ab positive. In 2015, all Organ Procurement Organizations were mandated to perform and report HCV Nucleic Acid Amplification Testing (NAT) results on all deceased and living donors. Studies are not yet available on how organs are being utilized based on NAT status and whether NAT status affects recipient outcomes. Further studies are needed to maximize the use of these organs.

Key words: Heptitis C organ utilization; Hepatitis C virus aviremic; Liver transplantation; Hepatitis C positive recipients

Core tip: For many years hepatitis C virus (HCV) positive livers have been used with caution in carefully selected mostly HCV-positive patients. With the introduction of the new highly effective antiviral therapies discard rate of HCV-positive livers, although improved, continues to be high. On August 10, 2015, the United Network for Organ Sharing mandated all Organ Procurement Organizations to perform and report HCV Nucleic Acid Amplification Testing (NAT) results on all deceased and living donors. We believe further research in the outcome of viremic and aviremic HCV livers is needed so that the utilization of these organs can be maximized in HCV NAT + and potentially HCV NAT - recipients.

Kling CE, Limaye AP, Sibulesky L. Changing landscape of hepatitis C virus-positive donors. *World J Hepatol* 2017; 9(20): 905-906 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i20/905.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i20.905

TO THE EDITOR

In the face of liver graft shortage, increasing numbers

of extended criteria or marginal grafts are being used. Such grafts include those from donors after circulatory death, older donors, livers with steatosis, and livers from donors infected with hepatitis C. For many years, hepatitis C (HCV) positive livers have been used with caution in carefully selected mostly HCV positive patients.

In the recent study Bowring *et al*[1] noted that with the introduction of the new highly effective antiviral therapies, there has been a dramatic increase, from 6.9% to 16.9%, in the use of the HCV-positive livers in HCV-positive recipients. The authors demonstrated that the allograft survival in HCV-positive recipients was similar for patients who received an HCV-positive liver and those who received an HCV-negative liver. Despite a better use of these organs, the reluctance to utilize these livers continues, demonstrated by the 1.7 times higher discard rate when compared to non-infected liver allografts[1].

In the majority of studies, HCV positivity is defined as a donor testing HCV Ab positive. However, there is variability among HCV Ab positive donors - some donors are actively viremic and hence are HCV Ab positive and RNA positive by Nucleic Acid Amplification Testing (NAT), while others are Ab positive but aviremic and NAT negative. Approximately 10%-25% of people will spontaneously clear the virus without treatment[2,3] and thus would be Ab positive NAT negative. Other donors have cleared the virus with treatment. Sustained virologic response, defined as aviremia 24 wk after completion of antiviral therapy for chronic HCV infection, would also result in Ab positive NAT negative serostatus, and relapse and thus transmission of infection is expected to be minimal.

On August 10, 2015, the United Network for Organ Sharing mandated all Organ Procurement Organizations perform and report HCV NAT results on all deceased and living donors[4]. As a result, transplant centers must specify whether candidates who are listed as accepting livers from HCV Ab positive donors are willing to accept organs from NAT positive and/or NAT negative donors. Studies are not yet available on how organs are being utilized based on NAT status and whether NAT status affects recipient outcomes, but given the difference in viremic status between the two populations, there likely is a difference.

As a result of these changes in donor testing and recipient listing, and in the era of new DAA therapies, we believe further research in the outcome of viremic and aviremic HCV livers is needed so that the utilization of these organs can be maximized in HCV NAT + and potentially HCV NAT-recipients.

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REVIEW

Chemotherapy for hepatocellular carcinoma: The present and the future

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary tumor of the liver. Its relationship to chronic liver diseases, in particular cirrhosis, develops on a background of viral hepatitis, excessive alcohol intake or metabolic steatohepatitis, leads to a high incidence and prevalence of this neoplasia worldwide. Despite the spread of HCC, its treatment it's still a hard challenge, due to high rate of late diagnosis and to lack of therapeutic options for advanced disease. In fact radical surgery and liver transplantation, the most radical therapeutic approaches, are indicated only in case of early diagnosis. Even local therapies, such as transarterial chemoembolization, find limited indications, leading to an important problem regarding treatment of advanced disease. In this situation, until terminal HCC occurs, systemic therapy is the only possible approach, with sorafenib as the only standard treatment available. Anyway, the efficacy of this drug is limited and many efforts are necessary to understand who could benefit more with this treatment. Therefore, other molecules for a targeted therapy were evaluated, but only regorafenib showed promising results. Beside molecular target therapy, also cytotoxic drugs, in particular oxaliplatin- and gemcitabine-based regimens, and immune-check-point inhibitors were tested with interesting results. The future of the treatment of this neoplasia is linked to our ability to understand its mechanisms of resistance and to find novel therapeutic targets, with the objective to purpose individualized approaches to patients affected by advanced HCC.

Key words: Hepatocellular carcinoma; Systemic therapy; Chemotherapy; Molecular targeted therapy; Cytotoxic therapy; Immunotherapy; Perspectives

Core tip: The aim of this review is to make a point on chemotherapeutic options for treatment of hepatocellular carcinoma (HCC) at advanced stage, the most frequent stage of presentation of this neoplasia, still characterized

by an important mortality rate. By now, sorafenib is the only standard treatment, but other options were recently studied and will be soon available for clinicians and patients affected by HCC. The review can be divided in four sections: The first one regards molecular target therapy and are described sorafenib, its open issues, but also other drugs with similar targets that have been evaluated for treatment of HCC. The second and the third parts regard cytotoxic drugs and immunotherapy, respectively, which were evaluated in recent years as possible alternatives or adjuvant to Sorafenib. In the last part of the review, future perspectives are described, in particular for what concerns resistance mechanism of the neoplasia, delivery methods or biological enhancers for drugs already in use, new drugs that will be probably evaluated and molecular targets that could soon become eligible for target therapy hopefully leading to the development of personalized therapy.

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INTRODUCTION

According to last EASL-EORTC guidelines, liver cancer is the sixth most common cancer, the third cause of cancer related death, and accounts for 7% of all cancers. Hepatocellular carcinoma (HCC) represents more than 90% of primary liver cancers and is a major global health problem. Its incidence reaches a peak at median age of 70 years, which results to be higher in Japanese population (70-79 years) and lower in Chinese and Black African populations. HCC appears to be more frequent in males than in females (2.4:1)[1].

HCC development is often related to the presence of a chronic liver, which represents one of the most important risk factors for this neoplasia. In particular cirrhosis, which can occur as a consequence of chronic viral hepatitis, excessive alcohol intake, nonalcoholic fatty liver disease or genetic diseases (*e.g.*, hemochromatosis), is a frequent setting for HCC onset as well as a cause of liver dysfunction.

Liver dysfunction, in addition to high heterogeneity regarding the mechanisms of carcinogenesis and to the frequent diagnosis of HCC at an advanced stage despite appropriate screening in particular regarding viral chronic hepatitis, lead to great difficulty in treating this neoplasia, as well as in developing new therapeutic alternatives.

Surgery and liver transplantation (OLT) in fact represent the only radical treatments of this disease, but, as mentioned, are not feasible in case of advanced disease or significant hepatic dysfunction[2]. In particular, according to EASL indications based on Barcelona-Clínic Liver Cancer (BCLC) classification related on prognostic variables, surgery is proposable in very early stage HCC (stage 0), while OLT is indicated for early stage disease (stage A). More advanced diseases are treated with, in order: Radiofrequency ablation (RFA), transarterial chemoembolization (TACE) or sorafenib, while terminal HCC (stage D) has best supportive care as unique therapeutic option[1]. RFA and TACE are treatment of choice in case of early stage disease (stage A) with associated diseases and in case of intermediate stage disease (stage B) respectively, while other non-surgical approaches as transarterial radiation, percutaneous ethanol injection and microwave ablation are still infrequently used in clinical practice because of partial or less encouraging results compared with TACE and RFA[3,4].

Of particular interest is the approach with TACE, which, in addition to its purely therapeutic indication, has shown utility for its ability to lead to the down-staging of the disease[4,5] and for its neo-adjuvant effect[6]. For this reason, the TACE has been subject to intense technical development, which has led to, in addition to the conventional method Lipiodol-TACE, new approaches such as drug-eluting beads TACE (DEB-TACE)[7], based on doxorubicin and on administration as microspheres, with encouraging results.

In case of TACE resistance or advanced stage HCC (stage C), compatibly with the residual liver function, systemic chemotherapy is indicated, but sorafenib is currently the only standard systemic treatment available[8,9]. In consideration of the frequent approach to advanced HCC, and given the lack of viable alternatives, many efforts in the field of research have been made to optimize the use of sorafenib, for example by using it together with TACE or with hepatic arterial infusion chemotherapy (HAIC), and to evaluate chemotherapy regimens and other small molecules already in use for other types of malignancies or under development. The aim of our review is to evaluate the available options and future possible strategies regarding systemic therapy for HCC.

MOLECULAR TARGETED THERAPY

As previously said, sorafenib is the only standard treatment available for advanced HCC. In the wake of the good results obtained with sorafenib, numerous other small molecules were evaluated for the treatment of this neoplasia.

Sorafenib

The action of sorafenib is expressed on various molecular targets involved in the mechanism of tumor growth and angiogenesis, leading to their inhibition: Serine-threonine kinases Raf-1 and B-Raf involved in RAF/MEK/ERK pathway, RET, FLT-3, the receptor tyrosine kinase activity of vascular endothelial growth factor receptors (VEGFRs) 1, 2 and 3 and platelet-

derived growth factor receptor β (PDGFR-β)[10-13]. The efficacy of this drug in treating Child-Pugh A stage C HCC was demonstrated in two phase Ⅲ, randomized, placebo-controlled clinical trials: the SHARP trial[8] and the Asia-Pacific study (ORIENTAL)[9]. The SHARP trial compared Sorafenib treatment (400 mg twice a day) to placebo among 602 patients, showing a significant difference in overall survival (10.7 mo *vs* 7.9 mo, $P < 0.001$), time to radiologic progression (5.5 mo *vs* 2.8 mo, $P < 0.001$) and disease control rate (43% *vs* 32%, $P = 0.002$), even if no significant difference was observed in time to symptomatic progression (4.1 mo *vs* 4.9 mo, $P = 0.77$). The observed side effects were diarrhea, weight loss, hand-foot syndrome and hypophosphatemia.

The ORIENTAL trial had a design similar to the SHARP study but was performed on 226 patients from the Asia-Pacific region: The overall survival was significantly increased in the Sorafenib-treated group (6.5 mo *vs* 4.2 mo, $P = 0.014$), even if the overall survival was lower compared to the SHARP study; more encouraging results were observed evaluating the time to progression, which was significantly higher in the Sorafenib group (2.8 mo *vs* 1.4 mo, $P = 0.0005$).

The eligibility criteria for treatment with sorafenib are still relatively restrictive and few data are available regarding its use in the presence of impaired liver function (Child-Pugh B/C) or in elderly patients. Regarding liver function, available data come from retrospective studies[14-18], that evaluated treatment with sorafenib in patients with liver function Child-Pugh B, showing shorter overall survival in these patients, compared with patients with Child-Pugh A. In addition, two studies[15-18] showed an increased incidence of severe adverse events in Child-Pugh B patients, that led to dose reduction or discontinuation of treatment. Thus, in the latest available guidelines there is no clear contraindication about sorafenib administration in patients with Child-Pugh B, but caution is advised due to the increased risk of side effects[19]. Sorafenib treatment in elderly (age > 70 years) was evaluated only in a retrospective study[20], which reported a progression free survival and overall survival similar to younger patients, associated to a higher incidence of some adverse events (neutropenia, malaise and mucositis); anyway, no clear indication about treatment of older patients was given in last guidelines. Beside the evaluation of therapeutic usefulness of sorafenib in single therapy, numerous studies have evaluated its use as adjuvant or neoadjuvant treatment. As previously said, potential down-staging effect was suggested, leading to a possible use of this drug as neo-adjuvant therapy or as bridge-to-transplantation therapy[21]; in particular some studies suggest a possible role of sorafenib in preventing tumor relapse after liver transplantation[22,23], even if available studies were performed on small samples not providing statistically significant results. Unfortunately, the same optimism placed in the use of this drug for a neoadjuvant therapy does not seem to be confirmed regarding its use with adjuvant intent. In 2015, the STORM trial, a randomized, double blind, placebo controlled trial, evaluated sorafenib efficacy as adjuvant after resection or local ablation, but no difference in median recurrence free survival was observed (33.3 mo *vs* 33.7 mo, $P = 0.26$)[24]. A more in-depth discussion should be done about the combination of sorafenib and TACE: Initial encouraging results came from retrospective studies[25,26] that evaluated sorafenib in case of TACE refractory or ineligibility (reduced efficacy of TACE itself, vascular devastation, involvement of complex extrahepatic blood supply routes, vascular invasion, distant metastases)[27]. Despite this, initial randomized trial to evaluate this combination did not confirm the efficacy of TACE + sorafenib. In particular, the SPACE trial[28] showed no difference between TACE + sorafenib *vs* TACE + placebo regarding time-to-tumor progression (169 d *vs* 166 d, $P = 0.072$) and overall survival (554 d *vs* 562 d, $P = 0.295$); a more recent phase Ⅲ randomized trial from Kudo *et al*[29] with a similar design confirmed those results (time to tumor progression 5.4 mo *vs* 3.7 mo, $P = 0.252$; overall survival 29.7 mo *vs* NE, $P = 0.072$). Recent observational studies[30,31] showed more encouraging results in terms of progression free survival and overall survival respectively, and a systematic review/meta-analysis[32] reported a significant different among TACE + sorafenib *vs* TACE in terms of response rate (OR = 3.59, 95%CI: 1.74-7.39, $I^2 = 21\%$, $P = 0.0005$), disease control rate (OR = 4.72, 95%CI: 1.75-12.72, $I^2 = 56\%$, $P = 0.002$), 1-year overall survival (OR = 3.10, 95%CI: 2.22-4.33, $I^2 = 41\%$, $P = 0.00001$), but further randomized trials are still ongoing with the aim to evaluate the effectiveness of this combination therapy (NCT01004978, NCT01324076, NCT01217034).

To develop novel systemic therapies for HCC, sorafenib was also evaluated as second-line therapy after fluoropyrimidine plus platinum-based chemotherapy[33]: The resulting disease control rate of 58.3%, with overall survival and progression-free survival of 7.1 and 2.3 mo, respectively, without increased incidence of adverse events, suggests a modest efficacy of sorafenib as second-line treatment after other systemic therapies. In consideration of new systemic therapeutic options, great importance has acquired the search for markers of resistance to sorafenib, with the intention to offer a personalized therapy for advanced HCC. An example is represented by c-Jun N-terminal kinase activity, related with the CD133 expression level and inversely correlated with the therapeutic response to the drug[8,34]. Thus, many efforts should be done to identify other markers of poor response to sorafenib, with the aim to give each patient a personalized therapeutic approach, based on the resistance profile of each single HCC and to choose among other drugs that will be hopefully soon available beside Sorafenib.

Brivanib

Brivanib is a small molecule acting as dual tyrosine kinase inhibitor (TKI) of VEGFR and FGFR. The drug, administrated orally (800 mg once daily), was initially evaluated as first line treatment in comparison with sorafenib in the BRISK-FL trial, then as second line treatment in comparison with placebo in patients who complained intolerance or lack of response to sorafenib in BRISK-PS trial. BRISK-FL trial[35] showed no difference regarding overall survival between brivanib and sorafenib (9.5 mo *vs* 9.9 mo, HR = 1.06, 95%CI: 0.93-1.22, *P* = 0.311). Even as second-line therapy, in comparison with BSC, Brivanib failed: BRISK-PS[36] trial showed no significant difference regarding overall survival between the two approaches (9.4 mo *vs* 8.2 mo, *P* = 0.3307). Finally, brivanib, like sorafenib, was tested in a randomized, double-blind, placebo-controlled trial[37] as adjuvant therapy after TACE in comparison with placebo, but even in this case it failed in improving overall survival of HCC patients (19.1 mo *vs* 26.1 mo, *P* = 0.5280). Thus, at this time evidences do not allow to consider brivanib an effective alternative to Sorafenib, but further studies may show better results, if we consider positive data about time to tumor progression (4.2 mo *vs* 2.7 mo; HR 0.56, 95%CI: 0.42-0.76, *P* < 0.001) from BRISK-PS and lack of cross tolerance with Sorafenib.

Sunitinib

Sunitinib is another small molecule acting as multikinase inhibitor which targets VEGFR, PDGFR and c-kit. Only one phase Ⅲ trial (SUN1170 trial)[38] studied the efficacy of the drug as first-line treatment for HCC, but was discontinued due to adverse events. Anyway sunitinib appeared to be inferior to sorafenib regarding overall survival (7.9 mo *vs* 10.2 mo, *P* = 0.0014). Based on current evidence, sunitinib is not to be considered as a viable therapeutic alternative to sorafenib.

Linifanib

Linifanib is a dual tyrosine-kinase inhibitor targeting VEGFR and PDGFR. LIGHT phase Ⅲ trial[39] compared the drug to sorafenib as first-line treatment, but overall survival between the two groups was similar (95%CI: 8.3-11.0, HR = 1.046, 95%CI: 0.896-1.221) and linifanib group showed higher rate of adverse events (*e.g.*, hypertension and hepatic encephalopathy).

Erlotinib

Erlotinib is a tyrosine kinase inhibitor targeting EGFR, which was evaluated in combination with sorafenib *vs* sorafenib alone in SEARCH phase Ⅲ trial[40]. This combination did not lead to an increased overall survival (9.5 mo *vs* 8.5 mo, *P* = 0.408) and was related to potent toxicity.

Everolimus

Everolimus acts inhibiting the mammalian target of rapamycin (mTOR). It was evaluated in comparison with placebo in EVOLVE-1 phase Ⅲ trial[41] in case of sorafenib failure or intolerance, but it did not increase overall survival (7.6 *vs* 7.3, HR = 1.05, 95%CI: 0.86-1.27, *P* = 0.68).

Ramucirumab

Ramucirumab is a recombinant IgG1 monoclonal antibody able to bind extracellular domain of VEGFR-2. REACH trial[42] failed in showing its efficacy as second-line treatment in comparison with placebo, because overall survival was similar between the two groups (9.2 mo *vs* 7.6 mo; HR = 0.87, 95%CI: 0.72-1.05, *P* = 0.14); however the promising results obtained in patients with alpha-fetoprotein > 400 ng/mL, led to an ongoing trial to verify its usefulness of this drug in this specific population.

Regorafenib

Regorafenib is a multi-target inhibitor acting on VEGFR1-3, TIE2, c-kit, Ret, wild type or V600-mutated B-RAF, PDGFR and FGFR, administered orally and derived from sorafenib. RESORCE[43] trial is a phase Ⅲ randomized, double-blind trial, that recently evaluated the drug as second-line treatment in comparison with placebo in patients who showed intolerance or failure to sorafenib. Regorafenib was related to positive results in terms of overall survival (10.6 mo *vs* 7.8 mo; HR = 0.63, 95%CI: 0.50-0.79, *P* < 0.0001). Adverse events reported are hypertension (15%), fatigue (9%), diarrhea (3%). It is possible to affirm, on the basis of this trial, that regorafenib appears to be the only alternative currently available regarding systemic therapy for the treatment of advanced HCC in case of progression on sorafenib treatment.

Other small molecules

Other small molecules are currently under evaluation for the treatment of HCC. Some of them act against targets already mentioned as factors involved in angiogenesis (*e.g.*, VEGF), other drugs act on pathways that are already targets of other drugs (*e.g.*, MEK, MET). It is important to emphasize that drugs that act on c-MET may have greater efficacy in cases of HCC with increased expression of the receptor[44,45]. Phase Ⅲ studies are required to define the clinical utility of these drugs, in particular in comparison with sorafenib; for some of them phase Ⅲ trial are under way. Table 1 shows a list of drugs under preliminary evaluation.

CYTOTOXIC CHEMOTHERAPY

Historically, traditional chemotherapy agents have not shown great efficacy in the treatment of HCC when used in advanced stage of disease, in particular in case of progression after locoregional therapy. This assessment comes from initial examination of single-arm, open-label studies evaluating the use of some chemotherapeutic, that did not lead in the past years to further evaluation

Table 1 Targeted drugs under evaluation in advanced hepatocellular carcinoma

Drug	Molecular target	Study design	DCR	PFS	OS	TTP	Tolerabilty	Phase III study
Lenvatinib[46]	VEGFR, FGFR, PDGFR,RET, KIT	Phase I/II (first line)	NR	NR	18.7 mo	12.8 mo	Favorable profile	Ongoing (E7080)
Cabozantinib[47]	VEGFR-2, MET, RET	Phase II (second line)	68% at 12 wk	4.2 mo	NR	NR	Favorable profile	Ongoing (NCT01908426 – CELESTIAL)
Tivantinib[48]	c-MET	Phase II (*vs* placebo, second line)	MET low NS MET high 50% *vs* 20%	NR	MET low NS MET high 7.2 mo *vs* 3.8 mo; $P = 0.01$	MET low NS MET high 2.7 mo *vs* 1.4 mo; $P = 0.04$	Severe neutropenia	Ongoing (NCT01755767)
Apatinib[49]	VEGFR2	Phase II (first line)	NR	NR	9.7 mo	4.2 mo	Favorable profile	Ongoing (NCT02329860)
Refametinib[50]	MEK	Phase II (+ sorafenib)	43%[1]	NR	290 d[1]	122 d[1]	High incidence of 3/4 grade adverse events	NR
Foretinib[51]	MET, RON, AXL, TIE-2, VEGFR	Phase I/II (first line)	79%	NR	NR	4.2 mo	Favorable profile	NR
Tepotinib[52]	c-MET	Phase Ib/II (*vs* sorafenib, first line) - Ongoing	NR	NR	NR	NR	Favorable profile	NR
Capmatinib[53]	c-MET	Phase I	NR	NR	NR	NR	Favorable profile	NR
Golvantinib[54]	c-MET	Phase I/IIb (+ sorafenib) - Ongoing	NR	NR	NR	NR	Favorable profile	NR
Emibetuzumab[55]	c-MET	Phase I (monotp *vs* emibetuzumab + erlotinib)	NR	NR	NR	NR	Favorable profile	NR
LY2157299[56]	TGF-β	Phase II (second-line)	NR	NR	36 wk[2]	12 wk[2]	Favorable profile	Ongoing
Pazopanib[57]	VEGFR1-3, PDGFRα-β, c-kit	Phase I	NR	NR	NR	NR	Favorable profile	NR
Axitinib[58]	VEGFR1-3	Phase II (*vs* placebo, second line)	NR	3.6 mo *vs* 1.9 mo; $P = 0.004$	12.7 mo *vs* 9.7 mo; $P = 0.287$	3.7 mo *vs* 1.9 mo; $P = 0.006$	Acceptable profile	NR

[1]Best clinical response was observed in case of RAS mutations; [2]Best clinical response was observed in case of AFP level decrease. DCR: Disease control rate; OS: Overall survival; TTP: Time-to-tumor progression; PFS: Progression free survival; NR: Not reported; NS: Not significant.

of this class of drugs and limiting their use to palliative approaches.

Recently, however, new chemotherapeutic agents, such as oxaliplatin, have shown efficacy in the treatment of cancers of the digestive tract (stomach, colorectal, pancreas). Based on these positive results, some of these drugs have also been evaluated for the treatment of advanced HCC, with promising findings.

Monotherapy regimens

This kind of regimen is indicated in case of worse general conditions or worse tolerance to systemic therapy. Doxorubicin was one of the first chemotherapic drugs used for HCC and showed interesting results[59], but its role is actually related to already mentioned DEB-TACE. Doxorubicin was also evaluated in combination with sorafenib (see below for details). The interest for doxorubicin is growing again due to the technological advance that allows a targeted release of the drug; this aspect will be discussed in another section of this review. Capecitabine is a drug converted to 5-fluorouracil (5-FU) which acts on DNA synthesis, slowing tumor growth. Currently its role in HCC treatment regards adjuvant therapy after surgery, based on a randomized, controlled trial, placebo-controlled[60], that showed lower recurrence rate (53.3% *vs* 76.7%) and higher time-to-tumor progression (40 mo *vs* 20 mo, $P = 0.0046$); 5-years-overall survival was better in capecitabine group, even if this result did not reach statistical significance (62.5% *vs* 39.8%, $P = 0.216$). From a point of view of safety profile, the drug showed a good tolerability. TS-1 (Titanium-silicate) is a newly developed chemotherapeutic agent that acts on metabolism of 5-FU, increasing its toxicity in neoplastic

cells. Its effect was observed for the treatment of other GI tumors, so it was evaluated as second line treatment for HCC in comparison with placebo in a phase Ⅲ trial (S-CUBE)[61]. This trial failed in proving the superiority of this drug over placebo, but a subanalysis[62] suggests that better results could be observed in a more specific population, characterized by TNM stage Ⅲ, Ⅳa or IVb, Child-Pugh liver function class A and low levels of tumor markers. In this subgroup, overall survival was significantly longer (426.0 d *vs* 375.5 d; HR = 0.69; 95%CI: 0.51-0.93, *P* = 0.0156), suggesting that more personalization in therapeutic approach should be aimed. Nonetheless this studies show how the best possible results for the systemic therapy are linked to good liver function and to a not too advanced disease.

Politherapy regimens

As previously said, newly developed chemotherapeutic agents, appear to be a valuable option for HCC. FOLFOX4 regimen (fluorouracil, leucovorin, oxaliplatin) was evaluated in comparison to doxorubicin alone for the treatment of advanced HCC ineligible for surgery or for local treatments in EACH trial (phase Ⅲ trial)[63]. FOLFOX4 was related to better results in terms of progression free survival (2.93 mo *vs* 1.77 mo, *P* < 0.001), response rate (8.15% *vs* 2.67%, *P* = 0.002), disease control rate (52.17% *vs* 31.55%, *P* < 0.001); beside these positive findings and a good safety profile, no significant difference in terms of overall survival, the primary endpoint of the study, was observed (6.40 mo *vs* 4.97 mo, *P* = 0.07), leading to a formal negativity of the study. Still, an unplanned subsequent analysis performed at 7 mo after the end of the previous study has shown an improvement in terms of overall survival (6.47 mo *vs* 4.90 mo, *P* = 0.04) and significant results regarding overall survival (5.9 mo *vs* 4.3 mo, *P* = 0.0281), but progression free survival, response rate and disease rate control in the Chinese population[64], leading to FOLFOX4 approval by Chines Food and Drug Administration for treatment of advanced HCC ineligible for surgery or local treatment. GEMOX regimen (gemcitabine, oxaliplatin) was firstly evaluated in a large, multicenter, retrospective study (AGEO)[65] for treatment of advanced HCC with notable results: 22% response rate, 66% disease control rate, 4.5 mo progression free survival, 8.0 mo time-to-tumor progression and 11.0 mo of overall survival. Two interesting aspects should be considered: As first, overall survival was related to cirrhosis stage and response to the regimen were associated to overall survival; in particular response to GEMOX led to a better overall survival in comparison with lack of response (19.9 mo *vs* 8.5 mo). As second, this regimen was related to a downstaging effect on the neoplasia, considering that 8.5% of patients became eligible for curative-intent treatments. Attention should be given to possible serious side effects of this regimen (neurotoxicity, thrombocytopenia, neutropenia and diarrhea). Another retrospective study[66] subsequently evaluated GEMOX as second-line treatment after failure of targeted therapy, reporting an overall survival of 8.3 mo, a 6-mo overall survival rate of 59% and a progression free survival of 3.1 mo. Even this study showed an association between overall survival and performance status, alpha-fetoprotein and BCLC score at diagnosis. Further studies are therefore required, in particular phase 3 trials, to assess the role of this regimen in the treatment of HCC. Some other oxaliplatin-based regimens have begun to be studied in phase Ⅱ trials for HCC treatment, showing interesting results, such as XELOX[67] (oxaliplatin plus capecitabine), GP[68] (gemcitabine plus cisplatin) and cisplatin plus capecitabine[69]. A meta-analysis study[70] tried to define the efficacy and safety of oxaliplatin-based regimens and to assess the best regimen for treatment of advanced HCC, but it as an important limitation having evaluated only small single arm studies, with the exception of the EACH study; anyway, it suggests that better results could be obtained with GEMOX combination. Given the yet ambiguous and preliminary available data, further efforts are necessary, performing randomized trials on extended samples, to define the role of these regimens for treatment of HCC.

Chemotherapy and sorafenib

The growing interest about chemotherapy for the treatment of HCC, has led to its comparison with the only available standard systemic treatment: Sorafenib.

As previously said, there are no significant data about comparison between sorafenib and chemotherapeutic drugs, being the lack of phase Ⅲ randomized trials a reason. As a matter of fact, this comparison was evaluated only retrospectively[71] with no significant difference in overall survival (23 wk *vs* 43.6 wk, *P* = 0.105) and progression free survival (11.1 wk *vs* 12.4 wk, *P* = 0.496). More efforts were done to assess a possible synergistic effect of sorafenib plus chemotherapeutic agents. After initial promising data from a phase Ⅱ study[72], a phase Ⅲ trial (CALGB80802)[73] was planned to assess the efficacy of doxorubicin plus sorafenib in comparison with sorafenib alone as first-line treatment, but it was interrupted after a planned interim analysis demonstrated a higher toxicity in combination group and because primary and secondary endpoints (overall survival and progression free survival, respectively) were not met. The main difference between this and the previous phase Ⅱ trial is represented by the use of sorafenib in the control group instead of doxorubicin, suggesting that sorafenib could be the determinant in the therapeutic effect of this combination, with a marginal role of doxorubicin. The GONEXT study[74], a phase Ⅱ study, evaluated the combination of GEMOX plus sorafenib *vs* sorafenib alone as first-line therapy, with moderately positive results: Response rate (16%), disease control rate (77%), median progression free survival (6.2 mo) e 4-mo progression free survival rate (61%), even if overall survival was similar to the

one reported for sorafenib monotherapy; tolerability resulted to be acceptable. The authors commented results pointing out that primary endpoint was met (4-mo progression free survival > 50%), while other results were encouraging. Another preliminary randomized study[75] evaluated this combination as first-line treatment (6 cycles) followed by maintenance treatment with sorafenib alone: objective response was 26.5%. The median time to progression was 10.3 mo (95%CI: 8.7-11.9 mo) and median overall survival was 15.7 mo (95%CI: 13.0-18.4 mo). Toxicity was manageable. Even this approach deserves further evaluations with phase Ⅱ and Ⅲ trials. Another phase-Ⅱ trial[76] studied SECOX regimen (sorafenib, capecitabine and oxaliplatin) in Asian HCC patients; the primary endpoint was time-to-tumor progression (5.29 mo), while secondary ones were response rate (16%), progression free survival (5.26 mo), overall survival (11.73 mo) and tolerance (good tolerance). Results were thus considerate promising and deserving of further evaluations. It is therefore possible to state that oxaliplatin based regimens plus sorafenib showed results suggesting a synergistic action between these drugs and a possible fundamental role in the future of treatment of HCC.

HAIC

HAIC was introduced in Japan before the advent of sorafenib and Japanese clinical guidelines suggested HAIC plus sorafenib in case of HCC with Vp4 or Vp3 (HCC with invasion of the main trunk or the left and right main branches of the portal vein) even in absence of phase Ⅲ trials supporting the efficacy of this approach. Available regimens are: IA-call (one-shot intra-arterial injection), LFP (repeated intra-arterial injection of cisplatin with a reservoir catheter system) and 5FU/IFN (5-fluorouracil continuous intra-arterial injection with a reservoir catheter system in combination with subcutaneous interferon administration). The best results from a single regimen came from IA-call, that was related to a response rate of 33.8% in a phase Ⅱ trial[77]. As previously said, these regimens are often used in combination with sorafenib, but only combination based on IA-call was associated to interesting results in terms of overall survival in comparison with sorafenib alone (9.5 mo *vs* 7.0 mo; HR = 0.74)[78]. On the other side, no significant difference was observed using sorafenib+LFP (11.8 mo *vs* 11.8 mo; HR = 1.0)[79].

IMMUNOTHERAPY

Tumor immune escape ad its mechanism brought to a growing interest from scientific community, resulting in development of tumor immunotherapy, that proved to be effective for the treatment of some malignant neoplasia (*e.g.*, melanoma, NSC lung cancer, renal carcinoma). Two immunological pathways are involved in tumor immunotherapy: The first one is related to T cells inhibition caused by the interaction between cytotoxic T lymphocyte-associated-4 (CTLA-4), a transmembrane receptor on T cells, and its molecular ligand B7, that may lead to a protective effect for tumor cells and its inhibition is the target of some immunotherapeutic drugs[80]. The second immunological pathway targeted by immunotherapy is the one started by programmed death receptor 1 (PD-1) and its ligands (PD-L1 and PD-L2). PD-1 is produced by several immunity cells (T cells $CD28^+/CD4^+$, B cells, NK cells, *etc*.) but it's often expressed by tumor cells with an immunosuppressive effect, caused by TCR receptor signal transduction inhibition by PD-1-PD-L1 that results in drop of proliferation and depletion of T-cells[81]. Tremelimumab is a humanized anti-CTLA-4 IgG2 antibody and it was evaluated for the treatment of HCC in patients with chronic HCV infection with encouraging results in terms of response rate (18%), disease control rate (76%) and time-to-tumor progression (6.48 mo); two interesting characteristics of this drug are its long half-life (22 d), which could lead to a more comfortable management for the patient, and its antiviral activity, represented by a drop in viral load[82]. An interesting important clinical aspect is the possible synergistic action of this drug with local treatments (TACE and RFA). This synergy might be explained by immune reaction against the tumor caused by local treatments, which improves the efficacy of immunotherapeutic drug. Only preliminary results[83] are available, but they appear to be promising: 40% of patients reached partial response, 5/7 patients affected by HCV infection showed a drop in viral load, histology evaluation showed immune cell infiltration in tumor and progression free-survival was 7.4 mo; in addition no worsening of safety profile was observed. Nivolumab is a fully humanized monoclonal IgG4 antibody against PD-1, recently studied in a phase Ⅰ/Ⅱ study[84] for treatment of patients affected by HCC with intolerance to, or inefficacy of, sorafenib. This study reported extremely positive results: 2/39 patients (5%) showed complete response and 8/39 (18%) showed partial response; 6-mo overall survival rate was 72%. On the other hand a moderate rate of adverse events was observed (71%), but only 17% of patients were affected by grade 3/4 adverse events (elevated AST, elevated ALT, elevated serum lipase). A phase Ⅲ trial (NCT02576509) to compare nivolumab to sorafenib is ongoing. It is safe to say that tumor immunotherapy is a very promising option among systemic therapies, especially because its targets are completely different from targets of the currently available systemic therapies. Furthermore, its effectiveness may allow a better understanding of the biology of HCC. In the near future it will be interesting to evaluate immunotherapy in comparison with standard treatments, but also in combination with them in consideration of possible synergy as seen in case of Tremelimumab and TACE.

FUTURE PERSPECTIVES

HCC appears to be still a tough opponent, if it is not possible to treat it by surgery or by transplantation. It this therefore necessary to improve medical therapy for this neoplasia to give a chance to patients affected by its more advanced stages. It is important to focus which are directions we should follow regarding research in this field.

Understanding why some drugs had partial results or were able to show improvements only in some groups of patients is very important and could allow us to understand resistance mechanisms of this neoplasia and to develop strategies to overcome them. On the other side, many efforts should be made to find new therapeutic targets and develop new drugs. Certainly, the future of advanced HCC treatment will be represented by personalized therapy based on a deep evaluation of the patients, to find out the better targets of disease to be attacked.

Resistance mechanisms

Not so much data is available about resistance mechanisms of HCC and practical ways to overcome them. Preliminary studies have shown that, as previously said, c-Jun N-terminal kinase activity could be related to sorafenib resistance, but this information did not lead to clinical consequences yet. Resistance could be related to systemic therapy in general or to the single drug. In the first case, altered pathways are fundamental for tumorigenesis, metastatic process and maintenance of stem cell properties; in particular molecules involved in autophagy (osteopontin[85]), apoptosis (Cofilin-1[86] and AKR7A3[87]) and stemness related mechanism of cancer stem stells (NRBP2[88]) seem to play an important role, as showed in some preliminary *in vitro* studies.

Particular mechanisms resulted to be involved in resistance to specific drugs. For example, aberrant expression of non-coding RNA was related to oxaliplatin-resistant profile: 421 differentially expressed mRNAs, 228 up-regulated and 193 down-regulated (fold change > 2, $P < 0.05$) in oxaliplatin-resistant (MHCC97H-OXA), were individuated and appear to be related not only to resistance to oxaliplatin, but also to tumor size, differentiation and poor prognosis[89]. On the other hand, TUC338\RASAL1 pathway was related by Jin *et al*[90] to sorafenib resistance: *in vitro* inhibition by non-coding RNA of TUC338 led to a sensitization to sorafenib and, in addition, to a decrease in proliferative and invasive ability. Of particular interest is the recent hypothesis of the role of tumoral microenvironment in chemotherapeutic resistance: Azzariti *et al*[91] described in their study the resistance to sorafenib induced by hepatic stellate cells, that produce laminin-332, an extracellular matrix protein, that is able to bind α3β1 integrin, if expressed, leading to protection of FAK, a target of sorafenib, from degradation.

New combinations of drug with delivery systems or biological enhancers

Another important field of research is the one regarding the development of new forms of drugs already used to enhance the effect and selectivity for HCC; an example is represented by nanoparticle-mediated targeted drug delivery system[92]. Doxorubicin is an example of drug that could soon have a new role in HCC treatment, as demonstrated by preliminary studies on animal models with modified forms of the drug. Lactosaminated albumin conjugate of doxorubicin showed rapid and selective accumulation in the liver[93], such as mesoporous magnetic nanocomposites wrapped with chitosan gatekeepers[94], that in addition exploit acidic pH of tumoral cells with a selective release of drug at pH 4.0. Even A54 peptide modified Doxorubicin glucolipid conjugate micelles[95] showed high selectivity for hepatic cells, in particular for tumoral ones because of redox-sensitivity.

Moreover the modification of cisplatin by the addition of a pH-sensitive polymer and HCC-targeting peptide, to obtain a higher selectivity to HCC and in particular to its stem cells, that are not sensitive to cisplatin alone, showed promising results[96]. On the other hand, elaboration of sorafenib was targeted to add molecules which could acts as biological enhancers in a synergistic way. Two examples of molecules used with this intent are C2-ceramide[97], a potent inducer of apoptosis in human neoplastic cells, and 2-Deoxyglucose[98], an inhibitor of glycolysis that leads to depletion of ATP.

Other drugs under evaluation

Pre-existing and new drugs were studied for treatment of HCC. Antiangiogenic drugs could have a role, because of important angiogenic activity of this neoplasia; in fact VEGFR is already a target of some drugs previously discussed. Unfortunately, bevacizumab was tested in combination with sorafenib in a phase I/II trial with consequent observation of high toxicity and low efficacy of this combination, that led to the interruption of the study[99,100]. It's necessary to mention drugs that have been studied *in vitro* and *in vivo* with promising results, awaiting for trials on humans. Some examples are ursolic acid derivates[101] and a B5G9[102] (piperazidine derivative of 23-hydroxy betulinic acid), that cause ROS-mediated apoptosis in HCC cells, EMMQ[103] (an indolylquinoline derivative), that causes DNA damage by activating p53 and γ-H2AX, and GL63[104] (a curcumine analogue), which was able to suppress the proliferation of HCC cells by inhibition of the JAK2/STAT3 signaling pathway. Even Valproic Acid[105], a well-known antiepileptic drug, showed potential anti-HCC effect *in vitro* by promotion of epithelial mesenchymal transition of hepatocarcinoma cells *via* transcriptional and post-transcriptional up regulation of Snail.

Another new therapeutic approach regards arginine, which cannot be produced by HCC cells; thus, pegy-

lated arginine diminase (ADI-PEG 20) was tested as arginine-degrading enzyme, with favorable tolerability[106] and encouraging disease control rate and median overall survival[107]; a phase Ⅲ trial to evaluate this drug is actually ongoing (NCT01287585). JX-594 is a recombinant vaccine virus able to cause virus replication-dependent oncolysis and tumor-specific immunity, after inserting human granulocyte-macrofaghe colony-stimulating factor (hGM-CSF) and β-galactosidase transgenes, with disruption of the viral thymidine kinase gene. This vaccine was tested in a low dose administration *vs* a high dose administration; this last one was related to a better median overall survival (6.7 mo *vs* 14.1 mo; HR = 0.39, *P* = 0.02), while response rate was 15% for both groups[108]. PHOCUS phase Ⅲ trial in combination with sorafenib is ongoing (NCT02562755).

New molecular targets

The advancement of knowledge of the biology of HCC is gradually allowing us to identify new potential molecular targets, which are an essential part of the development and the activity of this tumor. Rao *et al*[109] recently provided an article in which frequently mutated genes/pathways are described and can be source of inspiration to individuate new future therapeutic targets.

NF-κB has a key role in immune response and resulted to be altered in precancerous cirrhosis tissues and in a subset of HCCs. Ramesh *et al*[110] reported preliminary data about *in vitro* activity of ornithogalum against HCC. The importance of NF-κB in HCC biology and in relation to a potential clinical use, was suggested by Chen *et al*[111]: In his study, pretreatment of sorafenib with RT suppressed the expressions of NF-κB and its downstream proteins induced by radiation through downregulation of phosphorylated extracellular signal-regulated kinase (pERK), with a synergistic effect that could lead to a new role for radiotherapy for the treatment of HCC. Another target that has been evaluated in oncology is telomerase, which appears to be constitutively activated in many tumors. In a recent review by Picariello *et al*[112], inhibition of telomerase activity were evaluated. An interesting new approach is the exploitation of telomerase activity using nucleoside analogues that could be metabolized by telomerase. Acycloguanosyl-thymidyltriphosphate[113], a thymidine analogue pro-drug of Acyclovir, was tested *in vitro* and *in vivo* against HCC, leading to reduced tumor growth, increased apoptosis and reduced proliferation of tumor cells in transgenic and orthotopic mouse models. Further studies are necessary to test this kind of drugs on humans.

Other promising molecular targets are prothymosin-alpha[114], a negative regulator of apoptosis, *NEK2*[115], a critical regulator of centrosome structure and function, and *STARD13*[116], a positive regulator of apoptosis.

CONCLUSION

To date, the treatment of HCC is still a major surgical and medical challenge. This is even more true with regard to cases of advanced disease, treatable only with systemic therapy, which by now has few arrows available in its quiver. Sorafenib is today the only standard systemic treatment, but it presents still unsolved issues; this explains the urgency of finding new alternatives to be proposed to the patient. Molecular therapy has a key role: Many drugs are under development and under evaluation; furthermore another drug from this class, Regorafenib, showed positive results and for sure will be considered by future guidelines for the treatment of HCC; on the other hand, the number of available drugs is likely to increase with the rise of biological weaknesses of this neoplasia. Yet, cytotoxic drugs, in particular modified forms, and immunotherapeutic drugs are making a promising competition to sorafenib, acting on different routes. The future availability of a great number of different options with different mechanisms of action definitely gives much hope regarding the treatment of advanced HCC, in particular in terms of personalized therapy.

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MINIREVIEWS

Is the 25-year hepatitis C marathon coming to an end to declare victory?

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Abstract

Hepatitis C virus (HCV) which was originally recognized as posttransfusion non-A, non-B hepatitis has been a major global health problem affecting 3% of the world population. Interferon/peginterferon and ribavirin combination therapy was the backbone of chronic HCV therapy for two decades of the journey. However, the interferon based treatment success rate was around 50% with many side effects. Many chronic HCV patients with psychiatric diseases, or even cytopenias, were ineligible for HCV treatment. Now, we no longer need any injectable medicine. New direct-acting antiviral agents against HCV allowed the advance of interferon-free and ribavirin-free oral regimens with high rates of response and tolerability. The cost of the medications should not be a barrier to their access in certain parts of the world. While we are getting closer, we should still focus on preventing the spread of the disease, screening and delivering the cure globally to those in need. In the near future, development of an effective vaccine against HCV would make it possible to eradicate HCV infection worldwide completely.

Key words: Treatment; Therapy; Epidemiology; History; Prevention

Core tip: Spreading awareness about the need for screening and treatment of hepatitis C virus (HCV) will help identifying more cases to provide appropriate treatment. As more direct-acting agents are coming out of the pipeline, healthcare managers will face the major task of making those medicines available to HCV-infected patients. One of the efforts, successfully dismantling some of those barriers is the Extended Community Healthcare Outcomes project. Finally, efforts toward developing effective vaccines should be boosted as history tells us that most of success stories in eradicating infectious illness were made possible largely because of vaccines against the offending pathogen.

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INTRODUCTION

It has been less than three decades since the discovery of hepatitis C virus (HCV) in 1989 by Choo *et al*[1]. The process of discovering the virus was very daunting as described in Dr. Houghton's paper[2]. Recognized as the reason behind non-A non-B hepatitis, the big picture of the health and financial burden this virus would have caused became clear. With the high sustained virologic responses (SVRs) reported recently with the use of direct acting, soon will be forgotten the miserable quality of life patients of hepatitis C have had to endure with the not as effective and with unpleasant side effects interferon-based treatments. Not until five years ago when direct acting agents, protease inhibitors telaprevir (Incivec, Vertex) and boceprevir (Victrelis, Merck) were approved by the Food and Drug Administration, had we started seeing SVR rates above 70%. Since then, many direct acting agents have been approved with SVR rates above 90%. While very promising, challenges for treatment, such as access to medications and healthcare management, remain widely spread.

NATURAL HISTORY OF HEPATITIS C

After the acute infection, only 15%-25% of the patients get cured spontaneously and 75%-85% develop chronic infection with the diagnosis made if viremia persists 6 mo from the onset. Chronic inflammation will lead eventually to structural damage or fibrosis which, as it progresses, will lead to cirrhosis. From those chronically infected, 10% to 15% develop cirrhosis[3]. Progression of fibrosis can be influenced by host factors [such as older age at time of infection, male gender, coinfection with human immunodeficiency virus (HIV) or hepatitis B virus (HBV), immunosuppression, insulin resistance, non-alcoholic steatohepatitis, hemochromatosis, schistosomiasis, and the grade and stage on the liver biopsy] as well as external factors (such as excessive alcohol drinking). HCV and HBV play an etiologic role in acute liver failure[4]. HCV has mostly a dominant role in HBV and HCV coinfection[5]. However, HBV acute superinfection may cause spontaneous clearance of HCV RNA[6].

Liver biopsy remains the gold standard for the grading and staging of chronic hepatitis C. The grade, which reflects the inflammation activity, is determined by the severity of mononuclear inflammatory cells around the portal areas and by necrosis of the hepatocytes. The stage reflects the extent of the fibrosis which ranges from absent to mild or advanced in case of bridging fibrosis (fibrosis extending from a portal tract to another) or cirrhosis (fibrosis closing up in circles forming nodules).

Deaths usually are caused by complications of cirrhosis such as ascites, variceal bleeding, hepatic encephalopathy, hepatorenal syndrome, and hepatocellular carcinoma (HCC). Unfortunately, the disease can progress silently until it is advanced and complications ensue. For compensated cirrhosis, 3-, 5-, and 10-year survival rates were 96%, 91%, and 79%, respectively[7]. The 5-year survival rate drops to 50% once decompensated[7]. HCC risk increased 17-fold in HCV-infected patients[8]. This risk appears to have decreased in those with a sustained viral response rather than non-responders to interferon treatment[9]. The rates of progression to cirrhosis and HCC have been variable with a mean time to cirrhosis estimated at 20 years[3,10]. HCC can develop at a rate of 1% to 4% per year[11-14].

EPIDEMIOLOGY OF HEPATITIS C

The prevalence of HCV antibody in the United States is 1.6%[15]. In 1999-2002, the highest prevalence (4.3%) was in people 40 to 49 years of age[15] and two-thirds of those infected were born between 1945-1965. In 2007, HCV infection was associated with an estimated 15000 deaths in the United States[16]. This number has risen to 19695 in 2014 and this is now thought of as only a fraction of the actual number[17]. Decompensated chronic HCV is the most common indication for liver transplantation in the United States[18,19]. HCV and chronic kidney disease are associated. Hemodialysis patients have five time higher risk of chronic HCV infection compared to the healthy population. On the other hand, HCV has extrahepatic manifestations of cryoglobulinemia and glomerulonephritis[20]. Chronic hepatitis C is a leading cause of HCC[21]. The incidence of acute hepatitis C in the United States was estimated to be 180000 cases per year in the mid-1980s, but declined to approximately 30000 new cases per year in 1995[22], and to 16000 cases in 2009[23]. In a more recent surveillance, the incidence of acute hepatitis C in the United States has been on the rise since 2011. The estimated number on the actual new cases was 16500 in 2011 and has risen to 30500 in 2014[17].

SCREENING FOR HEPATITIS C

Because of the lack of symptoms in compensated disease and because 75% of patients chronically infected with hepatitis C are unaware of their infection[24], screening can help identify those infected before their disease progresses to a late stage. The United States Preventive Services Task Force has found

Table 1 Food and Drug Administration approved anti-hepatitis C virus tests[28]

Abbott HCV EIA 2.0	Abbott Laboratories, AbbottPark, IL	EIA (Manual)
ADVIA Centaur HCV	Siemens, Malvern, PA, United States	CIA (Automated)
ARCHITECT anti-HCV	Abbott Laboratories, AbbottPark, IL	CMIA (Automated)
AxSYM anti-HCV	Abbott Laboratories, AbbottPark, IL	MEIA (Automated)
OraQuick Rapid Test	OraSure Technologies,Bethlehem,PA	Immunochromatographic (Manual)
Ortho HCV Version 3.0 EIA	Ortho	EIA (Manual)
VITROS anti-HCV	Ortho	CIA (Automated)

Anti-HCV: HCV antibody; EIA: Enzyme immunoassay; CIA: Chemiluminescent immunoassay; MEIA: Microparticle enzyme immunoassay; CMIA: Chemiluminescent microparticle immunoassay.

Table 2 Milestones in the history of hepatitis C

1975	Non-A, non-B hepatitis was first described[36,37]
1989	Randomized controlled trials were carried out using interferon alpha to treat non-A, non-B hepatitis[38-40]
1989	HCV was identified[1]
1991	Ribavirin is used as a monotherapy for chronic hepatitis C[41,42]
1995	The combination of interferon alpha and ribavirin were tested[43,44]
1996	Hepatitis C serine protease structure was published[45]
1998	First randomized double-blind, placebo controlled study using recombinant interferon alpha alone or in combination with ribavirin[46,47]
1999	Structure of hepatitis C RNA-dependent RNA polymerase NS5B was identified[48,49]
2001	Pegylated interferon alpha and ribavirin were used in trials[50,51]
2005	Structure of NS5A was published[52]
2011	First direct acting agents: Protease inhibitors were used in combination with pegylated interferon and ribavirin to treat hepatitis C genotype 1[53,54]
2012	Pilot studies using combinations of direct-acting antiviral drugs without interferon[55]
2014	Several direct acting antiviral medications were released to the market to treat different hepatitis C genotypes with SVR exceeding 90% and with better tolerability

HCV: Hepatitis C virus; SVR: Sustained virologic response.

evidence that screening high risk population and one time screening of those born between 1945 and 1965 is of moderate benefit. The risk of stigmatization appeared small and, although there was evidence for harm from liver biopsy of 1% bleeding risk and < 0.2% death risk from liver biopsy, the use of liver biopsy to guide the management is becoming less. In light of the availability of effective antiviral agents that have rare and self-limited side effects, identifying patients with chronic hepatitis C and treating them is probably of benefit[25].

There is adequate evidence that anti-HCV antibody testing followed by confirmatory polymerase chain reaction testing accurately detects chronic HCV infection. The number needed to screen to identify 1 case of chronic hepatitis C in a high risk population, such as past or present injection drug use, sex with an injection drug user, or blood transfusion before 1992, is < 20 persons and anti-HCV antibody testing is associated with high sensitivity (> 90%)[26]. There is also evidence that different noninvasive tests have good diagnostic accuracy in detecting fibrosis[27].

IS THERE A VACCINE YET?

Unlike hepatitis A and B, no vaccine is available to protect against hepatitis C. The high variability among different strains and the fast rate at which mutations can develop made it very challenging to create an effective vaccine[28]. Several attempts are currently made to create a vaccine either by directing efforts at a relatively stable glycoprotein that is used by the virus to invade liver cells[28].

ASSAYS

Testing for hepatitis C has improved over the years. Anti-hepatitis C tests are accurate and with high sensitivity > 90% in high risk groups. The CDC recommends one of three immunoassays; two enzyme immunoassays (EIA) (Abbott HCV EIA 2.0, Abbott Laboratories, Abbott Park, Illinois, and Ortho® HCV Version 3.0 ELISA, Ortho-Clinical Diagnostics, Raritan, New Jersey) and one enhanced chemiluminescence immunoassay (CIA) (Vitros® Anti-HCV assay, Ortho-Clinical Diagnostics, Raritan, New Jersey) for the initial screening[29]. The OraQuick test has been also approved by the FDA for the initial screening in 2011[30]. A reactive initial test should be followed by a confirmatory nucleic acid test where the plasma is tested to detect (qualitative) or detect and quantify (quantitative) hepatitis C RNA. If HCV RNA is detected, that indicates active hepatitis C infection. If HCV RNA is not detected, that indicates a false positive HCV antibody test or resolved infection[31]. A single step, combined RT-PCR technique can detect HCV RNA from extracted liver tissue[32]. Occult HCV cases have only positive HCV RNA in the hepatocytes, while their plasma HCV markers are all negative[33,34]. Table 1 shows FDA approved anti-HCV tests[35], Table 2 shows the milestones in the history of hepatitis C.

Table 3 American Association for the Study of Liver Diseases/Infectious Diseases Society of America Guideline Recommendations: Genotypes 1, 2, 3, 4, 5 and 6 hepatitis C virus[58]

HCV genotype	Cirrhosis	Prior Tx	Recommended regimen	Alternative regimen	Notes
1a	No		LDV/SOF 12 wk		
			DCV + SOF 12 wk		
			SMV + SOF 12 wk		
			SOF/VEL 12 wk		
			GZR/EBR 12 wk		NS5A RAVs absent
				GZR/EBR 16 wk + RBV	NS5A RAVs present
			OBV/PTV/RTV + DSV 12 wk + RBV		
1b	No		LDV/SOF 12 wk		
			DCV + SOF 12 wk		
			SMV + SOF 12 wk		
			SOF/VEL 12 wk		
			GZR/EBR 12 wk		
			OBV/PTV/RTV + DSV 12 wk		
1a	Compensated	Naive	LDV/SOF 12 wk		
				DCV + SOF 24 wk ± RBV	
				SMV + SOF 24 wk ± RBV	No Q80K
			SOF/VEL 12 wk		
			GZR/EBR 12 wk		NS5A RAVs absent
				GZR/EBR 16 wk + RBV	NS5A RAVs present
				OBV/PTV/RTV + DSV 24 wk +RBV	
1a	Compensated	PR exp	LDV/SOF 12 wk + RBV or 24 wk		
				DCV + SOF 24 wk ± RBV	
				SMV + SOF 24 wk ± RBV	No Q80K
			SOF/VEL 12 wk		
			GZR/EBR 12 wk		NS5A RAVs absent
				GZR/EBR 16 wk + RBV	NS5A RAVs present
				OBV/PTV/RTV + DSV 24 wk + RBV	
1b	Compensated	Naive	LDV/SOF 12 wk		
				DCV + SOF 24 wk ± RBV	
				SMV + SOF 24 wk ± RBV	
			SOF/VEL 12 wk		
			GZR/EBR 12 wk		
			OBV/PTV/RTV + DSV 12 wk		
1b	Compensated	PR exp	LDV/SOF 12 wk + RBV or 24 wk		
				DCV + SOF 24 wk ± RBV	
				SMV + SOF 24 wk ±- RBV	
			SOF/VEL 12 wk		
			GZR/EBR 12 wk		
			OBV/PTV/RTV + DSV 12 wk		
1a or 1b	Decompensated	Naive or exp	SOF/VEL 12 wk + RBV		Child-Pugh B or C
2	No	Naive	SOF/VEL 12 wk		
				SOF + DCV 12 wk	
2	No	PR exp	SOF/VEL 12 wk		
				SOF + DCV 12 wk	
2	No	SR exp	DCV + SOF 24 wk ± RBV		
			SOF/VEL 12 wk + RBV		
2	Compensated	Naive	SOF/VEL 12 wk		
				SOF + DCV 16-24 wk	
2	Compensated	PR exp	SOF/VEL 12 wk		
				SOF + DCV 16-24 wk	
2	Compensated	SR exp	DCV + SOF 24 wk ± RBV		
			SOF/VEL 12 wk + RBV		
2	Decompensated	Naive or exp	SOF/VEL 12 wk + WB RBV		Child-Pugh B or C
			DCV + SOF 12 wk + low initial dose RBV		Child-Pugh B or C
3	No	Naive	SOF + DCV 12 wk		
			SOF/VEL 12 wk		
3	No	PR exp	SOF + DCV 12 wk		
			SOF/VEL 12 wk		
3	No	SR exp	DCV + SOF 24 wk + RBV		
			SOF/VEL 12 wk + RBV		
3	Compensated	Naive	SOF/VEL 12 wk		

			SOF + DCV 24 wk ± RBV		
3	Compensated	PR exp	SOF/VEL 12 wk + RBV		
			SOF + DCV 24 wk + RBV		
3	Compensated	SR exp	SOF + DCV 24 wk + RBV		
			SOF/VEL 12 wk + RBV		
3	Decompensated	Naive or exp	SOF/VEL 12 wk + WB RBV		Child-Pugh B or C
			DCV+ SOF 12 wk + low initial dose RBV		Child-Pugh B or C
4	No cirrhosis or compensated	Naive	SOF/LDV 12 wk		
			OBV/PTV/RTV 12 wk + RBV		
			GRZ/EBV 12 wk		
			SOF/VEL 12 wk		
4	No	PR exp	SOF/LDV 12 wk		
			OBV/PTV/RTV 12 wk + RBV		
			GRZ/EBV 12 wk		Relapse
			GRZ/EBV 16 wk + RBV		Others
			SOF/VEL 12 wk		
4	Compensated	PR exp	SOF/LDV 12 wk + RBV	SOF/LDV 24 wk	
			OBV/PTV/RTV 12 wk + RBV		
			GRZ/EBV 12 wk		Relapse
			GRZ/EBV 16 wk + RBV		Others
			SOF/VEL 12 wk		
5 or 6	No cirrhosis or compensated	Naive or PR exp	SOF/LDV 12 wk		
			SOF/VEL 12 wk		

AASLD: American Association for the Study of Liver Diseases; DCV: Daclatasvir; DSV: Dasabuvir; EBR: Elbasvir; GT: Genotype; GZR: Grazoprevir; LDV: Ledipasvir; OBV: Ombitasvir; PR: Peginterferon/ribavirin; PTV: Paritaprevir; RAV: Resistance associated variant; RBV: Ribavirin; RTV: Ritonavir; SMV: Simeprevir; SOF: Sofosbuvir; VEL: Velpatasvir; WB: Weight-based; SR: Sofosbuvir/ribavirin; exp: Experienced; Tx: Treatment.

DIRECT ACTING AGENTS ERA

In 2011, telaprevir and boceprevir became available as the first direct acting antivirals for the treatment of chronic hepatitis C with variable SVRs for the different genotypes. While the use of the protease inhibitors was a great milestone in the journey of treating hepatitis C with SVRs above 70%, it had limiting factors, such as the need to use in combination with pegylated interferon and ribavirin, and their limitations in treating those with decompensated cirrhosis. Pegylated interferon and ribavirin have exerted their antiviral activities by modulating the host immunity rather than directly inhibiting the virus. With the better understanding of the HCV non-structural proteins, agents that inhibited those proteins have shown promise by directly disabling the life cycle of the virus.

To overcome those species with resistance against the various agents, combination therapy with agents attacking different vital functions became available. Several trials have been conducted with many drugs and combinations of drugs tested in patients with different genotypes and subtypes as well as groups who were treatment naive and those with experience. Defining cure by achieving SVR, defined as the absence of virus detection with an acceptable HCV RNA assay at 12 wk after the end of the treatment, therapy outcomes were compared in those with and without cirrhosis, and between those with and without disease decompensation. While some data is still lacking in different special groups, we are learning more about how to treat those with comorbidities such as coinfection with HIV, those with renal disease, pediatric[56], and liver transplant patients[34,57]. Most of the regimens available at this time are interferon - free regimens and all oral medications with very limited side effects and with SVRs > 85% and in many cases > 95%. Table 3 summarizes the latest recommendations for treating the different HCV genotypes in different groups of patients[58-67].

SO DID WE FIND THE IDEAL CURE?

With manufacturing highly potent medications, we may be winning a battle against hepatitis C, but are we winning the war? The challenge in eradicating the disease lies in delivering and administering those medications to the appropriate patients and ensure their compliance with the treatment and follow up. Patient access to the medications is limited by cost, lack of healthcare services, and ignorance. While the AASLD recommends treating everybody with chronic hepatitis C, third party payers may restrict treatment to those with advanced disease. Payers may also limit the choice of medications authorized. Many healthcare providers choose not to treat hepatitis C because of the perception that such treatment is complex or at least time and resources consuming. Some providers and insurers will decline those with ongoing illicit drug or alcohol use. Shortening the treatment period in selected cases can significantly reduce the cost[68]. Many patients, an estimated 75% of those infected, do not know they have the disease and, therefore, do not seek the appropriate care. While such hindrances have been there all along, the challenge seems to be shifting more towards

socioeconomic nature.

WHAT IS NEXT?

As more direct acting agents are coming out of the pipeline, healthcare managers will have to face the major task of making those medicines available to chronic hepatitis C patients. Spreading awareness about the need for screening and for treatment if infected not only in the population at large, but also among the healthcare providers, will help identifying more cases of infection and help provide those with the appropriate treatment[69]. One of the efforts, successfully dismantling some of those barriers is the Extended Community Healthcare Outcomes project. Launched in New Mexico in 1993, the project has grown to encompass several national and international hubs. These hubs provide healthcare providers with the appropriate educational and coaching resources to empower them with the knowledge and with the How-To guidance to treat patients in their communities where no specialized care is available. This project has helped many patients in rural areas to receive treatment without the need to travel out of their own towns[70,71]. The project provides healthcare providers with direct access to specialized knowledge and provides them with step-by-step coaching which helps in alleviating the misperceptions about the complexity of the treatment and, hence, recruiting more providers in the war against hepatitis C.

On the preventative front, efforts should be also directed towards studying the increasing incidence of hepatitis C acute infection to identify the newer trends behind this surge and to try to eliminate them. While treating acute hepatitis C infection is still not recommended, we may need to revisit the guidelines to facilitate earlier treatment, particularly now that we have highly potent medications with few tolerable side effects.

Finally, efforts toward developing effective vaccines should be boosted as history tells us that most of success stories in eradicating infectious illness were made possible largely because of vaccines against the offending pathogen.

CONCLUSION

While we are getting closer, it is still early to declare victory against hepatitis C. We are armed with better ammunition, but we need to do better job in developing strategies that not only deliver cure to those in need, but also prevents the spread of the disease by educating the population about the risk factors for contracting the disease and how to avoid them, identifying the undiagnosed, and providing early treatment to those in need.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Small for size syndrome difficult dilemma: Lessons from 10 years single centre experience in living donor liver transplantation

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Abstract

AIM

To analyze the incidence, risk factors, prevention, treatment and outcome of small for size syndrome (SFSS) after living donor liver transplantation (LDLT).

METHODS

Through-out more than 10 years: During the period from April 2003 to the end of 2013, 174 adult-to-adults LDLT (A-ALDLT) had been performed at National Liver Institute, Menoufiya University, Shibin Elkoom, Egypt. We collected the data of those patients to do this cohort study that is a single-institution retrospective analysis of a prospectively collected database analyzing the incidence, risk factors, prevention, treatment and outcome of SFSS in a period started from the end of 2013 to the end of 2015. The median period of follow-

up reached 40.50 m, range (0-144 m).

RESULTS

SFSS was diagnosed in 20 (11.5%) of our recipients. While extra-small graft [small for size graft (SFSG)], portal hypertension, steatosis and left lobe graft were significant predictors of SFSS in univariate analysis (P = 0.00, 0.04, 0.03, and 0.00 respectively); graft size was the only independent predictor of SFSS on multivariate analysis (P = 0.03). On the other hand, there was lower incidence of SFSS in patients with SFSG who underwent splenectomy [4/10 (40%) SFSS *vs* 3/7 (42.9%) no SFSS] but without statistical significance, However, there was none significant lower incidence of the syndrome in patients with right lobe (RL) graft when drainage of the right anterior and/or posterior liver sectors by middle hepatic vein, V5, V8, and/or right inferior vein was done [4/10 (28.6%) SFSS *vs* 52/152 (34.2%) no SFSS]. The 6-mo, 1-, 3-, 5-, 7- and 10-year survival in patients with SFSS were 30%, 30%, 25%, 25%, 25% and 25% respectively, while, the 6-mo, 1-, 3-, 5-, 7- and 10-year survival in patients without SFSS were 70.1%, 65.6%, 61.7%, 61%, 59.7%, and 59.7% respectively, with statistical significant difference (P = 0.00).

CONCLUSION

SFSG is the independent and main factor for occurrence of SFSS after A-ALDLT leading to poor outcome. However, the management of this catastrophe depends upon its prevention (*i.e.*, selecting graft with proper size, splenectomy to decrease portal venous inflow, and improving hepatic vein outflow by reconstructing large draining veins of the graft).

Key words: Living donor liver transplantation; Outcome after living donor liver transplantation; Small for size syndrome; Small for size graft; Portal inflow; Venous outflow

Core tip: Small for size syndrome (SFSS) was diagnosed in 20 (11.5%) of our recipients where, small for size dysfunction affected 16 of patients (80%) and small for size non function was present in four patients (20%). Regarding graft size in patients with SFSS; 10, 5 and 5 of patients had extra-small graft [small for size graft (SFSG), graft recipient weight ratio (GRWR) < 0.8], small graft (GRWR ⩾ 0.8 and < 1) and medium sized graft (GRWR ⩾ 1) respectively. Extra small graft (SFSG), portal hyper-perfusion, severe portal hypertension (PHTN), and venous outflow obstruction were the main direct causes of SFSS in 10 (50%), 3 (15%), 4 (20%), and 3 (15%) of patients respectively. While extra-small graft, PHTN, steatosis and left lobe graft were significant predictors of SFSS in univariate analysis, only graft size was independent predictor of SFSS on multivariate analysis. On the other hand, there was non-significant lower incidence of SFSS in patients with SFSG when splenectomy was done, furthermore, there was non-significant lower incidence of the syndrome in patients with right lobe graft when drainage of the right anterior and/or posterior liver sectors by middle hepatic vein, V5, V8, and/or right inferior vein was done. The SFSS related mortalities were recorded in 13/20 of patients (65%). The 6-mo, 1-, 3-, 5-, 7- and 10-year survival in patients with SFSS were 30%, 30%, 25%, 25%, 25% and 25% respectively, while, the 6-mo, 1-, 3-, 5-, 7- and 10-year survival in patients without SFSS were 70.1%, 65.6%, 61.7%, 61%, 59.7%, and 59.7% respectively, with statistical significant difference.

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INTRODUCTION

Living donor liver transplantation (LDLT) is acceptable management option for end-stage liver disease (ESLD) patients to overcome organ shortage and waiting list death. On the other hand, adult-to-adults LDLT (A-ALDLT) is affected by the so-called SFSG[1]. Until now, there is debate about the least volume of the graft required for A-ALDLT[2,3]. The volume of liver graft is determined by either graft recipient weight ratio (GRWR)[4], or the ratio of graft volume relative to standard liver volume of the recipient (GV/SLV); SFSG are those with a GRWR < 0.8% and/or those with a GV/SLV < 35%[2,3]. So, if GRWR < 0.8 % or a GV/SLV < 35%, the graft should be regarded as SFSG[5-8]. As SFSS occurrence depends upon the liver graft volume as well as other different negative factors, SFSG and SFSS definitions differ in different institutes and at different times[9,10].

SFSS diagnosis is determined by persistent elevation of bilirubin and large volume of ascites during the early period post liver transplantation (LT) with absence of other possible causes[2,3,11]. Generally, it is characterized by occurrence of the followings at the end of the 1st week post LT: Persistent cholestasis, coagulopathy, ascites, encephalopathy and/or bleeding from gastrointestinal tract and/or renal failure in some severe conditions[4,11-19]. Moreover, SFSS can be defined as Total bilirubin > 10 mg/dL and/or output of ascites > 1 L/d on the 14th day after LT[7].

The loss of balance between the rapid liver regeneration and the increased demand of liver to do his function is the principal pathogenesis of SFSS[3,20], moreover, it has become evident that SFSS is not just caused by SFSG, but by multiple factors. These factors are divided into graft-related factors and recipient

related ones[19,21-23].

The graft related factors include: (1) high portal inflow[17,20,24]; (2) low venous outflow[25,26]; (3) Pree-xisting steatosis in the donor[27,28]; (4) advanced donor age[29]; and (5) both warm and cold ischemia times[16,30,31]. However, recipient-related causes include severe preoperative ESLD and poor health status[7,16,32,33].

As occurrence of SFSS is determined by the balance between the functional mass of the liver, inflow of portal venous (PV), and outflow of hepatic vein (HV), Strategies to prevent it depend upon increasing the volume of liver graft and controlling adequate PV inflow and HV outflow by the surgical and the non-surgical techniques[22,34]. For increasing graft volume, a larger-sized graft, such as the right lobe (RL) graft, is used as the standard strategy for A-A LDLT to fulfill the required metabolic demands of adult recipients[35-38]. There are different techniques for control of graft inflow (*i.e*., splenectomy, splenic artery embolization, splenic artery ligation, mesocaval - or portocaval shunts)[39,40]. For outflow modulation; any short HV (especially RIV, V5, V8) larger than 0.5 cm are preserved, to be anastomosed with the recipient inferior vena cava (IVC)[3].

Splanchnic vasoconstrictors, intravenous octreotides, and oral propranolol may improve the persistent hyperbilirubinemia and coagulopathy in SFSS adult recipients[40,41]. The purpose of this work was to analyze the incidence, risk factors, prevention, treatment and outcome of SFSS after LDLT.

MATERIALS AND METHODS

Patients

Two hundred ten LDLT operations were done between April 2003 and December 2013 in our surgical department, National Liver Institute, Menoufiya university, our study included 174 adult patients after exclusion of cases with data loss and pediatrics, after taking the approval of our institutional reviewers (IRB); we did this cohort study which is a single-institution retrospective analysis of a prospectively collected database that analyzed the incidence, risk factors, prevention, treatment and outcome of SFSS in a period started from the end of 2013 to end of 2015, with patients observation from the 1st post-operative day (POD 1) until December 2015 or until patient death. The median period of follow-up reached 40.50 m, range (0-144 m).

The characteristics of recipients and their donors (including operative parameters): Regarding recipient gender, males were 154 (88.5%) while females were 20 (11.5%); furthermore, the mean age of them reached 46.5 ± 8.1 years. As regard donor gender, male donors were 118 (67.8%) and females were 56 (32.2%); the donors mean age reached 27.2 ± 6.7 years. According to Child-Pugh score, child A, B, and C were 9 (5.2%) 53 (30.5%) and 112 (64.4%) respectively, on the other hand, the mean model for end stage liver disease score(MELD) was 16.09 ± 4.3, moreover, MELD < 18, MELD 18-24, and MELD > 24 were 114 (65.5%), 50 (28.7%) and 10 (5.7%) respectively. Pre LT portal hypertension (PHTN) affected 144 (82.8%) of them.

Steatosis affected nine (5.2%) of grafts. The RL graft was given to 166 (95.4%) and the LL was given to 8 (4.6%) of them. The MHV was reconstructed in 17 (9.8%) of patients, furthermore, there were single, double, three and four HV anastomoses in 110 (63.2%), 53 (30.5%), 10 (5.7%) and 1 (0.6%) of them respectively. However, drainage of right anterior and/or posterior sectors by MHV, V5, V8, and/or RIV in RL grafts occurred in 56/166 (33.7%) of patients. The mean actual graft weight and actual GRWR were 820.9 ± 174.2 g and 1.04 ± 0.2 g respectively, moreover, SFSG (GRWR < 0.8) was found in 17 (9.8%) of patients, where splenectomy was done in seven (41.2%) of them to decrease portal hyper-flow. The decision to do intra-operative splenectomy was as follow: 4 cases due to severe pre transplant PHTN and SFSG (GRWR = 0.7, 0.73, 0.74, and 0.75) and the other 3 cases due to extra SFSG (GRWR = 0.57, 0.65, and 0.66).

Regarding cold ischemia and warm ischemia times, their mean reached 74.9 ± 51.2 min and 52.9 ± 15.2 min respectively. On the other hand, the mean intra-operative plasma and blood transfusion reached 8.2 ± 8.9 units and 7.05 ± 7.4 units respectively. Lastly, operative time mean was 13.1 ± 3.2 h while the in-hospital stay mean after LT was 22.4 ± 15.9 d (Table 1).

Methods

We collected our data from the unit of LT of our Institute after obtaining written informed consents for operations and researches from recipients and their donors. Our donor's age was > 19 years, furthermore, they underwent the followings: Liver function tests, abdominal ultrasound, liver biopsy, CT angiography, CT volumetric study and psychological assessment. We studied the following.

Preoperative data: Age of donors and recipients, their gender, donors body mass index and liver biopsy, recipient Child Pugh, MELD scores and PHTN. For pre-operative prevention of SFSS; the following strategies were done: (1) appropriate donor selection: (2) steatosis < 10%; (3) donor diet program and/or daily exercise for controlling steatosis in donors; (4) younger donors; (5) in the early cases, estimated (by volumetric study) GRWR < 0.8 were refused, and then in late cases we refused estimated GRWR < 1 for obtaining actual GRWR < 0.8; and (6) appropriate recipient selection by refusing MELD scores < 30.

Intra-operative data: RL or LL grafts, graft with or without MHV, No of HV anastomoses, HV drainage of the RT anterior and/or posterior liver sectors, actual graft weight, and GRWR, performing splenectomy or not, cold ischemia and worm ischemia times per

Table 1 Characteristics of patients and their donors

Character	*n* (%) 174 (100%) (mean ± SD)
Donor age (yr) (mean ± SD)	27.2 ± 6.7
Recipient age (yr) (mean ± SD)	46.5 ± 8.1
Donor gender	
Males	118 (67.8)
Females	56 (32.2)
Recipient gender	
Males	154 (88.5)
Females	20 (11.5)
Child class	
A	9 (5.2)
B	53 (30.5)
C	112 (64.4)
MELD score	
< 18	114 (65.5)
18-24	50 (28.7)
> 24	10 (5.7)
MELD score (mean ± SD)	16.09 ± 4.3
Pre LT PHTN	144 (82.8)
Steatosis	9 (5.2)
Graft type	
Right lobe	166 (95.4)
Left lobe	8 (4.6)
MHV with the graft	
RL graft	10 (5.7)
LL graft	7 (4.1)
No of HV anastomoses	
1	110 (63.2)
2	53 (30.5)
3	10 (5.7)
4	1 (0.6)
Drainage of RT anterior and/or posterior sectors by MHV, V5, V8, and/or RIV in RT lobe grafts (*n* = 166)	56/166 (33.7)
Actual graft weight (g) (mean ± SD)	820.9 ± 174.2
Actual GRWR	1.04 ± 0.2
SFSG (GRWR < 0.8)	17 (9.8)
Splenectomy in SFSG (*n* = 17)	7/17 (41.2)
Cold ischemia time (min) (mean ± SD)	74.9 ± 51.2
Warm ischemia time (min) (mean ± SD)	52.9 ± 15.2
Intraoperative blood transfusion (units) (mean ± SD)	7.05 ± 7.4
Intraoperative plasma transfusion (units) (mean ± SD)	8.2 ± 8.9
Operative time (h) (mean ± SD)	13.1 ± 3.2
Hospital stay post LT (d) (mean ± SD)	22.4 ± 15.9

MELD: Model for end stage liver disease; PHTN: Portal hypertension; MHV: Middle hepatic vein; RIV: RT inferior vein; GRWR: Graft recipient weight ratio; SFSG: Small for size graft.

minutes, plasma and blood transfusion per units and operative time per hours.

For intra-operative prevention of SFSS, the following strategies were done: (1) in the donor operation, with RL graft without middle hepatic vein (MHV) (our standard technique), any short hepatic vein (specially RIV, V5, V8) > 0.5 cm was preserved for possible anastomosis with recipient veins, while MHV was taken with the graft in some cases (dominant MHV and/or SFSG), on the other hand, MHV was taken with all LL grafts except one of them[42]; (2) during back table preparation, the required interposition vein grafts (patch, pantaloon or jumping grafts) that were obtained mainly from the native PV or PUV were reconstructed with the graft veins and prepared for reconstruction with the recipient veins to maximize the liver graft outflow; (3) in the recipient operation, IVC was preserved during explantation of the native liver, the RL graft HV drainage pathways consisted of the RHV without MHV or with it in some cases, furthermore, the RIV, V5 and/or V8 veins were reconstructed in some cases when indicated (Figures 1-4). The standard technique used in reconstruction of the RHV was an end-to side anastomosis between RHV of the graft and the RHV of the recipient with caudal extension to the IVC[43]. However, the LL graft HV drainage pathways consisted of the MHV with the LHV in one stump or separately (N.B the standard technique of HV reconstruction was performing a wide end-to-side anastomosis, between the graft and recipient veins avoiding rotation with extended incision to the vena cava)[3]. It was fundamental to perform complete reconstruction of these pathways of HV outflow to avoid HV congestion of the RL or LL grafts.

The portal vein (PV) reconstruction was then performed in an end-to-end fashion using 3 loupe magnification and by using 6/0 prolene continuous stitches with the routine use of about 1 cm growth factor during tying[44]. After PV reconstruction, doppler ultrasonography (US) was done to assess PV flow (PVF).

Postoperative management: (1) based on our institutional policy and similar to other schools like Japanese school; immunosuppression protocol was as follow: Triple-drug regimen that included calcineurin Inhibitors (CNIs) as FK-506 or cyclosporin, mycophenolate mofetil (MMF), and steroids. Three months after LT, steroids were withdrawn while we performed withdrawal of MMF 6 mo after operation. In late cases, for minimizing the dose of CNI, we administered an interleukin-2 receptor blocker on the day of LT and on the 4th day postoperative; (2) Doppler ultrasonography (PV and HV patency, flow and velocities) was performed routinely just after vascular reconstruction and after closure of the abdomen and then twice daily until the 7th day after operation (POD7), and once per day until hospital discharge; (3) Diagnosis of SFSS: The patients laboratory and clinical parameters (*i.e.*, Serum bilirubin, INR, volume of ascites, and encephalopathy) were followed up to detect the occurrence of SFSS that was classified into small for size dysfunction (SFSD) and small for size non function (SFSNF) (N.B, SFSD is dysfunction of the graft (the presence of persistent hyperbilirubinemia, ascites and coagulopathy) during the early post LT period with absence of other possible causes like Immunological (*e.g.*, graft rejection), technical (*e.g.*, HA or PV obstruction, HV outflow occlusion or biliary leak), infection (*e.g.*, cholangitis). However, SFSS is SFSD or failure of the graft (SFSNF) (loss of graft function leading to patient loss or necessity of retransplantation) during the early post LT period with

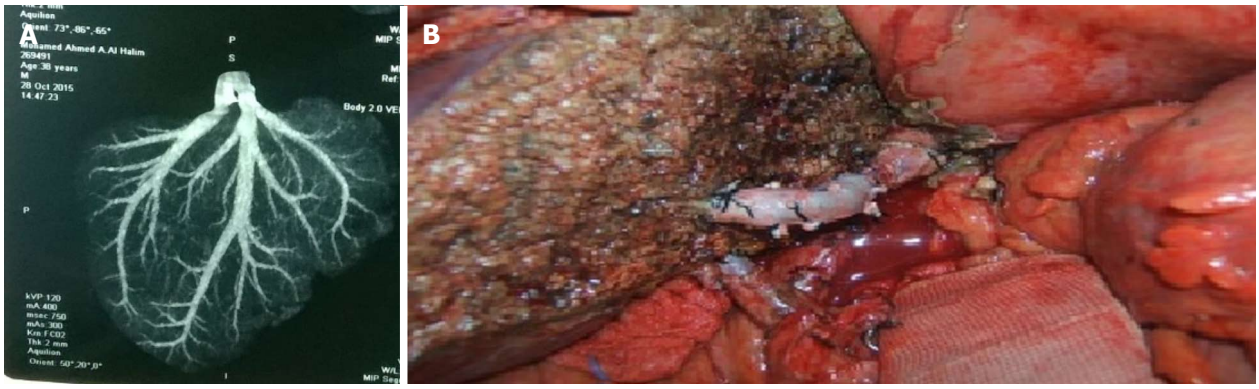

Figure 1 Graft with V5 to be anastomosed with recipient liver transplantation hepatic vein. A: Computed tomography venography showing large V5; B: A jumping graft between the V5 vein of liver graft and liver transplantation hepatic vein of recipient.

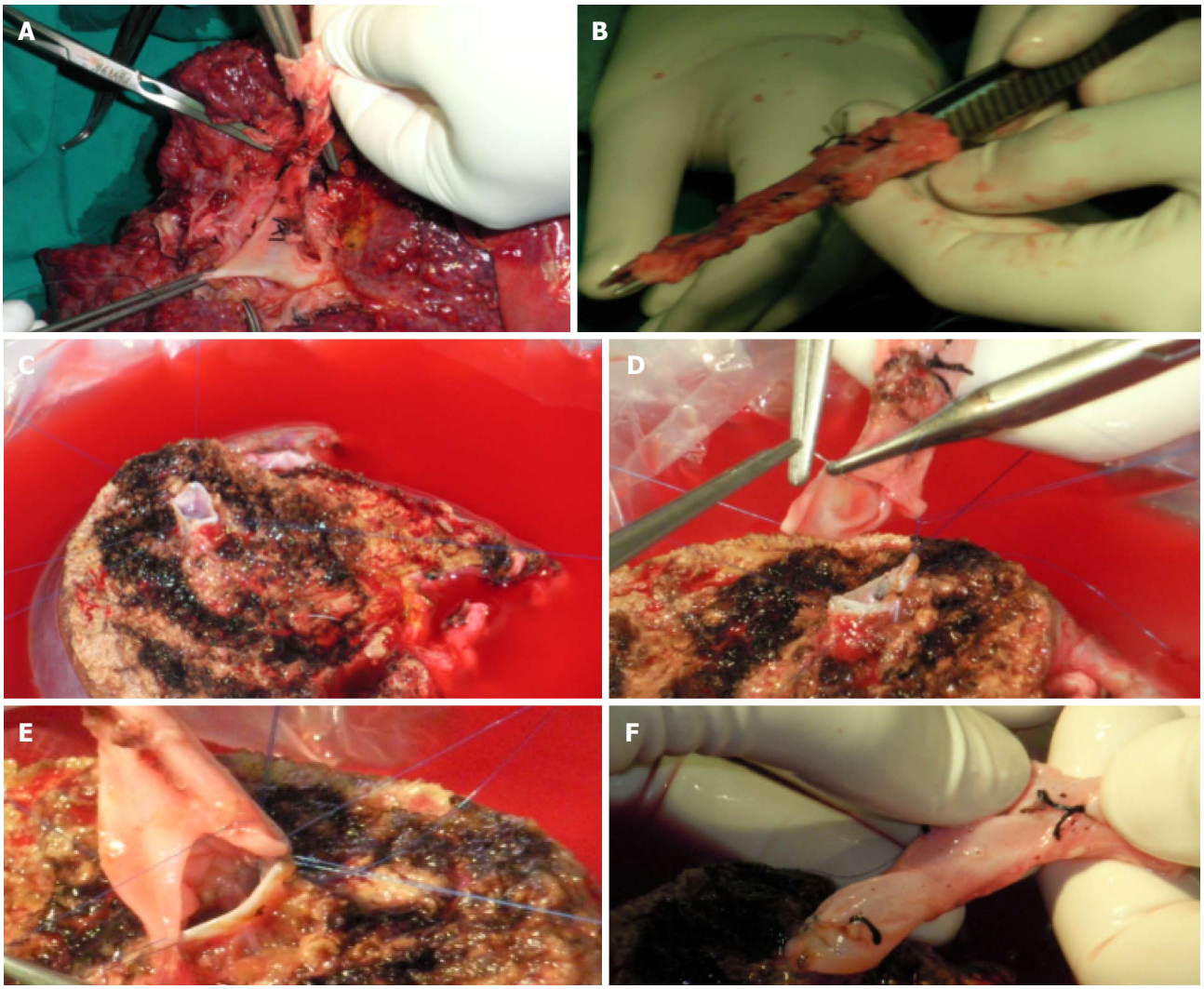

Figure 2 Graft with V8 to be anastomosed with recipient inferior vena cava by jumping graft. A: Obtaining the venous graft from native PV; B: The venous graft; C: V8 vein during back table preparation; D and E: Anastomosing the venous graft to V8; F: Preparation for anastomosing the venous graft to IVC. IVC: Inferior vena cava.

absence of those previously mentioned causes[11]; and (4) management of SFSS: Strategies for prevention were mentioned in the pre- and intra-operative data; furthermore, meticulous post-transplant care was taken in cases with SFSG; Treatment: Right now, very little literature payed attention on how to manage the SFSS after its development; however, oral propranolol (2 × 40 mg/d) and a somatostatin infusion (250-μg

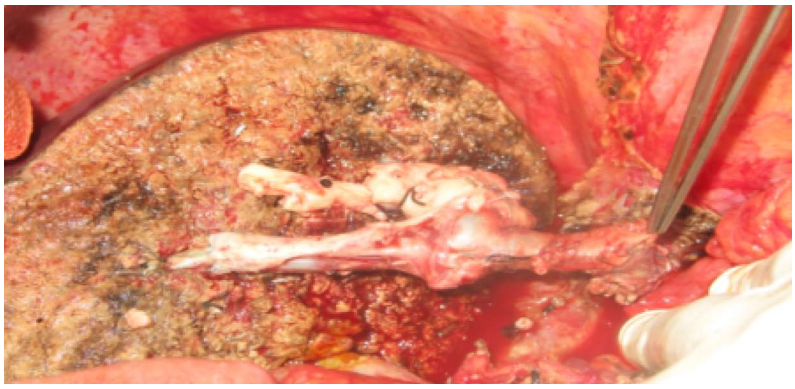

Figure 3 Venous graft obtained from native PUV, portal venous and hepatic vein to communicate 2 V5, 1V8 and right hepatic vein of liver graft with recipient inferior vena cava.

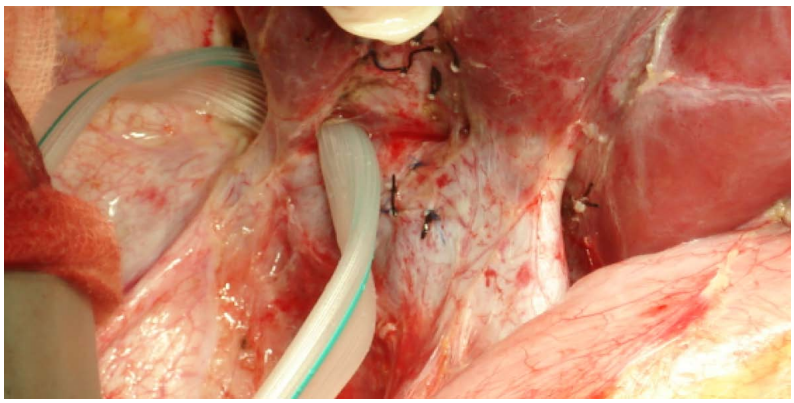

Figure 4 Small right hepatic vein (encircled) and large right inferior vein harvested and anastomosed with recipient inferior vena cava.

bolus followed by perfusion at a rate of 250-50 μg/h for 5 d were given to some of our patients with SFSS to decrease PVF[23,41,45]. Moreover, liver symptomatic support was taken by all patients with the syndrome[15].

Follow-up and outcome of patients: They were followed-up daily until hospital discharge, then weekly until the end of the 1st month then monthly until the end of the follow-up period to detect SFSS and its outcome regarding survival, mortalities, causes of deaths as well as the outcome of SFSG.

Statistical techniques

We used SPSS software (version 21, Chicago, IL, United States) for data processing. Categorical variables were analyzed with the χ^2 or Fisher exact tests. Continuous variables were compared using the student T or Mann whitney tests. The pre-operative, intra-operative and post-operative variables were descriptively studied. We did comparison between patients with and without SFSS regarding the pre- and intra-operative variables using univariate analysis and then multivariate analysis. Furthermore, their outcome as well as cause of death was compared by univariate analyses. On the other hand, Kaplan-Meier curve was applied and plotted for survival analysis (patient and graft survival) and the log-rank tests were used for comparing patient and graft survival according to SFSS and for comparing patient survival according to SFSG. In the previous tests, if *P* value was < 0.05, it was considered significant.

RESULTS

Some characteristics of patients with SFSS

SFSS was diagnosed in 20 (11.5%) of our recipients where, SFSD affected 16 of patients (80%) and SFSNF was present in four patients (20%). Persistent hyperbillirubinaemia, ascitis, and coagulopathy affected 100%, 90%, and 85% of our SFSS cases respectively, where; all the 16 patients with SFSD had persistent hyperbilirubinemia, ascites and coagulopathy during the early post-LT period; however, all the 4 cases with SFSNF had persistent hyperbilirubinaemia, 2 of them had massive ascites and one of them had coagulopsthy; furthermore, they developed graft failure and died from SFSS complications (*e.g.*, Sepsis, MOF, ARDS, DIC) during the 1st week post-transplant. Regarding graft size in patients with SFSS, 10, 5, and 5 of patients had extra-small graft (SFSG, GRWR < 0.8), small graft (GRWR ⩾ 0.8 and < 1) and medium sized graft (GRWR ⩾ 1) respectively. Extra small

Table 2 Some characteristics of patients with small for size syndrome

Character	*n* (%)
SFSS	20 (100)
Type of SFSS	
SFSD	16 (80)
SFSNF	4 (20)
Main presentation	
Hyperbilirubinaemia	20 (100)
Large volume of ascites	18 (90)
Coagulopathy	17 (85)
Graft size	
GRWR < 0.8 (SFSG)	10 (50)
GRWR ⩾ 0.8 and < 1	5 (25)
GRWR ⩾ 1	5 (25)
Main aetiology of SFSS	
Extra small graft (SFSG)	10 (50)
Portal hyperperfusion	3 (15)
Severe PHTN	4 (20)
Outflow obstruction	3 (15)

SFSS: Small for size syndrome; SFSD: Small for size dysfunction; SFSNF: Small for size non function; PHTN: Portal hypertension.

graft (SFSG), portal hyperperfusion, severe PHTN, and venous outflow obstruction were the main direct causes of SFSS in 10 (50%), 3 (15%), 4 (20%), and 3 (15%) of patients respectively. Moreover; Portal hyperperfusion was assessed by doppler US post operatively, severe PHTN was the persistent pre transplant severe PHTN that was assessed by complete history, clinical examination laboratory and imaging, lastly, venous outflow obstruction was known by post-transplant doppler ultrasonography US (Table 2).

Comparison between patients with and without SFSS

The following variables were statistically significant predictors of SFSS on univariate analysis, Pre LT PHTN, graft steatosis, LL graft, SFSG, mean actual graft weight 640.50 ± 211.049 g, mean actual GRWR 0.862 ± 0.2158 g and mean intra-operative plasma transfusion 11.40 ± 7.816 units. On the other hand, there was lower incidence of SFSS in patients with SFSG who underwent splenectomy [4/10 (40%) SFSS *vs* 3/7 (42.9%) no SFSS] but without statistical significance, However, there was none significant lower incidence of the syndrome in patients with RL graft when drainage of the RT anterior and/or posterior sectors by MHV, V5, V8, and/or RIV was done [4/10 (28.6%) SFSS *vs* 52/152 (34.2%) no SFSS], furthermore, there was lower incidence of the syndrome in patients with RL graft without MHV who underwent reconstruction of V5, V8 and/or RIV [3/13 (23.1%) SFSS *vs* 43/143 (30.1%) no SFSS] but without statistical significance. On the other hand, Child score, MELD score, cold and worm ischemia times had no effect on occurrence of the syndrome (Table 3).

On multivariate analysis, mean actual graft weight 640.50 ± 211.049 g, and mean actual GRWR 0.862

Table 3 Comparison between patients with and without small for size syndrome (Univariate analysis)

Character	SFSS, *n* (%) 20 (100) (mean ± SD)	No SFSS, *n* (%) 154 (100) (mean ± SD)	*P* value
Child class			< 0.05
A	1 (5)	8 (5.2)	
B	7 (35)	46 (29.9)	
C	12 (60)	100 (64.9)	
MELD score			< 0.05
< 18	16 (80)	98 (63.6)	
18-24	4 (20)	46 (29.9)	
> 24	0 (0)	10 (6.5)	
Pre LT PHTN			0.046
Yes	20 (100%)	128 (83.1%)	
No	0 (0)	26 (16.9%)	
Steatosis			0.035
Yes	3 (15)	6 (3.9)	
No	17 (85)	148 (96.1)	
Graft type			0
RL	14 (70)	152 (98.7)	
LL	6 (30)	2 (1.3)	
SFSG (GRWR < 0.8)			0
Yes	10 (50)	7 (4.5)	
No	10 (50)	147 (95.5)	
Actual graft weight (g) (mean ± SD)	640.50 ± 211.049	844.39 ± 154.888	0
Actual GRWR (g) (mean ± SD)	0.862 ± 0.2158	1.065 ± 0.1922	0.001
Cold ischemia time (min) (mean ± SD)	73.95 ± 55.350	75.13 ± 50.923	< 0.05
Warm ischemia time (min) (mean ± SD)	50.95 ± 14.248	52.08 ± 16.336	< 0.05
Intraoperative plasma transfusion (units) (mean ± SD)	11.40 ± 7.816	7.81 ± 8.943	0.021
No. of HV anastomoses			< 0.05
1	11 (55)	99 (64.3)	
2	8 (40)	45 (29.2)	
3	1 (5)	9 (5.8)	
4	0 (0)	1 (0.6)	
Splenectomy in patients with SFSG (*n* = 17)			< 0.05
Yes	4 (40)	3 (42.9)	
No	6 (60)	4 (57.1)	
Drainage of RT anterior and/or posterior sectors (MHV, V5, V8, RIV) in RL graft with or without MHV (*n* = 166)			< 0.05
Yes	4 (28.6)	52 (34.2)	
No	10 (71.4)	100 (65.8)	
MHV reconstruction in patients with RL graft (*n* = 166)			< 0.05
Yes	1 (7.1)	9 (5.9)	
No	13 (92.9)	143 (94.1)	
Drainage of RT anterior and/or posterior sectors (V5, V8, RIV) in RL graft without MHV(*n* = 156)			< 0.05
Yes	3 (23.1)	43 (30.1)	
No	10 (76.9)	100 (69.9)	

MELD: Model for end stage liver disease; Pre LT PHTN: Pre liver transplant portal hypertension; RL: Right lobe; LL: Left lobe; SFSG: Small for size graft; GRWR: Graft recipient weight ratio; MHV: Middle hepatic vein; RIV: Right inferior vein.

Table 4 Multivariate analysis of predictors of small for size syndrome (Binary logistic regression)

	P value	Exp(B)	95%CI for EXP (B)	
			Upper	Lower
Pre LT PHTN	0.998	0.00	0.000	
Steatosis	0.060	0.145	0.020	1.074
Graft type	0.166	6.407	0.463	88.717
Actual GRWR < 0.8	0.050	4.303	1.024	18.082
Actual graft WT	0.030	1.004	1.000	1.008
Intraoperative plasma transfusion (units)	0.235	0.963	0.905	1.025

Pre LT PHTN: Pre liver transplant portal hypertension; GRWR: Graft recipient weight ratio.

± 0.2158 g were the only independent predictors of SFSS, however, graft steatosis had trend towards independence (*P* = 0.06) (Table 4).

Outcome of patients

Patients with SFSG had statistically significant higher mortality than those without SFSG (76.5% *vs* 40.8%, *P* = 0.005), furthermore, mortality was significantly higher in SFSS patients than those without SFSS (75% *vs* 40.3%, *P* = 0.003), On the other hand, the most frequent cause of death in patients with the syndrome was the syndrome itself and its complications (*i.e.*, Sepsis, graft failure, DIC, renal failure, ARDS, and MOF), furthermore, the 4 cases with SFSNF died during the 1st week post LT due to the syndrome complications (*e.g.*, sepsis, MOF, ARDS, DIC) and the other 16 cases with SFSD were classified into: Five a live patients, 2 patients died from post LT bleeding, and 9 patients died from the syndrome complications (*i.e.*, Sepsis, graft failure, DIC, renal failure, ARDS, MOF). However, sepsis was the most frequent reason for mortality in non SFSS patients 19 (30.6%); moreover, MOF from causes other than SFSS, post-operative bleeding, intra-operative bleeding, PVT, renal impairment from causes other than SFSS, metastatic cholangiocarcinoma, early graft dysfunction from causes other than SFSS, HCC recurrence, ischemic reperfusion injury, HAT were the other causes of death in 11 (17.7%), 10 (16.1%), 8 (12.9%), 4 (6.4%), 2 (3.2%), 2 (3.2%), 2 (3.2%), 2 (3.2%), 1 (1.6%), and 1 (1.6%) of them respectively. Regarding clavien grading, all the previous causes of death in both groups were grade V. The 6-mo, 1-, 3-, 5-, 7- and 10-year survival in patients with SFSS were 30%, 30%, 25%, 25%, 25% and 25% respectively, while, the 6-mo, 1-, 3-, 5-, 7- and 10-year survival in patients without SFSS were 70.1%, 65.6%, 61.7%, 61%, 59.7%, and 59.7% respectively, with statistical significant difference. Lastly, graft survival in patients with SFSS was 20%, however it was 57.8% in patients without the syndrome with statistical significant difference (*P* = 0.001) (Table 5 and Figure 5).

DISCUSSION

SFSS limits LT expansion; furthermore, it is the major cause of worse short-term prognosis after LDLT[17]. Therefore, better understanding of its pathogenesis, risk factors, strategies for prevention and treatment may improve outcomes after LDLT.

The incidence of SFSS in LL LDLT is higher than RL LDLT (20% *vs* 10%)[46]; as the LL graft gives only about 40 % of the needed liver mass that affect the metabolic demands of adult recipients leading to SFSS[1,18,47]. Similarly, the syndrome rate was significantly higher in our LL LDLT than RL LDLT (75% *vs* 8.4%, *P* = 0.000), and this was due to small NO of our LL LDLT (eight cases), where six of them (75%) had SFSG. On the other hand, LL SFSG was the only independent predictor of graft dysfunction in Yi *et al*[48] (2008) study. However, SFSS rate was 22.2%, and 19.5% in LL LDLT of Soejima *et al*[7] (2003), and Soejima *et al*[49] (2012) studies respectively, and 11.5% in our study that included mainly RL LDLT(166 cases). On the other hand, it was 9.6% and 12.5% in LDLT of Gruttadauria *et al*[50] (2015) and Ben-Haim *et al*[32] (2001) studies respectively. In contrast, it was higher (22.7%, 50% and 37.5%) in RL LDLT of Goralczyk *et al*[15] (2011), LL LDLT of Katsuragawa *et al*[51] (2009) and LL LDLT of Lauro *et al*[52] (2007) studies respectively, and obviously lower (6.3%) in Botha *et al*[53] (2010) study.

SFSS is a disease related to partial liver grafts denoting its inability to perform the functional requirements of the adult recipients resulting in hepatic dysfunction and/or failure and usually manifests as hyperbilirubinemia, ascites, coagulopathy, and encephalopathy[15,19,23,40,46,47]. Furthermore, it is characterized microscopically by cholestasis, hemorrhagic necrosis around the central veins and ballooning of hepatocytes due to microcirculatory disturbances[54]. Similarly, the syndrome was presented by hyperbilirubinemia, ascites, and coagulopathy in 100%, 90%, and 85% of our patients respectively. Moreover, we had 4 (20%) cases with SFSNF and 16 (80%) patients with SFSD.

The principal pathogenesis of SFSS is the unbalance between regeneration of the liver and the increased liver function demand, resulting in graft dysfunction[1]. Furthermore, it is clear that the syndrome is not just caused by SFSG, but also by multiple factors including technical issues, quality of the graft, and recipient factors[3,18,22,32,55,56] (where, the balance between PV inflow, outflow of HV, and functional mass of liver determines its development)[17]. So, for preventing SFSS, it is important to increase the graft volume, and to control adequate PV inflow and HV outflow by the surgical and the none surgical techniques[34]. On the other hand we divided our strategies for preventing the occurrence of the syndrome into pre-operative and intra-operative ones.

The required graft size for successful LT is 30%-40% of the expected liver volume for the recipient (GV/SLV) or 0.8%-1.0% of the body weight (GRWR)[19]; as the insufficient graft size is the primary cause of SFSS due to the relative shortage of hepatic parenchymal cells[2,3,12,16,17,55]; furthermore, SFSG suffers from a

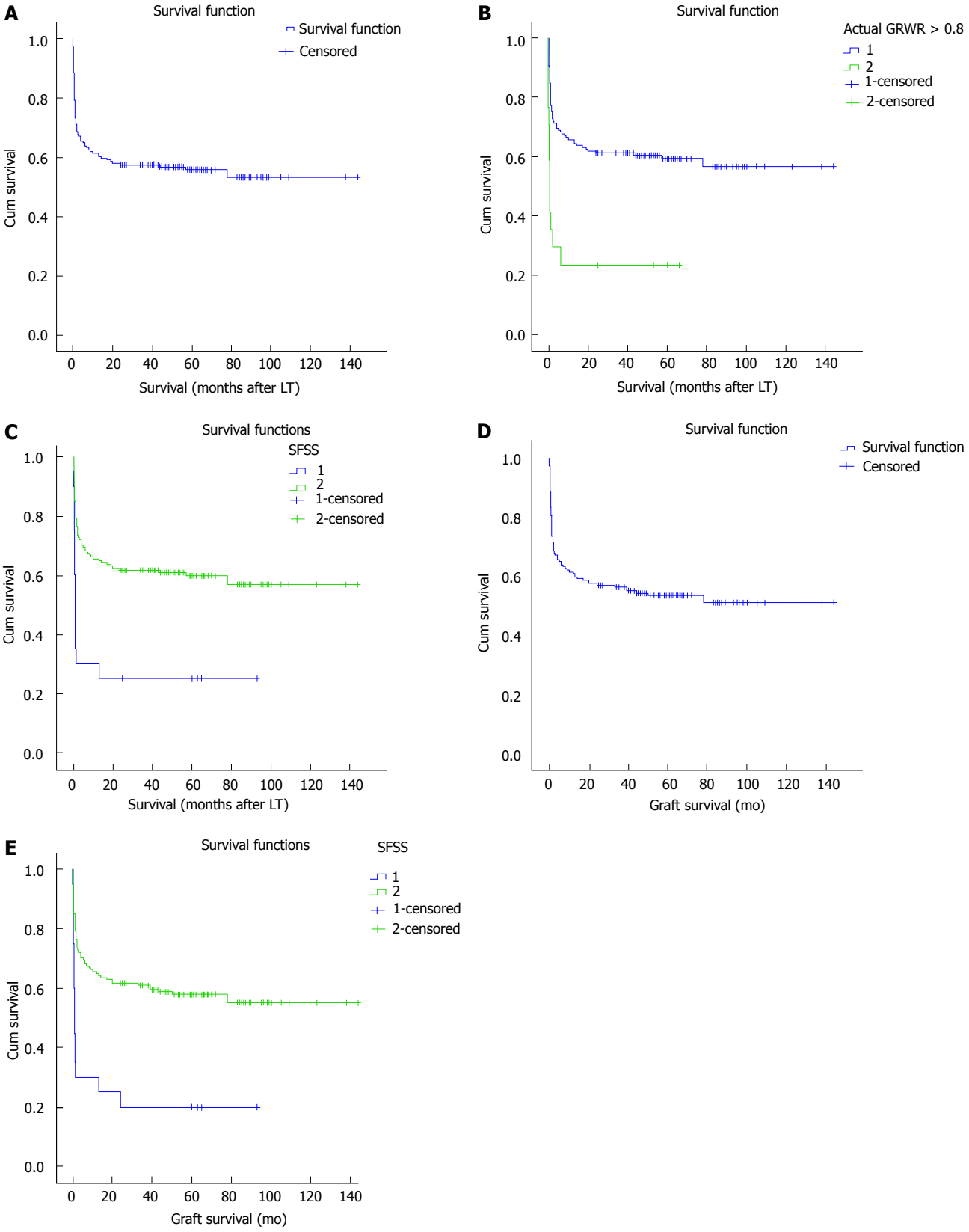

Figure 5 Kaplan-Meier survival curves (1, 2, 3). A: KM survival curve; B: SFSG and survival [SFSG (GRWR < 0.8) = 2, Log-Rank = 0.00]; C: SFSS and survival (SFSS = 1, Log-Rank = 0.00); D: KM graft survival curve; E: SFSS and graft survival (SFSS = 1, Log-Rank = 0.00). SFSG: Small for size graft; GRWR: Graft recipient weight ratio; SFSS: Small for size syndrome.

transient PHTN early after reperfusion, that is associated with up-regulation of endothelin-1 in the graft and ultra-structural evidence of sinusoidal damage[8], so, the incidence of SFSS increases when the graft

Table 5 Outcome of patients

Total number	SFSS *n* (%) 20 (100)	-	No SFSS *n* (%) 154 (100)	-	*P* value
Overall mortality	15 (75)	Grade	62 (40.3)	Grade	0.003
Cause of mortality and their Dindo-Clavien score					
Sepsis from causes other than SFSS	0	-	19 (30.6)	V	0
SFSS (sepsis, graft failure, DIC, renal failure, ARDS, MOF)	13 (86.7)	V	0	-	
MOF from causes other than SFSS	0	-	11 (17.7)	V	
Post-operative bleeding	2 (13.3)	V	10 (16.1)	V	
Intra-operative bleeding	0	-	8 (12.9)	V	
PVT	0	-	4 (6.4)	V	
Renal impairment from causes other than SFSS	0	-	2 (3.2)	V	
Metastatic cholangiocarcinoma	0	-	2 (3.2)	V	
Early graft dysfunction from causes other than SFSS	0	-	2 (3.2)	V	
HCC recurrence	0	-	2 (3.2)	V	
Ischemic reperfusion injury	0	-	1 (1.6)	V	
HAT	0	-	1 (1.6)	V	
6-mo survival	6 (30)	-	108 (70.1)	-	0.000
1-yr survival	6 (30)	-	101 (65.6)	-	0.002
3-yr survival	5 (25)	-	95 (61.7)	-	0.002
5-yr survival	5 (25)	-	94 (61)	-	0.002
7-yr survival	5 (25)	-	92 (59.7)	-	0.003
10-yr survival	5 (25)	-	92 (59.7)	-	0.003
Survival per months (mean ± SD)	16.3 ± 28.9	-	39.9 ± 34.3	-	0.002
Graft survival	4 (20)		89 (57.8)		0.001
Graft survival per months (mean ± SD)	16.2 ± 28.9		39.7 ± 34.3		0.003

SFSS: Small for size syndrome; DIC: Disseminated intravascular coagulation; ARDS: Adult respiratory distress syndrome; MOF: Multi organ failure; PVT: Portal vein thrombosis; HCC: Hepatocellular carcinoma; HAT: Hepatic artery thrombosis.

is SFSG[11,23,52,57]. In Similar, SFSG was independent predictor of SFSS in Lei *et al*[58] (2012) study, similarly, SFSG was the most frequent cause of SFSS (50%) in our series, and the only independent predictor of it in our multivariate analysis despite our efforts to decrease SFSG by selecting larger-sized RL graft and by selecting donors with estimated GRWR > 1(in our late cases) as a pre-operative strategy for preventing SFSS. In contrast, Graft size had no impact on SFSS in Shimazu *et al*[59] (2004), and Ikegami *et al*[60] (2009) studies.

Although, SFSS is frequently encountered in SFSG (GRWR < 0.8), it may also be found in recipients of larger grafts (GRW > 0.8)[9,10,61-64]. Similarly, in our work the incidence of SFSS in normal size graft (GRWR > 0.8) was 6.4% (10/157); and this was due to the effect of other negative factors.

Steatotic liver grafts should not be used if the graft volume is small to avoid SFSS[16,17]; furthermore, graft steatosis is an exclusion criterion for donation in LDLT[65]. The mechanisms of poor steatotic graft function after reperfusion include defective anaerobic metabolism of the fatty hepatocytes, decreased lumen of sinusoids by the fat droplets, and higher free radicals caused by lipid peroxidation[3,27]. In similar, severe steatosis was significantly associated with poor function post LDLT in Hayashi *et al*[66] (1999) study. In addition, despite our refusal of grafts with steatosis > 10% to avoid the occurrence of SFSS, steatosis was significant predictor of SFSS in our univariate analysis; moreover it had a trend towards being independent predictor in multivariate analysis. In contrast, graft steatosis had no impact on graft dysfunction in Yi *et al*[48] (2008) study. Similarly, Sterneck *et al*[67] (1995) reported that grafts with mild to moderate steatosis had good function, and Soejima *et al*[56] (2003) found that a graft with 20%-50% macrovesicular steatosis (moderate grade) was accepted for transplantation.

Because LDLT is a scheduled procedure, daily exercise and diet control are required for steatosis control in donors[1,4]. In similar, donor diet programs and/or daily exercise for controlling steatosis in our donors were parts of our preoperative strategies for avoiding SFSS.

The principal mechanism in SFSS seems to be sinusoidal shear stress secondary to increased PV pressure (PVP) and/or PVF which cause graft over-perfusion leading to hepatic microcirculatory disturbance, hepatocyte functional insufficiency, over-regeneration of the hepatocytes, hepatocellular damage and death[3,16,17,19,20,23,46,51,52,68]; furthermore, Portal hyper-perfusion and insufficient venous outflow decrease the arterial perfusion (the so-called hepatic arterial buffer response), with a reduced capacity for regeneration, resulting in impaired liver function[18,19,23,69]; Similarly, portal inflow volume was independent predictor of SFSS in Lei *et al*[58] (2012) study. In similar, in our work, pre LT PHTN was significant predictor of SFSS in univariate analysis; furthermore, severe pre LT PHTN that persisted post LT was the etiology of the syndrome in 4 (20%) of our cases of SFSS, however, portal hyper-perfusion (identified by doppler US) was the cause of it in 3 (15%) of them.

One of the ways to get portal decompression is depriving the splenic part of portal flow by splenectomy[3,17,19,39,46,51,52,68,70]. Furthermore, splenectomy increases the HA blood flow leading to increased oxygen supply[18]. Similarly, we did splenectomy in 7/17 of our patients with SFSG to decrease portal overflow that lead to non-significant lower incidence of the syndrome (40% SFSS *vs* 42.9% no SFSS).

Theoretically, a RL graft including MHV is the best graft for LDLT regarding the recipients; but, this type of graft is not performed in most major transplant centers due to increased donor risk by decreasing the residual volume of the liver[3,23]. So, the RL graft without MHV is the standard technique in A-ALDLT[1,15,71]; however deprivation of the anterior segment venous drainage cause graft congestion, leading to graft dysfunction in spite of the increased volume of the graft[1,25,26,36]. Therefore, reconstruction of the anterior segments drainage veins (V5/V8)[15,17,23,72,73] with or without the reconstruction of the RIV is frequently necessary to prevent this[3]. Similarly, in our series, RL graft without MHV was our standard technique of LT, moreover, we did reconstruction of V5, V8 and/or RIV in 46/156 of our patients with RL graft without MHV that lead to non-significant lower rate of the syndrome (23.1% SFSS *vs* 30.1% no SFSS). Nevertheless, venous outflow obstruction (Known by doppler US) was the reason for the syndrome in 3 (15%) of our SFSS cases. In addition, venous outflow capacity was independent predictor of SFSS in Lei *et al*[58] (2012) study.

Early graft function is better when the graft is given by a younger donor[74,75]; as, the grafts from older donors have diminished regenerative capacity[75,76], lower blood flow and poor function due to aging[18]. Similarly, Ikegami *et al*[77] (2000) in their LL LDLT, found that regeneration of grafts from older donors of LDLT were inferior to those of grafts from younger ones and Tanemura *et al*[78] (2012), in their RL LDLT reported that donor age equal or more than 50 years was independent predictor of impaired regeneration of remnant liver at 6 mo post LT, furthermore, donor age was significant predictor of graft dysfunction and poor graft survival in Yi *et al*[48] (2008) and Moon *et al*[79] (2010) studies, and was independent predictor of SFSS in Sanefuji *et al*[80] (2010) study, while Ikegami *et al*[29] (2008) found that grafts from younger donors had lower bilirubin levels and ascites production post LDLT. On the other hand, in their RL LDLT, the Kyoto group reported that the functional recovery of recipients from older donors was comparable to that of those from younger ones[81]. Similarly, donor age was not significant predictor of SFSS in our series where our donors had younger age (mean = 27.2 ± 6.7 years).

Both warm[30] and cold ischemia times[31] impair regeneration after LDLT. Conversely, in our series, there was no significant correlation between cold or worm ischemia times and SFSS occurrence. Similarly, ischemia time did not affect graft function in Yi *et al*[48] (2008) study.

A higher MELD score has negative insult on graft function that may cause its dysfunction or failure especially in SFSG; due to its inability to meet the increased metabolic and synthetic demands of those high-risk recipients with severely damaged liver function[1,16,17]. In similar, MELD score was independent predictive of SFSS in Lei *et al*[58] (2012) study. However, Yoshizumi *et al*[75] (2008) reported that a larger liver graft is necessary with older donors (> 50 years) and higher MELD score (> 20), and Emiroglu *et al*[82] (2007) mentioned that recipients with high MELD scores should be given grafts only when their GRWR is > 1 to improve graft survival also, Ikegami *et al*[77] (2000) recommended that patients with high-risk should be given a younger and larger grafts to minimize the risk of SFSS. On the other hand, pre-operative MELD score did not affect SFSS rate in our work.

The preoperative Child Pugh score is mostly associated the portal hyper-perfusion state after LT leading to SFSS[1,18]. Similarly, Ben-Haim *et al*[32] (2001) reported that patients with severe decompensation (Child B, C) require larger grafts to prevent occurrence of SFSS, while, Soejima *et al*[7] (2003) found that the rate of SFSS after A-A LDLT was higher in cirrhotic patients (43.8%) in comparison with non-cirrhotics (5%). Conversely, there was no significant correlation between Child score and SFSS in our work.

Most literature mentions how to prevent SFSS occurrence. However, very few literatures discuss the treatment of this syndrome after its occurrence. In Goralczyk *et al*[15] (2011) study, most SFSS cases were treated with successful symptomatic therapy. Furthermore, intravenous octreotide, and oral propranolol were found to decrease the hyperbilirubinemia and coagulopathy seen in patients with SFSS in Ozden *et al*[41] (2007) study. On the other hand, symptomatic liver support was given to all our patients with SFSS but with poor outcome; moreover, oral propranolol and a somatostatin infusion were given to some of our patients with SFSS to decrease portal flow and improve the syndrome outcome but also with poor outcome.

Approximately 50% of recipients with SFSG die of sepsis 4 to 6 wk after LT[83]; moreover survival rates of patients with SFSG are worse than those with adequate graft size[2,12]. In similar, SFSG was significant predictor of poor survival in our work (P = 0.005), also, it had negative impact on survival in Lo *et al*[84] (1999), Sugawara *et al*[55] (2001), and Lee *et al*[8] (2004) studies. Furthermore, it was independent predictor of graft loss in Katsuragawa *et al*[51] (2009) study. Conversely, SFSG did not affect survival in Shimazu and Kitajima[59] (2004), Shimada *et al*[85] (2004), Ikegami *et al*[60] (2009), Selzner *et al*[86] (2009), Moon *et al*[79] (2010), Kaido *et al*[47] (2011), and Li and Li[87] (2013) studies.

SFSS results in higher incidence of septic compli-

cations, pulmonary failure, renal failure, and increased mortality[22,23,46], furthermore, it causes prolonged hospitalization, graft and patient loss[15]. Similarly, in our series, SFSS lead to significant higher mortality rate (P = 0.003), and the most frequent cause of death was the syndrome itself and its complications (*i.e.*, sepsis, graft failure…). In similar, recipients who developed SFSS had inferior patient survival in Soejima *et al*[7] (2003), and Lauro *et al*[52] (2007) studies. In addition, it was the direct cause of 3 mortalities in Soejima *et al*[43] (2006) study. In conclusion: SFSG is the independent and main factor for occurrence of SFSS after A-ALDLT leading to poor outcome. However, the management of this catastrophe depends upon its prevention (*i.e.*, selecting graft with proper size, splenectomy to decrease portal venous (PV) inflow, and improving HV outflow by reconstructing large draining veins of the graft).

COMMENTS

Background

Small for size syndrome (SFSS) is dysfunction of the graft (the presence of persistent hyperbilirubinemia, ascites and coagulopathy) during the early post liver transplantation (LT) period with absence of other possible causes like technical, immunological or infection causes, or failure of the graft (loss of its function leading to patient loss or necessity of retransplantation) during the early post LT period with absence of the previously mentioned causes. Small for size graft (SFSG) is the independent and main factor for occurrence of this syndrome that limits LT expansion and leads to worse short-term prognosis after living donor liver transplantation (LDLT). Therefore, better understanding of SFSS pathogenesis, risk factors, strategies for prevention and treatment may improve outcomes after LDLT. Moreover, the management of this catastrophe depends mainly on its prevention by pre-, intra- and post- operative measures like selecting graft with proper size, proper control of portal vein (PV) inflow and hepatic vein (HV) outflow.

Research frontiers

SFSG is the independent and main factor for occurrence of SFSS after A-ALDLT leading to poor outcome; so it is crucial to select graft with proper size to avoid this catastrophic complication. Furthermore, proper control of PV inflow by splenectomy and HV outflow by reconstruction of large tributaries of graft HV may prevent occurrence of this syndrome, however, these conclusions need further studies.

Innovations and breakthroughs

The study goes with other literature studies that mentioned the correlation between SFSG and SFSS and their negative insult on outcome after A-A LDLT, however, the innovation and breakthroughs in the work is that the authors gave an idea about the important rule of intra-operative splenectomy (specially in SFSG) as well as the meticulous reconstruction of HV tributaries of liver graft in preventing the occurrence of this syndrome (despite the non-statistical significance), as the literature data is very few regarding these points.

Applications

The study emphasizes the rule of pre-, intra- and post-operative strategies for prevention of SFSS as selection of graft with proper size. Furthermore, the authors encourage performing further studies to emphasize the rule of intra-operative splenectomy as well as the rule of reconstructing large HV tributaries of the transplanted liver graft in preventing the occurrence of SFSS.

Terminology

SFSG: Is the graft where graft recipient weight ratio (GRWR) < 0.8; SFSD: It is dysfunction of the graft (the presence of persistent hyperbilirubinemia, ascites and coagulopathy) during the early post LT period with absence of other possible causes like Immunological (*e.g.*, graft rejection), technical (*e.g.*, HA or PV obstruction, HV outflow occlusion or biliary leak), infection (*e.g.*, cholangitis); SFSNF: Failure of the graft (loss of its function leading to patient loss or necessity of retransplantation) during the early post LT period with absence of those previously mentioned causes; SFSS: SFSD and/or SFSNF.

Peer-review

It is an interesting quite large series.

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ORIGINAL ARTICLE

Observational Study

Outcomes of pregnancy in patients with known Budd-Chiari syndrome

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Abstract

AIM

To analyse the risk of pregnancy (a prothrombotic state) in patients with Budd-Chiari Syndrome (BCS).

METHODS

Retrospective study of pregnancy in women with known BCS at single center from January 2001 to December 2015.

RESULTS

Out of 53 females with BCS, 7 women had 16 pregnancies. Median age at diagnosis of BCS in these women was 25 years (range 21-34 years). At least one causal factor for BCS was identified in 6 women (86%). Six women had undergone radiological decompressive treatment. All patients had anticoagulation. Six fetuses were lost before 20 wk gestation in 2 women. There were 9 deliveries over 32 wk gestation and one delivery at 27 wk. All infants did well. Seven babies were born by emergency caesarean section. There were no cases of thrombosis. Two patients had notable vaginal (PV) bleeding in 3 pregnancies. None of the patients had variceal haemorrhage. Two patients were diagnosed with pulmonary hypertension, one during pregnancy

and the other in the post-partum period. There was no maternal mortality.

CONCLUSION

Maternal outcomes in patients with treated BCS are favourable and fetal outcomes beyond 20 wk gestation are good. There has been increased rate of caesarean section. Pulmonary hypertension is an important finding that needs further validation. These patients should be managed in centers experienced in treating high-risk pregnancies.

Key words: Budd-Chiari syndrome; Pregnancy; Portal hypertension; Pulmonary hypertension; Thrombophilia

Core tip: Pregnancy is a prothrombotic state and can cause adverse outcome in patients with Budd-Chiari syndrome (BCS). In our study, maternal outcome in patients with known and treated BCS was good. However, most deliveries were carried out by emergency caesarean section (7/10). There was high incidence of placental disease leading to caesarean section. Fetal outcome beyond 20 wk gestation was also good. With careful monitoring of anti-coagulation, there were no cases of thrombosis and only a minority of patients had noteworthy bleeding complications. Development of pulmonary hypertension in two patients several years after TIPSS is an important finding that warrants further studies.

Khan F, Rowe I, Martin B, Knox E, Johnston T, Elliot C, Lester W, Chen F, Olliff S, Mehrzad H, Zia Z, Tripathi D. Outcomes of pregnancy in patients with known Budd-Chiari syndrome. *World J Hepatol* 2017; 9(21): 945-952 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i21/945.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i21.945

INTRODUCTION

Budd-Chiari syndrome (BCS) is a rare disorder caused by hepatic venous outflow obstruction and resulting hepatic dysfunction due to sinusoidal congestion, ischaemic injury to the liver and portal hypertension. The main mechanism for BCS is thrombosis of the hepatic veins or of the terminal portion of the inferior vena cava[1,2]. The management using a stepwise regimen is largely successful with anticoagulation and interventional radiology alone. Stepwise regimen includes; (1) anticoagulant therapy for an indefinite period of time; (2) angioplasty or stenting for stenosis of hepatic veins; and (3) decompressive techniques [surgical shunt or transjugular intrahepatic porto-systemic shunts (TIPSS)], for patients who are non-responsive to medical treatment or not candidates for angioplasty/stenting[3]. TIPSS has a lower morbidity and mortality rate than surgery and is a preferred approach**.** The outcomes are favourable with 10-year survival approaching 90%[4,5].

Usually multiple risk factors for venous thromboembolism are present in patients with BCS[1,6-8]. In one study, 84% of 163 patients with BCS had at least one thrombotic risk factor, and 46% of these patients had more than one prothrombotic risk factor; the most common was myeloproliferative neoplasia (MPN) (49% of 103 tested patients)[9]. In another study of 43 women with BCS, at least one thrombotic risk factor (not considering pregnancy as risk factor) was identified in 40 women (93%) including MPN in 56% of study participants[10]. Other thrombotic risk factors include mutation in Factor V Leiden and prothrombin gene, protein C, protein S or antithrombin deficiency, antiphospholipid syndrome, hyperhomocysteinemia and paroxysmal nocturnal haemoglobinuria. BCS may also be a complication of systemic vasculitides such as Bechet's disease[11].

BCS mainly affects women of childbearing age and pregnancy can be a crucial issue. There is conflicting data on prevalence of pregnancy related BCS. A systematic review and meta-analysis of twenty studies demonstrated a pooled prevalence of pregnancy-related BCS of 6.8%[12]. However another study showed that pregnancy is unlikely to cause BCS in the absence of other thrombotic risk factors[10].

Pregnancy is a hypercoagulable state and earlier studies reported that women with BCS could be at risk of developing severe exacerbation of their underlying disease during pregnancy[13,14]. Rautou *et al*[15] conducted a study on outcome of pregnancy in women with known and treated BCS and concluded that good maternal outcome could be achieved with current treatment modalities and close surveillance of BCS. Therefore, BCS cannot be considered a contraindication to pregnancy in stable patients with well-controlled disease.

As the available literature on pregnancy complications in women with known BCS remains scarce, we performed this study of women treated at our tertiary centre for BCS who had become pregnant.

MATERIALS AND METHODS

We used the definitions related to outcome of pregnancies as previously described by Rautou *et al*[15]: (1) date of diagnosis of BCS: the first imaging modality showing an obstructed venous outflow tract; and (2) miscarriages: A spontaneous loss of pregnancy before 20 weeks' of gestation. Outcome of the pregnancy: (1) favourable: Live birth occurred at 32 or more completed weeks of gestation, with a healthy infant and no serious obstetrical complication (bar intrahepatic cholestasis); and (2) poor: Otherwise pregnancy outcome. Rotterdam prognostic index was calculated as previously described[16].

The electronic records of all female patients dia-

gnosed with BCS between January 2001 and December 2015 at our tertiary care referral center were retrospectively analysed. The data was collected prospectively and radiology records of these patients were also searched. Those that became pregnant during the follow-up for BCS were included in the study. Patients in whom pregnancy occurred before BCS was diagnosed were excluded.

All patients were tested for the known prothrombotic factors. Combined oral contraceptive pill (OCP) use within the 3 mo preceding diagnosis of BCS was considered a thrombotic risk factor.

Where possible, patients had pre-pregnancy counselling and were made aware of the potential complications that may occur during pregnancy. Patients with known varices or portal hypertension had pre-pregnancy gastroscopy to ensure varices had been treated. These patients had further gastroscopies for variceal surveillance during second trimester. Patients with TIPSS had regular abdominal ultrasound to ensure patency of the TIPSS. The patients were monitored in a joint haematological/obstetric clinic.

Given the risk of embryopathy and fetal loss associated with warfarin, low molecular weight heparin (LMWH) was substituted for warfarin as soon as pregnancy was diagnosed, or prior to conception in one patient who had two *in-vitro* fertilisation treatments. The dose of LMWH was adjusted to maintain therapeutic factor Xa activity in selected cases under haematology supervision. LMWH treatment was replaced by warfarin following the delivery.

RESULTS

Baseline characteristics

Fifty-three female patients under follow-up for BCS were identified. Out of these, 7 patients had 16 pregnancies during the study period.

Median age of diagnosis of BCS was 25 years (range 21-34 years). Five (71%) patients had abdominal pain as the presenting complaint and symptoms were mainly chronic in nature. One patient had variceal haemorrhage and three patients had ascites on presentation of BCS. None of them had hepatic encephalopathy. None of the patients had other significant co-morbidities when the diagnosis of BCS was established. The characteristics of these patients including Rotterdam and Clichy scores at the time of diagnosis of BCS are given in Table 1. The laboratory values were stable at time of conception in all patients and ascites had resolved.

BCS was managed by anticoagulation therapy and radiological interventions with the aim to recanalise any outflow obstruction. Six out of the 7 patients underwent liver decompression procedures before conception. Procedures included dilatation of right hepatic vein (one patient), TIPSS (in four patients) and right hepatic vein stenting (one patient). One patient did not have any intervention for decompression and was managed with oral anticoagulation (warfarin) alone. All patients had anticoagulation. None of the patients in our series required surgical porto-systemic shunting or liver transplantation as a definite treatment of BCS.

At least one causal factor for hepatic vein obstruction was identified in 6 of these 7 women (86%). JAK 2V617F mutation alone was seen in 2 patients; factor V Leiden alone in one; JAK 2 mutation and factor V Leiden in one patient; JAK 2 mutation and OCP use in one patient; and factor V Leiden and OCP use in one patient**.** One patient did not have any identifiable risk factor.

Pregnancy course

Median age at conception was 32 years (range 23-39). Median time between diagnosis of BCS and conception was 5 years (range 3 mo-13 years). Follow up after the diagnosis of BCS in the seven women with pregnancies was for a median of 7 years (range 3-14 years). All patients that became pregnant had well compensated liver disease at the time of each conception and stigmata of decompensation of liver disease (ascites, in majority of patients at presentation) were no longer present at the time of any pregnancy. Gestational course is detailed in Table 2.

Aspirin (along with LMWH) was administered to one patient in 2 pregnancies (patient 6) for Essential Thrombocytosis. This patient was also treated with interferon for JAK 2 positive MPN. No patient was treated with beta-blockers during pregnancy.

Six out of the 16 (38%) pregnancies miscarried with fetal loss before 20 wk gestation. Six miscarriages/failed pregnancies occurred in 2 patients. One miscarried at 5 wk when she presented with vaginal bleeding. She was not aware of the pregnancy. The other patient had 5 miscarriages over a 9-year period. Two out of 5 were after the first trimester and these were attributed to cervical weakness and, therefore, she had cervical sutures in the following pregnancies (after 13 wk of gestation) leading to two successful deliveries.

Out of the 10 pregnancies reaching beyond 20 wk gestation, there were 3 vaginal deliveries and 7 caesarean sections. There was one very preterm birth at 27 wk and 5 preterm deliveries between 32 and 35 wk gestation, all with favourable neonatal outcomes. Four pregnancies resulted in delivery after 36-wk gestation, again all with favourable outcome.

Seven (70%) infants were delivered *via* emergency caesarean sections. Indications for caesarean section were varied, including fetal distress in three pregnancies; pre-eclampsia in one, breech presentation in one, bleeding from placenta praevia in one patient and difficult labour due to cervical suture in one patient.

Specific complications

Four patients developed intrahepatic cholestasis of pregnancy (ICP) in five pregnancies and they were treated with ursodeoxycholic acid. One patient had pre-eclampsia needing emergency caesarean section.

Table 1 Baseline characteristics of the patients at presentation

Patient ID	1	2	3	4	5	6	7
Age at diagnosis (yr)	34	21	30	21	31	24	25
Symptoms at presentation	Ascites	Oesophageal variceal haemorrhage, abdominal pain	Abdominal pain; ascites	Abdominal pain, ascites	Abdominal pain, fever, mouth ulcers	Ascites, renal failure and sepsis (ITU admission)	Abdominal pain
Risk factors for BCS	JAK 2 positive MPD; OCP	JAK 2 positive mutation	None identified	Factor V Leiden; OCP	JAK2 positive MPD (Essential Thrombocythaemia); Factor V Leiden	JAK 2 positive mutation	Factor V Leiden
Encephalopathy	None	None	None	None	None	None	None
Ascites	Moderate	Mild	Mild	Mild	None initially	Severe	Moderate
INR	1.7	1.4	1.2	1.3	1.7	1.4	1.5
Albumin (g/L)	28	37	49	49	49	25	26
Bilirubin (umol/L)	19	18	20	18	11	51	32
ALT (U/L)	-	31	-	57	-	-	-
AST (U/L)	134	49	20	34	27	277	43
Urea (mmol/L)	2.7	2.3	2.9	4.7	2.9	4.4	2
Creatinine (mmol/L)	72	43	70	68	51	92	70
Sodium (mmol/L)	143	137	143	142	140	130	133
MELD	19	14	6	10	12.37	14	17
UKELD	53	53	48	49	49	49	55
Hb (g/L)	137	121	155	128	150	147	88
WCC (10^9/L)	7.9	9.6	10.9	5.7	5.7	28.8	6.8
Platelets (10^9/L)	345	183	307	247	411	400	226
Rotterdam PI	1.116	0.072	1.12	0.07	1.08	1.244	1.168
Clichy PI	4.39	1.99	3.13	4.04	3.44	7.54	7.55
Liver biopsy	Not done	Not done	Not done	Suggestive of hepatic vein obstruction	Consistent with Hepatic venous outflow obstruction	Not done	Not done
Level of obstruction	Left hepatic vein	Hepatic vein	Hepatic vein	Hepatic Vein	Right Hepatic Vein	Left Hepatic vein	Hepatic vein
Radiological intervention	TIPSS	TIPSS	None	Angioplasty and Stenting to Hepatic vein	Right Hepatic Vein dilatation	TIPSS	TIPSS
Type of TIPSS	Viatorr (covered)	Viatorr (covered)	-	-	-	Memotherm, then Viatorr	Memotherm (Uncovered)
Medications post intervention	Warfarin	Warfarin	N/A	Warfarin	Warfarin	Warfarin, Interferon	Warfarin
Duration of follow up (yr)	4	5	7	3	13	14	14
Comments/ complications following intervention	TIPSS Stent redilatation after a week of insertion	TIPSS stent stenosis - needed to be re-dilated in 2 yr	Maintained on oral anticoagulation (warfarin) and did not require any intervention	Vascular Wallstent was re-canalized after 2 yr	Inferior RHV dilated 5 yr after the diagnosis (developed ascites and had compliance issues).	Bleeding from hepatic nodule (with INR > 9). Managed conservatively. Later stent was changed to a covered one for TIPSS stenosis	-

MPD: Myeloproliferative disorder; TIPSS: Trans-jugular intrahepatic posto-systemic shunt; OCP: Oral contraceptive pills; INR: International normalised ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; MELD: Model for end-stage liver disease; UKELD: United Kingdom model for end-stage liver disease; Hb: Haemoglobin; WCC: White cell count.

Significant PV bleeding occurred after 3 pregnancies in 2 patients (patients 3 and 6 in Tables 1 and 2). One patient (patient 3) had a primary post-partum haemorrhage secondary to a retained placenta that was surgically removed. The other patient (patient 6) had a complicated first pregnancy with placental abruption at 27 wk gestation and needed emergency caesarean section. In her second pregnancy, she had secondary postpartum haemorrhage following caesarean section for suspected placental abruption. It was treated with surgical evacuation of uterine clot and insertion of a Rusch Balloon. There were no cases of variceal haemorrhage.

One patient, (patient 5) underwent regular gastroscopies for banding of (non-bleeding) oesophageal varices. That patient was not treated with beta-blockers during pregnancy. There were no cases of thrombosis in any of the pregnancies.

Table 2 Gestational course and perinatal complications in 16 pregnancies

Patient No.	Pregnancy No.	Age at gestation (yr)	Anticoagulation during pregnancy	Mode of delivery	Weeks gestation	Birth weight	Foetal/infant condition	Maternal condition
1	1	37	LMWH	Vaginal	36	2645 g	Neonatal jaundice, treatment with antibiotics for suspected infection	
2	2	24	LMWH	Emergency caesarean section	35	2140 g	Fetal distress (reduced foetal movements)- Healthy baby	ICP OGDs during pregnancy, no varices seen
3	3	35	LMWH	Vaginal delivery	35	2600 g	Mild Jaundice	*In-vitro* fertilization treatment
3	4	37	LMWH	Vaginal delivery	37	2450 g	Healthy	*In-vitro* fertilization treatment Primary post-partum haemorrhage secondary to retained placenta that was surgically evacuated
4	5	23	LMWH	Caesarean section	37	2645 g	Fetal distress - Healthy baby post delivery	…
5	6	36	LMWH and Aspirin (switched from warfarin and Hydroxyurea at 22 wk when pregnancy was diagnosed)	Emergency caesarean section	37	3115 g	Breech presentation	Had several gastroscopies (OGD) and banding to Oesophageal Varices during pregnancy
5	7	39	Warfarin	Miscarriage	5	-	-	PV bleeding; was not aware of conception
6	8	31	LMWH	Emergency Caesarean Section	27	Not available	Healthy boy	Bleeding secondary to placental abruption ICP from 25 wk
6	9	37	LMWH, Aspirin, interferon for MPD (Myeloproliferative disorder)	Emergency Caesarean section	35	Not available	Fetal distress. Healthy baby	ICP Minor subchorionic bleeding at 12 and 23 wk. LMWH reduced, aspirin stopped temporarily. Changes resolved on subsequent scans. Presentation with PH and suspected placental abruption at 35 wk Secondary post-partum haemorrhage treated with surgical of uterine clot evacuation and Rusch Balloon
7	10	25	LMWH	Miscarriage	9	-	-	-
7	11	27	LMWH	Miscarriage	20	-	Congenital pneumonia and mild amnionitis	Weakness of cervix;
7	12	28	LMWH	Miscarriage	19	-	-	Placental abruption Weakness of cervix; Placental abruption
7	13	29	LMWH	Emergency Caesarean Section	35	2974 g	Healthy boy	Dyspnoeic during 3rd Trimester; ICP in 20 wk onwards; C-Section for difficult labour (cervical suture could not be removed)
7	14	31	LMWH	Failed Pregnancy	10	-	-	Surgical removal of retained products of Contraception
7	15	33	LMWH	Miscarriage	7	-	-	-
7	16	34	LMWH	Emergency Caesarean Section	35	2440 g	Healthy boy	Pre-eclampsia; Breathlessness during 3rd trimester, PH diagnosed after pregnancy

LMWH: Low molecular weight heparin; ICP: Intrahepatic cholestasis of pregnancy; PH: Pulmonary hypertension.

Two patients (patients 6 and 7) developed symptoms of pulmonary hypertension (PH) during the

course of pregnancy and are described as follows.

Case 1

This patient had second pregnancy at the age of 37 years (13 years after the diagnosis and treatment of BCS). She had minor subchorionic bleeding noted on ultrasound during pregnancy. At 35 wk of gestation, this patient had emergency caesarean section for suspected placental abruption and developed respiratory failure post operatively. Trans-thoracic echocardiography (TTE) suggested PH with pulmonary artery systolic pressure estimated at 60-65 mmHg. CT scan excluded pulmonary embolism and showed patent TIPSS and mild splenomegaly. Right heart catheterisation confirmed the presence of PH with mean pulmonary artery pressure (mPAP) of 37 mmHg and pulmonary artery wedge (PAWP) pressure of 12 mmHg. She is being treated with Sildenafil (phosphodiesterase inhibitor) and Macitentan (endothelin receptor antagonist) for PH. Follow up investigations demonstrated improved exercise tolerance with no significant limitations in activities of daily living (patient 6; Tables 1 and 2).

Case 2

This patient delivered her second child at 34 years of age, 9 years after the diagnosis and treatment of BCS. Caesarean section was performed at 35-wk gestation for pre-eclampsia. Dyspnoea on exertion was noted during the pregnancy and six months after delivery she was admitted with right heart failure. CTPA excluded pulmonary embolus; but noted dilatation of pulmonary artery, moderate to severe dilatation of right atrium and moderate dilation of right ventricle with a degree of right ventricular hypertrophy. TIPSS was shown to be patent. TTE demonstrated severe PH, severely dilated right ventricle with impaired systolic function. Right heart catheterisation confirmed PH (mPAP 53 mmHg, PAWP 11 mmHg). The patient has been treated with sildenafil and intravenous Iloprost (along with warfarin) for PH and is being considered for lung transplantation assessment (patient 7; Tables 1 and 2).

DISCUSSION

The majority of the patients affected by BCS in Western countries are women of childbearing age[1,16], with the peak incidence in the third decade for women and in the fourth decade for men[17]. Fertility is generally unaffected in women with BCS as only a minority becomes cirrhotic.

Several previously reported observations suggest that pregnancy in BCS women could cause deterioration of the liver disease and pregnancy was associated with development of ascites in several women with known BCS[17-19]. Rautou *et al*[15] showed that the maternal outcome, in 14 women with 24 pregnancies is good in women becoming pregnant after the diagnosis and treatment of BCS. All mothers were alive at a median follow-up of 34 mo after last delivery and only one of them required liver transplantation after 73 mo follow-up.

In our series, there were no thrombotic events occurring during pregnancy or the postpartum period. This is comparable to previous study[15] where 2 of 17 pregnancies on anticoagulation therapy were complicated by portal vein thrombosis[15]. Subclavian and portal venous thrombosis has been reported in a pregnant patient with known and treated BCS secondary to (JAK 2 negative) essential thrombocytosis on anticoagulation[20].

Two patients had notable bleeding related to 3 deliveries in contrast to 6 patients with 7 bleeding episodes during pregnancy or postpartum in the previous study[15], signifying the importance of careful management of anticoagulation in pregnancy.

Both of our patients who developed pulmonary hypertension (mPAP $\geqslant$ 25 mmHg at rest) had the diagnosis of BCS and insertion of TIPSS several years ago. TIPSS has been regarded as a cardiac stress by suddenly increasing the preload leading to increased cardiac diastolic volumes and diameters, and a transient PH for 3-6 mo[21,22]. It is usually accommodated rapidly and is then associated with a reduction in systemic vascular resistance and a reduction in afterload[22]. However, development of PH after one and half years following TIPSS insertion has been reported[23]. In a recent study looking at the long-term cardiopulmonary outcome following TIPSS in cirrhotic patients, authors found higher prevalence of PH in the TIPSS group, 1 to 5 years post TIPSS implantation[24]. Although the patients in that study[24] could could have had associated cirrhotic cardiomyopathy, conversely there appears to be a potential long-term risk of development of PH in non-cirrhotic patients with a patent, functional TIPSS. Therefore, further studies on the interactions of TIPSS and cirrhotic cardiomyopathy are warranted[25].

PH has also been reported as a common finding in MPN[26]. This possible association of PH with MPN has also been suggested by small case series and studies[27-30] and the exact incidence and prevalence of PH in this group of patients remain poorly defined[31]. MPN could possibly have had an impact on the development of PH in one of our patients (patient 6).

Current recommendations are to offer endoscopic screening for varices in patients with portal hypertension, when conception is planned and during the second trimester if not already on prophylaxis. One patient (patient 2) who had originally presented with variceal haemorrhage underwent gastroscopy in second trimester for variceal screening and was found not to have varices. Another patient (patient 5, who had right hepatic vein dilatation) had several gastroscopies for oesophageal variceal band ligation during pregnancy. None of the patients suffered variceal bleeding during pregnancy or were administered non-selective beta-blockers during pregnancy given concerns regarding

use of beta-blockers in pregnancy[32,33].

The number of deliveries by caesarean section was higher in our group of patients (7 in 10 deliveries, 70%) than in the general obstetric population in England (26%)[34] and the previous study (8 caesarean sections in 17 pregnancies, 47%)[15]. Although some of the indications for caesarean section were clearly not related to the presence of BCS (*e.g.,* breech presentation, placenta praevia), the high incidence of placental disease (abruption, pre-eclampsia, fetal distress) leading to caesarean section may be related to the underlying causative aetiology of the BCS. Therefore, close maternal and fetal surveillance for placental disease should be considered in these patients.

Interestingly, for unknown reasons, incidence of ICP has been higher in our patients (4 patients in 5 pregnancies) than the normal obstetric population (0.7%-1.5%)[35,36].

Our study supports that the maternal outcome is good in women becoming pregnant after the diagnosis and treatment of BCS. This favourable maternal outcome is likely to be attributable to improvement in management of BCS including effective decompressive treatment, management of the underlying conditions, anticoagulant therapy with careful follow-up; and management of pregnancy and delivery in multi-disciplinary settings. A possibly decreased level of significant bleeding and no thrombosis implies the benefits of very close monitoring of anticoagulation through joint clinics.

In contrast to the good overall maternal outcome seen in our set of patients, the livebirth rate of 62.5% is lower than in the general obstetric population (84%[37] and 85%-88%[38]), but is better than earlier reports and in line with the finding of Rautou *et al*[15]. Importantly, failed pregnancies occurred in only 2 out of 7 patients. One patient (patient 7) had 5 fetal losses over a 9-year period (83% of the incomplete pregnancies reported here).

Our study supports the conclusion that BCS cannot be considered a contraindication to pregnancy in stable patients. Development of PH is an important finding that needs further validation. Such patients should be managed at tertiary level care centres with multi-disciplinary involvement.

COMMENTS

Background

Budd-Chiari syndrome (BCS) is a rare condition that results from hepatic venous outflow obstruction mainly due to the thrombosis of the hepatic veins and leading to hepatic dysfunction and portal hypertension. Patients with BCS usually have risk factors for venous thromboembolism (VTE). BCS mainly affects young women. Pregnancy is one of the risk factors for VTE and earlier studies reported that women with BCS could be at risk of developing severe exacerbation of BCS during their pregnancies.

Research frontiers

Pregnancy is an important issue in young women with known BCS. There are very few literature sources concerning the pregnancy related complications in women with known BCS. This study hotspot is to look at the outcome of pregnancies in women treated at the centre for BCS and to help other peers understand this important relationship.

Innovations and breakthroughs

Several previously reported observations suggest that pregnancy in women with BCS could cause deterioration of the liver disease. In this series, maternal outcome was good. There were no thrombotic events occurring during pregnancy or the postpartum period, comparable to a large previous study. Only two patients had notable bleeding related to 3 deliveries signifying the importance of careful management of anticoagulation in pregnancy. Two out of 7 patients developed pulmonary hypertension several years after the diagnosis of BCS and insertion of TIPSS. Higher prevalence of PH up to 5 years post TIPSS in cirrhotic patients has been reported recently. There appears to be a potential long-term risk of development of PH in non-cirrhotic patients with a patent, functional TIPSS that needs further exploration. There was higher incidence of deliveries by caesarean section (7 in 10 deliveries) in this study group and was attributed to the placental disease that could be related to the underlying causative aetiology of BCS.

Applications

This study supports that the maternal outcome in women becoming pregnant after the diagnosis and treatment of BCS is good. Fetal outcome beyond 20 wk gestation is also good. Close maternal and fetal surveillance for placental disease should be considered in these patients. Development of PH post TIPPS is an important finding that needs further validation. Such patients should be managed at tertiary level care centres with multi-disciplinary involvement.

Terminology

The BCS is named after a British Physician, George Budd in 1845 and a pathologist Hans Chiari who first described the features of BCS caused by the hepatic venous outflow obstruction in 1899. TIPSS-transjugular intrahepatic portosystemic shunt or transjugular intrahepatic portosystemic stent shunting is an artificial connection within the liver between the inflow portal vein and the outflow hepatic vein. This procedure is usually performed to reduce the portal pressure.

Peer-review

This is an interesting observational analysis of BCS in relation to pregnancy. Previous data are scarce and heterogeneous. The manuscript is nicely written.

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MINIREVIEWS

Addictive behaviors in liver transplant recipients: The real problem?

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Abstract

Liver transplantation (LT) is the gold standard treatment for end-stage liver disease. Whatever the primary indication of LT, substance abuse after surgery may decrease survival rates and quality of life. Prevalence of severe alcohol relapse is between 11 and 26%, and reduces life expectancy regardless of the primary indication of LT. Many patients on waiting lists for LT are smokers and this is a major risk factor for both malignant tumors and cardiovascular events post-surgery. The aim of this review is to describe psychoactive substance consumption after LT, and to assess the impact on liver transplant recipients. This review describes data about alcohol and illicit drug use by transplant recipients and suggests guidelines for behavior management after surgery. The presence of an addiction specialist in a LT team seems to be very important.

Key words: Liver transplantation; Tobacco use; Illicit drugs; Behavior management; Severe alcohol relapse

Core tip: Liver transplantation is the best treatment for end-stage liver disease. However, some transplant recipients use or abuse alcohol, tobacco and illicit drugs during the post-transplant period. Given the scarcity of organs, this type of consumption, which can affect life expectancy and quality, must be addressed with kindness and without moralizing. Although specific behavior treatment does not exist in this indication, specialists in addiction should be part of the transplant team.

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INTRODUCTION

Liver transplantation (LT) is the gold standard curative treatment for end-stage liver disease, acute liver failure and hepatocellular carcinoma. The aim of LT is to improve life expectancy and quality. Hepatitis C is the most common indication for LT, and the major risk factor for hepatitis C virus (HCV) infection is intravenous drug abuse. Alcoholic liver disease (ALD) is the second most common indication for LT in the United States and Europe[1]. Except for alcohol consumption, addictive behavior is poorly studied in transplant patients[2], and there are many obstacles to obtaining pre- and post-transplant data for psychoactive substance consumption. Currently, the question of how to select transplant candidates is often posed; selection is intended to guarantee a survival probability of at least 50% at 5 years with good quality-of-life. How psychoactive substance consumption affects survival rates and post-surgery outcome are major questions that must be answered.

Firstly, the aim of this review is to describe psychoactive substance consumption of patients after LT; and secondly the various treatments available for patients presenting with substance abuse will be described.

PSYCHOACTIVE SUBSTANCE CONSUMPTION AFTER LT

Alcohol consumption in LT patients

Currently, ALD is the second most common indication of LT worldwide, with 30% to 50% of all LT in Europe and 17% in the United States[3]. The survival rates in Europe are 75% at 5 years and 68% at 10 years[1].

The rates of alcohol relapse vary from 7% to 95% because of the heterogeneity of its definition[4-7]. The notion of relapse goes from "slips" to severe relapse[8,9]. The moment and the intensity of alcohol relapse are both important. DiMartini *et al*[9] identified four distinct types of alcohol consumption in liver transplant recipients. Patients who drank low amounts infrequently, patients with early moderate use that decreased over time, patients with later moderate use that increased over time and patients with early and increasing use. Patients who died of recurrent alcoholic liver diseases were in groups with early alcohol relapse after LT[9,10].

Severe relapse consists in the consumption of more than 14 units of alcohol per week for women and more than 21 per week for men[8-12]. The frequency of severe relapse is estimated at 11% to 26%[13] and 5 years after LT this type of relapse decreases life expectancy regardless of the primary indication of surgery[14-17].

Previous studies have attempted to identify the risk factors of alcohol relapse such as the duration of pre-transplantation abstinence, the severity of alcohol dependence, neurocognitive data, male sex, polyaddiction, and social isolation[18-20]. These risk factors are not clearly adapted to the prediction of severe relapse. Some LT teams have suggested calculating a risk of relapse score[21], but their multi-center findings are not yet available[17]. The effect of addiction treatment before LT has been little studied as yet[22,23]; these studies used classical behavioral therapies and were limited with regard to medication, which is not indicated for patients with end-stage liver disease. More recently, baclofen, which is not metabolized by the liver, has demonstrated some effectiveness in maintaining abstinence in cirrhosis patients[24]. This pre-graft period is very special because the question of "life or death" is posed, and there is a serious deterioration in the quality-of-life. Patients on the waiting list are extremely anxious and some present symptoms of depression, stress or insomnia and are in denial of their disease[25]. Apart from a standard addictological follow-up, implementation of any new addictological procedures at this difficult time is neither suitable nor effective. Masson *et al*[26] tried to define an "alcohol contract" before LT in which patients awaiting transplant confirmed their abstinence. This contract did not have any effect on severe relapse rates after LT. In the general population there is a wide variety of alcohol use disorders (AUD), and most people with AUD go into remission after three years without any specific addiction treatment[27]. As Dom says very well[18] some patients with an AUD are more at risk of relapse than others and the course of LT tends to have selected those patients with a low risk of alcohol relapse.

For a minority of transplanted patients, severe relapse exists. The diagnosis of severe alcohol relapse after LT is very difficult for the transplant team. It can be made using several tools such as clinical, blood or urinary analysis, an interview with an addiction specialist or histological data[28,29]. Diagnosis and treatment of severe relapse requires the presence of an addiction unit within the LT center[30,31]. In Table 1, previous significant reports on alcohol relapse are given.

Tobacco consumption in LT patients

Tobacco use is the first preventable cause of mortality in the general population of the United States with a prevalence of 20.9%[2]. During the pre-transplant period, 57% of patients have a lifetime prevalence of smoking, and 27% of all patients are active smokers[32]. Tobacco use is associated with graft loss and higher mortality in kidney, pancreas, lung and heart transplant patients[33]. In LT patients, tobacco use is associated with an increase in the incidence of vascular complications, but this was not found in

Table 1 Previous significant reports on alcohol relapse after liver transplantation

Theme	Ref.	Year	Journal
Risk factors of alcohol relapse	De Gottardi *et al*[21]	2007	*Arch Intern Med*
	Dew *et al*[4]	2008	*Liver Transpl*
Types of relapse	Tome *et al*[8]	2003	*J Hepatol*
	DiMartini *et al*[9]	2010	*Am J Transplant*
	Faure *et al*[15]	2012	*Journal of Hepatology*
	Dumortier *et al*[10]	2015	*Am J Gastroenterol*
Treatment of alcohol relapses	Dimartini *et al*[28]	2001	*Psychosomatics*
	Weinrieb *et al*[23]	2007	*Liver Transpl*
	Addolorato *et al*[31]	2013	*Alcohol Clin Exp Res*
	Dom *et al*[17]	2015	*World J Hepatol*
	Donnadieu-Rigole *et al*[30]	2017	*Alcohol Clin Exp Res*

all the series[32,34,35]. *De novo* cancers are the second cause of late mortality after LT; during recent years, series of LT patients have shown an increase in upper aerodigestive tract, colon and kidney tumors[36-38]. Tobacco use before transplantation seems to be a risk factor for malignancies in LT patients presenting with alcoholic liver disease[39]. Other risk factors for malignancies are advanced age, alcohol consumption pre-and post-transplantation, viral infections, sun exposure, obesity, premalignant lesions and tacrolimus exposure levels[36,37]. Cardiac events in LT patients also limited long-term survival[40] and tobacco is a well-known risk factor for cardiovascular diseases[39].

Some authors believe that tobacco use should be a contraindication to organ allocation demanding smoking cessation before transplantation; other authors just recommended abstinence[41]. For kidney transplant recipients, a program for treating tobacco use was designed by Ehlers *et al*[42]. This program could be adapted to LT patients with systematic addiction consultations before and after LT.

Iruzubieta *et al*[43] proposed pre- and post-transplant follow-up during which tobacco use after LT should be taken care of.

Polysubstance abuse in LT patients

There are very few exact descriptions of the prevalence of polysubstance use in LT patients during pre- or post-transplant periods.

When a patient is dependent on a psychoactive substance they are at higher risk of being dependent on another one; this is true for tobacco and cannabis, so any detection of cannabis use must be systematically investigated in pre- and post-LT patients. Cannabis use is often associated with other psychoactive substance consumption in a context of polysubstance abuse[44]. In this series of polysubstance abuse in LT patients, the mean number of substances consumed was 3 before LT. The etiology of the end-stage liver disease was HCV infection and substance abuse had no impact on survival rates after LT. In the event of HCV infection as the primary indication, lifelong abuse of alcohol or other substances is often missed by the referent physician[45,46].

Patients on methadone maintenance therapy (MMT) for opiate dependence have not been well studied after LT; Weinrieb *et al*[47] and Tome *et al*[48] described more severe recurrent HCV infection and 20% of alcohol or illicit drug use after LT in these patients, but larger studies are necessary.

TREATMENT OF ADDICTIVE BEHAVIORS IN LT PATIENTS

Treatment of alcohol relapses

Although LT is the treatment of choice in the event of liver failure, some patients need specific follow-up post-surgery. No specific follow-up treatment is recommended for transplant recipients with addiction disorders, but motivational therapies have proved their effectiveness in this indication[49,50]. In the general population, they reduce mortality of liver diseases[51]. Psychotherapies include Twelve-step Facilitation Therapy, which is recommended by Alcoholics Anonymous; Cognitive-Behavioral Therapy and Motivational Enhancement Therapy can promote abstinence or help to reduce the amount of alcohol drunk[52-55].

Medication exists to treat alcohol dependence. Acamprosate is a medication that has proved its effectiveness in maintaining abstinence[55]. Naltrexone, an opioid receptor inhibitor, is effective on alcohol craving[56]. These two medicinal products are poorly studied in liver disease, so they are not currently approved in LT recipients and further studies are necessary[57]. Disulfiram (an acetaldehyde dehydrogenase) is a treatment which causes unpleasant sensations that prevent alcohol consumption. This treatment is potentially hepatotoxic and must be used with caution in LT patients[52]. Baclofen is the only treatment of alcohol dependence that has been studied in patients with alcoholic cirrhosis[24]. Pharmacotherapy should be associated with psychosocial support[58].

Treatment of opioid dependence

MMT for opiate-dependent patients at any dosage is not a contraindication for transplantation[59,60] but MMT patients continue to be discriminated against and it is very important to repeat that patients

should not be weaned from methadone before liver transplantation[59]. This treatment may be associated with anti-rejection drugs without specific supervision.

Treatment of tobacco cessation

For tobacco, patients transplanted for alcoholic liver disease often resume smoking very soon after surgery and the number of cigarettes smoked increases rapidly with patients smoking more than during the pre-transplantation period[61]. Nicotine replacement therapies can be used after LT. Bupropion should be used with caution in patients with liver disease and there are no contraindications for varenicline except allergy[62] but these medicinal products have not been studied in LT recipients.

In our LT center a systematic addiction consultation was made before LT and follow-up was proposed to patients with a high level of risk factors, but this did not result in the reduction of severe alcohol relapse.

A structured addiction consultation such as BRENDA[63] (B for biopsychosocial evaluation, R for restitution, E for Empathy, N for Needs identification, D for Direct counseling, A for Assess) in order to prevent and diagnose any alcohol relapse as soon as possible is now proposed systematically one month after LT.

Furthermore, these addiction consultations will promote tobacco cessation and/or prevention and treatment of psychoactive substance consumption.

CONCLUSION

There are many barriers to obtaining and documenting data about alcohol and illicit drug use by transplant recipients. One such barrier is the fear of patients and their referent physicians of judgment and medical sanction. But the objectives of addiction specialists are to improve life expectancy and quality without automatically obtaining total abstinence in patients. For all patients the period of LT surgery is a real "psychological earthquake" and causes behavioral changes that should be systematically evaluated after a few weeks of convalescence. Whatever the primary indication of LT, all transplanted patients should be seen at least once during the post-transplantation period to document present or past use of tobacco, alcohol, opiates, marijuana, cocaine and other drugs. There are no specific guidelines for behavioral management in LT patients, but non-judgmental care and a fostering attitude by the transplant team is recommended[64,65]. As well as transplant surgeons and anesthetists, addiction specialists must actively participate in the patient's clinical journey before, and especially after, LT.

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ORIGINAL ARTICLE

Retrospective Study

Low serum albumin predicts early mortality in patients with severe hypoxic hepatitis

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Abstract

AIM

To evaluate the incidence, etiology, and predictors of mortality of severe hypoxic hepatitis.

METHODS

We used computerized patient records to identify consecutive cases of severe hypoxic hepatitis admitted to a tertiary hospital in Singapore over a one-year period. We defined severe hypoxic hepatitis as elevation of serum transaminases more than 100 times upper limit of normal in the clinical setting of cardiac, circulatory or respiratory failure after exclusion of other causes of hepatitis. We used multivariable regression analysis to determine predictors for mortality.

RESULTS

We identified 75 cases of severe hypoxic hepatitis out of 71380 hospital admissions over one year, providing an incidence of 1.05 cases per 1000 admissions. Median age was 65 years (range 19-88); 57.3% males. The most common etiologies of severe hypoxic hepatitis were acute myocardial infarction and sepsis. Fifty-three patients (71%) died during the hospitalization. The sole independent predictive factor for mortality was serum albumin measured at the onset of severe hypoxic hepatitis. Patients with low serum albumin of less than 28 g/L have more than five-fold increase risk of death

(OR = 5.39, 95%CI: 1.85-15.71).

CONCLUSION
Severe hypoxic hepatitis is uncommon but has a high mortality rate. Patients with low serum albumin are at highest risk of death.

Key words: Severe; Mortality; Albumin; Incidence; Hypoxic hepatitis; Predictors; Etiology; Prognosis

Core tip: Hypoxic hepatitis is an important cause of liver injury that is associated with a high mortality rate. We sought to evaluate the incidence, etiology and predictors of mortality of severe hypoxic hepatitis in a large tertiary-level hospital in Singapore. Our findings confirm that the prevalence and mortality rate of severe hypoxic hepatitis in Asians is consistent with previous studies. Importantly, the unique finding from our study is that low serum albumin level is an independent predictive factor for mortality in severe hypoxic hepatitis, with a five-fold increase in risk of death in patients with serum albumin less than 28 g/L.

Chang PE, Goh BBG, Ekstrom V, Ong ML, Tan CK. Low serum albumin predicts early mortality in patients with severe hypoxic hepatitis. *World J Hepatol* 2017; 9(22): 959-966 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i22/959.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i22.959

INTRODUCTION

Hypoxic hepatitis - inflammation and necrosis of the liver due to hypoxia - can be a devastating disease. It is characterized by a substantial but transient increase in serum transaminase levels in the setting of cardiac, circulatory or respiratory failure, after exclusion of viral hepatitis and drug-induced liver injury[1]. Although initially referred to as "ischemic hepatitis", the term hypoxic hepatitis is now preferred as it is recognized that ischemia is not the sole contributing factor[2-4]. The typical presentation of hypoxic hepatitis is a sudden massive increase in serum transaminases, typically above ten times the upper limit of normal, due to massive hepatocyte necrosis. Characteristically there is predominant elevation of aspartate transaminase (AST) over alanine transaminase (ALT) followed by their rapid decline.

Severe hypoxic hepatitis can occur when there is massive elevation of serum transaminases, more than 100 times the upper limit of normal. The incidence, causes and predictive factors of mortality in patients with severe hypoxic hepatitis have not been well described. In a previous study examining the clinical outcomes of patients with extreme elevations of serum transaminases (ALT and/or AST more than 3000 U/L), we observed a high rate of mortality in patients with low serum albumin and advanced age[5]. In this present study, we sought to determine the incidence and predictors of mortality in patients with severe hypoxic hepatitis.

MATERIALS AND METHODS

Identification of patients with severe hypoxic hepatitis

Consecutive cases of severe hypoxic hepatitis were identified from the computerized database of patient admissions to a large tertiary care hospital in Singapore over a one-year period. Cases of severe hypoxic hepatitis were defined by the presence of the following three factors: Massive elevation of serum transaminases (either ALT and/or AST values greater than 3000 U/L), with rapid decline over 5 d, a typical clinical setting of cardiac, circulatory or respiratory failure, and the exclusion of all other causes of liver necrosis, particularly viral or drug-related hepatitis[1].

The ethics committee of the hospital approved waiver of informed consent for this retrospective study, which was conducted in accordance with the Declaration of Helsinki. Case records of patients fulfilling the above criteria were retrieved and two independent reviewers (Chang PE and Goh BBG) systematically extracted the relevant patient demographic data, clinical details and hemodynamic data. Relevant laboratory data was analyzed, including liver function, renal function and cardiac function tests. Specifically, the date of elevation of serum transaminases to > 3000 U/L and the trend of resolution were analyzed. Baseline laboratory data was defined as the laboratory values performed on the day of onset of elevation of serum transaminases to > 3000 U/L.

Analysis of precipitating factors for severe hypoxic hepatitis

Patient records were analyzed in detail to identify precipitating factors for hypoxic hepatitis. In particular, episodes of hypotension, arrhythmia, bradycardia, hypoxia and acidosis in the 48 h prior to the development of severe hypoxic hepatitis were analyzed. A hypotensive episode was defined as documented systolic blood pressure < 90 mmHg on at least two separate readings. Arrhythmia was defined as an abnormally rapid heart rate > 120 beats per minute, accompanied by electrocardiogram evidence of an abnormal heart rhythm. Bradycardia was defined as documented heart rate less than 60 beats per minute or a lowering of the heart rate by more than 25% of the baseline heart rate. Hypoxic episodes were defined as arterial oxygen saturation < 90% on pulse oximetry and/or partial pressure of oxygen < 60 mmHg on arterial blood gas. Metabolic acidosis was defined as a pH < 7.4 on arterial blood gas associated with serum bicarbonate of < 20 mmol/L. The inpatient medication records and clinical drug history were carefully analyzed to identify any potential hepatotoxic medications taken prior to the onset of severe hypoxic hepatitis.

Table 1 Clinical characteristics of patients with severe hypoxic hepatitis *n* (%)

	n = 75
Demographics	
Age	61.9 ± 16.6
Male gender	43 (57.3)
Race (Chinese/Malay/Indian)	51/20/4 (68/26.7/5.3)
Pre-existing co-morbidities	
Pre-existing ischemic heart disease	41 (54.7)
Pre-existing cardiac failure	27 (36.0)
Pre-existing renal failure	27 (36.0)
Pre-existing chronic viral hepatitis	5 (6.7)
Precipitating conditions	
Documented hypotension	55 (73.3)
Documented bradycardia	11 (14.7)
Documented metabolic acidosis	43 (57.3)
Baseline laboratory parameters[1]	
Albumin (g/L)	25.3 ± 6.9
Bilirubin (μmol/L)	76.1 ± 82.6
GGT (U/L)	126 ± 90
Creatinine (mmol/L)	321 ± 211
Prothrombin time (seconds)	27.8 ± 12.2
ALT (U/L)	2295 ± 1656
AST (U/L)	4896 ± 2986
ALT/AST ratio_D1[2]	0.70 ± 1.36
Peak ALT (U/L)	2834 ± 1938
Peak AST (U/L)	5894 ± 3148
Clinical outcome	
Admitted to ICU	50 (66.7)
Died within same admission	53 (70.7)

[1]Measured at onset of severe hypoxic hepatitis; [2]Ratio of the ALT to AST at the first documentation of either ALT or AST > 3000U/L. AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: Gamma glutamyl transferase; ICU: Intensive care unit.

Clinical course of severe hypoxic hepatitis

The clinical evolution of consecutive patients with severe hypoxic hepatitis was recorded for each subject. This included admissions to the intensive care unit (ICU), length of stay in ICU and survival to discharge from hospital. For patients who died, the cause of death was based on the diagnosis stated on the death certificate. In cases where post-mortem examination was performed, the cause of death was based on the final coroner's report. The etiology of hypoxic hepatitis was based on thorough review of clinical data, laboratory results, clinical evolution and autopsy details.

Study outcome

The data was analyzed to identify potential predictive factors for mortality, defined as death within the same admission as the episode of severe hypoxic hepatitis. Patients who survived to discharge were followed up for a further six months to determine the incidence of delayed mortality.

Statistical analysis

Clinical variables were compared between patients who died and those who survived to discharge. χ^2 analysis was performed for comparisons of discrete variables and Students *t*-test was used for comparison of continuous variables. Multivariable regression analysis was performed to identify independent predictors of early mortality. Survival comparisons were performed using Kaplan Meier analysis and compared using log rank statistics. All statistical analyses were performed using SPPS version 21 (Chicago, IL, United States). A P value of < 0.05 was considered statistically significant. Values in the text are described as mean ± SD or number (percentage of total) unless specified otherwise.

RESULTS

Incidence of severe hypoxic hepatitis

Of a total of 71380 admissions to the Singapore General Hospital over the course of one year, 75 patients fulfilled the predefined criteria for severe hypoxic hepatitis, providing an incidence of 1.05 cases of severe hypoxic hepatitis per 1000 admissions.

Clinical characteristics of severe hypoxic hepatitis

The clinical characteristics of the 75 patients are summarized in Table 1. Median age was 65 years, of which 57% of were male. All patients were Asians, with a predominance of Chinese followed by Indians and Malays respectively, in keeping with the multi-ethnic nature of the Singapore population. A pre-existing history of ischemic heart disease and cardiac failure was present in 55% and 36% respectively. A precipitating hypotensive event in the 48 h preceding the rise in liver enzymes was documented in 73% of cases. Precipitating episodes of bradycardia and metabolic acidosis were identified in 14.7% and 57.3% respectively. As expected, AST levels were higher than ALT levels at the onset of hypoxic liver injury with mean ALT and AST levels of 2295 ± 1656 U/L and 4896 ± 2986 U/L respectively. The mean ratio of ALT to AST at onset (ALT/AST ratio_D1) was 0.70 ± 1.36. Peak ALT and AST levels reached 2834 ± 1938 U/L and 5894 ± 3149 U/L respectively, typically within the first 3 d. Normalization of serum transaminases occurred in 82% of the 22 patients who survived. Mean number of days to normalization of ALT was 43 ± 46 d and 29 ± 23 d for AST.

Fifty patients (66.7%) with severe hypoxic hepatitis required admission to the ICU. All patients required vasopressor support. Of these, 37 (74.0%) died. Mean duration of stay in ICU was significantly longer in those who died compared to those who survived (9.4 ± 12.1 d *vs* 3.2 ± 2.0 d, $P = 0.02$). All 13 patients who were discharged from ICU recovered and were safely discharged from hospital.

Etiology of severe hypoxic hepatitis

The underlying etiology of severe hypoxic hepatitis was due to acute myocardial infarction (AMI) in 36% and septicemic shock in 32% (Figure 1). Congestive cardiac

Table 2 Comparison between patients with severe hypoxic hepatitis who died and survived *n* (%)

	Died (*n* = 53)	Survived (*n* = 22)	*P* value
Demographics			
Age	64.3 ± 17.6	56.3 ± 12.8	0.059
Male gender	35 (66.0)	8 (36.4)	0.018
Race (Chinese/Malay/Indian)	36/13/4	15/7/0	0.373
Pre-existing co-morbidities			
Pre-existing ischemic heart disease	27 (50.9)	12 (54.5)	0.793
Pre-existing cardiac failure	16 (30.2)	11 (50.0)	0.104
Pre-existing renal failure	19 (35.8)	8 (36.4)	0.966
Precipitating conditions			
Documented hypotension	45 (84.9)	14 (63.6)	0.041
Documented bradycardia	9 (17.0)	2 (9.1)	0.379
Documented metabolic acidosis	33 (62.3)	10 (45.5)	0.180
Baseline laboratory parameters[1]			
Albumin (g/L)	23.7 ± 6.8	29.1 ± 5.8	0.001
Bilirubin (μmol/L)	79.2 ± 84.4	68.8 ± 79.7	0.625
ALT (U/L)	2051 ± 1601	2880 ± 1674	0.048
AST (U/L)	5093 ± 2943	4440 ± 3103	0.395
ALT/AST ratio_D1[2]	0.50 ± 0.80	1.18 ± 2.15	0.051
GGT (U/L)	111 ± 67	158 ± 123	0.161
Creatine kinase	2949 ± 6180	395 ± 419	0.006
Creatine kinase MB	37.1 ± 56.6	13.5 ± 20.7	0.015
Troponin T (Trop T)	2.02 ± 4.21	0.49 ± 1.42	0.031
Lactate dehydrogenase	2503 ± 2552	4035 ± 3671	0.229
Creatinine (mmol/L)	297.3 ± 172.9	376.6 ± 276.3	0.143
Prothombin time (seconds)	27.8 ± 12.5	27.7 ± 11.9	0.980
Peak ALT (U/L)	2572 ± 2026	3464 ± 1575	0.069
Peak AST (U/L)	6223 ± 3138	5131 ± 3110	0.176

[1]Measured at onset of severe hypoxic hepatitis; [2]Ratio of the ALT to AST at the first documentation of either ALT or AST > 3000 U/L. AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: Gamma glutamyl transferase.

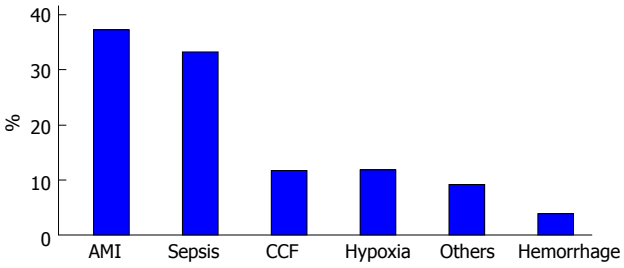

Figure 1 Etiology of severe hypoxic hepatitis. AMI: Acute myocardial infarction; CCF: Congestive cardiac failure.

failure, chronic respiratory failure and gastrointestinal hemorrhage accounted for the remaining cases of severe hypoxic hepatitis.

Mortality in severe hypoxic hepatitis

Severe hypoxic hepatitis was associated with a high mortality rate, accounting for 53 (71%) deaths within the same admission. The main causes of death were AMI in 39.6%, sepsis in 30.2%, metastatic cancer in 13.2% and gastrointestinal hemorrhage in 5.7%. Amongst the survivors who were discharged, there were no cases of delayed mortality in the 6-mo follow-up period. The ability to recover from the acute hypoxic injury was thus associated with an excellent prognosis. The clinical characteristics of patients who survived and died within the same admission are compared in Table 2.

On univariate analysis, four variables were found to be significantly different between cases of severe hypoxic hepatitis who survived and those who died within the same admission (Table 3). Mortality was more common in males and in those with a precipitating hypotensive event. Interestingly, baseline serum albumin level and ALT (but not AST) levels measured at onset of severe hypoxic hepatitis were significantly lower in patients who died. However, the peak ALT and AST levels did not have any discerning effect on mortality. Markers of cardiac infarction (CK, CKMB and troponin T) were significantly elevated in those who died whereas bilirubin and prothrombin time were not different in the two groups, suggesting that the underlying cause of death in severe hypoxic hepatitis is related to underlying cardiac ischemia and not to liver failure.

On multivariable analysis (Table 3), the sole independent predictor of early inpatient mortality in severe hypoxic hepatitis was baseline serum albumin. Using area under receiving operator curve (AUROC) statistics (Figure 2), serum albumin was determined to be an accurate predictor of mortality in severe hypoxic hepatitis with an AUROC of 0.740 (P = 0.001, 95%CI: 0.617-0.862). Baseline serum albumin < 28 g/L was an accurate predictor of mortality with a sensitivity of 75%, specificity of 64% and positive predictive value of 83%. Using logistic regression analysis, a baseline serum albumin lower than 28 g/L was associated with

Table 3 Univariate and multivariable analysis of predictive factors for mortality in severe hypoxic hepatitis

Variable	Univariate analysis		Multivariable analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age	1.03 (0.99-1.06)	0.069	1.07 (0.99-1.14)	0.056
Male gender	0.29 (0.10-0.83)	0.021	0.17 (0.03-1.19)	0.074
Precipitating hypotensive episode	3.21 (1.02-10.14)	0.046	4.40 (0.47-40.9)	0.192
Albumin	0.88 (0.80-0.96)	0.004	0.83 (0.71-0.96)	0.015
ALT	1.00 (0.99-1.00)	0.054	1.00 (1.00-1.00)	0.609
Creatine kinase	1.00 (1.00-1.00)	0.032	1.00 (1.00-1.00)	0.068
Creatine kinase MB	1.02 (0.99-1.05)	0.122	0.98 (0.94-1.02)	0.330
Troponin T	1.39 (0.88-2.16)	0.178	1.29 (0.74-2.27)	0.374
ALT/AST ratio_D1[1]	0.65 (0.35-1.19)	0.160	0.22 (0.04-13.1)	0.465

[1]Ratio of the ALT to AST at the first documentation of either ALT or AST > 3000 U/L. ALT: Alanine transaminase; AST: Aspartate transaminase.

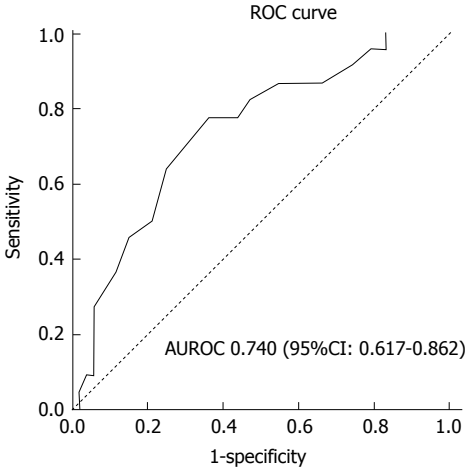

Figure 2 Receiver operating curve of baseline serum albumin to predict mortality in severe hypoxic hepatitis. AUROC: Area under receiving operator curve; ROC: Receiver operating curve.

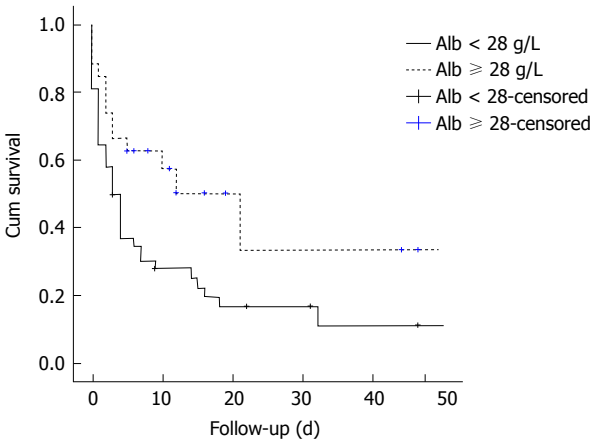

Figure 3 Kaplan-Meier comparison of survival between severe hypoxic hepatitis patients with baseline serum album < 28 g/L and ⩾ 28 g/L. Alb: Albumin.

a five-fold increased risk of early mortality (OR = 5.39, 95%CI: 1.85-15.71). Median survival was 85% lower in severe hypoxic hepatitis patients with baseline serum albumin less than 28 g/L compared to those with baseline albumin greater than 28 g/L (3.0 d *vs* 21.0 d, *P* = 0.015 by log-rank comparison, Figure 3).

DISCUSSION

To our knowledge, this is the first study to describe the clinical course and outcome of Asian patients with severe hypoxic hepatitis. Several key findings are noted - firstly, severe hypoxic hepatitis occurs with an incidence of 1.05 in every 1000 admissions to a tertiary level general hospital in Singapore. The clinical presentation of severe hypoxic hepatitis in Asian patients is not different from that reported in Western studies[6-8]. Secondly, the clinical outcome of severe hypoxic hepatitis is poor with a high mortality rate of 71%. Thirdly, most deaths associated with severe hypoxic hepatitis are due to acute myocardial infarction and sepsis and not due to liver failure. Finally and most importantly, the unique finding in our study is that low serum albumin at baseline is an independent predictor of early mortality in patients with severe hypoxic hepatitis.

There is a wide variation in the reported incidence of hypoxic hepatitis ranging from 1 per 1000 admissions to 4 per 100 admissions[6,7]. The reason for this wide variation is the denominator, *i.e.*, the population of patients studied. The incidence of hypoxic hepatitis is expectedly lower in studies including all general admissions compared to those focusing on admissions to intensive care units[8-10]. The denominator in our study was all admissions to a tertiary care hospital over a one-year period. The incidence of 1.05 cases of severe hypoxic hepatitis per 1000 admissions in our Asian center is consistent with the literature from studies performed in Western populations[11-14].

Our study demonstrates that the clinical profile of Asian patients with severe hypoxic hepatitis is similar to that reported in Western studies. A history of ischemic heart disease and cardiac failure are common and was present in 55% and 36% of our study cohort respectively. Pre-existing passive hepatic congestion has been proposed as a pre-requisite condition for the development of hypoxic hepatitis[15,16]. Patients with chronically elevated right heart pressures are

prone to developing congestive hepatopathy resulting in decreased hepatic perfusion to hepatocytes. In such patients, a slight decrease in the hepatic arterial perfusion pressure may be sufficient to cause hypoxic hepatitis. Indeed, Seeto *et al*[17] have demonstrated that as little as 15 min of transient systemic hypotension is sufficient to produce massive hepatocyte necrosis in patients with pre-existing congestive hepatopathy. However, as demonstrated in our study, a precipitating episode of hypotension is not necessarily seen in all patients with hypoxic hepatitis[16].

Severe hypoxic hepatitis is associated with a high mortality rate[18]. More than two-thirds of patients in our study cohort died, which is at the higher end of the range of 30%-77% reported in similar studies[6,9,10,18]. The underlying etiology of severe hypoxic hepatitis is strongly associated with risk of early mortality. Ninety-three percent of patients with severe hypoxic hepatitis due to AMI died compared to only 58% of non-AMI-related etiologies. However, neither the severity of elevation of liver transaminases nor the rate of resolution of elevated transaminases was associated with mortality. The cause of death has not been reported in meta-analysis of studies on hypoxic hepatitis[7]. In our study, the main cause of death was due to cardiovascular failure in 40%, followed by septic shock with multi-organ failure in 30%. In both conditions, the underlying pathophysiological process is inadequate perfusion pressure to the essential organs. Importantly, liver failure is not the cause of death in patients with severe hypoxic hepatitis. This reinforces the point that the primary strategy in the management of patients with severe hypoxic hepatitis is to focus on maintaining systemic perfusion pressure and to correct the underlying cause of hypoperfusion be it due to primary cardiac pump failure, massive peripheral vasodilatation due to sepsis or hypovolemia from hemorrhage[6].

The novel aspect of this study is the finding that baseline serum albumin is an independent predictor of early mortality in patients with severe hypoxic hepatitis. Duration of hypoxic hepatitis, INR, presence of septic shock, SOFA score, jaundice, need for renal replacement and vasopressor therapy have previously been suggested to be factors that predict mortality in hypoxic hepatitis[9,18-21]. However, serum albumin has never been previously reported to be an independent predictor for mortality in hypoxic hepatitis.

Hypoalbuminemia is a well-known predictor of mortality in patients with acute illness[22,23]. Serum albumin on admission has been shown to be a strong predictor of inpatient mortality in internal medicine wards[24,25]. Furthermore, a progressive decrement of serum albumin concentration is associated with a 24%-56% increase in odds of death[26]. Decline in albumin levels occurs in acute illnesses due to increased catabolism, reduced hepatic synthesis and renal loss. Albumin is often viewed as a non-specific negative acute phase protein that reflects the severity of the underlying illness. It is thus conceivable that low serum albumin may be associated with increased mortality in patients with hypoxic hepatitis, as in any other systemic illness. However, our study provides evidence that albumin is an independent predictor for mortality in severe hypoxic hepatitis, with a strong odds ratio. This suggests that low serum albumin levels may play a direct role in causing cardiovascular-related mortality in patients with severe hypoxic hepatitis.

Low serum albumin levels are associated with increased risk of mortality in coronary disease[27] and cardiac failure[28,29]. This is attributed to slower coronary flow in patients with low albumin levels[30]. In a study investigating the relationship between serum albumin and coronary flow rate following primary percutaneous coronary intervention[31], baseline serum albumin levels were significantly lower in the group with no coronary reflow compared to normal coronary reflow and serum albumin was identified as an independent predictor of no-reflow. Low levels of serum albumin cause an increase in coronary blood viscosity and disruption of endothelial function due to increased concentrations of free lysophosphatidylcholine[32]. In addition, albumin is an important inhibitor of platelet aggregation through increases in production of antiaggregatory prostaglandin PGD2 from cyclic endoperoxidases[33]. Low levels of serum albumin thus increase risk of platelet aggregation in the coronary vasculature, hence increasing risk of AMI. The novel association observed between low serum albumin levels and increased mortality risk in severe hypoxic hepatitis can thus be potentially explained by the effects of low albumin on slowing coronary flow and increased platelet aggregation, thus increasing risk of acute myocardial infarction and early death. However, this postulation remains speculative and needs to be evaluated in future prospective studies.

Low serum albumin levels are also associated with increased mortality in patients with severe sepsis[34]. Serum albumin plays an important role in modulating innate immune responses to systemic inflammation and sepsis[35]. The ability of serum albumin to modulate inflammation and oxidative stress as well as inhibit neutrophil adhesion could provide some protection from endothelial dysfunction mediated by these factors[36,37]. Although several studies have demonstrated efficacy of albumin infusions in improving outcome in septic patients[38], others have not demonstrated any meaningful benefit[39,40]. It thus remains unclear whether low serum albumin is a direct cause of mortality or a surrogate for severity of illness in patients with sepsis.

Our findings are clinically important because it provides the clinician with a simple way to identify patients with severe hypoxic hepatitis who are at highest risk of death. Patients with severe hypoxic hepatitis with a baseline serum albumin level less than 28 g/L can be fast-tracked for ICU care and early vasopressor therapy to maintain adequate central perfusion. Of interest is whether timely intervention

with intravenous albumin will result in a reduction of mortality in patients with severe hypoxic hepatitis.

There are several limitations to this study, including the retrospective study design. The study was limited to a single center, which may limit the generalizability of the findings. This study focused on patients with severe elevation of serum transaminases beyond 100 times the upper limit of normal. The findings of our study are thus limited to patients with severe hypoxic hepatitis and may not be applicable to milder degrees of hypoxic hepatitis.

In conclusion, severe hypoxic hepatitis is associated with high mortality. Most deaths are related to underlying cardiovascular failure and septic shock with multi-organ failure. Low serum albumin levels at the onset of severe hypoxic hepatitis is an independent predictor of mortality and is a useful clinical marker for early prognostication of patients at high risk of death.

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COMMENTS

Background

Hypoxic hepatitis is an important cause of acute liver injury that is associated with a high mortality rate.

Research frontiers

The novel aspect of this study is the finding that baseline serum albumin is an independent predictor of early mortality in patients with severe hypoxic hepatitis.

Innovations and breakthroughs

This is the first study to describe the clinical course and outcome of Asian patients with severe hypoxic hepatitis. The unique finding in this study is that low serum albumin at baseline is an independent predictor of early mortality in patients with severe hypoxic hepatitis.

Applications

These findings are important because it provides the clinician with a simple way to identify patients with severe hypoxic hepatitis who are at the highest risk of death.

Peer-review

It was a nice retrospective study and very interesting to read. What I especially liked that you did not forget to state that mortality actually depends on the underlying disease and not the condition of the liver itself.

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EVIDENCE-BASED MEDICINE

Serum cholinesterase: A predictive biomarker of hepatic reserves in chronic hepatitis D

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Abstract

AIM

To determine the predictive performance of cholinesterase compared to existing prognostic models in evaluating liver function in patients with chronic hepatitis D.

METHODS

In an observational, cross-sectional and retrospective study, consecutive patients with hepatitis D cirrhosis were evaluated. Demographic, clinical and laboratory parameters were recorded. Serum cholinesterase levels were correlated with existing scoring models for chronic liver disease and Liver function tests. Receiver operating characteristic (ROC) curves were constructed to find an optimal cholinesterase level predicting ascites, Child Turcotte Pugh (CTP) score $\geqslant$ 10, model for end stage liver disease (MELD) score $\geqslant$ 15, baseline-event-anticipation (BEA) score for hepatitis D $\geqslant$ 5 and the aspartate transaminase to Platelet Ratio Index (APRI) $\geqslant$ 1.5.

RESULTS

This study investigated 233 patients with chronic liver disease due to hepatitis D; 192 were male, median age 42 (16-69 years). Fifty patients had ascites and 15 had encephalopathy. One hundred and sixty-seven (71.7%) were in Child class A, 52 (22.3%) in Child class B and 14 (5.0%) in class C. A MELD score of 15 or more was seen in 24 patients. Cholinesterase levels correlated well with the INR, albumin, CTP score, MELD, MELD sodium, BEA and APRI scores (P < 0.001 each). Area under the ROC curve for ascites, CTP $\geqslant$ 10, MELD $\geqslant$ 15, BEA $\geqslant$ 5, APRI $\geqslant$ 1.5 was 0.836, 0.966, 0.913, 0.871 and 0.825 respectively (P < 0.001 each). Cut off values of cholinesterase (IU/L) for predicting ascites, CTP $\geqslant$ 10, MELD $\geqslant$ 15, BEA $\geqslant$ 5 and APRI $\geqslant$ 1.5

were < 3812, < 2853, < 2829, < 4719 and < 3954 with a sensitivity of 80%, 100%, 91.67%, 82.50%, 58.0% and specificity of 81.97%, 84.79%, 87.56%, 77.06% and 55.64% respectively.

CONCLUSION

Serum cholinesterase demonstrates promising correlations with serum albumin, INR and CTP, MELD, BEA and APRI scores and is predictive of liver reserves in hepatitis D cirrhosis.

Key words: Cholinesterase; Liver function; cirrhosis; Model for Endstage Liver Disease score; Aspartate transaminase-to-platelet ratio index; Hepatitis D; Child Turcotte Pugh score; Baseline-event-anticipation score

Core tip: Prognostic models to assess liver function in patients with chronic liver disease are used extensively in clinical settings. These systems employ multiple clinical and laboratory parameters to evaluate liver reserves and predict outcome. In our study we assessed cholinesterase as an independent predictor of hepatic reserves. We found that its values correlated strongly with Liver function tests and with the existing scoring models. Thereafter, we defined optimal cholinesterase levels corresponding to the different stages and classes of the scoring systems and hence the severity of chronic liver disease. The study's subjects were patients suffering from cirrhosis due to hepatitis D.

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INTRODUCTION

Prognostic models to evaluate liver function include the Child Turcotte Pugh (CTP) score, the Model for Endstage Liver Disease (MELD) score and the aspartate transaminase to Platelet Ratio Index (APRI). The CTP score is often used to assess the risk of surgery in patients with cirrhosis and it correlates with survival[1]. The MELD score is used by the United Network of Organ Sharing (UNOS) to prioritize patients awaiting cadaveric liver transplant[2]. An increase in the MELD score is associated with an increasing severity of hepatic dysfunction and an increased three-month mortality risk. The APRI is considered as an alternative to liver biopsy for predicting liver fibrosis[3]. However, its role in some etiologies is controversial. Increasing levels > 1.5 may show decreasing hepatocyte mass and increasing fibrosis. Recently, a baseline-event-anticipation (BEA) score has been developed for hepatitis D to define clinical parameters associated with worse outcomes[4].

Commonly used liver synthetic function tests include serum albumin and prothrombin time and international normalized ratio. Serum butyrylcholinesterase, commonly known as serum cholinesterase, is an enzyme synthesized by hepatocytes and has the half-life of eleven days[5]. Its serum level is decreased in chronic liver damage, infections, and malnutrition[6].

Chronic hepatitis D is a severe disease with rapid progression of fibrosis leading to cirrhosis, decompensation and hepatocellular carcinoma[7]. The role of cholinesterase to assess the liver reserves in hepatitis D patients has not been well defined. The objective of this study is to determine the performance of cholinesterase in predicting liver function compared to existing synthetic liver function tests and scoring models in patients with hepatitis D and cirrhosis.

MATERIALS AND METHODS

This observational, cross-sectional study examined the efficacy of cholinesterase as a liver function test to assess the synthetic reserve in a retrospective fashion. Two hundred and thirty-three consecutive patients presenting to the liver clinic with cirrhosis due to chronic hepatitis D were evaluated. Available baseline demographic and clinical parameters were recorded. Serum cholinesterase levels were checked as a routine test to evaluate liver function.

Data were expressed as the number of subjects with percentages for nominal variables. These variables were compared by χ^2 or Fisher exact test. Continuous variables were presented as means with standard deviation, and compared using Student *t* test, Mann-Whitney *U* test and ANOVA. Correlations were tested using tests Pearson's correlation test. Receiver operating characteristic (ROC) curves were constructed to determine optimal cholinesterase levels predicting multiple state variables such as MELD score $\geqslant$ 15. Areas under receiver operating characteristic curves, sensitivity and specificity were used to examine the accuracy of the cholinesterase for various predictions. The state variables examined included Ascites, MELD score $\geqslant$ 15, MELD score > 10, APRI $\geqslant$ 1.5, BEA $\geqslant$ 5, CTP $\geqslant$ Class B and CTP $\geqslant$ Class C. Cutoff cause were determined by Youden's J statistic (verified by a unit weighted ROC cutoff based on minimizing the distance from the point representing perfect classification to the ROC curve). Statistical analyses were performed using SPSS 23.0 software (IBM SPSS Statistics, New York, NY, United States). All tests were 2-tailed and a *P* value < 0.05 was required for statistical significance.

RESULTS

Out of 233 patients with chronic liver disease due to hepatitis D, 192 (82.4%) were male, the median age was 42 (range 16-69 years). Fifty (21.5%) patients had

Table 1 Baseline characteristics of the study patients

No. of patients	233
Male:female	192:41
Age (yr)	42 (16-69)
BMI (kg/m^2)	23.4 (14.3-40)
Ascites	50 (21.5)
Encephalopathy	15 (6.4)
Bilirubin (mg/dL)	0.90 (0.2-6.9)
Albumin (g/dL)	3.8 (1.8-5.0)
INR	1.13 (0.6-2.6)
Creatinine (mg/dL)	0.8 (0.4-1.96)
Sodium (mmol/L)	139 (120-150)
AST (IU/L)	53 (10-638)
Platelets (× 10^9/L)	120 (22-388)
Cholinesterase (IU/L)	5508 (861-12891)
Child class	
A	167 (71.7)
B	52 (22.3)
C	14 (5.0)
CTP score	5 (5-13)
MELD score	8 (6-24)
MELD sodium	9 (6-26)
MELD score 15 or more	24 (10.3)
APRI	1.26 (0.19-10.8)
APRI 1.5 or more	100 (42.9)
BEA class	
A	6 (2.6)
B	164 (70.4)
C	63 (27)
BEA score	4 (1-7)

Values are median (range) or *n* (%). BMI: Body mass index; INR: International normalization ratio; AST: Aspartate aminotransferase; CTP: Child Turgotte Pugh; MELD: Model for End Stage Liver Disease; APRI: AST to platelet ratio index; BEA: Baseline-event-anticipation.

Table 2 Mean cholinesterase levels

Parameter	Values (U/L)	*P* value
Child class		
A (CTP up to 6)	7058 ± 208	
B (CTP 7-9)	3773 ± 372	< 0.001 (A *vs* B)
C (CTP ⩾ 10)	1605 ± 129	< 0.001 (B *vs* C)
MELD score		
⩾ 15	2285 ± 373	
< 15	6423 ± 206	< 0.001
APRI		
⩾ 1.5	4002 ± 219	
< 1.5	7498 ± 251	< 0.001
BEA score		
A	8036 ± 967	
B	6993 ± 222	0.337 (A *vs* B)
C	3211 ± 258	< 0.001 (B *vs* C)

Values are mean ± SE. CTP: Child Turcotte Pugh; MELD: Model for End Stage Liver Disease; APRI: AST to platelet ratio index; BEA: Baseline-event-anticipation.

Table 3 Receiver operating characteristic analysis

State variable	AUC	Std. error	Asymptotic sig. (*P* value)	95%CI
Ascites	0.836	0.038	< 0.001	0.762-0.910
CTP ⩾ 7	0.889	0.029	< 0.001	0.832-0.946
CTP ⩾ 10	0.966	0.013	< 0.001	0.940-0.992
MELD ⩾ 10	0.798	0.034	< 0.001	0.731-0.864
MELD ⩾ 15	0.913	0.038	< 0.001	0.838-0.987
APRI ⩾ 1.5	0.825	0.026	< 0.001	0.773-0.877
BEA ⩾ 5	0.871	0.028	< 0.001	0.816-0.926

CTP: Child Turcotte Pugh; MELD: Model for End Stage Liver Disease; APRI: AST to platelet ratio index; BEA: Baseline-event-anticipation.

ascites and 15 (6.4%) encephalopathy. One hundred and sixty-seven (71.7%) were classified into Child class A, 52 (22.3%) into Child class B and 14 (5.0%) into class C. A MELD score of 15 or more was seen in 24 (10.3%). Cholinesterase levels (mean ± SEM) in males were 6177 ± 228 and in females were 5151 ± 452 (P = 0.06). A statistically significant difference was not found between gender and BMI at any stage of the disease. The baseline characteristics of the study patients are recorded in Table 1. Cholinesterase levels correlated with the albumin, INR, CTP score, MELD, MELD sodium, APRI and BEA scores with the Pearson correlation coefficient, r values of 0.724, -0.520, -0.624, -0.561, -0.533, -0.531, and -0.591 respectively (P < 0.001 each) (Figure 1). The mean cholinesterase levels for each class are shown in Table 2.

Area under the ROC curve for ascites, CTP ⩾ 10, MELD ⩾ 15, BEA ⩾ 5, APRI ⩾ 1.5 was 0.836, 0.966, 0.913, 0.871 and 0.825 respectively (P < 0.001 each) (Table 3 and Figure 2). Cut off values of cholinesterase (IU/L) for predicting ascites, CTP ⩾ 10, MELD ⩾ 15, BEA ⩾ 5 and APRI ⩾ 1.5 were < 3812, < 2853, < 2829, < 4719 and < 3954 with a sensitivity of 80%, 100%, 91.67%, 82.50%, 58.0% and specificity of 81.97%, 84.79%, 87.56%, 77.06 and 55.64% respectively (Table 4). Mean serum cholinesterase decreased with reduced hepatic reserves. For example patients in Child Class A had a mean cholinesterase of 7058 compared to those in Class B with 3773 and Class C with 1605. This is also reflected in the optimal cutoff values of < 3812 at Child Class B (CTP score 7-9) and < 2853 at Child Class C (CTP ⩾ 10) and similarly a value of < 4719 at MELD ⩾ 10 and < 2829 at MELD ⩾ 15.

DISCUSSION

Traditional liver function tests and scoring systems used to stage severity of the liver disease face several inherent limitations. For example, the LFTs investigated in this study maybe abnormal in illnesses not associated with liver dysfunction. Aminotransferase levels may increase in non-hepatic disease such as myocardial infarction[8] while bilirubin maybe altered by hemolysis. Moreover, the CTP score includes subjective parameters such as the degree of ascites and encephalopathy[9] and these findings may be altered substantially by medical interventions. Furthermore, its role is limited due to a ceiling and floor effect: An inability to discriminate values for bilirubin > 3.0 mg /dL, INR greater > 2.3 and albumin less < 2.8 g/dL. Finally, the CTP score does not include creatinine for the

Table 4 Optimal cholinesterase cutoffs

State variable	Cholinesterase cut off value (IU/L)	Sensitivity, % (95%CI)	Specificity, % (95%CI)	Likelihood ratio
Ascites	< 3812	80.00 (66.28- 89.97)	81.97 (75.62-87.25)	4.436
CTP ⩾ 7	< 3812	79.10 (67.43-88.08)	67.47 (59.78-74.53)	2.432
CTP ⩾ 10	< 2853	100.00 (79.41-100.00)	84.79 (79.31-89.29)	6.576
MELD ⩾ 10	< 4719	72.09 (61.38-81.23)	80.27 (72.91-86.37)	3.654
MELD ⩾ 15	< 2829	91.67 (73.00-98.97)	87.56 (82.31-91.71)	7.369
BEA ⩾ 5	< 4719	82.54 (70.90-90.95)	77.06 (70.00-83.15)	3.598
APRI ⩾ 1.5	< 3954	58.00 (47.71-67.80)	55.64 (46.78-64.25)	1.307

CTP: Child Turcotte Pugh; MELD: Model for End Stage Liver Disease; APRI: AST to platelet ratio index; BEA: Baseline-event-anticipation.

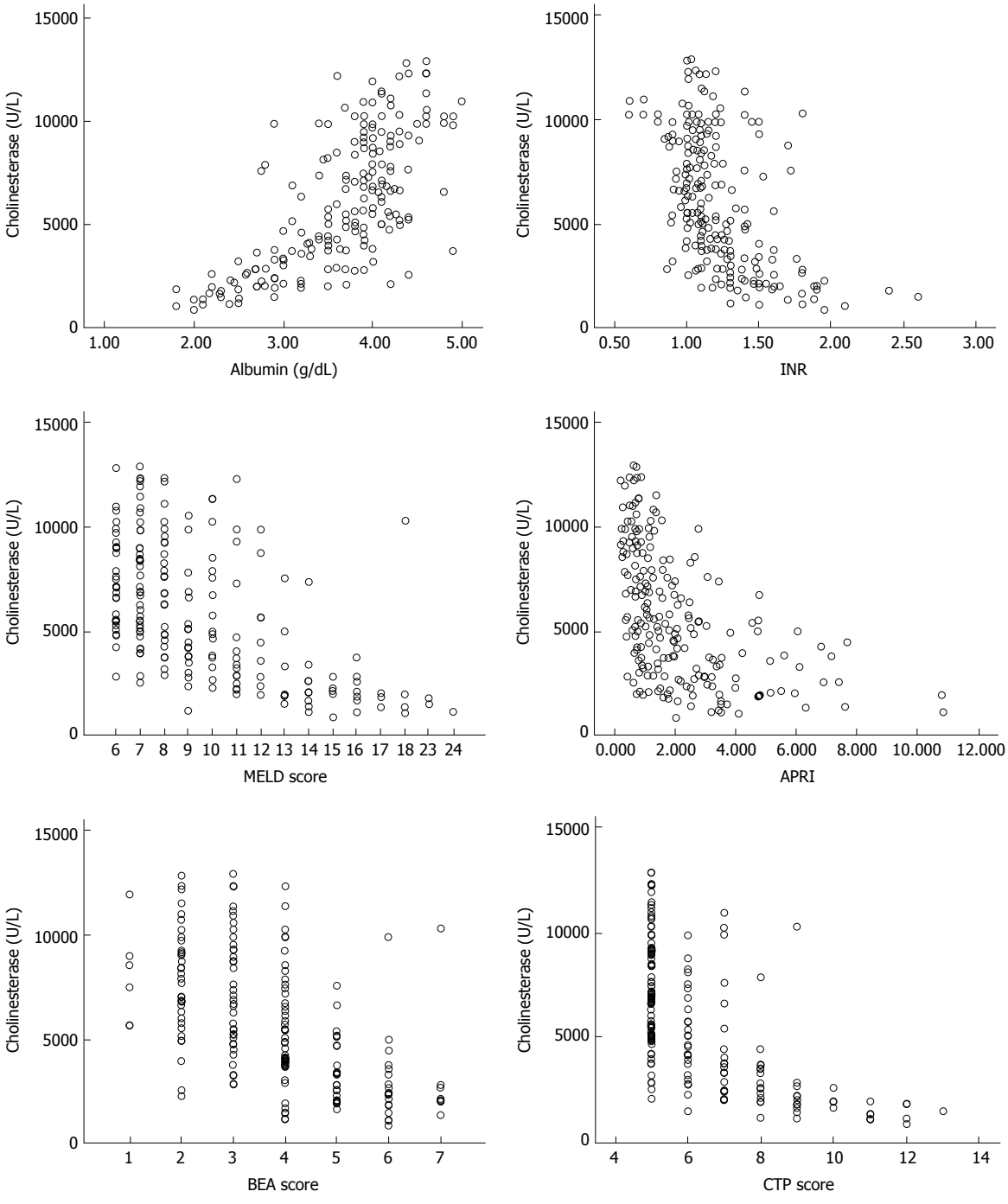

Figure 1 Correlations of cholinesterase levels with the synthetic liver function tests and liver function prognostic models. INR: international normalization ratio; MELD: Model for end stage liver disease; APRI: AST to platelet ratio index; BEA: baseline-event-anticipation; CTP: Child Turgotte Pugh.

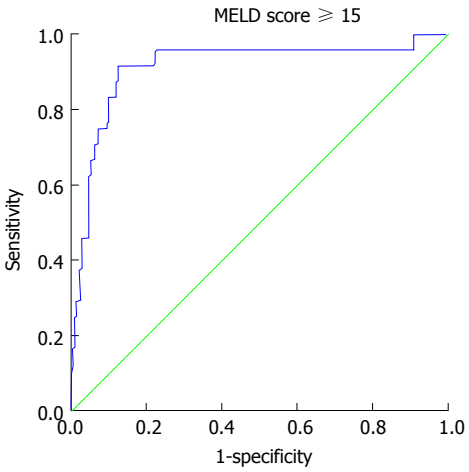

Figure 2 Receiver operating characteristic curve. Cholinesterase levels for model for end stage liver disease score 15 or more (A smaller test result indicates a more positive test). MELD: Model for Endstage Liver Disease.

assessment of renal function, another major marker of the severity of the disease.

The MELD score has been criticized for several different reasons[10-13]. It is vulnerable to variations in laboratory measurements and does not include portal hypertensive complications (*e.g.,* ascites, encephalopathy, variceal bleeding, and spontaneous bacterial peritonitis). Again, it suffers from a floor and ceiling effect: Patients with the combination of an INR of $\leqslant$ 1, creatinine $\leqslant$ 1 mg/dL, and bilirubin $\leqslant$ 1 mg/dL receive the minimum score of 6 MELD points, while UNOS set an upper limit for the MELD score at 40 points. Modifications of the MELD scoring system have been implemented by introducing the MELD sodium, by reweighting MELD components (lower weights ascribed to serum creatinine and international normalized ratio (INR) and a higher weight to serum bilirubin), by refitting MELD [by implementing new upper and lower bounds for creatinine (0.8 and 3.0 mg/dL, respectively) and for INR (1.0 and 3.0, respectively)], and by dynamic changes in MELD scoring (Delta MELD).

The scoring systems use multiple clinical and laboratory parameters to evaluate liver reserves and predict outcomes. In our study we assessed cholinesterase as an independent test for liver function and hepatic reserves.

Cholinesterase levels have been assessed to predict survival in patients with Parenchymal cirrhosis[14], predict outcome in graft-*vs*-host disease[15], distinguish between liver disease and non-liver disease aberration in liver function tests[16] and differentiate cirrhosis from non-cirrhosis[17]. Serum cholinesterase levels have also been found to correlate with CTP Class[18,19]. In addition, cholinesterase levels have been shown to recover with improvements in hepatic function[20] at a rate exceeding recovery from organophosphate poisoning.

Our study showed that cholinesterase levels could be used in conjunction with existing scoring systems as a prognostic marker of hepatic reserves. However, serum cholinesterase levels may be affected by gender, nutritional status and carcinomas[6]. We did not find any differences related to gender and body mass index. None of our patients had malignancy while all of the patients included in this study were suffering from cirrhosis related to hepatitis D. So differences in the etiology of the liver disease could not affect the results of this study. The prevalence of inherited atypical cholinesterase has been reported to be low in multiple studies[21]. So any genetic variations are less likely to influence the results of this study.

In conclusion, serum cholinesterase is an excellent biomarker of the synthetic function of liver in CLD with hepatitis D. Cholinesterase levels should be routinely checked to assess liver function and may be incorporated in MELD scoring. It can be effectively used to follow the staging of liver disease in hepatitis D. Our results should be validated in other cohorts and etiologies of CLD.

COMMENTS

Background

Chronic hepatitis D is a severe disease with rapid progression of fibrosis leading to cirrhosis, decompensation and hepatocellular carcinoma. Commonly used liver synthetic function tests include serum albumin and prothrombin time and international normalized ratio. The objective of this study was to determine the performance of cholinesterase levels in predicting liver function compared to the existing scoring models in patients with hepatitis D and cirrhosis.

Research frontiers

The authors defined optimal cholinesterase levels corresponding to the different stages and classes of the scoring systems assessing the severity of chronic liver disease.

Innovations and breakthroughs

Serum cholinesterase demonstrated promising correlations with serum albumin, international normalized ratio and Child Turcotte Pugh, Model for Endstage Liver Disease, baseline-event-anticipation and aspartate transaminase to Platelet Ratio Index scores.

Applications

Serum cholinesterase levels can be effectively used to monitor the staging of liver disease in hepatitis D. These results may be validated in other cohorts and etiologies of chronic liver disease to predict the liver reserves.

Terminology

Serum butyrylcholinesterase, commonly known as serum cholinesterase, is an enzyme synthesized by hepatocytes and has the half-life of eleven days

Peer-review

The authors investigated the role of cholinesterase levels as predictor of hepatic reserves in chronic hepatitis D patients. This paper is generally well conducted and straightforward. The authors concluded that cholinesterase levels can be considered a biomarker of liver function in these patients.

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CASE REPORT

Extrahepatic metastasis of hepatocellular carcinoma to the paravertebral muscle: A case report

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Author contributions: Takahashi K and Kim DY designed the report; Takahashi K, Putchakayala KG and Safwan M collected the data; Takahashi K and Kim DY wrote the paper; Putchakayala KG and Safwan M performed critical revisions of the paper.

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Abstract

Identification of extrahepatic metastases (EHM) of hepatocellular carcinoma (HCC) has been paradoxically increasing due to an increase in the survival of HCC patients. However, metastasis of HCC to the skeletal muscle tissue is extremely rare. We describe a unique case of HCC metastasizing to the paravertebral muscle. A 55-year-old man with a history of hepatitis B cirrhosis underwent partial liver resection with complete removal of HCC. Three months later, a computed tomography (CT) scan showed intrahepatic recurrence. The tumors were treated with yttrium-90 microspheres, trans-catheter arterial chemoembolization, and sorafenib. Six months later, a CT scan showed an enhancing lesion of the left paravertebral muscle that on biopsy were consistent with metastatic HCC. The tumor was treated with stereotactic hypo-fractionated image-guided radiation therapy (SHFRT). A follow-up scan 3 mo post-radiotherapy revealed a stable appearance of the paravertebral muscle metastasis. Because of the progression in the intrahepatic tumors, the patient was treated with capecitabine, which was changed to dasatinib 6 mo later. The patient passed away three years after the primary surgical resection. Management of EHM poses an extreme challenge. This is the first case of HCC with EHM to the paravertebral muscle in which stability of disease was achieved using SHFRT. This case highlights the importance of early detection of hepatitis B viral infection and initiation of anti-viral therapy to decrease recurrence of HCC and prevent EHM.

Key words: Hepatocellular carcinoma; Skeletal muscle; Paravertebral muscle; Extrahepatic metastasis; Stereotactic hypo-fractionated image guided radiation therapy;

Hepatitis B virus

Core tip: Extrahepatic metastases (EHM) of hepatocellular carcinoma (HCC) to skeletal muscle are extremely rare. We describe the first case of HCC with EHM to the paravertebral muscle, in which stability of disease was achieved using stereotactic hypo-fractionated image-guided radiation therapy. A literature review revealed the strong relationship between hepatitis B viral infection and EHM. This case highlights the importance of early detection of viral infection and initiation of anti-viral therapy to decrease recurrence of HCC and prevent EHM.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third most common cause of cancer-related death in the world[1-3]. Worldwide incidence is between 250000 and 1000000 new cases per year, and it has been rapidly increasing due to the prevalence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections[1-3]. In the United States, HCC related to HCV infection has become the fastest rising cause of cancer-related death, and the incidence has tripled during the past two decades. Survival time in patients with HCC has recently increased as a consequence of advanced diagnostic modalities and treatment methods; however, the 5-year survival rate still remains low at approximately 16%[1,3,4]. Current available treatment methods include surgical resection, radio-frequency ablation, trans-catheter arterial chemoembolization (TACE), yttrium-90 microspheres, liver transplantation, chemotherapy, and radiotherapy[5].

Because of the improvement in survival, extrahepatic metastases (EHM) are becoming more commonly recognized in patients with HCC, with a reported incidence of 15%-17%[6,7]. The most common sites of EHM are lungs, lymph nodes, bones, and adrenal glands; however, HCC can metastasize to the skeletal muscles and subcutaneous tissues, albeit rarely[7]. In this report, we describe a unique case of HCC metastasizing to the paravertebral muscle, which was treated with stereotactic hypo-fractionated image guided radiation therapy (SHFRT) and achieved disease stability. We report this case along with a review of the recent literature.

CASE REPORT

A 55-year-old male with a history of HBV-associated liver cirrhosis had an incidental right lobe liver mass 6.0 cm in size identified during a routine computed tomography (CT) scan. His serum alpha-fetoprotein (AFP) level was within the normal range. A magnetic resonance imaging (MRI) scan showed a hyper-intense irregular T2 focus, which distorted the contours of the liver. This focus demonstrated moderate enhancement on the initial phase post-Gadolinium images, with a central hypo-intense area. These imaging characteristics were most compatible with focal nodular hyperplasia, and follow-up at the outpatient clinic was advised. However, the patient was non-compliant and did not visit the clinic until three years later. MRI scan at that time showed that the tumor had increased in size to 9.4 cm, and the patient had a mild elevation in AFP level (15.1 ng/mL). His HBV DNA level was 12.7 × 10^6 copies/mL and he had not received any anti-viral therapies. The patient then underwent partial liver resection with complete removal of the tumor. Histopathological examination revealed the tumor to be a moderate-to-poorly differentiated HCC with vascular invasion. According to the Union for International Cancer Control guidelines, the final stage of the tumor was stage Ⅱ (pT2N0M0). Due to the elevated viral titer, entecavir 1 mg daily was instituted postoperatively.

Three months later, CT scan showed recurrence of the tumor as three foci: 4 mm in size along the resected plane, 7 mm at S4, and 6 mm at S7. The patient's HBV DNA level was less than 300 copies/mL. The tumors were treated with yttrium-90 microspheres (TheraSphere®, BTG IM, London, United Kingdom). A total dose of 90 Gy was delivered. One year later, he developed multiple enhancing lesions in the liver. He received three sets of TACE with adriamycin, and finally sorafenib (Nexavar®, Bayer HealthCare AG, Leverkusen, Germany) 200 mg twice daily. Six months later, he complained of back pain, and CT scan showed an enhancing lesion 3.7 cm in size in the left paravertebral muscle (Figure 1). A biopsy of the mass showed moderate-to-poorly differentiated HCC, consistent with metastatic HCC (Figure 2). The tumor was treated with four rounds of SHFRT at 10 Gy per fraction with a total dose of 40 Gy. A follow-up scan at 3 mo post-radiotherapy revealed a stable appearance of the paravertebral muscle metastasis. Because of progression of the intrahepatic tumors, the patient was switched to capecitabine (Xeloda®, Roche, Basel, Switzerland) 1500 mg twice daily once a week for 2 wk. He was later enrolled in a clinical trial and started on dasatinib. The patient passed away more than three years after the primary liver resection.

DISCUSSION

Despite significant advances in the treatment of HCC, the prognosis remains poor. Median survival times for

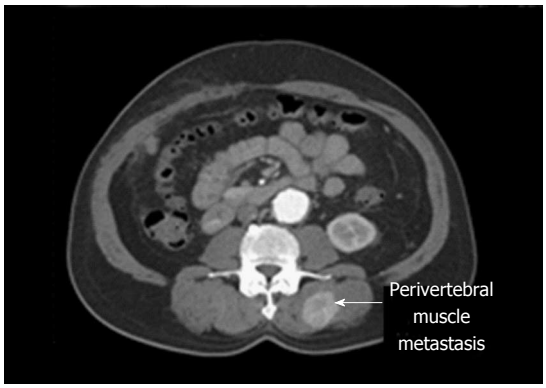

Figure 1 Computed tomography of the recurrent tumor. Computed tomography scan showing an enhancing lesion 3.7 cm in size in the left paravertebral musculature.

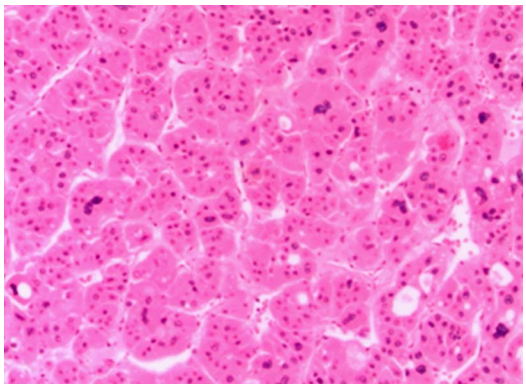

Figure 2 Histology of the paravertebral muscle tumor. A biopsy of the mass showed moderate-to-poorly differentiated hepatocellular carcinoma (HCC), consistent with metastatic HCC (hematoxylin and eosin, × 200).

patients with HCC who have EHM are 4.9-7.0 mo. One, three, and five year survival rates are 21.7%-31.0%, 7.0%-7.1%, and 4.0%, respectively[8]. Currently, there is no standardized treatment for HCC patients with EHM. Sorafenib is the first systemic agent that has demonstrated a significant improvement in survival time in patients with advanced HCC; however, the modest improvement of 3 mo is far from satisfactory[9]. Systemic cytotoxic chemotherapy agents, such as adriamycin, fluorouracil, cisplatin, etc. are considered palliative treatment options for advanced HCC but have low response rates of less than 10%. Recently, there have been some reports on the efficacy of capecitabine as a second-line treatment following sorafenib[10,11]. However, these studies are retrospective in nature with low levels of evidence. Other target agents such as regorafenib, c-Met inhibitor, and check point inhibitors are promising, but still under investigation. Dasatinib, an Src family kinase inhibitor, is reported to have effects on human HCC cell lines[12,13], however, the results of a recent clinical study showed insufficient response rates[14]. Due to lack of highly effective systemic chemotherapy for HCC, enrolling in a clinical trial with a new chemotherapeutic agent is the only option for patients with advanced HCC[15,16].

Several authors have reported long-term survivors after aggressive surgery for EHM[17,18]. From the viewpoint of reducing tumor burden, loco-regional therapy may be a reasonable strategy when the target lesions account for a major portion of the total tumor volume. These reports suggest a potential benefit to loco-regional treatment for intra and/or extrahepatic tumor in HCC patients with EHM. Patients with T1/2 primary tumor or less than two EHM were described as good candidates for aggressive local therapy[19,20]. A retrospective analysis reported that surgical resection of peritoneal or thoraco-abdominal wall implants from HCC in selected patients (limited number of implanted lesions; intrahepatic lesions absent or predicted locally controllable; and the absence of ascites with sufficient hepatic functional reserve) improved long-term survival, with 1, 3 and 5 year overall survival rate of 71%, 44% and 39%, respectively[21]. On the other hand, the cause of death in HCC patients with extrahepatic metastasis were mostly related to problems as a consequence of intrahepatic tumors, such as liver failure[8,18]. In our case, SHFRT was selected for local treatment of EHM, in addition to sorafenib as a systemic treatment, since the tumor invaded deeply into the paravertebral muscle and multiple intrahepatic recurrent HCC foci were identified, suggesting a poor prognosis even after the resection. Although the primary purpose for this radiation was for pain control, it was also effective in the control of disease progression. Our case is the first report of EHM treated by a non-surgical method which led to extrahepatic disease stability.

Vascular invasion of HCC has proven to be a strong determinant of EHM. Hematogenous spread to the lungs, lymph nodes, bones, and adrenals are reported to be the most common sites for EHM. Metastasis of HCC to muscle tissue is an infrequent phenomenon. Skeletal muscle and cardiac muscle are classified as striated muscles, which contain sarcomeres that are arranged into highly organized bundles. The infrequency of muscle metastasis seen in HCC may be attributed to the contractility of muscle, the local pH environment, and the presence of tumor suppressors in the muscle tissue[22]. Over 40 cases of cardiac muscle metastasis of HCC have been reported, whereas only found 17 cases of skeletal muscle metastasis of HCC have been reported (Table 1)[17,23-37]. All these cases were reported after 2005, two years before sorafenib was approved by the Food and Drug Administration for the treatment of HCC. Skeletal muscle recurrence occurred in various locations throughout the body, the trunk, and the peripheral musculature, with one case of extraocular muscle metastasis[28]. The majority of patients were male (16/18 cases) and had a history of HBV infection (10/13 cases, excluding 5 cases with unknown etiology). HBV viral load and anti-viral treatment were not recorded except in our case. Most cases underwent surgical resection as a local treatment (9/17 cases, excluding one case with unknown treatment), and some received radiation therapy as palliative therapy

Table 1 Skeletal muscle metastasis of hepatocellular carcinoma

Ref.	Year	Age/gender	Background	Treatment (primary lesion)	Muscle recurrence site	Recurrence time (mo)[1]	Treatment (metastasis)	Other lesions[2]	Simultaneous systemic treatment
This case	2017	55/M	HBV	Resection	Paravertebral muscle	21	SHFRT	Multiple intrahepatic HCC	Sorafenib
[23]	2014	36/M	Unknown	Chemo-radiotherapy	Chest wall	0	Chemo-radiotherapy	Liver, peripancreatic region, brain, cervical lymph node	Chemo-radiotherapy
[23]	2014	31/M	HBV HIV	Cisplatin/ adriamycin	Chest wall, pectoral muscles	0	Cisplatin/ adriamycin	Intrahepatic HCC	Cisplatin/ adriamycin
[24]	2014	47/M	Unknown	Resection	Rectus muscle	13	Resection	None	None
[17]	2013	55/M	HBV HCV	Resection	Pectoralis major Deltoid, left teres minor	54	Radiotherapy	Brain metastasis	Sorafenib
[25]	2013	61/M	Alcohol	None	Iliac muscle	0	Chemotherapy	Diffuse intrahepatic HCC	Chemotherapy
[26]	2012	65/M	HBV	RFA TACE	Intercostal muscle	24	Resection	None	None
[27]	2012	72/M	Alcohol	None	Medial pterygoid muscle	0	Radiotherapy	Multiple intrahepatic HCC	Sorafenib
[28]	2012	44/M	Unknown	Resection	Extraocular muscle	17	Radiotherapy	None	None
[29]	2011	70/M	HBV	Resection	Humorous muscle	108	Resection	None	Unknown
[30]	2009	82/M	Unknown	Resection	Diaphragm	30	Resection	None	None
[31]	2009	62/unknown	HBV	TACE resection	Pectineal muscle	96	Unknown	Multiple intrahepatic HCC	Unknown
[32]	2008	54/M	HBV	Resection	Rectus femoris muscle	60	Sorafenib	Multiple pulmonary metastasis	Sorafenib
[33]	2008	52/M	HBV	Liver transplant	Chest wall	60	Resection	None	None
[34]	2007	63/M	Unknown	Resection	Gastrocnemius muscle	18	Resection	None	None
[35]	2007	53/M	HCV	None	Gluteus maximus muscle	0	Resection	None	None
[36]	2006	50/M	HBV	Resection	Psoas muscle	12	Resection	None	None
[37]	2005	39/F	HBV	Resection	Chest wall	11	Resection	None	None

[1]Months after the primary treatment; [2]At the time of muscle recurrence. M: Male; F: Female; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; TACE: Transcutaneous chemoembolization; SHFRT: Stereotactic hypofractionated image-guided radiation therapy; HCC: Hepatocellular carcinoma.

(three cases). In cases with simultaneous recurrence similar to ours, sorafenib or another chemotherapeutic agent was used as systemic therapy[17,23,25,27,32]. However, even with these treatments, prognosis was extremely poor, ranging from a few weeks to 6 mo.

Previous studies have described the importance of controlling viral status to prevent HCC recurrence and improve survival after curative treatment for HBV-related HCC[38,39]. Huang *et al*[38] reported that preoperative antiviral treatment decreased viral reactivation rate, and pre- plus postoperative antiviral treatment achieved a better 5-year overall survival rate than postoperative antiviral treatment alone by decreasing HBV-related HCC recurrence. On the other hand, only one study described a correlation between HBV status and EHM. Sasaki *et al*[40] reported that HBV infection was an independent predictor for the occurrence of EHM in patients with large HCC tumors. In addition, the authors posit that HBV infection might promote the establishment of EHM through modulation of the adhesion-de-adhesion balance of HCC cells[40]. In our case, although the patient's HBV status was well-controlled by entecavir after hepatectomy, the patient did not receive any anti-viral treatment preoperatively despite a high viral load. No previous case reports of muscle recurrence included patient HBV status or antiviral treatments. Although the relationship between HBV infection and skeletal muscle recurrence has not been clarified, we consider controlling HBV viral load through antiviral treatment prior to surgical intervention important due to the high incidence of HBV infection among patients with HCC with EHM recurrence.

We report the first case of HCC with EHM to the paravertebral muscle. Though this is a single case, it raises interest in detecting EHM at an earlier stage and initiating therapy if the patient's overall health permits.

A study of surgical and non-surgical treatment with systemic *vs* loco-regional therapy may shed further light on this topic.

ACKNOWLEDGMENTS

We acknowledgements Transplant and Hepatobiliary Surgery Henry Ford Hospital, 2790 West Grand Boulevard, Detroit, MI 48202, United States.

COMMENTS

Case characteristics

A 55-year-old male with a history of hepatitis B virus (HBV) induced liver cirrhosis complained of back pain two years after removal of hepatocellular carcinoma (HCC).

Clinical diagnosis

Computed tomography (CT) scan showed a mass at the left paravertebral muscle, biopsy of which was consistent with moderate to poorly differentiated HCC.

Differential diagnosis

Rhabdomyosarcoma, fibromatoses, hemangioma, or metastatic tumor of HCC.

Laboratory diagnosis

A mild elevation of the alpha-fetoprotein level (15.1 ng/mL). HBV DNA counts of 12.7×10^6 copies/mL.

Imaging

CT scan showed an enhancing lesion 3.7 cm in size at the left paravertebral muscle.

Pathological diagnosis

A biopsy of the mass showed moderate to poorly differentiated HCC, consistent with metastatic HCC.

Treatment

The tumor was treated with four sessions of stereotactic hypo-fractionated image guided radiation therapy at 10 Gy per fraction with a total dose of 40 Gy.

Related reports

There were only 17 cases of the skeletal muscle metastasis of HCC. These were at various locations from the skeletal muscles of body trunk to peripheral muscles.

Term explanation

Extrahepatic metastases (EHM) of HCC to the skeletal muscle tissue are extremely rare. Median survival times for patients with HCC who have EHM are 4.9-7.0 mo. Currently, there is no standardized treatment for HCC patients with EHM.

Experiences and lessons

This case invites interest in detecting EHM at an earlier phase and the initiation of therapy if the patient's health and overall assessment permits. A study of surgical and non-surgical treatment with systemic *vs* loco-regional therapy may shed further light on this situation.

Peer-review

The paper is well-written.

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WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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REVIEW

Innate lymphoid cells in tissue homeostasis and diseases

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Abstract

Innate lymphoid cells (ILCs) are the most recently discovered family of innate immune cells. They are a part of the innate immune system, but develop from the lymphoid lineage. They lack pattern-recognition receptors and rearranged receptors, and therefore cannot directly mediate antigen specific responses. The progenitors specifically associated with the ILCs lineage have been uncovered, enabling the distinction between ILCs and natural killer cells. Based on the requirement of specific transcription factors and their patterns of cytokine production, ILCs are categorized into three subsets (ILC1, ILC2 and ILC3). First observed in mucosal surfaces, these cell populations interact with hematopoietic and non-hematopoietic cells throughout the body during homeostasis and diseases, promoting immunity, commensal microbiota tolerance, tissue repair and inflammation. Over the last 8 years, ILCs came into the spotlight as an essential cell type able to integrate diverse host immune responses. Recently, it became known that ILC subsets play a key role in immune responses at barrier surfaces, interacting with the microbiota, nutrients and metabolites. Since the liver receives the venous blood directly from the intestinal vein, the intestine and liver are essential to maintain tolerance and can rapidly respond to infections or tissue damage. Therefore, in this review, we discuss recent findings regarding ILC functions in homeostasis and disease, with a focus on the intestine and liver.

Key words: Innate lymphoid cells; Intestine; Liver; Homeostasis; Inflammatory diseases

Core tip: Receiving approximately 70% of blood through the portal vein, the liver represents one of the most important sites of defense against invading pathogens. In addition, the liver and the intestine are important immune organs, as they are often in contact with antigens and endotoxins produced by the gut microbiota. These organs are densely populated by innate immune cells such as natural killer cells, dendritic cells, macrophages, natural killer T cells and innate lymphoid cells (ILCs), which are rapidly activated by commensal and pathogenic antigens,

growth factors, cytokines and host metabolites. Recent studies have been focused on discovering the role of ILCs and how these cell populations can regulate the immune response. Our goal is to discuss innovative literature highlighting the importance of ILCs in the context of infectious disease, tissue repair, tolerance of gut microbiota and inflammatory diseases that affect the liver and intestine homeostasis.

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INTRODUCTION

Innate lymphoid cells (ILCs) are the most recent family of innate immune cells discovered among the myriad of factors that make up the immune system. They belong to innate immune system but develop from the lymphoid lineage. However, contrary to T and B lymphocytes, ILCs do not have RAG-mediated recombined antigen receptors[1,2]. Their distribution is ubiquitous, being found throughout the body and enriched in mucosal surfaces[3,4]. These cells are able to communicate with different cell types to orchestrate the immune system during homeostasis and inflammation[5-7].

The non-cytotoxic ILCs consist of three different groups: ILC1, ILC2 and ILC3[5,7-10]. The ILC3s also include the lymphoid tissue inducer (LTi) cells. These cells were uncovered in 1997 and are involved in the formation of secondary lymphoid tissues[4].

Mirroring the Th subsets, the non-cytotoxic ILCs are separated based on cytokine expression, transcription factors during development, surface markers and distinct effector functions[5,6]. Although parallels between ILCs and Th subsets have been observed, ILCs lack pattern-recognition receptors and therefore cannot directly mediate antigen specific responses[3,11]. In fact, given that these cells are not directly activated by pathogen-associated molecular patterns it was unclear how ILCs discern infection, tissue injury or disruption of homeostasis. It is now known that ILCs present within adult tissues constitutively express cytokines, alarmins and growth factor receptors making them more sensitive to these mediators in their environment, enabling immediate ILC activation[3,12]. Despite being present in very low numbers, the wide distribution of ILCs in lymphoid and non-lymphoid tissues across species was seen as an indicator of the fundamental role of these innate cells in regulating multiple physiological processes throughout the body[7].

Several studies have shown that ILCs are easily recovered in areas susceptible to microbial colonization or invasion by pathogens, such as barrier surfaces. Recently, it became known that ILC subsets play a key role in host immune responses to bacteria, fungi, viruses and extracellular parasites at these sites[6,13,14]. In addition, their interaction with the microbiota, nutrients and metabolites[6,13] highlighted important functions for ILCs in triggering tissue repair and inflammation which, if unregulated, can result in exacerbated immune responses.

Based on the emerging roles of ILCs in controlling tissue homeostasis, this review will highlight the advances in understanding how ILCs can participate in host defense in the context of immunity, microbiota, autoimmunity and tissue remodeling, focusing on the intestinal and liver pathophysiology.

ILCs DEVELOPMENT: AN OVERVIEW

Until the discovery of ILCs, conventional natural killer cells (cNKs) were the only innate cells able to respond to cytokines released by antigen presenting cells (APCs). Therefore, NK cells represent the prototypical member of the ILC family[1,15-18]. However, NKs have additional roles that set them apart from other ILCs, such as cytotoxicity and the ability to initiate immune responses against virus and tumor cells[18]. Besides, recent analysis of the progenitor cells and surface markers of the ILC family members indicate that NK cells and non-cytotoxic ILCs group do not come from the same lineage[7-10].

The identification of the ILC precursors and the key factors required for development of the different ILC subsets is quite recent. It was found that the ILCs arise from a common lymphoid progenitor (CLP). Downstream, the precursors can develop into different ILC subsets and NK cells expressing the integrin $\alpha4\beta7$ and the transcription factors Nfil3 (nuclear factor- interleukin 3 regulated) and TOX[7,9,19,20]. First, the common helper-like ILC progenitor (CHILP), that expresses the transcriptional regulator inhibitor of DNA binding 2 (Id2), gives rise only to ILC1, ILC2, ILC3 and LTi cells[9,10,19]. Downstream to CHILP, another ILC precursor is able to give rise all ILCs subsets, but not LTi or cNK cells[10]. This precursor can express the transcription factor promyelocitic leukemia zinc finger (PLZF)[21,22].

ILC SUBSETS

As mentioned before, ILC populations differ based on their transcription factors and production of signature cytokines, similar to Th cells. However, while ILC2s and ILC3s are well characterized, ILC1s are more complex to identify due to many shared characteristics with NK cells[23]. Both are responsive to inflammatory cytokines, such as interleukin (IL)-15 and IL-12, and produce interferon (IFN)-γ and tumor necrosis factor (TNF) after activation[24,25]. ILC1s are enriched in the liver, skin, salivary glands, uterus, thymus and the gut[23,26]. Regarding transcription factors, T-bet is the most important and regulates the ILC phenotype and functions, such as the production of IFN-γ. NK cells can

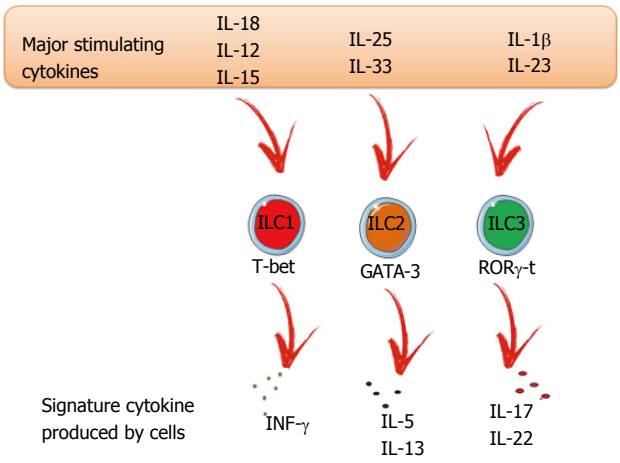

Figure 1 Innate lymphoid cell family. Each individual ILC subset is characterized by differential expression of transcription factors and patterns of expression of cytokines. ILCs can be activated by a diverse array of cytokines and can contribute to immunity, inflammation and maintenance of tissue homeostasis. IL: Interleukin; IFN-γ: Interferon γ; ILC: Innate lymphoid cell.

also express T-bet (Figure 1). However, in NK cells, this transcription factor is not expressed in the same level, nor is it important to their development, unlike ILC1s. The T-box transcription factor eomesodermin (Eomes) is the marker of NK cells, but in some organs ILC1s can also express this transcription factor[24,25,27,28]. The general ILC1 surface markers include CD69, NK1.1 and NKp46 but, depending on the environment, we can find distinct ILC1 phenotypes and, consequently, surface markers. ILC1s are important to promote immunity against intracellular bacteria and parasites[9,29-31].

ILC2s are known to produce Th2 signature cytokines, IL-5 and IL-13[32-35] and can also release IL-9, IL-5 and IL-6[36]. They are characterized by their responsiveness to IL-33, IL-25 and thymic stromal lymphopoietin (TSLP)[37-40]. Their main function is to promote type 2 inflammation, which is important during allergies, helminth infection, and tissue repair[32-34,41-43]. ILC2s are found in different tissues including lung and adipose tissue, as well as in the gut, liver and skin[39,44-46]. Their main surface markers are CD127, c-kit, Sca1 and ST2 (the receptor for IL-33)[1]. Regarding transcription factors, experiments with GATA-binding protein 3 (GATA-3) knockout mice have shown that this factor is essential for ILC2 differentiation and maturation (Figure 1)[47,48].

ILC3s are a heterogenous group found mainly in mucosal tissues[49]. However, a small number can be found in the spleen[50], lung[50] and liver[51]. Two different ILC3 subsets can be distinguished based on the expression of T-bet and CCR6. Both express RAR-related orphan receptor gamma (RORγt), but only LTi cells express CCR6$^+$. The LTi population can be further divided based on the expression of CD4 (LTi CD4$^+$ or LTi CD4$^-$). The CCR6- ILC3 population can express T-bet and consists of two subpopulations that are distinguished based on the expression of the natural cytotoxicity receptor (NCR) NKp46[52-54]. The ILC3 subset signature cytokines are IL-22 and IL-17 (Figure 1). The IL-22 acts selectively on stromal and epithelial cells leading to a rapid production of the antimicrobial peptides alpha and beta defensins. ILC3-derived IL-22 is crucial in preventing dissemination of commensal bacteria[52,55,56].

ROLES OF ILCs SUBSETS: FROM HOMEOSTASIS TO DISEASE

The strategic location of ILCs at the mucosal surfaces ensures the induction of an immune response and shapes the adaptive immune response against invading pathogens. In addition, the presence of these cells in non-lymphoid tissues suggests that, besides regulating the proinflammatory responses, ILCs can also play a role in tissue development and homeostasis (Table 1).

ILCs induce protective immunity in response to infections

Following pathogen invasion and tissue damage, epithelial cells and innate immune cells produce cytokines and alarmins which cooperatively mobilize and activate ILCs subsets[6]. Studies have shown that ILC-derived cytokines have an important protective function against *S. Typhimurium*[57], *C. rodentium*[54,58], *N. brasiliensis*[32,48], and *C. albicans*[59] infections.

IFNγ-producing ILC1s contribute to protection against *Salmonella enterica* subsp. *enterica* serovar Typhimurium infection in the colon. In addition, ILC3-derived IL-22 is required for the fucosylation of the intestinal epithelium which helps to protect against *S. Typhimurium* infection. Once bound to the receptor, IL-22 triggers a signaling cascade which induces fucosylation of epithelial cells, activation of the transcription factor STAT3 and consequently secretion of antimicrobial peptides[57,60]. Murine ILC1s are also important to immune function in response to *T. gondii*[9]. An increase of human ILC1s was shown in patients with chronic hepatitis B infection[61], indicating that this population can contribute to immunity in response to specific pathogens in both mice and humans.

Before the onset of adaptive immune responses, the innate immune response to the enteric pathogen *Citrobacter rodentium* is critically dependent on ILC3-derived IL-22. C. *rodentium* is a gram-negative bacterium which causes acute colitis in mice. As mentioned above, the expression of antimicrobial peptides, dependent on the STAT-3 pathway, is induced by IL-22 and contributes to maintenance of the epithelial barrier surface. In addition, mice deficient in IL-22 rapidly succumb to infection due to exacerbated intestinal inflammation, bacterial invasion and proliferation throughout the tissues[60]. IL-23 production by DCs or CX3CR1$^+$ mononuclear phagocytes is necessary for ILC3 activation and it has been shown that ILC3s are the predominant source of IL-22 in the first week of *C. rodentium* infection[60]. Satpathy *et al*[62] showed that *il23a*$^{-/-}$ mice are more susceptible to infection with high

Table 1 Innate lymphoid cell functions across the intestine during homeostasis and inflammatory diseases

ILC subtype	Function	Model	Evidence	Ref.
ILC1	*T. gondii* infection	Oral infection C57BL/6 mice	Immunity to *T. gondii* infection is IFNγ-dependent; mice lacking T-bet expression had virtually no IFN-g production in response to *T. gondii* infection and failed to control parasite replication	[9]
ILC2	*N. brasiliensis* infection	Balb/c subcutaneous infection	Combined absence of IL-25 and IL-33 signaling led to a defect in worm expulsion, that was rescued by ILC2-adoptive transfer	[34]
ILC3	*S. Thiphymurium* infection	Fut2-deficient C57BL/6 mice	Fucosylation of intestinal epithelial cells is catalyzed by Fut2; IL-22-derived ILC3s induce the expression of Fut2. Disruption of intestinal fucosylation led to increased susceptibility to infection by *S. Typhimurium*	[57]
ILC3	*C. rodentium* infection	Oral infection C57BL/6 mice	Mice lacking IL-22-producing ILC3 cells showed heightened susceptibility to the pathogen	[54,62]
ILC3	*C. albicans* infection	C57BL/6 and BALB/c mice	IL-22 mediates protection in IL-17RA-deficient mice; an early IL-22-dominated response is then followed by Th1/Treg reactivity	[65]
ILC2	Epithelium repair after intestinal inflammation	C57BL/6 DSS-induced colitis	Number of AREG-expressing ILC2s increases following intestinal inflammation. Disruption of the AREG-EGFR pathway exacerbated disease	[74]
ILC3	Repair of lymphoid tissue	C57BL/6 mice	LCMV infection induces the destruction of secondary lymphoid organs RORγ-deficient WT chimeras had impaired rebuilding of stromal cell compartment after LCMV infection	[70]
ILC3	Regeneration of intestinal epithelium	C57BL/6 mice	Intestinal microbiota represses the ILC3-producing IL-22 through the induction of IL-25 by IECs. RAG-2-deficient mice treated with IL-25 showed significant weight loss in response to DSS treatment	[68]
ILC3	Containment of the gut microbiota	C57BL/6 mice	Depletion of IL22-producing ILC3s resulted in peripheral dissemination of commensal bacteria and systemic inflammation, which was prevented by administration of IL-22	[80]
			Ablation of LTα in RORgt + cells abrogated IgA production in the gut and altered microbiota composition	[81]
ILC1	Crohn's disease	Human	ILC1 population is increased in the inflamed intestine of people with Crohn's disease	[29,30]
ILC1	Ulcerative colitis	Anti-CD40 colitis model	IELs from the small intestine of mice treated with anti-CD40 revealed a robust production if IFN-γ by ILC1s. Anti-Nk1.1 treatment reduced inflammatory infiltration and epithelial damage, suggesting that ILC1 can contribute to colitis through IFN-γ secretion	[85]
ILC3	Ulcerative colitis	Anti-CD40 colitis model	ILC3s secrete higher amounts of GM-CSF which in turn recruits pathogenic $Ly6C^+$ inflammatory monocytes, increasing inflammation and tissue damage	[86]
ILC3	Crohn's disease	Human	Inflamed tissue from patients with CD showed accumulation of IL-23-responive ILCs and increase expression of IL-17	[91]
ILC3	Colorectal cancer	C57BL/6 mice	Absence of IL-23 promotes tumor development accompanied by increased innate immune cell infiltration; tumorigenesis induced by IL23 could not be initiated in $RAG2^{-/-}IL\text{-}2R^{-/-}$ double knockout mice; IL-23R expression was identified in gut associated lymphoid tissue	[49] [93]

IFN: Interferon; Fut2: Fucosyltransferase 2; AREG: Amphiregulin; LTα: Lymphotoxin α; LCMV: Lymphocytic choriomeningitis virus; GM-CSF: Granulocyte-macrophage colony-stimulating factor; EGFR: Epidermal growth factor receptor; IL: Interleukin; ILC: Innate lymphoid cell.

concentrations of this bacterium than wild type mice. As IL-23 was found to be crucial for IL-22 production by ILC3s, but not by Th17 cells, this model suggests that ILC3s are essential for resistance to *C. rodentium* infection. To clarify this phenomenon, experiments with $Rag^{-/-}il2rg^{-/-}$ mice, which lack T cells and ILCs, showed that ILC-deficient mice are more susceptible to infection when compared to $Rag^{-/-}$ mice[54,58]. However, at a later infection stage, it was observed that T cell-derived IL-22 contributes substantially to *C. rodentium* clearance and tissue repair[63]. Therefore, whether ILC3s and T cells can perform redundant functions cannot be ruled out. In addition to IL-22 and IL-33, IL-17 was also described to play an important role during viral hepatitis. The intrahepatic subpopulation of ILC3s can induce IL-17 signaling to induce T cell responses in viral hepatitis, improving the clearance of the virus[64].

In a similar fashion, in the response against bacterial pathogens, ILC3s seem to be the primary source of IL-22 during *Candida albicans* infection. *C. albicans* is a commensal fungus found on mucosal and skin surfaces, but can also cause infection in children younger than 1 mo, elderly and in immuno compromised individuals. A recent study showed that IL-22 acts as the first-line of defense during candidiasis by controlling fungal overgrowth and epithelial integrity. In the second stage, the Th1 response is crucial to prevent fungal dissemination[65].

Interactions between the epithelium and ILC2s mediate immunity to helminth parasites. The type 2 immune response is characterized by the production of IL-4, IL-5, IL-9 and IL-13 cytokines. The immunity to the mouse nematode *Nippostrongylus brasiliensis* is IL-13-dependent, as this cytokine upregulates macrophage activation, development of goblet cells and smooth muscle contraction that together will induce

parasite expulsion[7]. Even though $CD4^+$ T cells are central in a type 2 immune response, it was shown that T cells are not the major producing cells of IL-13 for the expulsion of *N. brasiliensis.* $CD4^+$ T cells from wild type mice were unable to induce worm expulsion when transferred into IL-4- and IL-13-deficient *Rag2*$^{-/-}$ mice[66]. The secretion of IL-13 by ILC2s was demonstrated by adoptive transfer of IL-13$^{-/-}$ ILC2s, which were not able to promote worm elimination. Moreover, transferring of wild-type ILC2s into mice deficient in IL-13 supported the data whereby IL-13 secretion from ILC2s is enough for worm expulsion[33].

Although the trigger of the signals is not well understood, these recent studies suggest a crosstalk between epithelial cells and ILCs driving appropriate ILC response. In attempting to explain the mechanisms by which ILCs interact with the non-hematopoietic and other hematopoietic cells, the employment of genetic and imaging tools are necessary to clarify it.

ILCs maintain tissue integrity

ILCs also promote the maintenance of tissue integrity by contributing to tissue remodeling and healing of tissue injury. During embryonic development, a subset of ILC3s known as LTi, promote the formation of secondary lymphoid organs such as Payer's Patches in the gut. LTi cells induce the production of chemokines CXCL13, CCL21 and CCL19 by stromal cells and the upregulation of adhesion molecules (VCAM1, MadCam1 and ICAM1) that attract and bind leukocytes to constitute lymphoid structures[67]. ILC3s have also been implicated in repair of lymphoid tissue after damage as a result of graft-*vs*-host disease, acute viral infection[7], irradiation or treatment with methotrexate. The intestinal epithelial cell regeneration can also be launched through ILC3-derived IL-22 which mediates the regeneration of the cells by acting on intestinal stem cells that express the IL-22 receptor[60]. In addition, IL-22 can be produced in the liver, acting on hepatocytes and hepatic stellate cells (HSCs) exhibiting hepatoprotective properties by diminishing liver fibrosis and improving acute liver injury[68-71]. Kong *et al*[72] identified high levels of IL-22R1 expression on HSCs. This cell type is the most involved during liver fibrogenesis. They demonstrated that IL-22, *via* STAT3, SOCS3 and p53 activation, has an antifibrotic effect by inducing the senescence of HSCs, ameliorating liver fibrosis[72]. Matsumoto *et al*[73] reported that the IL-22-producing ILC3s play a protective role in a murine acute hepatitis model, by potentially blocking the hepatocyte cell death.

Corroborating the IL-22 protective role, Kudira *et al*[51] recently demonstrated that IL-22 contributes to liver regeneration in a partial hepatectomy (PH) model. However, they demonstrated that the source of IL-22, in this model, is ILC1s and cNK. They showed that IL-22 is essential for liver regeneration, and its production depends on the extracellular adenosine triphosphate (ATP) *via* P2X1 receptor[51].

The fact that dying and damaged epithelial cells discharge alarmins which can be sensed by ILC2s suggests a close interaction between these two cells types. In fact, ILC2s expressing amphiregulin can regulate cell differentiation and proliferation by binding to the epidermal growth factor receptor (EGFR). IL-33-stimulated ILC2s can induce the repair of intestinal epithelial lesions after DSS-induced colitis by amphiregulin secretion[74]. This cytokine, IL-33, as well as ILC2s, have been in the spotlight due to their contributions to the improvement of obesity-induced insulin resistance. IL-33 can bind the ST2 receptor and induce the production of large amounts of anti-inflammatory cytokines by ILC2s in adipose tissue. These cytokines lead to polarization of the adipose tissue macrophages to an M2 phenotype[75,76]. In the liver, unlike in DSS-induced colitis and adipose tissue, IL-33 was identified as a key mediator of hepatic fibrosis. It is released in response to chronic hepatocellular stress and, after binding to ST2, culminates in ILC2s activation, as mentioned above. These cells produce anti-inflammatory and tissue remolding cytokines, such as IL-13 and IL-4. In turn, IL-13 can activate HSCs in an IL-4Ra- and STAT6 transcription-factor-dependent fashion, a pro-fibrotic cascade. Accordingly, IL-33 plays a role in a profibrotic cascade as the apex of the signaling pathway[77]. Another study showed that HBV infected patients have higher concentrations of IL-33 in serum compared to healthy controls. In addition, that concentration decreases following 12 wk of treatment[78]. These findings indicate that, in certain conditions, ILC2s can be manipulated, avoiding excessive tissue remodeling, when IL-33-stimulated ILC2s secrete IL-13 and IL-4, inducing fibrosis mediated by liver stellate cells (Figure 2)[79].

Besides their tissue repair properties, ILC2s play a role in limiting exacerbation of inflammatory responses. This can occur through the production of type 2 cytokines, that can suppress type 1 and type 17 inflammation[3], showing the diverse roles that ILCs can play.

ILCs and the crosstalk with the intestinal microbiota

Complementary to their role in promoting immunity against pathogens, ILCs are also evolved with tolerance mechanisms regarding interactions between the host and the commensal microbiota. Recent studies have begun to disclose how ILC3s interact with gut bacteria, diet-derived factors and various cell types to maintain intestinal homeostasis.

Although the organization of ILC subsets in the gut-associated lymphoid tissues and murine intestinal tissues occur independently of microbiota colonization, the anatomical retention of lymphoid tissue resident bacteria seems to be related with ILC3s function[80]. For example, B cells can be activated by ILC3s through lymphotoxin $\alpha 1\beta 2$ which induces the proliferation and the production of immunoglobulin A (IgA), that

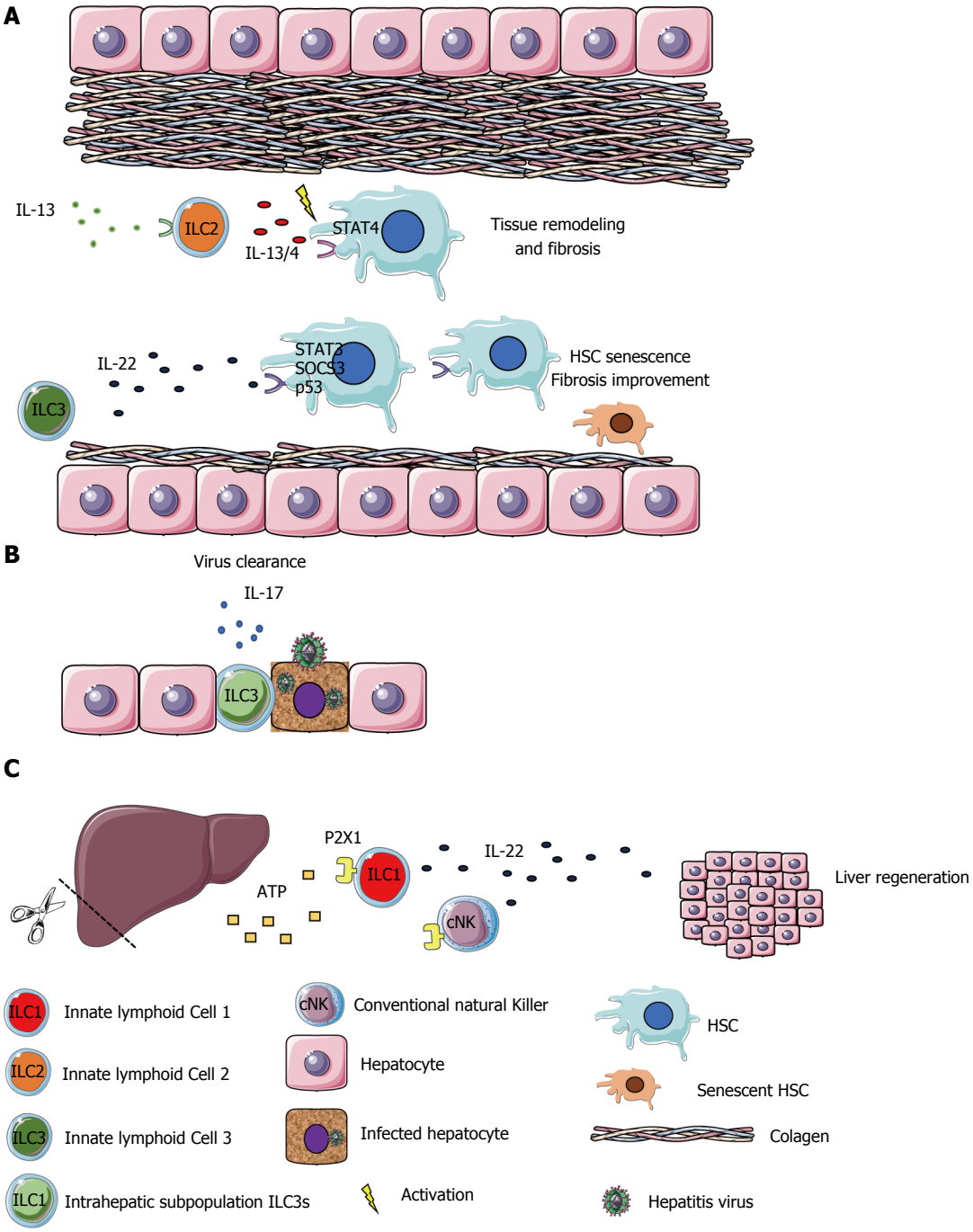

Figure 2 Innate lymphoid cell family plays different roles in the liver. ILCs can develop different functions depending on the organ and environment in which they are found. In the liver (A) IL-33, produced by hepatic cells, can act on ILC2s, promoting the release of anti-inflammatory cytokines, such as IL-13 and IL-4. These cytokines can activate HSCs, *via* STAT4, promoting tissue remodeling and fibrosis. On the other hand, IL-22, produced by ILC3s, acts on HSCs, *via* STAT3, SOCS3 and p53, promoting their senescence and ameliorating liver fibrosis; B: IL-17 can be released by the intrahepatic subpopulation ILC3s, during the virus infection promoting the clearance of the virus; C: IL-22 can be produced by ILC1s and cNK cells in the liver, contributing to liver regeneration *via* ATP-P2X1. Therefore, different cytokines can be manipulated, as therapeutic targets, in benefit of hepatic inflammation, fibrosis and tissue regeneration. IL: Interleukin; IFN-γ: Interferon γ; ILC: Innate lymphoid cell; HSCs: Hepatic stellate cells.

subsequently contributes to neutralization of commensal bacteria in the lumen and prevents an inappropriate immune response[81]. Furthermore, it was shown that ILC3s express MHC class Ⅱ and although they do not express co-stimulatory molecules necessary to activate T-cells, depletion of ILC3s or selective deletion of MHC class Ⅱ in these cells is associated with exacerbated bacteria-specific Th17 cell response and intestinal inflammation[82]. These data suggest that ILC3s can drive a host immune tolerogenic state in the intestine by controlling the functions of other immune cell types.

Controversial results have been found regarding the influence of microbiota on ILC3 function. The production of protective levels of IL-17A, IL-17F and IL-22 and the responsiveness to IL-23 suggest that both human and mice ILC3s contribute to intestinal homeostasis[14].

Despite some studies that have shown that murine ILC IL-22 production is not affected after alteration of bacterial communities[80], other works show that microbial products influence the level of IL-22 secretion in mice and humans[7,14], and that germ-free mice have decreased IL-22-expressing ILC3s[54]. In addition, epithelial cells stimulated by commensal microbiota release IL-25 which acts on $CD11c^+$ cells to limit IL-22 secretion derived from ILC3s[83]. Future work will be needed to elucidate the mechanisms by which this interaction occurs and how this process is regulated. In attempt to explain how ILC3s communicate with environmental factors in the intestine, recent studies have focused on whether dietary substances can be sensed by ILC3s. Fucose can be used as a carbohydrate source by commensal bacteria. ILC3s facilitate the transfer of fucose to the surface of intestinal epithelial cells which is critical for resistance to infection with *Salmonella Thyphimurium* and to maintain the appropriate number of bacteria in the lumen[57]. Another example is the relationship between the level of vitamin A and ILC3 functions, whereby vitamin A deficiency was related with impaired ILC3 responses[84], suggesting that these cells sense signals from host-derived nutrients and directly from the microbiota.

ILCs promote chronic inflammatory diseases

Besides their function in promoting tissue homeostasis, the chronic activation of ILCs can also induce inflammation at mucosal surfaces. IL-23 is a powerful activator of ILC3s and this axis is intimately linked to inflammatory bowel disease (IBD). Infection-induced and sterile inflammation models of colitis such as *S. typhimurium, Helicobacter hepaticus*, *Helicobacter typhlonius* infectious models or anti-CD40 models have been used to better understand the ILC3 functions, which are thought to be related to stimulation by IL-23 or IL-12 and consequently release of IL-17, GM-CSF and IFN-γ[85-87]. IL-17-producing ILC3s have been shown to play a key role in T-cell independent mouse models and, in this context, CD127 blockade seems to reduce ILC3 numbers and ameliorates disease. It is believed that activation of dendritic cells leads to TNF and IL-23 release which in turn results in an expansion of IL-17 producing ILCs[88]. In contrast, ILC3-derived IL-22 protects mice from intestinal inflammation trigger by *C. rodentium* infections, DSS-induced colitis and the transfer of T cells[55,86,89]. In some murine colitis models, the blockade of intra-epithelial ILC1s and IFNγ-producing ILC3 ameliorates the inflammation on the mucosal layer[6,90]. Conversely, although a consistent role for ILCs in human IBDs continues tobe discussed, several studies have reported varying numbers of these cells in intestinal samples. Patients with Crohn's disease presented an increase in ILC1 populations accompanied by decreased levels of IL-22-producing ILC3s in inflamed intestinal tissues[29,30]. In addition, in pediatric patients with Crohn's disease, a lower expression of MHC class Ⅱ on ILC3s was observed than in control subjects without IBD; a reduction of MHC class Ⅱ was correlated with increased numbers of Th17 cells[91]. Together, these data suggest that ILC1s and ILC3s might participate in the establishment and the development of inflammation, and ILC3s might reduce pathogenic T cells trough MHC class Ⅱ interactions.

Evidence regarding the function of ILCs in tumorigenesis are emerging from studies investigating the pro-carcinogenic role of cytokines and chronic inflammation. Human colorectal cancer (CRC) samples showed increased expression of IL-23 receptor, and the induced expression of IL-23 in mice led to the development of adenomatous tumors originating in the duodenum. Although the contribution of adaptive cells remains unclear, this model would indicate a potential role for ILC3s[92,93]. Moreover, IL-22-producing ILC3s might also be related in human CRC because uncontrolled IL-22 production facilitates tumor-infiltrating lymphocytes and IL-22 levels in the tumor were significantly higher than in non-tumor sections from the same patients[94].

Future perspectives

The liver and intestine are complex organs that have multiple interactions with the microbiota, nutrients, metabolites and diverse types of cell to maintain the host homeostasis. The importance of the ILC family in the immunity panel is growing fast. Many studies have been done to elucidate the specific ILCs function in different sites triggering immunity, tissue repair and inflammation. However, the molecular mechanism by which ILC subsets play specific roles and their consequences for the host homeostasis remain unclear. Future studies focusing on how ILC responses are regulated and how they integrate the immune cells in different organs might provide therapeutic potential in the treatment of diverse diseases.

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MINIREVIEWS

Use of everolimus in liver transplantation

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Abstract

In recent years, the use of mammalian target of rapamycin inhibitors has gained traction in their use as alternative or adjunct immunosuppressants in the post-liver transplantation (LT) setting. The efficacy of everolimus (EVR) in de novo LT is established and a reasonable time to initiate EVR is 30 d from LT surgery. Initiating EVR early post-LT allows for calcineurin inhibitor (CNI) reduction, thus reducing nephrotoxicity in LT recipients. However, data is inadequate on the appropriate timing for conversion from CNI to EVR maintenance in order to achieve optimal renoprotective effect without compromising drug efficacy. Adverse effects of proteinuria, hypercholesterolemia and hyperlipidemia are significantly higher as compared to standard CNI and long-term implications on graft and patient survival in LT is still unclear. Future research to explore strategies to minimise EVR adverse effects will be crucial for the success of EVR as an important alternative or adjunct immunosuppressive therapy in LT.

Key words: Everolimus; Mammalian target of rapamycin inhibitor; Immunosuppression; Liver transplantation; Nephrotoxicity

Core tip: Everolimus is the most recently approved immunosuppressant for use in liver transplantation (LT). Its renoprotective effect is an attractive option for LT recipients who have calcineurin inhibitor-induced nephrotoxicity. This review examines through data published, discovers gaps of evidences and discusses the place in therapy for everolimus (EVR) in LT. At the end of review, it summarises how EVR can benefit LT recipients as well as the caveat in using EVR.

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INTRODUCTION

Since the first liver transplantation (LT) surgery in 1963,

surgical techniques and immunosuppression therapy have evolved much and improved patient outcomes. Based on Organ Procurement Transplantation Network/ Scientific Registry of Transplant Recipients (OPTN/ SRTR) data in 2013, the 5-year graft survival rate in LT is as high as 76%[1]. In most transplant centers, LT immunosuppressive regimes include calcineurin inhibitors (CNI), antimetabolites, steroid with or without induction therapy[2]. For the past few decades, CNIs have been the cornerstone of immunosuppressant regimens for LT recipients. The overall patient survival at 1-, 5- and 10-years for LT with tacrolimus (FK) were in range of 81%-84%, 70%-72% and 57%-68% respectively[3,4]. Nonetheless, CNIs, both FK and cyclosporine (CsA), increase the risk of nephrotoxicity, diabetes, hypertension and neurotoxicity[2]. Ojo *et al*[5] reported as high as 18% of LT recipients developed renal impairment within 5 years post-LT. Therefore much research has been focused on finding strategies or alternatives to avoid or minimize nephrotoxicity in the past 10 years and one of the more recent drug classes to be used are the mammalian target of rapamycin (mTOR) inhibitors [sirolimus, everolimus (EVR)].

EVR was approved for the prevention of graft rejection in LT when used in combination with both FK and steroid in Europe (October 2012) and in the United States (February 2013).

PHARMACOLOGICAL PROPERTIES OF EVR

EVR is an mTOR inhibitor and has antiproliferative properties. It reduces protein synthesis and cell proliferation by binding to FK binding proten-12 to form a complex that inhibits activation of the mTOR serine threonine kinase activity (Figure 1). It also has antiangiogenic effects by inhibiting expression of hypoxia inducible factor and vascular endothelial growth factor. In addition, mTOR may have additional importance in neuroendocrine cells and EVR has been shown to block the action of IGF-1 in neuroendocrine cells[6].

EVR is a derivative of sirolimus, differing by one extra hydroxyethyl group at position 40 (Figure 2). Based on pharmacokinetics data, its absorption is rapid and bioavailability is variable, about 16%-20% (higher than sirolimus' 10%-14%)[7,8]. EVR requires twice daily dosing as its elimination half-life is 32 h, which is shorter than sirolimus' half-life of 62 h. Therefore, no loading dose is required for EVR and steady state can be achieved faster, in 4 d, *vs* 6 d for sirolimus. EVR is extensively metabolised in the liver *via* cytochrome P450-3A4 (CYP3A4) and has 6 wk metabolites. Similar to sirolimus, it is a substrate of p-glycoprotein (PgP) and CYP3A4 pathways. It interacts with strong and moderate inhibitors, inducers and substrates of CYP3A4 and PgP at different intensities[9,10]. CsA increases the maximum concentration of EVR by 82%, EVR however

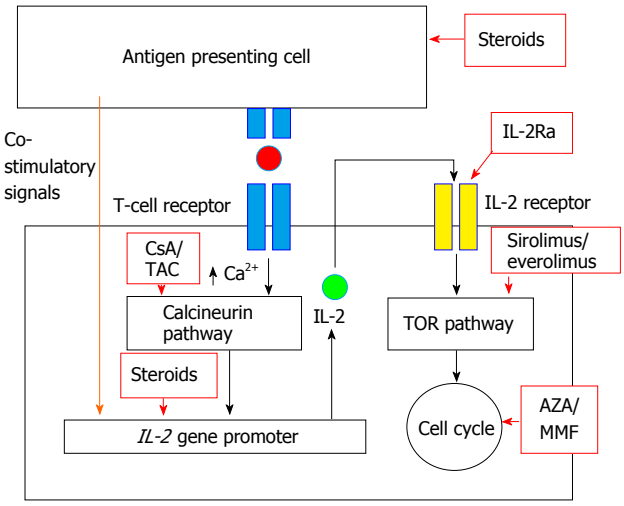

Figure 1 Mechanism of action of efficacy of everolimus and other immunosuppressants in solid organ transplantation (permission from Moini *et al*[2], *World J Hepatol* 2015). AZA: Azathioprine; CsA: Cyclosporine; IL-2: Interleukin-2; IL-2Ra: Interleukin-2 receptor antagonist; MMF: Mycophenolate mofetil; TAC: Tacrolimus; TOR: Target of rapamycin.

does not influence trough level nor drug exposure (area under the curve, AUC) of CsA[8]. EVR is excreted mainly (80%) *via* feces and only 5% in urine[7,8]. There is no dose adjustment required in renal impairment but dose reduction is recommended for moderate and severe liver impairment. As EVR has a narrow therapeutic index and immunogenicity varies post LT, therapeutic monitoring is essential for dose titration and monitoring. The EVR trough level (C_0) correlates well (correlation coefficient of 0.86-0.94) with drug exposure, *i.e.*, AUC, and has been recommended as the standard for EVR monitoring[7,11].

Key studies on the use of EVR in LT (Tables 1 and 2)

Several studies, both prospective and retrospective, on EVR in LT have been reported. In a phase II study, Levy *et al*[12] compared different dosing regimen of EVR (0.5 mg BD, 1 mg BD and 2 mg BD) to placebo. The study concluded that EVR in combination with CsA could be a safe and tolerable alternative in LT, despite the increased incidence of adverse effects. There are 3 main phase Ⅲ studies in the use of EVR in LT, *i.e.,* PROTECT, H2304 and RESCUE studies (Table 1). PROTECT, an open-label multi-center prospective randomised controlled trial (RCT) recruited 203 patients randomised to EVR plus withdrawal of CNI by month 4 post-LT *vs* continued standard CNI till month 11[13]. Steroid was optional in either group. The study concluded significant improvement in renal function (estimated glomerular filtration rate, *i.e.*, eGFR improved by 7.8 mL/min) in the group with EVR, despite similar mortality rates, biopsy-proven rejection (BPAR) rates and efficacy failure rates between both groups. However, it also reported a significantly higher incidence of adverse effects mainly oral herpes, leukopenia, hypercholesterolemia, hyperlipidemia and proteinuria in the EVR-treated

Table 1 Outcomes of everolimus-based immunosuppressant for *de-novo* liver transplantation recipients in prospective randomised controlled trial

Ref.	Treatment group	Time (d) from transplant EVR was initiated	Key inclusion and exclusion criteria	*n*	Follow-up period (mo)	Efficacy	Mean improvement in eGFR (mL/min per 1.73 m^2)	Safety
Fischer *et al*[13] 2012 (PROTECT Study)	EVR + eliminate CNI by month 4 (EVR C_0 5-12 ng/mL, if with CsA, EVR C_0 8-12 ng/mL)	from day 30 and by day 56	Inclusion: No rejection 2 wk before study, renal function > 50 mL/min	101	12	BPAR, graft loss or death: 20.8% *vs* 20.4% (P = 1.0)	7.8 (P = 0.021)	No HAT, no increased risk of delayed wound healing. Higher incidence of infections, leukopenia, hyperlipidemia, anemia, proteinuria and arterial hypertension in the EVR group
	Control: FK or CsA		Exclusion: Severe systemic infections, total cholesterol ≥ 9 mmo/L, TG > 8.5 mmol/L, significant renal dysfunction (eGFR < 50 mL/min)	102				
Sterneck *et al*[14] 2014 (PROTECT Study, extended to 36 mo)	Same as above	From day 30 and by day 56		41	36	BPAR, graft loss and death: 19.5% *vs* 2.5% (P = 0.029) at month 11 (baseline)	9.4 (P = 0.053)	Peripheral edema and back pain were significantly higher in EVR group
				40		BPAR, graft loss and death: 4.9% *vs* 5.0% (P = 1.0) at month 36		
Sterneck *et al*[15] 2016 (PROTECT Study, extended to 59 mo)	Same as above	From day 30 and by day 56		41	59	BPAR, graft loss and death: 9.8% *vs* 7.5% (P = 1.0) from month 11 to month 59	11.4 (P = 0.021)	Peripheral edema and back pain were significantly higher in EVR group
				40				
De Simone *et al*[16] 2012 (H2304 Study)	EVR + low FK (EVR C_0 3-8 ng/mL and FK C_0 3-5 ng/mL)	Day 30	Inclusion: eGFR ≥ 30 mL/min, FK trough ≥ 8 ng/mL.	245	12	BPAR, graft loss or death: 6.5% in EVR group *vs* 9.5% in control group (P < 0.001)	8.5 (P < 0.001)	Higher incidence of proteinuria, acute renal failure, hyperlipidemia, neutropenia, peripheral edema, stomatitis/mouth ulceration, and thrombocytopenia in the EVR group
	FK elimination (EVR C_0 3-8 ng/mL till month 4 then 6-10 ng/mL thereafter and FK elimination started at month 4 when EVR C_0 6-10 ng/mL achieved		Patent hepatic artery and veins, absence of rejection	231				
	Control: FK (C_0 8-12 ng/mL until month 4 and C_0 6-10 ng/mL thereafter)		Exclusion: HCC not fulfill Milan criteria, receipt of antibody induction therapy proteinuria ≥ 1 g/24 h	243				
Saliba *et al*[17] 2013 (H2304 Study, extended to 24 mo)	EVR + low FK (EVR C_0 3-8 ng/mL and FK C_0 3-5 ng/mL)	Day 30		245 243	24	BPAR, graft loss or death: 10.3% in EVR group *vs* 12.5% in control group (P = 0.452)	6.7 (P = 0.002)	No increased risk of wound healing. Higher incidence of proteinuria, acute renal failure, hyperlipidemia, neutropenia, peripheral edema, stomatitis/mouth ulceration, and thrombocytopenia in the EVR group
Fischer *et al*[18] 2015 (H2304 Study, extended to 36 mo)	Same as above	Day 30		106 125	36	BPAR, graft loss and death: 11.5% *vs* 14.6% (P = 0.334)	8.5 (P = 0.005)	Higher drop-out rate due to ADR and incidence of hyperlipidemia in EVR group

ADR: Adverse drug reaction; BPAR: Biopsy proven acute rejection; C_0: Trough level; CNI: Calcineurin inhibitor; CsA: Cyclosporine; EVR: Everolimus; FK: Tacrolimus; eGFR: Based on Modification of Diet in Renal Disease (MDRD) 4.

Table 2 Outcomes of everolimus-based immunosuppressant as maintenance for lt recipients in prospective RCT

Ref.	Treatment group	Time (mo) from transplant surgery EVR was initiated	Key inclusion and exclusion criteria	*n*	Follow-up period (mo)	Efficacy	Mean improvement in CrCl (mL/min)	Safety
De Simone *et al*[19] 2009 (RESCUE Study)	EVR with CNI reduction or elimination (EVR C_0 3-8 ng/mL, FK C_0 3-5 ng/mL or EVR C_0 6-12 ng/mL with FK elimination Control: Standard exposure of FK or CsA	12 to 60 mo	Inclusion: CrCl ⩽ 60 mL/min and ⩾ 20 mL/min Exclusion: Renal dysfunction not due to CNI toxicity, proteinuria ⩾ 1 g/24 h, acute rejection < 6 mo, hepatitis C infection need active antiviral therapy	72 73	12	BPAR, graft loss or death: 8.3% in EVR group *vs* 4.1% in control group	-1.1 (P = 0.463) at month 6	Higher incidence of hyperlipidemia, mouth ulceration, increased hepatitis C virus viral titer, dry skin, eczema, and rash in the EVR group

BPAR: Biopsy proven acute rejection; C_0: Trough level; CNI: Calcineurin inhibitor; CrCl: Creatinine clearance (based on Cockcroft-Gault formula); CsA: Cyclosporine; EVR: Everolimus; FK: Tacrolimus.

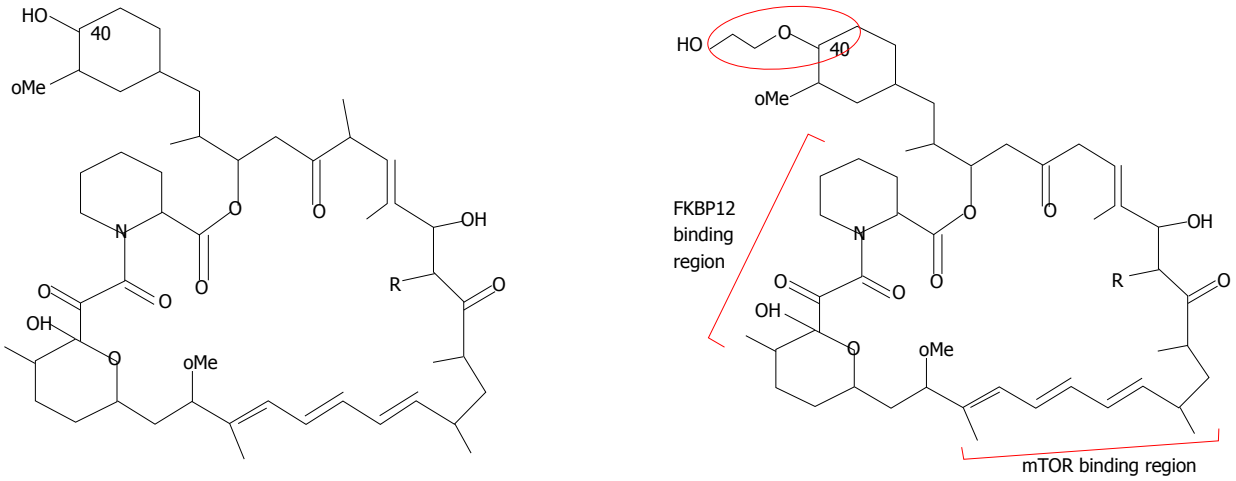

Figure 2 Molecular structure of sirolimus and everolimus.

group as compared to standard CNI. In its subsequent study, 81 patients were further followed-up till 3 years. A significant difference in renal function continued to be seen between the EVR with CNI-withdrawal *vs* the control group at month 35 from randomization, mainly due to the progressive deterioration of renal function in the standard CNI group[14]. Recently, the 5-year follow-up on these same 81 patients has been published, reporting a continued improved trend in renal function in the EVR-treated group (eGFR improved by 11.4 mL/min, P = 0.021) with comparable treatment failure rates (9.8% in EVR group *vs* 7.5% in standard CNI group, P = 1.000) in both groups[15].

In another open-label multi-center prospective RCT, H2304, 719 patients were randomised to receive EVR (EVR C_0 3-8 ng/mL) with reduced FK dosing (FK C_0 3-5 ng/mL) (n = 245) or control standard FK dosing (FK C_0 8-12 ng/mL till month 4 then C_0 6-10 ng/mL thereafter) (n = 243) or FK elimination (EVR C_0 3-8 ng/mL till month 4 then 6-10 ng/mL thereafter, FK elimination from month 4 when EVR C_0 6-10 ng/mL achieved) (n = 231) at 1 mo post liver transplant[16]. Steroid was initiated at time of transplant up till at least 6 mo from transplant while MMF was discontinued at the time of randomization. Recruitment to FK elimination group was terminated prematurely due to higher (19.5%) treated BPAR (tBPAR) episodes as compared to 6.5% and 9.5% of tBPAR in the EVR with reduced FK and control group, which clustered around the time of FK elimination at 4 mo post-randomization. At the end of both the first and second year, subjects in the EVR with reduced FK group had improved renal function significantly with comparable primary efficacy (tBPAR, graft loss and death) but a higher incidence of adverse effects (mainly hyperlipidemia, neutropenia, peripheral edema and stomatitis/mouth ulceration) than controls[16,17]. At the end of the third year, improvement in renal function was consistently significant in EVR with reduced FK group (n = 106) with comparable tBPAR rates and adverse effects as compared to the standard FK group (n = 125)[18].

The third Phase Ⅲ study of interest, RESCUE, provides evidence for converting to EVR 1 year post-LT (Table 2)[19]. In this 6 mo open-label multi-center

prospective RCT, 154 patient were followed-up for 12 mo. The studied group, EVR with CNI reduction or elimination was compared to standard CNI with or without MMF, azathioprine or steroid in both groups. While all concurrent immunosuppressants were kept the same in control group, MMF was discontinued at day 1 in the EVR group. Despite no graft loss, BPAR in EVR group at month 12 (4.2%) were higher than standard CNI group (1.4%). Furthermore, the improvement of renal function in the studied group was not statistically significant at 12-mo follow up.

EFFICACY OF EVR IN LT

De novo therapy

In the PROTECT study, the efficacy of EVR in LT was still doubtful with conflicting results. Initial results reported comparable composite BPAR, graft loss and death rates in the EVR-treated (20.8%) and the control (20.4%) group, up to month 11 of follow-up[13]. A similar comparable trend for its composite end-points in the extension study (from month 11 to month 35) results were reported at the end of 35 mo, despite a difference at baseline between both groups[14]. Treatment efficacy with EVR was difficult to analyse due to high discontinuation rates of drugs used in both groups due to adverse drug reactions (49.5% in EVR group and 38.2% in control CNI group). The discontinuation of CNI by end of month 4 could have compromised efficacy of immunosuppressive therapy. This similar finding was reported in the H2304 Study where efficacy failure (BPAR) in the FK elimination group was significantly higher (19.9%) as compared to control group (10.7%), P = 0.005. Hence, EVR monotherapy is not recommended in LT and EVR should instead be used in combination with CNI. EVR efficacy in *de novo* LT, and hence, United States FDA and Europe EMEA approval is based on results from De Simone's landmark H2304 study (Table 1)[16]. The reported outcomes of BAPR, graft loss and death in the treatment group were non-inferior to the control group on FK alone. In the post-hoc analysis for H2304 study, incidence of tBPAR was lower in those aged < 60 years and hepatitis C virus (HCV)-negative[20].

Based on phase Ⅲ studies (Table 1), EVR is approved for use 30 d from LT. However, there is emerging data on the safety and efficacy of EVR initiation within 30 d from LT. Masetti *et al*[21]'s prospective, single-center randomized trial described early initiation of EVR at day 10 from LT in 52 patients (EVR C_0 6-10 ng/mL till day 30, then C_0 8-12 ng/mL (when CsA was discontinued from day 30) till month 6 and then C_0 6-10 ng/mL thereafter) *vs* standard CsA in 26 patients (CsA C_0 225 ± 25 ng/mL till day 30, 200 ± 25 ng/mL till month 6 and 150 ± 25 ng/mL thereafter)[21]. There was no difference in BPAR nor patient survival rates in both groups. The study concluded that early withdrawal of CsA and early EVR use in *de novo* LT recipients significantly improved renal function (eGFR 87.7 mL/min in EVR group *vs* 59.9 mL/min in standard CsA group) and reduced incidence of chronic kidney disease (CKD) stage ⩾ 3 (15.4% in EVR group *vs* 52.2% in CsA group, P = 0.005) at 1 year post-LT.

In a single-center prospective cohort study, safety of EVR use in the early post-LT was evaluated in 43 living donor LT recipients[22]. All patients received basiliximab, steroid, FK and MMF as immunosuppressive therapy where steroid was discontinued after 2 wk from transplant and FK was maintained at C_0 of 8-10 ng/mL. EVR was introduced from low dose of 0.25 mg BD and titrated to 0.5 mg BD to achieve C_0 of 3-5 ng/mL while FK was kept at C_0 of 6-8 ng/mL. Mean time for EVR initiation was 12 ± 8 d (range: 4-20 d) from transplant where 33 patients were initiated within the 1st week, 9 patients within the 2nd week and 1 patient on day 20. EVR was continued for an average of 97 d (range: 26-190 d) from transplant. The mean follow up was 9 ± 6 mo (range: 3-15 mo) till discontinuation of EVR or death. No acute rejection episodes were reported.

In a retrospective study, Gastaca *et al*[23] reported 92.7% patient survival rates at 1 year post-LT for 28 patients who had EVR initiated early post-LT (median 14 d) where 85.7% was in combination with MMF or enteric-coated mycophenolate sodium and steroid. Nonetheless, more concrete data is warranted for EVR initiation within 30 d from LT.

Maintenance therapy

The efficacy data for EVR as maintenance immunosuppression in LT is sparse; with only one RCT to date (Table 2). De Simone *et al*[19]'s RCT reported results of conversion from CNI-based to EVR-based maintenance immunosuppression after 12 mo and up till 60 mo post-LT. Although the composite endpoint of BPAR, graft loss and death was low overall in both groups, it was double in the EVR group (8.3%) as compared to control group (4.1%)[19].

In another prospective cohort study by De Simone *et al*[24], 40 patients were converted to EVR at mean of 45.5 ± 31.2 mo from transplant and CNI was tapered by 50% every week and withdrawn over 4 wk with or without MMF or azathioprine and steroid. Concurrent MMF or azathioprine was discontinued at day 1 of conversion while steroid was remained unchanged in the EVR group. Indications for conversion to EVR included deterioration of renal function (90.0%), CNI-associated peripheral neuropathy (7.5%) and CNI-associated microangiopathy (2.5%). Despite a 100% patient and graft survival rate at 12 mo post-conversion, the incidence of BPAR was 15% and 4 of the patients (10%) had to be switched back to CNI for this reason.

Castroagudín *et al*[25] analysed impact on renal function post conversion to EVR at mean 62.4 ± 36.6 mo from LT in 21 patients with CKD. Twenty patients (95%) were able to have CNI completely withdrawn. From a baseline eGFR of 42.1 ± 8.7 mL/min, renal function improved to eGFR 49.8 ± 10.3 mL/min at the end of 360 d from conversion.

In a retrospective study, Saliba *et al*[26] described 240 patients who were successfully converted to EVR at median of 3 years from transplant with a low overall rejection rate (1.6%). At 12 mo post conversion, 61% of patients had CNI discontinued. Mean EVR C_0 was 7.3 ng/mL and 8.1 ng/mL at month 1 and 12 post conversion respectively while mean EVR C_0 was higher (8.8 ng/mL) in 40 patients who were kept on EVR monotherapy at month 12. Immunosuppression therapy was in combination with or without MMF and steroid in both groups. Renal function was markedly improved in patients who were converted within the first year from transplant (n = 68) as compared to conversion after 1 year from transplant (n = 172), calculated creatinine clearance 12.5 mL/min *vs* 5.5 mL/min based on Cockcroft-Gault formula.

In another retrospective study, 477 patients were recruited and 157 (33%) were converted to EVR for indication of renal dysfunction at median of 24 mo[27]. Significant improvement of renal function was observed in patients who were converted to EVR within 1 year from transplant but not in patients who were converted after 5 years from transplant. Of note, in patients who were converted in between 1-5 years from transplant, the improvement in renal function was only appreciable at month 3 and 6 but did not persist at 12 mo post conversion. Overall graft rejection rate was 5.9% which mostly occurred at 3 and 6 mo post conversion.

VALUE OF EVR IN LT

Renoprotective effect

Long-term renoprotective benefits of EVR in LT have been demonstrated in the H2304 study[16-18]. Based on the results, De Simone *et al*[16] showed that EVR with reduced FK dose is as efficacious as the FK standard regimen in the control group and improved patients' eGFR by 8.5 mL/min at month 12. The improvement trend in eGFR continued to be seen at month 24 (eGFR improved by 6.7 mL/min) and at month 36 (eGFR improved by 8.5 mL/min) of follow-up (Table 1). However, it can also be argued that the H2304 study had unintentionally recruited a majority of patients (72.3%) with better baseline renal functions of eGFR $\geqslant$ 60 mL/min, with mean baseline of 80.8 mL/min in EVR group and 78.9 mL/min in control group. Similar high baseline eGFR (78.0 mL/min and 74.9 mL/min in EVR and control groups respectively) were also seen in the PROTECT study. In the RESCUE study, baseline eGFR was 51.0 mL/min and 50.3 mL/min in EVR and control groups. Clearly, results in these studies should not be generalised to LT recipients with eGFR < 50 mL/min, where similar benefits might be doubtful. This was reaffirmed with the H2304 *post-hoc* analysis which showed that renal improvement was not observed in patients with eGFR of 30 to < 55 mL/min[20]. This analysis suggested that EVR renoprotective effect was observed particularly in patients aged < 60 years, female gender, HCV-negative and in those with baseline eGFR of 55 to < 70 mL/min.

The FK dose in the control group of the H2304 study was maintained at target C_0 of 8-12 ng/mL until month 4 and then tapered to target C_0 of 6–10 ng/mL for the remainder of the study. The FK C_0 in EVR group was targeted to be 3-5 ng/mL from 1 mo post-LT, though majority of patients maintained levels slightly above 5 ng/mL from month 3 onwards till month 12 of study period. Hence, the addition of EVR early post-LT allowed tapering of FK safely without an increased risk of rejection. The decrease in renal impairment was possibly contributed by the reduced CNI level. It has been proven that CNI minimization strategy improves renal function in LT recipients[28-30].

Before EVR was started in the H2304 study, majority (70%) of patients were also on mycophenolate mofetil (MMF) which was discontinued according to protocol. It would seem logical in clinical practice to have another non-CNI immunosuppressant, in combination with reduced CNI doses. In fact, the combination of MMF with reduced CNI has been a strategy which many clinicians adopt to minimize CNI nephrotoxicity[29,30]. A case-control study described 20 patients on *de novo* EVR plus MMF and steroids without CNI in comparison to 31 controls of FK plus MMF and steroids[31]. The eGFR in both groups were not statistically different at the end of 1- and 2-years follow-up but a 35% of rejection rate at 2 years from LT in EVR group was reported and attributed to difficulty achieving target drug levels. There is no head-to-head RCT comparing EVR-based and MMF-based LT immunosuppression regimes to date.

On the other hand, the evidence for EVR reno-protective effect in conversion after 6 mo post-LT is lacking. The RESCUE Study which showed an increased composite outcome of BPAR, graft loss and death demonstrated an improvement of eGFR by only 1.1 mL/min at the end of 1 year. In this study, the conversion to EVR occurred at mean 3.3 ± 1.7 years from transplant. In a retrospective observational study, Saliba *et al*[26] found the improvement in renal function to be greater when conversion to EVR was within first year post-LT (eGFR increased from 77.5 to 90.0 mL/min, P = 0.04) *vs* those who were converted beyond 1 year post-LT (eGFR increased from 59.1 to 64.6 mL/min, P = 0.01)[26]. The findings Castroagudín *et al*[25] reported in a retrospective study echoed the less remarkable renal improvement (eGFR improved by 7.7 mL/min at month 12) when conversion to EVR occurred at 5.2 ± 3.1 years from transplant.

Thus, the best time point for conversion to EVR for optimal renoprotective effect is still unclear and further studies are warranted. Although it would appear, from current available data, that earlier conversion (within 12 mo post-LT) is better than late conversion (beyond 3 years post-LT); and that renal protective effects are more prominent with mild renal impairment (eGFR > 60 mL/min) rather than with moderate-severe renal impairment (eGFR < 55 mL/min).

Alternative for CNI-induced neurotoxicity

Bilbao *et al*[32] reported a retrospective analysis on the use of EVR in 10 patients who experienced FK-neurotoxicity requiring the discontinuation of FK in the first 3 mo post-LT. Seven of the patients were converted to everolimus in the first month post-LT and the remaining 3 were converted in the second or third month.

However, within 80 d post conversion, graft rejection occurred in 4 of the 10 patients, all of whom were on triple immunosuppression (*i.e.*, EVR plus MMF plus steroids) at the time of graft rejection. All 4 patients subsequently had CNI (3 with FK, 1 with CsA) re-introduced, without recurrence of neurotoxicity. The findings suggest EVR use enables a temporary withdrawal of CNI in managing CNI-induced neurotoxicity. Re-introduction of CNI may be prudent after resolution of neurotoxicity in view of high rejection rates, especially within first 3 mo post-transplant, when EVR is not used in combination with CNI. Furthermore, in patients with acute rejection, the introduction of CsA or re-introduction of FK may be possible because the risk of further neurologic complications may be low.

Prevention of hepatocellular carcinoma recurrence

EVR has proven efficacy against breast cancer, renal cell carcinoma, neuroendocrine tumours and subependymal giant cell astrocytoma[33]. There is no data on *de novo* hepatocellular carcinoma (HCC). The HCC recurrence rate post-LT is 8%-20%, with most occurring within the first 2 years post-LT[34,35]. There is no RCT on EVR for the prevention of HCC recurrence. In Jeng *et al*[22]'s single-centre prospective non-randomised study, HCC recurred in 7% of the patients using EVR. In retrospective studies, it has been observed that EVR has no HCC recurrence post-LT during a mean follow-up of 11.2 ± 6.8 mo in 44 patients and 48 mo (range: 11-76 mo) in 21 patients respectively[26,36]. In a systematic review, LT recipients who were on mTOR inhibitors (sirolimus or EVR) had lower HCC recurrence rates[37]. However, the follow up period varied widely among the groups on CNI (42 mo), sirolimus (30 mo) and EVR (19 mo). No mortality data was presented in this review.

Hepatitis C and liver fibrosis

In an open-label multi-center randomised study, conversion to EVR delayed histological fibrosis progression in 43 LT recipients with HCV recurrence as compared to FK-based immunosuppressive therapy[38]. However, this potential benefit was not observed in the extended H2304 study, where no significant difference in histological fibrosis scores between the EVR and control groups was reported at the end of 3 years of follow-up[16]. Hence, more studies are warranted to confirm EVR benefit in delaying liver fibrosis progression of hepatitis C.

ADVERSE EFFECTS OF EVR

The most common adverse effects of EVR use in LT recipients are infections (50.6%), hyperlipidaemia (23.7%), hypertension (18.0%), peripheral oedema (17.6%), leukopenia (14.3%), and wound healing impairment (11.0%)[8,9]. In a phase Ⅱ study, the incidence of adverse effects was higher in patients with higher daily EVR doses, especially > 4 mg/d[11]. In *de novo* LT, the discontinuation of EVR due to adverse effects was higher in the EVR group, 25.7%, which was nearly double of the control (14.1%) in this study[16]. The common adverse effects that led to EVR discontinuation were proteinuria, delayed wound healing, pancytopenia, leukopenia and thrombocytopenia.

In maintenance therapy, 22% of patients discontinued EVR due to adverse effects while no patients in the control group discontinued study medication[18]. The adverse effects that led to EVR discontinuation included leukopenia, proteinuria, thrombotic microangiopathy, elevation in hepatic enzymes, increased HCV viral load, hypertriglyceridemia, renal impairment, interstitial lung disease, pneumonitis, pulmonary fibrosis and stomatitis. In a study on 94 patients converting to EVR at mean of 5 years from transplant, as many as 70% of patients experienced adverse reaction and 16% required EVR to be discontinued despite mean EVR C_0 level being at only 6 ng/mL[39].

Hepatic artery thrombosis

In February 2013, the United States Food and Drug Administration included a warning of hepatic artery thrombosis (HAT) in the EVR product insert. Most of the reported cases of HAT in the presence of mTOR inhibition occurred within the first 30 d from transplant surgery, leading to graft loss or death. Therefore, in most EVR studies, randomization was started only 30 d after transplant surgery.

One case of HAT was reported in the H2304 study, occurring in a subject who had a prior history of HAT before randomization. There were no reports of HAT in the PROTECT nor RESCUE studies. In the RESCUE study, although an adverse effect of thrombotic microangiopathy was reported, there were no specific details of its incidence nor eventual outcomes. Combination of EVR and CNI has also been reported to increase the risk of thrombotic microangiopathy elsewhere[40].

In Masetti *et al*[21]'s study on the early use of EVR within the first 30 d of LT, no HAT was reported with EVR use. This is in contrast to the control CsA group which had 2 (7.6%) patients with HAT and 2 (7.6%) patient with hepatic artery stenosis. Although there was a significant higher rate of hepatic stenosis and thrombosis in CsA group, it is important to note number of patients in CsA group (n = 26) was just half of patients in EVR group (n = 52).

In another prospective cohort study, no HAT was reported with EVR use in 43 patients in the early (33 patients within week 1, 9 patients within week 2 and 1 at day 20 from transplant) post-LT period[22]. Similarly, in a retrospective study, no HAT was observed in 28

patients when EVR was initiated at median of 14 (range 4-24) d[23].

Impaired wound healing

Wound healing is an important care issue for post-transplant surgery. Sirolimus has as high as 36% incidence rate of impaired wound healing[41]. Furthermore, it was reported that mTOR inhibitor is an independent risk factor for incisional hernia in LT[42]. Impairment of wound healing between EVR and control group was presented in the PROTECT study (3.0% *vs* 4.9% at 11 mo) and H2304 study (11.0% *vs* 7.9% at 1 year, RR = 1.40, 95%CI: 0.80, 2.45; 11.0% *vs* 8.3% at 2 year, P = 0.36)[13,16,17]. Both studies also reported an increase of incisional hernia with EVR exposure, although the difference did not reach statistical significance in either study[14,17]. Similarly, Masetti *et al*[21] reported a non-significant increase in incisional hernia in the EVR group. The findings in these studies were similar to the not statistically significant increase of wound healing impairment in renal transplant patients using EVR as compared to standard CNI reported by Nashan *et al*[43].

Generally, impaired wound healing rates with EVR use ranges from 11%-35%[41]. Despite the lack of statistical significance, further analyses or studies to guide the optimal time for initiation of EVR in LT are warranted, especially when EVR is used in combination with other immunosuppressants that may delay healing process as well.

Infection

The risk of infection is of concern in post-transplant care and the higher incidence of infection with EVR should not be overlooked. In the H2304 study, the overall incidence of any infection at 1 year was not statistically significant[16]. However, there was an increase for any serious infection (13.9% in EVR group *vs* 7.9% in control, RR = 1.76, 95%CI: 1.03, 3.00) which included pneumonia and hepatitis C. The overall incidence of any infection was also comparable between EVR and control groups in the 2-year and 3-year follow up (56.3% *vs* 51.7% and 70.8% *vs* 64.0% respectively) period, without a significant difference in the rates of serious infections.

In the PROTECT study, Kaplan-Meier survival plot showed the occurrence of any infection was higher in the EVR group as compared to standard CNI group (79.5% *vs* 68.3%, P = 0.050) at 11 mo from randomization particularly oral herpes, sinusitis and wound infection[13]. In the RESCUE study, 31.9% of patients in EVR group *vs* 21.9% in standard CNI group experienced infections which included stomatitis, herpes simplex, bronchitis and urinary tract infections[19]. Of note, it also reported a significant increase in HCV viral load in their EVR group (6.9%, P = 0.028) as compared to none in the control group. Although statistical difference was unknown, the authors also reported 15.3% (EVR group) in contrast to 1.4% (standard CNI group) of infections being related to studied drug.

Incidence of infection was the same in both EVR and control groups, 46.2% in Masetti's study[21]. In a retrospective observational study, infection was 60.7% with *de novo* EVR use in 28 patients. On the other hand, only 1 case of infection was reported in a single-center prospective study[22]. There was no clear definition of infection and the disparity could possibly be due to different definition among various studies.

Stomatitis

Stomatitis incidence was significantly higher (10.6%, 26.4%) in EVR group as compared to standard CNI group (1.2%, 0%) at 1-year and 2 year follow up of the H2304 (P < 0.001) and RESCUE (P < 0.010) studies[17,19]. Stomatitis has also been reported as one of the common adverse effects when EVR was used as maintenance immunosuppressive therapy[22,24]. Management strategies for stomatitis include the use of local anesthetic, intralesional and topical steroid to control stomatitis and reduce pain[41,44,45]. EVR has also been used as an alternative for renal transplant recipients who experienced sirolimus-induced stomatitis[46].

In general, mTOR inhibitor-associated stomatitis is generally not severe (< 5% is Grade 3 or 4)[41]. However, if nutrition status is compromised due to poor oral intake secondary to stomatitis, dose reduction or even withdrawal may be warranted.

Peripheral edema

mTOR inhibitor adverse effect of peripheral edema may be related to its anti-lymphangiogenetic effect, leading to lymphedema and capillary leak which may not be reversible[41]. In all 3 main phase Ⅲ studies, peripheral edema was reported to be significantly higher in EVR group in comparison to control group. In the PROTECT study, peripheral edema was consistently higher in EVR group (26.8%) *vs* in standard CNI group (12.5%), P = 0.162 at month 11[13]. The incidence of peripheral edema continued to increase in the extension study period from month 11-35 (22% in EVR group *vs* 5% in standard CNI group, P = 0.048) and from month 11-59 (31.7% in EVR group *vs* 7.5% in standard CNI group, P = 0.011)[14,15]. In H2304 study, 17.6% and 22.4% in EVR group as compared to 10.8% and 14.9% in the standard CNI group experienced peripheral edema at 1-year (RR = 1.63, 95%CI: 1.03, 2.56) and at 2-year (P = 0.036) respectively[16,17]. Similar trend was observed in the RESCUE study, with the incidence of peripheral oedema 5.6% in EVR group and 1.4% in the standard CNI group[19]. Nonetheless, peripheral edema was not reported as one of the adverse effects that led to drug discontinuation in all above studies.

Proteinuria

It is unclear how mTOR inhibitors influence glomerulus permeability and cause proteinuria[41]. Nonetheless, as proteinuria is an indicator of kidney injury and strong predicator for cardiovascular events, this adverse effect

Table 3 Recommendation for everolimus use in liver transplantation recipients

Indication and regimen	Renoprotective benefit
	EVR in combination with CNI to allow CNI dose reduction
	Management of CNI neurotoxicity
	EVR allows temporary withdrawal of CNI till resolution of neurotoxicity
Patients	LT recipients with renal function > 60 mL/min
	LT recipients proteinuria < 1 g/24 h
Timing	*De novo* therapy: Initiate EVR at 1 mo from transplant
	Maintenance therapy: Introduce EVR within 1 yr from transplant
	CNI neurotoxicity: Stop CNI and initiate EVR immediately

CNI: Calcineurin inhibitor; EVR: Everolimus; LT: Liver transplantation.

warrants clinical attention. Patients with proteinuria (≥ 1 g/24 h) were excluded in H2304 and RESCUE study[15,18]. At the end of 1 year of H2304 study, 2.9% in EVR group developed proteinuria as compared to 0.4% in standard CNI group (RR = 6.89, 95%CI: 0.85, 55.54)[16]. In the subsequent follow up year, proteinuria was the most frequent adverse event that resulting in discontinuation of EVR (3.3%) in contrast to standard CNI group (0.4%)[17]. In the RESCUE study, 2 out of 16 patients required discontinuation of EVR due to proteinuria, while no patients required drug discontinuation in the standard CNI group for the same reason[19]. Similarly, incidence of proteinuria was significantly higher in EVR group (9.9%) as compared to the standard CNI group (2.0%) at month 11 in the PROTECT study[13]. A similar trend in their extension study up to month 59 was seen, albeit without statistical difference[15].

The characteristic and long-term outcomes of patients experiencing proteinuria, which could possibly guide patient selection and risk-benefit consideration to use EVR in LT, are lacking.

Hyperlipidemia

Hyperlipidemia is one of the most common adverse effects of mTOR inhibitors[41]. From phase Ⅱ studies, a trend of dose-dependent hyperlipidemia was observed[11]. Masetti *et al*[21] reported significant increase in the incidence of hyperlipidemia but not hypertriglyceridemia with EVR use. In H2304, 23.3% in EVR group *vs* 17.8% in standard CNI group required lipid-lowing therapy (P = 0.944) at the end of 1 year and the incidence of hyperlipidemia was significantly higher (26.9% in EVR group *vs* 11.6% in standard CNI, P < 0.001) at the end of 2 years[16,17]. In the PROTECT study, EVR use was associated with an increased incidence of hyperlipidemia as compared to controls (11.9% *vs* 2.0%, P < 0.05) at month 11[13].

Although cardiovascular risk in LT is lower than renal and cardiac transplant, cardiovascular disease is still one of the leading causes of morbidity[47]. Undoubtedly, there is a range of effective lipid-lowing therapy in managing hyperlipidemia, and it is prudent to always weigh cardiovascular risks over the benefits before initiation or conversion to EVR.

RECOMMENDATION

A working group has recently consolidated recommendations for EVR use in LT based on consensus and experiences[48]. It provides some guidance while more outcome data is warranted to establish a comprehensive guideline for EVR use in LT. Based on current available data discussed in this review, EVR is an appropriate immunosuppressant for LT recipients as listed in Table 3.

The increased risk of adverse effects could offset the benefit of EVR particularly in preserving renal function. Although it has been mentioned that dose reduction was exercised in managing EVR adverse effects, but there were no details on the methods or outcomes[24,41,49]. Patient selection and strategies to reduce and minimise adverse effects will be key in determining the success of EVR use in LT.

CONCLUSION

EVR could be a viable alternative immunosuppressant in LT recipients who are at risk of renal impairment. Initiating EVR early (from 30 d post-LT and before eGFR < 55 mL/min) post-transplant allows CNI reduction and thus reduces CNI nephrotoxicity. Future research to strengthen EVR initiation, switch, or combination strategies and cost-effectiveness analyses would be important.

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Prospective Study

MicroRNAs and clinical implications in hepatocellular carcinoma

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Abstract

AIM

To assess the role of some circulating miRNAs (miR-23a, miR-203, miR338, miR-34, and miR-16) as tumor markers for diagnosis of hepatocellular carcinoma (HCC).

METHODS

One hundred and seventy-one subjects were enrolled, 57 patients with HCC, 57 patients with liver cirrhosis (LC) and 57 healthy subjects as control group. Severity of liver disease was assessed by Child Pugh score. Tumor staging was done using Okuda staging system. Quantification of Micro RNA (miR-23a, miR-203, miR338, miR-34, and miR-16) was performed.

RESULTS

All studied miRNA showed significant difference between HCC and cirrhotic patients in comparison to

healthy control. miR-23a showed statistically significant difference between HCC and cirrhotic patients being higher in HCC group than cirrhotic. miR-23a is significantly higher in HCC patients with focal lesion size equal or more than 5 cm, patients with multiple focal lesions and Okuda stage Ⅲ. At cutoff value $\geqslant 2^{10}$, miR-23a showed accuracy 79.3% to diagnose HCC patients with sensitivity 89.47% and specificity about 64.91%. At cut off level $\geqslant$ 200 ng/mL, serum alpha fetoprotein had 73.68% sensitivity, 52.63% specificity, 43.75% PPV, 80% NPV for diagnosis of HCC.

CONCLUSION

MicroRNA 23a can be used as a screening test for early detection of HCC. Also, it is related to larger size of tumour, late Okuda staging and multiple hepatic focal lesions, so it might be a prognostic biomarker.

Key words: Hepatocellular carcinoma; MicroRNA; Liver cirrhosis; MiR-23a

Core tip: MicroRNA is promising as diagnostic and prognostic biomarkers. miR-23a can be used in screening of hepatocellular carcinoma (HCC) and it gives better results than alpha fetoprotein. It is also related to more progressive HCC so it can be used as predictor of prognosis.

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INTRODUCTION

MicroRNAs (miRNA) are small, non-coding RNAs that negatively regulate gene expression at the post-transcriptional level[1]. miRNA is known to regulate the cell cycle, apoptosis and metastasis[2]. Aberrant miRNA expression contributes to tumorigenesis and cancer progression[3]. miRNA is involved in various biological processes that underlie hepatic tumor formation[1]. Murakami *et al*[4] was the first to report that hepatic cmalignancy exhibited an abnormal expression pattern of miRNAs as the dysregulation of miRNA expression has been identified as a common characteristic of liver cancer. Later on, a number of studies have confirmed that miRNAs possess important regulatory roles in hepatocarcinogenesis and malignant transformation[5].

Circulating miRNAs that are released from cancerous tissues are stable and readily available for clinical analysis, and therefore may be useful for the first-line detection of cancer[6]. Studies concerning miRNAs appear to show a novel perspective for cancer diagnosis and treatment[2].

Hepatocellular carcinoma (HCC) is characterized by significant morbidity and high mortality rates worlwide[7]. Because of the difficulty of clinical diagnosis at the early stage, only 30%-40% of cases can undergo curative resection[8]. As there are currently no reliable tumor markers or imaging technologies that can accurately diagnose early HCC, the use of circulating miRNAs as a potential tool for HCC detection has become an emerging area of study[6,9]. Many circulating microRNAs were evaluated in liver diseases including miR-122, miR-21, miR-34a, miR-221, miR-23a, miR-216, miR-155, miR-186, miR-150, miR-130b, and miR-214[10]. Our aim is to detect the possibility of using some circulating miRNAs as tumor markers for diagnosis of HCC namely miR-23a, miR-203, miR-338-3p, miR-34, and miR-16.

MATERIALS AND METHODS

This prospective cross sectional study included 171 subjects divided into 3 groups: Group Ⅰ comprising 57 patients with HCC, Group Ⅱ comprising 57 patients with liver cirrhosis, Group Ⅲ 57 healthy subjects as a control group.

Informed written consent was obtained from all participants prior to enrollment in the study and approved by ethical committee of Faculty of Medicine, Tanta University. Patients with other cancers or metastatic liver cancer were excluded. All patients were submitted to detailed history and clinical assessment. Liver cirrhosis was diagnosed on the basis of history, clinical examination, laboratory findings, and abdominal ultrasonography. Severity of liver disease was assessed by Child Pugh score[11]. HCC was diagnosed by abdominal ultrasonography, abdominal triphasic computed tomography and serum Alpha fetoprotein (AFP). Tumor characteristics were detected including tumor size, focal lesion number, site, and portal vein invasion. Tumor staging was done using Okuda staging system[12].

Fasting venous blood samples (5 mL) were collected by trained laboratory technicians. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total bilirubin levels and creatinine were measured by using SynchronCX4 clinical system. Serumα-fetoprotein levels and viral status (HCV-Ab and HBs Ag) were estimated by serological techniques (Axyam System, Abbott Laboratories). Prothrombin time measurements were performed for all patients; normal time was 12 s [100% concentration and International normalization ratio (INR) of 1]. Complete blood count was done using Automatic blood cell counter model PCE-210N (ERMA INC).

RNA isolation

Total RNA was isolated according to the instructions of the supplier and was further purified using an RN easy

Table 1 Demographic data and Child Pugh scoring of the studied groups *n* (%)

Variable(s)	Group Ⅰ HCC group (*n* = 57)	Group Ⅱ Cirrhotic group (*n* = 57)	Group Ⅲ Control group (*n* = 57)	*P* value
Gender				
Male	37 (64.91)	39 (68.42)	40 (70.18)	0.8289
Female	20 (35.09)	18 (31.58)	17 (29.82)	
Age (mean ± SD) (yr)	55.9 ± 5.194	54.88 ± 9.907	54.3 ± 6.34	0.5087
Child Pugh classification				
A	10 (17.54)	15 (26.32)	-	0.527
B	18 (31.58)	16 (28.07)	-	
C	29 (50.88)	26 (45.61)	-	

HCC: Hepatocellular carcinoma.

Table 2 Imaging characteristics of hepatocellular carcinoma cases

Variables		*n* (%)
Size (mean ± SD) (range) cm	7.61 ± 3.037 (3.2-14)	
Portal veins thrombosis	Yes	10 (17.54)
	No	47 (82.46)
No. of focal lesions	Single	32 (56.14)
	Multiple	25 (43.86)
Site of focal lesions	Right lobe	32 (56.14)
	Left lobe	17 (29.82)
	Both lobes	8 (14.04)
Okuda stage	Ⅰ	7 (12.28)
	Ⅱ	23 (40.35)
	Ⅲ	27 (47.37)

mini kit (Qiagen, Valencia, CA, United States).

Quantitative real-time PCR

Quantification of Micro RNA was performed using Taq Man Gene Expression (Applied BiosystemsInc, Foster City, CA, United States). RNAU6 was used as housekeeping gene (endogenous reference cDNA) for all micro RNA in this study. Fractional threshold cycles (CT) were expressing the initial concentration of target sequence. Relative mRNA quantification was calculated using the arithmetic formula $2)^{-\Delta CT}$, where $^{\Delta}CT$ is the difference between the CT of a given target cDNA and an endogenous reference cDNA. Thus, this value yields the amount of the target normalized to an endogenous reference.

Statistical analysis

The collected data were tabulated and analyzed using SPSS version 23 software (SPSS Inc, Chicago, ILL Company). Categorical data were presented as number and percentages while quantitative data were expressed as mean, standard deviation and median. Comparison of continuous data between two groups was made by using unpaired *t* test for parametric data and Mann-Whitney test for non-parametric data. Comparison of continuous data between more than two groups was made by using one way ANOVA for parametric data and Kruskal-Wallis test for nonparametric data. χ^2 test was used for comparison between categorical data. Receiving operating characteristic (ROC) analysis curves and the corresponding area under the curve were calculated for providing the accuracy of the microRNAs and AFP, in diagnosis of HCC. ROC curve was used for estimation of sensitivity (*i.e.*, true positive rate), specificity (*i.e.*, true negative rate), positive predictive value (PPV), negative predictive value (NPV) and cutoff values showing the best equilibrium between sensitivity and specificity were evaluated. The accepted level of significance in this work was stated at 0.05 ($P < 0.05$ was considered significant).

RESULTS

The demographic data and Child-Pugh scoring of the studied groups are shown in Table 1. Symptoms were elicited by 85.96% of the HCC. Out of the recruited patients, 73.68%, 14.04% and 12.28% were HCV patients, HBV patients and non-HCV non-HBV respectively. Regarding Okuda staging system, 12.28% of HCC patients presented in stage Ⅰ, 40.35% of HCC patients presented in stage Ⅱ and 47.37% of HCC patients presented in stage Ⅲ. Imaging showed that all HCC occurred on top of cirrhosis, ascites was present in 82.46% of the HCC patients and portal vein thrombosis was found in 17.54%. Focal lesions were single in 56.14% of cases, affected the right lobe in 56.14% of cases and their size ranged from 3.2 to 14 cm with a mean of 7.61 ± 3.037 as shown in Table 2. Comparison between all studied groups as regard liver functions tests, and other investigations are shown in Table 3.

Regarding miRNA values, the tested miR-23a, miR-203, miR-338, miR-34, and miR-16 showed a statistically significant difference between patients group Ⅰ and Ⅱ *vs* group Ⅲ. It was found that 23a, 34 and 16 microRNAs were significantly higher in HCC group and cirrhotic group when compared with the control group; but 203 and 338 microRNAs were significantly lower in HCC group and cirrhotic group when compared with the control group. But only miRNA 23a showed statistically significant difference between group Ⅰ and Ⅱ, being higher in the HCC group than the cirrhotic group as shown in Table 4.

When miRNAs were studied according to the focal lesion characteristics, it was found that 23a microRNA

Table 3 Laboratory characteristics among the studied groups

Variable(s)	Group Ⅰ HCC group (n = 57) mean ± SD	Group Ⅱ Cirrhotic group (n = 57) mean ± SD	Group Ⅲ Control group (n = 57) mean ± SD	*P*	*P*1	*P*2	*P*3
ALT (U/L)	60.75 ± 32.63	59.04 ± 68.74	30.18 ± 5.48	< 0.0001	< 0.05	< 0.001	< 0.001
AST (U/L)	86.7 ± 35.1	66.77± 32.07	32.79 ± 7.2	< 0.0001	< 0.01	< 0.001	< 0.001
Total bilirubin (mg/dL)	4.48 ± 4.7	5.2 ± 5.59	0.77 ± 0.18	< 0.0001	> 0.05	< 0.001	< 0.001
Serum albumin (g/dL)	2.54 ± 0.38	2.72 ± 0.53	4.05 ± 0.47	< 0.0001	> 0.05	< 0.001	< 0.001
INR	1.48 ± 0.3	1.54 ± 0.72	0.99 ± 0.07	< 0.0001	> 0.05	< 0.001	< 0.001
Serum α-feto protein (ng/mL)	1418.55 ± 2953.2	41.61 ± 15.78	5.8 ± 1.65	< 0.0001	> 0.05	< 0.001	< 0.001
Serum creatinine (mg/dL)	2.2 ± 1.77	1.64 ± 1.23	0.95 ± 0.16	< 0.0001	> 0.05	< 0.001	< 0.001
Hemoglobin (g/dL)	9.72 ± 1.22	10.02 ± 0.89	12.62 ± 1.1	< 0.0001	> 0.05	< 0.001	< 0.001
Platelet (× 10^9/L)	98.33 ± 30.83	102.32 ± 33.24	220.93 ± 53.14	< 0.0001	> 0.05	< 0.001	< 0.001
Total leucocytic count (× 10^9/L)	3.17 ± 0.47	3.39 ± 0.50	6.83 ± 2	< 0.0001	> 0.05	< 0.001	< 0.001

Table 4 MicroRNAs levels among the studied groups

Variable(s)	Group Ⅰ HCC group (n = 57) Median (range)	Group Ⅱ Cirrhotic group (n = 57) Median (range)	Group Ⅲ Control group (n = 57) Median (range)	*P*	*P*1	*P*2	*P*3
MicroRNA 23a	2^{14} (2^1-2^{18})	2^{11} (2^1-2^{18})	2^6 (2^0-2^{19})	< 0.0001	< 0.05	< 0.001	< 0.001
MicroRNA 34	2^{14} (2^2-2^{20})	2^{11} (2^4-2^{18})	2^5 (2^0-2^{19})	< 0.0001	> 0.05	< 0.001	< 0.001
MicroRNA 203	2^6 (2^1-2^{19})	2^6 (2^0-2^{19})	2^{14} (2^1-2^{25})	< 0.0001	> 0.05	< 0.001	< 0.001
MicroRNA 338	2^6 (2^1-2^{19})	2^6 (2^1-2^{19})	2^{12} (2^9-2^{17})	< 0.0001	> 0.05	< 0.001	< 0.001
MicroRNA 16	2^{14} (2^1-2^{25})	2^{13} (2^4-2^{18})	2^5 (2^0-2^{19})	< 0.0001	> 0.05	< 0.001	< 0.001

*P*1: Group Ⅰ *vs* Ⅱ; *P*2: Group Ⅰ *vs* Ⅲ; *P*3: Group Ⅱ *vs* Ⅲ.

Table 5 Comparison between microRNAs levels in relation to tumour characteristics, α-feto protein level, Okuda staging, and the etiology of liver cirrhosis

MicroRNA 16			MicroRNA 338			MicroRNA 203			MicroRNA 34			MicroRNA 23a			n	Variable(s)	
P-value	Range	Median	*P*-value	Range	Median	*P*-value	Range	Median	*P*-value	Range	Median	*P*-value	Range	Median			
0.3910	2^4-2^{17}	2^{12}	0.3890	2^4-2^{19}	2^7	0.2571	2^2-2^{17}	2^4	0.0558	2^4-2^{15}	2^{13}	0.0008[a]	2^1-2^{13}	2^{11}	11	Less than 5 cm	Size of focal lesions
	2^1-2^{25}	2^{14}		2^1-2^{15}	2^5		2^1-2^{19}	2^6		2^2-2^{20}	2^{15}		2^3-2^{118}	2^{14}	46	Equal or more 5 cm	
0.3026	2^3-2^{21}	2^{14}	0.5352	2^1-2^{19}	2^6	0.5040	2^2-2^{19}	2^6	0.8975	2^4-2^{20}	2^{14}	0.0001[a]	2^1-2^{18}	2^{11}	32	Single	No. of focal lesions
	2^1-2^{25}	2^{14}		2^2-2^{15}	2^6		2^1-2^9	2^6		2^2-2^{119}	2^{15}		2^{14}-2^{18}	2^{15}	25	multiple	
0.9581	2^9-2^{18}	2^{14}	0.9247	2^2-2^9	2^6	0.7767	2^3-2^9	2^6	0.8255	2^2-2^{19}	2^{14}	0.0795	2^{10}-2^{17}	2^{15}	10	Present	Portal vein thrombosis
	2^1-2^{25}	2^{14}		2^1-2^{19}	2^6		2^1-2^{19}	2^6		2^4-2^{20}	2^{14}		2^1-2^{18}	2^{13}	47	Absent	
0.8881	2^2-2^{25}	2^{14}	0.8295	2^1-2^{19}	2^6	0.0807	2^1-2^{17}	2^7	0.3367	2^2-2^{20}	2^{15}	0.9736	2^1-2^{18}	2^{14}	36	Less than 200 ng/mL	AFP level
	2^1-2^{21}	2^{14}		2^1-2^{15}	2^6		2^1-2^{19}	2^5		2^7-2^{19}	2^{14}		2^3-2^{17}	2^{14}	21	Equal or more 200 ng/mL	
0.9347	2^5-2^{17}	2^{14}	0.1145	2^4-2^9	2^7	0.4488	2^3-2^{17}	2^6	0.4396	2^4-2^{15}	2^{14}	0.0001[a]	2^4-2^{13}	2^{11}	7	Stage Ⅰ	Okuda stage
	2^3-2^{21}	2^{14}		2^1-2^{19}	2^4		2^2-2^{19}	2^7		2^9-2^{20}	2^{14}		2^1-2^{18}	2^{11}	23	Stage Ⅱ	
	2^1-2^{25}	2^{14}		2^2-2^{15}	2^7		2^1-2^9	2^5		2^2-2^{19}	2^{14}		2^{11}-2^{18}	2^{15}	27	Stage Ⅲ	
0.1603	2^3-2^{25}	2^{14}	0.4405	2^1-2^{19}	2^6	0.4007	2^1-2^{19}	2^7	0.7827	2^4-2^{20}	2^{14}	0.5433	2^1-2^{18}	2^{14}	42	HCV	Etiology of liver cirrhosis
	2^1-2^{15}	2^{11}		2^1-2^9	2^6		2^4-2^{15}	2^5		2^{10}-2^{17}	2^{14}		2^3-2^{15}	2^{14}	8	HBV	
	2^5-2^{16}	2^{14}		2^4-2^9	2^7		2^3-2^6	2^4		2^2-2^{19}	2^{15}		2^{10}-2^{17}	2^{14}	7	None	

[a]The first column is variable but is here in this copy the last column. HCV: Hepatitis C virus; HBV: Hepatitis B virus.

levels were higher in patients with focal lesion size equal to or more than 5 cm, in patients with multiple focal lesions; and in Okuda stage Ⅲ as shown in Table 5.

At a cut off level of 200 ng/mL, serum AFP in the studied patients had 73.68% sensitivity, 52.63% specificity, 43.75% PPV, and 80% NPV for the diagnosis of HCC. At a cut off level of 2^{10}ct, serum microRNA 23a had 89.47% sensitivity, 64.91% specificity, 56.04% PPV, and 92.5% NPV for diagnosis of HCC as shown in Table 6 and Figures 1 and 2.

DISCUSSION

microRNA is differentially expressed in development of different types of malignancies, including hepatic malignancy[13], which suggests that microRNAs may

Table 6 Sensitivity, specificity, positive prediction value, negative prediction value and accuracy of microRNAs and α-feto protein

Variable(s)	Cutoff value	Sensitivity%	Specificity%	Positive predictive value (PPV%)	Negative predictive value (NPV%)	Accuracy
MicroRNA 23a	$\geqslant 2^{10}$	89.47%	64.91%	56.04%	92.5%	79.3%
MicroRNA 34	$\geqslant 2^{10}$	89.47%	55.26%	50%	91.3%	79.3%
MicroRNA 203	$\geqslant 2^{4}$	80.7%	10.53%	31.08%	52.17%	29.6%
MicroRNA 338	$\geqslant 2^{5}$	63.16%	16.67%	27.48%	47.5%	26.4%
MicroRNA 16	$\geqslant 2^{10}$	87.72%	57.89%	51.02%	90.4%	75.7%
α-feto protein	⩾ 200	73.68%	52.63%	43.75%	80%	78.5%

PPV: Positive predictive value; NPV: Negative predictive value.

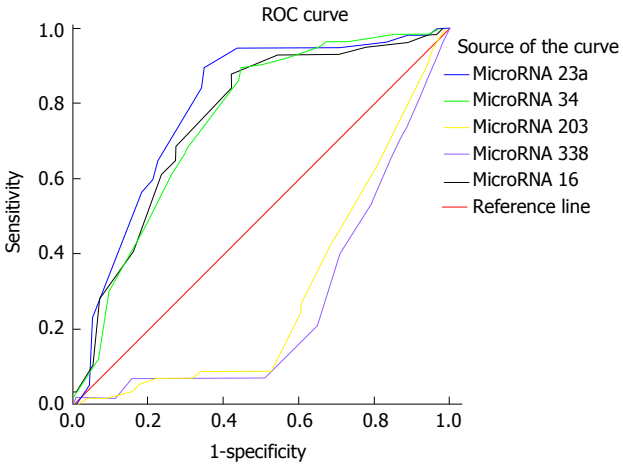

Figure 1 Receiving operating characteristic curve of microRNAs. ROC: Receiving operating characteristic.

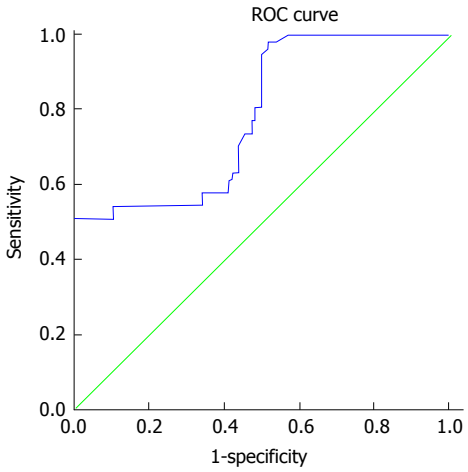

Figure 2 Receiving operating characteristic curve of α-feto protein. ROC: Receiving operating characteristic.

have a role in carcinogenesis as new oncogenes or tumor-suppressor genes. The presence of microRNAs in serum was first reported in 2008 in cases with large B cell lymphoma[14]. They could be a potential biomarker for diagnosis of tumors[15]. The present study was designed to evaluate the role of some microRNA (23a, 34, 203, 338 and 16) in early diagnosis of HCC. To fulfill this aim 57 HCC patients, 57 patients with liver cirrhosis and 57 healthy controls were enrolled.

In the present work, serum microRNA 23a level was significantly higher in the HCC group in comparison to other groups. Also, it is significantly higher in liver cirrhosis group in comparison to healthy controls. A similar result was obtained by Li *et al*[16]. They reported up regulation of microRNA 23a in HCC patients in comparison to liver cirrhosis patients and healthy control. This up-regulation in liver cirrhosis than healthy controls and further up-regulation in HCC patients in comparison to viral hepatitis patients suggests a role of microRNA 23a in the pathogenesis of HCC. A study on resected human HCC tissues found that microRNA-23a down-regulates the expression of interferon regulatory factor-1 in HCC cells[17].

Serum microRNA 34 and microRNA 203 levels were similarly elevated in HCC and liver cirrhosis groups. Many authors found that microRNA 34 expression is increased in hepatic fibrosis[18], HCV infection[19], alcoholic liver disease[20], NAFLD[21,22] and HCC tissues[23-25].

On the contrary, studies on microRNA 203 in HCC tissues found that microRNA 203 is down-regulated in HCC tissue[26,27]. Moreover, studies correlate this down-regulation with recurrence of HCC in liver transplantation[26] and bad prognosis in HCC patients[27]. None of these studies was done on serum; they assessed tissue level of HCC in resected HCC tissues.

Serum microRNA 338 and microRNA 16levels are similarly reduced in HCC patients and liver cirrhosis patients. In line with these findings, many studies concluded reduced tissue expression of miR-338-3p in different types of cancers[28-30]. Studies on HCC showed that miR-338-3pmiR-338-3p was significantly down-regulated in HCC tissues and cell lines compared to the corresponding matched adjacent normal tissues[31,32].

On the contrary, other researchers found that circulating microRNA 338 level increased in HCC patients than liver cirrhosis and controls[33] but their study has a limitation of small sample size (37 HCC patients, 29 cirrhosis patients, and 31 healthy controls).

Serum microRNA 23a level at cutoff value $\geqslant 2^{10}$ showed accuracy of 79.3% to differentiate HCC patients from cirrhotic patients and healthy control with high sensitivity about 90%, specificity about 65%, PPV 56% and NPV 92.9. These values are better than those elicited by alpha fetoprotein. The later at a cut off level of 200 ng/mL, had 73.68% sensitivity, 52.63% specificity, 43.75% PPV, and 80% NPV for

the diagnosis of HCC. So, serum microRNA 23a can be used as a screening test to diagnose HCC as it showed high sensitivity. To the best of our knowledge no previous studies elicited such finding. A single study that used combination of 13 microRNA including 23a found that HCC on top of chronic HBV infection could be differentiated from chronic HCV infection and healthy control[16]. MicroRNA 23a levels were significantly higher in patients with focal lesion 5 cm or more in size, patients with multiple focal lesions; and Okuda stage Ⅲ when compared with patients with less advanced HCC disease. Thus it could be used as a prognostic biomarker.

Other studied microRNA factors showed insignificant difference between HCC and liver cirrhosis patients, so they cannot be used as diagnostic markers of HCC. In conclusion, microRNA 23a can be used as a screening test for early detection of HCC. Also, it is related to larger size of tumour, late Okuda staging and multiple hepatic focal lesions, so it might be a prognostic biomarker. Validation study on large scale is needed to confirm these results.

COMMENTS

Background

MicroRNAs are small, non-coding RNAs that negatively regulate gene expression at the post transcriptional level including cell cycle, apoptosis and metastasis. Aberrant miRNA expression contributes to tumorigenesis and cancer progression. Number of studies have confirmed that miRNAs possess important regulatory roles in hepatocarcinogenesis and malignant transformation. Hepatocellular carcinoma (HCC) is characterized by significant morbidity and high mortality rates. Because of the difficulty of early clinical diagnosis, only 30%-40% of cases can undergo curative resection. Circulating miRNAs released from cancerous tissues are stable and readily available for clinical analysis and appear to show a novel perspective for cancer diagnosis.

Research frontiers

Many microRNAs have been studied as biomarkers for diagnosis of malignancies. Yet, role of microRNA in early diagnosis of HCC is not confirmed.

Innovations and breakthroughs

This work demonstrates that miR-23a can be used in screening of liver cancer and it gives better results than alpha fetoprotein. This work showed first demonstration that microRNA 23a could be used as a promising biomarker for HCC patients, even though large scale examination is required. This manuscript would provide important clues for the development of microRNA as biomarkers for HCC.

Applications

MicroRNA 23a can be used as a screening test for early detection of HCC. Also, it is related to larger size of tumour, late Okuda staging and multiple hepatic focal lesions, so it might be a prognostic biomarker. Validation study on large scale is needed to confirm these results.

Peer-review

This manuscript would provide important clues for the development of microRNA as biomarkers for HCC.

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CASE REPORT

Association of autoimmune hepatitis type 1 in a child with Evans syndrome

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Abstract

Autoimmune hepatitis (AIH) is a progressive liver disease that is often associated with extrahepatic autoimmune disorders. Evans syndrome (ES) is a rare autoimmune disorder, which is characterized by immune thrombocytopenia and autoimmune hemolytic anemia. Association of AIH with ES is rare, especially in children. We report a 3-year-old female with a past medical history of ES who presented with jaundice and significant transaminitis due to AIH type 1. She required multiple treatments with steroids as well as azathioprine, intravenous immunoglobulin and a course of rituximab.

Key words: Evans syndrome; Autoimmune hepatitis type 1; Child

Core tip: We report a 3-year-old female with a past medical history of Evans syndrome (ES) who presented with jaundice and significant transaminitis due to autoimmune hepatitis (AIH) type 1. To our knowledge, this is a rare association of concurrent AIH and ES in a child who responded well to rituximab. The patient also demonstrated short-term response to intravenous immunoglobulin, methylprednisolone, azathioprine and oral prednisone. We conclude that ES may evolve over a period of several months therefore evaluation for associated autoimmune conditions should be considered in these patients.

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INTRODUCTION

Autoimmune hepatitis (AIH) is characterized by chronic necroinflammatory liver disease of unknown cause, circulating non organ-specific autoantibodies and increased levels of immunoglobulin G. The epidemiology of pediatric AIH is unknown. Most patients are diagnosed before the age of 18 years and 75% are girls- the peak incidence being prior to puberty. Currently two types of AIH are recognized according to seropositivity for smooth muscle and/or anti-nuclear antibody for AIH type 1 or liver kidney microsomal antibody and/or to a liver cytosol antigen for AIH type 2. AIH type 1 accounts for two-thirds of the cases and presents usually during adolescence, whereas AIH type 2 presents at a younger age especially during infancy[1]. Liver biopsies remain essential for diagnosis and evaluation of disease severity in patients with AIH. In children, AIH often presents acutely and has a more aggressive course than in adults[2]. If left untreated, it generally progresses rapidly to cirrhosis and liver failure.

Evans syndrome (ES) is a rare autoimmune disease, which is characterized by immune thrombocytopenia (ITP) and autoimmune hemolytic anemia (AIHA). Both diseases are mediated by autoantibodies, though in some cases it is considered a T-lymphocyte disorder. It was first described in 1951. The incidence of ES in children has not been calculated[3]. ES has a chronic and relapsing course, and patients usually depend on prolonged immunosuppressive treatments. ES is more difficult to treat and has a higher mortality than AIHA alone[4]. In approximately half of those diagnosed with ES, no other immune disorder is recognized but in rest of the patients it may be a manifestation of systemic lupus erythematosus, common variable immune deficiency[5], autoimmune lymphoproliferative disorder[6] or another immune disorders[7]. The first line of treatment is intravenous immunoglobulin or steroids. The second-line immunosuppressive therapies are rituximab, azathioprine, cyclosporine and mycophenolate mofetil. ES in children should be considered a severe disease because the risk of life threatening hemorrhage is greater than in classic ITP[3].

AIH cases have been reported concomitantly with extrahepatic immune disorders such as systemic lupus erythematous, rheumatoid arthritis, Sjögren's syndrome, chronic thyroiditis, ulcerative colitis, celiac disease, connective tissue disorder, proliferative glomerulonephritis, Myasthenia Gravis or ITP. Association with ES is rare, especially in children. We report a child with past medical history of ES who presented with jaundice and significant transaminitis due to AIH type 1.

CASE REPORT

A previously healthy two-year-old African American female presented with a two-month history of epistaxis and easy bruising. She was admitted to the University of South Alabama Children's and Women's hospital in May 2015. Examination was remarkable for few healing bruises without hepatosplenomegaly. Laboratory tests (Table 1) showed hemoglobin of 8.4 g/dL, mean corpuscular volume of 75 fL, white blood cell count of 14800/μL, platelet count of 61000/μL and reticulocyte count of 8%. Her aspartate aminotransferase (AST) was 387 IU/L and alanine aminotransferase was 449 IU/L. Coagulation studies were normal and the viral panels including anti-HAV-IgM, HBsAg, anti-HBc, anti-HCV, CMV-IgM, EBV-VCA-IgG, EBV-VCA-IgM, EB early Ag, EBnA, Parvovirus, and HSV-IgM were negative. Further laboratory evaluations yielded a negative anti-nuclear antibody, positive antiplatelet antibody and direct Coomb's test positive for both IgG and anti-compliment factor 3 antibody. Thus, a diagnosis of ES was made. After treatment with single 1 g/kg dose of intravenous immunoglobulin (IVIg) followed by oral prednisone at 2 mg/kg per day, her hemoglobin improved from 8.4 to 10.9 g/dL and corticosteroids were discontinued but she was lost to follow-up overtime. She was hospitalized a few times for intravenous antibiotics due to a bacterial pneumonia and acute bacterial sinusitis. Her ES remained stable during this time. Immune work up showed normal immunoglobulin levels. (Immunoglobulin G 1.090 mg/dL, Immunoglobulin A 114 mg/dL, Immunoglobulin M 86 mg/dL and Immunoglobulin E 148 kU/L), normal absolute lymphocyte counts and sub-set population (including CD3, CD4, CD8, CD56, no double negative T cells) *via* flow cytometry without evidence of autoimmune lymphoproliferative disorder.

One year later, she developed jaundice and pruritus, hepatomegaly with a liver span of 13-cm and increased echogenicity without gallstones on abdominal ultrasound. Her laboratory findings included AST 547 IU/L, alanine transaminase (ALT) 600 IU/L, albumin 2.6 g/dL, total protein 7.9 g/dL, total bilirubin 10.2 mg/dL and direct bilirubin 8.8 mg/dL, prothrombin time (PT) 13.5 s, partial thromboplastin time (aPTT) 31 s, International Normalized Ratio (INR) 1.02, positive anti-nuclear antibody (1:40), positive smooth muscle antibody (1:40), positive F actin antibody (39 units) and elevated total serum IgG (1090 mg/dL). The anti-liver-kidney-microsome antibody, anti-HAV-IgM, HBsAg, anti-HBc and anti-HCV were all negative. The serum alpha-1-antitrypsin and ceruloplasmin concentrations were normal. Prior to percutaneous liver biopsy, she received packed red blood cell (for associated AIHA flare with Hb 4.9 g/dL and reticulocyte count 44%) and fresh frozen plasma. Her pre-biopsy hemoglobin was

Table 1 Laboratory tests during the disease course

Laboratory tests	0 mo	12 mo	13 mo	14 mo	16 mo
Hemoglobin (g/dL)	8.4	4.9	9.6	9.8	14
Reticulocyte count (%)	8	44	34.2	32	4.7
Platelet (cells/μL)	61000	187000	303000	327000	502000
Albumin (g/dL)	2.7	2.6	3.5	3.7	4.1
Aspartate aminotransferase (IU/L)	387	547	45	49	87
Alanine transaminase (IU/L)	449	600	51	188	104
Total bilirubin (mg/dL)	0.8	10.2	1.3	0.5	0.4

0 mo: Diagnosis of Evans syndrome; 12 mo: Diagnosis of autoimmune hepatitis; 13 mo: One month after treatment of methylprednisolone and oral prednisolone; 14 mo: Prior to rituximab; 16 mo: Present.

11.5 g/dL with platelet count 101000 /mcL, PT 10.9 s, INR 1.0, and aPTT 31 s. She received high doses of intravenous methylprednisolone (30 mg/kg per day for 3 d) and oral ursodiol after percutaneously liver biopsy due to suspected AIH type 1. She was discharged with oral prednisone therapy after liver biopsy. Before discharge, her AST was 677 IU/L and ALT 1094 IU/L.

Liver biopsy revealed interface hepatitis with a mixed inflammatory infiltrate including lymphoid cells, eosinophils, neutrophils, histiocytic cells and plasma cells in addition to periportal fibrosis with rare portal-portal septa (stage 2 fibrosis) along with canalicular and hepatocytic cholestasis, indicating AIH. One month later after a high dose of methylprednisolone and oral prednisone, her AST improved to 194 IU/L and ALT to 424 IU/L. Shortly after, she was started on oral azathioprine at a dose of 1.5 mg/kg per day. Currently (4 mo after diagnosis of AIH), her AIH is controlled very well with oral azathioprine and oral prednisone, her present AST is 87 IU/L and ALT is 104 IU/L.

During her hospitalization for AIH, she also had a flare up of ES, with a drop in hemoglobin to 4.9 g/dL and elevated reticulocyte count up to 44% but stable normal platelet counts. She eventually received intravenous rituximab 375 mg/m^2 every week as an outpatient for four doses and she is currently on a replacement IVIg course once a month for six months. Her present labs show hemoglobin of 14 g/dL, reticulocyte count of 4.7% and a normal white blood cells and platelets count. She has not been hospitalized since starting rituximab and IVIg for 7 mo.

DISCUSSION

This report describes an unusual case of ES and AIH type 1 in a child. The diagnosis of ES preceded that of AIH for over a year. Patients with ES have a relapse rate of 74%, with a median delay of eight months (41 d to 9.5 years). Among those, 52% relapse with ITP and AIHA, 40% with ITP alone and 8% with AIHA alone[3]. In a French study ES was found to be secondary to an underlying disease in 10% of patients. No secondary disease was diagnosed over the entire course of study in 30% of children[3]. In addition, 60% of patients with ES demonstrated other associated immune manifestations such as autoimmunity and lymphoproliferation. This suggests that ES occurs within the context of a poorly understood autoimmune dysfunction[3]. Tokgoz *et al*[8] published a case report of a 12-year-old female presenting with ES, AIH and nephrotic syndrome. She differed from our patient by having lymphopenia, leukopenia, low IgA, IgG and IgM levels; low CD3, CD4, CD8 and low TCR alpha/beta expression. Finally, she was diagnosed with CD3γ (gamma) deficiency. CD3 chain deficiency is a heterogeneous group of immunodeficiencies responsible for a small proportion of Severe Combine Immune Deficiency (SCID). Our patient had a history of recurrent infections but her immunoglobulin levels were not low, CD3, CD4 and CD8 were also unremarkable. Flow cytometry also showed no evidence of autoimmune lymphoproliferative disorder. Therefore, our patient demonstrated AIH and ES without evidence of CD3γ deficiency.

Patients with ES are difficult to manage. Although ES may initially respond well to corticosteroids, it usually runs a chronic course with intermittent exacerbations. Interestingly, the effectiveness of rituximab for adults in ES has been established in a number of cases[9,10]. The effects of a weekly infusion with rituximab for four weeks would be effective for up to one year[11]. Experience with the use of Rituximab for treatment of concurrent ES and AIH is limited, especially in children. Carey *et al*[11] reported successful treatment of refractory AIH and ES with rituximab in an adult. Rituximab has been explored in children for a number of hematologic conditions including treatment of AIHA, ITP, factors Ⅷ and Ⅸ inhibitors in patients with hemophilia, post-transplant lymphoproliferative disease, Burkitt's lymphoma and so on. It is overall well tolerated except for occasional symptoms of chills, fever, headache, occasional dyspnea, nausea, pruritus, angioedema, and/or hypotension[12].

Lastly, long-term treatment of pediatric AIH is usually required, with roughly 20% of AIH type 1 patients able to discontinue therapy successfully[1]. Interestingly, our case had elevated levels of immunoglobulin G during diagnosis of AIH type 1. Immunoglobulin G is usually raised at presentation in both types of AIH, although 15% of AIH types 1 and 25% of AIH type 2 have normal levels[13].

To our knowledge, this case report is a rare concurrent association of AIH and ES in a child who responded well to rituximab. The patient also demonstrated short-term response to IVIg, methylprednisolone, azathioprine and oral prednisone. We conclude that ES may evolve over a period of several months therefore evaluation for associated autoimmune conditions should be considered periodically in these patients. Most of the published literature consists of either case reports or small case series. International collaboration is essential in order to better understand the association and treatment of ES and AIH in children and adults.

COMMENTS

Case characteristics

A 2-year-old African American female with past medical history of Evans syndrome (ES) presented with jaundice and significant transaminitis.

Clinical diagnosis

A two-month history of epistaxis and easy bruising at diagnosis of ES and one year later she developed jaundice, pruritus, and hepatomegaly.

Differential diagnosis

Viral hepatitis, cholelithiasis, alpha-1-antitrypsin deficiency, Wilson's disease, glycogen storage disease or congenital hepatic fibrosis.

Laboratory diagnosis

Aspartate aminotransferase 547 IU/L, alanine transaminase 600 IU/L, albumin 2.6 g/dL, total protein 7.9 g/dL, total bilirubin 10.2 mg/dL and direct bilirubin 8.8 mg/dL, prothrombin time 13.5 s, partial thromboplastin time 31 s, International Normalized Ratio 1.02, positive anti-nuclear antibody (1:40), positive smooth muscle antibody (1:40), positive F actin antibody (39 units) and elevated total serum IgG (1090 mg/dL). The anti-liver-kidney-microsome antibody, anti-HAV-IgM, HBsAg, anti-HBc and anti-HCV were all negative. The serum alpha-1-antitrypsin and ceruloplasmin concentrations were normal.

Imaging diagnosis

Abdominal ultrasound showed a liver span of 13-cm and increased echogenicity without gallstones.

Pathological diagnosis

Liver biopsy revealed interface hepatitis with a mixed inflammatory infiltrate including lymphoid cells, eosinophils, neutrophils, histiocytic cells and plasma cells in addition to periportal fibrosis with rare portal-portal septa (stage 2 fibrosis) indicating autoimmune hepatitis (AIH).

Treatment

High doses of methylprednisolone (30 mg/kg per day for 3 d) and then oral prednisone, oral ursodiol, oral azathioprine, intravenous immunoglobulin and intravenous rituximab.

Related reports

AIH is characterized by chronic necroinflammatory liver disease of unknown cause, circulating non organ-specific autoantibodies and increased levels of immunoglobulin G. AIH cases have been reported concomitantly with extrahepatic immune disorders. Association with ES is rare, especially in children.

Term explanation

ES may evolve over a period of several months therefore evaluation for associated autoimmune conditions should be considered periodically even if negative initially, especially AIH.

Experiences and lessons

This is the rare case report of concurrent AIH and ES in a child who responded well to rituximab. The patient also demonstrated short-term response to intravenous immunoglobulin, methylprednisolone, azathioprine and oral prednisone. International collaboration is essential in order to better understand the association and treatment of ES and AIH in children and adults.

Peer-review

Authors report an interesting case of 3-year-old child with ES associated with type 1 AIH.

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MINIREVIEWS

Role of endoscopic ultrasound in liver disease: Where do we stand in 2017?

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Abstract

Endoscopic ultrasound (EUS) was first introduced into medical practice in 1980s as a diagnostic imaging modality for pancreatic pathology. EUS has the unique advantage of combining ultrasound and endoscopy to obtain detailed information of the gastrointestinal tract. Over the past decade, the use of EUS in liver diseases has been increasing. EUS, which was initially used as a diagnostic tool, is now having increasing therapeutic role as well. We provide a review of the application of EUS in the diagnostic and therapeutic aspects of liver disease. We also look at the evolving future research on the role of EUS in liver diseases.

Key words: Endoscopic ultrasound; Liver disease; Portal hypertension; Liver lesions

Core tip: We have summarized the up-to-date literature on the emerging role of endoscopic ultrasound (EUS) in liver disease. This brief review summarizes both the diagnostic and therapeutic role of EUS in focal hepatic lesions, portal hypertension, liver abscess and hepatic cysts. We have also summarized the future research on this subject.

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INTRODUCTION

The evaluation of liver disease has been progressively changing over the last few decades with advancement of new technologies. Computed tomography (CT), conventional ultrasound and magnetic resonance imaging has have been the principal means for evaluating hepatic disease for long time[1].

Endoscopic ultrasound (EUS) was first introduced into medical practice in 1980s as a diagnostic imaging modality for pancreatic pathology[2]. It is distinctive in its ability to differentiate the histological layers of the gastrointestinal (GI) tract wall as well as the periluminal structures[3]. EUS has the unique advantage of combining ultrasound and endoscopy to obtain detailed information of the GI tract. With recent advances in technology, advanced physicians' training and the expanding use of EUS, its role has grown dramatically to include both diagnostic and therapeutic aspects in gastrointestinal, pancreatic and hepatobiliary tree disease[1].

In this review, we aim to summarize the application of EUS in diagnostic and therapeutic aspects of liver diseases. EUS performances in diagnostic and therapeutic aspects of liver disease include diagnosis and management of focal hepatic lesions, simple hepatic cysts, hepatic abscesses and portal hypertension. Limitations of EUS include limited access to the right hepatic lobe and increased risk of complications in those with anatomical alteration of the GI tract. Complications, although rare, can happen during EUS-guided fine needle aspiration (FNA) and include esophageal and duodenal perforation. We also look at the evolving future research on the role of EUS in liver diseases.

DIAGNOSTIC USE OF EUS, CONTRAST ENHANCED HARMONIC EUS, EUS-GUIDED FNA IN FOCAL HEPATIC LESIONS

Focal hepatic lesions are divided into benign lesions (such as hepatic cysts, focal nodular hyperplasia, regenerative nodular hyperplasia, abscess, adenoma or hemangioma) and malignant lesions (such as hepatocellular carcinoma, intrahepatic cholangiocarcinoma, biliary cystadenoma and metastatic liver disease)[4]. Those lesions were classically diagnosed with combination of conventional imaging such CT and transabdominal ultrasound and percutaneous liver. EUS was first used in liver imaging in 1997[5] and since then its use has become increasing popular.

EUS, especially when combined by cytology, has been used not for evaluating intra-abdominal masses only, but also for staging purposes[6-9]. In recent review by Srinivasan *et al*[4], EUS has shown superiority in detecting focal hepatic lesions compared with conventional CT and trans-abdominal ultrasound, especially for small lesions. A recent study comparing the diagnostic sensitivity of EUS and CT scan showed that of 574 patients, 14 had liver lesions that were visualized by EUS, however, only 3 of those 14 patients had their lesions visualized by CT scan prior to the use of EUS[10]. Another study by Awad *et al*[11] showed that EUS could detect additional hepatic lesions in 28% of patients with a history of known liver mass that were detected initially by CT scan. Similarly, other reports have shown that EUS can detect liver lesions that were missed by conventional imaging modalities[12]. Fuijii-Lau *et al*[13] proposed diagnostic criteria to differentiate between benign hepatic lesions and malignant metastatic lesions according to the lesion's characteristics on EUS. These criteria include lesion's shape, borders, echogenicity, homogeneity and size. These EUS criteria were applied to 200 patients who were diagnosed with malignancy using EUS-FNA. The authors concluded that EUS criteria may help in distinguishing benign from malignant hepatic lesions with a positive predictive value of 88%. The authors also suggested that the use of EUS criteria can guide the decision to perform EUS-FNA on a liver mass or not. The limitations of their study was that it was a signle center study and the EUS criteria was validated by one expert endosonosgrapher only.

The use of contrast-enhanced harmonic endoscopic ultrasound (CH-EUS) for liver disease has evolved recently. Since the liver cells have a dual blood supply, CH-EUS is divided into three phases according to timing from contrast injection; arterial phase, portal phase and late phase[14]. According to contrast enhancement imaging, increased arterial enhancement and late-phase contrast washout indicate hepatocellular carcinoma, while peripheral-rim like hyper enhancement followed by subsequent washout is visualized in metastatic liver cancer[15]. In cases of hemangioma, peripheral nodular hyper enhancement associated with sustained enhancement in the late phase is usually visualized[15]. A comparable study by Liu *et al*[16] showed that CH-EUS is the same if not superior to CT scan in characterization and visualization of focal hepatic lesions.

The use of EUS was not limited to visualization only, but also in obtaining tissue biopsy for diagnostic purpose. EUS guided fine needle aspiration (FNA) has played a major role in revolutionizing the diagnosis of focal hepatic lesions. EUS-FNA is a minimally invasive procedure that is utilized for procurement of tissue

Table 1 Complication of endoscopic ultrasound guided fine needle aspiration compared with percutaneous fine needle aspiration

EUS guided FNA	Percutaneous FNA
Bleeding[18]	Bleeding[21,22]
Pain[18]	Severe pain[21]
Fever[18]	Punctured gall bladder[21]
Hemoperitoneum[23]	pneumothorax[21]
Death[23]	Syncope[21]
	Hemoperitoneum[24]
	Hypovolemic shock[24]
	Death[22]

EUS: Endoscopic ultrasound; FNA: Fine needle aspiration.

of hepatic lesions. Currently, its use is limited to the left lobe, the proximal right lobe, the hilum and part of the intrahepatic biliary tract[17]. EUS-FNA has a theoretical advantage over classical percutaneous biopsy in patients with cirrhosis, since percutaneous approach may be difficult in these patients owing to the presence of ascites and coagulopathy[4]. Previous reports on the safety and efficacy of EUS-FNA have yielded encouraging results. In a survey by tenBerge *et al*[18], which included data from twenty-one centers of 167 cases of EUS-FNA of the lives lesions, it was shown that EUS-FNA was able to diagnose malignancy in 23 out of 26 (89%) of cases after a non-diagnostic trans-abdominal ultrasound guided FNA. Safety of EUS-FNA was also tested, with only 1% rate of major complication was reported. EUS-FNA was also shown to be safe with only 1% rate of major complications. Several other studies have shown the sensitivity of EUS-FNA for diagnosis of malignancy in liver lesions ranging from 82%-94%[19,20]. Table 1 sumarizes the complications of EUS guided FNA and percutaneous FNA[18,21-24].

EUS-GUIDED LIVER BIOPSY

Liver biopsy remains the cornerstone in the diagnosis of liver diseases[25]. Percutaneous liver biopsy was first described in 1923[26] before the transjugular approach was suggested in 1973[27]. Limitations of percutaneous approach are significant sample variability[25] and risk of adverse events that include pain at site of biopsy, bleeding, marked hypotension and pneumothorax[21]. The transjugular approach for liver biopsy entails accesses to the liver parenchyma through superior vena cava and hepatic vein, hence the liver capsule is not punctured[25]. This approach is preferred in those with coagulopathy, marked ascites and in morbidly obese patients[25]. Recently, EUS was used to obtain liver biopsy. EUS-guided liver biopsy (EUS-LB) was first described in animal studies in 2002[28], with favorable outcome and safety profile. EUS-LB in humans was described by Dewitt *et al*[29]. A case series of 21 patients who underwent a transgastric EUS guided Tru-cut biopsy with a 19-gauge needle. Histologic diagnosis was successfully obtained in 90% of specimens (19/21), however, only 71% (15/21) were helpful for clinical diagnosis. No adverse events were reported in any of the patients. In another retrospective study of 9 patients, Gleeson *et al*[30] were able to show that Tru-cut biopsy is safe and at the same time yields suitable tissue for diagnostic purposes of liver disease.

THERAPEUTIC EUS-FNA OF FOCAL HEPATIC LESIONS

Recently some case reports have highlighted the therapeutic role of EUS in liver lesions as well[31-34]. This includes the use of EUS to guide alcohol injection and laser ablation of hepatic lesions. Barclay *et al*[31] described a case of 3.3 cm metastatic liver lesion treated with multiple EUS-guided ethanol injections. Follow-up imaging showed a decrease in tumor size to less than 2 cm. Hu *et al*[32] also reported a patient with pancreatic adenocarcinoma with metastasis to retroperitoneal lymph nodes and left hepatic lobe. Following pancreatoduodenectomy and chemotherapy, patient underwent successful ethanol injection of left hepatic lesion with no significant post-procedure complications. Other examples of therapeutic intervention include EUS-guided Nd:YAG (neodymium-doped yttrium aluminum garnet; Nd:$Y_3Al_5O_{12}$) laser ablation of hepatocellular carcinoma[35].

THERAPEUTIC USE OF EUS IN SIMPLE HEPATIC CYSTS

Hepatic cysts are mostly asymptomatic, and estimated to occur in 5% of population[36]. The female: Male is approximately 1.5:1 among those with asymptomatic simple hepatic cysts (SHC) while it is 9:1 in those with symptomatic or complicated SHC[36]. SHC is generally diagnosed incidentally on abdominal imaging. Only 10%-16% of such cysts are symptomatic[4]. Symptoms are due to mass effect, rupture, hemorrhage and infection[36], and include abdominal pain, nausea, vomiting, early satiety, obstructive jaundice and hepatomegaly[4,36]. Management of SHC has varied over the years. Treatment options include surgical approach (open deroofing, laparoscopic deroofing, complete cyst resection and hepatectomy), percutaneous aspiration and sclerotherapy[4,36-40]. Prior reports have shown that percutaneous aspirations is associated with recurrence rate, as high as 100%, that can be seen as early as two weeks[38,40]. A recent systematic review by Wijnands *et al*[39] evaluated the role of percutaneous sclerotherapy in the management of SHC. The authors included 16 studies and reported cysts volume reduction ranged between 76% to 100% after a median follow-up period

of one to fifty-four months. In 10 of these studies, 72% to 100% patients reported improvement of symptoms, while 56% to 100% patients reported symptoms resolution. In regards to safety, three studies reported ethanol intoxication incidence, manifested as headache, nausea and flushing, with frequency of intoxication as high as 93%. The risk of intoxication increased with increased sclerotherapy duration, and increased volume of ethanol used[39].

In recent years, EUS guided ethanol lavage has emerged as a popular treatment modality of SHC. In 2014, Lee *et al*[41] did a single center retrospective cohort study comparing EUS guided and percutaneous ethanol lavage for treatment of large hepatic cysts. A total of 10 cysts were drained by percutaneous approach with placement of drainage catheter, while 8 cysts were drained using EUS guided ethanol lavage. In EUS-guided group, cysts were drained in a 1-step approach without the placement of a catheter. Both approaches were efficacious. Results revealed a 97.5% and 100% reduction in cysts size at 11.5-mo follow-up and 15-mo follow-up, respectively. The authors concluded that there is an excellent symptomatic and radiological response in both groups. EUS-guided approach is more effective for left liver lobe cysts while percutaneous approach is better in right sided liver cysts[41]. Despite positive results, further multi-center trials are needed to confirm these findings, since this was a single center study.

THERAPEUTIC USE OF EUS IN LIVER ABSCESSES

Liver abscesses are defined as encapsulated collection of suppurative material within the liver parenchyma[42]. They are the most common intra-abdominal abscesses with a reported incidence of 8-20 cases per 100000 hospitalized patients per year in the United States[43]. Historically, pyogenic liver abscess has been managed with either surgical or percutaneous interventions[44]. Since 2001, the number of percutaneous procedures has doubled, while the number of surgical procedures has decreased by about 20%[45]. Percutaneous abscess drainage has a success rate of up to 100%[46], hence making it the first line drainage technique. On the other hand percutaneous drainage is associated with side effects including catheter dislodgment, subscapsular hematoma, drainage from catheter exit site[47], hepato-venous fisulas[48] and hepato-colic fistulas[49]. In recent years, EUS guided drainage for liver abscesses has emerged an alternative approach since it was first proposed by Seewald *et al*[50] in 2005. The authors reported a case of an 11 cm hepatic abscess within the left lobe of the liver that was successfully drained through trans-gastric approach using EUS with no complications or recurrence on follow-up. Since then, several other case reports and series have described successful EUS guided drainage of liver abscess *via* trans-gastric and trans-duodenal approaches[51-56]. In a retrospective report by Ogura *et al*[57], 27 patients who underwent either EUS-guided abscess drainage or percutaneous abscess drainage, the clinical success rate of EUS-guided group was superior to that of the percutaneous group, at 100% and 82%, respectively. Safety and hospital stay was also superior in EUS guided group[57]. Although this data is encouraging, more prospective studies are still needed to compare the safety and efficacy of both interventions.

EUS AND PORTAL HYPERTENSION

Diagnostic aspect

Portal hypertension is the hallmark of end stage liver disease or advanced fibrosis. Hepatic venous pressure gradient (HVPG) greater than 5 mmHg is defined as portal hypertension. Esophageal varices (EV) form when HVPG is greater than 10 mmHg and the chances of EV bleeding occurs when HVPG exceeds 12 mmHg[58,59]. Esophagogastroduodenoscopy (EGD) has been the cornerstone for diagnosis, surveillance and treatment of EV[60]. Over the last decade EUS has emerged as an important tool for evaluation of gastroesophageal varices[61].

EUS can effectively measure the size of EV by using the sum of the cross-sectional surface area of all the EV in the distal third of the esophagus[62]. While upper gastrointestinal endoscopy continues to be the gold standard in detecting EV, EUS has better sensitivity in detecting gastric varices[63]. In one study EUS was able to detect gastric varices twice more than conventional EGD[63]. Since EUS can detect vascular changes better, some experts believe that EUS can easily differentiate thickened gastric folds from small gastric varices that can be difficult to diagnose *via* EGD[64]. EUS like EGD can not only diagnose esophageal and gastric varices but can also predict the risk of bleeding. One report showed that the detection of hemocystic spots *via* EUS predicted the chance of variceal hemorrhage[65].

The other advantage of EUS is increased sensitivity in detection of collateral veins around the esophagus. These veins can be small in size, called peri-esophageal collateral veins, or large in size; para-esophageal collateral veins[61]. In one study from China, EUS was able to detect extra-luminal venous abnormalities in greater than 90% of patients with cirrhosis[66]. Some gastroenterologists argue that the early detection of gastroesophageal varices, and other venous abnormalities in cirrhosis *via* EUS might reduce the need of liver biopsy if the etiology of cirrhosis is clear, *e.g.,* alcohol use and long standing viral hepatitis[67].

The detection of collateral vasculature does not only have diagnostic significance, but also has prognostic value. Prior studies have shown that the presence of severe collateral and perforating veins can help predict

Table 2 Animals studies regarding endoscopic ultrasound-guided intrahepatic portosystemic shunt placement

Ref.	Animals	Type of needle	Success rate
Schulman *et al*[79]	5 pigs	19-G-needle	100%
Buscaglia *et al*[80]	10 pigs	19-G-needle	100%

the chance of recurrence of esophageal varices before and after treatment[68-70]. Konishi *et al*[70] performed a study evaluating the risk of recurrence of esophageal varices after band ligation based on presence of vascular structures around the gastric cardia detected *via* EUS. They reported that over 90% of patients with severe perforating veins seen on EUS prior to variceal band ligation had recurrence of varcies[70]. In another study by Masalaite *et al*[71], severe esophageal collateral veins seen during EUS were shown to be independent risk factors for recurrence of varices. This suggests that this subset of patients might need closer follow-up as compared to patients who do not have perforating veins.

Therapeutic aspect

Over recent years, EUS has found role in management and treatment of gastroesophageal varices as well. The role of sclerosing therapy under EUS guidance is becoming increasingly popular. One randomized trial from Brazil showed encouraging results demonstrating that EUS guided sclerotherapy was equally effective as compared to standard endoscopic sclerotherapy for esophageal collateral vessels[72]. Where treatment of esophageal varices *via* EGD continues to be the standard of care, bleeding from gastric varices continues to be a challenge for endoscopists around the globe. Gastroesophageal varices type 2 (GOV-2) are usually large in size and lead to significant bleeding. These varices cannot be effectively treated by band ligation, and therapy targeting the accompanying perforating and collateral veins is needed. Due to these challenges, EUS guided therapy with precise localization of these veins is becoming exceedingly popular[73]. The two common modalities include EUS guided cyanoacrylate injection and EUS guided coil embolization[73,74]. Lee *et al*[66] performed a study in which 54 patients with bleeding due to gastric varices underwent EUS every two weeks, with injection of cyanoacrylate until obliteration of gastric varices. The authors reported that this intervention lead to decrease in recurrence of bleeding and improved survival in this group of patients[66]. A multi-center study also compared the use of cyanoacrylate injection (CI) with EUS guided coil embolization (CE) for treatment of bleeding gastric varices[75]. The results of this study were promising and showed that both EUS guided CI and CE were effective in treatment of gastric varices, however, CE had less side effects and needed less number of sessions for eradication of gastric varices. EUS guided sclerosis has also been successfully used to treat bleeding rectal varices in some cases[76].

The role of EUS in portal hypertension seems to be growing even more. Recently an animal study reported comparable results of portal pressure gradient measurement by EUS guided manometer approach with interventional radiology guided portal pressure measurement[77]. The same group of investigators also performed a pilot human study in which 28 patients underwent EUS guided portal pressure measurement with a hundred percent success rate and no adverse events[78]. Whereas further studies with larger sample size are needed in this regard, EUS guided portal pressure measurement might be a breakthrough for gastroenterologists and hepatologists in taking care of patients with cirrhosis. Animal studies (Table 2) have also shown that EUS can potentially be used for creation of intra-hepatic portosystemic shunts[79,80]. Historically the intra-hepatic portosystemic shunt has been placed using a trans-jugular approach under angiography (TIPS). Although this procedure as suggested has been technically feasible in animals, major concerns should be addressed before its application in patients with advanced liver disease. Those concerns include high risk of bleeding, severe infections and technical difficulties in stent placement[81].

COMPLICATIONS OF EUS

Due to specific mechanical properties of echoendoscopes used for EUS and the evolving training of advanced endoscopy specialists, there is a low, and yet noteworthy risk of complications with EUS. Majority of the complications related to EUS occur during EUS-FNA[82]. The mortality associated with EUS and EUS-FNA is 0.02%[82]. The major adverse complication with EUS is perforation. Gastrointestinal perforation can happen, especially at areas of angulation and in the presence of unexpected anatomical changes[82]. A survey conducted in Germany, including 67 centers, reported 32 complications associated with EUS. Esophageal perforation occurred only in 8 of almost 85000 diagnostic EUS procedures[83]. Another survey among members of American endosonography club in 2002 reported 16 esophageal perforations that occured after almost 44000 EUS procedures were performed, and more than half of those occurred with endoscopists who had less than one year of experience performing EUS[84]. Duodenal perforations occur more frequently than esophageal perforation[82]. In a prospective EUS online registry, 10 events of gastrointestinal perforations in 13988 diagnostic EUS procedures were noted, with duodenal perforation accounting for 60% of these cases[82]. A survey by Lachter[85] investigated the mortality in patients who had a complication during EUS. The authors reported that 13 out of 18

(73%) fatalities resulted from duodenal tears causing retroperitoneal perforations, with four of those thirteen patients having duodenal diverticula.

CONCLUSION

The role of EUS has evolved greatly in recent years. Initially thought to be a great tool for diagnostics, EUS has now several therapeutic implications as well. Since expansion of EUS in liver diseases, it is emerging as a great tool for gastroenterologists and hepatologists to manage several liver related conditions. Focal hepatic lesions have always been a challenge for hepatologists. With recent advancements in EUS, it has shown superiority in detecting focal liver lesions as compared to conventional CT scan and ultrasound imaging modalities. Moreover, recently several therapies including EUS guided ethanol and EUS-guided Nd:YAG (neodymium-doped yttrium aluminum garnet; Nd:$Y_3Al_5O_{12}$) laser ablation are also used to treat focal hepatic lesions. Similarly, recent data is showing that EUS guided liver biopsy may potentially be more safer than percutaneous liver biopsy when done by an experienced endosonographer. In regards to portal hypertension, EUS can detect early changes of portal hypertension and hence provides early and accurate assessment of overall clinical status. Despite encouraging results from available data, further research including randomized control trials is needed, before the use of EUS can be generalized in liver diseases.

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MINIREVIEWS

Liver transplantation in the treatment of severe iatrogenic liver injuries

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Abstract

The place of liver transplantation in the treatment of severe iatrogenic liver injuries has not yet been widely discussed in the literature. Bile duct injuries during cholecystectomy represent the leading cause of liver transplantation in this setting, while other indications after abdominal surgery are less common. Urgent liver transplantation for the treatment of severe iatrogenic liver injury may-represent a surgical challenge requiring technically difficult and time consuming procedures. A debate is ongoing on the need for centralization of complex surgery in tertiary referral centers. The early referral of patients with severe iatrogenic liver injuries to a tertiary center with experienced hepato-pancreato-biliary and transplant surgery has emerged as the best treatment of care. Despite widespread interest in the use of liver transplantation as a treatment option for severe iatrogenic injuries, reported experiences indicate few liver transplants are performed. This review analyzes the literature on liver transplantation after hepatic injury and discusses our own experience along with surgical advances and future prospects in this uncommon transplant setting.

Key words: Urgent liver transplantation; Acute liver failure; Iatrogenic liver injury; Vascular injury; Surgical complication; Biliary injury; Tertiary referral center; Liver transplantation

Core tip: Liver transplantation may represent the only option to manage severe iatrogenic liver injuries. Despite

widespread interest, reported experiences indicate only a minority of liver transplants are performed, and the place of liver transplantation in this setting has not yet been widely discussed. Causes other than severe bile duct injuries during cholecystectomy are less common indications for liver transplantation. Urgent liver transplantation for the treatment of severe iatrogenic liver injury may require technically difficult and time-consuming surgical procedures. The centralization of complex surgery in tertiary centers and the early referral of patients with severe iatrogenic liver injuries are crucial.

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INTRODUCTION

At the end of the line, liver transplantation (LT) may represent the only curative and life-saving option to manage severe iatrogenic liver injuries. Whereas many recent articles have focused on different strategies in the multidisciplinary management of iatrogenic bile duct injuries (BDI) after cholecystectomy[1-4], the place of LT in the treatment of other severe iatrogenic liver injuries after hepatobiliary (HPB) surgery has not yet been widely discussed in the literature. This review analyzes the cases reported to date and discusses our own experience along with surgical advances and future prospects in this uncommon transplant setting.

TYPE OF INJURY

There are basically two main types of severe iatrogenic liver injury requiring urgent LT: Biliary or vascular injuries, or a combination of the two. Some patients were indicated for LT due to acute liver failure (ALF) resulting from vascular injury secondary to a first biliary injury or other less common severe iatrogenic liver injuries.

BDI and vasculobiliary injuries during cholecystectomy

The incidence of BDI during cholecystectomy varies from 0.1% to 0.3%, rising to 0.6% when considering the laparoscopic approach[5,6]. The type and extent of BDI play an important role in surgical planning for appropriate timing and treatment.

Different systems have been proposed to classify and grade the severity of BDI. In 1982, Professor Bismuth[7] first classified postoperative bile duct strictures in a chapter of the "Blumgart book". He subsequently proposed a useful classification of biliary strictures based on the principles of surgical treatments[8]. Like the Bismuth classification, Strasberg's scale[9] incorporates other biliary injuries commonly encountered after laparoscopic cholecystectomy. To prevent bile duct injury, the Stewart-Way classification incorporates the mechanism of injury as well as its anatomy, separating resectional damage from stricture and providing a guide to pre-operative evaluation and biliary reconstruction[10]. Although other classifications of BDI after laparoscopic cholecystectomy have been reported and recently reviewed by Chun[11], the Strasberg scale remains the classification of choice for defining the types of BDI.

Some recently reported series on LT for cholecystectomy-induced BDI provide important insights. In 2011, Ardiles *et al*[2] analyzed their experience using LT as a definitive treatment for BDI, reporting data from a retrospective national survey performed in 18 LT centers over 20 years in Argentina. Among 2766 LT performed from 1990 to 2009, 19 (0.7%) were secondary to BDI arising during 16 cholecystectomies (open in 10, and laparoscopic in 6), two hydatid cyst resections, and one right hepatectomy. Seven patients had associated vascular injuries. The indication for LT was liver cirrhosis in 18 cases and ALF in the remaining one. No intraoperative mortality was reported but four patients died during the first month after LT, and another four died in the late postoperative period. The remaining 11 patients showed a good quality of life in the long-term follow-up and recipient survival rates at one, three, five and ten years were 73%, 68%, 68% and 45% respectively. The authors reported a higher rate of major post-operative complications (52%), according to the Clavien classification[12], compared with other etiologies and secondary biliary cirrhosis[13]. Interestingly, the significant decrease over time in the incidence of LT for this indication in their cohorts (3.1% of all LT in the period 1990-1994; and 0.2% in the period 2005-2009 - $P < 0.001$) reflects improvements in the prevention and management of BDI related to a multidisciplinary and specialized approach to injury-related complications.

In 2013, Parilla *et al*[4], on behalf of the Spanish Liver Transplantation Study Group, reviewed the indications and outcome of 27 patients with BDI after cholecystectomy and listed for LT in Spain over a 24-year period. Emergency LT for ALF was indicated in seven patients all after laparoscopic cholecystectomy. Two of them died while on the waiting list, one from multiorgan failure (MOF) secondary to BDI-related sepsis, and the other was anhepatic after a total hepatectomy required for massive liver necrosis. Another 20 patients underwent elective LT for secondary biliary cirrhosis after BDI (13 after open and 7 after laparoscopic cholecystectomy). Four of the five recipients who underwent emergency LT for ALF died within 30 d after LT, and the estimated overall five-year survival rate was 68%. The Spanish study confirms that BDI after laparoscopic cholecystectomy tends to be more severe than that after the open approach.

Very recently, an Italian group from Genoa reviewed the literature and reported another two cases of LT for

iatrogenic injuries among 12 patients referred to their tertiary center for the management of complicated cholecystectomy[14]. The timing for LT differed in this series. The first patient was transplanted after several endoscopic and radiological attempts to solve recurrent cholangitis that led to secondary biliary cirrhosis five years after BDI. He initially underwent open cholecystectomy with a biliary lesion described as type E2 (according to the Strasberg-Bismuth classification), and referred to the tertiary center five years after the first injury. Conversely, the second patient was listed for an emergency LT after a laparoscopic cholecystectomy converted to the open approach because of bleeding from the liver parenchyma. Eight days after surgery the patient had bile leaks and underwent endoscopic biliary stent placement complicated by a large intrahepatic hematoma and bleeding initially treated by right hepatic embolization. The patient required emergency surgical exploration and a total hepatectomy with temporary portocaval shunt (TPCS) was required to overcome the bleeding after a right hepatectomy. The intraoperative field showed a massive liver hematoma involving the right lobe, deep parenchyma lacerations, and a type D injury. After a two-day anhepatic bridging period the patient was successfully transplanted and underwent long-term follow-up. The same authors also described another patient with chronic cirrhosis who underwent LT after acute liver decompensation caused by open cholecystectomy for common bile duct lithiasis.

In addition to biliary damage, severe vascular iatrogenic injuries during HPB surgery can result in devastating complications. While the BDI rate after cholecystectomy is estimated up to 0.6% (6), and concomitant hepatic artery damage has been reported in 12%-47% of patients[15], isolated portal vein (PV) injury is uncommon. In 2011, Strasberg *et al*[16] published an analytical review of vasculobiliary injury in cholecystectomy, evaluating frequencies, causes clinical implications, and their management. A year later, the same team addressed the pathogenesis of "extreme" vasculobiliary injury and reported on outcomes after cholecystectomy for severely inflamed gallbladders in eight patients[17]. Unfortunately, one patient developed infarction of the bile ducts after injury to the proper hepatic artery and died of sepsis in the postoperative period after urgent LT. In author's opinion, in presence of inflammation a fundus-down cholecistectomy should be avoided for the prevention of extreme vasculobiliary injuries.

In 2013, Wang *et al*[15] analyzed the therapeutic strategies for iatrogenic PV injury after cholecystectomy, reporting their experience of 11 patients with vascular injuries in the absence of biliary damage. One of these patients, a 50-year-old woman, underwent LT due to chronic liver failure four months after the initial injury to the right branch of PV after an open cholecystectomy. In the authors' opinion, delayed diagnosis and treatment may have led to difficult vein repair and liver revascularization resulting in PV thrombosis and hepatic necrosis. They highlighted the major role of thrombolytic and anticoagulation therapy in the treatment of acute massive thrombus. We agree with them that an immediate attempt to repair severe PV injury should be preferred in a hemodynamically stable patient.

Other causes of severe iatrogenic liver injuries

Indications for LT to treat severe iatrogenic liver injuries after abdominal surgery or causes other than injuries during cholecystectomy are certainly less common, and very few cases have been reported.

In 2006, Huerta *et al*[18] described three lethal complications resulting from severe iatrogenic injuries during bariatric surgery performed in a high-volume bariatric center. They also described details of three cases of PV thrombosis that led to LT after two Roux-en-Y gastric bypass (RYGBP) procedures and one vertical banded gastroplasty. In the two cases of RYGBP, the porta hepatis was inadvertently stapled, while in the patient who underwent vertical banded gastroplasty the PV was divided and promptly reconstructed, but caused irreversible ischemic liver damage. Although the iatrogenic injuries were immediately recognized, a transplant surgeon consulted, and patients referred for emergency LT, the postoperative course was complicated by sepsis, MOF, and other severe medical complications resulting in the deaths of the patients. The authors claimed that PV ligation with immediate patient referral to a LT center for emergency transplant may improve the outcome in case of severe PV injury.

In 2009, the group from the University Medical Center, Nashville, Tennessee (United States) reported two cases of iatrogenic porta hepatis transection requiring an urgent two-stage liver LT[19]. In the first case, severe porta hepatis transection occurred during an open adrenalectomy in a 39-year-old woman with a history of cholecystectomy. Before transferring the patient to the authors' tertiary LT center, primary PV repair was attempted, and a Roux-en-Y hepaticojejunostomy performed, while the hepatic artery was left divided. Due to progression of the hepatic dysfunction and worsening hemodynamics, the patient underwent urgent total hepatectomy and portocaval shunt, and was listed for an emergency LT. In the other case, severe iatrogenic injury occurred during a laparoscopic cholecystectomy converted to an open operation to control a massive bleed and complete cholecystectomy before emergency transfer of the patient to the authors' tertiary center. A computed tomography (CT) scan showed infarction of the right hepatic lobe, transection of the right hepatic artery and right PV. Arterial perfusion of the left lobe was provided through a replaced left hepatic artery. A right hepatic lobectomy was planned and an urgent surgical re-exploration performed. Unfortunately, the extent of the

left PV injury precluded successful reconstruction of the PV flow and a total hepatectomy with a portocaval shunt was performed. The patient underwent LT 20 h later. We agree with the author that patients presenting with severe portal transection cannot be treated expectantly, and prompt radiological evaluation and surgical intervention are mandatory to attempt to restore hepatic flow. Hepatic resections should not be the only options entertained and LT should be promptly evaluated on a case-by-case basis.

Another case of severe hepatic injury resulting from an open right adrenalectomy was reported in the same year by Tessier *et al*[20] in a review of high-grade complications after adrenalectomy. The surgical procedure was complicated by an unrecognized injury to and ligation of the proper hepatic artery. Three months after adrenalectomy, the patient underwent a Roux-en-Y hepaticojejunostomy for the treatment of multiple liver abscesses, recurrent episodes of cholangitis and later a bleeding cholecysto-enteric fistula. The patient was ultimately referred to a tertiary center where LT was performed because of recurrent cholangitis and bile duct sclerosis.

Interestingly, in 2010 Di Benedetto *et al*[21], reported details of their experience in the treatment of severe injuries after transjugular intrahepatic portosystemic shunt placements in two cirrhotic patients where surgical and radiological attempts had failed to stop the bleeding after parenchymal and vascular rupture. Although the indications for LT were liver failure after artery embolization, and uncontrollable hemobilia, this experience highlights the ability of a tertiary referral center to offer LT as the only curative option.

OUR EXPERIENCE

Our tertiary referral center offers both a specialist HPB referral service and an abdominal organ transplantation service with more than 1800 LTs performed by the end of 2016. Out of 64 patients referred to our center with BDI after cholecystectomy only four underwent LT for secondary biliary cirrhosis, while the injuries were repaired by surgical operations or radiological and endoscopic approaches in the other cases. Another three patients were listed for LT to manage severe iatrogenic liver injuries occurring during HPB surgery.

The first case of life-saving LT performed by our institution has been described in detail elsewhere together with a full description of the surgical technique adopted[22]. A 46-year-old man was initially considered for a liver resection due to a giant symptomatic hepatic hemangioma arising from the caudate lobe with compression of the retrohepatic inferior vena cava (IVC), and thrombosis of the left and middle hepatic veins. An uncontrollable bleeding from the confluence of the suprahepatic veins occurred during the liver resection and a total hepatectomy with retrohepatic IVC resection after a venous-venous by-pass was carried out to overcome the hemodynamic instability. The extensive liver congestion excluded any attempt to proceed to an *ex-vivo* major hepatectomy, and a request for urgent LT was launched. A Dacron interposition prosthesis replaced the retrohepatic vena cava, and an end-to-side TPCS was performed between the recipient PV and the Dacron prosthesis. The LT was carried out with a side-to-side cavocaval anastomosis between the graft retrohepatic vena cava and the Dacron interposition graft. There were no postoperative complications, and the patient was discharged 26 d after LT.

The second patient was a 52-year-old woman referred to our center from another HPB tertiary center without a LT program. She had ALF resulting from a radiologically assisted hepatic artery embolization in a patient initially affected by bilobar intrahepatic calculosis treated by bile duct exploration and a Roux-en-Y hepaticojejunal anastomosis. Before referral, after surgical bile duct exploration an intrahepatic bleed occurred with a rapid deterioration of the patient's clinical status due to hemorrhagic shock. The CT scan showed a massive intrahepatic hematoma involving the right hepatic lobe and segment Ⅳ (Figure 1). After right hepatic artery embolization the bleeding stopped, but the patient developed severe ALF due to acute ischemic liver necrosis (Figure 2). After the patient was referred to our center, a conservative liver resection such as right extended hepatectomy was excluded because of the liver failure and the massive hepatic infarction extending to the left lobe. In our opinion, a liver resection could be a surgical option only when the hepatic infarction and necrosis is limited and liver function preserved, because any surgical or infectious complication after a major hepatectomy could represent a contraindication to proceed to LT. An urgent LT was planned and a liver graft from a deceased donor was immediately requested on a top priority basis from the Italian national organ sharing network. An AB0-compatible graft became available 16 h later, and the patient underwent LT. The intraoperative findings are summarized in Figure 3. Despite the huge right lobe hematoma extending to segment Ⅳ with signs of extrahepatic rupture, the hepatectomy was carried out with hemodynamic stability and a TPCS and a venovenous by-pass. The liver implant was performed in a piggy-back fashion, and a Roux-en-Y reconstruction carried out using the same intestinal loop created during the first surgery. The patient was transferred to the floor after two days spent in the ICU, discharged after 12 d, and alive three years after LT.

Another patient, a 42-year-old woman, was referred to our center the day after a complicated Whipple procedure for an ampullary adenoma with subsequent total pancreasectomy due to pancreatic fistula and hemoperitoneum. After surgical re-exploration patient was transferred to the ICU. Liver function tests, lactate, and her hemodynamic conditions continued to worsen and a CT scan showed massive liver necrosis with multiple abscesses excluding any attempt to proceed to a liver resection. A request for an urgent LT was

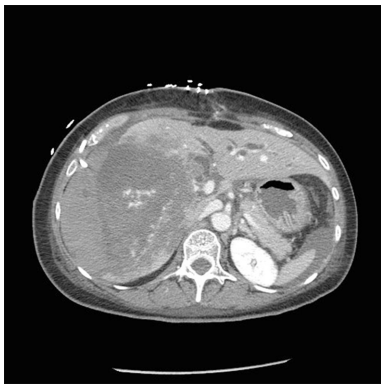

Figure 1 Computed tomography scan show a massive intrahepatic hematoma involving the right hepatic lobe and segment Ⅳ.

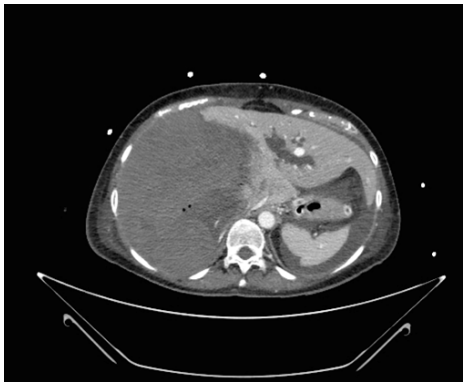

Figure 2 Computed tomography scan show the ischemic liver necrosis after the right hepatic artery embolization.

launched, and a compatible donor was available eight hours later. Recipient laparotomy revealed massive intestinal necrosis, and complete hepatic artery and PV thrombosis. These findings, associated with severe MOF and hemodynamic instability, made the indication for LT impracticable and futile. Unfortunately, the patient failed to overcome MOF and the available liver graft was connected to oxygenated hypotermic machine perfusion after 12:15 h of static cold storage before the transplant in a back-up recipient[23].

SURGICAL CONSIDERATIONS

Urgent LT to solve severe iatrogenic liver injuries may represent a surgical challenge requiring technically difficult and time-consuming procedures. Although a TPCS improves hemodynamic stability during LT, its role is still controversial and its use has remained limited since the technique was recommended in the early 1990s for recipients with portal hypertension caused by acute or subacute liver failure expected not to have adequate portosystemic collaterals[24]. A total hepatectomy and subsequent LT could be a useful strategy for patients presenting massive ischemic liver or exsanguinating hepatic injuries with uncontrollable vascular or parenchymal bleeding. In addition, urgent

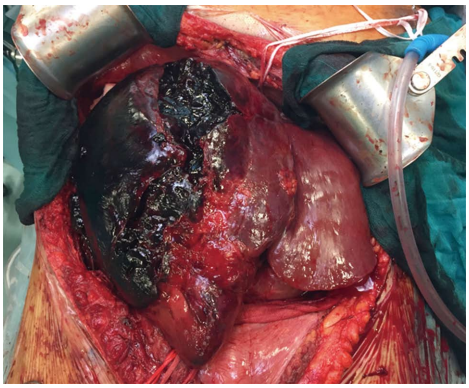

Figure 3 Intraoperative findings: A huge right lobe hematoma extended to segment Ⅳ with signs of extrahepatic rupture.

total hepatectomy and a TPCS may be performed awaiting a compatible deceased liver donor, or in the event of "toxic hepatic syndrome" secondary to massive hepatic necrosis. It is well known that total hepatectomy might improve the metabolic, coagulation and hemodynamic profiles of these patients while waiting for a suitable liver donor[21,25].

From a surgical point of view, portal blood could be shunted to the systemic circulation performing an end-to-side anastomosis between the main PV and the anterior wall of the anterior surface of the suprarenal IVC or performing a portosuprahepatic anastomosis[26].

Alternatively, an extracorporeal portocaval shunt-catheter connecting the PV to the femoral vein can be applied as described by the Munich transplant group[27] who reported the feasibility of this shunt technique, which does not require anticoagulation or an additional pump supply.

A venovenous by-pass may represent another possible option especially when a patient becomes hemodynamically unstable after a massive bleed and resection of the IVC required as previously reported by our Institution[22].

Vascular reconstruction in patients with severe iatrogenic injuries of hepatic hilum elements could be challenging, and extra-anatomical reconstruction with the use of arterial conduits remains an important tool in the transplant surgeon's armamentarium. Banked or freshly procured vascular grafts from deceased donors should be considered for supraceliac or infrarenal aortohepatic conduits.

The use of aortohepatic conduits using deceased donor iliac artery as an interpositional graft in LT have already been investigated and recently reviewed[22-30].

In addition to deceased arterial grafts, the use of cryopreserved arterial grafts as conduits has been recently proposed in living donor LT[31].

A recently published paper by Hibi *et al*[32] advised proceeding with caution in primary adult LT, where the placement of an aortohepatic conduit should be strictly limited because of the greater risk of late hepatic artery thrombosis and impaired graft survival.

Nevertheless, the use of arterial conduits could provide the only alternative option for graft vascularization during LT after severe iatrogenic injury of the hepatic artery. Baylor's group recently published their center experience after twenty years' follow-up of PV conduits in LT[33]. More than two thousand adult LTs were evaluated. All PV conduits were the donor's iliac vein procured during liver retrieval. PV conduits were required during the first LT in 35/2370 patients (1.5%). Long-term graft survival after LT using PV conduits was excellent and comparable to that of the control group (65% with the conduit *vs* 66% without the conduit at five-year follow-up, 58% *vs* 51% at ten years, and 48% *vs* 35% at 15 years). The authors reported excellent long-term results proving the longevity of the PV conduits using the donor's iliac vein. The reported results may also be applicable to other complex surgical settings such as severe iatrogenic vascular injuries requiring LT.

Resection and replacement of the IVC could occasionally be required during LT for severe iatrogenic injury of the liver or the vena cava. A variety of reconstruction strategies and materials including biological (autologous and heterologous) and synthetic grafts such as polytetrafluoroethylene (PTFE) and polypropylene (Dacron) have been reported to replace the vena cava[22]. Pulitanò *et al*[34] recently highlighted some important technical aspects in the use of biological tissues for IVC replacement. They reported advances in the use of glutaraldehyde-treated bovine pericardium and an autogenous peritoneo-fascial graft from a flap of parietal peritoneum backed by the posterior rectus sheath as alternatives to prosthetic IVC reconstruction. After 32 IVC reconstructions, the authors claimed that biological grafts allow greater flexibility and biocompatibility and long-term patency without permanent anticoagulation.

As previously mentioned for arterial and PV reconstructions, especially in LT centers, the use of cryopreserved banked or freshly procured venous allografts from deceased donors offers an option in IVC replacement. The use of allografts was first described long ago by Starzl *et al*[29] and is still common practice in the field of LT[28,29,35].

TIMING OF REFERRAL AND THE ROLE OF TERTIARY CENTERS

HPB surgery has had an extraordinary evolution and diffusion in recent years thanks to the success in reducing mortality and morbidity rates[36], especially in high-volume centers. A debate is ongoing on the need for centralization of complex surgery in tertiary referral centers. Clinicians are constantly reminded about the importance of early referral for patients with severe iatrogenic liver injuries to a tertiary center with experienced HPB and transplant surgery. Patients initially and repeatedly treated in non-specialist hospitals and referred for LT in the ALF setting have been reported to have worse outcomes[4].

The role of surgical experience in the repair process has been widely explored and demonstrated in the past[37]. In 2008, Silva *et al*[38] from the Queen Elisabeth Hospital, United Kingdom reported their experience as a specialist outreach service for on-table repair for iatrogenic BDI after laparoscopic cholecystectomy. They highlighted the role of this new kind of "travelling surgeon" reporting repeatable outcomes with no post-operative mortalities in 22 procedures avoiding transfer of the patient to a tertiary center, prolonged bile drainage, and a reoperation with a shorter hospital stay and a reduced risk of sepsis and liver failure. They also claimed that the proposed immediate approach has potential medicolegal advantages reducing the risk of litigation and costs.

Our experience highlighted the crucial role of a liver transplant program when referring a patient with complex and severe injuries after HPB surgery because LT may represent the patient's only curative option in a small number of cases.

FUTURE PERSPECTIVES

The literature lacks reports on severe iatrogenic liver injuries, likely because negative outcomes tend to be under-reported, and we have no information on those patients with severe iatrogenic liver injuries who died before referral to a tertiary center. This is detrimental to surgical education, and the topic was recently voiced by Cheah *et al*[39] who discussed improvement in care by close examination of "near-miss" cases.

Reported experiences on the place of LT in the treatment of severe iatrogenic injuries indicate few LTs are performed in this uncommon setting. Without an official comprehensive registry, it is exceedingly difficult to determine appropriate indications and long-term outcomes as detailed data are confined to individual case reports in the literature.

All the clinicians involved in the care of patients with severe iatrogenic liver injuries should clearly spell out information on their outcomes honestly and swiftly so that others can learn a lesson and not repeat the same errors.

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ORIGINAL ARTICLE

Basic Study

Novel synthetic adhesive as an effective alternative to Fibrin based adhesives

Pramod Kadaba Srinivasan, Vera Sperber, Mamdouh Afify, Hirokazu Tanaka, Kenji Fukushima, Babette Kögel, Felix Gremse, René Tolba

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Institutional review board statement: The governmental care and use committee (LANUV), Recklinghausen, NRW, Germany, granted official permission.

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Abstract

AIM

To compare a novel, fully synthetic, polyurethane based glue (MAR-1) to fibrin sealant in a partial liver resection rat model.

METHODS

After 50% resection of the lateral left liver lobe in male Wistar rats (n = 7/group/time point), MAR-1, Fibrin or NaCl was applied. After 14, 21 and 90 postoperative days, sealant degradation, intra-abdominal adhesions were scored, and histological examination of liver tissue was performed.

RESULTS

(Mean ± SEM) (MAR-1 *vs* Fibrin *vs* NaCl). Bleeding mass was significantly higher in NaCl (3.36 ± 0.51 g)

compared to MAR-1 (1.44 ± 0.40 g) and Fibrin (1.16 ± 0.32 g). At 14 and 90 d, bleeding time was significantly lower in MAR-1 (6.00 ± 0.9 s; 13.57 ± 3.22 s) and Fibrin (3.00 ± 0.44 s; 22.2 ± 9.75 s) compared to NaCl (158.16 ± 11.36 s; 127.5 ± 23.3 s). ALT levels were significantly higher in MAR-1 (27.66 ± 1 U/L) compared to Fibrin (24.16 ± 0.98 U/L) and NaCl (23.85 ± 0.80 U/L). Intrabdominal adhesions were significantly lower in MAR-1 (11.22% ± 5.5%) compared to NaCl (58.57% ± 11.83%). Degradation of the glue was observed and MAR-1 showed almost no traces of glue in the abdominal cavity as compared to the Fibrin (10% ± 5% 14 d; 7% ± 3% 21 d). Survival showed no significant differences between the groups.

CONCLUSION

Compared to Fibrin, MAR-1 showed similar hemostatic properties, no adverse effects, and is biocompatible. Further studies on adhesion strength and biodegradability of synthetic sealants are warranted.

Key words: MAR-1; Fibrin; Liver resection; Hemostasis; Polyurethane

Core tip: This study evaluates the effectiveness of a novel, polyurethane based, surgical adhesive on a liver resection model. This study will further help in better sealing of wounds in a trauma model in comparison to Fibrin glue.

Srinivasan PK, Sperber V, Afify M, Tanaka H, Fukushima K, Kögel B, Gremse F, Tolba R. Novel synthetic adhesive as an effective alternative to Fibrin based adhesives. *World J Hepatol* 2017; 9(24): 1030-1039 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i24/1030.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i24.1030

INTRODUCTION

Hemorrhage due to traumatic injury is one of the leading causes of death worldwide. It is estimated that hemorrhage is responsible for more than 35% of pre-hospital mortality and 40% of mortality in the first 24 h[1]. In case of abdominal trauma, the liver is one of the most commonly injured organs; anatomical position and its delicate parenchyma make it susceptible to injury and hemorrhage[2]. Despite modern surgical techniques, management of hemorrhage after liver trauma still remains a challenge, with major liver trauma resulting in high morbidity and mortality rates[3]. Furthermore, surgeries involving liver resection are known to be of high risk; nevertheless, it is the one of the curative treatment options for hepatocellular cancer patients[4].

Management of liver injury has progressed tremendously in the last three decades[2]. Advancement in biotechnological research has resulted in a variety of hemostatic agents[5]. These hemostatic agents are either biological or synthetic in nature[5]. They are based on components including cellulose, collagen, glutaraldehyde, fibrin, and dihydroxyacetone[5-7]. Fibrin sealants (also known as fibrin adhesive or glue) are the most widely used hemostatic agents as a complimentary adjunct in various surgical procedures. Fibrin sealants comprise of two components, human-thrombin and fibrinogen, usually plasma derived[5]. During application, these two components interact to form a stable fibrin clot[5]. However, most require 2 ℃-8 ℃ storage, extensive preparation, and, once taken out of refrigeration, have to be used within 9 h[6]. Notably, fibrin sealants are less effective in events of strong bleeding, as they can be washed away with blood or other liquids and there is a risk of re-bleeding, due to fibrin sealants' limited sealing strength[8,9]. Due to their biological origin, fibrin sealants are associated with risk factors including immune reactions, viral transmission, and potential embolism risk[6,10,11].

In the early forties, cyanoacrylate based glues were marketed under brand names, such as, Superglue and Krazy glue. Cyanoacrylate glues are neither biocompatible nor bioabsorbable[12]. Additionally, upon degradation, cyanoacrylates form cyanoacetate and formaldehyde, which are toxic to humans[5,12]. Other options for synthetic products include urethane based polymers, such as polyurethane. Polyurethanes (PUs) are known for their tensile strength of 4-60 MPa; thus, making them highly elastic[13]. Research has shown that several factors, such as hydrolysis and enzymatic action, contribute to their degradation[14]. Because of their non-biological components, there is no risk of virus transmission or antigenic reaction like with fibrin based adhesives.

The aim of this study was to evaluate MAR-1, determining hemostatic properties, functionality, and prevention of intra-abdominal adhesions, tissue compatibility as well as biodegradation. In comparison, we tested the clinically used fibrin sealant Beriplast® P (CSL Behring GmbH, Marburg, Germany) and Sodium Chloride (NaCl) as a control solution.

MATERIALS AND METHODS

MAR-1 and Fibrin sealant

MAR-1 is a polyurethane based sealant that consists of two different components: A isocyanate-functional polyester-ether pre-polymer and an amino-functional asparagine acid ester. This adhesive technology and its polyaddition reaction are well-known.

The two components were stored at 22 ℃ in a double chamber syringe and combined upon application (Adhesys Medical GmbH, Aachen, Germany). The Fibrin sealant used was the commercially available Beriplast® P (CSL Behring GmbH, Marburg, Germany), which consists of fibrin and thrombin mixed prior to application.

Animals and surgical procedure

All experiments were conducted in accordance with German Federal Law regarding the protection of animals and the DIRECTIVE 2010/63/EU on the protection of animals used for scientific purposes. The Guide for the care and use of laboratory animals (8th edition, NIH Publication, 2011, United States) was also followed. The governmental care and use committee (LANUV), Recklinghausen, NRW, Germany, granted official permission. Male Wistar rats weighing between 200-260 g were used. The animals were housed in Type 2000 rat filter top cages (Tecniplast, Hohenpreisenberg, Germany) under specific pathogen free (SPF)-conditions according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines (www.felasa.eu), in a temperature (22 ℃) and humidity controlled environment (55% relative humidity) with a 12-h light/dark cycle and allowed food (standard rat diet, Ssniff-Spezial Diäten GmbH, Soest, Germany) and water ad libitum.

Sixty-three rats were randomly allocated to the following groups: MAR-1, Fibrin and NaCl. The groups were further classified into three time points: 14 d, 21 d, and 90 d. Rats received general anesthesia by inhalation of 1.5% isoflurane (Abbott GmbH and Co.KG, Wiesbaden, Germany) and administration of 0.1 mg/kg body weight Buprenorphine (Temgesic®, Essex Pharma GmbH, Munich, Germany) subcutaneously as analgesic. For perioperative anti-biotic prophylaxis, rats received 16 mg/kg bodyweight Cefuroxime s.c. (Fresenius SE and Co. KGaA, Homburg, Germany). Using a vessel loop for compression, 30% of the left lateral lobe was removed, and sealant was applied in an amount sufficient to cover the wound area. Pre-weighed gauze was placed under the liver lobe prior to resection. Post resection, the blood absorbed by the gauze was weighed and subtracted from the pre-weight of the gauze to calculate the bleeding mass. The animals were euthanized under anesthesia after 14, 21 and 90 d respectively.

μCT to visualize the biodegradation

μCT data was measured using Tomoscope 30 s Duo (CT-Imaging GmbH, Erlangen, Germany) using a protocol (HQD-6565-90-360) that took 720 projections (1032 × 1012 Pixel) in 90 s during one rotation with radiation dose of 421 mGy[15]. Several sub-scans were taken and reconstructed using a Feldkamp algorithm with a voxelsize of 70 μm × 70 μm × 70 μm and were assembled into one volume data set. Volumetric image data was analyzed and visualized using the Imalytics Preclinical Software[16].

Histological evaluation

Tissue samples of the liver were collected at the time when the rats were euthanized. The samples were immediately fixed in 4% neutral buffered formalin (Roti®-Histofix 4%, Roth, Karlsruhe-Germany), and then were shaken overnight on a shaker (Lab net, International Inc., United States). The specimens were processed in grading series of alcohol and xylene, embedded in paraffin and sectioned at 4-6 μm thin slices using a microtome and were stained with hematoxylin and eosin (H and E). Paraffin-embedded liver sections were used for H and E staining and analysed using a Leica DM 2500 microscope (Leica, Bensheim, Germany).

Immunohistochemistry was performed as per manufacturer's instructions. CD68 macrophages were identified by a 1:50 mouse monoclonal antibody from Dako (Glostrup, Denmark), pre-treatment of the fixed specimen with microwave three times, citrate-buffer pH 6, and as secondary antibody rabbit anti-mouse 1:300 from Dako (Glostrup, Denmark).

Serum analyses and hematology

Serum was withdrawn at 14, 21 and 90 d post operation and analyzed with a clinical chemistry analyzer (Ortho Clinical Diagnostics GmbH, Neckar-gemünd, Germany). Liver enzymes, ALT and AST were measured from serum. In addition, blood count of leukocytes (10^3/μL), erythrocytes (10^6/μL), platelets (10^3/μL), and hemoglobin were measured using the MEK6450K automatic cell counter (Nihon Kohden, Rosbach, Germany).

Statistical analysis

Statistical review was performed by Professor René Tolba. All results are expressed as mean ± SEM and the data was analyzed by Graph Pad Prism® Version 5 (Graph Pad, San Diego, CA, United States). Significance between different groups was measured with one-way analysis of variance (ANOVA) and posttest: Tukey-Kramer. Survival analysis was carried out by Kaplan-Meier curve and Mantel-Cox test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Bleeding mass

Bleeding mass (Figure 1) was assessed in order to record the amount of blood lost after liver resection. After 21 d, NaCl (3.36 ± 0.51 g) showed significantly higher levels of blood loss in comparison to MAR-1 (1.44 ± 0.40 g) and Fibrin (1.16 ± 0.32 g) treated animals. However, there were no significant differences between the animals in 14 d (MAR-1: 2.08 ± 0.30 g; Fibrin: 1.02 ± 0.29 g; NaCl: 1.02 ± 0.29 g) and 90 d (MAR-1: 2.21 ± 0.44 g; Fibrin: 2.03 ± 0.28 g; NaCl: 3.04 ± 0.50 g) group.

Bleeding time

Duration of blood loss (Figure 2) was recorded to evaluate the bleeding time in different groups. NaCl (158.16 ± 11.36 s) (127.5 ± 23.3 s) showed significantly higher bleeding times on 14 and 90 d in comparison to MAR-1

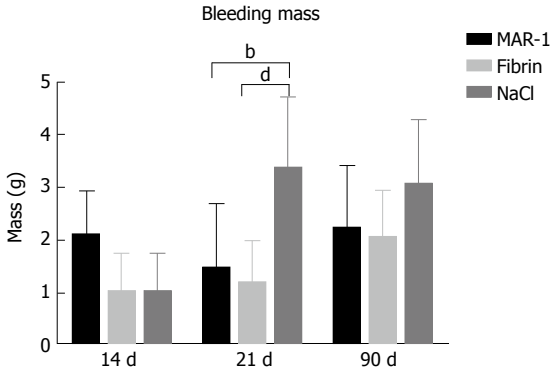

Figure 1 Amount of blood loss was measured at three different time points. $^{b}P < 0.001$ MAR-1 *vs* NaCl; $^{d}P < 0.01$ Fibrin *vs* NaCl in 21 d group ($n = 7$).

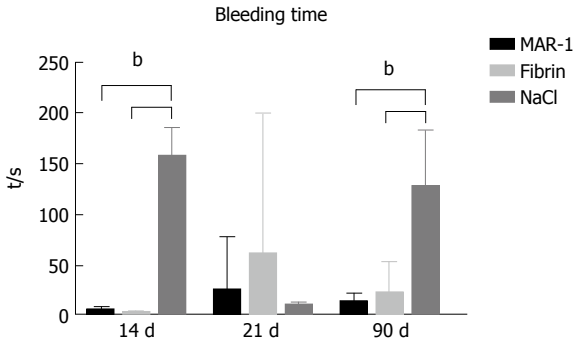

Figure 2 Bleeding time was recorded after liver resection. $^{b}P < 0.001$ MAR-1 and Fibrin *vs* NaCl in both 14 and 90 d group ($n = 7$).

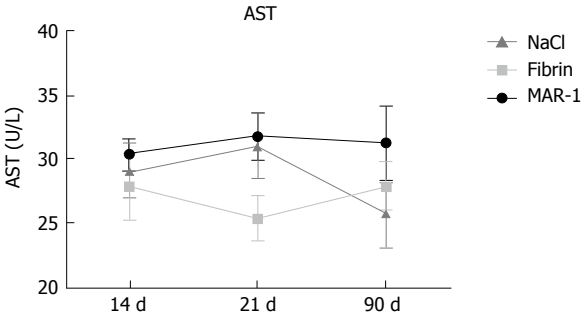

Figure 3 Aspartate transaminase release was measured after 21 and 90 post-operative days ($n = 7$). AST: Aspartate transaminase.

(6.0 ± 0.9 s) (13.57 ± 3.22 s) and Fibrin (3.0 ± 0.44 s) (22.2 ± 9.75 s) groups respectively. However, the groups showed no significance at 21 d time point (MAR-1: 25.33 ± 17.53 s; Fibrin: 61.16 ± 56.77 s; NaCl: 10.71 ± 1.19 s).

Aspartate transaminase

Aspartate transaminase (Figure 3) was measured as parameter for liver injury. There were no significant differences noticed in the groups at 14 (MAR-1: 30.37 ± 1.23 U/L; Fibrin: 27.83 ± 2.54 U/L; NaCl: 29.16 ± 2.12 U/L), 21 (MAR-1: 31.77 ± 1.80 U/L; Fibrin: 25.33 ± 1.70 U/L; NaCl: 31.00 ± 2.46 U/L) or 90 d (MAR-1: 31.28 ± 2.86 U/L; Fibrin: 27.90 ± 1.86 U/L; NaCl: 25.83 ± 2.71 U/L).

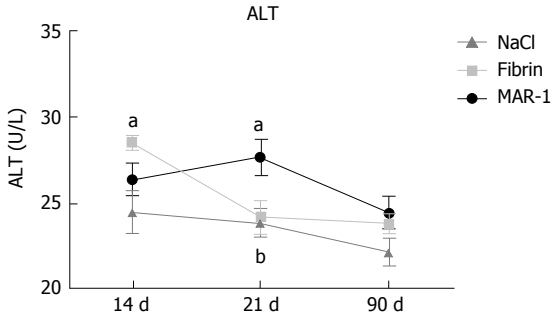

Figure 4 Alanine transaminase release was measure after 14, 21 and 90 post-operative days. $^{a}P < 0.05$ Fibrin *vs* NaCl after 14 d; $^{a}P < 0.05$ MAR-1 *vs* Fibrin; $^{b}P < 0.01$ MAR-1 *vs* NaCl after 21 post-operative days ($n = 7$). ALT: Alanine transaminase.

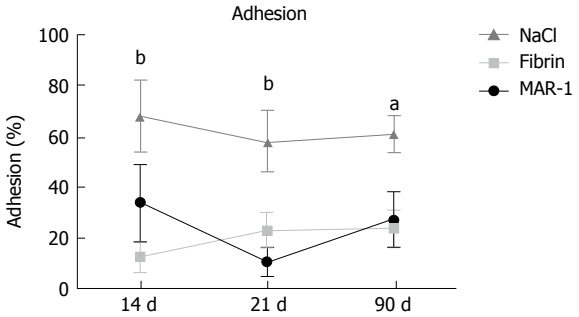

Figure 5 Percentage of tissue adhesion after 14, 21 and 90 post-operative days was tabulated. $^{b}P < 0.01$ Fibrin *vs* NaCl after 14 d, $^{b}P < 0.01$ MAR-1 *vs* NaCl after 21 d; $^{a}P < 0.05$ Fibrin *vs* NaCl after 90 d ($n = 7$).

Alanine transaminase

Alanine transaminase (Figure 4) release was measured as a parameter for liver parenchymal damage. Significant differences between the treatment groups were seen after 14 and 21 post-operative days. Fibrin (28.5 ± 0.42 U/L: 14 d) (24.16 ± 0.98 U/L: 21 d) showed a significantly higher release of ALT compared to NaCl (24.5 ± 1.23 U/L: 14 d) (23.85 ± 0.80 U/L: 21 d) group after 14 d. Meanwhile, MAR-1 (26.37 ± 0.92 U/L: 14 d) (27.66 ± 1 U/L: 21 d) showed significantly higher levels after 21 d in comparison to both NaCl and Fibrin treated animals.

Adhesions

Intra-abdominal adhesions (Figure 5) were visualized and the extent of adhesions was evaluated. After 14 d, Fibrin (13.33% ± 6.1%) treated animals showed significantly lower percentage of adhesions in comparison to NaCl (68.33% ± 14.24%). MAR-1 (11.22% ± 5.5%) showed significantly lower adhesion compared to NaCl (58.57% ± 11.83%) after 21 d. After 90 d, Fibrin group (24% ± 7.29%) showed significantly lower levels of adhesions compared to NaCl group (61.66% ± 7.03%). Whereas, there were no significant differences found between Fibrin and MAR-1 groups at any given time point.

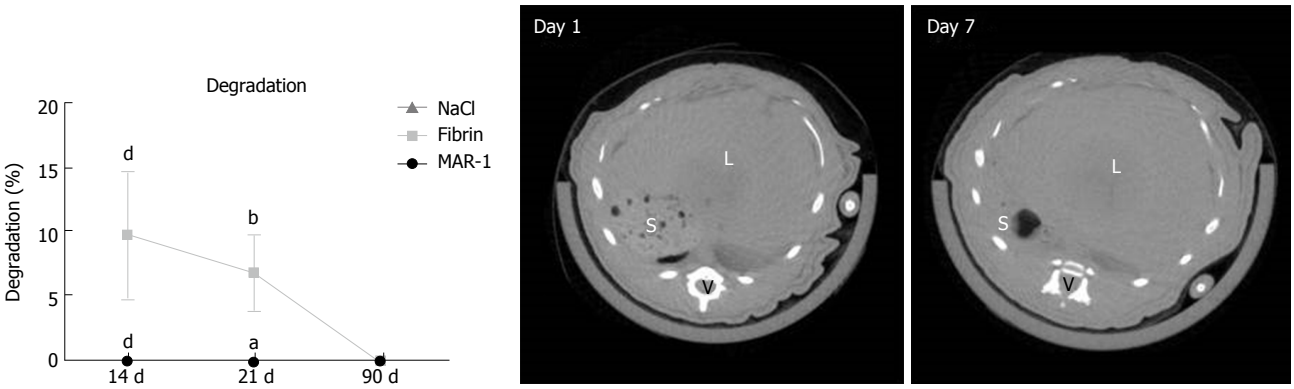

Figure 6 µCT scans of MAR-1 rat on day 1 and day 7. Percentage of adhesive degradation was evaluated after 14, 21 and 90 post-operative days. $^{d}P < 0.001$ Fibrin *vs* MAR-1 and NaCl after 14 d; $^{b}P < 0.01$ Fibrin *vs* MAR-1; $^{a}P < 0.05$ NaCl *vs* Fibrin 21 d ($n = 7$). L: Liver, S: Stomach, V: Vertebra.

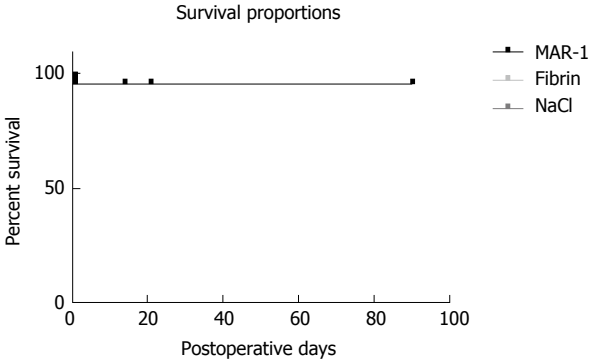

Figure 7 Survival proportions between the treatment groups were calculated during 14, 21 and 90 post-operative days. $P = 0.9906$ as per Mantel-Cox test ($n = 7$).

Degradation

µCT scans were performed on day 1 and day 7 to visualize the glue. Interestingly, due to its hydrogel like properties, the glue could not be distinguished from the liver tissue in the µCT images (Figure 6). Degradation of MAR-1 and Fibrin were noted and compared to NaCl treatment. MAR-1 (0% ± 0% at all time points) and NaCl (0%) were absent or negligible compared to Fibrin (10% ± 5% 14 d; 7% ± 3% 21 d; 0% 90 d). Fibrin glue levels were significantly higher compared to MAR-1 and NaCl groups after 14 and 21 d. Fibrin glue was completely metabolized after 90 d.

Survival rate

Percentage survival (Figure 7) was calculated for each treatment group. MAR-1 showed a survival percentage of 95.83% in comparison to Fibrin with 95.65% and NaCl with 95%. As per Mantel-Cox test, the *P* value was 0.9906 and there was no statistical significance seen between MAR-1 and other the groups.

Histopathology

Histopathological evaluation (Figure 8) was performed on the tissue section after 90 post-operative days. There was a slight inflammation due to foreign body reaction in both MAR-1 and Fibrin groups. The reaction zone showed granulation tissue along with some collagen structures. A dense collagenous fibrotic tissue along with histiocytic inflammation was noticed. Whereas, in MAR-1 and Fibrin treated animals inflammation was noticed initially; however, the reaction was absent after 90 d. In case of NaCl treated animals, a thicker liver capsule was seen and occasional inflammation due to bleeding remnants.

CD68

Immunohistochemical staining is an ideal tool to identify the presence of CD68 positive cells (Figure 9). It specifically stains macrophages as well as Kupffer cells, Giant cells, and Monocytes. This helps in recognizing cell proliferation in tissues. The CD68 cell count at 14 d (8.6 ± 1.0, 9.0 ± 1.0 AU, 6.8 ± 0.8 AU), 21 d (5.4 ± 0.6 AU, 5.6 ± 0.67 AU, 2.4 ± 1.0 AU), and 90 d (1.6 ± 0.5 AU, 2.4 ± 0.6 AU, 2.4 ± 1.0 AU) showed no significant differences within the groups.

Elastic van Gieson

Elastic van Gieson staining (Figure 10) protocol specifically stains elastic fibers, which helps in differentiating between normal and pathological elastic fibers. Due to the chemical reaction in the staining process, the elastic fibers and cell nuclei are stained black, collagen fibers are stained red, and other tissue elements including cytoplasm are stained yellow. We noticed the width of the reaction zone along with the proliferative tissue reduced with time and there were no significant changes noticed in the structural integrity.

Hematological parameters

Leucocytes, Erythrocytes, Hematocrit, and Platelets were measured and the groups showed no significant differences (Table 1). However, Hemoglobin levels (Table 1) were measured in all the groups. There was a significant difference noted between Fibrin (11.69 ± 0.21 g/dL) and NaCl groups (12.48 ± 0.17 g/dL) at baseline level. Whereas, MAR-1 (12.09 ± 0.29 g/dL) showed significantly lower haemoglobin levels compared to NaCl group (13.68 ± 0.26 g/dL).

Table 1 Leucocytes in 103/μL; Erythrocytes in 106/μL; Hemoglobin in g/dL; Hematocrit in %; Platelets in 103/μL; (mean ± SEM); 1-way ANOVA, Posttest: Tukey Kramer

		MAR-1	Fibrin	NaCl	*P* value
Leucocytes	0 d	7.48 ± 0.33	6.82 ± 0.30	7.26 ± 0.39	NS
	14 d	6.78 ± 0.66	7.18 ± 0.67	8.65 ± 0.75	NS
	21 d	7.56 ± 0.75	7.20 ± 0.72	6.23 ± 0.50	NS
	90 d	6.11 ± 0.78	5.76 ± 0.52	5.41 ± 0.32	NS
Erythrocytes	0 d	5.71 ± 0.09	5.68 ± 0.07	5.68 ± 0.08	NS
	14 d	6.07 ± 0.17	6.48 ± 0.20	6.03 ± 0.18	NS
	21 d	6.33 ± 0.14	7.03 ± 0.10	6.50 ± 0.17	NS
	90 d	7.58 ± 0.19	7.51 ± 0.12	7.72 ± 0.17	NS
Hemoglobin	0 d	12.29 ± 0.14	11.69 ± 0.21[a]	12.48 ± 0.17[a]	[a]$P < 0.05$
	14 d	12.09 ± 0.29[b]	13.13 ± 0.43	13.68 ± 0.26[b]	[b]$P < 0.01$
	21 d	13.08 ± 0.22	13.84 ± 0.28	14.17 ± 0.28	NS
	90 d	14.14 ± 0.33	13.88 ± 0.18	13.90 ± 0.29	NS
Hematocrit	0 d	35.09 ± 0.48	34.59 ± 0.30	34.05 ± 0.42	NS
	14 d	35.66 ± 0.96	38.30 ± 0.94	35.13 ± 0.50	NS
	21 d	35.50 ± 0.76	36.98 ± 0.62	36.31 ± 1.02	NS
	90 d	39.69 ± 0.91	39.26 ± 0.55	39.75 ± 0.94	NS
Platelets	0 d	923 ± 32	960 ± 42	998 ± 30	NS
	14 d	907 ± 67	985 ± 63	1035 ± 34	NS
	21 d	1104 ± 50	1024 ± 57	970 ± 41	NS
	90 d	924 ± 42	874 ± 25	916 ± 48	NS

[a]$P < 0.05$; [b]$P < 0.01$.

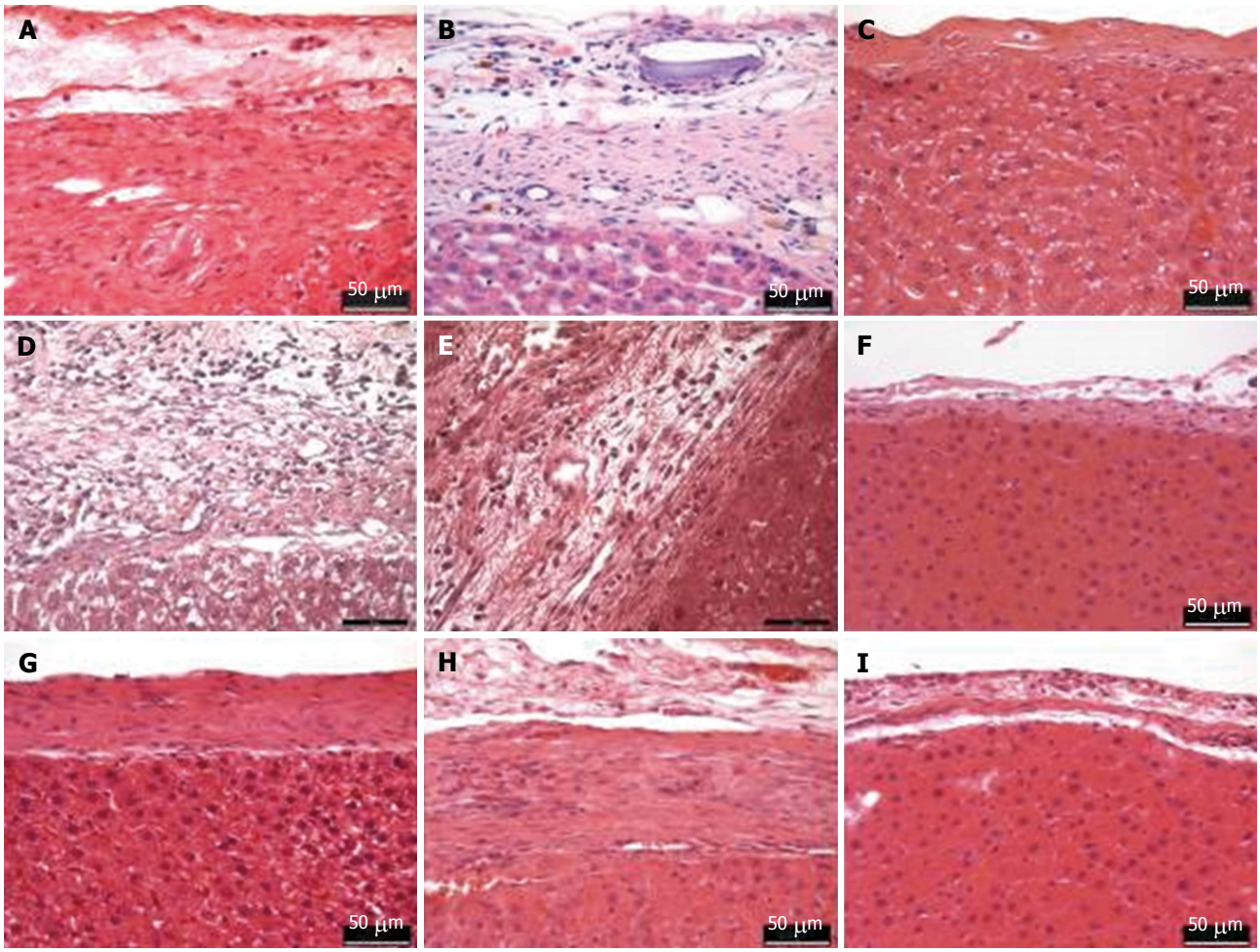

Figure 8 Histopathological evaluations of H and E stained liver tissue section shows the resected area and structural intergrity at different time points, MAR-1 (A: 14 d, B: 21 d, C: 90 d); Fibrin (D: 14 d, E: 21 d, F: 90 d); NaCl (G: 14 d, H: 21 d, I: 90 d).

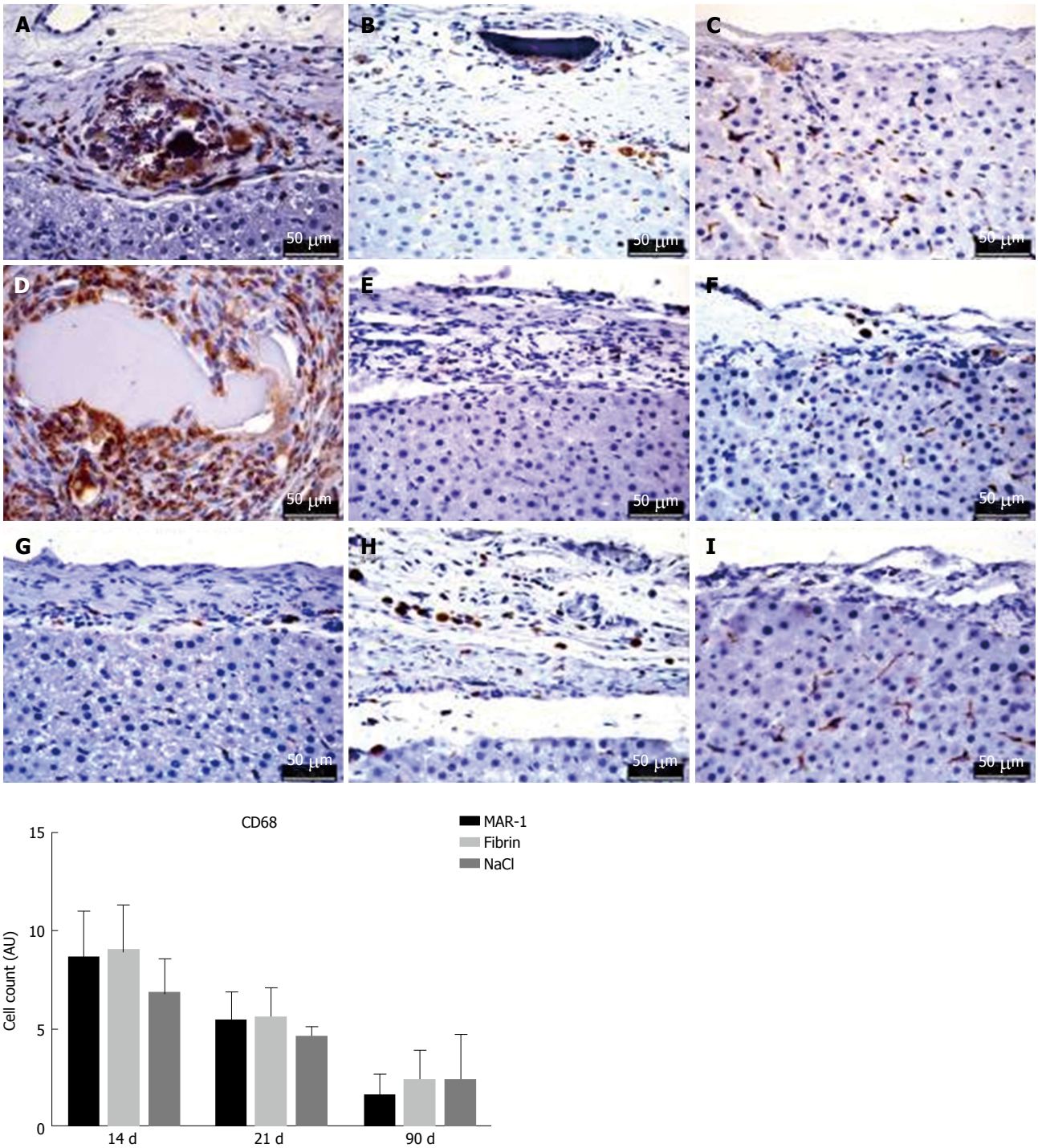

Figure 9 Immunohistochemical staining for CD68 shows a few darkly stained positive cells. The graph represents the CD68 positive cell count with no significant differences between the groups, MAR-1 (A: 14 d; B: 21 d; C: 90 d); Fibrin (D: 14 d, E: 21 d, F: 90 d); NaCl (G: 14 d, H: 21 d, I: 90 d).

DISCUSSION

According to WHO 2010 database, 5.8 million deaths due to injuries were recorded worldwide[17]. A quarter of these were due to trauma and hemorrhagic shock due to injuries; thus, making it a leading cause of death across the globe[1,17]. Liver injury is most commonly observed in abdominal trauma cases[18]. Apart from trauma, liver resection in hepatocellular carcinoma patients carries a high risk of hemorrhage[19]. Hemorrhage during liver surgery is directly associated with extensive use of vascular occlusion techniques, which leads to post-operative complications and eventually hepatic failure[19]. During liver surgery, it is vital to minimize bleeding, especially from small blood vessels of liver parenchyma, in order to prevent intraoperative blood loss and to better visualize the surgical field[19].

In this study, we compared the efficacy, haemostatic properties, and biocompatibility of a novel, polyurethane based synthetic adhesive, MAR-1, with that of

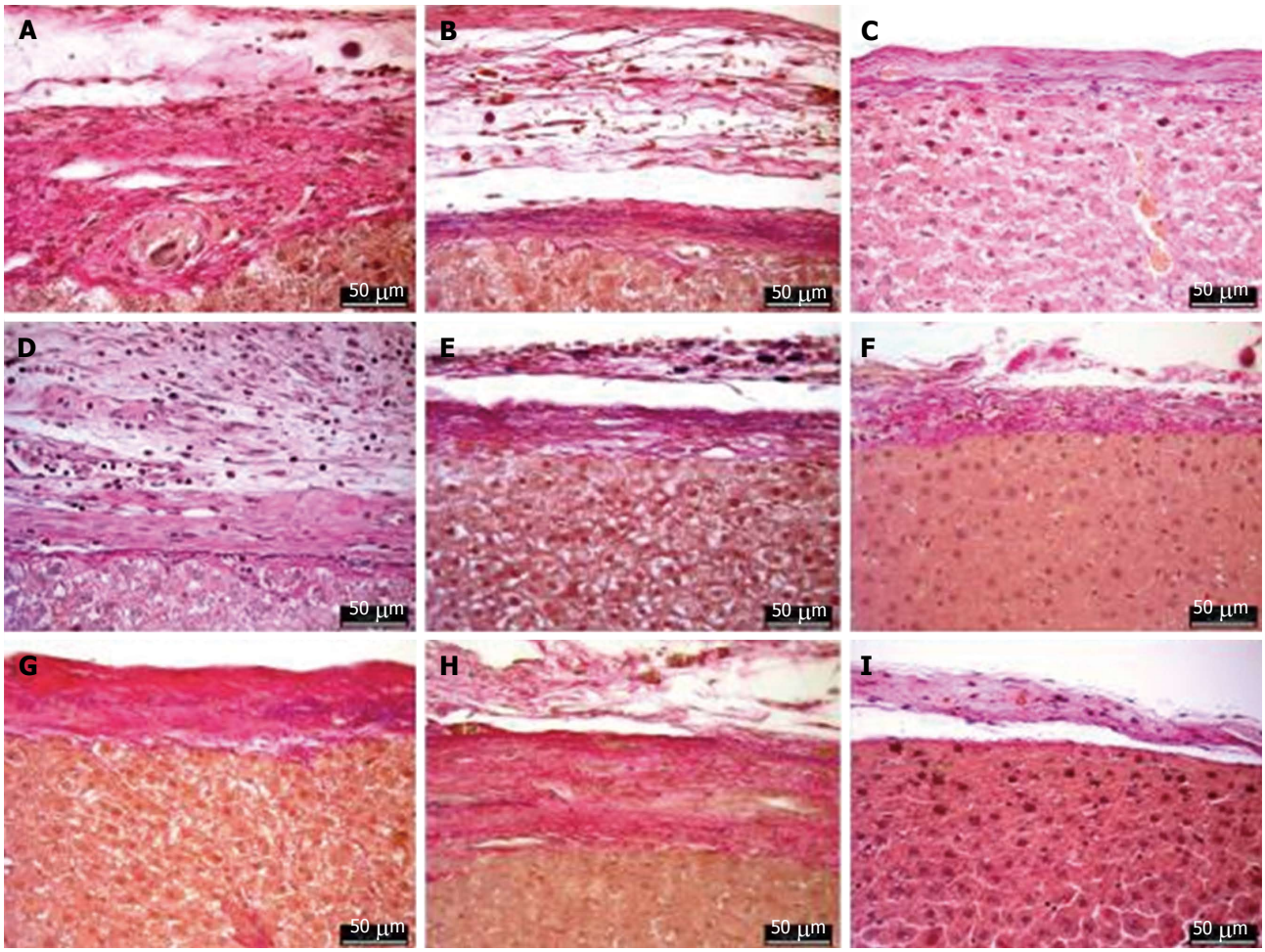

Figure 10 Elastic van Gieson staining shows the resected area and proliferative tissue, MAR-1 (A: 14 d, B: 21 d, C: 90 d); Fibrin (D: 14 d, E: 21 d, F: 90 d); NaCl (G: 14 d, H: 21 d, I: 90 d).

Fibrin, which is a clinically used medical adhesive.

Fibrin sealants mimic the coagulation cascade, which depends on various factors such as enzymes, proteins, and co-factors[20]. Polyurethane-based adhesives mainly react with the amino groups of proteins in the tissue, which enables the formation of urea linkages and eventually adhesion[12]. Polyurethanes are known to activate platelets, which enhances the blood clotting process[21]. Moreover, polyurethanes have demonstrated strong thrombogenic properties due to their hydrophobic nature, this promotes the proteins to adhere and initiate the coagulation cascade[12]. We measured the bleeding mass and time to assess the capacity of these sealants to stop bleeding after liver resection. The results showed no significant differences between the two sealants and the results were comparable. However, we noticed a significant difference between MAR-1, Fibrin and NaCl groups, this clearly showed the effectiveness of a sealant in minimizing blood loss, thereby reducing the bleeding time. On the other hand, liver parenchymal enzymes, AST and ALT, were measured and we noticed no significant changes in AST levels throughout the time course; whereas, a significant increase in ALT levels were seen in MAR-1 group after 21 d, in comparison to Fibrin and NaCl groups. AST and ALT levels are routinely measured to assess the functionality of liver and their ratio between the concentrations is of clinical relevance. AST/ALT ratio of 2:1 or more is considered as a sign of liver damage. The elevated ALT levels in the MAR-1 group was probably due to repeated manipulation of the liver lobe during the surgical procedure. Nevertheless, the values were within the physiological range and did not increase at a later time point.

Depending on the origin of thrombin in the fibrin sealants, severe immune reactions have been observed, leading to anaphylactic shock in some cases[22-24]. When extracted from human pooled blood, it carries a high risk of viral contamination[25,26]. Despite improved methods of viral inactivation[27], it still carries a risk of parvovirus infection[28]. Whereas, MAR-1, the polyurethane based adhesive, showed no adverse reaction in this study. Polyurethanes in general are considered biocompatible and biodegradable; they are polymers consisting of urethane links[13]. Research has

shown that polyurethanes containing biodegradable diisocyanates degrade into non-cytotoxic decomposition products[13,29,30]. After 14 d, the quantity of MAR-1 was either negligible or absent in the abdominal cavity, suggesting the rapid and efficient degradation of the glue. These results were significant in comparison to Fibrin glue, which was present even after 21 d. Nevertheless, both the glues were efficiently degraded by the end of 90 d. Meanwhile, it was difficult to visualize MAR-1 with the help of μCT, which can be attributed to its hydrogel like properties causing low contrast to the adjacent liver tissue. Studies have suggested that degradation of polyurethanes was mainly dependent on the polyester polyol composition[13,31,32]. Polyurethanes exhibit great versatility in their polymeric properties. Rapid degradation of MAR-1 proves its biocompatibility without any adverse effects. This also supported the previously established properties of polyurethanes such as toughness, durability, elasticity, biocompatibility, which is not achieved by any other available material[33].

Intra-abdominal adhesions are commonly noticed after abdominal surgery. Their incidence is estimated at 67%-93%, which affects the final outcome of the surgery[34]. When a foreign body is introduced into the abdominal cavity it leads to fibrosis and adhesion formation[35]. Demirel *et al*[36] showed that fibrin sealant drastically reduced adhesions in comparison to primary suture. In general, polyurethanes have been known to exhibit strong adhesion to the tissue[37], as mentioned earlier, their interaction with the amino acids results in the adhesion of the glue to the tissue[12]. We noticed the formation of adhesions during the time course; however, there were no significant differences between MAR-1 and Fibrin treated animals. However, significantly more adhesions were noticed in NaCl group compared to MAR-1 group. These results supports our hypothesis, which is the biocompatibility and non-inferiority of MAR-1 compared to Fibrin glue, the clinical gold standard. Furthermore, the survival rate showed no significant differences between the groups. Meanwhile, the histopathological examination revealed a few structural changes, however, the tissue sections failed to show any significant differences between the groups.

In summary, MAR-1 has been shown to be non-inferior to Fibrin in terms of effective and safe sealing of a liver in a resection model. Based on the obtained results, MAR-1 is biocompatible and showed no adverse effects. We agree that further research is needed to study the chemistry and biodegradability. Nevertheless, MAR-1 is ready to be used in its current form as a topical wound sealant. Moreover, due to the fully synthetic nature, there is no risk of increased immune reactions or viral transmission like with Fibrin.

ACKNOWLEDGMENTS

The authors thank Mr. Pascal Paschenda for his skillful technical assistance.

COMMENTS

Background

Despite advanced surgical techniques, hemostasis after liver trauma is a major cause for morbidity and mortality. Fibrin glue is the current gold standard for managing hemostasis; however, there are some disadvantages like production costs, allergic reactions, and storage conditions. In this study, the authors introduce a novel, polyurethane based, synthetic adhesive, which is biocompatible and controls bleeding effectively.

Research frontiers

Hemostasis in trauma and surgery is of prime importance, their study primarily focuses on management of blood loss during surgical and trauma procedures.

Innovations and breakthroughs

Novel polyurethane based adhesive, MAR-1, helps in managing blood loss effectively in rat partial liver resection model. This study shows variety of parameters, which plays an important role during traumatic situations.

Applications

Partial liver resection model in rats is an established model for liver trauma studies. Results from this study shows the effectiveness of fully synthetic, polyurethane based novel adhesive. This model provides all the necessary information to study the application of surgical adhesives.

Terminology

MAR-1: Medical adhesive revolution-1 is a novel polyurethane based adhesive; PU: Polyurethanes.

Peer-review

The manuscript is well-written and the data shown in the manuscript was understandable.

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LETTERS TO THE EDITOR

Lurking epidemic of hepatitis C virus infection in Iran: A call to action

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Abstract

Despite having a relatively low prevalence in the Iranian general population, the burden of hepatitis C virus (HCV) infection is on the rise, and hepatitis C is predicted to be the most important leading cause of viral hepatitis-related mortality in the near future in Iran. The recent population-based epidemiological studies have revealed the predominant role of injecting drug use in increasing prevalence of HCV infection. Undoubtedly, new management paradigm is required to drive down the rising wave of hepatitis C in Iran. Priority should be given to young injecting drug users as the cornerstone of the lurking epidemic of HCV infection in Iran.

Key words: General population; Injecting drug user; Epidemiology; Hepatitis C virus; Iran

Core tip: Iran is known as a low-endemic country for hepatitis C virus (HCV) infection, while the recent population-based epidemiological studies have revealed the increasing burden of HCV infection in the Iranian population. The asymptomatic nature of HCV infection and the undiagnosed HCV-infected injecting drug users have fueled this increase. Obviously, the current management paradigm is inadequate if control of HCV infection is aimed to be achieved.

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TO THE EDITOR

Less than 0.5% of the population, as many as 186500 patients are infected with hepatitis C virus (HCV) in Iran[1]. The majority of HCV-positive patients have been infected by injecting drug use, equivalent to 75% of the HCV-infected population[2]. The burden of HCV infection shows a rising trend, and HCV infection is projected to be the most important leading cause of viral hepatitis-related mortality in the near future in Iran[1,3]. Obviously, the current management paradigm is inadequate if control of HCV infection is aimed to be achieved.

Mandatory screening of all blood donors for hepatitis C resulted in a remarkable decrease in the prevalence of HCV infection[1,2,4,5]. In view of the success in the Iranian Blood Transfusion Organisation, the talk of HCV elimination has been intensified. However, all hopes came to knot due to rising wave of HCV infection among injecting drug users (IDUs), those whom the control of HCV transmission among is the most difficult. The shared use of drug paraphernalia and lack of awareness among young IDUs regarding the risk of acquiring HCV infection *via* needle-sharing are the root cause of the increasing prevalence of HCV infection among IDUs community[1]. At the same time, the asymptomatic nature of HCV infection and the undiagnosed HCV-infected IDUs would accelerate this increase[1].

The recent changes in the genotype distribution of HCV have also fueled this epidemic[6]. High rates of mutation in HCV genome have resulted in the emergence of seven major genotypes and at least 67 subtypes[7]. Each geographic region has a distinct genotypic pattern, which depends on the predominant mode of transmission, risk factors, life style, the source of infection, disease transmission patterns and age distribution in that particular region[8,9]. These genotypic patterns are not constant, change overtime and influence the epidemiology of HCV infection in that region[10,11]. The most prevalent subtype in Iran is 1a, followed by 3a and 1b. Over the last decade, however, a gradual decrease in the frequency of subtypes 1a and 1b and an increase in subtype 3a have been reported due to changes in the routes of transmission of HCV from blood transfusion to injecting drug use[6,9-12]. These changes should be taken in to consideration to establish better strategies for managing the silent epidemic of hepatitis C in Iran.

Another challenge is treatment of HCV-infected population. Despite having poor tolerability, prolonged treatment course and frequent side effects, interferon (IFN)-based therapy is still recommended as the first-line therapy in Iran due to affordability and local availability[3,9]. Annually, 2.4% of the Iranian HCV-infected population is treated by pegylated IFN plus ribavirin, with approximately 58%-78% of patients showing a sustained virological response (SVR) depending on the HCV genotype[2]. Introduction of IFN-free direct-acting antivirals (DAAs) has revolutionized the treatment course of HCV infection due to superior rates of SVR, favorable tolerability, fewer side effects and shorter treatment period[13-15]. However, in reality, the restricted accessibility and high price of DAAs outweigh these benefits. Recently, the production of a domestic DAA, the combination of daclatasvir and sofosbuvir, with health insurance coverage has been announced in Iran, paving the way for low-cost access to DAAs and subsequently widespread use of these drugs in the near future[1,3]. This domestically produced DAA, Sovodak, has shown favorable SVR rates in Iranian patients infected with genotypes 1 or 3 HCV, the most predominant genotypes in Iran, providing an opportunity to improve the treatment rate and subsequently eliminate HCV infection in the future[1].

These challenges in the management of hepatitis C epidemic cannot be neglected any longer. Resent changes in the epidemiology of HCV would demand changes in health policies, prevention and management strategies. In view of the success of the transfusion-safety measures implemented in the Iranian Blood Transfusion Organization[4,9], screening of high-risk populations for hepatitis C, new therapeutic strategies with an emphasis on timely diagnosis and treatment, expansion of harm-reduction interventions, public education regarding the risk of HCV infection, as well as comprehensive cooperation and mobilization of health care providers are required to drive down the rising wave of HCV infection in Iran once again. Priority should be given to young IDUs as the cornerstone of this silent epidemic. Furthermore, national health policies should be prioritized in a way to curb the lurking epidemic of HCV infection once and for all.

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MINIREVIEWS

Hepatitis B in patients with hematological diseases: An update

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Abstract

Hepatitis B virus (HBV) reactivation (HBVr) in patients undergoing immunosuppressive therapy is still a hot topic worldwide. Its prevention and management still represents a challenge for specialists dealing with immunosuppressed patients. Aim of this paper is to provide a critical review of the relevant information emerged in the recent literature regarding HBV reactivation following immunosuppressive treatments for oncohematological tumors. A computerized literature search in MEDLINE was performed using appropriate terms arrangement, including English-written literature only or additional relevant articles. Articles published only in abstract form and case reports not giving considerable news were excluded. Clinical manifestation of HBVr can be manifold, ranging from asymptomatic self-limiting anicteric hepatitis to life-threatening fulminant liver failure. In clusters of patients adverse outcomes are potentially predictable. Clinicians should be aware of the inherent risk of HBVr among the different virological categories (active carriers, occult HBV carriers and inactive carriers, the most intriguing category), and classes of immunosuppressive drugs. We recommend that patients undergoing immunosuppressive treatments for hematological malignancies should undergo HBV screening. In case of serological sign(s) of current or past infection with the virus, appropriate therapeutic or preventive strategies are suggested, according to both virological categories, risk of HBVr by immunosuppressive drugs

and liver status. Either antiviral drug management and surveillance and pre-emptive approach are examined, commenting the current international recommendations about this debated issue.

Key words: Reactivation; Lymphoma; Hematology; Immunosuppressive therapy; Prophylaxis; Hepatitis B virus; Chemotherapy; Occult/active/inactive carrier; Entecavir; Lamivudine

Core tip: Despite the increasing awareness regarding the issue of hepatitis B virus reactivation (HBVr) in patients undergoing immunosuppressive treatments, there are still some many debated items concerning this potentially fatal but preventable complication. Both hepatitis B surface antigen (HBsAg) patients and subjects with serological signs of previous resolved exposure to the virus (HBsAg negative/anti-core antibody positive patients) are at risk of HBVr. Purpose of our work was to analyze the current international literature and dedicate guidelines, providing evidences and strategies that have been proposed to manage these patients.

Coluccio C, Begini P, Marzano A, Pellicelli A, Imperatrice B, Anania G, Delle Fave G, Marignani M. Hepatitis B in patients with hematological diseases: An update. *World J Hepatol* 2017; 9(25): 1043-1053 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i25/1043.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i25.1043

INTRODUCTION

Hepatitis B virus (HBV) infection represents a significant global health problem, since almost one third of the world's population has serological signs of previous or present infection, and that 240 million individuals are chronic hepatitis B surface antigen (HBsAg) carriers[1]. Worldwide, low rates of serological HBsAg positivity (0.2%-0.5%) and signs of previous HBV contact [4%-6% HBsAg negative/anti-hepatitis B core antigen antibodies (anti-HBc) positive subjects] are registered in north western and central Europe, north America and Australia. On the contrary, the highest prevalences are reported in China, Southeast Asia and tropical Africa (chronic infection 8%-20%, and previous exposure 70%-95%, respectively)[2].

It is presently well known that medications such as glucocorticoids and anticancer treatments can interfere with the host immune system and blunt the control that it exerts over HBV replication, with the potential to cause viral reactivation (HBVr) in both HBsAg positive patients and individuals with serological signs of previous resolved HBV exposure. HBVr can assume various manifestations, spanning from asymptomatic hepatitis to life threatening fulminant liver failure. This risk is most common among patients undergoing treatment for hematological tumors or those receiving hematopoietic stem cell transplantation (HSCT). Nevertheless, also patients with solid tumors (such as breast cancer), immunological diseases and inflammatory bowel diseases are exposed to the risk of HBVr[1,3-5].

In this paper, we will critically review the relevant information emerged in the recent international literature regarding HBVr, focusing on patients undergoing immunosuppressive treatments for hematological malignancies.

LITERATURE SEARCH

A computerized literature MEDLINE search was done adopting several combinations of these terms: HBsAg, reactivation, lymphoma, hematology, immunosuppressive therapy, anti-HBc, occult carrier, including only papers in English language. Literature on hematopoietic stem cell transplantation recipients was not considered. Articles published only in abstract form were excluded. Case reports have been included only if adding significant contributions.

HBV INFECTION, HOST IMMUNE RESPONSE AND VIROLOGICAL PROFILES

When the HBV virus encounters the human host, in the presence of a competent immune system, three outcomes relevant to our discussion can be observed: (1) the infection can be rapidly cleared, as it is to be expected in most immunocompetent adults. However, in a part of these individuals, the covalently closed circular (ccc) viral DNA can integrate and persist indefinitely as an immune template in the host hepatocyte; (2) The host immune response might create a dynamic equilibrium in which viral replication either stops or is minimally active; and (3) the host immune system is unable to either eradicate or control viral replication and a state of chronic liver disease ensues, potentially leading to the development of liver cirrhosis and its consequences. These different immunological and clinical scenarios of host-virus interplay constitute the basis to define the corresponding virological HBV categories, summarized in Table 1[5].

Active carriers (AC) are those HBsAg positive patients in whom HBV replication prevails over the control of host immune system, and are characterized by elevated HBVDNA levels ($\geqslant$ 2000 IU/mL). On the other extreme are the occult HBV carriers (OBI), individuals in whom the immune system has successfully cleared the acute viral infection. These individuals however still harbor the viral DNA inside the hepatocytes, integrated in the form of cccDNA but under the effective replicative control of the immune

Table 1 Virological categories of hepatitis B virus infected patients (adapted from[5])

	AC	IC	pOBI
HBsAg	+	+	-
Anti-HBc	+	+	+
Anti-HBs	-	-	-/+
qHBsAg	⩾ 1000	< 1000	-
ALT	Increased	Normal	Normal
HBV DNA in the blood	⩾ 2000	< 2000	-
Liver stiffness (kPa)	> or < 6	< 6	< 6

HBsAg: Hepatitis B surface antigen; AC: Active carrier; IC: Inactive carrier; OBI: Occult hepatitis B virus (HBV) infection; Anti-HBc: Anti-hepatitis B core antigen antibodies; Anti-HBs: Antibodies to HBV surface antigen.

system, only showing serological signs of previous viral exposure (*i.e.*, presence of anti-HBc), very low (< 200 IU/mL) or absent circulating HBVDNA, positive or negative antibodies to HBV surface antigen (anti-HBs), and normal transaminases[6]. The third, more intriguing and elusive category, is currently that of inactive carriers (IC), HBsAg and anti-envelope antigen antibody (anti-HBe) positive patients with indosable or < 2000 IU/mL HBVDNA levels. Their classical definition is completed by the concurrent presence of persistently normal levels of serum transaminases, no signs of HBV-induced liver inflammation/fibrosis and a clinically benign course. The IC state was generally ratified by the stability of these parameters during the course of an extended (usually 12-mo) observation period[7]. However, this lengthy mandatory observation period is awkward in settings requiring a rapid categorization, such in those in which a decision regarding the start of antiviral drugs to protect from HBVr is to be taken.

In the Asian pacific region, the benignity of this entity has been debated, and the term of "low replicative chronic HBV infection" proposed, favored over the "inactive carrier" definition, as the latter can give the patients an incorrect sense of confidence. Considering that hepatitis B infection should be considered a dynamic interplay between the host and the virus, the activity profile can modify over time and virological category can change at different time points[8]. However, this scenario is based on the virological characteristics of the Asian population, while in the Mediterranean basin up to one third of IC individuals present levels of HBVDNA between 2000 and 20000 IU/mL with normal transaminases and absence of liver fibrosis during long term observation. To further sharp the definition of this virological HBV category, recent studies have focused their attention on the role of quantitative HBsAg testing (qHBsAg), HBVDNA cut-offs, and use of fibroelastometry[9-11].

Recent studies have in fact provided data to allow a timely identification of IC group of patients with an acceptable approximation, without the need of a prolonged observation.

In the study by Brunetto *et al*[7], 209 genotype D carriers were enrolled, and the capacity of qHBsAg testing to discriminate between active and inactive HBV carriers and patients with active chronic hepatitis B (CHB) was tested. It was demonstrated that a one-time (so called "spot") quantification of HBVDNA below 2000 IU/mL and HBsAg less than 1000 IU/mL was able to single out IC with good sensitivity, specificity, positive and negative predictive values (91.1%, 95.4%, 87.9%, 96.7% respectively) concluding that this single observation approach obtains the same results of long term monitoring with an acceptable approximation[7]. Raimondo *et al*[6] recently evaluated the reliability of serum HBVDNA and qHBsAg testing, along with liver stiffness measurements (LSM) in identifying the IC status at a spot point investigation among 147 HBsAg and anti-HBe positive patients, including 57 IC and 90 individuals with CHB. The overall evaluation of all parameters allowed to recognize 23 out of 57 (40.3%) ICs, with good specificity, sensitivity, positive and negative predictive values, and diagnostic accuracy (100%, 96%, 100%, 92% and 97%respectively). Even removing from the analysis CHB or cirrhotic patients, the results were similar. It was concluded that combined assessment of HBVDNA level, liver stiffness along with quantitative surface antigen measurements, provide a dependable working instrument, correctly identifying a large portion of IC with a spot assessment only[12]. In genotype B and C patients the validation of a one-time dosage of qHBsAg and HBV DNA to predict IC state was performed in a population of 1529 subjects. When HBsAg < 1000 IU/mL was associated with HBVDNA < 2000 IU/mL, the one-time evaluation was able to discriminate IC from patients with chronic hepatitis B with slightly lower diagnostic accuracy[13]. Thus, it can be concluded by these observations that by using serological and elastographic testing, IC can be currently identified with an acceptable approximation in those instances when prolonged observation is unfortunately not an option.

HBV REACTIVATION AND FACTORS INFLUENCING ITS OCCURENCE

HBVr during immunosuppressive treatments can occur as the result of a loss of control over viral replication induced by these drugs, since they can modify the competence of the host immune system[3]. In this setting, the virus rapidly replicates infecting multiple hepatocytes, however in this phase usually no damage occurs since the immunological response is blunted by immunosuppressive medication. When the immunosuppressive therapy is concluded, a progressively restored immune system can activate the search, destroy and eradication of the HBV infected hepatocytes, and this can cause massive liver necrosis and acute liver failure. This event process can occur at different time points, usually ranging from a few months but also potentially developing years after the end of the immunosuppressive therapeutic cycle, after

Table 2 Incidence of hepatitis B virus reactivation without prophylaxis (adapted from[21])

Disease	HBsAg+ (%)	HBsAg-/anti-HBc+ (%)
Lymphoma	18-73	34-68
Acute leukaemias	61	2.8-12.5
Multiple myeloma	Not available	6.8-8
Breast cancer	21-41	Not available
Hepatocellular cancer (systemic chemotherapy)	36	11
Inflammatory bowel disease	36	0-7
Autoimmune diseases	Not available	17

HBsAg: Hepatitis B surface antigen; Anti-HBc: Anti-hepatitis B core antigen antibodies.

immune response is completely restored[14,15].

HBVr has been variably defined overtime and a consensus has not been reached. According with the American Gastroenterological Association (AGA) guidelines, in HBsAg carriers reactivation occurs when there is either a *de novo* detection of viremia or a one $\log_{10}$ or greater increase in HBVDNA as compared to baseline levels (obtained before starting therapy). Hepatitis flare is considered when there is at least a two-three fold rise of ALT above baseline or a predetermined multiple of the upper normal limit. In HBsAg negative/anti-HBc positive patients reactivation is defined by the reverse seroconversion to HBsAg-positive condition[16]. Similar definitions are also suggested by the Italian association for the study of the liver (AISF).

Since different levels of baseline HBVDNA influence the occurrence of HBVr, the different virological classes, proceeding from OBI to IC and then to AC, are at a progressively higher risk of reactivation. It is in fact widely accepted that subjects with high level of viremia before immunosuppressive therapy are at an increased risk for the development of HBVr as compared to those with undetectable or low levels of HBVDNA[17-19].

Accordingly, many studies have estimated that the risk of HBVr is 5- to 8-fold higher among HBsAg positive patients[20] and that HBeAg positive patients are at higher risk of developing HBVr as compared to HBeAg negative ones[17]. Compared to other diseases groups, patients with hematological malignancies are reported to be those characterized by the highest risk of experiencing HBVr (Table 2)[21] with figures ranging between 24%-88%[22]. It is speculated that this difference could be due to the intrinsic immunosuppression typical of hematological malignancies and to the treatments used to cure them. Interestingly, the first cases of HBVr were actually recorded among patients with lymphoma[23]. In a large multicenter case-control study conducted in Italy, the prevalence of HBsAg positivity among 400 B-cell non-Hodgkin's lymphoma (NHL) cases was higher than in 392 controls (8.5% *vs* 2.8%, respectively)[24]. Thirty-eight to 73% of HBsAg positive NHL cases undergoing chemotherapy for NHL can experience HBVr[25,26].

Also multiple myeloma patients are at risk of HBVr as reported in several recent papers, since in the advanced stages of this disease the occurrence of a more critical immune dysregulation might predispose to the development of viral reactivation[27].

A substantial risk of HBVr, not different from that of lymphoma patients, has also been described among patients undergoing treatment for acute myeloid leukemia. Recently Chen *et al*[28] observed that HBVr and HBV-related hepatitis occurred in 9.5 and 8.3 per 100 person-years, respectively. There is now clear evidence that different classes of immunosuppressive drugs are characterized by different risks of inducing HBVr. Medications used for hematological malignancies are frequently marked by a severe immunosuppressive effect, as the case of rituximab (RTX), an anti-CD20 monoclonal antibody acting as a potent B-cells depleting agent, mostly used in hematological malignancies during the last two decades[29] and well known to increase the chance of HBVr of more than five-fold[30]. This high risk is justified by the marked B-cell reduction, which interferes with the production of anti-HBs and their neutralizing effect on serum HBsAg. Moreover, RTX worsens the imbalance of antigen-presenting B-cells typical of chronic HBV infection, determining a lower activity of CD4 T-cell in generating an adequate immune response[31].

The rate of HBVr inherent to these B-cell depleting agents (RTX, but also ofatumumab) is roughly 16.9% among patients with serological signs of previous HBsAg exposure, and their seroreversion percentage is 20%-40%. With these drugs HBVr can be a late event, even up to 60 mo after the cessation of immunosuppressive therapy, further marking the strong and lengthened influence of these drugs on the recovery of immune competence[16,32,33].

Considering these evidences antiviral prophylaxis of these patients have to be prolonged up to 10-24 mo after the discontinuation of the B-cell depleting agents and a careful surveillance has to be activated after the antiviral therapy withdrawal[3,5]. Among the B-cell depleting agents, more drugs are or will soon be available. A possible example is Obinutuzumab, a new humanized monoclonal antibody to CD20[34] which, in association with other chemiotherapics, has been shown to be more effective than RTX in the treatment of chronic lymphatic leucemia (CLL)[35], but at the cost of determining a more profound immunosuppression than RTX. Even though no HBVr cases have been registered following the use of this drug, it is conceivable that the concerns developed during the experience with RTX should also be extended to the other members of this class of drugs.

Corticosteroids are also widely used in the treatment of hematological malignancies and combined to cytotoxic agents in several therapeutic schedules for the treatment of lymphoma and multiple myeloma. These dugs are able to influence the activity of T-cells but also to directly intensify HBV replication[36]. It has in

Table 3 Risk of hepatitis B virus reactivation according to different immunosuppressive drug classes (adapted from[21])

Risk	Drug class
High (> 10%)	B-cell depleting agents
	Anthracycline
	Corticosteroids high dose
Moderate (1%-10%)	TNFα inhibitors
	Cytokine and integrin inhibitors
	Tyrosine kinase inhibitors
	Corticosteroids moderate dose
Low (< 1%)	Corticosteroids low dose
	Traditional immunosuppression (*e.g.*, azathioprine or methotrexate)

fact been demonstrated that the prolonged assumption of prednisolone increases HBsAg and HBVDNA levels in liver cells, and that the withdrawal of corticosteroid seems to determine a rebound in immune T-cell function resulting in hepatocyte destruction[37]. Corticosteroids have the potential to cause HBVr, but with different percentages of risk depending on dosage, duration of treatment and route of administration; in fact high-dose (> 20 mg/d) prednisolone, and prolonged treatment extension (> 1 mo), correlate with higher risks of reactivation.

AGA evaluated the risk of HBVr according to distinct drug categories, basing its conclusions on an extensive systematic review of the available studies. However, on some medication, data were limited and extrapolated only from either case series or caser reports. This risk stratification is reported in Table 3[21]. A gradation of the HBVr risk (high > 10%, moderate 1%-10% and low < 1%) has been proposed and currently accepted in the western countries[1,3,5].

PREVENTION OF HBVr

To prevent HBVr, it is crucial to identify patients at risk for the development of this potentially severe event before starting immunosuppressive drugs. Most international scientific associations such as the European Association for the Study of the Liver (EASL), AGA, the Asian-Pacific Association for the Study of the Liver (APASL) and AISF suggest to screen for HBV all patients scheduled to undergo immunosuppressive treatment by testing HBsAg, anti-HBc and anti-HBs[1,3,5,8].

On the other hand the American Association for the Study of Liver Diseases (AASLD) and the American Society of Clinical Oncology (ASCO) recommend to limit HBV screening to patients with high or moderate risk of HBVr risk factors[38,39]; for patients at low risk, screening strategies should follow instead the indications produced by the Center for Disease Control and Prevention[40] and the United States Preventive Services Task Force[41,42].

It has been demonstrated in various studies that HBsAg positive patients should undergo antiviral treatment started before (2-4 wk) and continued during chemotherapy, regardless of baseline HBVDNA level, and not on a pre-emptive based strategy, considering that if hepatitis has already developed, it could be more difficult to control the extent of the reactivation process[32,43].

Currently, guidelines worldwide indicate treatment with nucleot(s)ide analogs (NA) for patients with hematological malignancies, positive for the HBsAg and receiving cytotoxic chemotherapeutic drugs[1,3,5,8]. The duration of the antiviral treatment in these patients has been the matter of long debates in the last decade, but actually a higher concordance is registered. In patients with CHB or cirrhosis antiviral therapy has not to be discontinued. However in IC it should be continued during the immunosuppressive treatment and for 12 mo after its discontinuation. Patients with serological signs of resolved past exposure to the virus and detectable viremia should be managed as surface antigen positive subjects, while those with undetectable serum HBVDNA should be carefully followed by ALT, HBsAg and/or HBVDNA testing (regardless of anti-HBs status), and promptly treated with nucleoside analogues upon confirmation of HBV reactivation before ALT elevation. However, when patients with this serological pattern (HBsAg negative/anti-HBc positive) are treated with RTX or similar immunosuppressive drugs, especially when low/absent serum hepatitis B surface antibodies are detected or if close HBVDNA surveillance is not feasible, many experts acknowledge their higher risk of viral reactivation and recommend prophylaxis[8]. In case of monitoring aimed at the prompt activation of pre-emptive therapy, ALT, HBsAg and/or HBVDNA testing is performed every 1-3 mo during the immunosuppressive treatment in the early phase, depending on the type of immunosuppressive drug and comorbidities. When prophylaxis is instead chosen, lamivudine (LAM) is usually suggested[44]. The 2007 Italian AISF guidelines and its recent implementation are in agreement with the international indications previously reported. In particular, among HBsAg-positive patients, AC are treated as their immunocompetent counterparts with the more potent antivirals available, while viremic IC, which received LAM in the past, are now preferentially treated with entecavir (ETV). In these patients monitoring of drug efficacy was performed by HBVDNA and ALT testing. In hematological anti-HBc positive subjects undergoing severely immunosuppressive regimens of various kind (see[4] for a complete list), universal prophylaxis with LAM has been advocated and recently confirmed. In these patients monitoring in prospective of pre-emptive therapy or of response to treatment is advised with ALT and HBsAg testing for their high specificity and maneuverability during the very long period at risk after the immunosuppressive treatment[4,5].

CHOICE OF ANTIVIRAL AGENTS

Regarding the antiviral to use in HBsAg positive sub-

jects, the 2017 American and European guidelines suggest the use of a NA with high potency and high genetic barrier (ETV or tenofovir disoproxil or alafenamde, respectively TDF and TAF)[1,3]. In these patients the role of LAM remains marginal in the very few IC patients without detectable viremia or in developing countries[3,5]. In HBsAg negative/anti-HBc positive subjects with hematological diseases and/or treated with B-cell depleting drugs high barrier antivirals can be obviously considered but the antiviral treatment with LAM is yet accepted[1,5].

In the face of such indications, the most part of data derived from the historical experience with LAM. Seminal papers considered LAM prophylaxis as an efficient agent to decrease the event of reactivation and hepatitis flare, to reduce the risk of HBV-related liver failure, and prevent the delay or discontinuation of chemotherapy as a consequence of HBVr[45]. The influential systematic review by Loomba demonstrated that LAM prophylaxis exerted a protective role against HBVr and death attributable to hepatitis B (relative risk 0.0-0.21 and 0.0-0.2 respectively)[22].

A later review concluded that antiviral LAM prophylaxis during cytotoxic treatment influenced HBVr, determining both a 87% reduction of this event, and a 92% decrease in treatment delay/early interruption of chemotherapy as compared to patients not given prophylaxis[45].

The systematic review and metanalysis of five randomised controlled trials contained in the recent AGA technical review, compared LAM prophylaxis to treatment at the beginning of viral reactivation (pre-emptive strategy)[16,43,46-49]. Antiviral prophylaxis was more effective than the pre-emptive strategy [overall risk ratio (RR) = 0.13], and also determined a significant decrease of hepatitis flare risk (RR = 0.16)[16]. Nevertheless, it has currently been suggested that LAM prophylaxis provides a suboptimal protective action for IC with detectable HBVDNA. The supposed superior efficacy of ETV as compared to LAM in the prevention of HBVr among patients undergoing treatment for hematological malignancies is supported by the results of the registrative studies in patients with CHB[50,51], in which ETV was shown to be more powerful than LAM in terms of histological amelioration, control of viremia, and reversal of ALT values to normal range in either HBeAg positive or negative chronic active hepatitis patients.

Additionally, in patients with NHL has been suggested that LAM provides a suboptimal preventive approach also in low viremic patients. A randomized multicenter study compared the efficacy of prophylactic therapy with LAM and ETV among HBsAg positive subjects and diffuse large B-cell lymphoma treated with RTX-CHOP (Cyclophosphamide, Hydroxydanorubicin, Oncovin, Prednisone); in low viremic (HBVDNA < 2000 IU/mL) patients it was demonstrated that the virological events were significantly lower in the ETV group considering hepatitis (8.2% *vs* 23.3%), HBVr (6.6% *vs* 30%) delayed hepatitis B (0% *vs* 8.3%) and chemotherapy disruption (1.6% *vs* 18.3%). However, at the moment this is the only available prospective study, burdened by some relevant limitations, such as the high prevalence of low viremic HBeAg positive patients in the Asiatic population evaluated[52]. However, a recent systematic review with network meta-analysis has suggested that prophylactic therapy with tenofovir or ETV may represent the most potent intervention to prevent HBVr and HBV-related morbidity and mortality in HBsAg-positive patients undergoing chemotherapy[53]. In two meta-analysis aimed to HBsAg-negative/anti-HBc-positive patients treated with RTX without antiviral prophylaxis, HBVr developed in 6.3%-16.9% of cases[16,54].

LAM was the drug most used for the universal prophylaxis in antiHBc-positive patients with hematological disease. In this setting viral breakthrough and loss of response during the antiviral treatment is very rare, while the risk of HBVr is significant during the first 6-12 mo after the discontinuation[5,16].

A unique randomized prospective study was performed in anti-HBc positive patients treated with RTX, comparing 3 mo of prophylaxis with LAM or ETV. HBVr was significantly higher in the LAM group (P = 0.19); however all the clinical events developed after (0.5-14 mo) the discontinuation of the drug without demonstrating a higher protective effect of ETV during the therapy[46].

LATEST NEWS AND COMPARISON BETWEEN THE MOST RECENT INDICATIONS

As previously reported, in the last few months some relevant indications on the management of HBV reactivation among immunosuppressed patients have emerged and published.

The Italian guidelines[5] are the result of the continuously updated work produced by a team of hepatologists dedicated to the management of immunosuppressed patients at risk for HBV reactivation. Its contents have been widely cited in this paper, as for instance the controversies regarding the best strategies to manage inactive carriers. Guidelines are discussed and developed in single topic events endorsed by AISF. Statements are produced after revision and discussion of the specific literature by hepatologists and other specialists such as hematologists, oncologists, immunoreumathologists, nephrologists and transplantologists. Virological classes and their relative diagnostic criteria are addressed as are screening and diagnostic approaches. Definitions of clinical and virological events are provided. Management and follow up strategies are also thoroughly scrutinized with the aim to promote the awareness regarding this issue, and collaboration among specialists. HBV screening is recommended in all patients undergoing

treatment for hematological malignancies with the use of HBsAg, anti-HBs, and anti-HBc. HBVDNA is then tested to both distinguish AC and IC and to identify potential false OBI. The different classes of risk for HBV reactivation proposed by the 2015 AGA guidelines have been incorporated. For patients with a high risk of reactivation, evaluation by an expert in liver disease is required. For HBsAg positive patients with hematological malignancies the risk of reactivation emerges to be clearly significant (24%-88%, median 50%), and the particular increase of HBVr associated with the use of RTX has been definitely stated. Also, the increased risk of HBVr due to the use of RTX in the OBI group has also been clearly recognized. In this latter virological category, the actual risk of reactivation as the result of treatment with several recently introduced biologics (imatinib, bortezomib, mogamolizumab, ofatumumab, carfilzomib, romidepsin, *etc*.) remains debated. As far as the treatment of HBsAg-positive patients is concerned, even if most available data came from the experience developed with LAM, the presence of newer drugs with greater potency and high genetic barrier, has imposed ETV and tenofovir (especially in the new form to be commercialized in Italy, TAF, with an improved safety profile) as the drugs of choice in viremic patients. In OBI treated with RTX for lymphoma, or with detectable HBVDNA LAM still maintains its role, in the absence of a proven greater protective effectiveness over other antivirals. Antiviral treatment with either ETV or TDF (TAF) is recommended indefinitely for AC patients, while in IC patients, LAM (HBVDNA negative) or ETV (HBVDNA positive) prophylaxis is indicated for at least 12 mo from the end of the immunosuppressive treatment. In OBI subjects duration of LAM prophylaxis it is indicated to extend prophylaxis for at least 18 mo after immunosuppressive regimen has been stopped. In LAM treated pOBI, the monitoring of ALT and HBsAg is indicated every three months. Monitoring in AC during and after the immunosuppressive treatment is similar to that of immune-competent; for IC in prophylaxis, monitoring should be performed dosing ALT and HBVDNA, every 12 wk in the case of LAM; every 6-12 mo, after virological response, with ETV and TDF(TAF). In case of viral breakthrough during prophylaxis or therapy with LAM or ETV, the prompt activation of a rescue therapy with either TDF or TAF is advised; during therapy with TDF/TAF or ETV a partial virological response requires a combined therapy with a nucleoside and a nucleotide. A similar monitoring (HBsAg in OBI and HBVDNA in AC) is recommended in the first month and every three months after the discontinuation of prophylaxis for the first year and every six months thereafter.

Another goal of the team is to provide practical indications for the working physician. To this purpose, statements are then published as a full report illustrating the management of the different subclasses of immunosuppressed patients. These indications have been published for the first time in 2007 and have been constantly updated thereafter during the course of the years. The most recent paper has been published online on the AISF web site in February 2017, and a further meeting is scheduled by the end of this year, with the aim of producing and English version of the newly discussed statements.

The EASL has published in April 2017 the updated guidelines on the management of hepatitis B infection[1]. In this paper, as in its previous 2012 version, the issue of immunosuppressed patients with signs of current or past infection with the HBV are addressed in the section dedicated to the treatment of various special patients groups with HBV infection. Also in this paper, the different classes of risk for HBV reactivation proposed by the 2015 AGA guidelines have been accepted. Vaccination of HBV seronegative immunosuppressed individuals is endorsed. Similarly to the AISF guidelines, it is suggested that all patients scheduled to undergo cytotoxic and/or immunosuppressive treatments should firstly perform a serological screening based on HBsAg, anti-HBs and anti-HBc testing. Evidence and grade of recommendation are very strong. All HBsAg-positive candidates for immunosuppressive therapies should undergo evaluation by a specialist to define their virological class. All HBsAg positive patients should start potent NA as a treatment or prophylaxis. A clear cut approach is proposed for AC, and they should be treated with ETV, TDF or TAF, similarly to the immunocompetent patients. Controversial remains the management of IC. Prophylactic LAM has been shown to obtain a reduction of both HBV reactivation risk and of associated morbidity and mortality. Nevertheless, a residual risk of HBV reactivation remains (approximately 10%) in patients with low viremia (HBV DNA < 2000 IU/mL). Thus, a simplified approach recommends ETV, TDF, TAF in all HBsAg positive patients, both as treatment and prophylaxis (Evidence level Ⅱ-2, grade of recommendation 1). The EASL guidelines also suggest long term prophylaxis (at least 12 mo, and 18 mo in case of rituximab-based regimens) after the cessation of the immunosuppressive treatment, and NA prophylaxis should be stopped only in case the underlying disease is in remission. During prophylaxis, liver function tests and HBVDNA should be tested every 3 to 6 mo. Testing should be performed with the same schedule also after NA withdrawal, since a relevant proportion of HBV reactivations develops after their discontinuation. It is not defined when testing should be stopped.

The risk of HBV reactivation in OBI varies widely according to underlying disease and the type and duration of immunosuppressive regimen. HBVDNA testing should be performed before immunosuppression. If viremic, they should be treated similarly to HBsAg-positive patients. As in the Italian guidelines, in patients at high risk (10%) of HBV reactivation (*i.e.*, anti-HBc

positive subjects undergoing treatment with rituximab in the oncohematological setting; those undergoing stem cell transplantation), antiviral (universal) prophylaxis is recommended. This should be continued for at least 18 mo after stopping immunosuppression and monitoring should continue for at least 12 mo after prophylaxis withdrawal. LAM may be used although cases of HBV reactivation due to LAM resistance have been reported. Interestingly, the EASL guidelines suggest that prophylaxis with ETV or TDF or TAF can also be considered in HBsAg-negative, anti-HBc positive patients receiving highly immunosuppressive regimens of extended duration. So it is concluded that these patients should receive anti-HBV prophylaxis if they are at high risk of HBV reactivation (Evidence level Ⅱ-2, grade of recommendation 1). In isolated anti-HBc positive subjects with either moderate (< 10%) or low (< 1%) risk of HBV reactivation, pre-emptive therapy and not prophylaxis is recommended. Also, the EASL guidelines consider HBsAg reappearance (seroreversion) the main virological event in these patients, constantly associated with hepatitis flare. As also indicated in the Italian guidelines, HBVDNA detection leads to seroreversion and hepatitis in only 50% of cases, thus being less specific as compared to HBsAg testing. However, with an apparent contradiction or a conservative prudence, both HBsAg and/or HBVDNA are monitored every 1-3 mo during and after immunosuppression, and therapy with ETV, TDF or TAF started in case of detectable HBVDNA or HBsAg seroreversion following a pre-emptive strategy. Since after HBsAg seroreversion a severe, even fatal, acute hepatitis could ensue, NA should be started as early as possible, independently of ALT levels. Interestingly the opportunity of using universal prophylaxis rather than pre-emptive therapy is recommended for selected clinical settings, characterized by long duration of immunosuppression, limited compliance to monitoring or unknown risk of viral reactivation for new biological. Limited are the indications on how and when follow-up should be performed after NA withdrawal.

A very recent review by Loomba and Liang[3] also needs to be mentioned. It further stresses and perfects the 2015 position of the AGA regarding patients with signs of current or past HBV infection undergoing immunosuppressive treatments at risk for viral reactivation. The authors accurately scrutinize the most recent data regarding this issue, updating the risk of reactivation associated to other immunosuppressive treatments such as cytokine and integrin inhibitors, immune checkpoint inhibitors such as ipilimumab (anti-CTLA4) and nivolumab (anti-PD-L1), and histone deacetylase inhibitors (HDIs). Complementary information is also provided on tyrosine kinase and proteasome inhibitors. Fine mechanisms of reactivation are reviewed. As in the AISF guidelines, a thorough baseline evaluation of liver status is recommended, and screening for HBV infection by testing HBsAg, anti-HBc and anti-HBs suggested for all patients who are receiving therapies that have either a high or moderate risk of reactivation. Evaluation by a HBV specialist is recommended. Even if LAM might be considered in resource-limited countries, especially in HBsAg-positive individuals with either undetectable or very low HBVDNA serum levels, high potency and high genetic barrier antiviral drugs such as ETV and tenofovir are preferred. Patients with CHB (HBsAg positive HBVDNA ⩾ 2000 IU/mL, elevated transaminases) should be treated as their immunocompetent counterpart. IC (HBsAg positive, HBV-DNA < 2000 IU/mL, normal transaminases) should undergo prophylaxis when exposed to high- and moderate-risk immunosuppressive therapy. Prophylaxis should ideally be initiated 14-30 d prior the initiation of immunosuppressive treatment and maintained for a minimum of 12 mo after its discontinuation.

For IC exposed to low-risk immunosuppressive treatments and OBI patients, surveillance with ALT and HBsAg (adding HBVDNA in those who are HBsAg positive) is recommended. To reduce the event of reactivation, OBI treated with RTX or other high risk treatments should undergo prophylaxis. For OBI at a moderate risk, anti-HBV prophylaxis should be considered, but they could also be monitored for serum ALT and HBsAg levels (and not by HBVDNA testing, similarly to the AISF indications) every 3 mo up to 6 mo after the discontinuation of immunosuppressive treatments. However, since HBV reactivation may occur up to 1-2 years after the last dose of RTX, patients treated with this medication may continue prophylaxis for up to 2 years after its discontinuation.

DISCUSSION AND CONCLUSION

Management of patients with HBV infection undergoing immunosuppressive therapy for hematological malignancies is still a challenge. It is necessary to be aware and vigilant about the risk of HBVr and its potential dire consequences and complications. Baseline screening for HBV infection before treatment initiation it is thus mandatory for these patients. HBV serum markers (HBsAg, anti-HBc and anti-HBs) must be checked, in order to stratify the risk of reactivation and decide which category of patients needs therapy and what is the best option for them.

Management with appropriate antivirals is indicated for their marked propensity to reactivate. Antiviral therapy is necessary in patients with moderate or high risk for reactivation. For HBsAg positive patients antiviral therapy is mandatory; for HBsAg negative/anti-HBc positive patients (OBI) it is possible to consider either prophylactic antiviral management (especially in patients undergoing high-risk therapies), or a pre-emptive approach monitoring ALT, HBsAg and/or HBV-DNA level and starting antiviral therapy as soon as it becomes detectable in the blood.

For several years LAM has been the only antiviral available to treat and manage hepatitis B and its reactivation, but during the last few years several

studies have been published to demonstrate the efficacy of antivirals with superior characteristics of potency and genetic barrier as ETV and TDF (waiting for the availability of TAF, a less nephrotoxic prodrug). Today in the setting of hematology these high barrier drugs have to be used in HBsAg-positive patients and it should be clear that LAM maintains a role only for the universal prophylaxis of HBVr in HBsAg-negative/anti-HBc positive (OBI) patients.

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MINIREVIEWS

Hepatitis B in renal transplant patients

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Abstract

Hepatitis B virus (HBV) poses a significant challenge for both dialysis patients and kidney transplant recipients despite its decreasing rates, especially in developed countries. The best preventive method is vaccination. Patients with chronic renal disease should ideally be vaccinated prior to dialysis, otherwise, reinforced vaccination practices and close antibody titer monitoring should be applied while on dialysis. HBV infected dialysis patients who are renal transplant candidates must be thoroughly examined by HBV-DNA, and liver enzyme testing and by liver biopsy. When needed, one must consider treating patients with tenofovir or entecavir rather than lamivudine. Depending on the cirrhosis stage, dialysis patients are eligible transplant recipients for either a combined kidney-liver procedure in the case of decompensated cirrhosis or a lone kidney transplantation since even compensated cirrhosis after sustained viral responders is no longer considered an absolute contraindication. Nucleoside analogues have led to improved transplantation outcomes with both long-term patient and graft survival rates nearing those of HBsAg(-) recipients. Moreover, in the cases of immunized HBsAg(-) potential recipients with concurrent prophylaxis, we are enabled today to safely use renal grafts from both HBsAg(+) and HBsAg(-)/anti-HBc(+) donors. In so doing, we avoid unnecessary organ discarding. Universal prophylaxis with entecavir is recommended in HBV kidney recipients and should start perioperatively. One of the most important issues in HBV(+) kidney transplantation is the duration of antiviral prophylaxis. In the absence of robust data, it seems that prophylactic treatment may be discontinued in selected stable, low-risk recipients during maintenance immunosuppression and should be reintroduced when the immune status is altered. All immunosuppressive agents in kidney transplantation can be used in HBV(+) recipients. Immunosuppression is intimately associated with increased viral replication; thus it is important to minimize the total immunosuppression burden long term.

Key words: Hepatitis B virus (+) donor; Hepatitis B virus (+) recipient; Renal transplantation; Viral reactivation; Immunosuppression; Nucleoside analogues; Antiviral discontinuation; Antiviral prophylaxis; Hepatitis B

Core tip: Though decreasing, hepatitis B still remains a considerable problem, especially in high-risk patient populations as kidney transplant recipients. The widespread use of new antivirals and the introduction of universal prophylaxis immediately after transplantation have changed the picture in hepatitis B virus (HBV) (+) transplantation. Long term survival rates of HBV(+) recipients are approaching those of HBV(-), altering HBV(+) kidney transplantation from a "high risk" procedure into routine practice. Furthermore, accumulating evidence confirms the safety of transplantation from HBsAg(+) donors into immunized recipients. All immunosuppressants can be used in HBV(+) transplantation and total immunosuppression must be kept at the lowest possible levels long term.

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HEPATITIS B PREVALENCE AND PREVENTION IN HEMODIALYSIS PATIENTS

Acute and chronic hepatitis are caused by a double stranded DNA type of virus, hepatitis B virus (HBV). Although a safe and effective vaccine has been available for at least twenty years now, infection of HBV remains an enormous problem of public health worldwide[1].

Because of increased skin breaching, significant exposure to blood products, the sharing of dialysis machines, the nature of the dialysis process that allows great access to the bloodstream and underlying immunodeficiency problems, hemodialysis patients are at a greater risk for HBV infection. Fortunately, a number of prevention measures have in the last thirty years effectively resulted in the significant reduction of HBV infection incidence amongst hemodialysis patients. These include but are not limited to stricter adherence to general hygiene rules, mandatory separation of these patients during dialysis, aggressive vaccination protocols as well as erythropoietin use. However, hepatitis B prevalence remains a challenge in dialysis[2]. USRDS data indicates that 1% of dialysis patients tested positive for hepatitis B surface antigen (HBsAg) while in a registry study of Asian-Pacific countries the prevalence of HBsAg in hemodialysis populations ranged from 1.3% to 14.6%[3,4]. In general the incidence of HBsAg positivity among dialysis patients ranges from 0%-7% in low-prevalence countries to 10%-20% in endemic areas.

As in most public health challenges, immunization is the most critical move in preventing HBV infection. It is preferable that chronic kidney disease patients are vaccinated at an early stage and certainly prior to going on dialysis, because vaccine immunogenicity is higher in the general population in comparison to dialysis patients (90% *vs* 70%). Still, dialysis patients should also be vaccinated against HBV infection and have an annual test regarding their hepatitis B antibody (anti-HBs) titer. If it is lower than 10 IU/mL, an intensified protocol should be followed vis a vis a booster vaccine dose should be administered. Such protocols have shown very good responses in hemodialysis patients[5].

HBV EVALUATION IN THE PRETRANSPLANTATION SETTING

HBsAg (+) kidney transplant candidate

All dialysis patients should be routinely checked for HBsAg. In case of seropositivity, additional serologic markers including anti-HBc (IgM and IgG), HBeAg/anti-HBeAb, anti-HbsAb, quantitative HBV-DNA PCR and liver biochemistry including transaminases, ALP, GGT and bilirubin are considered necessary in order to differentiate between active and inactive liver infection.

Active carrier state is defined as HBsAg(+) in the presence of HBeAg(+) or HBeAb, with HBV viral load above 20000 IU/mL with or without elevated alanine aminotransferase (ALT) levels whereas inactive carriers are HBsAg(+) and negative for HBeAg(-) with persistently low viral load, normal liver enzymes and low anti-HBc IgM or anti-HBc IgG levels[6]. The occult HBV carrier state refers to a rare subgroup of patients who are HBsAg(-), most often with detectable anti-HBc but low viral load without liver enzyme elevation[7].

According to these definitions, the most cost-effective strategy is to screen and monitor all dialysis patients with basic serology which includes HBsAg, anti-HBc and anti-HBs. HBV PCR should be performed in the few cases of isolated anti-HBc positivity in order to detect occult carriers, especially among those on the waiting list[8].

In active HBV carriers on hemodialysis, therapy with one of the available antiviral agents is indicated until HBeAg becomes negative and viral replication is suppressed. Inactive carriers should be monitored with HBV-PCR and liver enzymes.

By interpreting HBV serology and virology in hemodialysis patients, it is essential to take into consideration the altered natural history of hepatitis B in this patient setting. HBV infection is usually asymptomatic even in the acute phase, transaminase levels are lower compared to the general population and seroconversion

from HBeAg to anti-HBeAb or from anti-HBc IgM to IgG is delayed or does not occur, even after resolution of the active infection[9]. About 80% of HBV infected dialysis patients progress silently to a chronic carrier state[10].

While on the waiting list, dialysis patients should be monitored every 6-12 mo with HBV-DNA and transaminase levels. Wait-listed transplant candidates must be either inactive carriers or sustained viral responders (SVR) with persistently low, or undetectable HBV-DNA.

Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommend performing a liver biopsy in hemodialysis patients that are candidates for a kidney allograft and are positive for HBsAg, so that hepatitis' severity is assessed. After baseline histological evaluation, candidates should repeat liver biopsy every 3-5 years, if there is ongoing viral replication[11].

Currently, non-invasive tools for the assessment of hepatitis stage are available. The biochemical indices as the APRI score, though useful in the general population, have a reported diagnostic accuracy of about 50% in dialysis patients[12]. The same applies for transient elastography, a routine applied noninvasive tool aiming to assess hepatic fibrosis by liver stiffness measurement (LSM). Unfortunately, both in HBV infected hemodialysis patients and kidney recipients it has not yet been validated. Liver stiffness measurement is influenced by the fluid volume of the patient, which complicates the interpretation of the results due to the discrepancy between pre- and postdialysis values[13]. In a single center cohort of 284 dialysis patients with hepatitis C transient elastography demonstrated high diagnostic accuracy without diminishing the need for further validation, especially in pre-transplant control[14]. Still, in regards to kidney transplant candidates, performing a liver biopsy continues to be considered the "gold standard".

Liver cirrhosis has been regarded for a long time as a definite contraindication for lone kidney transplant with a combination of kidney-liver transplantation being considered the established therapy option. On the other hand nowadays, using new nucleotide analogues often leads to sustained viral response, fibrosis regression and the eventual evolution to a stage of septal inactive cirrhosis. In such cases, a follow up biopsy - 12 mo after the original SVR-must be performed and if the disease remains inactive, the patient may move to the waiting list and possibly undergo lone kidney transplantation[15].

A recent single center study provided data of an excellent five-year survival rate (94%) in 12 cirrhotic patients with hepatitis B after kidney transplantation alone[16].

Routine evaluation for hepatocellular carcinoma (HCC) with liver ultrasound and alfa-fetoprotein values every 6 mo is recommended in all dialysis patients with advanced fibrosis or pre-cirrhotic stage[11].

HBsAg(+) prospective kidney donor

HBV transmission from donor to recipient may occur in kidney transplantation as in all solid organ transplantations. HBV-infected donors' kidneys may be safely used under certain conditions and thus avoid unnecessary organ discarding especially in countries with organ shortage and low HBV prevalence. The routine serologic evaluation of a potential living or deceased donor includes HBsAg, antiHBc and HBsAb. The risk of HBV transmission *via* donation depends on the donor's serologic status.

HBsAg(+)/antiHBc(-)/antiHBs(-): Kidney transplantation is not suggested when the donor is HBsAg(+) and the recipient is HBV naïve since it poses an increased chance of an acquired infection which in most cases has an aggressive progression[17]. Jiang *et al*[18], however, have shown that allografts from HBsAg (+) donors may safely be used in transplantation when the recipient is HBsAg(-) independent of immunity type. This applies to all HbsAg(+) patients with a titer count of more than 10 IU/mL simultaneously receiving hepatitis B hyperimmune globulin (HBIG) independently of whether they are receiving an additional vaccine dose. Even though the probability of transmission is relatively small, it is imperative in such cases to obtain a written informed consent after fully briefing the patient prior to moving along with kidney transplantation. Singh *et al*[19], describe a successful transplantation in 104 anti-HBs(+) patients. Twenty seven recipients received only the original vaccination whereas, the rest concurrently received additional vaccine dose, HBIG and other antiviral medication.

At Laiko hospital in Athens, this kind of renal transplantations from seropositive donors to seronegative or HBs antibody positive patients independent of immunization type (past infection, vaccine) are only allowed when the recipient's titers are at least 10 IU/mL. All recipients receive one booster vaccination dose combined with HBIG just before transplantation. After the introduction of Entecavir such recipients receive post transplantation antiviral prophylaxis for 6 mo. Following this protocol, we have performed 13 transplantations from HBsAg(+) donors to immunized recipients with excellent long term results (unpublished data).

Another safe way to avoid unnecessary organ discarding especially in endemic areas, is to transplant kidneys from HBsAg(+) donors into HBsAg(+) recipients, a practice which offered successful results. In Greece, the allocation policy allows such transplantations, which are also performed in our center with good results.

HBsAg(-)/antiHBc(+)/antiHBs(+): Kidney transplant donors with this serologic profile are considered safe, since there is no way to transmit HBV to the kidney recipient**.** A single case report describes HBV transmission from a multiorgan donor only to the reciepient of the liver graft[20].

HBsAg(-)/antiHBc(+)/antiHBs(-), *i.e.*, isolated

presence of anti-HBc: The risk of HBV transmission from donors with this serological profile, though very low, has not been completely clarified. A recent analysis that examined transplants from anti-HBc(+) donors to 1385 HBsAg(-) recipients found seroconversion to HbsAg-positivity only in four recipients (0.28%) and to anti-HBc-positivity in 32 patients (2.3%)[21]. These donors should preferably be checked for the presence of anti-HBcIgM in order to exclude recent infection. Unfortunately, in relation to deceased donors, such testing is due to time constraints practically impossible. Renal transplantation should however be at the very least considered, since transmission risk is significantly smaller than from HBsAg(+) donors[22,23]. If one selects the safer side, it is preferable to apply the protocol relevant to HBsAg(+) donors.

OUTCOMES OF HBV INFECTED PATIENTS AFTER KIDNEY TRANSPLANTATION

HBV infection is associated with worse survival rates for seropositive patients in comparison to seronegative ones. In a 2005 study with an overall population of 6050 seropositive renal transplant recipients, Fabrizi *et al*[24] calculated a relative death risk of 2.49. The respective graft loss risk was 1.44.

On histological level, the severity of chronic hepatitis B increases during the post-transplantation period and is characterized by higher rates of progression to cirrhosis and mortality due to liver failure. Moreover, HBV(+) renal transplant patients are at increased risk of hepatits B reactivation which may rarely manifest as fulminant hepatitis with massive necrosis or as severe cholestatic hepatitis[25].

The only study of renal transplant patients' liver biopsies did not detect histological worsening in only 15% of seropositive recipients. Following the kidney transplantation, 28% of the patients progressed to liver cirrhosis whereas none had developed it beforehand. Twenty-three percent of the cirrhosis patients also developed hepatocellular cancer[26].

Survival rates for HBV infected kidney transplant recipients have since 1986 significantly increased due to the extensive use of antiviral agents. In a small Italian study, the authors reported that 67% out of the 42 HbsAg(+) patients that received a renal transplant from 1976 to 1982, achieved a survival rate of 12 years[27]. Similarly, Yap *et al*[28], reported that 81% amongst 63 seropositive kidney allograft recipients that received nucleoside/nucleotide analogues therapy, achieved a survival rate of 10 years. Liver failure, however, is still the leading cause of death for this cohort.

ANTIVIRAL TREATMENT IN KIDNEY TRANSPLANTATION

Goal of antiviral treatment

The therapeutic aim is to effectively suppress viral replication, prevent hepatic fibrosis, and at the same time minimize drug resistance. In order to systematically measure the patients' response to therapy, we must measure HBV DNA levels because ALT has a low reliability as a marker of liver disease activity.

Antiviral treatment strategies in kidney transplant recipients: Preemptive administration or prophylaxis?

The introduction of antivirals after transplantation aims to prevent immunosuppression-induced increase of viral replication which may lead to hepatitis B reactivation. The latter is defined by high viral load and or biochemical hepatitis. Virus reactivation is diagnosed by redetection of previously negative HBV-DNA using a highly sensitive assay with a cut off level less than 20 IU/mL, while "hepatitis" diagnosis relies on > 3 fold increase of ALT levels or an absolute increase in ALT above 100 IU/mL. Reverse seroconversion means redetection of HBsAg or anti-HBcAg when previously negative[29].

Antiviral prophylaxis means that treatment is initiated in inactive carriers in order to prevent HBV reactivation. The term "universal prophylaxis" is used when treatment is applied to the entire population at risk as for example to all kidney recipients under treatment with immunosuppression. Preemptive treatment defines antiviral administration after the reappearance of viral load or after the occurrence of seroconversion. According to recent guidelines, universal prophylaxis is recommended for all patients of moderate to high risk for viral reactivation during immunosuppression[30].

Treatment initiation: When should antiviral prophylaxis start?

Antiviral prophylaxis must begin before or at worst immediately after transplantation. A study of 15 patients with normal transaminase levels before transplantation, showed that the 7 that started LAM therapy along with the procedure had undetectable HBV DNA levels for the duration of the observation period. Half of the patients that didn't receive early treatment presented transaminase elevation during the first post-transplantation year[31].

Currently available antiviral agents and their use in kidney transplantation

A number of antivirals agents are available to treat hepatitis B. They include: Pegylated interferon alfa 2a, interferon alfa-2b as well as the nucleoside analogues LAM, telbivudine, tenofovir, entecavir, and adefovir.

Interferon and PEG-INF

The use of interferons following kidney transplant procedure is no longer advised since these agents have led to immunomodulatory effects and ultimately either to graft rejection or to hepatitis reappearance at a rate of almost 80% after suspending treatment[32].

LAM

LAM is a nucleoside reverse transcriptase inhibitor

and has been considered the best therapeutic option and it was the first such agent to be approved for clinical use in HBV infected kidney allograft recipients. The prophylactic use of LAM post-transplantation has offered long-term efficacy. A meta-analysis of 14 clinical trials with a total of 184 recipients that received LAM, indicated in 91% of them untraceable viral cargo and normal liver enzyme in 81%, for a significantly long time[33].

Prolonged treatment with LAM, however, eventually leads to the treatment resistance. In most cases resistance occurs due to a mutation in the tyrosine-methionine-aspartate-aspartate (YMDD) locus of HBV DNA polymerase[34]. The clinical presentation of resistance varies. Some patients show only reappearance of serum HBV DNA while others present with HBV reactivation.

The rate of LAM resistance varies from 20% up to 60% in different studies[35,36]. Following 29 kidney allograft recipients for a mean period of 69 mo, Fabrizi *et al*[34], reported that 48% of them (14/29) developed LAM resistance, whereas all 14 of them had YMDD mutation. Out of these patients that presented resistance, 79% had a disease flare.

Prolonged period of therapy is positively linked with resistance to LAM with the cumulative probability reaching 60% after 69 mo of therapy[33,35]. Patients with LAM resistance should be treated preferably with adefovir or tenofovir, if renal function permits or alternatively with entecavir.

Even though LAM is not nephrotoxic, it is removed by the kidney, and therefore the dose ought to be adjusted to the patient's renal function. The recommended dose for patients with estimated GFR > 50 mL/min per 1.73 m^2 is 100 milligram per day and 100 milligram every second day for those that present kidney injury/failure.

Most importantly, after systematic use of LAM prophylaxis, survival rates in HBV infected kidney transplant recipients have increased progressively with 81% of them reaching a survival rate of ten years, which is very similar to that of seronegative patients[37].

Entecavir

Entecavir is an analog for guanosine and is considered to be much more effective compared to LAM. It has a high antiviral potency, a high genetic barrier for resistance, a good safety profile and is effective in treatment of naïve as well as of LAM treated patients without resistance.

There is significant evidence of its ability to successfully suppress the virus for a prolonged time. Hu *et al*[38] recently, in 2012, studied 18 (67% of total cases) naïve renal transplant recipients and 9 (33%) recipients that had been previously treated with LAM but without resistant mutations, entecavir was successful in clearing HBV DNA in 70%, 74%, 96% and 100% of patients after 12, 24, 52 and 104 wk respectively. Moreover, compared to LAM, entecavir reached at the same time of treatment higher rates of undetectable HBV DNA (32% *vs* 70%, 37% *vs* 74%, 63% *vs* 96% and 63% *vs* 100% of patients at 12, 24, 52 and 104 wk respectively; $P < 0.005$).

LAM resistant HBV patients, however, do not show similar results. Complete response to entecavir may take more than 6 wk and may not be achieved at all. Entecavir use in LAM or adevofir resistant kidney allograft recipients, was studied by Kamar *et al*[39], examining 10 patients with solid organ transplantation, that included eight renal transplant recipients. After 16.5 mo of therapy, there was a variable decrease in HBV DNA viral load with 50% succeeding in clearing HBV reporting no important unwanted reactions.

Between kidney allograft recipients there are no reported Entecavir-resistant cases. Similarly in the general population Entecavir-resistant patients after 5 years of therapy is minimal (1.2%) in naïve patients. On the contrary, in cases with LAM resistance the chance of entecavir-resistant cases increases annually from year 1 to year 5 (6%, 15%, 36%, 46% and 51% respectively)[40]. According to recent guidelines, entecavir has displaced LAM as first line prophylaxis in HBV(+) kidney transplant recipients[30].

Adefovir dipivoxil

Adefovir, an acyclic nucleoside, is an adenosine analog and is used both in a single agent therapy or combined to entecavir in HBV infected patients and LAM-resistant cases[41]. It is mainly used in LAM resistant HBV patients either as monotherapy or as "add on" therapy to LAM[42].

It is, however, potentially nephrotoxic. Research on HIV patients indicates that high daily doses of adefovir (60-120 mg) could result in renal tubular injury[43]. In a study of 11 renal transplant recipients with LAM resistance that were treated solely with adefovir by Fontaine *et al*[44], dosage was adjusted according to renal function. After 12 mo, serum HBV DNA declined satisfactorily and no hepatitis B reactivation was observed. There was no evidence of nephrotoxicity with no significant adverse events and the drug seemed to be well tolerated. In an analogous study of 11 kidney LAM resistant transplant recipients, adefovir was administered at very low doses according to GFR (2.5-10 mg/d) and showed good efficacy in terms of reducing HBV DNA viral load and normalizing liver enzymes after two years of therapy. Renal parameters were closely monitored and showed a slight increase in creatinine (from 125 ± 35 to 141 ± 32 mmol/L, $P = 0.02$), an increase in proteinuria as well as slight impairment of proximal tubular reabsorption[45]. In a series of 14 LAM resistant transplant recipients, adefovir was administered to 5 patients as monotherapy and to 9 as "add on" to LAM. Five out of 14 patients (29%) had a significant decline in GFR (loss of 10 mL/min or more after 32 mo therapy) which led to treatment

discontinuation in 4 of them[46].

Tenofovir disoproxil fumarate

Tenofovir DF as a nucleotide analog reverse-transcriptase inhibitor (NtRTI) selectively inhibits viral reverse transcriptase, a crucial enzyme in retroviruses such as human immunodeficiency virus and hepatitis B virus, while showing limited inhibition of human enzymes, such as DNA polymerases. Tenofovir has a strong antiviral effect, prevents viral replication and is used in the therapy of naïve patients and those that present LAM resistance[47,48]. In a study with HBV infected patients of the general population, this nucleotide analog had a strong effect when used to treat patients with LAM resistance, while no tenofovir-resistant cases appeared during a forty eight month post-therapy follow up[49]. Still, the shortage of data referring to kidney transplant recipients leads to concerns for potential kidney injury. In a pilot study by Daudé *et al*[50], 7 solid organ recipients - 3 with kidney transplantation - received tenofovir as rescue therapy after resistance to other nucleoside analogues. After 12 mo, there was effective suppression of viral replication with HBV clearance in 3 out of 7 patients.

Telbivudine

Telbivudine is ineffective in LAM resistant HBV renal transplant recipients, due to cross-resistance to entecavir and LAM. There is not enough information regarding telbivudine in the area of kidney transplant recipients.

Treatment duration: Is discontinuation of antivirals feasible?

In the general population the duration of antiviral treatment with nucleoside analogues still remains unclear, since nucleoside analogues cannot completely eradicate HBV[51]. The duration of antiviral therapy for renal transplant patients is even more difficult to assess, while data referring to long term outcomes after nucleoside analog withdrawal in immunosuppressed patients including kidney transplant recipients are lacking. The prophylactic or preemptive use of LAM initially and the newer nucleoside analogues later on, have indeed changed the picture in kidney transplantation, with HBV(+) recipients reaching significantly better long term outcome worldwide. Nevertheless, there are still unresolved issues concerning the use of antivirals in transplantation. Solid organ recipients including kidney, are receiving lifelong immunosuppression. Consequently, one logical assumption might be that they also need lifelong prophylaxis to prevent viral breakthrough or reactivation. On the other hand, "lifelong" antiviral prophylaxis, besides cost, is associated with various problems. The main issue is the development of resistance, primarily to LAM but *via* cross-resistance also to the newer agents as entecavir and to a lesser degree adefovir and tenofovir. Rates of LAM resistance increase with increased therapy duration and approach 60% after 5 years of treatment[35]. Therefore, the prophylactic use of entecavir as first line prophylaxis has already been implemented following recent guidelines. Unfortunately, entecavir is much more expensive and has not been widely approved, especially in developing countries. Furthermore, adefovir and tenofovir are both nephrotoxic[43,46] and with the lower doses used as prophylaxis in kidney transplantation, their long term therapeutic efficacy has not yet been proven.

After the development of resistance, combination therapies are indicated either by switching from LAM to entecavir and tenofovir or as "add on" to LAM. Combination therapies have the same adverse effects and are even more expensive than single agents. Last but not least, nucleoside analogues interfere with immunosuppressive agents as calcineurin inhibitors, making patient monitoring after transplantation even more complicated.

For all these reasons, the feasibility of treatment discontinuation remains one of the most important, yet unresolved issues in HBV(+) kidney transplantation. The first attempt for LAM discontinuation was published by Chan *et al*[52] in 2002. LAM was discontinued in 12 low-risk kidney recipients after stabilization. Withdrawal was successful in 5 patients (41.7%)[53]. Another study retrospectively followed a small cohort of of 14 HBsAg(+) renal transplant recipients. In six of them, antiviral therapy seized after a median of 14 mo. Each of them was on stable maintenance of immunosuppression without any sign of viral activity. After discontinuing antiviral treatment and following the patients for a median of 60 mo, 4 of them (67%) presented no sign of viral breakthrough or HBV re-activation. In the last 2 cases who presented HBV reactivation, antiviral treatment was subsequently reinstated leading to HBV clearance[54]. Despite the small number of cases in both studies, they provide promising results for further investigation.

To sum it up, post renal transplantation antiviral therapy could be withdrawn in cautiously chosen subsets of patients that fulfil certain criteria: Stable renal function, low immunological rejection risk, a minimum of 6-9 mo low-dose maintenance immunosuppression, no evidence for HBV activity and a minimum of 12 mo therapy with antiviral agent without developing resistance. Frequent measurement of HBV-DNA levels and 3-6 mo testing of liver enzymes are essential while antiviral treatment ought to be reinstated if immunosuppression grows, *i.e.,* in the case of antirejection therapy.

IMMUNOSUPPRESSION IN THE COURSE OF HBV AFTER KIDNEY TRANSPLANTATION

There is an association between immunosuppression and HBV reappearance, both in seropositive patients,

and in those positive for anti-HBc/anti-HBs, most frequently in a titer count that is quite low, *i.e.,* previously infected patient[55]. Most data derive from HBV infected patients that receive treatment for either solid organ or hematological malignancies[55,56].

Recipient's immunocompetence as well as the overall level of immunosuppression are highly associated with HBV reactivation after transplantation. Immunosuppression affects the relationship between the host and HBV possibly resulting in serious liver damage. Immunosuppressionmay lead to liver injury through two distinct routes. One pathogenetic pathway is virus hepatotoxicity due to unrestrained intracellular viral replication resuting from diminished host immunosurveillance. Such a risk is intimately associated with the initial phase, during which the overall burden of immunosuppression is elevated while the most severe clinical manifestations are fibrosing cholestatic hepatitis (FCH) and fulminant liver failure. FCH has been initially described as complication of HBV infection in liver grafts. A small number of FCH cases with dismal course have been reported in renal transplant recipients as well without differing histologically from FCH manifesting in liver allografts[57-59].

The second pathway involves secondary immune mediated liver injury occuring when immunosuppressants are withdrawn and immune efficiency is reconstituted. The host immune response destroys HBV infected hepatocytes leading to extensive parenchymal necrosis. This pathway has mainly been observed in solid organ and hematologic malignancies cases even after 6 to 12 mo having completed chemotherapy. In renal transplantation, this process may lead to accelerated liver damage after rapid reduction of immunosuppression, usually after tapering of the high corticosteroid-doses given for anti-rejection therapy[56].

Immunosuppressants

The traditional immunosuppressive agents that may be prescribed in different permutations for renal transplant recipients are: Corticosteroids, azathioprine, mycophenolate acid derivates (MMF/MPA), calcineurin inhibitors (cyclosporin, tacrolimus), and the well known inhibitors of mammalian target of rapamycin (mTORi's: Everolimus, sirolimus). There are two more groups of immunosuppressants; Monoclonal antibodies (anti-CD20 Rituximab, anti-IL2 Basiliximab) and polyclonal antibodies as ATG (antithymocyte globulin) that may be prescribed for either induction or rejection therapy.

According to the KDIGO guidelines all immunosuppressive agents currently used for induction and maintenance immunosuppression in kidney transplantation can be used in HBV(+) recipients[11]. They all increase replication of the virus and may lead to increased chances of HBV reactivation. The American Gastroenterological Association (AGA) has assessed the HBV reactivation risk depending on the use of particular immunosuppressants[30].

Rituximab

Rituximab is considered to have the most elevated risk for HBV reactivation (> 10%) from all immunosuppressive agents that are used in renal transplantation, according to AGA guidelines[30]. Furthermore, this risk may continue up to 12 mo, due to the prolonged duration of the antibody's immune reconstitution. Rituximab is linked to HBV reactivation in HBsAg(+) but also in recipients with anti-HBc possitive and those with anti-HBs positive (reverse seroconversion). In a retrospective analysis, 24.3% between 230 B-cell lymphoma patients, HBsAg-negative patients that received rituximab, were anti-HBc(+). Reactivation oft he virus was observed in 8.9% of patients. Entecavir use led to HBV DNA clearance and allowed for the re-administration of rituximab[60].

Polyclonal antibodies (Antithymocyte globulin)

After administering antithymocyte globulin to patients with severe aplastic anemia, increased rates of viral replication have been reported in HSV, EBV and CMV infections. More specifically, in those cases ATG was given concomitantly with cyclosporin[61]. There is a shortage of reliable data in relation to HBV reactivation after ATG therapy.

Corticosteroids

Corticosteroids (CS) are the most commonly used immunosuppressant in the world. They are, however, undeniably related to elevated viral replication. HBV reactivation risk is dependent upon the dose as well as on the duration of CS use. High CS doses increase viral load even though ALT may decrease. During steroid tapering, one finds the opposite effect with influenced liver enzymes four to six weeks following withdrawal[56,62]. As stated by American Gastroenterological Association, doses of prednisone of 20 mg per day or/and long periods of administration (> 3 mo) could increase the risk for reactivation of hepatitis B along with quick reduction, because of immune modification[30].

In relation to renal trnsplantation, increased doses of corticosteroids are used in the first wk post transplantation; the doses are reduced from that point on and for the next 3-6 mo, eventually leading to a prednisone standard of 5 mg every day or second day. Corticosteroids can totally be sidestepped (steroid-avoidance regimens) or at least could be retracted at four to six weeks or more (steroid-sparing regimens), in stable and low immunological risk cases with outstanding outcomes. In HBV renal transplant patients, CS should be administered at the lowest possible doses and ideally should be withdrawn or even totally abandoned in low immunological risk cases.

Calcineurin inhibitors

Tacrolimus and ciclosporine continue to be the mainstays of immunosuppressive regimens in renal transplant

recipients. There is enough evidence that cyclosporin leads to *in vitro* reduction of viral replication[63,64]. Today, most immunosuppressive treatments are based on tacrolimus. Despite the lack of definitive guidelines, many people support the use of cyclosporin instead of tacrolimus in HBV infected renal transplant patients. Some others prefer to withdraw steroids from a tacrolimus-based regimen. Due to the lack of definite guidelines, choosing between the two calcineurin inhibitors depends on each hospital's practice.

Antimetabolites

Even though, azathioprine is considered to be hepatotoxic, it has not been linked when administered as monotherapy to elevated HBV reactivation risk. Still, the use of more potent and more selective antimetabolites as MPA's, has limited azathioprine use in renal transplantation to patients with special indications[65].

Mycophenolate acid derivates

Azathioprine has been replaced by mycophenolate mofetil and its most recent derivate mycophenolate sodium in the majority of immunosuppressive treatments. There is no definite data about MPA's and HBV reactivation. They are, however, generally considered to be safe for HBV renal transplant patients.

Mamalian target of Rapamycin inhibitors

The reactivation of HBV under treatment with mamalian target of Rapamycin (mTOR) inhibitors has not been examined in kidney transplantation but normally their safety is not disputed. Everolimus when used as a chemotherapeutic agent has been reported to lead to HBV reactivation. The doses, in those cases, however were more elevated compared to accustomed ones prescribed as standard immunosuppressive regimen in renal transplant recipients[66].

Summarizing, all immunosuppressive agents used in renal transplantation could be administered in HBV positive patients. There is no evidence for any specific effect of a particular immunosuppressive agent on viral replication since it is associated with the total amount of immunosuppression. Efforts to minimize immunosuppression-induced viral reactivation should focus on minimization of the total immunosupression burden long term, which is more important than the choice of one single agent over another. Minimization protocols, especially corticosteroid-avoiding or sparing protocols, are preferable and should be applied to low-immunological risk HBV(+) recipients. Close HBV monitoring is mandatory whenever the total immunosuppression status is altered.

CONCLUSION

In the era of potent antivirals and with evolving knowledge and mounting evidence in the areas of both kidney transplantation and hepatitis B, HBsAg(+) renal transplant candidates and recipients can be monitored and successfully treated, reaching survival rates that are comparable to their HBsAg(-) counterparts. Furthermore, under certain conditions kidneys from HBsAg(+) donors can be safely transplanted into immunized recipients thus avoiding unnecessary organ discard.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Real-world efficacy of daclatasvir and asunaprevir with respect to resistance-associated substitutions

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Abstract

AIM

To investigate daclatasvir (DCV) and asunaprevir (ASV) efficacy in hepatitis C (HCV) patients, with respect to resistance-associated substitutions (RASs).

METHODS

A total of 392 HCV-infected patients from multiple centers were included in this study. We evaluated their clinical courses and sustained virologic responses (SVR) according to pretreatment factors (gender, age, history of interferon-based regimens, platelet counts, level of viremia, pretreatment NA5A:L31, and Y93 substitutions). We also analyzed the pretreatment and post-treatment major RASs of NS3:D168, NS5A:L31 and Y93 substitutions using a direct-sequencing method in 17 patients who were unable to achieve SVR at 12 wk after treatment completion (SVR12).

RESULTS

The overall SVR12 rate was 88.3%. Thirty-one patients discontinued treatment before 24 wk because of adverse events, 23 of whom achieved SVR12. There were no significant differences in SVR12 rates with respect to gender, age, history of interferon-based regimens, and platelet counts. The SVR12 rate in patients with viral loads of $\geqslant$ 6.0 log IU/mL was significantly lower than those with viral loads of < 6.0 log IU/mL (P < 0.001). The SVR12 rate in patients with Y93 substitution-positive was significantly lower than those with Y93 substitution-negative (P < 0.001). The L31 substitution-positive group showed a lower SVR12 rate than the L31 substitution-negative group, but the difference was not statistically significant. Seventeen patients who did not achieve SVR12 and had available pretreatment and post-treatment sera had additional RASs in NS3:D168, NS5:L31, and Y93 substitution at treatment failure.

CONCLUSION

Combination of DCV and ASV is associated with a high SVR rate. Baseline RASs should be thoroughly assessed to avoid additional RASs after treatment failure.

Key words: Hepatitis C; Asunaprevir; Combination therapy; Resistance-associated substitutions; Daclatasvir

Core tip: Hepatitis C - infected patients treated with daclatasvir and asunaprevir were evaluated for sustained virological response (SVR) according to pretreatment factors. The overall rate of SVR12 was 88.3%. The SVR12 rate in the $\geqslant$ 6.0 log IU/mL group was significantly lower than in the < 6.0 log IU/mL group. The SVR12 rate in Y93 substitution-positive patients was significantly lower than that in non-Y93 substitution patients. The L31 substitution-positive group had a lower SVR 12 rate than the L31 substitution-negative group. Seventeen patients who did not achieve SVR 12 had additional RASs in NS3:D168, NS5:L31, and Y93 post-treatment. Baseline RASs should be thoroughly assessed to avoid additional RASs after treatment failure.

Fujii H, Umemura A, Nishikawa T, Yamaguchi K, Moriguchi M, Nakamura H, Yasui K, Minami M, Tanaka S, Ishikawa H, Kimura H, Takami S, Nagao Y, Shima T, Itoh Y. Real-world efficacy of daclatasvir and asunaprevir with respect to resistance-associated substitutions. *World J Hepatol* 2017; 9(25): 1064-1072 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i25/1064.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i25.1064

INTRODUCTION

Hepatitis C virus (HCV) is one of the most important chronic infections worldwide. An estimated 170-200 million people are infected with HCV worldwide[1], with approximately 1.0-1.5 million infected people in Japan[2]. HCV treatments have dramatically changed recently. Pegylated interferon (PEG-IFN) and ribavirin (RBV) dual therapy has long been the standard treatment for genotype 1 chronic hepatitis C (CHC). Recently, however, newer anti-HCV drugs, termed direct-acting antiviral agents (DAAs), have become available[3].

Telaprevir (TVR) was the first nonstructural protein 3 (NS3) protease inhibitor (PI)[4] approved in Japan, followed by the second generation NS3 PIs, simeprevir (SMV) and vaniprevir[5-8]. These drugs were scheduled to be administered in combination with PEG-IFN and RBV, and could enhance treatment efficacy. However, both PEG-IFN and RBV can cause various side effects, and they are contraindicated in elderly patients and/or patients with certain comorbid conditions.

The combination of oral daclatasvir (DCV), a NS5A inhibitor, and asunaprevir (ASV), a second generation NS3 PI, has been the first drug combination approved in Japan for the treatment of HCV genotype 1-infected patients. This drug combination showed high rates of sustained virologic response (SVR) and better tolerability[9,10]. Thus, many patients for whom conventional IFN-based treatment was intolerable or incurable have been medicated. We performed a retrospective cohort study to evaluate the safety, tolerability, and effectiveness of DCV and ASV combination therapy in real-world clinical practice. Moreover, we evaluated the presence of pretreatment and post-treatment major resistance-associated substitutions (RASs) (NS5A: L31 and Y93 substitution, and NS3:D168 substitution) using a direct-sequencing method in 17 patients who did not achieve SVR12.

MATERIALS AND METHODS

Patients

Patients were enrolled at Kyoto Prefectural University of Medicine and seven affiliated hospitals in the Kinki area of Japan (Kyoto, Osaka, Nara, Shiga Prefecture)

from 2014 to 2015. Study protocols were approved by the ethics committee of each institution and conformed to the provisions of the Declaration of Helsinki. Eligible patients were those aged at least 20 years with HCV genotype 1 infection diagnosed by board-certified hepatologists. Patients with decompensated liver cirrhosis, chronic hepatitis B, HIV, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, or Wilson's disease, were excluded. Patients with uncontrollable hypertension, those with a history of alcohol abuse or clinically significant medical conditions (severe renal disease, severe heart disease, active drug users, pregnancy, and those receiving drugs which interact with DCV or ASV) were also excluded. Patients were followed up monthly or every 2 wk to assess liver function and virological markers during treatment and until 12 wk after the completion of DCV and ASV therapy. All patients gave informed consent to participate in this study. Five patients were lost to follow-up or underwent extreme protocol deviation (*e.g.,* death by accident). Those lost to follow-up or had extreme protocol deviation were excluded from the analysis.

Study design

Patients received oral daclatasvir (Daklinza; Bristol-Myers Squibb Company) 60 mg once daily with oral asunaprevir (Sunvepra; Bristol-Myers Squibb Company) 200 mg twice daily, in accordance with prescribing information, for 24 wk. Patients were followed up until at least 12 wk after final treatment administration to assess SVR12.

HCV RNA responses during therapy were classified into the following groups: The non-response group (NR), patients whose HCV RNA remained detectable during treatment, resulting in treatment discontinuation; the breakthrough group (BT), patients whose HCV RNA was once undetectable but reappeared during treatment; and the relapse group (REL), patients whose HCV RNA was undetectable at the end of the 24-wk treatment but became detectable again during follow-up. SVR12 was defined as undetectable serum HCV RNA levels at 12 wk after the end of treatment (EOT). Therapeutic effects were evaluated using per-protocol analysis and included patients who received at least 2 wk of this therapy.

Continuation of treatment was decided by board-certified hepatologists. In general, patients whose serum HCV RNA was positive at 8 or 12 wk were judged as NR, and the treatment was terminated at that time. Patients whose serum HCV RNA reappeared were diagnosed as belonging to the BT group, and the treatment was stopped at the time. Dose reduction or discontinuation of DCV or ASV was determined by board-certified hepatologists. Discontinuation of the treatment was generally considered when grade 3-4 adverse events according to Common Terminology Criteria for Adverse Events v4.0 occurred.

Laboratory assessments

Blood samples were obtained for routine biochemical and hematological assessments at treatment initiation, on treatment weeks 4, 8, 12, 16, 20, 24, at EOT, and at 12 wk after EOT. Antiviral effects were mostly assessed by measuring serum HCV RNA levels using the COBAS TaqMan HCV test (Roche Molecular Diagnostics, Tokyo, Japan) with a lower limit of quantitation (LLOQ) of 15 IU/mL (with a quantitation range of 1.2-7.8 $\log_{10}$ IU/mL). Missing data points were deemed a success if the immediately preceding and subsequent time points were successful; otherwise, data points were termed as failures. Patients who had missing data because of premature discontinuations were considered failures from the point of discontinuation.

Pretreatment major RASs of NS5A, L31 or Y93 substitutions were assessed using commercially available assays of direct-sequencing method, the cycleave probe method, or invader assays. Furthermore, the pretreatment and post-treatment major RASs of NS3:D168, NS5A:L31, and Y93 substitution were investigated in 21 patients with virological failure by using a direct-sequencing method. In brief, HCV RNA was extracted from blood serum using a commercially available kit (QIAamp viral RNA kit; QIAGEN, Valencia, CA, United States). This sample was used for reverse transcription with random hexamer primers (SuperScript Ⅲ First-Strand Synthesis System for RT-PCR cDNA synthesis kit; Invitrogen, Carlsbad, CA, United States). The NS3 and NS5A regions were amplified by nested PCR using Takara Ex Taq HS (Takara Ex Taq, Otsu, Japan). The PCR primer sequences were NS3 forward primer: gccgcgatgccatcatcctcc, gtccaaatggccttcatgaagctgg, caatgtagaccaggacctcgtcgg and reverse primer: tggtgaaggtgggatccaagctgaa; or NS5A forward primer: atcctctccagccttaccatcact and reverse primer: ccatgaccaactcgggctggacctt. The PCR products were separated by electrophoresis on 1% agarose gels. These were purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and sequenced with second-round PCR primers using a dye terminator sequencing kit (BigDye Terminator v 1.1 cycle sequencing kit; Applied Biosystems, Foster City, CA, USA) and ABI PRISM 310 genetic analyzer (Applied Biosystems).

Statistical analysis

Baseline continuous data were expressed as median with interquartile ranges in parentheses, and categorical variables were expressed as numbers. Some baseline data were categorized, and univariate analyses were performed using the χ^2 or Mann-Whitney *U*-tests as appropriate. All *P*-values of < 0.05 of two-tailed tests were considered significant. All statistical analyses were performed using the SPSS 22.0 statistical package

Table 1 Baseline patient characteristics

No. of patients	*n* = 392	
Gender (male/female)	159/233	
Age, yr	71.0	(64.0-77.0)
< 65 yr *vs* ⩾ 65 yr	99/293	
Laboratory data		
Level of viremia (log IU/mL)	6.2	(5.8-6.5)
< 6.0 *vs* ⩾ 6.0	137 *vs* 255	(35.1% *vs* 64.9%)
Platelet count (× 10^4/mm^3)	12.6	(9.2-16.7)
10 < *vs* ⩾ 10	114/278	(29.0% *vs* 70.4%)
ALT (IU/L)	41	(29-65)
γ-GTP(IU/L)	34	(22-57)
Other data		
Prior treatment		
IFN *vs* PEG plus RBV *vs* TVR *vs* SMV	70/147/13/9	
NS5A polymorphisms, *n* (%)		
L31 substitution, *n* = 288	10 (3.5)	
Y93 substitution, *n* = 321	27 (8.4)	
L31 and/or Y93, *n* = 321	35 (10.9)	

Data are presented as numbers. Percentages or medians with interquartile ranges are presented in parentheses. ALT: Alanine aminotransferase; γ-GTP: Gamma-glutamyltransferase; IFN: Interferon; PEG plus RBV: Pegylated interferon plus ribavirin; TVR: Pegylated interferon plus ribavirin plus telaprevir triple therapy; SMV: Pegylated interferon plus ribavirin plus simeprevir triple therapy; L31: NS5A: L31 substitution patients. A total of 288 patients were assessed at pretreatment; Y93: NS5A: Y93 substitutions. A total of 321 patients were assessed at pretreatment.

(SPSS Incorporated, Chicago, IL, United States).

RESULTS

Baseline characteristics

The baseline patient characteristics are shown in Table 1. In total, 392 patients were included in this study. Female patients were predominant. Enrolled patients were generally older (median, 71.0 years) and had lower platelet counts. As for prior treatments, 70 patients had received IFN, 147 patients had received PEG-IFN or IFN plus RBV, 13 patients had a history of PEG-IFN plus RBV plus TVR, and 9 patients had a history of PEG-IFN plus RBV plus SMV. Concerning RASs, L31, and Y93 substitutions at pretreatment were seen in 3.5% (10/288) and 8.4% (27/321) of patients, respectively (Table 1). Two patients had both L31M and Y93H RASs.

Virological response to therapy and loss of HCV RNA during treatment

Undetectable HCV RNA levels were achieved in 79.7% (299 of 375), 94.1% (367 of 390), 94.1% (369 of 392), 92.8% (363 of 391), 92.2% (355 of 385), 91.3% (358 of 392), and 88.3% (346 of 392) of patients at treatment weeks 4, 8, 12, 16, 20, 24, and at 12 weeks' post-treatment, respectively (Figure 1). Because two of the SVR12 patients experienced late relapse of chronic hepatitis C and two additional patients were lost to follow-up, the final SVR24 resulted in 87.2%. Treatment was discontinued in 8 NR patients (2.0%) because the therapeutic effect was hardly expected, and in 18 BT patients (4.6%). Twelve patients (3.0%) received 24 wk of treatment but ended in REL. Thirty-one patients discontinued treatment before 24 wk because of adverse events. Reasons for discontinuation included liver dysfunction (15 patients), fever increase (6), detection of HCC (2), edema or ascites (2), and other reasons (6). Of these, 23 patients (15 liver dysfunction, 2 fever increase, 2 HCC, 1 edema and ascites, and 3 with other reasons) eventually achieved SVR12. Eight patients who received treatment less than 8 wk due to adverse events achieved SVR12. There were no treatment-related deaths.

SVR12 rates according to age, platelet counts, level of viremia, and substitutions in NS5A:L31 and Y93

We assessed the SVR12 rate according to gender, age (< 65 *vs* ⩾ 65), history of IFN-based treatment, platelet counts (< 10 × 10^4/mm^3 *vs* ⩾ 10 × 10^4/mm^3), level of viremia (< 6.0 log IU/mL *vs* ⩾ 6.0 log IU/mL), and pretreatment L31 and Y93 substitution (negative or positive). The SVR12 rate in the ⩾ 6.0 log IU/mL group was significantly lower than in the < 6.0 logIU/mL group (P < 0.001). As for Y93 substitutions, the SVR12 in the Y93 substitution-positive group was significantly lower than that in the Y93 substitution-negative group (P < 0.001). As for L31 substitutions, the L31 substitution-positive group showed a lower SVR 12 rate than their negative counterparts, but without statistical significance (P = 0.28). Other parameters were similar between the two groups (Figure 2).

Pretreatment and post-treatment RASs and fibrosis-4 index

We investigated the pretreatment and post-treatment major RASs (NS5A:L31 and Y93 substitutions and NS3:D168 substitution) using a direct-sequencing method in 21 patients who did not achieve SVR12. The results of direct-sequencing were of poor quality in four patients, leaving 17 patients who could be investigated completely. Five patients had NS3:D168 substitution, three had NS5A:L31 substitution, and six had NS5A:Y93 substitution before treatment. Five patients had neither NS3:D168 nor NS5A:L31 or Y93 substitutions before treatment. Analysis at the time of virological failure revealed that 14, 14 and 13 patients had NS3:D168, NS5A:L31, and NS5A:Y93 substitutions, respectively. All 17 patients whose pretreatment and post-treatment sera were available had one of the major RASs at the time of virological failure. Moreover, many patients had additional amino acid substitutions like NS5A Q54H (Table 2).

We compared the Fibrosis-4 (FIB-4) index[11] at baseline and at 12 wk after the end of treatment. Baseline FIB-4 index was 4.14 and it remarkably decreased to 3.78 at 12 wk after the end of treatment in the SVR12 group (P < 0.001). Meanwhile, bseline FIB-4 index was 3.84 and it slightly decreased to 3.57 in the non-SVR12 group (P = 0.03) FIB-4 index was

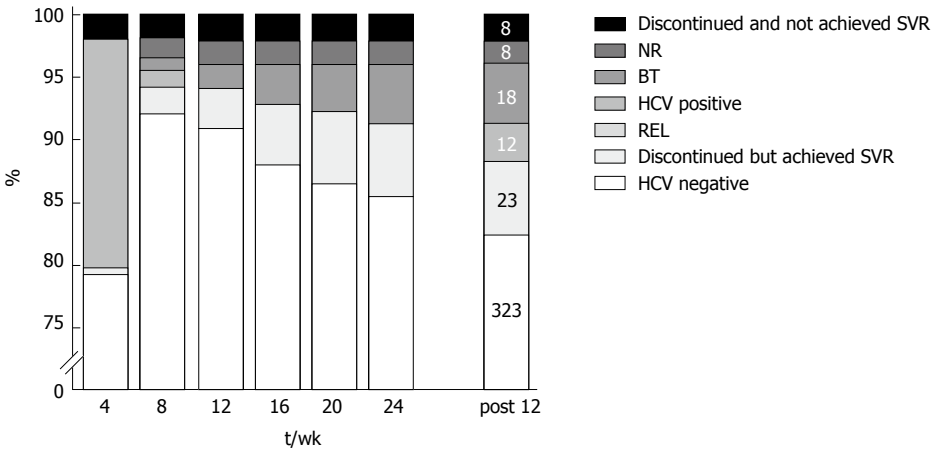

Figure 1 Virological response and treatment outcomes. Black closed squares indicate the proportion of patients who discontinued treatment because of adverse events and unachieved SVR12. Non-response (NR), where HCV RNA remained detectable during treatment, prompting treatment discontinuation. Breakthrough (BT), where HCV RNA was undetectable but reappeared during treatment. Relapse (REL), where HCV RNA was undetectable at the end of the treatment but became quantifiable again during follow-up. Gray closed squares indicate the proportion of patients with HCV RNA detected at the time of measurement. Light gray square indicate the proportion of patients who discontinued treatment because of adverse events but nevertheless achieved SVR12. White closed squares indicate the proportion of patients whose HCV RNA viral loads were undetected at the time of measurement. The Post 12 wk bar indicates the number of patients in each square.

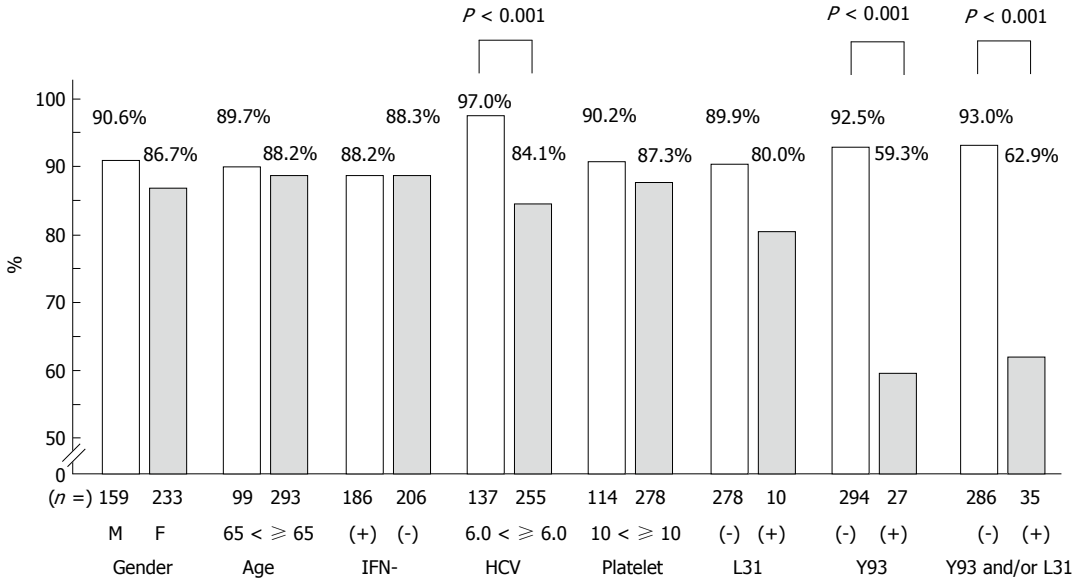

Figure 2 Bars in this graph indicate SVR12 rates according to gender (male, female), age (< 65 year *vs* ⩾ 65 year), history of interferon-based regimen treatment (+ *vs* -), level of viremia (< 6.0 logIU/mL *vs* ⩾ 6.0 logIU/mL), platelet counts (< 10 × 10^4/mm^3 *vs* ⩾ 10 × 10^4/mm^3), pretreatment existing L31 substitution [(-): substitution negative, (+): substitution positive], pretreatment existing Y93 substitution [(-): substitution negative, (+): substitution positive], and Y93 and/or L31 [(-): both L31 and Y93 substitution negative, (+): either L31 or Y93 substitution positive, or both L31 and Y93 substitution positive]. M: Male; F: Female; IFN: Interferon.

more markedly reduced in the SVR12 group.

DISCUSSION

In the present study, we presented the efficacy of DCV and ASV in real-world clinical practice. Most patients were rapidly cleared of HCV and achieved SVR12 with this combination of DAAs. Interestingly, some patients who discontinued treatment after a short duration because of side effects also achieved SVR. Resistance analyses revealed that patients who did not carry both Y93H and L31 mutations before treatment achieved 93.0% SVR12. All 17 patients who could not achieve SVR 12 and who were investigated for major RASs had either NS3-D168 substitution, or NS5A L31 and/or Y93 substitutions, including five patients who did not carry any RASs before treatment.

The high SVR rate of 88.3% in our present study is consistent with previous reports. Serum HCV RNA levels decreased rapidly, and was undetectable by 12 wk in the majority of patients[9,10]. In general, the therapeutic efficacy of a novel IFN-based HCV therapy

Table 2 Pretreatment and post-treatment major RAVs of 17 patients who did not achieve SVR12 (NS3:D168, NS5A:L31, and Y93 substitution)

No.	Pretreatment						Post-treatment				
	C.C.	D168	L31	Y93	Other NS3	Other NS5A	D168	L31	Y93	Other NS3	Other NS5A
1	BT	E	-	-	-	-	E	-	-	-	R30H
2	REL	-	L/I	-	-	A92T	-	M	-	-	A92K
3	NR	-	-	H	V170I	Q54Y	-	I	H	V170I	Q54Y
4	BT	Y	-	-	-	Q54H	Y	F	H	-	Q54H
5	NR	E	-	-	Q80R, V170I	Q54H	E	V	H	Q80R, V170I	Q54H
6	BT	E	-	-	-	Q54H	E	V/M	H	V170I	Q54H
7	BT	-	M/L	-	-	Q54V	V	M/V	H	-	Q54V
8	BT	-	-	H	-	Q54H	V	M	H	-	Q54H
9	REL	-	-	H	-	Q62E	V	I	H	-	Q62E
10	BT	-	-	H/Y	-	-	T	M	H	-	-
11	BT	-	-	H/Y	-	-	V	V/F	H	V170I	-
12	BT	Y	F	H	-	-	D	F	H	-	Q54H
13	BT	-	-	-	-	Q54H, A92T	V	-	-	-	Q54H, A92K
14	REL	-	-	-	-	Q54H	E	-	-	-	P32L, Q54H, A92K
15	BT	-	-	-	-	Q62N	V	V	H	-	Q62N
16	BT	-	-	-	-	-	-	V	H	-	-
17	REL	-	-	-	-	-	E	M	H	-	-

C.C.: Virological clinical course; NR: Non-response, HCV RNA was still detectable during treatment so treatment was discontinued; BT: Breakthrough, HCV RNA became undetectable but reappeared during treatment; REL: Relapse, HCV was undetectable at the end of the 24-wk treatment but became quantifiable again during follow-up; D168: NS3:D168 substitution; L31: NS5A:L31 substitution; Y93: NS5A:Y93 substitutions. Other NS3, other NS3 substitution except D168 substitution; Other NS5A, other NS5A substitution except L31 and Y93 substitution. Analyses were performed by using a direct-sequencing method.

in real-world clinical practice demonstrates lower SVR rates and higher rates of adverse events than observed in clinical trials. The stable therapeutic effect in a real-world setting is one of the notable benefits of the DCV and ASV combination therapy.

Baseline characteristics of gender, advanced age, history of IFN-based regimens, and low platelet counts (suggestive of advanced fibrosis or cirrhosis) did not influence SVR12 rates. An important finding was that patients with pretreatment viral loads of $\geqslant$ 6.0 log IU/mL showed a significantly lower rate of SVR12 than patients with pretreatment viral loads of < 6.0 log IU/mL. As for DCV and ASV treatment, Wang *et al*[12] showed that patients with lower viral loads (< 8×10^5 IU/mL: 8.0×10^5 IU/mL = 5.9 log IU/mL) seemed to have higher SVR rates than those with higher viral loads ($\geqslant 8 \times 10^5$IU/mL) in their meta-analysis[12]. Comparing the background characteristics of patients with viral loads of $\geqslant$ 6.0 logIU/mL and < 6.0 logIU/mL in this study showed that they were not significantly different with respect to gender, age, platelet counts, number of treatment discontinuations, and preexisting major RASs (L31 substitution or Y93 substitution). The number of patients with a history of IFN-based treatment was significantly greater in patients with viral loads of $\geqslant$ 6.0 log IU/mL than those with < 6.0 log IU/mL (P = 0.04). PEG-IFN and RBV-based treatment was covered by public health insurance only for high viral loads ($\geqslant$ 5.0 log IU/mL) in Japan. This might have caused the background difference in our study. The HCV RNA loads alone may have influenced treatment efficacy.

Twenty-three patients who experienced adverse events discontinued the treatment but nevertheless achieved SVR12. It is notable that eight patients who were treated for < 8 wk still achieved SVR12. The shortest treatment duration was only 2 wk. The factors that contributed to HCV clearance in such a short treatment duration are unknown. Because alanine aminotransferase (ALT) elevation was the main cause (15 in 23 patients) of stopping treatment early, elevated blood levels of ASV may be both a cause of ALT elevation and an enhanced treatment efficacy[13]. Interestingly, in our study, patients who discontinued treatment because of ALT elevation tended to have lower viral loads. This background factor of low viral load might influence treatment effectiveness. Patients with adverse events such as ALT elevation can still achieve SVR even with short treatment periods.

Resistance analyses before treatment revealed that patients who did not carry both Y93 and L31 substitutions using commercially available tests achieved 93.0% SVR12 (Figure 2). The SVR ratio of the Y93 substitution-positive group was significantly lower than in the Y93 non-substitution group. However, the SVR ratio of the L31 substitution-positive group was not significantly different from that of the L31 non-substitution group. The baseline prevalence of L31 substitution in our study was lower than that in other studies and this might have affected our statistical analyses.

We investigated RASs before treatment and after failure in 17 patients who failed to achieve SVR12. All 17 patients had major well-known substitutions (either NS5A L31 substitution and/or Y93 substitution, or NS3 D168 substitution) at the time of failure. The

appearance pattern of these RASs was different in each patient, but can be classified into one of two patterns. The first pattern (Cases 13-17) had no major substitutions before treatment, but carried more than one major variant after treatment. The other (Cases 4-11) had one major substitution before treatment but carried three major substitutions after treatment. The mechanism of occurrence for the first pattern is obscure, but it might have been influenced by the detection sensitivity of direct sequence methods. The mechanism of the second pattern is also obscure, but it revealed an important problem of this therapy for the next generation of DAA treatments from the viewpoint of drug resistance. At any rate, there are still many problems to be solved concerning RASs.

The first problem of pretreatment RAS analysis is that there are no available commercial assays for NS3 mutations in the real world. We did not check the RASs in NS3 for two reasons. One reason is that naturally occurring NS3 RASs are reported to be rare[14,15]. Another reason is that the guideline for the treatment of hepatitis C edited by the Japan Society of Hepatology do not recommend to check NS3 RASs, but recommend to check NS5A RASs before starting DCV/ASV treatment. The second problem is the difference in sensitivities of available assays[16,17]. We could measure NS5A variants using three different methods: Direct-sequencing, the cycleave probe method, and invader assay. Although ultra-deep sequencing is the most sensitive method and can detect minor variants with frequencies of < 1% (Miura *et al*[18]), this method is too expensive and complicated. The appropriate proportion of RASs to predict this treatment's efficacy has been reported in several studies. Ide *et al*[19] reported that SVR rates were clearly altered by the proportion of Y93 substitutions. In our unpublished data, patients who had > 10% pretreatment Y93 substitutions by the cycleave probe method tended to experience virological failure. Thus, this may be the appropriate cutoff value in our study (data not shown). Except for L31 and Y93 substitutions which can be checked commercially, other rare NS5A RASs were reported to have a rather small influence on therapeutic effect[20]. After all, although RASs have a great influence on the therapeutic efficacy of DCV and ASV combination treatment, we cannot determine the best method at present. Further larger-scale studies are needed to clarify this point.

The eradication of HCV can ameliorate liver inflammation as well as liver fibrosis[21]. We calculated the values of FIB4 index both at baseline and after SVR12. We found that FIB-4 index was more markedly reduced in the SVR12 group. This data indicate that liver fibrosis is improved in SVR12 group in the future

A major limitation of the present study was the inability to evaluate several factors known to influence HCV treatment efficacy. We did not examine IL28B[22], amino acid substitutions of the HCV core region 70 and 91[23], NS5A interferon sensitivity determining region[24], interferon/ribavirin resistance determining region[25]. These factors were mainly related to the efficacy of IFN based therapy and were not easily available in real-world.

DCV and ASV combination therapy is associated with a high SVR rate in real-world clinical practice. The SVR12 rate in patients with viral loads of HCV RNA $\geqslant$ 6.0 log IU/mL was significantly lower than that in patients with HCV RNA < 6.0 log IU/mL. The ratio of SVR12 in the Y93 substitution-positive group was significantly lower than that in the Y93 substitution-negative group. The pretreatment and post-treatment NS3:D168 substitution, and NS5A:L31 and Y93 substitutions were evaluated in 17 patients who did not achieve SVR 12 using direct-sequencing method. All 17 patients had increased RASs after treatment failure. Baseline RASs should be thoroughly assessed to avoid additional RASs after treatment failure.

COMMENTS

Background

The modality for treating hepatitis C has rapidly progressed in a recent years. The usage of directly acting antiviral (DAA) has changed the treatment dramatically. The combination of oral daclatasvir (DCV), a NS5A inhibitor, and asunaprevir (ASV), a second generation NS3 PI, is the first drug combination approved in Japan for the treatment of hepatitis C (HCV) genotype 1-infected patients. This drug combination showed high rates of sustained virologic response (SVR) and better tolerability. They performed a retrospective cohort study to evaluate the effectiveness of DCV and ASV combination therapy in real-world clinical practice. Moreover, they evaluated the presence of pretreatment and post-treatment major resistance-associated substitutions (RASs) (NS5A:L31 and Y93 substitution, and NS3:D168 substitution) using a direct-sequencing method in 17 patients who did not achieve SVR12.

Research frontiers

In the era of DAA treatment for hepatitis C, drugs are designed targeting NA3, NA5A, and NS5B of HCV. Evaluation of the efficacy/tolerability of the DAAs regimens as well as the acquisition of RASs in DAAs treatment are major interest in the field of hepatology. Above all, attention is paid for RASs (NS5A: L31 and Y93 substitution, and NS3:D168 substitution) in DAC/ASV treatment.

Innovations and breakthroughs

All 17 patients who failed to achieve SVR12 in DAC/ASV treatment had major well-known RASs (either NS5A L31 RAS and/or Y93 RAS, or NS3 D168 RAS) after treatment failure. The appearance pattern of these RASs was different, but can be classified into two patterns. The first pattern: No major RASs before treatment, but more than one major RASs after treatment failure. The second pattern: One major RAS before treatment but three major RASs after treatment failure.

Applications

DCV/ASV combination therapy is associated with a high SVR rate in real-world clinical practice, but appearance of RASs were seen in patients with treatment failure. Baseline critical RASs should be checked to avoid additional RASs after treatment failure.

Peer-review

Very interesting paper on DCV and ASV efficacy in HCV patients.

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ORIGINAL ARTICLE

Observational Study

CD4$^+$ T cells and natural killer cells: Biomarkers for hepatic fibrosis in human immunodeficiency virus/hepatitis C virus-coinfected patients

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Abstract

AIM

To characterize peripheral blood natural killer (NK) cells phenotypes by flow cytometry as potential biomarker of liver fibrosis in human immunodeficiency virus (HIV)/hepatitis C virus (HCV) coinfected patients.

METHODS

Peripheral mononuclear cells from 24 HIV/HCV (HBV

negative) coinfected and 5 HIV/HCV/HBV seronegative individuals were evaluated. HIV/HCV coinfected patients were divided in to groups: G1, patients with METAVIR F0-F2 and G2, patients with METAVIR F3-F4. NK surface cell staining was performed with: Anti-CD3(APC/Cy7), anti-CD56(PE/Cy5), anti-CD57(APC), anti-CD25(PE), anti-CD69(FITC), anti-NKp30(PE), anti-NKp46(PE/Cy7), anti-NKG2D(APC), anti-DNAM(FITC); anti-CD62L (PE/Cy7), anti-CCR7(PE), anti-TRAIL(PE), anti-FasL(PE), anti CD94(FITC). Flow cytometry data acquisition was performed on BD FACSCanto, analyzed using FlowJo software. Frequency of fluorescence was analyzed for all single markers. Clinical records were reviewed, and epidemiological and clinical data were obtained.

RESULTS

Samples from 11 patients were included in G1 and from 13 in G2. All patients were on ARV, with undetectable HIV viral load. Liver fibrosis was evaluated by transient elastography in 90% of the patients and with biopsy in 10% of the patients. Mean HCV viral load was (6.18 ± 0.7 $\log_{10}$). Even though, no major significant differences were observed between G1 and G2 regarding NK surface markers, it was found that patients with higher liver fibrosis presented statistically lower percentage of NK cells than individual with low to mild fibrosis and healthy controls (G2: 5.4% ± 2.3%, G1: 12.6% ± 8.2%, P = 0.002 and healthy controls 12.2% ± 2.7%, P = 0.008). It was also found that individuals with higher liver fibrosis presented lower CD4 LT count than those from G1 (G2: 521 ± 312 cells/μL, G1: 770 ± 205 cells/μL; P = 0.035).

CONCLUSION

Higher levels of liver fibrosis were associated with lower percentage of NK cells and LTCD4$^+$ count; and they may serve as noninvasive biomarkers of liver damage.

Key words: CD4$^+$ T cell; Human immunodeficiency virus/hepatitis C virus-coinfection; Fibrosis; Biomarker; Natural killer cells

Core tip: Approximately 2.3 million individuals with human immunodeficiency virus are coinfected with hepatitis C virus (HCV). The high cost of HCV treatment restricts its use. It is crucial to identify patients with advanced liver fibrosis with an urgent need of treatment. The aim of this study was to identify natural killer (NK) phenotypes as a biomarker for liver fibrosis. We observed that those subjects with higher fibrosis are those with lower percentage of NK cells and also with lower LTCD4$^+$ count. These constitute two simple parameters that might be performed in a routine laboratory test and used in clinical practice as biomarkers for liver fibrosis.

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection affects 115 million individuals worldwide and is a common cause of chronic hepatitis, which may eventually progress to cirrhosis and hepatocellular carcinoma[1]; whereas currently 36.9 million people are living with human immunodeficiency virus (HIV)/aids [2]. Because of overlapping pathways of transmission, approximately 2.3 million individuals worldwide are estimated to be coinfected with both viruses[3]. Direct antiviral agents (DAA) are a major development in the treatment of HCV infection, with cure rates higher than 90%[4]. However, the high cost of DAA regimens and competing public health priorities have prompted a worldwide discussion whether all patients should have access to the new therapies without restriction. In many countries, new DAA regimens are therefore reserved for patients with advanced fibrosis or cirrhosis[5,6].

Liver fibrosis is a response to a wound-healing process triggered by various types of chronic liver injuries, among them HCV infection[7]. Liver fibrosis is well characterized by abnormal accumulation of extracellular matrix, and hepatic stellate cells (HSCs) are considered to be the major type of cells responsible for liver fibrosis. Such profibrotic role might be down-regulated by natural killer (NK) cells either directly through induction of HSC apoptosis or indirectly *via* production of IFN-γ. Increased peripheral NK cell-mediated cytotoxicity has been associated with less liver fibrosis during HCV infection and likely reflects this mechanism[7]. HIV infection *per se* has a strong suppressive effect on anti-HCV activity of NK cells[8].

NK cells are lymphoid cells that are primary responders to microbial infections and tumor cells[9]. Phenotypically, NK cells are defined as CD3$^-$CD56$^+$cells with variable expression of CD16, depending on cell subpopulation of NK cells. They comprise approximately 5%-20% of peripheral lymphoid cells, but up to 30%-50% of intrahepatic lymphoid cells. NK cell activation is regulated by cell surface receptors that become engaged by cognate ligands expressed on target cells by cytokines, and by Toll-like receptors (TLRs)[9-11].

Different techniques to asses liver fibrosis have been developed, from liver biopsy (gold standard) to non-invasive studies (transient liver elastography; patented and nonpatented biomarkers - FIB4, FibroTest, APRI, *etc*). Liver biopsy is invasive and has risk of complications[12]. In addition, liver biopsy may be limited by the size of the specimen obtained as well as sampling,

Table 1 Fluorochrome-conjugated antibodies panels

Antibody	Fluorochrome	Clone	Provider
All panels			
Anti-CD3	APC/Cy7	SK7	BioLegend
Anti-CD56	PE/Cy5	679.1Mc7	Beckman Coulter
Panel 1			
Anti-CD57	APC	HNK-1	BioLegend
Anti-CD25	PE	BC96	BioLegend
Anti-CD69	FITC	FN50	BioLegend
Panel 2			
Anti-NKp30	PE	P30-15	Biolegend
Anti-NKp46	PE/Cy7	9E2	Biolegend
Anti-NKG2D	APC	1D11	Biolegend
Anti-DNAM	FITC	TX25	Biolegend
Panel 3			
Anti-CD62L	PE/Cy7	DREG-56	Biolegend
Anti-CCR7	PE	G043H7	Biolegend
Panel 4			
Anti-TRAIL	PE	S35-934	BD Bioscience
Panel 5			
Anti-FasL	PE	NOK-1	Biolegend
Anti CD94	FITC	DX22	Biolegend

BioLegend, San Diego, California United States. BD Bioscience, San Jose, California, United States. NK: Natural killer.

intraobserver, and interobserver variability[13]. On the other hand, there are many unresolved issues regarding the accuracy (especially in HIV/HCV-coinfected patients) of noninvasive studies[14], and in low-resource countries there is an important barrier to access to these methods (in particular liver elastography and patented biomarkers).

The identification of noninvasive liver fibrosis biomarkers is still an open research area. In this context, we reasoned that the study of the phenotype of peripheral blood cells may unravel interesting clues towards the identification of such biomarkers. Some evidence indicates that the characteristics of the immune cells, including NK cells, observed in peripheral blood are similar to those seen in liver with relatively lower levels of magnitude. Accordingly, the aim of this study was to characterize peripheral blood NK cell phenotypes by flow cytometry as potential biomarker for liver fibrosis in patients chronically coinfected with hepatitis C and HIV.

MATERIALS AND METHODS

Study cohort

Informed consent was obtained from each subject. The study protocol is in line with the ethical guidelines of the Declaration of Helsinki and was approved by the ethics review committee of Fundación Huésped (Buenos Aires, Argentina).

Cryopreserved peripheral blood mononuclear cells (PBMC) from 24 HIV/HCV-coinfected individuals and 5 HIV/HCV-seronegative individuals (healthy controls, HC) were used in this study. HIV/HCV-coinfected patients and healthy control individuals enrolled in this study were not acutely or chronically infected with HBV; they denied current use of recreational drugs or alcohol intake. HIV/HCV-coinfected patients were divided into two groups based on their level of liver fibrosis (group 1: Patients with METAVIR score F0 to F2 on liver biopsy or transient elastography - FibroScan®-; and group 2: Patients with METAVIR score F3-F4). Hepatic fibrosis was evaluated by liver biopsy in 10% of patients and by transient hepatic elastography in 90% of patients. All healthy control individuals presented F0-F1 fibrosis according to transient liver elastography (less than 5 kPa). Clinical records were reviewed, and epidemiological and clinical data were obtained.

Multicolor flow cytometry

Cryopreserved PBMC were thawed and stained with fluorochrome-conjugated antibodies distributed in five different panels (depending on PBMC availability) to evaluate expression of different markers on NK cells detailed in Table 1. Staining was performed for 30 min at 4 ℃. Samples were washed, fixed in 1% paraformaldehyde and acquired in a FACS Canto flow cytometer (BD Biosciences). Data were analyzed using the FlowJo software (TreeStar, Ashland, Oregon, United States). NK cell populations were defined according to the corresponding isotype control.

Plasma viral load levels (Abbott RealTime HIV-1 RNA version 3; Abbott Molecular, Inc., Des Plaines, IL, United States) were assessed in HIV-infected subjects and $CD4^+$ T-cell counts (flow cytometry double platform, BD FACSCanto; BD Biosciences, San Diego/California, United States) were assessed in HIV and HIV-negative individuals.

Statistical analysis

For categorical variables, both χ^2 and Fisher's exact test were applied. For continuous variables, the nonparametric Kruskal-Wallis and Mann-Whitney test were used. Area under the receiving operating curve (ROC) was used to calculate the cut-off point in NK cell percentage with the best sensitivity of high liver fibrosis. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 19.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Patient characteristics at the time of liver fibrosis assessment are shown in Table 2. Individuals from Group 1 (n = 11, 46%) presented low to mild liver fibrosis (METAVIR F0-F2) whereas patients included in Group 2 (n = 13, 54%) had severe fibrosis (METAVIR F3-F4). Forty percent of patients had previously received HCV treatment with pegylated interferon and ribavirin (with no differences between groups); a median of 6.25 ± 1.48 years before sample collection; none of them achieved sustained virological response. The mean age was 46.9 years (± 8.4); 83% were male. Patients from group 2 were older than those with lower METAVIR score (P = 0.028). No differences were

Table 2 Characteristics of the population and divided according the level of fibrosis

Characteristics	Group 1 *n* = 11	Group 2 *n* = 13	Control *n* = 5	*P* value
Age (yr)[1]	46.3 (3.9)	52.2 (4.5)	31 (4.8)	0.02
Male/female	9/2	11/2	3/2	0.85
CD4 cell count[1]	770 (205)	521 (312)	910 (251)	0.03
CD8 cell count[1]	1079 (475)	657 (339)	NA	0.24
METAVIR F0-F2	100%	0	100%	NC
METAVIR F3-F4	0	100%	0	NC
TGP[1]	79.7 (74.2)	99 (71.4)	NA	0.41
Time of known HIV infection[1]	18 (6.52)	17.3 (3.8)	NC	0.60
Time of known HCV infection[1]	14.3 (7.0)	13.8 (4.6)	NC	0.65

[1]mean ± SD. Beckman Coulter, Marseille, France. *P* values, correspond to comparison between group 1 and 2. NA: Not available; NC: Not correspond; HIV: Human immunodeficiency virus; HCV: Hepatitis C virus.

found between groups regarding gender or mean time of known HIV and HCV infection. The mean HCV viral load was 6.18 ± 0.70 log, with no differences between the two groups (G1: 6.54 ± 0.24; G2: 6.18 ± 0.7). HCV genotype 1 was identified in 90% of the patients, the rest presented infection by genotype 3. All patients were on antiretroviral treatment with undetectable HIV viral load, with no differences in the time on ARV therapy between groups. Patients with higher fibrosis presented lower $CD4^+$ T cell count (521 ± 312 cells/μL) than those from group 1 (770 ± 205 cells/μL, P = 0.035) There was no difference in the $CD4^+$ T cell count between group 1 and healthy controls (P = 0.49).

Regarding NK cells, a lower percentage was found in samples from patients of group 2 (5.4% ± 2.3%) compared both with patients from group 1 (12.6% ± 8.2%, P = 0.002) and healthy controls (12.2% ± 2.7%, 0.008) (Figures 1 and 2). With ROC curve analysis a cut-off of a NK cell percentage lower than 6.6% was determined to have 90% sensitivity and 77% specificity to predict the presence of METAVIR F3-F4 (Figure 3).

The percentage of $CD56^{bright}$ NK cells (G1: 11.7% ± 8.0%, G2: 7.1% ± 4.0%, HC: 6.8% ± 3.6%) and $CD56^{dim}$ NK cells (G1: 88.2% ± 7.6%, G2: 73.7% ± 40.1%, HC: 92.9% ± 3.6%) did not present differences among the three groups studied.

As the function of NK is regulated by an array of activating and inhibitory receptors, we also evaluated the NK cell activating receptors[15] NKp46 (CD335), NKp30 (CD337), NKG2D (CD314) and DNAM (CD226), the activation markers CD69 and CD25, and other molecules involved in NK cell effector functions, terminal differentiation and cytotoxicity such as CD94, TRAIL, CD57[16], Fas-L (CD178), CCR7 (CD197) and CD62L.

When compared with healthy controls, samples from patients included in group 2 presented a higher frequency of $CD56^{bright}$ $DNAM\text{-}1^+$ NK cells (76.2% ± 18.5% *vs* 4.6% ± 8.5%, P = 0.008) and $CD56^{dim}$ $DNAM\text{-}1^+$ NK cells (42% ± 29% *vs* 6.0% ± 8.1%, P = 0.018). The same differences were observed between group 1 and healthy controls, both in the percentage of $CD56^{bright}$ $DNAM\text{-}1^+$ NK cells (71.2% ± 23% *vs* 4.6% ± 8.5%, P = 0.003) and in the percentage of $CD56^{dim}$ $DNAM\text{-}1^+$ NK cells (41% ± 28% *vs* 6.0% ± 8.1%, P = 0.013).

Additionally, samples from group 1 exhibited higher percentage of $CD56^{bright}$ $CD25^+$ NK cells (53.1% ± 16.6% *vs* 19.4% ± 18.9%, P = 0.029) and $CD56^{dim}$ $CD25^+$ NK cells (28.3% ± 10.2% *vs* 7.1% ± 5.6%, P = 0.001) than healthy controls. These results show the possible consequence of a higher activation degree in NK cells from subjects with chronic infection. Of note, there were no differences in the frequency of these NK cells subsets between group 1 and 2. Moreover, no differences were observed in the other activator molecules evaluated (NKp46, NKp30, NKG2D, CD69) neither between group 1 and 2 nor between controls and HCV-infected subjects.

No differences in surface expression of CD94 were observed between the 3 groups. The frequency of these molecules was very high in $CD56^{bright}$ NK cells in all the samples evaluated (G1: 77.1% ± 28.2%, G2: 91.7% ± 1.2%, controls: 77.4% ± 36.7%), whereas in $CD56^{dim}$ NK cells this molecule was stained in less than 50% (G1: 48.4% ± 21.4%, G2: 36.9% ± 19.7%, controls: 31.6% ± 16.9%).

The frequency of $CD56^{dim}$ $TRAIL^+$ NK cells was higher in samples from group 2 than those from group 1 (29.4% ± 31.7% *vs* 7.5% ± 3.1%, P = 0.04), while no differences were observed between coinfected patients and healthy controls.

Nevertheless, the percentage of $CD56^{dim}$ $FasL^+$ NK cells was lower in samples from HCV/HIV-coinfected patients (G1: 27.2% ± 19.8%, P = 0.001; G2: 36.9% ± 19.7%, P = 0.01) than those from healthy controls (69.3% ± 18.2%), without detecting differences between groups 1 and 2.

In addition, there was a trend towards a higher percentage of $CD56^{dim}$ $CCR7^+$ NK cells in samples from patients with advanced fibrosis than in samples from patients with lower fibrosis (G2: 56.4% ± 36.2% *vs* G1: 24.4% ± 14.6%; P = 0.05). Regarding the CD62L expression, there were no differences in $CD56^{bright}$ NK cells between groups (G1: 61.8% ± 24.9%; G2: 87% ± 15.1%; P = 0.09).

DISCUSSION

In this study we found that patients with advanced fibrosis presented lower LT $CD4^+$ cell counts than subjects with low to mild fibrosis. All the patients were on successful antiretroviral treatment. Even though there are controversial data whether the presence of HCV is a factor that alters LT CD4 recovery with ARV, it can be hypothesized that patients with higher chances to develop liver fibrosis are those with lower LT $CD4^+$ cell recovery after HIV treatment. Such a poor HAART-mediated LT $CD4^+$ cell recovery may

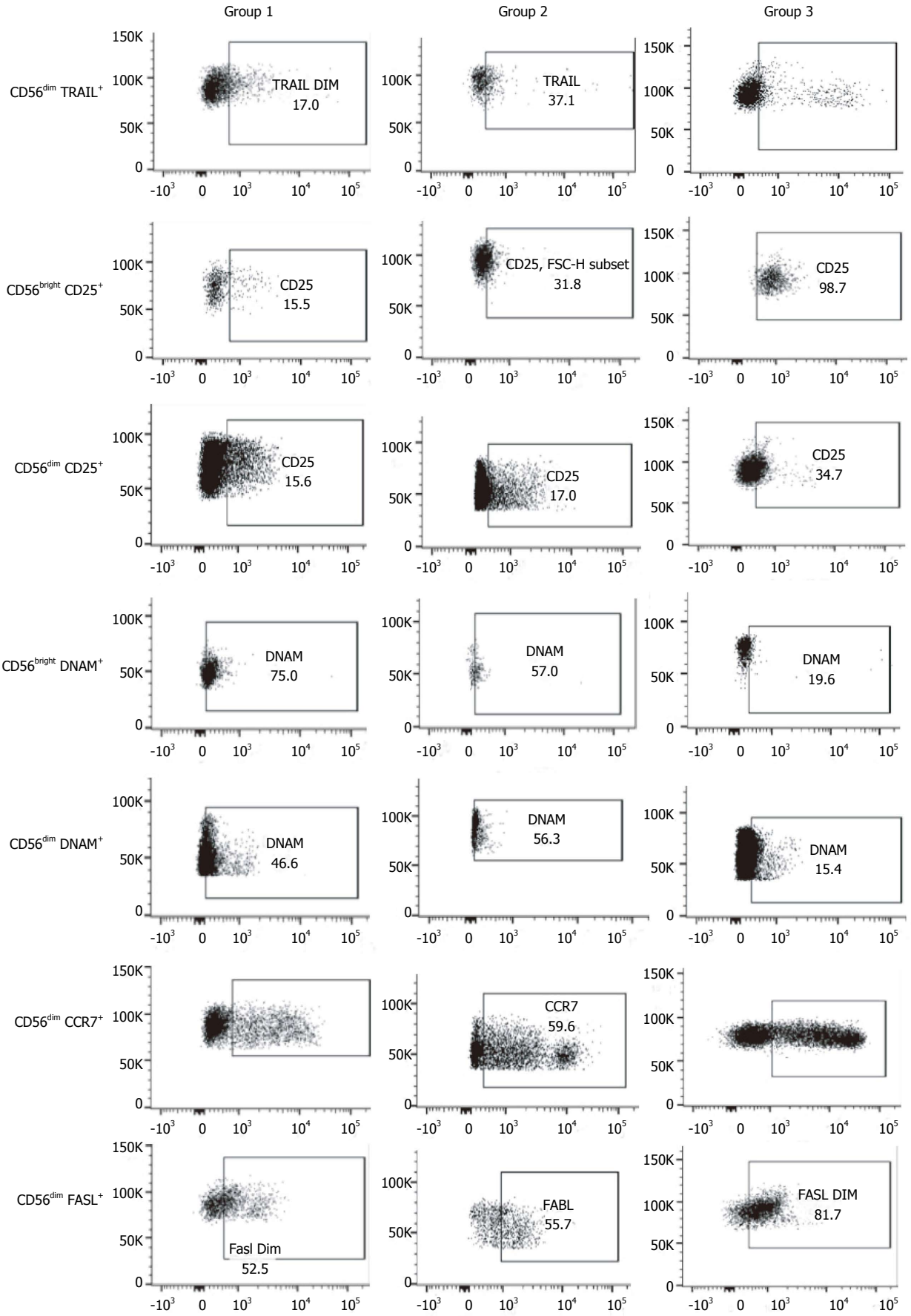

Figure 1 Representative dot plots of each of groups evaluated.

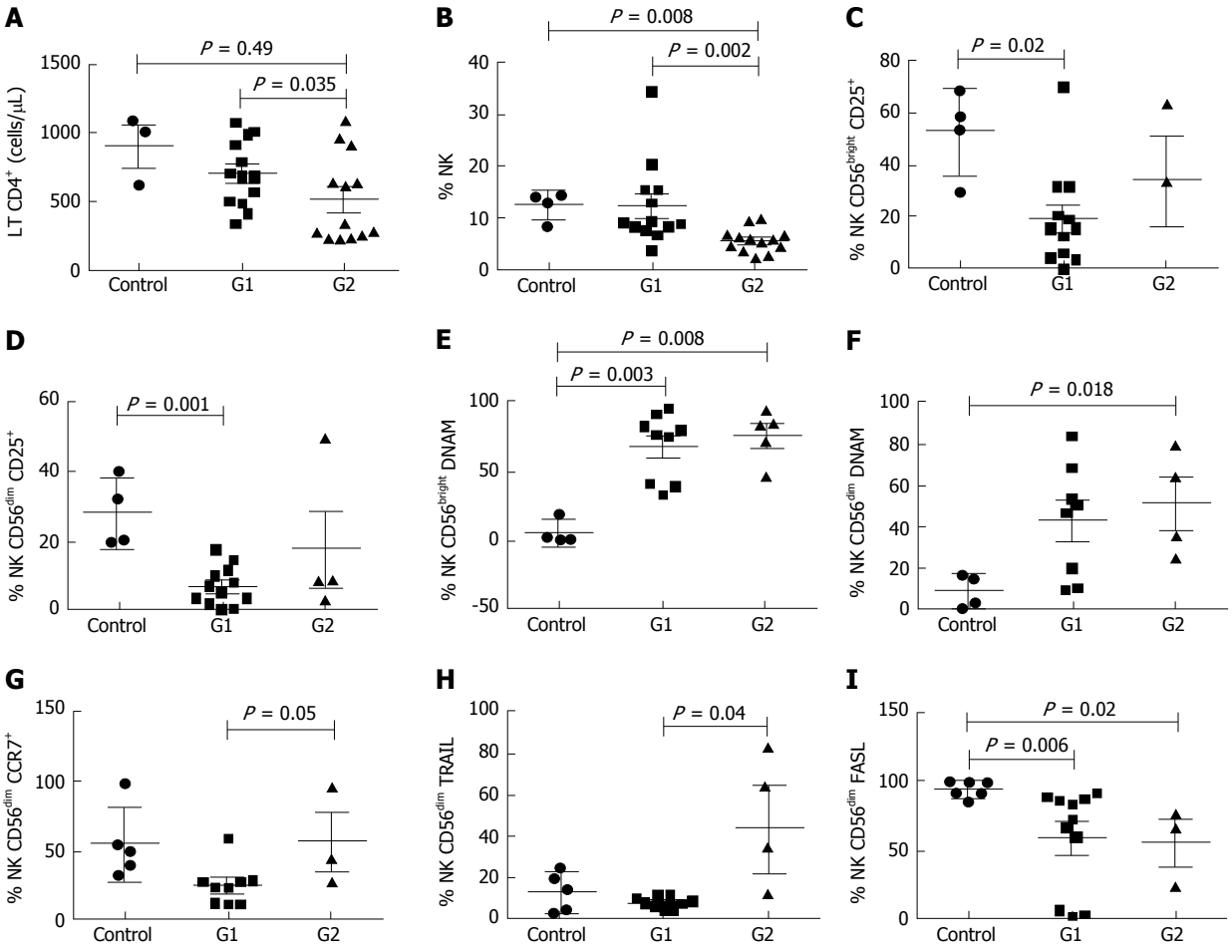

Figure 2 Differences between groups 1, 2 and controls regarding. A: Total CD4 cell count; B: Percent of NK cells; C: Percent NK CD56^{bright} CD25+; D: Percent CD56^{dim} CD25^{+} NK cells; E: Percent CD56^{bright} DNAM^{+} NK cells; F: Percent CD56^{dim} DNAM^{+} NK cells; G: Percent CD56^{dim} CCR7^{+} NK cells; H: Percent CD56^{dim} TRAIL^{+} NK cells; I: Percent CD56^{dim} FasL^{+} NK cells. The *P* values are shown in each graphic and the line below the *P* value connects the two groups compared. NK: Natural killer.

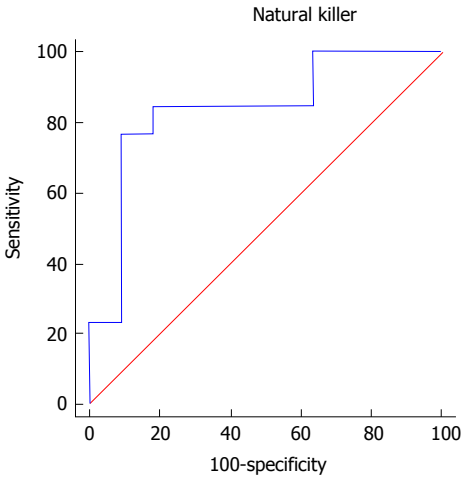

Figure 3 Area under the receiver operating characteristic curve, to evaluate the performance of natural killer cell % with liver fibrosis as the state variable.

contribute to an impaired stimulation of NK cells, and consequently a diminished anti-fibrotic activity by their action on hepatic stellate cells, favoring an accelerated liver fibrosis progression in HIV/HCV patients[17]. Yi *et al*[18] and other groups have observed that NK cells negatively regulated liver fibrosis. NK cells isolated from HCV-infected patients efficiently induced apoptosis of activated HSCs in TRAIL-, FasL-, and NKG2D-dependent manners[19]. NKp46^{high} NK cell subset potentially suppresses HCV replication and HCV-associated liver damage, leading to amelioration of liver fibrosis.

It has been described that HIV/HCV coinfection can modulate the peripheral NK phenotype[20]. In our study, we also observed differences in the NK phenotype particularly between control and HIV/HCV-coinfected patients which resemble those reported previously[21,22]. We found a lower percentage of CD56^{dim} FasL^{+} NK cells in HCV/HIV-coinfected patients compared to healthy controls. This finding could reflect a lower NK cell capacity to exert cytotoxic activity in patients with chronic HIV and HCV infection compared to non-infected individuals that could ultimately lead to a decreased capacity to regulate HSC.

Regarding HIV/HCV-coinfected individuals, no differences were observed in NK cell phenotypes according to the different degrees of liver fibrosis. Nevertheless, we could observe a statistically significant difference in the percentage of peripheral

blood NK cells in patients with high scores compared to patients with low liver fibrosis. Patients with advanced fibrosis have lower percentage of NK cells than those with low fibrosis scores. Moreover, we observed that a percentage of NK cells lower than 6.6% had 90% sensitivity and 77% specificity to predict the presence of advance fibrosis (METAVIR F3-F4). This observation could indicate, for the first time, that the evaluation of the NK cells compartment is a potential biomarker for fibrosis staging in HIV/HCV-coinfected patients.

In the era of direct antiviral agents with high efficacy for the treatment of chronic HCV, one of the main treatment access barriers for many patients is the high cost of these drugs, and where these barriers exist the assessment of liver fibrosis is mandatory to ensure treatment access. In this study, we have observed that those subjects with higher fibrosis are those with lower absolute count both of LT CD4$^+$ and lower percentage of NK cells. Although additional research is needed to confirm our findings, the evaluation of these two parameters that can be performed in a routine laboratory test may be helpful in improving the available noninvasive methods for liver fibrosis staging.

COMMENTS

Background

Different techniques to assess liver fibrosis have been developed, from liver biopsy (gold standard) to non-invasive studies (transient liver elastography; patented and non-patented biomarkers - FIB4, FibroTest, APRI, *etc*). Liver biopsy is invasive and has risk of complications. In addition, liver biopsy may be limited by the size of the specimen obtained as well as sampling, intra and inter-observer variability. On the other hand, there are many unresolved issues regarding the accuracy [especially in human immunodeficiency virus (HIV)/hepatitis C virus (HCV)-coinfected patients] of noninvasive studies, and in low-resource countries there is an important barrier to access to these methods (in particular liver elastography and patented biomarkers).

Research frontiers

The identification of non-invasive liver fibrosis biomarkers is still an open research area. In this context, the authors reasoned that the study of the phenotype of peripheral blood cells may unravel interesting clues towards the identification of such biomarkers. Some evidence indicates that the characteristics of the immune cells, including natural killer (NK) cells, observed in peripheral blood are similar to those seen in liver with relatively lower levels of magnitude. Accordingly, the aim of this study was to characterize peripheral blood NK cell phenotypes by flow cytometry as potential biomarker for liver fibrosis in patients chronically coinfected with hepatitis C and HIV.

Innovations and breakthroughs

In the era of direct antiviral agents with high efficacy for the treatment of chronic HCV, one of the main treatment access barriers for many patients is the high cost of these drugs, and where these barriers exist, the assessment of liver fibrosis is mandatory to ensure treatment access. In this study, the authors have observed that those subjects with higher fibrosis are those with lower absolute count both of LT CD4$^+$ and lower percentage of NK cells. Although additional research is needed to confirm their findings, the evaluation of these two parameters that can be performed in a routine laboratory test may be helpful in improving the available noninvasive methods for liver fibrosis staging.

Applications

The data in this study suggested that LTCD4 and NK cells could be used as potential non-invasive biomarkers of the level of liver fibrosis in HIV-HCV coinfected patients. These parameters could improve the accuracy of the available non-invasive methods to measure liver fibrosis.

Terminology

NK cells, and CD4 T lymphocytes (LTCD4) are involved in the immunological control of hepatic stellate cells that are the responsible of liver fibrosis development.

Peer-review

The identification of noninvasive liver fibrosis biomarkers is still an open research area. NK cells and LTCD4$^+$ count; are two simple parameters, that might be perform in a routine laboratory test and may serve as noninvasive biomarkers of liver fibrosis, identifying patients in need for HCV therapy in the short term.

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Abstract

Diffusion-weighted imaging (DWI), a functional imaging technique exploiting the Brownian motion of water molecules, is increasingly shown to have value in various oncological and non-oncological applications. Factors such as the ease of acquisition and ability to obtain functional information in the absence of intravenous contrast, especially in patients with abnormal renal function, have contributed to the growing interest in exploring clinical applications of DWI. In the liver, DWI demonstrates a gamut of clinical applications ranging from detecting focal liver lesions to monitoring response in patients undergoing serial follow-up after loco-regional and systemic therapies. DWI is also being applied in the evaluation of diffuse liver diseases such as non-alcoholic fatty liver disease, hepatic fibrosis and cirrhosis. In this review, we intend to review the basic principles, technique, current clinical applications and future trends of DW-MRI in the liver.

Key words: Liver imaging; Diffusion weighted imaging; Magnetic resonance imaging; Focal liver lesion; Diffuse liver disease; Response assessment

Core tip: This article reviews the current role of diffusion weighted imaging for various oncological and non-oncological applications in the liver.

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INTRODUCTION

Diffusion-weighted imaging (DWI) is a functional imaging technique, allowing qualitative and quantitative assessment of the diffusion properties of various types of tissues[1,2]. Numerous studies over the past decade have validated the role of DWI in oncologic and non-oncologic applications in the body[1,3-6]. Multiphase contrast enhanced MRI is an established technique for evaluation of a wide spectrum of liver diseases including focal lesions and diffuse parenchymal abnormalities. DWI compliments routine MRI of the liver by providing both qualitative and quantitative assessment for both focal and diffuse hepatic parenchymal processes. Factors such as the ease of acquisition and ability to obtain functional information in the absence of intravenous contrast, especially in patients with abnormal renal function, have contributed to the growing interest in exploring clinical applications of DWI. DWI improves sensitivity in detection of focal lesions, helps differentiate benign from malignant focal hepatic lesions, and also permits evaluation of treatment response to systemic and loco-regional therapies in primary and secondary hepatic malignancies. This review article focused on the basic principles, technique, current clinical applications and recent updates in DWI of the liver.

DWI: BASIC PRINCIPLES AND TECHNIQUE

DWI exploits the regional differences in the motion of water molecules within the extracellular/extravascular compartment of tissues. In highly cellular tissues (*e.g.,* lymphoma, carcinoma and abscess), the compact nature of the extracellular space causes increased impediment to motion of water molecules and the resultant water diffusion in such tissues is said to be "restricted". On the contrary, in tissues that are necrotic or fluid filled (*e.g.,* cysts), there is unrestricted motion of water molecules and water diffusion in such tissues, which is said to be "free". Therefore, the diffusion properties in different tissues provide information on tissue cellularity and the integrity of cellular membranes[1,2]. DWI is basically a modified T2 weighted sequence where the signal intensity depicts the tissue diffusion characteristics.

Single-shot spin-echo (SE) echo-planar technique is the most commonly utilized technique to acquire DW-MRI in combination with fat suppression[7]. To obviate the effect of motion, it can be acquired either using breath-hold or free breathing sequences with multiple signal acquisitions (in combination with respiratory and/or cardiac triggering). Free breathing sequences provide improved signal to noise ratios (SNR), thinner image sections, and higher number of b-values obtainable compared to breath-hold sequences. However, these take longer time (3-6 min) to acquire than breath hold sequences to evaluate the liver compared to free breathing EPI which takes (40-60 s)[8]. The free breathing technique has been shown to have better reproducibility of ADC values than other acquisition techniques like breath-hold, respiratory-triggered (RT), and navigator-triggered DWI[9,10]. Although cardiac motion also impacts quantitative ADC measurements, cardiac triggering is not routinely used in clinical practice[11].

Intravoxel incoherent motion (IVIM) imaging is a technique that has been introduced to quantitatively study the effects of tissue perfusion on the signal acquired with DWI and it resolves DWI measurements into true molecular-based (*D*) and perfusion-related (*D**, *f*) diffusion[12].

In patients with renal failure, gadolinium is contraindicated due to risk for developing nephrogenic systemic fibrosis (NSF)[13]. These patients also have a risk of worsening renal failure with iodinated CT contrast. MRI without contrast is a reasonable option for these patients but non-contrast protocols do not have a diagnostic accuracy comparable to multi-phase contrast MRI. DWI does not require administration of intravenous contrast, and because of its performance in oncological applications in general, it has generated much interest recently. The diagnostic performance of DWI has been tested in metastatic liver disease and HCC, and the results were comparable to contrast MRI[14-16].

CLINICAL APPLICATIONS IN LIVER

Imaging of focal liver lesions

Lesion detection: Multiphase contrast enhanced-MRI is currently the state-of-the-art imaging method for liver lesion detection and characterization. DWI at high *b*-values (≥ b100) provides a low background signal from normal liver parenchyma and thereby results in increased contrast between the background liver and lesions, enhancing the detection of focal liver lesions[17]. DWI is especially useful in detection of small lesions around vessels and in the periphery of liver which can be challenging to detect on routine T2 weighted images[18,19]. The DW-MRI can be particularly valuable in oncologic patients with compromised renal function who cannot get intravenous gadolinium based contrast agents[14-16]. DWI adds value in oncologic patients (Table 1)[15,20-22] by depicting more metastatic liver lesions when combined with multiphase contrast enhanced-MRI protocols, and improves reader confidence in lesion detection[22-25]. DW-MRI alone is less sensitive than gadoxetic acid-enhanced MRI for detecting liver metastases, but increases the sensitivity of detection for liver metastases (90.6%-95.5%) when combined with multiphase contrast enhanced MRI[25]. A major impact has been noted in the detection of metastases measuring ≤ 10 mm[17,22,24-27] (Figure 1). DWI has been used in detection of metastatic liver lesions from colorectal, pancreatic and neuroendocrine primaries[25,28,29].

DWI has also been found to be useful in detection

Table 1 Comparison of SSEPI diffusion-weighted magnetic resonance imaging *vs* conventional magnetic resonance sequences for detection of hepatic metastases[15,20-22,27]

Ref.	*b* value (s/mm^2)	Compared with (Seq)	Sensitivity of DWI *vs* other sequences	Accuracy of DWI *vs* other sequences	Advantages of DWI
Bruegel *et al*[27]	50, 300, 600	5 different T2-TSE (Turbo Spin Echo) sequences	0.88-0.91 compared to 0.45-0.62	0.91-0.92 compared to 0.47-0.67	Better sensitivity and accuracy
Zech *et al*[21]	50	Fat suppressed T2WI	83% *vs* 61%	-	Better image quality Fewer artifacts Better sensitivity
Hardie *et al*[15]	0, 50, 500	Gadolinium enhanced T1WI	66.3% *vs* 73.5%	88.2% and 88.2% for DW-MRI, 90.2% and 92.2% for CE MRI, respectively, for observers 1 and 2	Not significantly different
Donati *et al*[20]	0, 150, 500	Combined (Gd-EOB-DTPA) enhanced MRI/DWI *vs* Gd-EOB-DTPA enhanced MRI and DWI alone	-	Gd- EOB-DTPA/DWI: 0.84 and 0.83 *vs* 0.73 and 0.72 for DWI alone	Increase in diagnostic confidence No significant increase in diagnostic accuracy
Colagranade *et al*[22]	0-500	Added value of DWI for lesion detection in unenhanced and Gd-EOB-DTPA enhanced MRI	-62.5% for unenhanced MRI w/o DWI -85.0% for unenhanced MRI+ DWI -95.6% for CE MRI -97.3% for CE MRI + DWI	-81.1% for unenhanced MRI w/o DWI -89% for unenhanced MRI + DWI -92.9% for CEMRI -95.5% for CE MRI + DWI	DWI improved all statistical parameters in the unenhanced examinations, as for nodules either smaller or greater than 1 cm. In EOB-enhanced examinations DWI increased specificity/negative predictive value

DWI: Diffusion-weighted imaging; MRI: Magnetic resonance imaging.

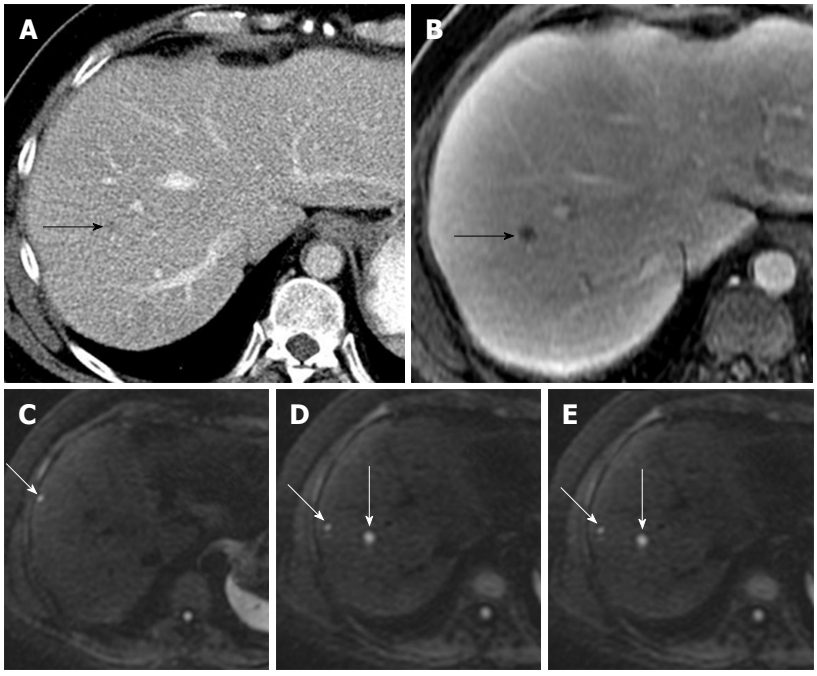

Figure 1 Value of diffusion-weighted magnetic resonance imaging in lesion detection in a 51-year-old male with metastatic leiomyosarcoma of the thigh. A: Axial contrast enhanced CT scan demonstrated a subtle hypodensity in the right lobe of liver (black arrow); B: Axial post gadolinium T1-weighted MR image demonstrates a single metastatic lesion (black arrow); C-E: DW-MR image at b-600 demonstrates additional lesions (white arrows). DW-MR: Diffusion-weighted magnetic resonance; CT: Computed tomography.

of primary hepatic malignancies such as hepatocellular carcinoma (HCC) and cholangiocarcinoma both in cirrhotic and non-cirrhotic livers (Figure 2). A combination of DW hyper-intensity and arterial hyper-enhancement results in increased sensitivity for diagnosis of HCC as compared to traditional criteria, particularly for small HCC < 20 mm[30,31].

A low cost abbreviated MRI (AMRI) protocol for HCC screening and surveillance has been proposed based on a simulation study using DWI and T1-weighted imaging obtained at the hepatobiliary phase (HBP) after gadoxetic acid injection[32]. The AMRI

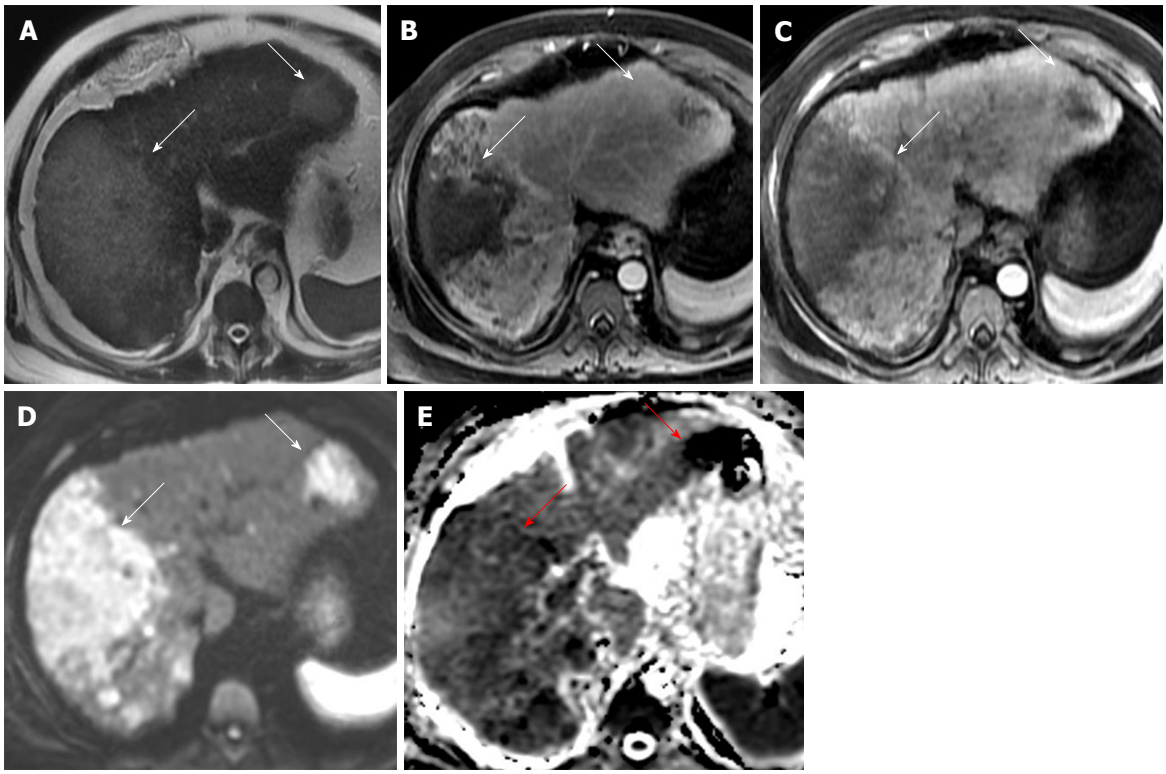

Figure 2 A 66-year-old lady with multifocal infiltrative hepatocellular carcinoma with improved detection on diffusion-weighted imaging. (A) Axial T2 weighted image demonstrates multifocal areas of T2 hyperintense masses (white arrows) which demonstrate heterogeneous arterial hyperenhancement on post gadolinium late arterial phase images (B) and washout appearance on portal venous phase images (C). (D) Axial DWI image at b-600 and (E) ADC image show that these masses demonstrate restricted diffusion and are better appreciated than the dynamic phase images. Serum Alpha feto-protein value of 1552. DWI: Diffusion-weighted imaging.

shows sensitivity and negative predictive values of 80.6% and 80% (for DWI + T1W HBP) compared to 90.3% and 94.9% for a full dynamic contrast enhanced data-set[32].

Lesion characterization: Several studies have attempted characterization of liver lesions using DW-MRI[33-38]. A general assumption is that ADC values are higher in benign lesions and lower in malignant liver lesions[33-36]. In fact, studies have found statistically significant difference in ADC values between benign and malignant liver lesions[3]. Different studies have reported variable success using various ADC cut-off values with high variability likely due to the difference in scanners and parameters used to obtain DW-MRI and ADC maps[39-43]. Moreover, there is a high degree of overlap between solid benign and malignant lesions[44,45]. Hence, the use of absolute ADC values or ADC value cut-off for characterization of focal hepatic lesions should be avoided and DWI should always be interpreted as a complimentary technique to conventional MR sequences[42,46,47]. It is also important to note that solid benign lesions such as hemangioma, FNH and hepatocellular adenoma can also show diffusion restriction compared to normal liver parenchyma. ADC values for these lesions are intermediate, generally greater than solid malignant lesions but with a significant degree of overlap[44,45]. Hepatic abscesses show lower ADC values than solid malignant lesions, and restriction pattern may be different from malignant lesions[42] (Table 2).

DWI has also been used to assist in differentiation of cirrhotic hepatocellular nodules[48]. Lesion hyperintensity on DWI, especially in association with hypointensity on delayed hepatocellular phase images, and low lesion-to-liver ratios should raise the suspicion of HCC or high-grade dysplastic nodules[49]. The HCCs have a tendency for angio-invasion and can present with filling defects in the portal or hepatic veins. Angio-invasion carries a high risk of distant metastasis and recurrence after transplantation. HCC invasion into the portal vein is considered as a contraindication for liver transplantation. It is important to distinguish tumor thrombus from a bland thrombus that is also common in chronic portal hypertension and has different clinical implications. In patients with locally advanced HCC, DW-MRI has been shown to be useful in characterization of the venous thrombus as bland *vs* tumor thrombus[50]. The mean ADC ratio of tumor thrombus and HCC has been reported to be < 2 (0.998) as compared to bland thrombus (2.9)[50].

Tumor grade and prognostication: Recently, there have been attempts to predict the histopathological grades of HCC using DWI. ADC values have been found to correlate with histopathological differentiation and microvascular invasion with poorly differentiated HCCs showing significantly lower ADC than well-differentiated

Table 2 Liver lesion characterization based on ADC values[33,35,44,45,102]

Ref.	Lesion type	Mean ADC (10^{-3}mm^2/s)	Sample size	*b*-values	Conclusion
Parsai *et al*[44]	Cyst	2.66	2	100, 200, 500, 750, and 1000 mm^2/s	ADC cutoff value threshold of 1.6 × 10^{-3} mm^2/s yielded higher accuracy for differentiating benign from malignant lesions. DWI is not reliable to differentiate malignant from benign solid lesions
	HCC	1.07	26		
	Metastases	1.04	39		
Taouli *et al*[98]	Cyst	3.63	52	0, 500	Threshold ADC value of 1.5 × 10^{-3} mm^2/s to differentiate between benign and malignant lesions, but with a significant overlap between benign hepatocellular lesions and HCCs
	HCC	1.33			
	Metastases	0.94			
Parikh *et al*[35]	Cyst	2.54	211	0, 50, 500	Accuracy of 75.3% for differentiating benign from malignant, by using a threshold ADC of less than 1.60 × 10^{-3}mm^2/s. Equivalent performance of DW imaging and T2- weighted imaging for lesion characterization
	HCC	1.31			
	Metastases	1.5			
Bruegel *et al*[33]	Cyst	3.02	204	50, 300, 600	88% of lesions were correctly classified as benign or malignant using a threshold value of 1.63 × 10^{-3} mm^2/s. Measurements of the ADCs of focal liver lesions on the basis of a respiratory triggered DW-SS-EPI sequence may constitute a useful supplementary method for lesion characterization
	HCC	1.05			
	Metastases	1.22			
Gourtsoyianni *et al*[102]	Cyst	2.55	37	0, 50, 500, 1000	Sensitivity and specificity of 100% for differentiating benign from malignant lesions using a cutoff ADC value of 1.47 × 10^{-3} mm^2/s
	HCC	1.38			
	Metastases	0.99			

HCC: Hepatocellular carcinoma; DWI: Diffusion-weighted imaging.

and moderately differentiated HCCs[51-54]. A cut-off value of 1.175 × 10^{-3} mm^2/s has been recommended as a predictor of microvascular invasion[52]. Additionally, the recurrence-free survival has been found to be significantly shorter in low-ADC group than in high-ADC group[52].

The association of ADC and histopathological grades has shown conflicting results in few other studies[55,56]. This might be a result of tumor necrosis, as it can result in reduced cellularity and increased ADC in high-grade lesions. Higher signal intensity on DWI has been reported to be associated with higher pathological grades despite insignificant correlation with ADC values[54,56].

Diffuse liver diseases

Evaluation of NAFLD: Non-alcoholic fatty liver disease (NAFLD) is the most common liver disorder in western industrialized countries with a prevalence of 6%-35% worldwide[57]. The severe form of this disease is steatohepatitis which can progress to cirrhosis in 15% of the patients[58]. Currently, the diagnosis of NAFLD is established based on histopathological evaluation of liver biopsy specimens. Liver biopsy is invasive and has risks of complications and sampling error, and cannot be frequently repeated.

The feasibility of DWI and IVIM was first tested in animal models with early results showing that the IVIM diffusion parameters, in particular the "*f*" values, might be potential biomarkers of NAFLD[59]. The correlation between histologic features of NAFLD and quantitative measures derived from IVIM-DWI was later tested in humans which showed that the true molecular diffusion was significantly decreased with steatosis[60,61]. ADC was not found to be associated with any histological feature[60]. Although these early results are promising, standardization of acquisition and post-processing techniques of IVIM DW-MRI is needed.

Evaluation of liver fibrosis and cirrhosis: Aubé *et al*[62] reported early benefits of DWI in the evaluation of diffuse liver diseases, particularly in the detection and quantification of hepatic fibrosis. Several authors thereafter have tried to find a simple, reliable and non-invasive method to detect and monitor hepatic fibrosis, thereby avoiding the existing gold standard involving liver biopsy and its complications[63,64]. A recent meta-analysis suggests that DWI and IVIM parameters can reliably stage hepatic fibrosis[65,66]. However, IVIM measurements and ADC values have been reported to be influenced by presence of fat or iron within the liver that can impact their accuracy for staging of fibrosis[67-69] and ascites[70]. Recent studies comparing MR elastography (MRE) and DWI in characterizing hepatic fibrosis demonstrate higher predictive ability of MRE in distinguishing stages of fibrosis compared to DWI[71,72]. Gadoxetate disodium enhanced liver MRI is also more strongly correlated with fibrosis stage as compared to DWI[73,74]. Considering the conflicting evidence, it can be concluded that at present, DWI cannot replace liver biopsy in liver fibrosis. Further investigations and analysis are needed to increase the reliability of the technique.

Monitoring treatment response

There has been a lot of interest in using DWI as an imaging biomarker for monitoring treatment response to various locoregional and systemic therapies in hepatic malignancies (Table 3)[75-79]. In comparison to conventional morphological methods of monitoring response such as RECIST and WHO which rely on changes in tumor dimensions for quantitating tumor response, DW-MRI allows evaluation of treatment response to novel targeted therapies which cause

Table 3 Role of diffusion-weighted magnetic resonance in assessment of treatment response[75-79]

Ref.	Treatment modality	Tumor type	DW-MR parameter evaluated	Study results/teaching point
Chapiro *et al*[79]	TACE	HCC	(3D) quantitative enhancement-based and DW volumetric MR	High accuracy and intermethod agreement of 3D quantitative techniques in the assessment of tumor necrosis after TACE is clinically relevant High diagnostic performance of qEASL criteria and qADC may help in triaging patients for repeat treatment after a TACE session
Mannelli *et al*[87]	TACE	HCC	ADC measured with DWI in treatment response	Pre TACE ADC obtained at 0, 50, 500 s/mm^2 *b* values before and after treatment may be used to predict HCC response to TACE
Park *et al*[42]	Radiotherapy	HCC	DW MR *vs* conventional MR for treatment response	Improved detection of viable tumor when DW MR is added to conventional sequences
Yu *et al*[76]	Radiation therapy	HCC	DW MR	Change in ADC value before and after RT is related to local progression free survival. Hence ADC value and RECIST may substitute for mRECIST in patients who cannot receive contrast agents
Schraml *et al*[77]	Radiofrequency Ablation	*n* = 16 HCC, 1 = cholangiocarcinoma, and 37 = metastases (28 colorectal cancer, 3 melanoma, 3 breast cancer, 1 pancreatic cancer, 1 gastric cancer, esophageal cancer)	DW MR and mean ADC values	ADC-based evaluation of signal alterations adjacent to the ablation zone may contribute to the identification of local tumor progression and nontumoral post- treatment tissue changes

HCC: Hepatocellular carcinoma; DW MR: Diffusion-weighted magnetic resonance; TACE: Trans-arterial chemoembolization.

early changes in tumor physiology prior to change in tumor size. The increase in post-treatment ADC values precedes a decrease in size of tumor which has been the traditional method of measurement for post-treatment response, especially in systemic therapy[80-82].

Percutaneous ablation: ADC-based evaluation of signal alterations adjacent to the ablation zone may contribute to the identification of local tumor progression and non-tumoral post-treatment tissue changes after radiofrequency ablation of hepatic primary tumors and metastases[77]. Early post-ablation zone may show heterogeneous signal on non-enhanced T1 and T2 weighted images due to edema, hemorrhage and inflammatory reaction. These changes resolve within 4-6 mo after ablation leaving behind a characteristic homogenous high T1 signal and low T2 signal (coagulation necrosis). Nodular enhancing foci within the ablation zone are considered as a sign of local recurrence. Low ADC values at 1 mo (< 1.145 × 10^{-3} mm^2/s) after RFA have been shown to be associated with an early local recurrence of HCC[83].

Intra-arterial therapies: The utility of DWI has been assessed in treatment response after trans-arterial chemoembolization (TACE) of HCC[84-87]. DWI has been shown to perform equally[78] or better than gadolinium-enhanced MRI in quantifying the area of tumor necrosis after chemoembolization[78,86,88]. Increased ADC values in non-enhancing tumors show a high correlation to the degree of tumor necrosis at pathology[86,88]. Mannelli *et al*[78] showed excellent performance of ADC for prediction of complete tumor necrosis after TACE (sensitivity of 75% and specificity of 88%) which was comparable to 100% sensitivity, and 58%-79% specificity for contrast-enhanced MRI.

Transarterial radioembolization (TARE) using yttrium-90 (^{90}Y)-loaded resin microspheres is a treatment option for various liver malignancies (including liver-dominant breast metastases). Early arterial blood flow stasis with consecutive incomplete dose administration may occur in 12%-25% of resin-based radioembolization procedures. The perfusion-sensitive IVIM parameter "*f*" may predict early blood flow stasis in patients undergoing TARE for liver-dominant breast metastases[89].

Image-guided radiation therapy: Image-guided targeted external beam radiation therapy is emerging as an alternative option in the treatment of advanced unresectable HCC. Accurate post-radiation response assessment can be challenging due to the concomitant changes occuring in the radiation zone. MRI is the preferred modality for response assessment. Inclusion of DWI in the imaging protocol has been shown to significantly enhance the diagnostic accuracy (91%-97% *vs* 72%) for detection of viable tumors after radiation treatment with improved sensitivity, specificity, and negative predictive value as compared to routine MR sequences (90%-97%, 91%-97% and 91%-97% *vs* 41%-55%, 86%-97% and 67%-70%, respectively)[75]. ADC values have also been shown to correlate with local progression-free survival[76]. Another group demonstrated that ADC values correlate with local progression-free survival and proposed that ADC and RECIST criteria could be substituted for mRECIST in

post-radiation evaluation of patients not amenable to receiving contrast agents[76].

Systemic chemotherapy: DWI can detect the effects of chemotherapy combined with antiangiogenetic treatment on liver metastases in patients with advanced colorectal cancer[90]. An increase in ADC values following systemic chemotherapy can be a sign of tumor response with non-responders showing lower ADC values than responders[91]. In addition to monitoring therapeutic response, DWI has also been found to be useful in prediction of response to chemotherapeutic agents[92,93].

Limitations of DWI

Diffusion imaging has several limitations, mostly attributable to the EPI based nature of the sequence[94,95]. SS EPI provides a limited image quality with low spatial resolution and poor SNR and is susceptible to several artifacts, including blurring, ghosting and distortions. Although modern scanners with multichannel coils, strong gradients, high magnetic fields and advanced software have been successful in reducing such effects to a great extent[96]. In addition, parallel imaging techniques improve SNR by allowing a decrease in acquisition time (TE)[97,98]. 3T MRI offers an advantage due to an inherent high SNR, but suffers from several limitations. Uniform fat suppression for liver DWI has always been a challenge with 3 Tesla magnets and susceptibility artifacts are also more pronounced at 3 Tesla scanners[99].

The reproducibility of quantitative ADC values has also been questioned. ADC values have been reported to vary significantly depending on the hardware, human or biologic factors[100]. There has been considerable effort, however, to "industrialize" this important biomarker across vendor platforms[101].

CONCLUSION

DWI is useful for focal liver lesion detection and is a desirable tool in patients who cannot receive intravenous contrast. In patients receiving systemic and local therapies for hepatic malignancies, DWI acts as a clinical tool for monitoring treatment response and predicting prognosis. Its utility in the assessment of diffuse hepatic parenchymal diseases is still at a research level. Further investigation and analysis are needed to increase the reliability of the technique for these indications. DWI has certain limitations and remains an adjunct and not a replacement to conventional sequences.

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MINIREVIEWS

Risk of liver disease in methotrexate treated patients

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Abstract

Methotrexate is the first line drug treatment for a number of rheumatic and non-rheumatic diseases. It is effective in controlling disease activity and preventing disease-related damage, and significantly cheaper than many alternatives. Use in rheumatoid arthritis infers a significant morbidity and mortality benefit. Methotrexate is generally well tolerated but can cause symptomatic adverse events. Multiple serious adverse events have been attributed to methotrexate, based largely on older reports using high or daily doses, and subsequent case reports and circumstantial evidence. The risk with modern dosing regimens: Lower doses, weekly schedules, and concomitant folic acid is less clear. Clarification and dissemination of the actual risk is crucial so appropriate judgements can be made for patients who may benefit from this treatment. Methotrexate has been associated with a range of liver related adverse events ranging from asymptomatic transaminase elevations to fibrosis and fatal hepatic necrosis. Concern over potential liver toxicity has resulted in treatment avoidance, cessation, or recommendations for investigations which may be costly, invasive and unwarranted. Modern laboratory monitoring of liver blood tests may also influence the risk of more serious complications. The majority of present day studies report an approximate doubling of the relative risk of elevated transaminases in methotrexate treated patients but no increased risk of symptomatic or severe liver related adverse events. In this article we will review the evidence around methotrexate and liver related adverse events.

Key words: Liver disease; Transaminases; Fibrosis; Cirrhosis; Methotrexate; Hepatic

Core tip: Methotrexate is a highly effective treatment for many diseases. In rheumatoid arthritis it controls symptoms, prevents damage, and reduces mortality. The risks of methotrexate use are often over-estimated. Methotrexate may result in asymptomatic transaminase elevations. Historically methotrexate has been infrequently associated with more severe liver adverse

events. With modern monitoring and management of liver blood tests serious liver related adverse events related to methotrexate use appear to be avoidable.

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INTRODUCTION

Methotrexate, formerly known as amethopterin, is one of several folic acid antagonists originally utilised in children with acute leukaemia[1]. Successful use in adults, and children with other tumours followed shortly thereafter[2]. Use has increased dramatically since that time both in volume and in scope; methotrexate is now commonly used in the treatment of a wide range of malignant and non-malignant diseases[3]. The importance of methotrexate as a treatment option is emphasised by its prominent place on the World Health Organisation's List of Essential Medicines, a list of those critical basic medications which should be available to every healthcare system[4]. Indeed methotrexate is arguably one of pharmaceuticals greatest success stories, a medication which has found indications widely disparate from its original intention. Methotrexate is highly effective for a range of diseases which had been difficult to treat prior to its introduction, including rheumatoid arthritis, psoriasis, and Crohn's disease[3]. Methotrexate has transformed the management of rheumatoid arthritis (RA), dramatically altering the disease course, patient's quality of life, and reducing RA-related mortality[5,6]. Modern reviews and meta-analyses show methotrexate has similar or better efficacy to other available agents including biologic therapies[7]. No treatment is superior to methotrexate monotherapy for inhibiting radiographic progression, but combination therapy is superior for methotrexate failures[6].

Despite this success, the potential adverse events associated with methotrexate attract considerable attention. Several reasons account for this, mainly stemming from notable toxicity with early use employing daily or high-dose therapeutic regimes. The translation of the adverse events associated with oncological dosing to those of modern low-dose methotrexate regimes for the treatment of autoimmune disease should not be automatic. Recent studies suggest methotrexate carries a similar risk of adverse events and toxicity to other agents, but combination therapy may have a higher rate of infection and liver-related adverse events[6-11].

METHOTREXATE RELATED ADVERSE EVENTS

The first papers on methotrexate use detailed the acute toxicity associated with high dose therapy for cancer, and later long-term sequelae[1,12]. Later studies in non-malignant disease showed similar problems with high dose therapy but not so with lower doses, weekly regimens and concomitant use of folic acid[13,14]. The adverse events associated with methotrexate use can be divided into two broad subsets; those symptomatic but rarely life-threatening adverse events experienced by patients, and those rarely symptomatic (at least until the latter stages) but potentially life-threatening adverse events which require careful monitoring by physicians.

Symptomatic adverse events associated with methotrexate are reported relatively commonly. These include symptoms such as nausea, headaches, fatigue, mucositis, and hair loss[3]. For the majority of patients they are an acceptable accompaniment to their treatment, and a minor inconvenience compared to their previously debilitating disease symptoms. These adverse events are common with many medications and also shared by many of the diseases which methotrexate is used to treat. This can make it difficult in many cases to know if methotrexate is definitively the cause of the symptoms the patient is experiencing. However, distinguishing the precise source of the symptoms is often not needed, provided they can be tolerated or managed with symptomatic interventions. The occurrence rate using modern dosing schedules appear similar to the placebo arm in some clinical trials yet the perception of toxicity persists, and often apportioned to methotrexate[15]. More rarely patients will ultimately be unable to tolerate methotrexate due to intractable symptomatic adverse events leading to drug cessation.

The more serious and infrequent adverse events attributed to methotrexate use present more of a clinical dilemma. The evidence linking many of these events to methotrexate use is sometimes weak and circumstantial. Methotrexate has been utilised in clinical practice for a considerable period and the origin of the attribution of many of its associated adverse events lies in older studies which used daily dosing, or much higher doses for example 3 mg/kg per week, or 100 mg/wk[1,2,12-14]. More recent reports of isolated cases, case series, and observational studies where established beliefs may bias the findings and/or conclusions are inconclusive. There is no doubt that very high doses are toxic to the marrow, gingiva, and long-term the liver, hence the introduction of lower dose, less frequent schedules[1,12,13]. Examination of these potential risks using modern regimens requires thorough exploration in well designed and performed studies in order to establish robust evidence for what the risks are. Adverse events falling into this category include cytopenias, interstitial lung disease (or methotrexate pneumonitis), and indeed methotrexate related liver disease.

Methotrexate-induced lung disease is a good example, an entity widely believed to be common, serious and potentially fatal[3]. Incorrectly apportioning

blame on methotrexate can result in two potential risks to the patient: (1) denying them an effective drug; and (2) delaying the appropriate investigation and treatment of the real cause of their symptoms. Recent studies show this is in fact a rare occurrence and may not exist at all[8,9,11]. Furthermore, it appeared that any increased risk was likely due to a small increase in respiratory infections with methotrexate use, rather than interstitial lung disease[8]. This knowledge has the potential to significantly change clinical practice as cessation of methotrexate frequently occurs as a knee jerk reaction to any cough or dyspnoea.

The nature of liver disease related to methotrexate is similarly complex. It is well established that patients treated with methotrexate may develop abnormal liver blood tests, but the long-term consequences of modern dosing regimens in people with normal renal function are unknown[16]. Many such patients not treated with this agent can develop abnormal liver enzymes, potentially confounded by alcohol use, non-steroidal anti-inflammatory drugs, non-alcoholic fatty liver disease, and both related and unrelated de novo liver diseases[16,17]. Patients prescribed methotrexate have liver blood tests performed at intervals far in excess of the general population so the significance of minor or transient abnormalities in these test results remain uncertain[18]. This is of course a vital issue regarding what action, if any, should be taken when faced with an abnormal liver blood tests, as it clearly depends on what it signifies. Cessation of an effective drug due to a transient unrelated transaminase elevation is potentially harmful to patients, as is continuation of that agent in the face of a developing significant drug induced liver injury[19].

EPIDEMIOLOGY OF METHOTREXATE RELATED LIVER DISEASE

Reported rates of liver blood abnormalities during methotrexate treatment vary. Initial reports of hepatic toxicity, and death from hepatic toxicity, as well as cumulative incidences of 48.9% for elevated transaminases and 16.8% for transaminases elevated more than twice the upper limit of normal have been reported[12,16,20]. Hepatic toxicity is not universal with prolonged chemotherapeutic regimes and some demonstrated normal liver histology despite several months of therapy[12]. The reported rates of hepatic toxicity appear to have decreased progressively over time, likely related to refinements in dosing and monitoring strategies[13]. A 2009 systematic review of observational studies up to that time reported that elevated transaminases were found in 20% of patients treated with methotrexate for 1 year, with transaminases greater than twice the upper limit of normal in 13%[21]. Present day monitoring strategies and treatment regimens appear to have significantly lower risks than those which have been historically associated with methotrexate use. Two high quality recent studies reported elevated transaminases in 22% but with as little as 1% having transaminases greater than twice the upper limit of normal[22,23]. A higher rate occurs when used in combination with other therapies[6,7,17]. A number of other risk factors for hepatotoxicity have been identified including obesity and hypercholesterolaemia[17].

In contrast to the frequently reported transaminase elevations in methotrexate treated patients, reports of serious adverse liver outcomes in appropriately treated patients are harder to find in more recent times. An estimated 5-year risk of 1/1000 patients is likely to be an over-estimate based on the limited histological information available[19]. A study reported rates of mild liver fibrosis, severe fibrosis and cirrhosis based on liver biopsies performed before and after methotrexate use. Rates prior to methotrexate use were 9.1%, 0% and 0.3%. The corresponding results after methotrexate use were 15.3%, 1.3% and 0.5% respectively[16]. A literature review on biopsy proven liver abnormalities found that 3% of methotrexate treated patients developed histological abnormalities after one year of treatment. Importantly however, when the results were confined to those controlled studies of patients with baseline biopsies prior to the introduction of methotrexate no biopsy proven histological abnormalities were identified after 4 years of treatment[21].

A recent meta-analysis of clinical trials demonstrated a cumulative incidence of liver adverse events of 11.2% in methotrexate treated patients compared to 6.3% in patients on other treatments[10]. Calculated incidence rates from this were 20/100 patient-years in methotrexate treated patients and 9/100 patients-years in patients on other treatments[10]. The majority of these adverse events were low grade liver enzyme elevations with an incidence rate of 16/100 patient-years in methotrexate treated patients compared to 8/100 patient-years in others[10]. The incidence rate of major liver enzyme elevations was 4/100 patient-years in methotrexate treated patients and 1/100 patient-years in other patients, which is concerning[10]. Reassuringly more serious liver complications did not occur in any methotrexate treated patients in these studies[10]. The short duration of clinical trials is universal, the mean duration of studies in this meta-analysis were 47 wk, therefore data from long-term registries with robust unbiased analyses are required.

PATHOGENESIS

Any discussion of the mechanisms of potential methotrexate toxicity must begin with an appreciation of our understanding of methotrexate's mode of action. It is here that we reach a major impediment, though perhaps an informative one. We simply do not fully understand how methotrexate, and in particular low dose methotrexate, achieves its clinical effects[11].

The oft quoted explanation that methotrexate is a dihydrofolate reductase inhibitor, while of course true, does not fully explain either the clinical efficacy or potential toxicities which we see with this agent. Low dose methotrexate has a multitude of biochemical effects at the most basic level including influences on T-cell apoptosis, cell proliferation and cytokine production[24]. Despite methotrexate's long historical use this remains an active area of research, in part due to a certain neglect of exploration of these pathways in the past, and in part due to the increasingly evident complexities of the effects of methotrexate.

A reduction in hepatic folate stores and toxicity due to a local folate deficiency is one possible toxic effect of methotrexate on the liver. A definitive relationship between folate depletion and hepatotoxicity has not been experimentally confirmed. However folic acid supplementation has been associated with a lower incidence of elevated transaminases[25].

Early animal and clinical studies of high dose methotrexate demonstrated the development of liver fibrosis and cases of cirrhosis but subsequent low dose weekly regimens failed to demonstrate a similar effect[13,26].

The histological appearance of the liver in methotrexate treated patients is generally graded according to the Roenigk classification system[27]. The system progressively classifies changes from early fatty infiltration and pleomorphism, through inflammation and necrosis, varying degrees of fibrosis and ultimately cirrhosis. Importantly none of these findings are unique to methotrexate and can be seen in other disease processes.

LIVER ADVERSE EVENTS WITH ANALAGOUS MEDICATIONS

One of the key tenets of causation is specificity[28]. If patients given alternative agents to methotrexate do not develop liver disease than this facet of evidence would strongly implicate methotrexate as a causative agent. If they do however, than this, while be no means definitive, raises a potential warning flag that we should reconsider our hypothesis. In inflammatory bowel disease thiopurines (azathioprine and 6-mercaptopurine) are the most commonly used alternatives to methotrexate. Hepatotoxicity due to thiopurines has been reported in 10%-17% of patients[29,30]. Risk factors for thiopurine induced hepatotoxicity appear to be similar to methotrexate with age, obesity, and concomittent medications implicated[29,30]. In randomised controlled trials comparing thiopurines with methotrexate hepatotoxicity appears to occur at a similar rate[31-33]. Leflunomide is often used in rheumatoid arthritis as an alternative to methotrexate and has been associated with a variety of similar complications to methotrexate. Pulmonary disease in particular has been associated with both agents, however our work has illustrated that leflunomide may not be causative in this regard[34]. Leflunomide has also been associated with transaminase elevations with a similar frequency to methotrexate with elevations in 17% and elevations greater than twice the upper limit of normal in 1%-2%[22]. Combining both agents appears to have additive effects with transaminase elevations seen in 31% and those greater than twice the upper limit of normal in 5%[7,22]. Sulfasalazine, another agent used in similar settings also appears to show similar effects to leflunomide (and to methotrexate)[7]. Anti-tumour necrosis factor alpha agents have been reported as a relatively frequent cause of mild transaminase elevations, however, as with methotrexate, significant elevations occur relatively infrequently and are reported in less than 1% of patients[35]. Again similar to methotrexate, serious liver adverse events seen in association with these agents appear infrequent[36].

All of this begs the question, what is the rate of transaminase elevations in a healthy population? Most laboratory tests define normality as lying within 2 standard deviations of the mean in a Gaussian distribution. In a normally distributed sample approximately 95% of values will lie within 2 standard deviations of the mean. Therefore 2.5% of the population will have transaminase levels above the normal range and 2.5% will have transaminase levels below the normal range. The importance of this is that the rate of "abnormality" is not zero and never can be if normality is defined in this manner. This must be born in mind in evaluating any reported rate of abnormalities. Since studies show a higher incidence of liver enzyme abnormalities and since there is well-documented hepatotoxic potential, understanding the relationship between the mild enzyme rises and long-term outcomes is necessary, but unclear at this time.

EFFICACY OF METHOTREXATE IN AUTOIMMUNE LIVER DISEASE

Methotrexate is a well-established treatment for a wide variety of autoimmune diseases[3]. Given the concern over the association between liver adverse events and methotrexate use it is perhaps understandable that the evaluation of methotrexate efficacy in autoimmune liver diseases has proceeded more slowly than in other disciplines. However the treatment depends on the cause and early studies in malignancy showed dramatic improvements in hepatic manifestations, coupled with longer term toxicity in some cases[1,12].

Primary biliary cholangitis (previously primary biliary cirrhosis) (PBC) is perhaps the liver disease with the best established evidence for an autoimmune basis. PBC is characterised by early lymphocytic infiltration and granulomatous inflammation progressing to chronic damage and scarring resulting in the ultimate clinical manifestations of the disease. PBC is more common

in a variety of rheumatic diseases including Sjogren's syndrome, rheumatoid arthritis, and a number of other connective tissue diseases[37]. However, the full importance of autoimmunity in the disease pathogenesis has been questioned given the apparent lack of response of PBC to many traditional immunosuppressants[38]. Ursodeoxycholic acid is recommended as the first-line treatment option in PBC, however even its benefits are at best modest and a substantial number of patients do not respond[39-43]. There is therefore a significant unmet therapeutic need for safe and effective treatment options for PBC.

Given the suggested autoimmune basis of the disease and the proven efficacy of methotrexate in a number of the conditions associated with PBC it was perhaps a natural development to progress to studying this agent in PBC. The ultimate trigger for the initial use of methotrexate in PBC however was its apparent efficacy in early studies in primary sclerosing cholangitis (PSC)[44]. Methotrexate has been demonstrated to improve liver blood tests and liver histology in a long-term open label study of PBC patients with an inadequate response to ursodeoxycholic acid[45]. Despite these apparent benefits the more widespread use of methotrexate in these diseases is difficult to recommend given the lack of evidence for improvements in important outcomes such as mortality and progression to transplantation[46]. Of even greater difficulty is the lack of convincing evidence for efficacy in the randomised controlled trials of methotrexate in PBC[38,47]. The only commonality across the studies of methotrexate in PBC has been a lack of evidence of adverse events, including transaminase elevations[38,45]. This picture is complicated by the inherent difficulties in studying treatment efficacy in PBC, a disease with widely variable outcomes, a prolonged course prior to the development of end-stage disease, and a lack of definitive surrogate markers of disease progression. It has been suggested that another aspect of the difficulty may be related to subsets of responders and non-responders among patients with PBC[47]. While the use of methotrexate in PBC remains controversial, the lack of alternative treatment options and the good evidence regarding the drug's safety in this patient population may justify a therapeutic trial.

Primary sclerosing cholangitis (PSC) is in many ways even more challenging than PBC. In contrast to PBC, PSC is not a classical immune disease, lacking characteristic autoantibodies, but does certainly have an immune component, with evidence of T-lymphocyte driven inflammation[48]. The use of immunosuppressants in PSC has not demonstrated convincing evidence of favourable responses[49]. Methotrexate was first used in PSC in the 1980's with initial reports of good responses with early treatment initiation[50,51]. Results from a subsequent randomised controlled trial and case series were not encouraging however with evidence of improvement only in alkaline phosphatase levels[52,53].

The utility of methotrexate in cholestatic liver diseases remains uncertain. Based on the clinical trials in these diseases however we can obtain some reassurance about the overall liver safety of methotrexate given the lack of evidence of significant adverse events in this group predisposed to liver adverse outcomes.

META-ANALYSIS

In view of the ongoing uncertainty over the risk of liver disease in methotrexate treated patients we recently performed a comprehensive meta-analysis of randomised controlled trials evaluating this issue[10]. We choose to limit our assessment to double-blind randomised controlled trials in order to eliminate the potential bias, both overt and covert, inherent in any situation in which a physician knows that a patient is prescribed, or potentially prescribed methotrexate[54]. Pre-existing perceptions among physicians regarding the liver toxicity of methotrexate are a major confounder in many of the previous assessments of methotrexate toxicities. An additional advantage of this methodology is that the very nature of a randomised controlled trial provides a large number of patients with similar clinical and demographic characteristics as a control group. Of course randomised controlled trials, and meta-analyses of such trials, have their own inherent limitations, including issues with generalizability to heterogeneous real world patient populations, and a limited period of follow-up[55]. Hence it is important to interpret such studies in conjunction with other forms of evidence such as that from observational studies[21].

In our meta-analysis we included randomised controlled trials in which patients were prescribed methotrexate for rheumatoid arthritis, psoriasis, psoriatic arthritis, or inflammatory bowel disease[10]. A total of 32 studies with 13177 participants were included in the analysis, 6877 of these were prescribed methotrexate and 6300 comparator agents. The majority of included studies used active comparators to methotrexate, predominantly synthetic disease-modifying antirheumatic drugs (DMARDs) or biologic agents; there were also 5 studies with placebo comparators. The trial durations ranged from 24 to 104 wk with a mean duration of 47 wk. Liver adverse events were common in both cohorts, the cumulative incidence was 11.2% in methotrexate treated patients and 6.3% in the comparator group. This translated to an incidence rate of liver adverse events of 20/100 patient-years in methotrexate treated patients compared to 9/100 patient-years in the comparators giving an attributable risk of 11/100 patient years in methotrexate treated patients.

Our meta-analysis demonstrated that methotrexate use was associated with an increased relative risk (RR) of liver adverse events in this population of 2.19 (95%CI: 1.73-2.77). Additionally methotrexate use was associated with an increased risk of transaminase

Study or subgroup	Methotrexate Events	Methotrexate Total	Comparator Events	Comparator Total	Weight	Risk ratio M-H, Random, 95%CI
Total liver adverse events	866	6303	467	5732		1.69 (1.52, 1.88)
Major transaminase elevations	251	6303	81	5732		2.82 (2.20, 3.61)
Minor transaminase elevations	615	6303	383	5732		1.46 (1.29, 1.65)
Liver fibrosis, cirrhosis, failure, death	0	6303	3	5732		0.13 (0.01, 2.51)

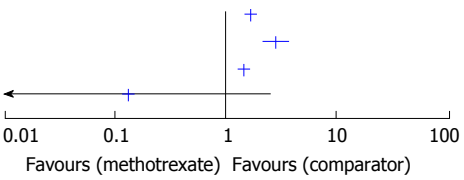

Figure 1 Risk of liver adverse events with methotrexate use.

elevation both less than or equal to three times the upper limit of normal, RR = 2.16 (95%CI: 1.67-2.79) and transaminases greater than three times the upper limit of normal, RR = 2.63 (95%CI: 1.90-3.64). The consistency in the increase risk across the various categories demonstrated by this portion of the meta-analysis was concerning, particularly given the utility of transaminases in predicting drug induced liver injury. We went on to analyse the hard endpoint of more serious liver outcomes, defined as hepatic failure, hepatic fibrosis, cirrhosis, or death due to liver disease. This was far more reassuring; methotrexate was not associated with any increased risk in these outcomes, RR = 0.12 (95%CI: 0.01-1.09). Indeed while not reaching statistical significance there was a strong trend towards less of these serious outcomes in methotrexate treated patients. The reasons why methotrexate could be associated with a possible reduction in serious outcomes but an increase in transaminase elevations are not immediately apparent. Methotrexate has shown potential efficacy in treating some autoimmune liver diseases[45,49]. Methotrexate induced transaminase elevations frequently prompt further investigations, potentially identifying concomitant diseases at an earlier stage, allowing earlier treatment and thus less progression to the hard endpoints evaluated in this outcome. However caution is required as only having surrogate measures of hepatic toxicity (transaminase elevations) with very few serious events is another major limitation. The main findings of the meta-analysis are summarised in Figure 1.

MANAGEMENT OF ABNORMAL LIVER BLOOD TESTS IN METHOTREXATE TREATED PATIENTS

The management of abnormal liver blood tests in patients treated with methotrexate is a common clinical query. As with any management plan the key first step is in ensuring the correct diagnosis. Abnormal liver blood tests should never be presumed to be due to methotrexate. The available evidence indicates that methotrexate related liver adverse events are rarely serious, particularly in the short term, while many other causes of abnormal liver blood tests may be. An evaluation for other potential causes should follow identical pathways and similar rigor to that applied to a patient who is not taking methotrexate. This investigative approach has been covered in detail elsewhere[56].

If after exhaustive investigation no cause other than methotrexate is identifiable than the treatment approach recommended in guidelines depends on the degree of transaminase elevation. The baseline transaminase levels prior to methotrexate institution are also important; a previously elevated transaminase level that hasn't changed following institution of methotrexate is unlikely to need further intervention. The threshold for immediately interrupting methotrexate use differs by the respective guideline, however levels greater than 3 times the upper limit of normal are often used[57]. Persistent lower grade elevations may also require intervention particularly if the trend is for a progressive increase in the transaminases[18,57].

Widely differing recommendations regarding the indication for a liver biopsy in methotrexate treated patients exist[58-60]. Increasingly a welcome move away from the routine performance of liver biopsies in methotrexate treated patients has accompanied a wider appreciation of the relative safety of this agent. Liver biopsy is the gold standard investigation as it allows direct assessment of liver histology, however it is imperfect and has a relatively high sampling error rate of 20%-30%[61]. In addition it is an invasive procedure, and like any such procedure carries with it risks of morbidity and indeed mortality; therefore it should only be performed when the results will be clinically useful[59]. In our practice a liver biopsy is infrequently clinically indicated and when it is performed is most commonly to investigate for another potential cause rather than investigation of suspected methotrexate induced hepatotoxicity.

Alternative methods of assessing for liver toxicity including procollagen Ⅲ aminopeptide, multibiomarker scores, and transient elastography in our opinion have potential but remain experimental and we do not recommend their use in routine clinical practice at the present time[62]. A proposed approach to suspected methotrexate hepatotoxicity is outlined in Table 1. All of the suggestions in this table must be interpreted and modified in the light of the clinical scenario.

CONCLUSION

Methotrexate is a highly effective treatment for a

Table 1 Management of suspected methotrexate toxicity

Transaminase monitoring	Commencing	Every 2 wk
	Adjusting dose	Every 2 wk
	Stable dose	Every 12 wk
Elevated transaminases	New persistent elevation	Reduce methotrexate, investigate
	New elevation greater than 3 times upper limit normal	Withdraw methotrexate, investigate, methotrexate may be restarted after normalisation
Liver biopsy	Indication	Investigation of other potential causes of elevated transaminases
		Very rarely for confirmation of methotrexate induced toxicity

broad range of diseases. Concern over potential adverse events has limited the use of methotrexate in certain populations. Robust evidence of the true risk of the majority of methotrexate associated adverse events with modern dosing regimens in patients with normal renal function have been lacking. Methotrexate use is associated with an increased risk of elevated transaminase levels; however the risk of an increased risk of serious liver adverse events with modern methotrexate monitoring protocols appears to be extremely low at present. Long-term follow-up studies of patients with mild transaminase elevations are needed. Large increases are rare, should be taken seriously, and the medication stopped. Physicians and patients should be comfortable using methotrexate where clinically indicated.

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ORIGINAL ARTICLE

Case Control Study

Regional differences in genetic susceptibility to non-alcoholic liver disease in two distinct Indian ethnicities

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Author contributions: Bale G and Steffie AU performed research; Rao PN, Sharma M and Reddy DN recruited patients; Ravi Kanth VV, Sasikala M and Rao PN designed the research; Ravi Kanth VV monitored the study, performed statistical analyses, and drafted the manuscript.

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Abstract

AIM

To validate the association of variants in *PNPLA3* (rs2281135) and *TM6SF2* (rs58542926) genes with ultrasound detected non-alcoholic fatty liver disease (NAFLD).

METHODS

A total of 503 individuals with and without fatty infiltration were recruited. Fatty infiltration was confirmed based on ultrasound findings. Anthropometric data and blood samples were collected from the study group. DNA was isolated from peripheral blood, quality and quantity was assessed by gel electrophoresis and spectrophotometer respectively. Genotyping of the variants in *PNPLA3* and *TM6SF2* genes was carried out by employing taqman probes (C_15875080_10 for PNPLA3 and C_8946351_10 for TM6SF2 SNP) on real time PCR (Stepone-Lifetechnologies). Genotype data was tested for deviations from Hardy-Weinberg

equilibrium. χ^2 test was used to analyze the statistical significance of the difference in genotype distribution of the studied variants in patients and controls and the strength of association was expressed as odds ratio (95%CI). A two-tailed *P* value of $\leqslant$ 0.05 was considered statistically significant.

RESULTS

The study group comprised of 503 individuals of which 256 had fatty infiltration and 247 without fatty infiltration and thus formed the patient and control groups respectively. As the patient group could be divided in to two distinct ethnicities (ancestral South Indians-ASI and North-East Indians-NEI), further recruitment of control cohort and association analyses was carried out based on ethnicities. Of the 256 with fatty infiltration 93 were ASI and 163 were NEI and of the 247 controls 138 were ASI and 109 were NEI. As expected, there were significant differences in the anthropometric and other clinical data between the control and the patient groups. However significant differences within the ethnicities were also noted. While rs2281135 in *PNPLA3* gene was significantly associated (*P* = 0.03) with higher risk (odds 1.9, 95%CI: 1.5-3.14, *P* = 0.03) of NAFLD in NEI ethnicity, rs58542926 in *TM6SF2* gene was significantly associated with NAFLD with a 2.7 fold higher risk (odds 2.7, 95%CI: 1.37-5.3, *P* = 0.0004) of the disease. There were significantly higher proportions of individuals with variants in both the genes in the patient group in both ASI (patients - 14/93 and controls - 7/138; *P* = 0.009) and NEI ethnicities (patients - 17/163 and controls - 7/109; *P* = 0.01).

CONCLUSION

Although the study identified distinct genetic susceptibility in the two ethnicities, transheterozygosity of the variants suggests higher risk of NAFLD in individuals with both the variants.

Key words: Transmembrane 6 superfamily 2; Patatin-like phospholipase domain-containing protein 3; Fatty infiltration; Genetic susceptibility; Ethnicity; Non-alcoholic fatty liver disease; Cirrhosis; Single nucleotide polymorphism

Core tip: Non-alcoholic fatty liver disease has become the leading cause of liver damage contributing to considerable mortality. The spectrum spans from simple steatosis, through non alcoholic steatohepatitis, fibrosis, cirrhosis and finally to hepatocellular carcinoma. Genetic variants have now been recognized to contribute to a substantial extent to the onset of the disease. Reliable genetic markers that confer susceptibility to the disease have to be identified for better management of the disease. Identification of at risk individuals at a younger age by screening for genetic susceptibility will aid in better management by early interventions and lifestyle changes. This study identified regional differences and ethnicity based genetic susceptibility for non-alcoholic liver disease.

Bale G, Steffie AU, Ravi Kanth VV, Rao PN, Sharma M, Sasikala M, Reddy DN. Regional differences in genetic susceptibility to non-alcoholic liver disease in two distinct Indian ethnicities. *World J Hepatol* 2017; 9(26): 1101-1107 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i26/1101.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i26.1101

INTRODUCTION

Non-alcoholic liver disease (NAFLD) describes a range of liver conditions beginning with fatty liver (accumulation of fat in the liver) that progresses to non-alcoholic steatohepatitis (NASH; fat accumulation along with inflammation and scarring) and cirrhosis (scar tissue replaces hepatic cells)[1], that may finally lead to hepatocellular carcinoma (HCC)[2]. While conditions up to NASH are reversible[3], progression beyond NASH to cirrhosis is irreversible[4]. Therefore it is very important to identify individuals with genetic susceptibility to fat accumulation at an early stage so that appropriate interventions can be planned to curtail/avoid progression to higher stages. Environmental factors including intake of calories[5], processed food[6] and sedentary lifestyles[7] have an impact on the predisposition of an individual to fatty liver and progression. Apart from environmental factors various studies have now confirmed the role of genetics in conferring susceptibility to the disease. Diseases with complex traits including NAFLD result from interactions between environment and polygenic genetic susceptibility made up of many independent modifiers[8]. Family aggregation, studies on twins and differences in susceptibility and progression suggest a significant heritable component to NAFLD that may be classified under "common disease-common variant" hypothesis[9].

The first Genome wide association study for NAFLD identified a SNP in *PNPLA3* gene (rs738409; c.444 C > G, p.I148M). Carrier of the minor allele and 148M was associated with a twofold increase in HTGC (Hepatic triglyceride content)[10]. Subsequent to this, the SNP was replicated in almost all the ethnicities successfully[8]. Further, two exome wide association studies[11,12] carried out independently in African-American and Norwegian ethnicities identified that a variant rs58542926 (p.E167K) in *TM6SF2* gene was associated with susceptibility to NAFLD, influencing total cholesterol levels and enhanced risk of myocardial infarction. Subsequently, functional studies identified *TM6SF2* as a regulator of liver fat metabolism influencing secretion of triglycerides and lipid droplet content in the liver[13]. A recent review suggested that male sex, *PNPLA3* I148M, *TM6SF2* E167K and low birth weight as important predictors of adult NAFLD[14] reiterating the importance

of variants in both *PNPLA3* and *TM6SF2* genes.

Our earlier pilot study[15] identified variants in *PNPLA3* (rs738409), *PARVB* (rs2073080), *SAMM50* (rs2143571) and *PZP* (rs6487679) genes to be associated with a higher risk of fatty infiltration in individuals of NEI ethnicity. In the present study we replicated variants namely rs58542926 in *TM6SF2* and rs2281135 in *PNPLA3* genes, identified earlier[12] to confer susceptibility to NAFLD in two distinct ethnicities. While one ethnicity belonged to South India, the other belonged to the North-Eastern region of the country. An earlier study on South Indians has reported that the genomic affinity is proportionate to caste rank-the upper castes being most similar to Europeans, while the lower castes are more similar to Asians[16]. However, the Northeast region's population results from ancient and continuous flows of migrations from Indo-Gangetic India, Tibet, the Himalayas, present day Bangladesh and Myanmar[17].

MATERIALS AND METHODS

A total of 503 individuals were recruited for the present study from the Hepatology clinics of Asian Institute of Gastroenterology. Although liver biopsy is considered to be the gold standard for identifying NAFLD, risk of complications, costs involved and ethical concerns limit its use, hence, patients with fatty infiltration were recruited based on ultrasound findings. Ethnicity, age and sex matched healthy subjects who volunteered to be part of the study were recruited as controls based on the sole criteria of the absence of liver fat on ultrasonography with normal liver function tests and negative for other viral indications. Written informed consent was obtained from individuals and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review (Scientific) Board (AIG/AHF IRB: 16/2014). Demographic and anthropometric details (height, weight, BMI and waist circumference) were collected. Whole blood (3 mL) was collected in pre coated EDTA containers from the study group and stored at -20 ℃ until further analysis. Biochemical investigations like ALT, viral markers and lipid profiles were estimated as per standard methods.

Genotyping

DNA was isolated from blood using a commercial kit (Bioserve Biotechnologies, Hyderabad) following manufacturers protocol. DNA with high molecular weight on agarose gel and A260/280 ratios between 1.8-2.0 were included for genotyping analyses. All the samples were genotyped for SNPs namely rs2281135 in *PNPLA3* and rs58542926 in *TM6SF2* genes using Taqman single nucleotide genotyping assay (Life Technologies, United States) on the Realtime polymerase chain reaction (PCR) platform. PCR for genotyping consisted of 5 μL of 2 × Taqman genotyping master mix, 0.5 μL of 1 × assay mix (C_15875080_10 for *PNPLA3* and C_8946351_10 for *TM6SF2* SNP) and 4.5 μL consisting of 8-10 ng of DNA in a final volume of 10 μL. PCR was performed on Step One Realtime PCR (Life technologies, United States) with the following cycling conditions: 95 ℃ for 10 min, 95 ℃ for 15 s and 60 ℃ for 1 min with fluorescence read after each cycle for a total of 40 cycles. Genotyping calls were made using the allelic discrimination software (Life Technologies, United States) and only auto calls made by the software were considered for further analysis. A known heterozygous and homozygous variant sample was replicated across all the plates and these known genotypes were verified manually during analysis in all the plates.

Statistical analysis

Data was entered in to MS-EXCEL and edited for consistency. Continuous variables were expressed as mean (95%CI) and categorical variables as proportions. Patient characteristics were compared using Student's *t* test for continuous variables and χ^2 test for categorical variables. χ^2 goodness-of-fit was used to confirm the agreement of the observed genotype frequencies with those of expected (Hardy-Weinberg equilibrium). χ^2 test was used to analyze the statistical significance of the difference in genotypic distribution of the studied SNPs in patients and controls. The association of the studied SNPs with the disease and various clinical parameters was expressed as odds ratio (95%CI). For transheterozygoisty analysis chi-square test was applied to compare the number of variant carriers in both the genes between patients and controls. A two-tailed *P* value of $\leqslant$ 0.05 was considered statistically significant. The analyses were carried out using Med cal C package.

RESULTS

Although categorization of the study group based on the ultrasound findings yielded two groups, ethnicity was identified as a major confounder for further analysis. Samples were therefore sorted based on ancestry and classified in to Ancestral South Indians (ASI; *n* = 231; Controls-138 and patients-93) and North-East Indians (NEI; *n* = 272; controls-109 and patients-163). All the clinical characteristics as shown in Table 1 namely waist circumference, hip circumference, waist/hip ratio, BMI, ALT, AST, Triglycerides were significantly different between the cohorts from both the ethnicities. Further, there was significant difference in the HDL levels only in the NEI group but not in the ASI group.

Genotyping and association with clinical traits

While rs58542926 in *TM6SF2* gene was significantly associated (*P* = 0.0004) with a 2.7 fold higher risk of fatty infiltration in ASI ethnicity, rs2281135 in *PNPLA3*

Table 1 General characteristics of the study

Characteristics	Ancestral South Indians			North-East Indians		
	Controls (n = 138) (mean ± SD)	Patients (n = 93) (mean ± SD)	P value[1]	Controls (n = 109) (mean ± SD)	Patients (n = 163) (mean ± SD)	P value[1]
Age (yr)	34.2 ± 11.9	35.3 ± 8.0	0.43	38.5 ± 12.7	36.5 ± 9.2	0.13
Gender male/female (n)	95/43	87/6	0.64	72/37	150/13	0.84
Waist circumference (cm)	83.3 ± 9.4	94.7 ± 10.2	0.01	81.1 ± 10.7	93.8 ± 10.1	0.01
Hip circumference (cm)	93.0 ± 7.1	100.5 ± 8.6	0.01	91.2 ± 6.9	95.1 ± 8.5	0.01
Waist/hip ratio	0.89 ± 0.06	0.95 ± 0.13	0.01	0.89 ± 0.07	0.99 ± 0.12	0.01
BMI (kg/m^2)	23.2 ± 4.0	27.7 ± 4.1	0.01	22.1 ± 3.5	25.7 ± 4.0	0.01
ALT (IU/L)	19.8 ± 7.6	88.1 ± 49.5	0.01	24.6 ± 7.9	119.3 ± 68.3	0.01
AST (IU/L)	21.2 ± 5.4	55.3 ± 25.6	0.01	24.6 ± 6.9	72.3 ± 39.8	0.01
Triglycerides (mg/dL)	134.8 ± 72.6	169.7 ± 82.1	0.01	131.4 ± 60.8	180.3 ± 93.7	0.05
HDL (mg/dL)	38.7 ± 8.5	36.3 ± 6.9	0.09	47.9 ± 28.7	40.5 ± 13.8	0.02

[1]Unpaired students t test (two tailed). BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HDL: High density lipoprotein.

Table 2 Genotype distribution of *Tm6SF2* and *PNPLA3* variants in the two ethnicities studies

	Ancestral South Indians TM6SF2 rs58542926						North-East Indians TM6SF2 rs58542926					
	Controls (n)	Patients (n)	Odds	95%CI	χ^2	P value[1]	Controls (n)	Patients (n)	Odds	95%CI	χ^2	P value[1]
Wild (CC)	110	61	2.7	1.37-5.3	15.28	0.0004	80	110	1.51	0.86-2.66	2.29	0.31
Heterozygous (CT)	18	22					22	44				
Homozygous (TT)	0	7					2	6				
PNPLA3 rs2281135												
Wild (GG)	79	49	1.34	0.78-2.33	2.12	0.34	63	71	1.9	1.5-3.14	6.48	0.03
Heterozygous (GA)	45	35					32	71				
Homozygous (AA)	4	6					9	17				

[1]χ^2 test. Odds: Odds ratio.

gene was associated with 1.9 fold higher risk in the NEI ethnicity (Table 2). rs58542926 in *TM6SF2* gene was associated with higher ALT, AST levels in the ASI ethnicity and higher BMI in NEI ethnicity. rs2281135 in *PNPLA3* gene was associated with ALT, AST levels in the NE ethnicity (Table 3).

Transheterozygosity analysis

On transheterozygosity analysis (χ^2 test), it was seen that there was a significant difference in individuals who carried variants in both the genes in the patient group as compared to control group in ASI ethnicity (P = 0.009), but not NEI ethnicity (P = 0.26) and increased the risk of the disease by 3 fold (OR = 3.11, 95%CI: 1.20-8.04) in the ASI ethnicity. Further, there were significantly higher proportion of individuals with variants in both the genes in the patient group in ASI (patients - 14/93 and controls - 7/138; Z proportion test P = 0.009) and NEI ethnicities (patients - 17/163 and controls - 7/109; Z proportion test P = 0.06).

Comparison of controls and patients within the ethnicities

There were significant differences in BMI (higher) AST, ALT and HDL levels (lower levels) in ASI controls as compared to NEI controls. While patients of ASI ethnicity had higher hip circumference, BMI and lower HDL levels patients of NEI ethnicity had higher waist-hip ratios, ALT and AST levels. Likewise, there were significant differences in hip circumference, BMI (higher levels in ASI as compared to NEI patients), waist-hip ratio, ALT, AST levels (higher levels in NEI patients as compared to ASI patients). It was also interesting to note that the HDL levels were significantly lower in the ASI patients (Table 4).

DISCUSSION

In a cohort of 503 individuals comprising individuals with and without NAFLD belonging to two distinct Indian ethnicities, we show here that rs58542926 in *TM6SF2* in South Indian and rs2281135 in *PNPLA3* in North-East Indian ethnicities confer higher susceptibility to ultrasound measured NAFLD. Further, there were a significant proportion of individuals with variants in both the genes in the patient group as compared to controls, in both the ethnicities, suggesting that although individually the variants may not confer susceptibility in the ethnicity, however carrying an additional variant might compound the risk of the disease. Our earlier pooled genetic association study in a predominantly North-East Indian ethnicity identified that rs738409 in *PNPLA3* gene was associated with higher risk of NAFLD apart from variants in *PARVB*, *SAMM50* and *PZP*

Table 3 Association of variants with clinical data

	Ancestral South Indians								North-East Indians							
	CC	CT	TT	GG	GA	AA	χ^2	*P* value[1]	CC	CT	TT	GG	GA	AA	χ^2	*P* value[1]
TM6SF2 rs58542926																
BMI																
< 22.9	49	12	2				0.03	0.98	75	18	5				8.24	0.01
> 22.9	110	28	4						94	47	2					
PNPLA3 rs2281135																
BMI																
< 22.9				38	23	2	0.59	0.74				48	37	10	0.077	0.96
> 22.9				82	52	8						71	58	14		
TM6SF2 rs58542926																
ALT																
< 30	89	17	1				6.52	0.038	65	20	1				2.45	0.29
> 30	56	19	5						108	42	7					
PNPLA3 rs2281135																
ALT																
< 30				64	39	4	1.3	0.52				54	28	4	10.27	0.005
> 30				42	33	5						66	71	19		
TM6SF2 rs58542926																
AST																
< 30	90	18	0				10.19	0.006	68	20	3				0.96	0.61
> 30	55	18	6						105	42	5					
PNPLA3 rs2281135																
AST																
< 30				65	40	3	2.89	0.23				54	30	7	5.55	0.06
> 30				41	32	6						66	69	16		
TM6SF2 rs58542926																
TG																
< 150	54	13	4				1.64	0.43	58	25	6				3.89	0.14
> 150	33	12	1						60	29	1					
PNPLA3 rs2281135																
TG																
< 150				42	25	8	0.29	0.86				49	32	8	2.49	0.28
> 150				29	14	3						39	40	11		
TM6SF2 rs58542926																
HDL																
> 40	26	5	0				2.03	0.36	49	22	3				0.05	0.97
< 40	57	18	3						59	27	3					
PNPLA3 rs2281135																
HDL																
> 40				20	11	0	2.52	0.28				39	28	7	0.5	0.77
< 40				46	26	6						71	58	14		

[1]χ^2 test. BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TG: Triglycerides; HDL: High density lipoprotein.

Table 4 Comparison of clinical data within the ethnicities

Characteristics	Controls			Patients		
	ASI controls (*n* = 138) (mean ± SD)	NEI controls (*n* = 109) (mean ± SD)	*P* value[1]	ASI patients (*n* = 93) (mean ± SD)	NEI patients (*n* = 163) (mean ± SD)	*P* value
Age (yr)	34.2 ± 11.9	38.5 ± 12.7	0.006	35.3 ± 8.0	36.5 ± 9.2	0.29
Gender male/female (*n*)	95/43	72/37	-	87/6	150/13	-
Waist circumference (cm)	83.3 ± 9.4	81.1 ± 10.7	0.27	94.7 ± 10.2	93.8 ± 10.1	0.54
Hip circumference (cm)	93.0 ± 7.1	91.2 ± 6.9	0.23	100.5 ± 8.6	95.1 ± 8.5	0.01
Waist/hip ratio	0.89 ± 0.06	0.89 ± 0.07	1.0	0.95 ± 0.13	0.99 ± 0.12	0.03
BMI (kg/m^2)	23.2 ± 4.0	22.1 ± 3.5	0.02	27.7 ± 4.1	25.7 ± 4.0	0.003
ALT (IU/L)	19.8 ± 7.6	24.6 ± 7.9	0.01	88.1 ± 49.5	119.3 ± 68.3	0.01
AST (IU/L)	21.2 ± 5.4	24.6 ± 6.9	0.01	55.3 ± 25.6	72.3 ± 39.8	0.01
Triglycerides (mg/dL)	134.8 ± 72.6	131.4 ± 60.8	0.79	169.7 ± 82.1	180.3 ± 93.7	0.44
HDL (mg/dL)	38.7 ± 8.5	47.9 ± 28.7	0.02	36.3 ± 6.9	40.5 ± 13.8	0.01

[1]Unpaired students *t* test (two tailed). ASI: Ancestral South Indians; NEI: North-East Indians; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HDL: High density lipoprotein.

genes[15].

The first Genome wide association study for NAFLD identified rs738409 in *PNPLA3* gene conferring susceptibility to NAFLD[10]. Subsequent to this, the variant

was found to be associated with the disease in various ethnicities across the world including our own and other studies from India[8,15,18]. *PNPLA3* is a 481-residue protein, exhibiting lipase activity against triglycerides in hepatocytes and a missense variant (I148M; rs738409-C>G) results in loss of function promoting hepatic steatosis by limiting triglyceride hydrolysis[19]. Further, another variant (rs2281135) in *PNPLA3* gene was identified, that conferred higher risk for NAFLD[11]. rs2281135 is an intronic variant and is known to be in tight linkage disequilibrium with rs738409 in ethnicities including African, Caucasian, Mexican Americans and East African (HapMap data). Apart from variants in *PNPLA3*, recent research has identified rs58542926 in *TM6SF2* gene to be associated with NAFLD. Recombinant protein expression in cultured hepatocytes confirmed that 50% less Glu167Lys TM6SF2 protein was produced relative to wild-type TM6SF2[11]. Further a study identified that TM6SF2 regulates liver fat metabolism and influences triglyceride secretion and lipid droplet content[13]. There is compelling evidence by now that variants in *PNPLA3* and *TM6SF2* genes are associated with progressive fatty infiltration (steatosis and cirrhosis) and further have a higher risk of progressing to HCC. It is therefore very important to understand the genetic susceptibility an ethnicity carries, so that appropriate lifestyle interventions can be planned to minimize the risk of progression, more so in the absence of reversing the genetic defect.

The intronic SNP (rs2281135) in *PNPLA3* gene was associated with a higher risk of fatty infiltration only in NEI ethnicity but not ASI. In an earlier study with a predominant NEI ethnicity we identified that rs738409 in *PNPLA3* conferring a higher susceptibility to fatty infiltration. It is known in literature that rs2281135, an intronic variant and rs738409 a functional variant are in tight LD in ethnicities including African, Caucasian, Mexican Americans and East African (HapMap data).

Although the general characteristics between patients and controls were significantly different as expected, it was interesting to note ethnicity based differences in the patient cohorts that could be predictive of higher susceptibility to NAFLD. While, higher hip circumference, BMI, and lower HDL levels could be predictive of a higher risk for NAFLD in the SI ethnicity, higher Waist-Hip ratio could be predictive in NE ethnicity. Further, higher BMI and lower HDL levels were seen in the controls of SI ethnicity and higher AST and ALT levels were seen in the controls of NE ethnicity suggesting cohort based differences and cutoffs in the clinical characteristics. Further, interestingly there were higher ALT and AST levels in the NEI ethnicity as compare to ASI ethnicity both between control and patient cohorts suggesting a higher necroinflammatory state in the patients of NEI ethnicity. Earlier genome wide studies have ascribed higher levels to genetic predisposition apart from other influencing factors including demographic such as age, sex, ethnicity, anthropometric features (waist circumference, BMI) and diurnal variation[20].

The genotype data in general did not deviate from Hardy-Weinberg equilibrium. However, it was interesting to note that there was a significant difference ($P = 0.02$) in the observed and expected genotype frequencies from the patient cohort of ASI ethnicity. Although the samples were represented in sufficient numbers, genotypes visually checked and manually re-scored, non-random mating and population structure excluded, the deviation persisted suggesting that the variant may contribute to disease risk in this ethnicity.

The genotyping data from this study suggests that while *TM6SF2* variant was significantly associated with susceptibility to fatty infiltration in the ASI ethnicity, *PNPLA3* variant was associated in the NEI ethnicity. However, it was interesting to see that there were a higher proportion of individuals in the patient group who were transheterozygous for *PNPLA3* and *TM6SF2* variants as compared to the control group suggesting that although there might be individual susceptibility in the two ethnicities, it is important to genotype the individuals for both the variants as there might be additive risk in the presence of the other risk allele. A recent study from Chinese ethnicity corroborated the same[21].

In conclusion, our study has identified distinct genetic susceptibility for ultrasound detected NAFLD in the two ethnicities. However, it is suggested that both the variants have to be genotyped for assessing the risk of the disease, as transheterozygosity of the studied variants seems to confer a higher risk in the population.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) with an incidence of 25%-30% is an epidemic that is on the rise globally. There are significant differences in the prevalence, severity and outcome of the disease in various ethnicities that suggests a genetic background to it. Approximately 26%-35% of NAFLD may be contributed by genetic susceptibility according to a study. Therefore it is important to identify genetic susceptibility an individual carries for better management of the disease.

Research frontiers

Understanding and identifying ethnicity based variants that confer higher risk of disease will aid in imparting lifestyle and nutrient based recommendations to an individual with fatty infiltration for better management of the disease.

Innovations and breakthroughs

The authors have identified distinct genetic susceptibility for NALFD in the two ethnicities that were studied. However, it was interesting to note that transheterozygosity of both the variants conferred a higher risk of the disease irrespective of ethnicity.

Applications

Individuals can be screened for these variants to assess their risk of developing NAFLD. Further, life style based modifications can be suggested to delay the onset/progression of the disease.

Terminology

NAFLD describes a range of liver conditions that begins with accumulation

of fat in the liver (fatty liver) and progresses to fat accumulation along with inflammation and scarring non-alcoholic steatohepatitis, hepatic cells replaced by scar tissue (cirrhosis) finally leading to hepatocellular carcinoma.

Peer-review

The present work deals with a human study in which genetic susceptibility to NAFLD in two Indian ethnicities is evaluated. This study constitutes an interesting work as the identification of population at risk is always desirable.

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ORIGINAL ARTICLE

Retrospective Study

Conjugated hyperbilirubinemia presenting in first fourteen days in term neonates

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Author contributions: Chiou FK and Kader A contributed equally to this work; Chiou FK contributed to study design, collected and analyzed the data, and drafted and revised the manuscript; Kader A is the principal investigator who designed and supervised the study, provided direction and guidance in data analysis, and reviewed and revised the manuscript; Ong C and Phua KB contributed to study design and reviewed the manuscript for intellectual content; Chedid F contributed to data analysis and provided expertise in statistical analysis; all authors have read and approved the final version of the manuscript.

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Data sharing statement: Dataset is available from the corresponding author at ajmalkader@dha.gov.ae. Consent for data sharing from study participants was not obtained as presented data are anonymized and risk of identification is low.

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Abstract

AIM

To describe the etiology and characteristics of early-onset conjugated hyperbilirubinemia (ECHB) presenting within 14 d of life in term neonates.

METHODS

Retrospective review was performed of term infants up to 28-d-old who presented with conjugated hyperbilirubinemia (CHB) at a tertiary center over a 5-year period from January 2010 to December 2014. CHB is defined as conjugated bilirubin (CB) fraction greater than 15% of total bilirubin and CB greater or equal to 25 μmol/L. ECHB is defined as CHB detected within 14 d of life. "Late-onset" CHB (LCHB) is detected at 15-28 d of life and served as the comparison group.

RESULTS

Total of 117 patients were recruited: 65 had ECHB, 52

had LCHB. Neonates with ECHB were more likely to be clinically unwell (80.0% *vs* 42.3%, $P < 0.001$) and associated with non-hepatic causes (73.8% *vs* 44.2%, $P = 0.001$) compared to LCHB. Multifactorial liver injury (75.0%) and sepsis (17.3%) were the most common causes of ECHB in clinically unwell infants, majority (87.5%) had resolution of CHB with no progression to chronic liver disease. Inborn errors of metabolism were rare (5.8%) but associated with high mortality (100%) in our series. In the subgroup of clinically well infants ($n = 13$) with ECHB, biliary atresia (BA) was the most common diagnosis (61.5%), all presented initially with normal stools and decline in total bilirubin but with persistent CHB.

CONCLUSION

Secondary hepatic injury is the most common reason for ECHB. BA presents with ECHB in well infants without classical symptoms of pale stools and deep jaundice.

Key words: Conjugated hyperbilirubinemia; Biliary atresia; Cholestasis; Direct hyperbilirubinemia; Neonatal jaundice

Core tip: Conjugated hyperbilirubinemia (CHB) is not routinely checked before 14-21 d of life, hence incidence and etiology of early-onset CHB (ECHB) before 14 d are not well-documented. Nearly three-quarters of ECHB have non-hepatic cause and are expected to recover with supportive treatment, while biliary atresia and metabolic disorders are important etiologies associated with significant morbidity. In our study, BA presenting before 14 d were detected solely from low levels of CHB without pale stools or worsening jaundice. Further studies are needed to determine if CHB screening before 14 d would lead to improved detection and outcome in neonatal liver disorders.

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INTRODUCTION

Conjugated hyperbilirubinemia (CHB) in a neonate signifies an underlying hepatobiliary dysfunction. A significant proportion of neonates with CHB do not have a primary liver disease[1,2]. According to current recommendations, serum conjugated bilirubin (CB) is checked when neonatal jaundice is prolonged beyond 14-21 d, prior to that only total bilirubin (TB) is checked[3,4]. The detection of CHB presenting before 14 d of life is usually triggered by specific clinical situations, therefore the real incidence and etiology of CHB in neonates below 14 d are unknown.

Even with well-established guidelines for the screening of neonatal CHB, actual referral for evaluation of CHB is frequently delayed to beyond 45 to 60 d of age[5-7]. Substantial observational evidence show that earlier diagnosis and surgical repair of biliary atresia (BA) result in better outcomes[8-11]. Early diagnosis of many of the other cholestatic conditions may also lead to improved outcomes[4]. Studies on infants with liver diseases including BA have shown that CB is often elevated in the first week of life[12-14]. Researchers have also found that CB level performed during the early newborn period is a useful "screening tool" for liver disorders especially biliary atresia[15].

We studied term newborns with CHB within 14 d of life, aiming to describe the etiology, clinical features and outcome in this poorly studied group, and to find out how they compare to those presenting with CHB between 15 to 28 d of life. To date, our study is the first to address CHB in full-term infants aged below 14 d.

MATERIALS AND METHODS

Retrospective data was collected from consecutive term infants with CHB below 28 d of age within a 5-year period from January 2010 to December 2014. Study was conducted at KK Women's and Children's Hospital which is the largest tertiary pediatric and neonatal facility in Singapore. The study was approved by Singhealth Centralised Institutional Review Board.

CHB is defined as CB fraction greater than 15% of TB, and CB $\geqslant$ 25 μmol/L[16-18]. We define "early-onset" as detection of CHB within 14 d of life (ECHB). Cases were identified through a search in the laboratory database using the inclusion criteria "conjugated bilirubin $\geqslant$ 25 μmol/L", "conjugated bilirubin/total bilirubin > 15%," "test performed at patient age $\leqslant$ 14 d." Infants born at less than 36 wk gestation were excluded.

Consecutive term neonates presenting with CHB aged 15-28 d within the same period served as the comparison group. For the purpose of this study, this group presenting after 14 d of life is referred to as "late-onset" CHB (LCHB).

CB was measured using an automated diazo dye reaction method from venous blood obtained by venipuncture in all patients. Blood samples were delivered immediately to the laboratory in covered specimen tubes to minimize the effect of light on the samples. Blood samples underwent an automated estimation of the hemolysis index, and samples that were found to be hemolysed based on established laboratory criteria were rejected, and repeat samples were taken.

Infants with CHB underwent a variety of investigations that included liver enzyme measurements, hepatobiliary ultrasonography, hepatobiliary iminodiacetic acid (HIDA) scan, liver biopsy, tests for inborn errors of metabolism (IEM), thyroid functions,

Table 1 Baseline clinical characteristics and biochemical indices at onset of conjugated hyperbilirubinemia

Baseline characteristics	ECHB (n = 65, %)	LCHB (n = 52, %)	P value
Ethnic origin			
Chinese	34 (52.3)	27 (51.9)	0.547
Malay	15 (23.1)	18 (34.6)	
Indian	8 (12.3)	4 (7.7)	
Others	8 (12.3)	3 (5.8)	
Male gender	38 (58.5)	40 (76.9)	0.035
Gestational age (wk)	38 (37-39)	38 (37-39)	0.303
Birth weight (g)	2918 (2570-3245)	3068 (2753-3416)	0.114
Apgar			
At 1 min	9 (6-9)	9 (9-10)	0.217
At 5 min	9 (8-9)	9 (9-10)	0.134
Cesarean section	25 (38.5)	14 (26.9)	0.190
Clinically ill status on presentation	52 (80.0)	22 (42.3)	< 0.001
LFT (at diagnosis)			
Total bilirubin (μmol/L)	147 (100-201)	120 (91-163)	0.033
Conjugated bilirubin (μmol/L)	46 (32-65)	38 (30-74)	0.310
Conjugated fraction (%)	35.7 (24.0-51.4)	37.4 (26.3-61.5)	0.159
ALP (IU/L)	160 (119-261)	322 (238-418)	< 0.001
ALT (IU/L)	20 (13-42)	23 (16-32)	0.377
AST (IU/L)	35 (26-75)	35 (25-52)	0.512
GGT (IU/L)	142 (74-334)	199 (131-273)	0.045

ECHB: Early-onset conjugated hyperbilirubinemia; LCHB: "Late-onset" conjugated hyperbilirubinemia; LFT: Liver function test; ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; GGT: Gamma-glutamyl transferase.

bacterial cultures and viral serologies depending on the judgement of the treating physician. Surgical conditions such as BA and choledochal cysts were diagnosed from biochemical tests, radiologic findings and intra-operative cholangiography. IEM were diagnosed if confirmed report of an abnormality was found on appropriate testing. Multifactorial liver injury (MLI) was defined in our study as secondary hepatic insult in an unwell neonate with any combination of the following: Severe cadiorespiratory instability, hepatoxic medications and parenteral nutrition. Sepsis was defined as infection in which a viral or bacterial agent was isolated, and the infection was the primary cause of illness in the child. CHB was categorized as idiopathic if no cause was identified.

Data on patient demography, clinical history, co-morbid conditions, drug history, clinical status at time of detection of CHB, laboratory parameters, radiologic investigations and histologic studies, final diagnoses as well as outcome were retrospectively obtained from medical records. An infant was classified as clinically unwell when the admitting physician documented that the infant appeared unwell.

Data analysis was performed using IBM SPSS Statistics for Windows, version 19 (IBM Corp, Armonk, NY, United States). Continuous variables were expressed as mean ± SD or median (25%-75% interquartile range). Categorical variables were expressed as number (proportion). Comparisons were performed using two sample t-test in normally distributed data with equal variance or Mann-Whitney U test when the assumptions of two sample t-test were not met. χ^2 test or Fisher's exact test was used to compare categorical variables. Statistical significance was set at $P < 0.05$.

RESULTS

Total of 117 neonates with CHB were included in the study. Sixty-five had ECHB, and 52 LCHB. Baseline characteristics and liver function tests at presentation are summarized in Table 1. There was a significant male preponderance in both groups, and higher proportion of clinically unwell neonates in ECHB.

Etiology of CHB was identified in about 93% and 60% of cases in ECHB and LCHB groups respectively, rest were classified as idiopathic. Non-hepatic cause for CHB was 73.8% *vs* 44.2% (P = 0.001) in ECHB and LCHB respectively. MLI was an attributable cause of ECHB in 60%, followed by primary sepsis (13.8%) and BA (12.3%) (Table 2). In contrast, the most common cause found in LCHB was idiopathic (40.4%), followed by MLI (34.6%) and BA (9.6%). Factors associated with MLI in both ECHB and LCHB groups are summarized in Table 3.

There was a significantly higher proportion of unwell infants in ECHB group, 80.0% *vs* 42.3% in LCHB group (P < 0.001) (Tables 1 and 4). In the subgroup of patients who were clinically well within the ECHB group, BA was the most common diagnosis (61.5%), the remaining were idiopathic. The most common etiology/association found in well infants in the LCHB group was idiopathic (70.0%), followed by surgical causes (23.4%). No patient with BA was clinically unwell.

Out of the 65 patients with ECHB, 47 (72.3%) resolved within a mean period of 1.9 ± 1.4 mo with eventual normalization of liver tests, 8 (12.3%) had

Table 2 Comparison of causes between early-onset conjugated hyperbilirubinemia and "late-onset" conjugated hyperbilirubinemia groups *n* (%)

Etiology	ECHB (*n* = 65)	LCHB (*n* = 52)	Total (*n* = 117)	*P* value
Non-surgical causes	56 (86.2)	45 (86.5)	101 (86.3)	0.962
Multifactorial liver injury	39 (60.0)	18 (34.6)	57 (48.7)	0.007
Sepsis	9 (13.8)	3 (5.8)	12 (10.3)	0.154
Inborn errors of metabolism	3 (4.6)	1 (1.9)	4 (3.4)	0.428
CMV infection	0 (0)	2 (3.8)	2 (1.7)	0.112
Idiopathic	5 (7.7)	21 (40.4)	26 (22.2)	< 0.001
Surgical causes	9 (13.8)	7 (13.5)	16 (13.7)	0.952
Biliary Atresia	8 (12.3)	5 (9.6)	13 (11.1)	0.647
Choledochal cyst	1 (1.5)	2 (3.8)	3 (2.6)	0.435
Non-hepatic causes	48 (73.8)	23 (44.2)	71 (61)	0.001

ECHB: Early-onset conjugated hyperbilirubinemia; LCHB: "Late-onset" conjugated hyperbilirubinemia; CMV: Cytomegalovirus.

Table 3 Factors associated with multifactorial liver injury *n* (%)

Factors associated with multifactorial liver injury	ECHB (*n* = 39)	LCHB (*n* = 18)	Total (*n* = 57)
Antibiotics	38 (97.4)	18 (100.0)	56 (98.2)
Parenteral nutrition	35 (89.7)	16 (88.9)	51 (89.5)
Sedatives/opioid	29 (74.4)	14 (77.8)	43 (75.4)
Mechanical ventilation	26 (66.7)	12 (66.7)	38 (66.7)
Inotropic support	23 (59.0)	9 (50.0)	32 (56.1)
Recent surgery	20 (51.3)	12 (66.7)	32 (56.1)
PPHN	19 (48.7)	4 (22.2)	23 (40.4)
Intestinal obstruction	13 (33.3)	7 (38.9)	20 (35.1)
Congenital heart disease	12 (30.8)	4 (22.2)	16 (28.1)
HFOV	11 (28.2)	3 (16.7)	14 (24.6)
Pneumothorax	8 (20.5)	1 (5.6)	9 (15.8)
CDH	5 (12.8)	4 (22.2)	9 (15.8)
MAS	6 (15.4)	2 (11.1)	8 (14.0)
Renal impairment	6 (15.4)	2 (11.1)	8 (14.0)
Seizures/anti-epileptic	4 (10.3)	2 (11.8)	6 (10.7)
Perinatal asphyxia	3 (7.7)	3 (16.7)	6 (10.5)
Intracranial haemorrhage	2 (5.1)	1 (5.6)	3 (5.3)
Trisomy 21	2 (5.1)	1 (5.6)	3 (5.3)
ECMO	2 (5.1)	1 (5.6)	3 (5.3)
Turner's syndrome	1 (2.6)	0	1 (1.8)
Trisomy 18	1 (2.6)	0	1 (1.8)

ECHB: Early-onset conjugated hyperbilirubinemia; LCHB: "Late-onset" conjugated hyperbilirubinemia; PPHN: Persistent pulmonary hypertension of the newborn; HFOV: High frequency oscillatory ventilation; CDH: Congenital diaphragmatic hernia; MAS: Meconium aspiration syndrome; ECMO: Extra-corporeal membrane oxygenation.

Table 4 Comparison of causes of early-onset conjugated hyperbilirubinemia and "late-onset" conjugated hyperbilirubinemia between subgroups of clinically well and unwell infants *n* (%)

	ECHB (*n* = 65)		LCHB (*n* = 52)	
	Unwell (*n* = 52)	Well (*n* = 13)	Unwell (*n* = 22)	Well (*n* = 30)
Non-surgical causes				
Multifactorial liver injury	39 (75.0)	0 (0)	18 (81.8)	0 (0)
Sepsis	9 (17.3)	0 (0)	3 (13.6)	0 (0)
Inborn errors of metabolism	3 (5.8)	0 (0)	1 (4.5)	0 (0)
CMV infection	0 (0)	0 (0)	0 (0)	2 (6.7)
Idiopathic	0 (0)	5 (38.5)	0 (0)	21 (70.0)
Surgical causes				
Biliary atresia	0 (0)	8 (61.5)	0 (0)	5 (16.7)
Choledochal cyst	1 (1.9)	0 (0)	0 (0)	2 (6.7)

ECHB: Early-onset conjugated hyperbilirubinemia; LCHB: "Late-onset" conjugated hyperbilirubinemia; CMV: Cytomegalovirus.

surgery for BA and 8 (12.3%) died. Five deaths were due to multi-organ failure and three due to IEM. In the subgroup of patients with ECHB due to non-hepatic causes (*n* = 48), 42 (87.5%) achieved complete

resolution of CHB without progression to chronic liver disease. In comparison, in the LCHB group overall (n = 52), 41 (78.8%) had complete resolution, 7 (13.5%) underwent surgery for BA and choledochal cyst, 2 (3.8%) patients died, one due to IEM and the other died with multi-organ failure. Two patients from each group, ECHB and LCHB, were lost to follow-up. Death occurred in all 4 patients with IEM, three of them in the ECHB group (two mitochondrial disorders and one organic aciduria) and one in LCHB group with urea cycle defect. In both ECHB and LCHB groups, all patients with MLI who survived and all those with idiopathic CHB had complete resolution of liver dysfunction on follow-up.

The reasons for measuring serum CB in the well-looking ECHB cases were atypical "bronze" appearance of skin (38.5%), screening at physician's discretion (30.8%), antenatally detected hepatobiliary anomalies (15.4%) and non-specific symptoms such as vomiting, abdominal distension, respiratory distress and hypoglycemia (15.4%). In eight infants with biliary atresia who presented with ECHB, four had atypical "bronze" appearance, two had antenatally detected hepatobiliary anomalies, and two were screened on physicians' discretion. None of these BA infants had acholic stools at presentation. They also had an initial declining trend of TB, reaching below 50% of initial values in 5 of them, while their CB remained persistently elevated.

DISCUSSION

CHB is often detected when infants are investigated for prolonged neonatal jaundice beyond 14-21 d of life[4]. Although less routinely encountered, neonatal CHB presenting within 14 d of life can pose considerable diagnostic and management challenges. In one study, the most common etiology of CHB (mean age 10 d) admitted to neonatal intensive care unit (NICU) was culture-proven sepsis (35.5%) and 30 out of 42 (71%) had non-hepatic cause[1]. In our study, the proportion of neonates with non-hepatic cause for CHB was similar (61%). However, the incidence of sepsis was much lower (10.3%), this difference is because 36% of neonates in that study were preterm requiring NICU care who were more likely to be predisposed to sepsis. Reported etiology of CHB differed depending upon age distribution, geographical region, type of study center and diagnostic approach[19]. We excluded preterm infants and focused on CHB in term neonates, including those who did not require hospitalization. Most studies on infantile cholestasis focus on BA but we did not find any study looking specifically into the clinical course of neonates with CHB aged below 14 d.

Similar to several other studies, MLI was an important etiology in our series and accounted for almost fifty percent[2,19-22]. Neonates are predisposed to MLI and cholestasis due to the relative immaturity of the hepatobiliary system, exacerbated by a wide variety of neonatal events such as hypoxia, prolonged fasting, parenteral nutrition, drug toxicity and sepsis[2-3,22-25]. Liver injury in such cases is part of multi-organ involvement. The severity and persistence of liver dysfunction depend on underlying disorders, and the dysfunction is usually reversible after resolution of the primary problem[21-23]. Standard intensive care management of the sick infant and close monitoring of liver function are the mainstays of treatment in these cases. In our study, CHB resolved without any long term liver complications in all the surviving infants with MLI and sepsis, majority of them (91%) recovered within 3 mo.

A significantly higher proportion of newborns who presented before 14 d were clinically unwell compared to those presenting later (80% *vs* 42%), (Table 4). As per guidelines, healthy infants below 14 d with jaundice are rarely tested for CB, potentially missing CHB in healthy patients and over-estimating the proportion of unwell patients. We observed that about three-quarters of clinically unwell CHB patients presenting within 14 d had non-hepatic cause for CHB. Importantly, no clinically unwell patient had BA (Table 4). The presence of IEM was an important risk factor for mortality. IEM have been reported to account for about 20% of all cases of neonatal cholestasis[16,19]. It is therefore recommended to maintain a high level of suspicion for IEM in unwell infants with CHB[26].

Excluding clinically unwell infants, the most common cause of ECHB is BA (61.5%). Notably all infants with BA had pigmented stools at this early stage. Prognosis of BA is dependent on timely diagnosis and surgical intervention. Despite data from BA case series suggesting presence of jaundice before 14 d[27,28], a significant proportion of cases are referred after 6 to 8 wk of life[5], and the age at which the Kasai operation is performed has not decreased over the years[8-11].

In our study, all patients with BA in the ECHB group had a significant initial decline of TB, and in 5 out of 8, TB fell by over 50% from presentation levels, reaching clinically undetectable levels (below 70 μmol/L). It can be argued that BA cases may initially have unconjugated hyperbilirubinemia, and CHB develops later. In our study the subset of infants with ECHB who were diagnosed to have BA continued to have persistently raised CB, and this observation was also seen in other studies[15,28]. Measuring CB in all patients with neonatal jaundice regardless of age, and investigating those with CHB could potentially discover BA at an earlier stage. A recent study examined the potential utility of newborn direct bilirubin measurements performed prior to 60 h of life when infants are still in the hospital as a screen for BA. Authors predicted sensitivity of 100%, based on 35 subjects with BA and predicted specificity of 98.2% based on 9102 subjects without BA[15].

A few indications to measure CB in well looking neonates below 14 d are antenatally detected hepatobiliary anomalies, pale stools, dark urine and bronze baby syndrome[29]. In our study approximately

one-third developed bronze baby syndrome, 15% had antenatally detected hepatobiliary anomalies, while none had pale stools or dark urine. This highlights that even with good antenatal ultrasonogram and careful clinical evaluation, a significant proportion of ECHB can be missed.

Delayed detection of neonatal CHB and BA in particular is unlikely to be confined to lack of training and awareness of guidelines among healthcare providers, as despite having guidelines for over 2 decades, cases continue to be missed and treatment delayed[7]. This is likely to be due to subjectivity in assessment of jaundice. Firstly, it is difficult for parents and physicians to detect minimal jaundice. In addition, as shown in our study, the initial decline of TB may give a false reassurance and the well-looking infant may not be followed-up with blood tests[5]. Parents may also avoid clinic visits if the infant appears to be improving, this may be for economic reasons or to protect infants from the discomfort of venipuncture.

Hypothetically, if CB is checked with TB measurement during neonatal jaundice screening, or within 60 h of life in all infants[15], we believe liver disorders and BA can be detected earlier. However, there is no data on the cost-effectiveness of such an approach. It is worthwhile to study the increased economic and logistic burden that arises from over-investigating the self-resolving cases and weigh it against the benefits of earlier detection of CHB. We acknowledge that this approach may not be applicable in centers relying on transcutaneous bilirubin (TcB) or in areas where BA prevalence is low. Hussein *et al*[6] discussed screening for CHB and suggested checking urine for conjugated bilirubin, its usefulness as an adjunctive test could be explored in scenarios where blood testing is deemed unnecessary and/or in units relying on TcB.

The main limitation of this study is the single-center retrospective data that could result in selection bias, particularly over-representation of unwell infants with ECHB and under-representation of untested well-looking infants with BA. Another limitation is the non-availability of liver biopsy data in all the cases which could potentially influence the accuracy of diagnosis. This study serves as a primer for prospective studies to evaluate the role of routine measurement of CB in neonatal jaundice and its impact on the outcomes of CHB.

In conclusion, non-hepatic etiology is the most common reason for ECHB in term neonates aged below 14 d. In clinically unwell neonates who do not have IEM, CHB is expected to resolve with supportive management.

BA is an important cause of ECHB in well-looking, jaundiced term infants; it is also an unlikely diagnosis in clinically unwell neonates. Low level of CHB is present in all cases of BA who had CHB tested prior to 14 d of life; large population-based studies may be able to provide the answer whether routine measurement of conjugated bilirubin in all neonates with jaundice regardless of age, may potentially lead to earlier detection of biliary atresia and other neonatal liver disorders.

COMMENTS

Background

Conjugated hyperbilirubinemia (CHB) in a neonate may be indicative of serious hepatobiliary pathology, such as biliary atresia (BA) or inborn errors of metabolism (IEM). Based on current guidelines, conjugated bilirubin (CB) is screened when neonatal jaundice persists beyond 14-21 d. Hence, incidence and etiology of neonatal CHB before 14 d are not well-defined. Published data suggest that diagnosis of neonatal liver diseases including BA is frequently delayed, and earlier detection can lead to improved outcomes for these infants.

Research frontiers

Early-onset CHB (ECHB) presenting in the first 14 d of life in neonates remain poorly-defined. At the time of writing, there is no other study looking specifically into the clinical course of term neonates presenting with ECHB. The results of this study may contribute to understanding the etiologies of ECHB in term infants and earlier detection/diagnosis of neonatal liver disorders.

Innovations and breakthroughs

This study shows that non-hepatic etiology is the most common reason for ECHB in term neonates aged below 14 d, particularly in the subgroup who are clinically unwell. IEM are rare but associated with high mortality. On the other hand, BA is an important cause of ECHB in well-looking, jaundiced term infants who may not exhibit classical symptoms and signs at this early stage, making the diagnosis of BA difficult if current guidelines are followed. Low level of CHB was found to be present in all cases of BA who had CHB tested prior to 14 d of life.

Applications

In clinically unwell infants with ECHB, if rare IEM are excluded early, majority of cases with non-hepatic causes are expected to resolve with supportive management without progression to chronic liver disease. However, BA should be suspected in well infants presenting with ECHB, even in the absence of pale stools or deep jaundice. This study serves as a primer for larger population-based studies to evaluate the cost-effectiveness of earlier screening for conjugated bilirubin before 14 d in term infants, and its impact on the outcome of neonatal liver disorders including BA.

Terminology

CHB is defined as conjugated bilirubin CB fraction greater than 15% of TB, and CB $\geqslant$ 25 μmol/L. ECHB is defined as CHB detected within 14 d of life.

Peer-review

This retrospective single-center study may contribute to early detection of the cause of conjugated hyperbilirubinemia in term infants.

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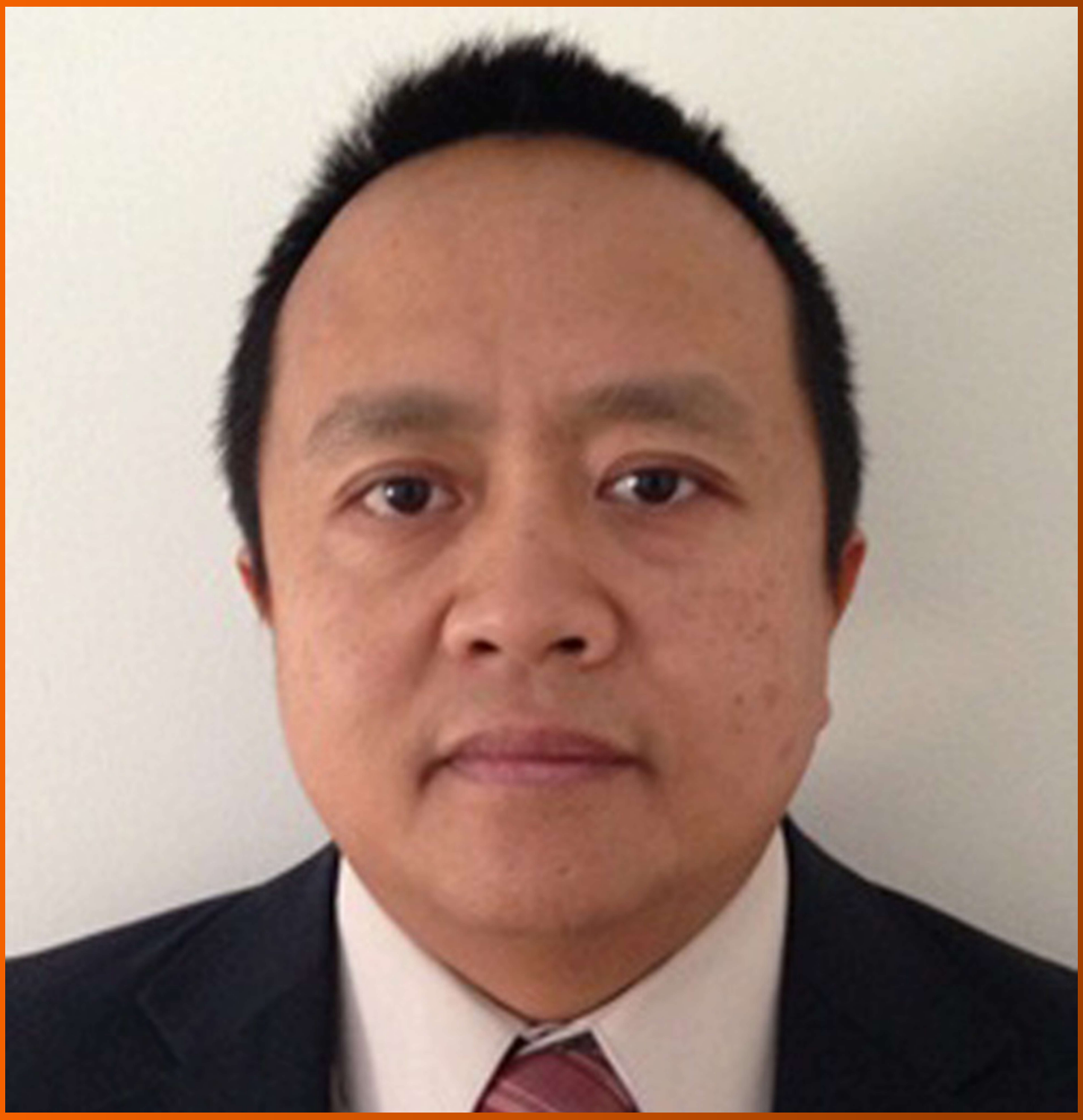

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ORIGINAL ARTICLE

Retrospective Study

T-cell allorecognition of donor glutathione S-transferase T1 in plasma cell-rich rejection

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Abstract

AIM

To investigate the role of glutathione S-transferase T1 donor-specific T lymphocytes in plasma cell-rich rejection of liver allografts.

METHODS

The study group included 22 liver transplant patients. Among them, 18 patients were mismatched for the glutathione S-transferase T1 (GSTT1) alleles (don+/rec-), and 4 were matched (don+/rec+). Seven of the mismatched patients produced anti-GSTT1 antibodies and developed plasma cell-rich rejection (former *de novo* immune hepatitis). For the detection of specific T

lymphocytes, peripheral blood mononuclear cells were collected and stored in liquid nitrogen. The memory T cell response was studied by adding to the cell cultures to a mix of 39 custom-made, 15-mer overlapping peptides, which covered the entire GSTT1 amino acid sequence. The specific cellular response to peptides was analyzed by flow cytometry using the markers CD8, CD4, IL-4 and IFNγ.

RESULTS

Activation of $CD8^+$ T cells with different peptides was observed exclusively in the group of patients with plasma-cell rich rejection (3 out of 7), with production of IL-4 and/or IFNγ at a rate of 1%-4.92% depending on the peptides. The $CD4^+$ response was most common and not exclusive for patients with the disease, where 5 out of 7 showed percentages of activated cells from 1.24% to 31.34%. Additionally, two patients without the disease but with the mismatch had cells that became stimulated with some peptides (1.45%-5.18%). Highly unexpected was the finding of a double positive $CD4^+CD8^{low}$ T cell population that showed the highest degree of activation with some of the peptides in 7 patients with the mismatch, in 4 patients with plasma cell-rich rejection and in 3 patients without the disease. Unfortunately, $CD4^+CD8^{low}$ cells represent 1% of the total number of lymphocytes, and stimulation could not be analyzed in 9 patients due to the low number of gated cells. Cells from the 4 patients included as controls did not show activation with any of the peptides.

CONCLUSION

Patients with GSTT1 mismatch can develop a specific T-cell response, but the potential role of this response in the pathogenesis of plasma cell-rich rejection is unknown.

Key words: Donor-specific glutathione S-transferase T1 antibodies; Indirect presentation; Glutathione S-transferase T1-memory T cells; *De novo* immune hepatitis; Donor/recipient mismatch

Core tip: In solid organ transplants, donor recipient mismatch of glutathione S-transferase T1 (GSTT1) alleles triggers a specific immune response with the production of IgG antibodies. In a proportion of mismatched liver and kidney transplants, the clinical outcome is rejection. However, detection of GSTT1-specific T lymphocytes has not been documented. We provide the first evidence of T cells able to become activated by GSTT1 peptides in patients who develop plasma cell-rich (PC-rich) rejection after GSTT1-mismatch liver transplantation. Interestingly, not only $CD8^+$ or $CD4^+$ cells but also double positive $CD4^+CD8^{low}$ cells reacted to the antigenic stimulation *in vitro*.

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INTRODUCTION

In the context of liver transplantation, both glutathione S-transferase T1 (GSTT1) mismatch and the presence of GSTT1 antibodies have been associated with the development of *de novo* immune hepatitis[1-3], recently accepted as a rejection of the liver allograft in which allogeneic hepatocytes that express GSTT1 constitutively in their cytoplasm are the main targets of the immune response. The Banff Working Group on Liver Allograft Pathology has recently updated the terminologies of post-transplant complications and encourages the use of "plasma cell-rich rejection" instead of the former "*de novo* autoimmune hepatitis"[4]. Therefore, in this manuscript, we will use the new terminology.

Plasma cell-rich (PC-rich) rejection is a liver disorder of unclear pathogenesis that is usually diagnosed within the first two years after liver transplantation. A common feature of all the patients diagnosed in our hospital is the presence of GSTT1 antibodies due to the recognition of GSTT1 as a foreign antigen expressed in the graft when the recipient lacks this gene. Although it is a very specific anti-donor response, it is unclear whether these antibodies have a pathogenic effect since some patients with sustained antibody-titers will never develop PC-rich rejection.

Pregnancy, transfusion and transplantation are circumstances where the host immune system is able to recognize foreign major and minor histocompatibility antigens. This is the case for GSTT1, a drug metabolizing enzyme that is abundantly expressed in the liver and kidney. Recipients who lack this gene (*GSTT1*0/0*) might generate antibodies against GSTT1 after blood transfusion and/or organ transplantation from GSTT1-positive donors (*GSTT1*A/0 or *A/A*)[5,6]. It has been reported that the GSTT1 protein is able to induce a memory B cell response in *GSTT1*0/0* women after pregnancy with GSTT1-positive offspring[6]. Moreover, it has been demonstrated that GSTT1-specific plasma cells are quickly activated when a GSTT1-positive patient receives an infusion of hematopoietic cells from an HLA-identical sensitized donor[7].

The liver is a very special organ with a variety of important cell types able to function as APCs. Hepatocytes, which represent 60% of the liver cells, express MHC class Ⅰ at low levels and have the ability to serve as antigen presenting cells (APCs). Furthermore, under some pathological circumstances in a pro-inflammatory environment, parenchymal cells and biliary epithelial cells can express MHC class Ⅱ antigens[8]. Some studies in mouse models

have indicated that both $CD4^+$ and $CD8^+$ T cells can independently initiate hepatocyte rejection, more rapidly in the case of $CD8^+$ cells, somehow preceding the $CD4^+$ mediated response[9]. In humans, patients with chronic allograft failure of kidney grafts have significantly higher frequencies of $CD4^+$ T cells indirectly activated by allogeneic peptides when compared with controls, whereas $CD4^+$ T cells activated in a direct manner reduced the cytotoxic T cell response[10]. However, there are variables such as immunosuppression therapy that can alter the immunological response in different ways.

In this study, we aim to explore the role of T cells in the context of PC-rich rejection. We have compared the T cell response in PBMCs collected from 18 GSTT1-mismatched liver transplant patients, 7 of which had a diagnosis of PC-rich rejection, with 4 GSTT1-matched transplanted patients after re-stimulation *in vitro* with the whole set of GSTT1 peptides. In summary, we have the first evidence of GSTT1-specific memory T cells ready to become activated after recall with the antigen, but further studies will be needed to test the potential role of these cells in the pathogenesis of PC-rich rejection.

MATERIALS AND METHODS

Patients

The study group included 22 liver transplant patients, 10 females and 12 males, who had transplants between June 1996 and April 2011. Eighteen of the patients lacked the GSTT1 gene and received a liver from a GSTT1 positive donor (rec-/don+). Consequently, all of them were candidates to develop a specific immune response against this foreign antigen. Four additional patients without the GSTT1 mismatch (rec+/don+) were included as a control group. Within the mismatched patients, we observed three different types of immune and clinical responses regarding the GSTT1 antigen. Group 1 consisted of 7 patients who produced anti-GSTT1 antibodies and developed PC-rich rejection. Group 2 included 2 patients who produced anti-GSTT1 antibodies but did not develop PC-rich rejection. Group 3 included 9 patients who did not produce anti-GSTT1 antibodies (which always precede clinical manifestations) and consequently did not develop the disease. Written informed consent was obtained from all of the participants, and the procedures were in accordance with the Helsinki Declaration. The study protocol was approved by the Ethics Committee of the University Hospital Virgen del Rocío, Seville, Spain. Patient characteristics are described in Table 1. Baseline immunosuppression was cyclosporine in 13 cases and tacrolimus in 9 cases, either alone or combined with mycophenolate mofetil and steroids during the first months. Cells were obtained at a mean time of 6.68 years after the transplant (1-16). Changes in the immunosuppression therapy at the time of cell extraction are described in Table 1. Six of the patients with PC-rich rejection were also receiving prednisone as a specific treatment, and one patient was not adequately diagnosed and died in 2014.

GSTT1 genotyping

Peripheral blood samples from the patients and their donors were collected, and genomic DNA was purified using the QIAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Primers and conditions for the GSTT1 PCR reaction have been described in detail elsewhere[11].

Detection of GSTT1 antibodies

Following the manufacturer's protocol, total IgG antibodies in sera were analyzed using a commercially available ELISA, which contains the human GSTT1 recombinant protein (Biomedal, Seville, Spain).

GSTT1 peptides

We selected 15-mer peptides that overlapped by 9 amino acids and spanned the GSTT1 protein. In total, there were 39 peptides (Table 2). Peptides were synthesized by Innovative Peptide Solutions, JPT (Berlin, Germany). Peptide purity was higher than 80%, as assayed by HPLC, and the peptide composition was verified by mass spectrometry. Lyophilized peptides were dissolved at 10 mg/mL in DMSO, aliquoted, and stored at -20 ℃.

Cell isolation and culture with the GSTT1 peptides

Post-transplant PBMCs were isolated using BD Vacutainer CPT ficoll tubes (BD Biosciences, CA, United States), frozen in FCS containing 10% DMSO, and stored in liquid nitrogen. For stimulation experiments, 3-4 × 10^5 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Biochrom AG, Berlin, Germany), Penicillin/Streptomicin (100 U penicillin/mL, 100 μg streptomycin/mL), 1 mmol/L Na-Piruvate (Sigma Aldrich, MI, EEUU) and L-Glutamine (2 mmol/L, Irvine Scientific, Wicklow, Ireland) in the presence of 8 pools, each one containing 5 peptides and the last one containing only 4 (10 μg/mL each peptide). Next, 10 μg/mL anti-CD28/CD49d (BD Biosciences, CA, United States) was added for 48 h at 37 ℃ 5% CO_2, and 10 μg/mL Brefeldin A was added to the samples during the last four hours (Golgi Plug: BD Biosciences). A negative control (without peptide but with the proportional amount of DMSO) and a positive activation control with 10 ng/mL PMA + 1 μg/mL ionomycin (Sigma Aldrich) were included in each assay. Pre-transplant samples were not available.

In silico analysis of MHC-peptide binding affinity

HLA class Ⅰ and Ⅱ binding affinity to GSTT1 peptides was analyzed by the Immune Epitope Database (IEDB) and Analysis Resources NetMHCI/IIpan.

Flow cytometry

Immunofluorescence staining was performed after

Table 1 Patient characteristics

Group	Patient	Gender	LT date	Original disease	Baseline IS	PBMC extraction date	Years after Tx	Treatment at PBMC extraction
1	1	M	06-05-99	Alcoholic cirrhosis	CYA (N), MMF, ST	12-04-12	13	CYA (N), MMF, ST
	2	F	07-05-07	Cirrhosis probably autoimmune	CYA, MMF, ST	16-04-12	5	CYA (N), MMF, ST
	3	F	02-07-00	HCV cirrhosis	CYA (N), ST, BASILISIMAB	13-03-12	12	TAC, AZA, ST
	4	F	18-09-03	Alcoholic cirrhosis	CYA, MMF, ST	09-05-12	9	TAC, MMF, ST
	5	M	02-11-01	HCV + alcoholic cirrhosis	CYA (N), ST, BASILISIMAB	14-06-12	11	MMF, ST
	6	F	27-03-09	Primary biliary cirrhosis	CYA, MMF	12-04-12	3	MMF, ST
	7	F	18-11-06	Secondary biliary cirrhosis	CYA (N), MMF, ST	08-05-12	6	CYA, MMF
2	8	M	23-11-96	HBV cirrhosis	CYA (N), MMF, ST	19-06-12	16	CYA (N), MMF
	9	F	03-06-96	Agenesis of the bile ducts	CYA, ST	21-05-12	16	TAC
3	10	M	12-7-06	Alcoholic cirrhosis + hepatocarcinoma	TAC, MMF, ST	16-04-12	6	MMF, SIR
	11	M	12-02-09	HBV cirrhosis	TAC, MMF, ST	16-04-12	3	MMF, SIR
	12	M	06-07-10	Non-alcoholic steatohepatitis	TAC (10 d) CYA, RAPA	17-04-12	2	MMF, SIR
	13	M	19-04-11	HCV cirrhosis+ hepatocarcinoma	CYA, ST	18-04-12	1	CYA, ST
	14	M	18-01-09	HCV cirrhosis	TAC, MMF, ST	02-05-12	3	TAC
	15	F	18-06-04	Alcoholic cirrhosis	TAC	07-05-12	8	MMF, EVE
	16	M	30-07-08	HCV cirrhosis	TAC, MMF, ST	08-05-12	4	MMF, EVE
	17	M	20-12-04	HCV cirrhosis	TAC, MMF, ST	22-05-12	7	TAC, MMF
	18	F	20-09-99	HCV cirrhosis	CYA, ST	18-06-12	13	CYA
4	E	F	09-03-09	Alcoholic cirrhosis	CYA, ST	21-03-12	3	CYA, MMF
	G	M	16-11-08	HCV cirrhosis	TAC, DACLIZUMAB	30-04-12	3	CYA
	J	F	22-05-10	Hepatocarcinoma	CYA, ST	02-05-12	2	CYA
	L	M	28-07-11	Primary biliary cirrhosis	TAC, MMF, ST	19-06-12	1	TAC

HBV: Hepatitis C virus; HCV: Hepatitis C virus.

fixation and permeabilization using lysing solution (BD Biosciences, CA, United States) with the following surface and intracellular markers: Anti-human CD4-PerCP/CD8-APC/IFNγ-FITC/IL-4-PE (Becton Dickinson BD Biosciences, CA, United States). Lymphocyte cytokine release patterns were analyzed by flow cytometry (FACSort; BD Biosciences) using CELLQuest software. The specific cellular response to the different pools was calculated by subtracting the percentage of activation of T cells cultured without GSTT1 peptides (negative control). Typically, 50000 events were collected using FL3 (CD4PerCP-Cy5) or FL4 (CD8-APC) as a fluorescent trigger. A second set of gating was drawn to include $CD8^-$ or $CD8^{low}$ and IFNγ and IL-4 expression.

RESULTS

Identification of memory T cell subsets specific for the GSTT1 antigen

We have categorized as positive the populations with more than 1% of activated cells. All of the patients who showed stimulation revealed a polyclonal T cell response since we observed stimulation with more than one peptide. For simplicity reasons, we have represented the highest percentage of cell activation among the positive values obtained with each peptide (Figure 1 and Table 3). The group of patients with PC-rich rejection (group 1) was the only group in which activation of $CD8^+$ T cells was detected in 3 patients, expressing IL-4, IFNγ or both cytokines simultaneously. This group also shows the most abundant and diverse patterns of $CD4^+$ T cell activation exhibiting Th0 (IL-4/IFNγ), Th1 (IFNγ) and Th2 (IL-4) pathways, although cellular activation is not exclusive of group 1 and was also observed in two patients included in group 3 (Table 3). The most striking result was the presence of $CD4^+CD8^{low}$ double positive (DP) cells that seem to be enriched in GSTT1-specific cells, especially cells with a secretion profile of both cytokines tested (3.44% patient 2, 78.95% patient 3, 9.54% patient 4, 4.56% patient 5). Unfortunately, DP cells are not abundant, and only 4 of the 7 patients with PC-rich rejection could be analyzed due to the low number of double positive cells gated in the remaining 3 cases.

The patients with antibodies but without PC-rich rejection (group 2) did not show $CD4^+$ or $CD8^+$ T cell activation, whereas five of the 9 patients included in group 3, without antibodies and therefore without disease, exhibited stimulation with some peptides. Again, the higher percentages of activation occurred in the double positive $CD4^+CD8^{low}$ cells (6.63% patient 10, 29.58% patient 17 and 43.05% patient 18), although in some cases the number of double positive $CD4^+CD8^{low}$ cells was too low to perform further analysis (Table 3). The four patients included as the control group with recipients and donors that were matched for the GSTT1 positive allele did not become activated with any of the peptides assayed.

In summary, 12 out of 18 liver transplant recipients with the GSTT1 mismatch showed different degrees of T lymphocyte activation upon exposure to the GSTT1 peptides. Although we could not test for memory markers, the short time of stimulation (48 h) indicates that this is not a primary response but a reactivation of pre-existing GSTT1-specific lymphocytes. There are 3 cell types involved, including $CD4^+$, $CD8^+$ and

Table 2 Glutathione S-transferase T1 peptides and amino acid position and sequence

Pool	Amino acid #	Amino acid sequence
1	1-15	MGLELYLDLLSQPCR
	7-21	LDLLSQPCRAVYIFA
	13-27	PCRAVYIFAKKNDIP
	19-33	IFAKKNDIPFELRIV
	25-39	DIPFELRIVDLIKGQ
2	31-45	RIVDLIKGQHLSDAF
	37-51	KGQHLSDAFAQVNPL
	43-57	DAFAQVNPLKKVPAL
	49-63	NPLKKVPALKDGDFT
	55-69	PALKDGDFTLTESVA
3	61-75	DFTLTESVAILLYLT
	67-81	SVAILLYLTRKYKVP
	73-87	YLTRKYKVPDYWYPQ
	79-93	KVPDYWYPQDLQARA
	85-99	YPQDLQARARVDEYL
4	91-105	ARARVDEYLAWQHTT
	97-111	EYLAWQHTTLRRSCL
	103-117	HTTLRRSCLRALWHK
	109-123	SCLRALWHKVMFPVF
	115-129	WHKVMFPVFLGEPVS
5	119-133	MFPVFLGEPVSPQTL
	125-139	GEPVSPQTLAATLAE
	131-145	QTLAATLAELDVTLQ
	137-151	LAELDVTLQLLEDKF
	143-157	TLQLLEDKFLQNKAF
6	149-163	DKFLQNKAFLTGPHI
	155-169	KAFLTGPHISLADLV
	161-175	PHISLADLVAITELM
	167-181	DLVAITELMHPVGAG
	173-187	ELMHPVGAGCQVFEG
7	179-193	GAGCQVFEGRPKLAT
	185-199	FEGRPKLATWRQRVE
	191-205	LATWRQRVEAAVGED
	197-211	RVEAAVGEDLFQEAH
	203-217	GEDLFQEAHEVILKA
8	209-223	EAHEVILKAKDFPPA
	215-229	LKAKDFPPADPTIKQ
	221-235	PPADPTIKQKLMPWV
	226-240	TIKQKLMPWVLAMIR

$CD4^+CD8^{low}$ cells, all of them with diverse cytokine expression patterns whose role is not easy to interpret, although DP cells are known to appear in situations of long-term exposure to antigens.

Antigenic areas of the GSTT1 protein

When we analyzed the relative contribution of each pool to the activation of T lymphocytes in each patient, we found that pools 3 and 4 seemed especially antigenic for the DP cells, whereas pool 4 did not stimulate any of the single $CD4^+$ or $CD8^+$ T cells of any patient in which other pools seem to have a more relevant role (Figure 2). A representative plot of flow cytometry data with cells gated on $CD4^+$ first and then CD8, selecting those cells with a low expression of CD8, is shown in Figure 3. We have selected 3 patients with different degrees of activation after stimulation with the pools of peptides; the negative control (without peptide) is also shown and was subtracted to obtain the final values (Figure 3).

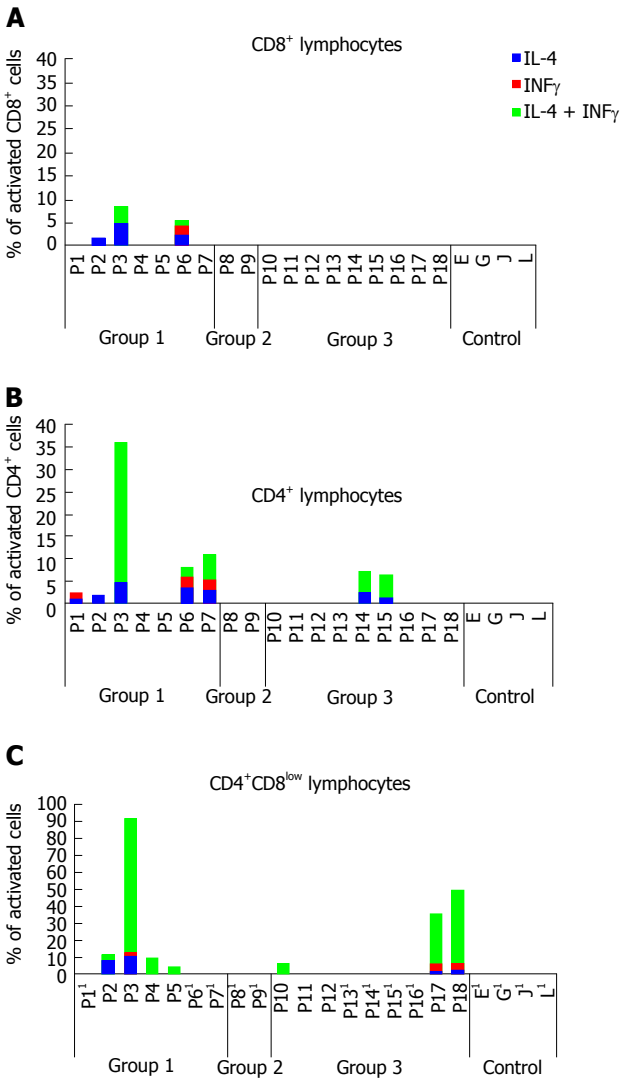

Figure 1 Polyclonal response of T cells upon recall with peptides of the glutathione S-transferase T1 protein. Percentages of activation upon exposure to the different pools are represented. Three cell types showed activation at different levels. A: Only $CD8^+$ lymphocytes of patients included in group 1 showed activation. The numbers in the figure represent the highest value obtained for each pathway when activation was induced by different pools of peptides; B: Activation of $CD4^+$ lymphocytes was observed in patients of groups 1 and 3; C: $CD4^+CD8^{low}$ cells showed the highest percentage of activation. [1]Indicate that the analysis could not be performed in these patients.

Indirect presentation pathway

These results have to be interpreted in the context of an indirect allo-recognition pathway since the experiments were performed only in the presence of recipient cells. The recipients' HLA genotypes are described in Table 4, highlighting in bold HLA class Ⅰ and Ⅱ alleles with the best percentile ranks for presentation of GSTT1 peptides, as concluded from the *in silico* analysis. We found a good correlation with part of the experimental results of T cell activation measured in terms of IL-4 and/or IFNγ production by $CD8^+$, $CD4^+$ and $CD4^+CD8^{low}$ T cells upon exposure to GSTT1 peptides. However, the fact that HLA genotyping was performed by low resolution methods constitutes a limitation of the analysis. When we placed the *in silico*-proposed

Table 3 Specific immune response after stimulation of T lymphocytes in culture with glutathione S-transferase T1 peptides[1]

Group	Pat #	$CD8^+$			$CD4^+$			$CD4^+CD8^{low}$		
		IL-4	IFNγ	IL-4/IFNγ	IL-4	IFNγ	IL-4/IFNγ	IL-4	IFNγ	IL-4/IFNγ
1	1	-	-	-	1.24%	1.41%	-	Δ	Δ	Δ
	2	1.7%	-	-	2.04%	-	-	8.23%	-	3.44%
	3	4.92%	-	3.54%	4.93%	-	31.34%	10.77%	2.25%	78.95%
	4	-	-	-	-	-	-	-	-	9.54%
	5	-	-	-	-	-	-	-	-	4.56%
	6	2.36%	2.03%	1.15%	3.71%	2.45%	2.14%	Δ	Δ	Δ
	7	-	-	-	3.19%	2.35%	5.63%	Δ	Δ	Δ
2	8	-	-	-	-	-	-	Δ	Δ	Δ
	9	-	-	-	-	-	-	Δ	Δ	Δ
3	10	-	-	-	-	-	-	-	-	6.63%
	11	-	-	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	-	-
	13	-	-	-	-	-	-	Δ	Δ	Δ
	14	-	-	-	2.65%	-	4.71%	Δ	Δ	Δ
	15	-	-	-	1.45%	-	5.18%	Δ	Δ	Δ
	16	-	-	-	-	-	-	Δ	Δ	Δ
	17	-	-	-	-	-	-	1.68%	4.6%	29.58%
	18	-	-	-	-	-	-	2.51%	4.13%	43.05%
Control	E	-	-	-	-	-	-	Δ	Δ	Δ
	G	-	-	-	-	-	-	Δ	Δ	Δ
	J	-	-	-	-	-	-	Δ	Δ	Δ
	L	-	-	-	-	-	-	Δ	Δ	Δ

[1]Pathways of activation are defined by the production of IL-4, IFNγ or both cytokines simultaneously. "-": No activation; "Δ": Analysis was not possible due to the low number of gated cells. Group 1: don+/rec-, with Abs, with PC-rich rejection; Group 2: don+/rec-, with Abs, without PC-rich rejection; Group 3: don+/rec-, without Abs, without PC-rich rejection; Control group: don+/rec+.

Table 4 Class Ⅰ and class Ⅱ human leukocyte antigen genotypes of the patients

Pat #	HLA-A*		HLA-B*		DRB1*	
1	01	03	07	57	11	15
2	01	66	08	41	03	13
3	11	29	07	35	07	13
4	02	11	51	60	04	13
5	30	-	13	18	03	07
6	02	11	35	44	07	08
7	26	29	38	44	01	03
8	23	24	14	52	01	11
9	11	68	35	44	01	14
10	02	33	14	35	01	07
11	01	29	57	61	01	04
12	01	33	44	64	01	07
13	01	-	08	18	04	07
14	29	-	44	-	07	-
15	01	30	08	51	03	07
16	02	29	39	44	07	11
17	03	32	37	44	03	12
18	03	11	14	49	07	-

peptides along the GSTT1 amino acid sequence, we were able to define very clearly a highly antigenic zone of the protein that basically shared amino acids from positions 60 to 80 (Table 5). Interestingly, the selected peptides are long, not only for HLA class Ⅱ, as expected, but also for HLA class Ⅰ alleles.

DISCUSSION

In this study, we have demonstrated the existence of memory T cells specific for the GSTT1 antigen in patients with PC-rich rejection after GSTT1-mismatched liver transplants. The results support our initial hypothesis in which both specific B and T cells are required to function simultaneously in the development of the immune response leading to PC-rich rejection. In fact, only patients diagnosed with the disease showed a combined T and B cell response, whereas those patients with specific T cells but lacking the humoral response never experienced this type of rejection.

The fact that GSTT1 is a drug metabolizing enzyme found in the cytoplasm of hepatocytes and cholangiocytes makes it difficult to explain a pathogenic role of anti-GSTT1 antibodies. Although it cannot be assumed that cytosolic antigens are never expressed on the cell surface[12], the presence of antibodies in all of the patients with a diagnosis of PC-rich rejection is evidence of specific B cells capable of presentation of GSTT1 to specific T cells. B cells are known to be critical for alloreactive T cells to differentiate into memory T cells[13,14]. In fact, a very interesting report by Zeng *et al*[15] demonstrated in an animal model of chronic allograft vasculopathy (CAV) that mice deficient in both B cells and antibodies were protected from CAV, while mice that were deficient for antibodies but not for B cells developed CAV. The conclusion was that B cells contributed to CAV by enhancing T-cell responses[15]. Very recently, Shiu *et al*[16] demonstrated that B cells are involved in supporting T-cell responses in patients with antibody-mediated rejection in a B-cell-dependent indirect T-cell alloreactivity.

$CD4^+$ cells seem to have a predominant role in the

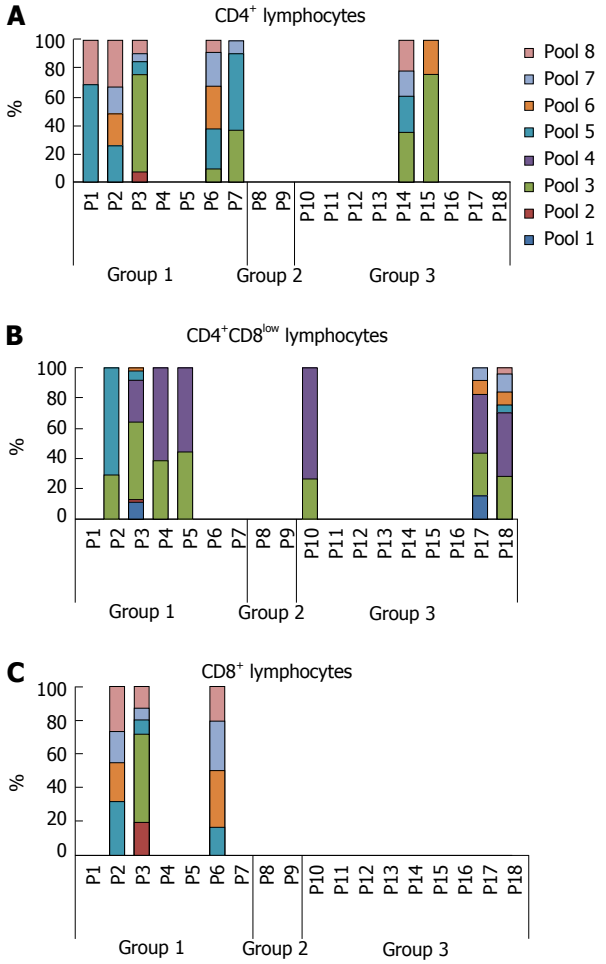

Figure 2 Different areas of the glutathione S-transferase T1 protein induce different degrees of activation in patients' cells. A: The majority of $CD4^+$ T cells that showed some degree of activation recognized peptides in pools 5 (blue) and 3 (green) but never in pool 4 (purple); B: $CD4^+CD8^{low}$ cells became stimulated almost homogeneously with in pools 3 (green) and 4 (purple); C: $CD8^+$ lymphocytes that showed activation recognized peptides in different pools but never in pool 4 (purple).

Table 5 Identification of several regions of the glutathione S-transferase T1 protein, whose peptides would be suitable to be presented by human leukocyte antigen class Ⅰ and Ⅱ alleles of the patients based on in silico predictions

Recipient's HLA	Peptide sequence	Aa position	Length	Percentile rank
Class Ⅰ				
A*01:01	FTLTESVAILLY[1]	62-73	12	0.1
A*02:01	YIFAKKNDIPFEL	18-30	13	0.1
	CLRALWHKVMFPV	110-122	13	0.1
	IKQKLMPWVLAMI	227-239	13	0.1
A*03:01	SVAILLYLTRKYK[1]	67-79	13	0.2
A*11:01	ESVAILLYLTRK[1]	66-77	12	0.1
A*29:02	FLTESVAILLY[1]	62-73	12	0.2
B*07:02	SPQTLAATLAEL	129-140	12	0.1
	RPKLATWRQRVEAA	188-201	14	0.2
B*08:01	FAQVNPLKKVPAL	45-57	13	0.2
	LAWQHTTLRRSCL	99-111	13	0.2
	DPTIKQKLMPWVL	224-236	13	0.2
B*35:01	YPQDLQARARVDEY	85-98	14	0.1
B*44:02	TESVAILLY[1]	65-73	9	0.15
	AELDVTLQL	138-146	9	0.15
Class Ⅱ				
DRB1*01:03	GDFTLTESVAILLYL[1]	60-74	15	0.6
DRB1*07:01	ALKDGDFTLTESVAI[1]	56-70	15	0.4
DRB1*11:01	AILLYLTRKYKVPDY[1]	69-83	15	0.5
DRB1*12:01	SVAILLYLTRKYKVP[1]	67-81	15	0.72
DRB1*13:01	LTESVAILLYLTRKY[1]	64-78	15	0.17
DRB1*14:01	SVAILLYLTRKYKVP[1]	67-81	15	0.48
DRB1*15:01	ESVAILLYLTRKYKV[1]	66-80	15	0.41

[1]Highly antigenic areas of the GSTT1 protein whose peptides are shared by HLA class Ⅰ and Ⅱ alleles. GSTT1: Glutathione S-transferase T1; HLA: Human leukocyte antigen.

context of GSTT1 mismatch in the patients described in this study. Mouse models have provided evidence of the role of $CD4^+$ T cells acting as effectors that directly mediate injury in renal allografts, while $CD8^+$ T cells had very little influence in promoting graft dysfunction[17]. Similarly, $CD4^+$ cells were sufficient to mediate rapid rejection of a cardiac allograft through the indirect pathway of alloantigen recognition[18]. Hence, $CD4^+$ specific T cells are key elements for the progression of allograft immunity, especially within the $CD4^+$ T cell indirect response. In the liver of mice with clinical manifestations of hepatitis, MHC class Ⅱ-expressing hepatocytes are able to act as APCs and activate specific $CD4^+$ T lymphocytes[19].

The pathogenic role of GSTT1-specific $CD8^+$ T cells in PC-rich rejection has not been explored. The results obtained in this study reveal the existence of reactive $CD8^+$ cells in the group of patients with PC-rich rejection, with percentages of activation that range from 1.1% to 8.46%, which is not as low as expected in immunosuppressed patients. A substantial difference between the percentages of IFNγ-producing $CD8^+$ T cells at diagnosis and during treatment with prednisolone has been demonstrated in patients with type 2 autoimmune hepatitis[20]. We should keep in mind that the patients with PC-rich rejection described in this study are under successful treatment with prednisone that has to be maintained throughout life. It would be very interesting to know the level of stimulation of cells obtained at diagnosis, before initiation of the treatment, since cells from immunosuppressed patients exhibit much lower levels of activation than immunocompetent cells. For that reason, it is even more remarkable that certain types of T lymphocytes from the patients with PC-rich rejection showed high percentages of activation.

The results of this study leave many questions about the function of GSTT1-specific $CD4^+CD8^{low}$ T cells in the context of transplantation. Subgroups of $CD4^{hi}CD8^{low}$ T cells have been described in chronic viral infections, with antigen specificity and memory phenotype[21,22], or in parasitic infections where the frequency of $CD4^+CD8^{low}$ T cells was higher in Chagasic patients than in healthy donors[23]. In a study performed with human cells from CMV-seropositive patients, the $CD4^+CD8^{low}$ population contained a two- to eight-fold higher frequency of antigen-specific $IFN\gamma^+$ cells than

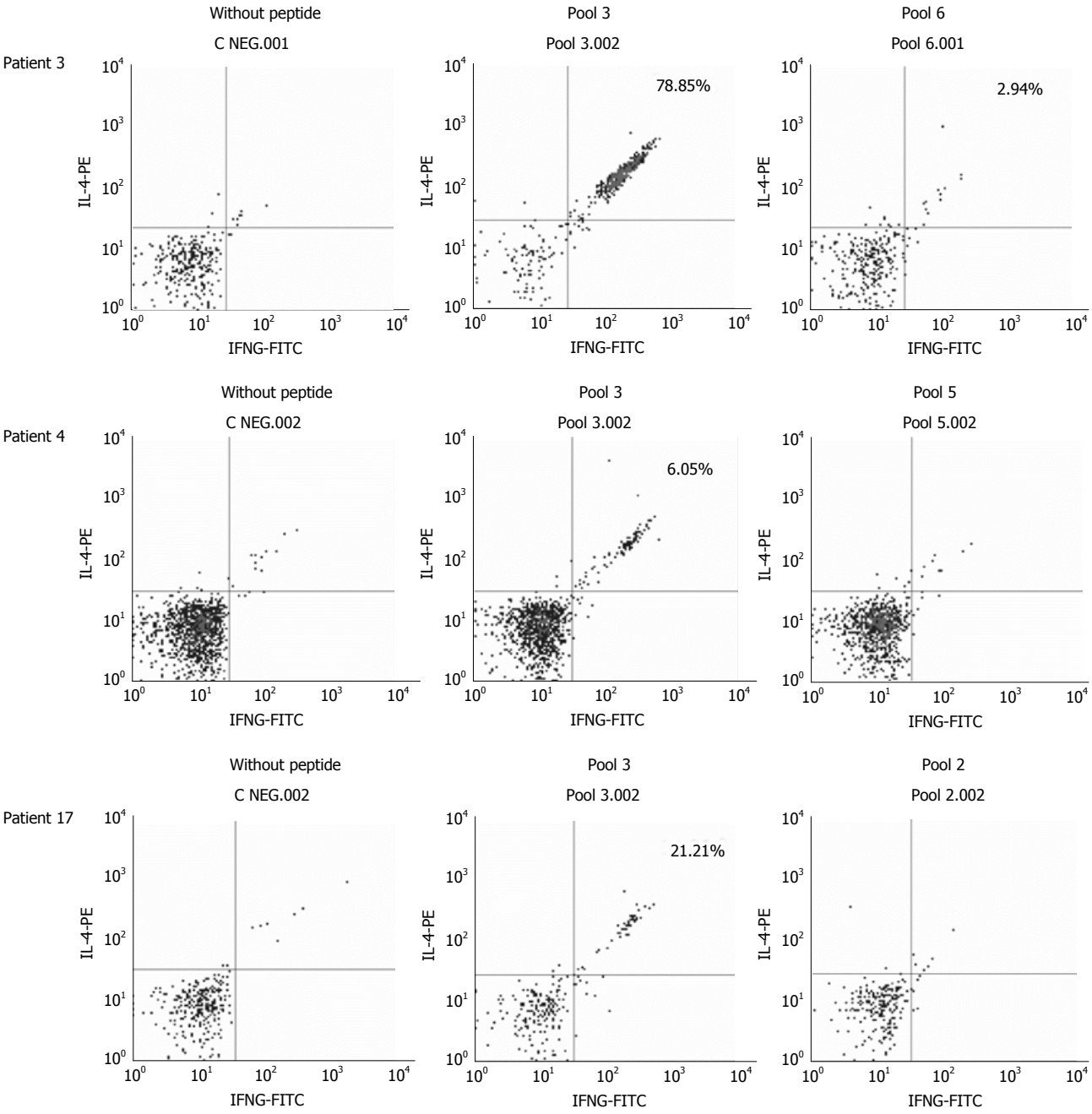

Figure 3 Representative plots showing $CD4^{+}CD8^{low}$ T lymphocyte activation in terms of cytokine production. Production of IL-4 and/or IFNγ was analyzed after exposure of the cells to the glutathione S-transferase T1 peptides. As an example, patient 3 had 78.85% of the double positive cells activated with pool 3 and 2.9% with pool 6. Patient 4 had 6.05% of cells activated with pool 3, whereas pool 5 did not have any effect. Patient 17 showed 21.21% of cell activation with pool 3, but no response was observed when the cells were cultured with pool 2. In all the cases, the cells produced both cytokines, indicating a Th0 type of response.

the $CD4^{+}CD8^{-}$ population[24]. It seems that this type of cell appears in chronic processes, mainly in viral infections, but this scenario could also be extended to the transplant setting where sustained expression of a foreign antigen, such as GSTT1, might lead to chronic rejection.

The terminologies used to describe post-transplant clinical situations with overlapping manifestations might be confusing. Late rejection, *de novo* autoimmune/alloimmune hepatitis or idiopathic post-transplant hepatitis may all be part of immune-mediated injury[25]. The underlying pathology of the formerly called *de novo* autoimmune hepatitis was poorly understood, and diagnoses were based mainly on histological findings such as the presence of plasma cell rich infiltrates or hepatocyte rosette formation; however, because rosettes are poorly reproducible, some groups do not consider them a diagnostic feature[26].

Although we did not have enough samples to check for memory markers, based on the short time of stimulation *in vitro* (48 h), we can say that GSTT1-specific lymphocytes are memory cells. It is still too soon to propose a model, as we have not yet tested what would be the response when recipients' cells are

confronted with GSTT1 peptides presented *via* the direct pathway. Apparently, there is not a predominant HLA class Ⅰ or Ⅱ allele among the donors of the patients with PC-rich rejection that could explain why some patients develop rejection and others do not. Given that donor cells are not available, in future studies we will have to design strategies to demonstrate the existence of donor HLA-restricted GSTT1-specific T lymphocytes through the use of artificial molecules such as pentamers, as well as cytotoxicity assays on "donor-like" target cells.

COMMENTS

Background

Antibody-mediated rejection of the liver allografts has never been considered a main problem after liver transplantation until now. The Banff Working Group on Liver Allograft pathologies published last year a new report in which the role of HLA as well as glutathione S-transferase T1 (GSTT1) donor specific antibodies is discussed. In this report, they have included new criteria and have suggested changes in the terminology of post-transplant complications. The process termed *de novo* autoimmune hepatitis is now defined as plasma cell-rich rejection.

Research frontiers

The authors' group has studied *de novo* immune hepatitis for years. The authors identified the target antigen as a donor protein expressed in the graft but absent from the donor. A genetic mismatch between a GSTT1+ donor and a GSTT1- recipient constitutes a risk factor to produce GSTT1 antibodies and to develop PC-rich rejection (former *de novo* immune hepatitis) but the pathogenic mechanisms leading to this type of rejection are still unknown.

Innovations and breakthroughs

The existence of T lymphocytes specific for GSTT1 in patients with PC-rich rejection has never been explored. The immune response requires collaboration between GSTT1-specific B and T lymphocytes. The hypothesis contemplated that the patients might have memory T cells able to become activated after recall with the antigenic stimulus. This is the first study in which GSTT1-specific T cells have been found in patients with PC-rich rejection in conjunction with anti-GSTT1 antibodies.

Applications

Although ultimate diagnosis of PC-rich rejection relies on histological examination, the fact that some histological features are common to different post-transplant outcomes makes a reliable diagnosis a complicated task. Understanding the mechanisms leading to PC-rich rejection would contribute to a correct diagnosis and appropriate therapy.

Terminology

Glutathione S-transferase T1 is a drug metabolizing enzyme highly expressed in liver and kidney.

Peer-review

This manuscript investigated the role of GSTT1 donor-specific T lymphocytes in plasma cell-rich rejection of liver allografts in patients, and found that T cells were able to become activated by GSTT1 peptides in patients who develop plasma cell-rich rejection after GSTT1-mismatch liver transplantation. The research design and detecting methods are reasonable, data analysis is correct, writing is fluent, written informed consent was obtained from all of the participants, and the study protocol was approved.

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ORIGINAL ARTICLE

Prospective Study

Evaluation of Doppler-ultrasonography in the diagnosis of transjugular intrahepatic portosystemic shunt dysfunction: A prospective study

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Author contributions: Nicolas C drafted the manuscript, and assisted with data analysis; Le Gouge A participated in design of the study and performed statistical analysis; d'Alteroche L participated in design and oversight of the study; Ayoub J, Georgescu M, Vidal V, Castaing D, Cercueil JP, Chevallier P, Roumy J, Trillaud H, Boyer L, Le Pennec V and Perret C were involved with data collection; Giraudeau B assisted with data analysis; Perarnau JM drafted the manuscript, participated in design and oversight of the study; the STIC TIPS group was involved with data collection.

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Clinical trial registration statement: This study is registered with ClinicalTrials.com. The registration identification number is 00593528.

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Abstract

AIM

To prospectively evaluate the performance of Doppler-ultrasonography (US) for the detection of transjugular intrahepatic portosystemic shunt (TIPS) dysfunction within a multicenter cohort of cirrhotic patients.

METHODS

This study was conducted in 10 french teaching hospitals. After TIPS insertion, angiography and liver Doppler-US were carried out every six months to detect dysfunction (defined by a portosystemic gradient $\geqslant$ 12 mmHg and/or a stent stenosis $\geqslant$ 50%). The association between ultrasonographic signs and dysfunction was studied by logistic random-effects models, and the diagnostic performance of each Doppler criterion was estimated by the bootstrap method. This study was approved by the ethics committee of Tours.

RESULTS

Two hundred and eighteen pairs of examinations performed on 87 cirrhotic patients were analyzed. Variables significantly associated with dysfunction were: The speed of flow in the portal vein (P = 0.008), the reversal of flow in the right (P = 0.038) and left (P = 0.049) portal branch, the loss of modulation of portal flow by the right atrium (P = 0.0005), ascites (P = 0.001) and the overall impression of the operator (P = 0.0001). The diagnostic performances of these variables were low; sensitivity was < 58% and negative predictive value was < 73%. Therefore, dysfunction cannot be ruled out from Doppler-US.

CONCLUSION

The performance of Doppler-US for the detection of TIPS dysfunction is poor compared to angiography. New tools are needed to improve diagnosis of TIPS dysfunction.

Key words: Transjugular intrahepatic portosystemic shunt; Dysfunction; Doppler-ultrasonography

Core tip: This large multicentric prospective study evaluates the performance of Doppler-ultrasonography (US) for the detection of transjugular intrahepatic portosystemic shunt dysfunction within a cohort of cirrhotic patients. Although many Doppler-US variables were significantly associated with dysfunction, the diagnostic performances of these variables were low compared to angiography.

Nicolas C, Le Gouge A, d'Alteroche L, Ayoub J, Georgescu M, Vidal V, Castaing D, Cercueil JP, Chevallier P, Roumy J, Trillaud H, Boyer L, Le Pennec V, Perret C, Giraudeau B, Perarnau JM; STIC-TIPS group. Evaluation of Doppler-ultrasonography in the diagnosis of transjugular intrahepatic portosystemic shunt dysfunction: A prospective study. *World J Hepatol* 2017; 9(27): 1125-1132 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i27/1125.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i27.1125

INTRODUCTION

Transjugular intrahepatic porto-systemic shunt (TIPS) is now routinely used for the treatment of complications of portal hypertension[1-4]. One of the main disadvantages of this technique is the frequent occurrence of stent dysfunction. Indeed, with bare-stents, a reintervention is necessary in more than half of the cases at 1 year[5-8]. Thus, strict and scheduled monitoring to search for dysfunction is usually recommended. However, the use of polytetrafluoroethylene covered stents (e-PTFE) since 2000 has improved shunt patency[9-13]. Nevertheless, shunt dysfunction can still arise in more than 25% of cases after one year with covered stents[13].

Portography to measure portal pressure gradient is the gold-standard for the detection of TIPS dysfunction[5]; however, it is an invasive procedure which cannot be conducted routinely. Doppler ultrasonography (Doppler-US) has been proposed as an alternative to angiography. Many studies have tried to define valid criteria for shunt dysfunction[8,12,14-17] but sensitivity and specificity are very different from one study to another. Among these criteria, the velocity of the portal flow, the direction of the intrahepatic portal flow and the velocity of the flow in the shunt were the most studied, but no threshold was defined. Given the inter-individual variability of portal velocity, some authors preferred an individual criterion such as the decrease of baseline value[17]. An association of many criteria may also be more relevant[14-18] but none has been properly validated so far.

The aim of our study was to evaluate prospectively the performance of Doppler-US for the detection of

shunt dysfunction assessed by portography, in a multicentric cohort of cirrhotic patients.

MATERIALS AND METHODS

The study protocol was approved by the ethics committee of Tours and each patient gave written consent. This study was funded by the French Ministry of Health and by the Société Nationale Française de Gastroentérologie. This study has been registered on Clinical Trials.com under # 00593528.

Patients

This is an ancillary study from a randomized trial comparing covered and bare stents[13]. Patients were prospectively included in the cohort between February 2008 and July 2009. Cirrhotic patients who needed a TIPS for refractory ascites, hydrothorax or to prevent variceal rebleeding and were treated in 10 French tertiary teaching hospitals were included. The inclusion criteria were: (1) age between 18 and 75 years; (2) cirrhosis previously documented on histological or typical clinical signs; (3) Child-Pugh score < C12 at inclusion; (4) affiliation to the social security system; and (5) provision of informed consent to participate in the study. The exclusion criteria were total portal thrombosis, known hepatocellular carcinoma, cardiac failure, pulmonary hypertension (MAP > 40 mmHg), hepatic polycystosis, dilatation of intrahepatic bile ducts, history of recurrent spontaneous hepatic encephalopathy (HE), hepatic arterial insufficiency, pregnancy, breastfeeding, inadequate contraception for patients of childbearing age.

TIPS procedure

The TIPS procedure was performed with covered or bare stent randomly assigned.

Protocol: For each patient, a Doppler-US was performed by a radiologist working in the center which included the patient. Doppler-US was carried out before the TIPS procedure, during the days following TIPS insertion, at 1 mo, and every 3 mo thereafter up to 2 years. During this follow-up, portography with portosystemic pressure gradient measurements was scheduled every six months and was performed if dysfunction was suspected from clinical signs or ultrasound. Only Doppler-US performed the day of portography, or during the 15 d before, were compared with portography in this study.

Dysfunction: Shunt dysfunction was defined as an increase of portosystemic gradient $\geqslant$ 12 mmHg and/or a stent stenosis $\geqslant$ 50% of the lumen, during angiography. Cases of shunt stenosis without portal hypertension were examined by two independent radiologists. These radiologists were not aware of the Doppler-US results and had no practice at all with vascular stents.

Doppler-US variables: Different Doppler-US variables were collected for each patient, every three months, by the same operator, on the same ultrasound unit. Patients were fasted for four hours at the time of examination: (1) flow velocity in the main portal vein and within the stent. Patients were asked to have a quiet and regular respiration, and velocities were recorded during a blockpnea. Reported result was the mean of three measurements (cm/s); (2) direction of blood flow in the intrahepatic portal vein branches. The flow was characterized as hepatopetal or hepatofugal in the left branch and the right branch; (3) portal flow modulation induced by the right atrium. The phasicity of portal blood flow was recorded and was classified as demodulated when absent *vs* modulated; (4) stent filling in color Doppler. The wall to wall color flow within the stent was classified as incomplete *vs* complete; (5) presence of ascites. Ascites was quantified as absent or moderate (peritoneal effusion in the pouch of Douglas and/or in perihepatic area) *vs* severe (peritoneal effusion in the abdominal cavity); (6) the relative change of the flow velocity in the main portal vein. The portal velocity was compared to the one measured at one month (considered as the baseline value). Indeed, at one month after TIPS insertion, hemodynamic disturbances are stabililized and neointimal hyperplasia within the stent is not yet significant; and (7) the conclusion of the operator. The conclusion of the physician performing the examination was also recorded (suspected dysfunction; yes or no).

Blinding: The Doppler-US examination was performed before the portography; therefore, it could not be influenced by it. Furthermore, angiography and Doppler-US were performed by different operators and the operator who performed angiography was unaware of the results of Doppler-US.

Statistical analysis

Associations between shunt dysfunction defined by angiography and Doppler-US variables were analyzed with logistic random-effects models to account for the correlation of data (each patient had several measures).

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of binary criteria were estimated by a bootstrapping method with 95%CI. This non-parametric method uses the patient as a unit of resampling, to account for the correlation of data and avoid cluster effect.

For quantitative variables (flow velocities), the areas under the curves were estimated punctually and with a bootstrapping method[19], with 95%CI.

Analyses were performed with SAS, version 9.2 (SAS Institute, Cary, North Carolina, United States) and R 2.12.1 (R Development Core Team. R: A language and environment for statistical computing R) by a

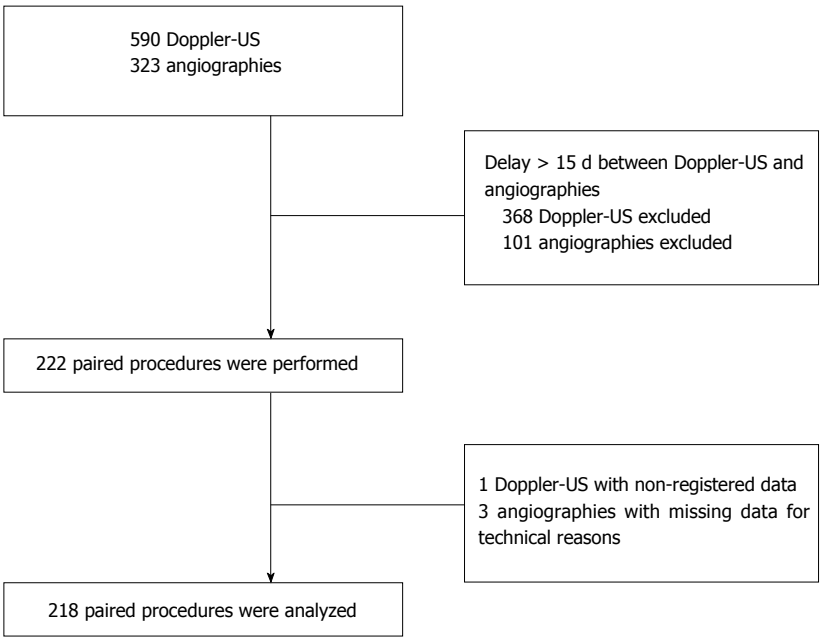

Figure 1 Flowchart describing pairing procedure of Doppler-ultrasonography and angiography among the 129 patients of the initial STIC-transjugular intrahepatic portosystemic shunt cohort. Doppler and angiography were paired if they were carried out with an interval of 15 d or less. US: Ultrasonography.

biomedical statistician.

RESULTS

Patients

In the original study, 137 patients were included and 129 were finally analyzed[20]. Forty one patients were excluded because Doppler-US was not performed within the 15 d before portography. From these 88 patients, 222 paired Doppler-US and angiographies were selected. Some Doppler and angiography data were not registered for technical reasons. Therefore, we analyzed 218 paired Doppler-US and angiographies from 87 patients (Figure 1).

The main characteristics of the patients are presented in Table 1. The number of patients for each listed characteristic varies because of missing data. In our cohort, causes of cirrhosis were largely dominated by alcohol (81.6% *vs* 13.8% for viruses and 8% for NASH) and the patients were predominantly classified as Child B (68.8% *vs* 18.8% Child A and 12.5% Child C).

Dysfunction

Among the 218 angiographies analyzed, 79 revealed a TIPS dysfunction in 51 patients.

Among these 79 dysfunction events, only 31 were suspected from Doppler-US, based on operator conclusions. Patency problems were detected for the first time with a median delay of 7.5 mo (6.2-18.3). The first event of dysfunction occurred in almost half of the cases (22/51) 6 ± 1 mo after TIPS insertion. Among these cases, less than half (10/22) were suspected from Doppler-US.

During portography, stenosis was located in the lower part of the stent in eight cases (20.5%), in the middle part of the stent in eight cases (20.5%), in the upper part of the stent in 16 cases (41%) and in the hepatic vein in seven cases (17.9%). Stenosis was suspected on Doppler-US in 62.5% cases (10/16) when the stenosis was located in the low or middle part of the stent, whereas it was suspected in 50% cases (8/16) when located in the upper part of the stent (Table 2).

Doppler-US variables

The performances of each Doppler-US criterion to discriminate TIPS dysfunction are summarized in Table 3.

Portal flow modulation

Loss of portal flow modulation induced by the right atrium was more frequent in case of dysfunction 29/65 (44.6%) *vs* 23/112 (20.5%) (P = 0.0005). The sensitivity of this variable was 44.4% (Table 3).

Intra hepatic portal flow direction: In the right portal vein, the flow was hepatopedal in 35/61 cases of dysfunction (57.3%), whereas it was hepatopedal in 43/112 cases (38.3%) in the absence of dysfunction. This variable (hepatopedal *vs* hepatofugal flow) was associated with TIPS dysfunction for both right (P = 0.038) and left branches (P = 0.049). The sensitivity and specificity of this variable were 57.2% and 61.7%, respectively for the right branch, and 54.7% and 66.8%, respectively for the left branch (Table 3).

Stent filling: Stent filling was incomplete in 18/57 cases of dysfunction (31.5%) and in 15/83 cases in the absence of TIPS dysfunction (18%) (P = 0.155).

Table 1 Baseline characteristics of the 87 patients at enrollment

Characteristic	
Age (yr)	58.1 ± 7.6
Male	68 (78.2)
Etiology of cirrhosis	
Alcohol	71 (81.6)
Viruses	12 (13.8)
NASH	7 (8.0)
Others	2 (2.3)
TIPS indication	
Recurrent bleeding	30 (34.5)
Refractory ascites	59 (67.8)
Hydrothorax	4 (4.6)
Child-Pugh score, *n = 86*	7.8 ± 1.6
Child-Pugh score, *n = 86*	
A	18 (20.9)
B	58 (67.4)
C	10 (11.6)
MELD score	11.6 ± 3.4

Data are mean ± SD or *n* (%). MELD: Model for end-stage liver disease.

Ascites: Ascites was severe in 22/79 cases of dysfunction (27.8%) *vs* 13/139 cases in the absence of dysfunction (9.3%) (P = 0.001). The sensitivity and specificity of this variable were 27.9% and 90.6%, respectively (Table 3).

Stent velocity: The mean velocity within the stent was 76.6 ± 52.5 cm/s in cases of dysfunction and 76.8 ± 35.8 cm/s in the absence of dysfunction (P = 0.753).

Portal vein velocity: The mean portal vein velocity was 25.1 ± 14.9 cm/s in cases of dysfunction and 34.3 ± 19.9 cm/s in the absence of dysfunction (P = 0.008). AUC is presented in Table 4.

The mean change of portal velocity relative to that measured 1 mo after TIPS insertion, called portal velocity delta, was -8.8 ± 18.1 cm/s in the dysfunction group, and -2.1 ± 22.5 cm/s in the absence of dysfunction group (P = 0.045). However, AUC of this variable is 0.577 (Table 4).

Portal velocity delta combined with right portal vein flow direction: The AUC of this association was 0.626 (Table 4).

Operator conclusion: Dysfunction was suspected from Doppler-US in 31/79 patients with certified dysfunction (39.2%), and in 18/139 patients in the absence of dysfunction (12.9%) (P = 0.0001). The sensitivity of this variable was 39.1% and its specificity was 87.1% (Table 3).

DISCUSSION

In our study, low portal vein velocity, hepatopedal flow in portal vein branches, loss of portal flow modulation, severe ascites and operator conclusion were associated with TIPS dysfunction. Nevertheless, the performance of these Doppler-US criteria for the diagnosis of TIPS dysfunction was poor.

Table 2 Suspicion of dysfunction based on the conclusion of the operator, according to the localization of the stenosis *n* (%)

	Dysfunction suspected (*n* = 21 stenosis)	Not suspected (*n* = 18 stenosis)
Lower part of the stent	6 (28.6)	2 (11.1)
Middle part of the stent	4 (19.1)	4 (22.2)
Upper part of the stent	8 (38.1)	8 (44.4)
Hepatic vein	3 (14.3)	4 (22.2)

Many studies have shown that dysfunction is associated with low main portal vein velocity[8,15,20]. Some authors have tried to define a threshold value to discriminate patent from non-patent shunts; however, results were inconsistent[8,15,20]. In our study, the AUC of main portal vein velocity was 0.655, so we cannot propose a relevant cut-off value. These results underline the difficulties to obtain a reproducible cut-off value, possibly due to the inter-individual variability of this variable. However, in our study, temporal change in main portal vein velocity relative to its baseline value was not more relevant than main portal vein velocity itself. Similarly, other authors[15,17] have reported poor sensitivity for a decrease of 33% in portal vein velocity.

The change of flow direction in the portal vein branches was significantly associated with dysfunction, both in the right and left branch. However, the sensitivity and the specificity of these variables were insufficient (all below 70%). This association has been already reported[8,14,15,18] with variable results. Kanterman *et al*[15] concluded this variable has a low sensitivity because intra hepatic flow reversal is a late sign of dysfunction.

Some authors have associated intrahepatic flow direction with another variable, portal vein velocity or stent velocity[14,18]. In our study, we evaluated the diagnostic accuracy of hepatopedal flow in the right portal branch combined with the decrease in portal vein velocity, but the AUC was mediocre. This is consistent with the low sensitivity we observed for each variable.

In our study, we did not find a significant modification of velocity within the shunt in cases of dysfunction. These results are consistent with some studies[16,18], whereas other authors have reported intra-stent velocity as a predictive variable[15,17]. These differences can be explained by the poor reproducibility of this measurement. Indeed, the stent velocity increases from the portal extremity to the hepatic end[21] and consequently depends on the measurement site.

The lack of cardiac modulation of the portal flow was strongly associated with TIPS dysfunction. These

Table 3 Performance of Doppler-ultrasonography binary criteria for the diagnosis of transjugular intrahepatic portosystemic shunt dysfunction

Variables	*n* paired	*n* patients	Sensitivity (95%CI)	Specificity (95%CI)	PPV	NPV (95%CI)
Portal flow modulation	177	73	44.4 (31.2-57.6)	79.6 (67.3-91.4)	56.4 (37.8-76.3)	71.1 (62.6-78.8)
Direction in right branch	173	76	57.2 (44.6-70.1)	61.7 (48.9-73.1)	45.2 (33.7-58.3)	72.4 (62.6-80.5)
Direction in left branch	171	77	54.7 (39.6-69.7)	66.8 (54.4-78.1)	48.5 (34.8-63.8)	72.1 (61.3-82.3)
Stent filling	140	64	31.3 (18.8-44.2)	81.8 (72.4-89.8)	54.1 (36.0-71.0)	63.4 (52.2-73.6)
Ascites	218	87	27.9 (16.9-39.7)	90.6 (84.0-95.4)	62.9 (43.7-79.1)	68.7 (61.0-76.1)
Conclusion	218	87	39.1 (27.6-51.4)	87.1 (79.3-93.3)	63.5 (45.8-79.8)	71.5 (63.6-78.5)

PPV: Positive predictive value; NPV: Negative predictive value.

Table 4 Performance of Doppler-ultrasonography quantitative criteria for the diagnosis of transjugular intrahepatic porto-systemic shunt dysfunction

	Patients	Paired procedures	AUC	95%CI
Portal velocity	80	192	0.655	0.553-0.749
Stent velocity	80	195	0.536	0.454-0.634
Portal velocity delta	63	150	0.577	0.485-0.679
Delta + right direction	58	128	0.626	0.530-0.726

AUC: Area under the curve.

results are consistent with those reported by some authors[22,23].

As others[5], we observed that detection of ascites during Doppler-US examination was associated with shunt dysfunction with a high specificity (90.6%). This is consistent with the fact that ascites is a late sign of dysfunction and not a predictive one.

The conclusion of the operator was associated with dysfunction with high specificity but with low sensitivity. The negative predictive value of this variable was 71.5%, thus a dysfunction cannot be ruled out when Doppler-US examination does not suggest dysfunction. In other studies[8,15,17], this variable predicted shunt dysfunction more accurately than in our study, probably because of the monocentric design of these studies. Indeed, Doppler-US is an operator-dependent examination[24] which explains differences observed from one study to another, and difficulties to identify objective and reproducible predictors of TIPS dysfunction. Moreover, this underlines the importance of the experience of the operator. Most of the Doppler-US were performed by experienced and specialized operators in this study. In only 2 centers, some examinations have been occasionally realized by residents.

In our study, we found lower sensitivities and specificities than those reported in literature, probably because we avoided institution bias. Indeed, this study was designed as a pragmatic study and represents the reality of current practice, with about half of the French centers realizing TIPS procedure included in this study.

Moreover, dysfunctions observed in our study were mostly located in the upper part of the stent and may be more difficult to diagnose in Doppler-US.

Other authors reported similar results to ours, and failed to identify Doppler-US variables relevant to diagnose shunt dysfunction[16,20]. Interestingly, these studies were also prospective and double-blinded but included fewer patients than our study.

In our study, some procedures were realized with bare stents and other with covered stent but this has no incidence on the results as we took in account only dysfunction. Shunt dysfunction occurs frequently, even with covered stents[13]; therefore, it is still necessary to monitor shunt patency, especially to avoid the recurrence of digestive bleeding as it is a life-threatening complication. Given its poor diagnostic performance, Doppler-US is not a good diagnostic tool for routine screening across centers. Clinical supervision may be sufficient for TIPS indications such as refractory ascites, whereas early detection of shunt dysfunction appears crucial for TIPS indications such as variceal bleeding. New tools, more efficient than Doppler-US and less invasive than angiography, are needed. Contrast-enhanced ultrasound[25], as well as the measurement of azygos blood flow by magnetic resonance imaging[26] may be of interest but further studies are needed. In the meanwhile, an angiography should still be proposed, especially for bleeding indications of TIPS, sixth months after TIPS insertion because the first event of dysfunction occurs in almost half of cases at 6 mo.

In conclusion, this pragmatic study shows that the performance of Doppler-US for the detection of TIPS dysfunction is poor in current practice.

COMMENTS

Background

Angiography is the gold-standard procedure to evaluate transjugular intrahepatic porto-systemic shunt (TIPS) dysfunction. However, it is an invasive technic performed only in limited specialized centers. Thus, Doppler ultrasonography (Doppler-US) is frequently used for TIPS monitoring.

Research frontiers

Despite frequent use of Doppler-US for TIPS monitoring, to date, no criterion of TIPS dysfunction have been prospectively evaluated.

Innovations and breakthroughs

The authors conducted the first large prospective multicentric evaluation of the performance of Doppler-US for the detection of TIPS dysfunction.

Applications

In routine practice, the performance of Doppler-US for the detection of TIPS dysfunction is insufficient. Thus, the gold standard remains angiography. Future researches have to focus on developing less invasive tools.

Peer-review

The article is aimed to assess the factors related to the prognosis of intra-abdominal liposarcoma and find the optimal minimum duration for remnant tumor screening.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Acute-on-chronic liver failure in a multi-ethnic Asian city: A comparison of patients identified by Asia-Pacific Association for the Study of the Liver and European Association for the Study of the Liver definitions

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Abstract

AIM

To explore the applicability of the Asia-Pacific Association for the Study of the Liver (APASL) and European Association for the Study of the Liver (EASL) guidelines for acute-on-chronic liver failure (ACLF) in profiling patients and determining the outcome.

METHODS

Patients admitted to a tertiary hospital in Singapore with acute decompensation of liver disease from January 2004

to July 2014 are screened for ACLF according to the APASL and EASL criteria. The patients' data (including basic demographics, information about existing chronic liver disease, information about the acute decompensation, relevant laboratory values during admission, treatment, and outcome) are retrospectively analyzed to determine the background, precipitating factors and outcome.

RESULTS

A total of 458 liver patients is analyzed, and 78 patients with ACLF are identified. Sixty-three patients (80.8%) meet the APASL criteria, 64 patients (82.1%) meet the EASL criteria, and 49 patients (62.8%) fulfilled both criteria. The most common causes of acute liver injury are bacterial infections (59.0%), hepatitis B flare (29.5%), and variceal bleeding (24.4%). The common aetiologies of the underlying chronic disease included hepatitis B (43.6%), alcoholic (20.5%) and cryptogenic (11.5%) liver disease. The overall mortality rate is 61.5%. Increased age, the number of organ failures (as per CLIF-SOFA score), peak creatinine, INR, and amylase levels are associated with increased mortality or the need for liver transplantation. 14.3% of patients undergo liver transplantation with a 100% 1-year survival rate.

CONCLUSION

Both APASL and EASL criteria have identified ACLF patients with high three-month mortality, but those who fulfill APASL criteria alone have a better survival.

Key words: Acute-on-chronic liver failure; Survival; Prognosis; Liver decompensation; Liver cirrhosis

Core tip: Acute-on-chronic liver failure (ACLF) is a distinct disease entity with a high short-term mortality. Utilizing both the Asia-Pacific Association for the Study of the Liver (APASL) and European Association for the Study of the Liver criteria, our study shows that the clinical profile of ACLF patients in Singapore appears to have mixed features compared with similar studies reported in the rest of Asia and the West. Patients with ACLF fulfilling only the APASL criteria in our study had significantly better survival rates. We also analyzed the prognostic factors of ACLF in our study.

Selva Rajoo A, Lim SG, Phyo WW, Tun T, Dan YY, Lee YM, Low HC, Lim K, Tan PS, Lee GH. Acute-on-chronic liver failure in a multi-ethnic Asian city: A comparison of patients identified by Asia-Pacific Association for the Study of the Liver and European Association for the Study of the Liver definitions. *World J Hepatol* 2017; 9(28): 1133-1140 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i28/1133.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i28.1133

INTRODUCTION

Acute-on-chronic liver failure (ACLF) is a distinct disease entity characterized by the acute deterioration of liver function in patients with chronic liver disease[1]. It describes a condition in which two hepatic insults liver operate simultaneously, one of them being ongoing and persistent (*e.g.,* chronic hepatitis C) while the other being an acute precipitating event (*e.g.,* hepatotoxic drug, variceal bleed)[2]. Patients with ACLF have a statistically higher mortality rate (30%-40%) compared with patients without ACLF, at the same baseline Model for End-Stage-Liver Disease (MELD) score[3].

There are currently two widely accepted diagnostic criteria for ACLF: The Asia-Pacific Association for the Study of the Liver (APASL) in 2014[2], and the European Association for the Study of the Liver (EASL) consensus definitions in 2011[4]. Although these definitions describe the same disease entity, there are some crucial differences between them (summarised in Figure 1). APASL focuses more on signs of ascites and encephalopathy within a time frame of 4 wk with chronic liver disease. EASL underlines the occurrence of organ failure in patients with cirrhosis resulting in 3-mo mortality. Furthermore, these two definitions are based on populations with different disease patterns[5].

The objectives of this study are first, to understand the clinical profile of the patients with ACLF in Singapore. From this, the precipitating risk factors for ACLF could be treated or prevented. Secondly, this study aims to analyze the prognostic indicators of ACLF thereby discussing ways to improve the outcome.

There is an increasing concern about ACLF, due to its high short-term mortality and lack of clear understanding of the natural history and clinical profile of the patients, which vary across different countries and regions in the world. This study provides preliminary data on the local ACLF patient profile and outcome of this condition. We also examined the relevance and applicability of the current two guidelines for ACLF diagnosis and prognosis in the local context.

MATERIALS AND METHODS

Study design

This retrospective cohort study was performed with existing data of patients admitted to the National University Hospital (NUH) in Singapore from January 2004 to July 2014. The data is part of an approved database of all patients admitted to the hepatology service or referred to liver transplant service. All patients were followed up for at least three months. All relevant data had been recorded in the hospital electronic medical records, Intensive Care Unit (ICU) monitoring system, and the patients' case files. In this study, the diagnosis of ACLF was made by utilizing either the APASL or EASL definitions.

Data collection

Data were retrospectively analyzed, but the clinicians prospectively collected the data through their inpatient lists and anonymously transferred to the study administrator.

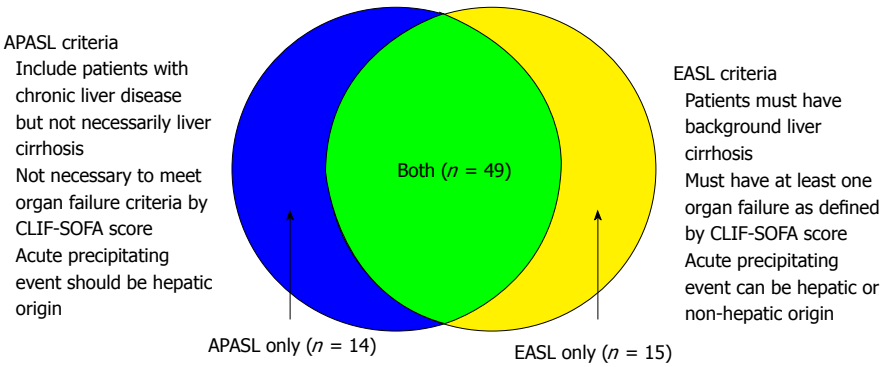

Figure 1 Main differences between Asia-Pacific Association for the Study of the Liver and European Association for the Study of the Liver criteria and the number of subjects fulfilling either or both criteria[2,5,7]. APASL: Asia-Pacific Association for the Study of the Liver; EASL: European Association for the Study of the Liver.

Confidentiality of the patients was preserved by anonymising the data collected. The subject data was assigned code numbers which do not reflect personal identifiers and were entered into a computerized database. Data collection included basic demographic information (age, gender, body mass index), information about existing chronic liver disease, information about acute decompensation, other relevant laboratory values of the patient during admission (white cell count, creatinine, bilirubin, international normalized ratio, C-reactive protein, *etc*.), treatment and outcome. This study protocol had been reviewed and approved by the National Healthcare Group Domain Specific Research Board (Domain E) (DSRB reference: 2014/01194).

Statistical analysis

Data entry and analysis were carried out using SPSS 20. Inter-group comparisons for categorical variables were made using the χ^2 test or Fisher's exact test, and those for quantitative variables were compared using the Student's *t*-test, one-way ANOVA. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

A total of 458 liver patients were screened. One hundred and forty-seven were found to have an acute decompensation of chronic liver disease, and 78 of these patients were found to have ACLF that fulfilled either the APASL or EASL criteria. Sixty-three patients (80.8%) met the APASL criteria, and 64 patients (82.1%) met the EASL criteria. Forty-nine patients (62.8%) fulfilled both criteria (summarized in Figure 1 and Table 1).

Patient demographics

Table 1 shows the profile of patients with ACLF. The age range of the 78 patients included in the study was 55 to 61 years. Sixty-nine point two percent of these patients were Chinese, which is consistent with the local racial demographics of the population (74.3% Chinese, 13.4% Malays, and 9.1% Indians[6]). Seventy-five point five percent of the patients were male. Patients with ACLF meeting the EASL criteria were more likely to be older than those in the other two groups ($P = 0.003$).

Events leading to acute insult and underlying etiology

Bacterial infection (59.0%), hepatitis B flare (29.5%) and variceal bleeding (24.4%) were the most common causes leading to the acute insult. Patients fulfilling ACLF-EASL criteria were more likely to have had a bacterial infection triggering ACLF compared to those in the other two groups ($P = 0.002$). On the other hand, patients fulfilling ACLF-APASL criteria were more likely to have had hepatitis B flare triggering ACLF compared to those in the other two groups ($P = 0.001$). Patients admitted with ACLF most frequently have hepatitis B (43.6%), alcoholic liver disease (20.5%) and cryptogenic liver disease (11.5%) as their underlying chronic liver diseases. Patients fulfilling the APASL criteria were more likely to have Hepatitis B compared to patients in the EASL group.

Outcome

Table 2 shows the outcome of patients with Acute-on-Chronic Liver Failure. The overall mortality rate at the point of admission and three-month mortality rate were expectedly high at 57.7% and 61.5% respectively. Patients with ACLF fulfilling both criteria were more likely to have a fatal outcome at the point of admission (67.3% mortality) as well as in 3 mo [71.4% mortality ($P = 0.033$ and $P = 0.041$ respectively)]. Transplant rate was 14.3%, and all the transplant patients survived and lived for more than one year ($P \leqslant 0.0001$).

Patients were further classified by ACLF grade (ACLF 0-3) according to the EASL-CLIF Consortium definitions[5,7], which classifies the severity of ACLF by the number of organ failures[8]. Table 3 shows information regarding organ failures, laboratory parameters and outcome of ACLF patients to the ACLF grade. Higher mortality rates were associated with an increased ACLF grade. Three-month mortality for ACLF 0 to 3 was 0%, 42.9%, 41.7% and 84.8% respectively. Patients with 3 or more organ failures (*i.e.*, ACLF 3) had a significantly higher mortality

Table 1 Profile of patients with acute-on-chronic liver failure *n* (%)

	APASL only (*n* = 14)	EASL only (*n* = 15)	Both (*n* = 49)	*P* value[3]	Total (*n* = 78)
AGE (mean)	53 (48-57)	66 (61-71)	57 (54-60)	0.003	58 (55-61)
Race					
Chinese	9 (64.3)	9 (60)	36 (73.5)	0.171	54 (69.2)
Malay	1 (7.1)	2 (13.3)	5 (10.2)		8 (10.3)
Indian	0	3 (20)	5 (10.2)		8 (10.3)
Others	4 (28.6)	1 (6.7)	3 (6.1)		8 (10.3)
Male gender	12 (85.7)	11 (73.3)	36 (73.5)	0.625	59 (75.6)
Diabetes mellitus	7 (50)	5 (33.3)	16 (32.7)	0.478	28 (35.9)
Cause of acute liver injury[4]					
Infection	4 (28.6)	14 (93.3)	28 (57.1)	0.002	46 (59)
Hepatitis B flare	9 (64.3)	0	14 (28.6)	0.001	23 (29.5)
Variceal bleeding	4 (28.6)	2 (13.3)	13 (26.5)	0.535	19 (24.4)
Unknown cause	2 (14.3)	1 (6.7)	8 (16.3)	0.642	11 (14.1)
Alcohol	1 (7.1)	0	5 (10.2)	0.429	6 (7.7)
TCM	1 (7.1)	0	5 (10.2)	0.429	6 (7.7)
TIPSS	0	0	1 (2)	0.741	1 (1.3)
Underlying chronic liver disease					
Hepatitis B	11 (78.6)	1 (6.7)	22 (44.9)	< 0.0001	34 (43.6)
Alcohol	2 (14.3)	3 (20)	11 (22.4)	0.799	16 (20.5)
Cryptogenic	0	3 (20)	6 (12.2)	0.234	9 (11.5)
Hepatitis C	0	2 (13.3)	3 (6.1)	0.339	5 (6.4)
NASH	0	2 (13.3)	2 (4.1)	0.23	4 (5.1)
Hepatitis B + alcohol	1 (7.1)	0	1 (2)	0.444	2 (2.6)
Others[1]	0	4 (26.7)	5 (10.2)	0.072	9 (11.5)
Liver cirrhosis	6 (42.9)	15 (100)	49 (100)	< 0.0001	70 (89.7)
HCC (Milan's criteria)	0	1 (6.7)	4 (8.2)	0.627	5 (6.4)
Other cancers[2]	0	1 (6.7)	4 (8.2)		5 (6.4)
No malignancy	14 (100)	13 (86.7)	41 (83.7)		68 (87.2)
Previous decompensation	4 (28.6)	10 (66.7)	20 (43.5)	0.11	34 (45.3)

[1]Other chronic liver diseases include Wilson's disease, Primary Sclerosing Cholangitis, Primary Biliary Cirrhosis, Congenital fibrosis, Drug-induced chronic liver disease and autoimmune liver disease; [2]Other cancers: Colon cancer, renal cell carcinoma, bladder cancer and ovarian cancer; [3]*P*-value of comparison of patients falling under APASL, EASL and both criteria; [4]Patients may have more than one cause of acute liver injury. TCM: Traditional Chinese Medicine; TIPSS: Transjugular intrahepatic portosystemic shunt; NASH: Non-alcoholic steatohepatitis; HCC: Hepatocellular carcinoma; APASL: Asia-Pacific Association for the Study of the Liver; EASL: European Association for the Study of the Liver.

Table 2 Outcome of patients with acute-on-chronic liver failure *n* (%)

	APASL only (*n* = 14)	EASL only (*n* = 15)	Both (*n* = 49)	*P* value	Total (*n* = 78)
MELD score (mean-range)	27 (23-31)	18 (13-23)	25 (22-28)	0.020	24 (33-26)
Transplant	4 (28.6)	0	7 (14.6)	0.089	11 (14.3)
Mortality (during admission)	4 (28.6)	8 (53.3)	33 (67.3)	0.033	45 (57.7)
Three month mortality	5 (35.7)	8 (53.3)	35 (71.4)	0.041	48 (61.5)

P value of comparison of patients falling under APASL, EASL and both criteria. APASL: Asia-Pacific Association for the Study of the Liver; EASL: European Association for the Study of the Liver.

rate than all other patients at the point of admission and at three months ($P \leqslant 0.0001$, $P < 0.0001$ respectively). Patients who fulfill the APASL criteria for ACLF exclusively (*i.e.*, no organ failures or ALCF 0) had a 0% mortality rate.

The demographics, type of organ failure and laboratory parameters of ACLF patients who survived *vs* those who met a fatal outcome have been analyzed and summarised in Table 4. Patients with fatal outcomes were more likely to be older (mean age 60 *vs* 55, $P = 0.044$). Patients with renal (68.9%, $P = 0.001$), cerebral (37.8%, $P = 0.012$), circulatory (63.6%, $P \leqslant 0.0001$) and respiratory (17.8%, $P = 0.044$) failure were more likely to have a fatal outcome compared to those without these organ failures. Also, higher serum creatinine and INR and baseline amylase were strongly associated with the poor prognosis compared to other laboratory tests ($P \leqslant 0.0001$, 0.018 and 0.026 respectively).

DISCUSSION

One of the compelling reasons for the lack of a unifying definition for ACLF is the difference in etiologies for both the acute insults and underlying chronic liver diseases in the East and West[9], and much of this can be attributed to the socioeconomic status of the countries in Asia. In Singapore, with endemic chronic hepatitis B as the dominant chronic liver disease, coupled with a Westernised lifestyle and standard of living,

Table 3 Organ failures, laboratory parameters and outcome of acute-on-chronic liver failure patients with respect to acute-on-chronic liver failure grade *n* (%)

	ACLF0 (*n* = 6)	ACLF1 (*n* = 7)	ACLF2 (*n* = 24)	ACLF3 (*n* = 33)[1]	*P* value
Organ failures-clif-sofa score					
Liver	1 (16.7)	1 (14.3)	12 (50)	27 (81.8)	< 0.0001
Kidney	0	4 (57.1)	10 (41.7)	25 (75.8)	0.002
Cerebral	0	1 (14.3)	2 (8.3)	13 (39.4)	0.018
Coagulation	1 (16.7)	1 (14.3)	16 (66.7)	25 (75.8)	0.002
Circulation	0	0	7 (30.4)	26 (78.8)	< 0.0001
Respiration	0	0	2 (8.3)	7 (21.2)	0.22
Chronic renal disease	0	2 (28.6)	5 (20.8)	3 (9.1)	0.245
Laboratory data (mean)					
Leucocyte count at baseline, $\times 10^9$/L	8 (6-11)	11 (6-16)	10 (7-12)	12 (10-13)	0.256
Platelet count at baseline	127 (42-212)	61 (40-82)	104 (78-129)	149 (105-193)	0.105
Bilirubin at baseline, mg/dL	7.0 (4.2-9.8)	7.7 (-3.1-18.5)	10 (4.9-15.1)	13.9 (9.9-17.8)	0.29
Peak bilirubin, mg/dL	11.3 (4.1-18.5)	10.7 (-4.2-25.5)	14.1 (9.1-19)	23 (18.7-27.3)	0.009
Creatinine at baseline, mg/dL	0.9 (0.4-1.3)	2.1 (1.3-2.9)	1.4 (1.1-1.8)	1.8 (1.1-2.4)	0.232
Peak creatinine, mg/dL	0.9 (0.5-1.2)	2.2 (1.4-3.1)	1.8 (1.4-2.1)	3.1 (2.6-3.7)	< 0.0001
Lactate at baseline, mmol/L	2.1 (1.4-2.7)	2.3 (2.1-2.6)	2.4 (1.7-3.2)	4.1 (2.4-5.7)	0.225
MELD score at baseline (mean)	23 (20-26)	21 (11-31)	20 (17-24)	26 (23-30)	0.113
Liver transplantation	1 (16.7)	0	6 (26.1)	1 (3)	0.043
Mortality during admission	0	3 (42.9)	10 (41.7)	28 (84.8)	< 0.0001
90-d mortality	0	3 (42.9)	11 (45.8)	29 (87.9)	< 0.0001

[1]Only patients with cirrhosis were included in this analysis. *P*-value of comparison of patients with ACLF grade 0, 1, 2 and 3. ACLF: Acute-on-chronic liver failure.

understanding the mixed profiling of local ACLF patients and the prognostic factors will be important in better prevention and management of such high-risk patients.

Bacterial infection, hepatitis B flare, and variceal bleeding are the most common causes for the acute component of ACLF. Patients fulfilling the EASL criteria are more likely to have bacterial infections that triggered ACLF while patients meeting the APASL criteria are more likely to have a hepatitis B flare as the trigger. This difference is in keeping with the differences in underlying etiologies of acute deterioration of liver disease between the East and the West published in the literature. In the Asia-Pacific region, which is the demographic that the APASL guidelines are based upon, the majority of ACLF is precipitated by viral hepatitis. In developed European countries, these viral etiologies are mostly supplanted by non-viral insults such as bacterial infections[10]. We note that more than half of the study population had a bacterial infection as the precipitating factor of ACLF, reflecting the developed health care standards enjoyed by the Singaporean population. There is no consensus as to whether variceal bleeding qualifies as a precipitant of ALCF under the APASL guidelines[10]. However, this study shows that it is a prominent cause of ACLF (24.4%) should it be included.

Patients with ACLF in this study most frequently have chronic hepatitis B infection, alcoholic liver disease, and cryptogenic liver cirrhosis as their underlying chronic liver disease. The prevalence of HBV is expected given that Singapore lies within the Asia-Pacific region and in most Asian countries, hepatitis B constitutes about 70% of all chronic etiologies of ACLF. Alcoholic liver cirrhosis represents 50%-70% of all underlying etiologies of ACLF in Western countries[5,11,12]. The fact that alcoholic liver cirrhosis constitutes such a high proportion of the study population suggests a Western influence on the local community as well. However, some studies do indicate that alcohol-related ACLF is equally represented worldwide[10,13].

One of the unexpected findings of this study is the narrow age range (52-64 years) of all 78 consecutive ACLF subjects. All 45 deaths were older (mean 60 years, range 56-64 years), with little overlap with the survivors (mean age 55 years, range 52-58 years) who were younger. Thus, a higher index of suspicion for progression to ACLF should be applied when patients above 50 years of age present with acute liver decompensation. Deterioration of the patient should be expected and pre-empted, especially for those between 55 to 65 years of age who are still eligible for liver transplantation. This age-related incidence and prognosis still await future validation studies for confirmation.

Patients with ACLF have a high mortality rate at 57.7% and 61.5% (at 0 and 3 mo respectively), which is comparable to the documented mortality rate of 50%-90%[14]. Higher mortality rates have been associated with an increase in ACLF grade based on the CLIF-SOFA score (*i.e.,* more organ failures) in line with existing literature[15]. Furthermore, patients with ACLF and no organ failure had a 0% mortality rate. These results suggest that the CLIF-SOFA organ failure score may be a useful predictor of death in our local ACLF population, in keeping with publications which identify the correlations between the number of organ failure(s) in patients with cirrhosis with mortality[5]. In this study, peak creatinine, INR, and amylase levels are independently associated with increased mortality or the need for liver transplantation.

Table 4 Demographics, type of organ failure and laboratory parameters of acute-on-chronic liver failure patients with respect to outcome *n* (%)

	Deaths (*n* = 45)	Survivors (*n* = 33)	*P* value
Age (mean-range)	60 (56-64)	55 (52-58)	0.044[1]
Liver transplantation	0	11 (34.4)	< 0.0001[2]
Race			
Chinese	33 (73.3)	21 (63.6)	0.004[1]
Malay	6 (13.3)	2 (6.1)	
Indian	6 (13.3)	2 (6.1)	
Others	0	8 (24.2)	
Male gender	33 (73.3)	26 (78.8)	0.579[1]
Diabetes mellitus	13 (28.9)	15 (45.5)	0.132[1]
Previous hepatic decompensation	16 (37.2)	18 (56.2)	0.101[1]
Potential events leading to acute insult			
Infection	28 (62.2)	18 (54.5)	0.496[1]
Hepatitis B flare	15 (33.3)	8 (24.2)	0.384[1]
Variceal bleeding	10 (22.2)	9 (27.3)	0.608[1]
Unknown cause	5 (11.1)	6 (18.2)	0.287[2]
Alcohol	2 (4.4)	4 (12.1)	0.204[2]
TCM	5 (11.1)	1 (3)	0.189[2]
TIPSS	1 (2.2)	0	0.577[2]
Underlying chronic liver disease			
Hepatitis B	20 (44.4)	14 (42.4)	0.859[1]
Alcohol	10 (22.2)	6 (18.2)	0.662[1]
Cryptogenic	4 (8.9)	5 (15.2)	0.307[2]
Hepatitis C	3 (6.7)	2 (6.1)	0.646[2]
NASH	2 (4.4)	2 (6.1)	0.567[2]
Hepatitis B + alcohol	1 (2.2)	1 (3)	0.670[2]
Others[1]	6 (13.3)	3 (9.1)	0.419[2]
Liver cirrhosis	41 (91)	29 (87.9)	0.459[2]
HCC+ (Milan criteria)	3 (6.7)	2 (6.1)	0.709[2]
Other cancers[2]	2 (4.4)	3 (9.1)	
None	40 (88.9)	28 (84.8)	
Organ failures-clif-sofa score			
Liver	31 (68.9)	17 (51.5)	0.119[1]
Kidney	31 (68.9)	10 (30.3)	0.001[1]
Cerebral	17 (37.8)	4 (12.1)	0.012[1]
Coagulation	31 (68.9)	19 (57.6)	0.303[1]
Circulation	28 (63.6)	7 (21.2)	< 0.0001[1]
Respiration	8 (17.8)	1 (3)	0.044[2]
Chronic renal disease	9 (20)	2 (6.1)	0.075[2]
Leucocyte count at baseline (mean-range)	11 (10-12)	10 (8-12)	0.273[3]
Platelet count at baseline (mean-range)	135 (109-160)	120 (79-160)	0.528[3]
Amylase (mean-range)	117 (90-144)	72 (38-105)	0.026[3]
Maximal total bilirubin (mean-range)	20.2 (16.3-24)	15.9 (11.5-20.2)	0.137[3]
Maximal creatinine (mean-range)	2.9 (2.4-3.3)	1.6 (1.3-1.9)	< 0.0001[3]
Maximal INR (mean-range)	4.3 (3.5-5.1)	3 (2.5-3.6)	0.018[3]
MELD at baseline (mean-range)	26 (23-29)	22 (19-25)	0.119[3]
Lactate at baseline (mean-range)	3.5 (2.4-4.6)	2.8 (2-3.7)	0.311[3]

[1]χ^2 test; [2]Fisher exact test; [3]Independent *t*-test. TCM: Traditional Chinese Medicine; TIPSS: Transjugular intrahepatic portosystemic shunt; NASH: Non-alcoholic steatohepatitis; HCC: Hepatocellular carcinoma; APASL: Asia-Pacific Association for the Study of the Liver; EASL: European Association for the Study of the Liver.

Peak creatinine level, in particular, is most strongly associated with increased mortality, which is expected given the association between renal failure and death in ACLF[16].

Liver transplantation is an important definitive treatment for patients with severe ACLF, who usually have underlying liver cirrhosis[17-20]. This study has shown that patients with ACLF who subsequently underwent liver transplantation had a 100% 1-year survival rate. This promising result suggests that high-urgency allocation of liver transplantation should be considered for ACLF patients[19,21]. Nonetheless, we note that not all patients with ACLF are transplant candidates for numerous reasons, which may include advanced age, active alcoholism, or concomitant diseases. The presence of non-liver organ failures may sometimes be a contraindication to liver transplantation[3].

In conclusion, ACLF is a life-threatening syndrome and both the APASL and EASL criteria have identified ACLF patients with high short-term mortality. The clinical profile of ACLF patients in Singapore appears to have mixed features compared with similar studies reported in

the rest of Asia and the West. This would not be unique to Singapore, but applicable to many growing cities in Asia undergoing a rapid transformation from traditional disease epidemiology and lifestyle to improved living standards and widespread modern healthcare standards. Each region will have to re-evaluate their changing patterns of ACLF and address the new needs accordingly. The multi-ethnic composition of the Singapore population also has implications for understanding the variations in the Asian-Pacific region.

Patients with ACLF fulfilling only the APASL criteria in our study had significantly better survival rates compared with patients meeting the EASL criteria only, largely due to the APASL criteria accepting subjects who had chronic hepatitis but not liver cirrhosis (42.9%), while EASL-defined ACLF subjects must be cirrhotic. It is interesting to note that patients meeting the only APASL criteria had a higher MELD score than patients fulfilling the EASL criteria. These patients may have had a more severe acute insult leading to acute decompensation, but they still had higher survival rate due to better baseline liver function. Patients with ACLF fulfilling both criteria were more likely to have a fatal outcome (71.4% 3-mo mortality (P = 0.041). CLIF-SOFA organ failure score, complemented by laboratory parameters such as creatinine, amylase, and INR appear to be promising tools in determining the prognosis of patients with ACLF. Early diagnosis of ACLF and identification of indicators predictive of poor outcome (as discussed above) will help to distinguish between patients with ACLF that would require transplantation from those that will survive with only organ support and intensive medical care[14] and thus optimise treatment and survival.

COMMENTS

Background

Acute-on-chronic liver failure (ACLF) is a distinct disease entity with a high short-term mortality. There are two widely accepted diagnostic criteria for ACLF. However, there are crucial differences between them. There is also currently a lack of clear understanding of the natural history and clinical profile of the patients, which vary across different regions in the world.

Research frontiers

This is the first study to explore the applicability of the Asia-Pacific Association for the Study of the Liver (APASL) and European Association for the Study of the Liver (EASL) guidelines for ACLF in profiling patients and determining the outcome in Singapore.

Innovations and breakthroughs

EASL criteria may identify patients with a higher mortality. CLIF-SOFA organ failure score, complemented by laboratory parameters such as creatinine, amylase, and INR appear to be promising tools in determining the prognosis of patients with ACLF.

Applications

Early diagnosis of ACLF and identification of indicators predictive of poor outcome will help to distinguish between patients with ACLF that would require transplantation from those that will survive with only organ support and intensive medical care and thus optimise treatment and survival.

Terminology

ACLF: A distinct disease entity characterized by the acute deterioration of liver function in patients with the chronic liver disease.

Peer-review

The manuscript describes a retrospective study investigating ACLF in patients from Singapore. The study compared the EASL and APASL ACLF guidelines in patients with an acute decompensation of liver disease. The manuscript overall is of interest, and the results are enlightening.

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Abstract

AIM

To analyze liver tests before and following treatment with herbal Traditional Chinese Medicine (TCM) in order to evaluate the frequency of newly detected liver injury.

METHODS

Patients with normal values of alanine aminotransferase

(ALT) as a diagnostic marker for ruling out pre-existing liver disease were enrolled in a prospective study of a safety program carried out at the First German Hospital of TCM from 1994 to 2015. All patients received herbal products, and their ALT values were reassessed 1-3 d prior to discharge. To verify or exclude causality for suspected TCM herbs, the Roussel Uclaf Causality Assessment Method (RUCAM) was used.

RESULTS

This report presents for the first time liver injury data derived from a prospective, hospital-based and large-scale study of 21470 patients who had no liver disease prior to treatment with herbal TCM. Among these, ALT ranged from 1 × to < 5 × upper limit normal (ULN) in 844 patients (3.93%) and suggested mild or moderate liver adaptive abnormalities. However, 26 patients (0.12%) experienced higher ALT values of ⩾ 5 × ULN (300.0 ± 172.9 U/L, mean ± SD). Causality for TCM herbs was RUCAM-based probable in 8/26 patients, possible in 16/26, and excluded in 2/26 cases. Bupleuri radix and Scutellariae radix were the two TCM herbs most commonly implicated.

CONCLUSION

In 26 (0.12%) of 21470 patients treated with herbal TCM, liver injury with ALT values of ⩾ 5 × ULN was found, which normalized shortly following treatment cessation, also substantiating causality.

Key words: Traditional Chinese Medicine; Herbal medicine; Liver injury; Roussel Uclaf Causality Assessment Method; Herb induced liver injury

Core tip: Worldwide research on herbal medicine safety is still limited. Adverse effects are range from clinically not relevant to more severe ones including suspected liver injury. We conducted a prospective hospital-based study to report the number of new liver injury in patients with no liver disease prior to treatment with herbal Traditional Chinese Medicine. Liver injury was detected in 26/21470 patients (0.12%) with alanine aminotransferase values of ⩾ 5 × upper limit normal. The Roussel Uclaf Causality Assessment Method assessed the causality of suspected cases and showed a causality level of "possible" for the majority of the liver injury cases.

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INTRODUCTION

Traditional Chinese Medicine (TCM) with the focus on its herbal constituents is an individual treatment option with growing worldwide popularity[1,2], despite still insufficiently documented efficacy[3] and known adverse reactions[4,5]. In particular, the risk of liver injury in patients under therapy using TCM herbs has appeared as a major problem for many decades[6]. This issue has been known at least since 1983[7] and is in line with many subsequent case reports and case series[8-15]. However, there have been attempts to downgrade the hepatotoxic risk of herbal TCM but such proposals were vague and rejected since no proof for this claim was provided[16]. Other problems were recognized as variables, which confounded establishing valid causality[17-19]. Among these variables were co-medication with other herbal products or synthetic, potentially hepatotoxic Western drugs, low case data quality, incomplete consideration of alternative causes, and questionable quality of herbal TCM products. Indeed, some herbal TCM products are confronted with problems of misidentified herbs, impurities, pesticides, heavy metals, or adulteration by Western drugs to enhance or provide efficacy[20-24].

Other challenges included the fact that not all publications used a sophisticated, robust causality assessment method. Nevertheless, Roussel Uclaf Causality Assessment Method (RUCAM)[25] was successfully applied in many cases of suspected liver injury by TCM herbs[25,26], including as examples some more recent reports[9,27-31]. Further, there was also uncertainty as to whether the observed liver disease could have been present prior to the initiation of the TCM use rather than caused by the herbal TCM therapy itself. Meeting the objections regarding pre-existing liver disease would have required an analytical approach whereby a study protocol is prospectively applied to patients without any liver disease, in whom therapy with herbal TCM is intended and liver tests can be analyzed under such treatment conditions. In patients with new abnormal liver tests under the therapy, causality for the suspected herbal TCM product can easily be assessed using RUCAM. So far, such a systematic prospective, large-scale investigation has not been published on liver-healthy individuals, at least not in the scientific literature available in the English language.

In this report, we present for the first time liver injury data derived from a prospective, hospital-based and large-scale study of 21470 patients, who had no liver disease prior to treatment by herbal TCM. This study focused on the effect of herbal TCM use on the liver integrity of patients with normal liver test results of alanine aminotransferase (ALT), used as a diagnostic biomarker to exclude liver disease. Since this study followed a strict protocol and was conducted under clinical conditions, the risk of confounding variables appeared low and should provide valid data.

MATERIALS AND METHODS

Study design

TCM study cohort: To assess to what extent herbal

TCM treatment leads to liver injury, we designed a protocol for a prospective study in consecutive patients, who were admitted to the Hospital for TCM in Bad Kötzting, Germany. Hospital admission was commonly arranged by patients' general practitioners or medical specialists with the intention of a TCM-based therapy. No restrictions on admission exist for patients residing in Germany, as hospital costs are covered by most German statutory sickness funds. Treatment modalities including indications, choice of specific herbal TCM products, daily dosage, and duration of therapy are based on the recommendations of the Beijing University of Chinese Medicine (BUCM), China[32,33].

Included in the TCM study cohort were all inpatients with normal ALT values on admission or the following day, who had received treatment with TCM herbs during their hospital stay, and were discharged between January 1, 1994, and December 31, 2015. Initial ALT results were obtained along with a routine blood sampling analysis. The inclusion criteria of normal serum ALT activities on admission ensured a lack of a preexisting liver disease that could later confound the potential diagnosis of liver injury along with herbal TCM treatment. For reasons of transparency, these patients represent the TCM study cohort. ALT was chosen as a specific diagnostic biomarker to clearly exclude or establish a liver disease[25,34]. Patients with increased ALT values on admission were excluded from the study.

To ensure the good medical care of the patients, six German hospital physicians and eight Chinese physicians who trained at the University of Chinese Medicine in Beijing were in charge of the patients at the 80-bed TCM hospital[35]. Also included in the team was also a pharmacist. On admission, hospital physicians provided a complete physical examination for all patients and recorded their past medical history. They also assessed all normal and elevated laboratory values and documented these together with any adverse or medical event during hospitalization in a standardized adverse event record as part of a hospital-based safety and quality assurance program. During the last three days before discharge, the occurrence of liver injury was tested using serum ALT as the appropriate diagnostic tool.

Treatment with TCM was carried out with TCM herbs, given as decoctions from raw materials[36,37]. Overall TCM treatment may also include acupuncture, Chinese manual therapy, and relaxation therapy, as outlined previously[19,35]. Western therapies were continued or prescribed if necessary. Details of prescriptions, each single Western drug, all specific TCM treatment modalities, and the duration of treatment were documented systematically in the hospital files.

Herbal TCM products were obtained from China[38,39]. Prior to use in patients, all herbal TCM products delivered to the hospital underwent a comprehensive preclinical drug control program under the guidance of the Center for Drug Research of the Ludwig-Maximilian University Munich and other drug control centers in China. For herbal TCM product quality and safety assessment, established methods were used that included HPLC, colored TLC photographs, and botanical authenticity proof[40]. This approach aimed to reduce the risk of possible falsification of the herbal products and to ensure concentrations of heavy metals, aflatoxins, and microbial contamination were within the allowed limits. Some of the herbal products were thus rejected for human use before being prescribed to any patient, mostly due to a lack of pharmaceutical quality criteria or detection of contaminants outside the regulatory requirements. All herbal TCM products were also analyzed for microbial contamination[41].

Liver injury study cohort: The liver injury cohort consists of and is limited to those patients of the TCM study cohort who experienced liver injury in connection with treatment with TCM herbs. Liver injury is defined by an elevated serum ALT activity of at least 5 × upper limit normal (ULN) in patients with normal ALT values on admission[25]. Case data of this liver injury cohort were recruited by further scrutinizing the files and adverse event reports supplied by the hospital physicians. Case identification covers age, sex, diagnosis, past medical history, treatment with herbal TCM drugs and conventional drugs, a course of laboratory data, and any adverse or medical event during hospitalization. The case details were recorded and summarized in individual narratives as part of the patients' hospital documents.

For patients identified with newly emerging liver injury, the suspected herbal TCM products were analyzed and closely reviewed. The aim was to highlight TCM medications that might be associated with an increased risk of liver injury. As this safety analysis of herbal TCM drugs was an outcome study within a routine quality assurance program, approval by an ethical review board was not requested. All patients on admission provided informed written consent prior to study enrollment.

Causality assessment using RUCAM

In line with a previous report[19] and the recommendations of the Chinese Society of Hepatology (GSH)[42], a causality assessment of herb induced liver injury (HILI) for individual cases and herbal TCM products was achieved using RUCAM[25]. This is the most commonly used liver-specific, and validated tool for liver injury cases, and a standard form was used to extract core elements of RUCAM[25]. This assessment requires the initial evaluation of liver injury criteria and its pattern in each suspected case. The core elements of RUCAM include: The time period from the beginning until the cessation of herb intake in relation to disease onset or from the cessation of herb use to the onset of the liver injury; de-challenge characteristics with a course of ALT values after cessation or continuation of the herb use; risk factors such as alcohol abuse, age and pregnancy; co-medication with synthetic drugs or other

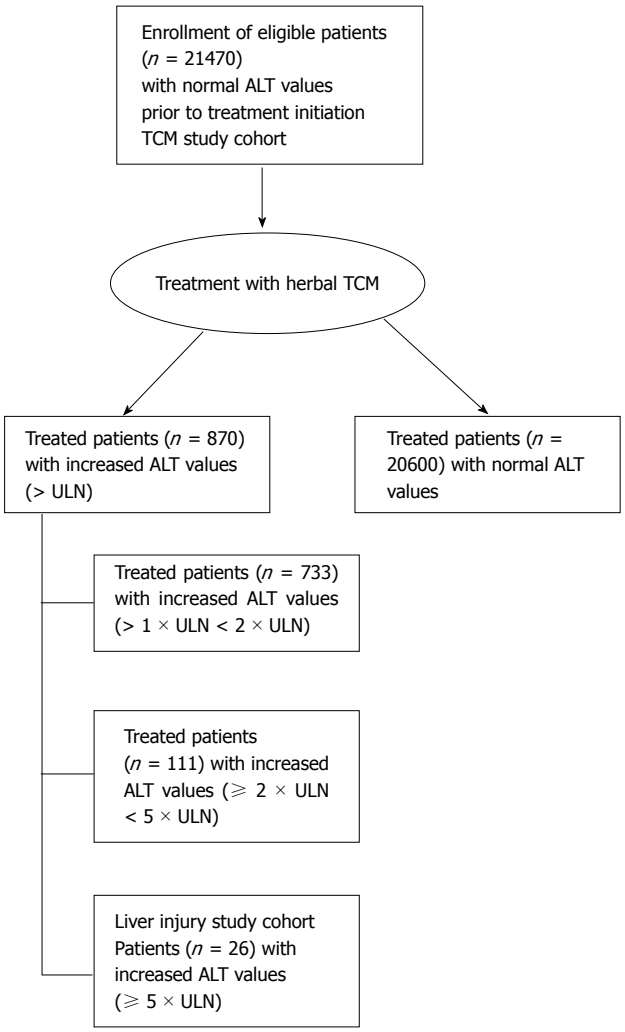

Figure 1 Flow chart of increased alanine aminotransferase values of patients treated at the Traditional Chinese Medicine Hospital Bad Kötzting between 1994 and 2015. TCM: Traditional Chinese Medicine; ALT: Alanine aminotransferase; ULN: Upper limit normal.

herbs; search for alternative causes with special care for all hepatitis types; available information on previous herbal hepatotoxicity; and response to unintentional re-exposure. RUCAM was performed for the hepatocellular type of injury, and scoring was independently conducted by three hospital physicians (Stefan Hager, Sabine Albrecht, Dieter Melchart). Final RUCAM scores commonly range from -5 to + 14 points and the resulting causality levels are defined as follows: ⩽ 0 points, excluded causality; 1-2, unlikely; 3-5, possible; 6-8, probable; and ⩾ 9, highly probable[25].

RESULTS

Flowchart

The inclusion criteria of the TCM study cohort were strict, especially the criterion of normal ALT values on admission and before the initiation of the treatment with herbal TCM. During the study period from 1994 to 2015, overall, 21896 patients were admitted to the hospital, but 426 patients of these had increased ALT values and were not eligible for inclusion in the study, which corresponds to 1.91%. Consequently, 21470 patients fulfilled the inclusion criteria of the TCM study cohort and were treated with herbal TCM (Figure 1). Among these patients of the TCM study cohort, ALT values remained in the normal range in 20600 patients (95.94%) under the TCM treatment. However, treatment led in 733 patients (3.41%) to abnormal ALT values (> 1 × ULN < 2 × ULN), in 111 patients (0.51%) to ALT values of ⩾ 2 × ULN < 5 × ULN, and 26 patients (0.12%) showed ALT ⩾ 5 × ULN, representing the liver injury study group (Figure 1).

Table 1 Traditional Chinese Medicine study cohort with elevated values of alanine aminotransferase as multiples of upper limit normal

ALT as multiples of the ULN	Patients (*n*) with ALT elevation
Total > 1 × ULN < 2 × ULN	733
⩾ 2 × ULN < 3 × ULN	71
⩾ 3 × ULN < 4 × ULN	32
⩾ 4 × ULN < 5 × ULN	8
Total ⩾ 2 × ULN < 5 × ULN	111
⩾ 5 × ULN < 6 × ULN	6
⩾ 6 × ULN < 7 × ULN	2
⩾ 7 × ULN < 8 × ULN	4
⩾ 8 × ULN < 9 × ULN	3
⩾ 9 × ULN < 10 × ULN	2
⩾ 10 × ULN	9
Total ⩾ 5 × ULN	26
Total number of elevated values of ALT	870

ALT: Alanine aminotransferase; ULN: Upper limit normal.

TCM study cohort

ALT abnormalities with values in a range from 2 × up to 5 × ULN observed in 111 patients in the TCM study cohort are clearly caused by the herbal TCM treatment (Figure 1 and Table 1), with a preference of the ALT range between 2 × and 3 × ULN (Table 1). These small increases are commonly without clinical relevance and likely due to metabolic adaptation caused by events associated with the metabolism of TCM plant chemicals.

Analysis of the TCM study cohort showed that the age was 52.7 ± 14.0 years (mean ± SD), and females accounted for 71.9% (Table 2). All patients in this cohort suffered for about 7.8 years (median) from chronic disorders that led to hospital admission (Table 2).

Chronic diseases or health conditions prevailed in the patients of the TCM study cohort (Table 2), whereby the majority experienced psychosomatic diseases as well as chronic pain syndromes. Additional diagnoses were, for example, hypertension and sleep disturbance. Herbal TCM decoctions were provided with four to five prescriptions of about 11 TCM herbs (ranging from a minimum of 6 to a maximum of 19 different herbs) per prescription during the hospital stay. The dosage of each herb was 6-15 g/d. The total

Table 2 Comparison of Traditional Chinese Medicine study cohort with liver injury study cohort

Parameter	TCM study cohort	Liver injury study cohort	*P* (difference between both cohorts)
Patients (*n*)	21470	26	
ALT (U/L, mean ± SD)	NA	300.0 ± 172.9	
Females (%)	71.9	84.6	NS
Age (yr, mean ± SD)	52.7 ± 14.0	57.6 ± 10.5	NS
Chronic diseases (%)	58.9	66.6	NS
Duration of complaints (yr, median)	7.8	8.5	NS
Duration of herbal TCM treatment (d, median, range)	20 (8-77)	19.5 (7-28)	NS
Total dosage of herbal TCM (g, mean, range)	88 ± 18 (18-208)	95 ± 30 (43-155)	< 0.05
Hospital stay (d, mean ± SD)	26.2 ± 5.2	26.1 ± 4.0	NS

NA: Not available; NS: Not significant; ALT: Alanine aminotransferase; TCM: Traditional Chinese Medicine.

daily dosage per prescription was mean 88 ± 18 g (range 18-208), provided by two dosages a day.

Liver injury study cohort

The liver injury study cohort consisting of 26 patients with serum ALT ⩾ 5 × ULN (Table 1) merits further consideration (Table 2). Compared with the large TCM study cohort, patients in the liver injury study cohort were older (52.7 ± 14.0 years *vs* 57.6 ± 10.5 years) and contained a higher percentage of women (71.9% *vs* 84.6%), whereas the duration of the hospital stay was similar in both cohorts (Table 2). There is a long list of indications for herbal TCM treatment in the patients in the liver injury study group, along with individual TCM herbs that were used as medication (Table 3). For these patients with confirmed liver injury, details are given for maximum ALT values, which range from 140 U/L to 1052 U/L (Table 3).

TCM herbs were rarely applied as a mono-preparation, but mostly as mixtures consisting of several herbs adding up to 35 different drugs during the patients' four-week stay. The daily dosage was 95 ± 30 g and thus slightly higher than in the TCM study cohort (Table 2). Among the many herbal TCM used by the 26 patients in the liver injury cohort, Bupleuri radix and Scuterllariae radix were the two TCM herbs most frequently implicated in liver injury, with variable RUCAM-based causality gradings. Most of the patients received one to six TCM drugs that were associated with potential liver injury as evidenced from the scientific literature, *e.g.*, one patient (case 8) received six potentially hepatotoxic herbal TCM drugs during their hospital stay (Table 4).

Narratives

Narratives are essential for case details including treatment conditions and are presented for reasons of transparency and possible re-evaluation by peers or regulatory agencies. The narratives were documented in the hospital case records and are provided for all 26 patients in the liver injury study cohort (Table 5). In only one patient (case 8), none of the potential hepatotoxic TCM herbs was prescribed. Half of the patients were also under co-medication with synthetic drugs, initiated prior to admission, and only a few of these drugs are known for their hepatotoxic potential. The RUCAM analysis excluded all co-medicated drugs as the cause of liver injury in the cases under consideration (Table 5).

Among the liver injury study cohort, 12/26 (46%) of the patients experienced one or more gastrointestinal symptoms such as abdominal pain, diarrhea (6/12), nausea (4/12), vomiting (3/12), and intestinal colicky cramps (3/12) (Table 2). These symptoms are most likely the result of incipient liver injury due to herbal TCM and may be interpreted as a clinical warning signal. Following the discontinuation of herbal TCM treatment, the symptoms rapidly vanished and ALT values normalized in virtually all patients in the liver injury study cohort.

RUCAM-based causality assessment and grading

For all 26 cases in the liver injury study cohort, causality for the used herbal TCM and co-medicated synthetic drugs used was assessed using RUCAM. RUCAM-based causality for TCM herbs was probable in 8/26 patients, possible in 16/26, and excluded in 2/26 cases. All details are presented to facilitate thorough information and reassessment by other groups or regulators (Table 6).

Assessing causality in the 26 cases is indeed challenging, but RUCAM can handle this condition fairly well. All patients used a mixture of several TCM herbs (Table 5). The exposure conditions of the suspected herbs are identical, especially regarding start of use and discontinuation. Therefore, basic causality gradings should be identical, unless some herbs have a record of known previous liver injury, which gives two extra RUCAM points, as compared to other herbs without such records, which do not allow two extra points. Therefore, differences in causality grading for TCM herbs can be achieved considering the criteria of known hepatotoxicity. In the absence of such criteria, causality must be attributed to all the herbs together that were used, without the possibility of differentiating between the various herbs. Some patients in the liver injury study cohort also used conventional drugs, which were prescribed either before they were included in the study or during hospitalization. RUCAM was also applied to

Table 3 Indication for treatment, maximum value of alanine aminotransferase, suspected Traditional Chinese Medicine herbs and Roussel Uclaf Causality Assessment Method-based causality grading in liver injury cases 1-26

Cases	Indication for TCM treatment	Maxi-mum ALT (U/L)	Suspected TCM herbs	RUCAM-based causality
Patient 1	Asthma Depression Lower back pain syndrome	341	Bupleuri radix Glycyrrhizae radix Scutellariae radix	Possible (score +4)
Patient 2	Posttraumatic paralysis of both legs	140	Bupleuri radix Glycyrrhizae radix Scutellariae radix	Possible (score +3)
Patient 3	Chronic bronchitis Emphysema Sleeping disorder	234	Bupleuri radix Ephedrae herba Glycyrrhizae radix Scutellariae radix	Probable (score +7)
Patient 4	Chronic migraine	168	Bupleuri radix Glycyrrhizae radix	Probable (score +6)
Patient 5	Post herpes zoster state Hypertension Diabetes mellitus	330	Bupleuri radix Dictamni radicis cortex Scutellariae radix	Excluded (score -1)
Patient 6	Chronic migraine Cervico-brachial pain syndrome Low back pain syndrome Diarrhoea	530	Bombyx batryticatus (t) Psoraleae fructus (semen) Scutellariae radix	Possible (score +3)
Patient 7	Lumbosacral plexus syndrome Cervicobrachial pain syndrome	132	Bupleuri radix Dictamni radices cortex Ephedrae herba Scutellariae radix	Possible (score +5)
Patient 8	Polyneuropathy	193	Decoction; none identified suspected herb	Possible (score +3)
Patient 9	Polymyalgia rheumatica Fibromyalgia	162	Bupleuri radix Scutellariae radix	Possible (score +4)
Patient 10	Chronic migraine Tension headache	195	Bombyx batryticatus (t) Bupleuri radix Meliae toosendan fructus Scutellariae radix	Possible (score +3)
Patient 11	Difficulty of walking Polyneuropathy Low back pain syndrome	325	Glycyrrhizae radix	Excluded (score -1)
Patient 12	Chronic fatigue Depressive episodes Gastrointestinal symptoms	751	Meliae toosendan fructus	Probable (score +7)
Patient 13	Low back pain syndrome Sleeping disorder	389	Cassiae semen	Possible (score +5)
Patient 14	Chronic osteomyelitis	1052	Meliae toosendan fructus Scutellariae radix	Probable (score +7)
Patient 15	Chronic fatigue Chronic cephalgia	290	Bupleuri radix Meliae toosendan fructus Scutellariae radix	Possible (score +4)
Patient 16	Lichen sclerosus Cervical spondylosis	715	Bombyx batryticatus (t) Bupleuri radix Scutellariae radix	Possible (score +5)
Patient 17	Chronic migraine Depression	252	Bombyx batryticatus (t) Bupleuri radix Cassiae semen Scutellariae radix	Probable (score +6)
Patient 18	Spondylosis cervicalis Depression Migraine	233	Bombyx batryticatus (t) Bupleuri radix Scutellariae radix	Probable (score +6)
Patient 19 (2011)	Carcinophobia Tinnitus	249	Bombyx batryticatus (t) Bupleuri radix	Probable (score +6, 2011)
(2014)	Allergic sensitivity syndrome	295	Ephedrae herba Puerariae radix Polygoni multiflora caulis Scutellariae radix	Probable (score +7, 2014)
Patient 20	Migraine Lower back pain syndrome Depressive episodes	207	Bupleuri radix Ephedrae herba Glycyrrhizae radix Polygoni cuspidate rhizoma Scutellariae radix	Possible (score +4)
Patient 21	Neurasthenia Fibromyalgia	221	Bupleuri radix Glycyrrhizae radix Rhei radix et rhizoma Scutellariae radix	Possible (score+5)

Patient 22	Tension headache Somatoform pain disorder Polyarthritis	361	Glycyrrhizae radix Scutellariae radix	Possible (score +3)
Patient 23	Alopecia cranialis totalis Hashimoto-Thyroiditis	268	Bupleuri radix Polygoni multiflori radix Psoraleae fructus (semen)	Possible (score +4)
Patient 24	Chronic migraine Depression	210	Bombyx batryticatus (t) Bupleuri radix Polygoni multiflori caulis Scutellariae radix	Probable (score +6)
Patient 25	Chronic pain syndrome	359	Bupleuri radix Scutellariae radix	Possible (score +5)
Patient 26	Somatization Gastrointestinal symptoms	182	Bupleuri radix Glycyrrhizae radix Meliae toosendan fructus Scutellariae radix	Possible (score +3)

For ALT until 2002, the normal range was ≤ 24 U/L for males and females, thereafter ≤ 35 U/L for females and ≤ 50 U/L males. Causality levels are as follows: ≤ 0 points, excluded causality; 1-2, unlikely; 3-5, possible; 6-8, probable; and ≥ 9, highly probable. TCM: Traditional Chinese Medicine; RUCAM: Roussel Uclaf Causality Assessment Method.

Table 4 Frequency of herbal Traditional Chinese Medicine use in patients with liver injury with Traditional Chinese Medicine herbssuspected to cause and case number correlation to Roussel Uclaf Causality Assessment Method based causality grading

Potentially hepatotoxic TCM herbs	Total herbs (*n*)	RUCAM-based causality grading: Probable Cases	RUCAM-based causality grading: Possible Cases	RUCAM-based causality grading: Excluded Cases
Bombyx batryticatus(t)	7	17, 18, 19, 24	6, 10, 16	
Bupleuri radix	19	3, 4, 17, 18, 19, 24	1, 2, 7, 9, 15, 16, 17, 20 21, 23, 25, 26	5
Cassiae semen	2	17	13	
Dictamni radicis cortex	2		7	5
Ephedrae herba	4	3, 19	7, 20	
Glycyrrhizae radix	9	3, 4	1, 2, 20, 21, 22, 26	11
Meliae toosendan	5	12, 14	10, 15, 26	
Polygoni cuspidate rhizoma	1		23	
Polygoni multiflori caulis	2	19, 24		
Polygoni multiflori radix	1		23	
Psoraleae fructus (semen)	1		23	
Puerariae radix	1	19		
Rhei radix et rhizoma	1		21	
Scutellariae radix	20	3, 14, 17, 18, 19, 24	1, 2, 6, 7, 9, 10, 15, 16, 20, 21, 22, 25, 26	5

TCM: Traditional Chinese Medicine; RUCAM: Roussel Uclaf Causality Assessment Method.

these co-medicated drugs, which may differ regarding their previous hepatotoxicity and their duration of use. However, it is unlikely that drugs may have caused the increases of ALT during the hospitalization since at the time of inclusion in the study, the ALT values were normal.

DISCUSSION

This report provides liver injury data derived from a prospective, hospital-based and large-scale study of 21470 patients, who had no liver disease prior to treatment with TCM herbs for the first time. Clinically relevant liver injury with ALT ≥ 5 × ULN developed in 26 patients (0.12%) (Figure 1 and Tables 1-5). These data suggest that TCM herbs carry a risk of liver injury in line with other reports[9,27,28,43] and concomitantly dismiss contrarian claims that TCM herbs lack hepatotoxic potency[16]. However, the surprisingly low frequency of liver injury caused by herbal TCM in this study (Figure 1) is at a variance with several reports implying that liver injury cases due to these herbs occur at a high frequency[2,9,11,17,19,27,28,43]. The rarity of liver injury cases found in the present investigation may be explained by the strict study protocol: (1) prospective rather than retrospective study approach; (2) valid exclusion of pre-existing liver disease prior to the start of the therapy with TCM herbs; (3) hospital-based treatment with specifically trained TCM physicians from Germany and China; (4) use of good quality TCM herbal products specifically ascertained by appropriate analyses; (5) therapy with a median of 19.5 d, avoiding prolonged treatment; (6) selective inclusion in the study only of those patients meeting the liver injury criteria ALT ≥ 5 × ULN; and (7) causality assessment using RUCAM and ascertaining ALT dechallenge following

Table 5 Narratives of the cases 1-26 of the liver injury study cohort

Patients	Narratives
Case 1 male 51 yr (1994)	Patient with asthma (ICD-9 493.9), chronic low back pain (ICD-9 724.2), and reactive depression (ICD-9 300.4) treated with TCM decoctions with 8 drugs for 23 d: Angelicae sinensis radix, Asari herba, Astragali radix, Atractylodis macrocephalae rhizoma, Bupleuri radix, Glycyrrhizae radix, Paeoniae rubrae radix, Poria (parts), Scutellariae radix. Total daily dose: 80 g. Co-medication theophylline, fluocortolon. No alcohol abuse. Adverse events: nausea after drinking the decoction. ALT 293 U/L. First control after 5 d: ALT 341 U/L. Second control 14 d after discharge: ALT 17 U/L. No hepatitis serology RUCAM-based causality for Bupleuri radix, Glycyrrhizae radix and Scutellariae radix: Possible (score +4)
Case 2 male 73 yr (1994)	Patient suffered from unclear paralytic symptoms in both legs after trauma (ICD-9 344). Herbal TCM treatment with 9 drugs: Angelicae sinensis radix, Asari herba, Astragali radix, Atractylodis macrocephalae rhizoma, Bupleuri radix, Glycyrrhizae radix, Paeoniae rubrae radix, Poria (Stücke), Scutellariae radix for 22 d. Total daily dose: 60 g. Co-medication: digoxine, carbochol, nitrofurantoin, and sulfadiazine. No alcohol abuse. No adverse event symptoms. At discharge: ALT 140 U/L. First control 3 d later: ALT 100 U/L. Second control 3 wk later: ALT 22 U/L. No hepatitis serology RUCAM-based causality for Bupleuri radix, Glycyrrhizae radix, and Scutellariae radix: Possible (score +3)
Case 3 female 68 yr 1995	Patient with chronic bronchitis (ICD-9 491), emphysema (ICD-9 492), and sleeping disorder (ICD-9 780.50) was treated with herbal TCM decoctions (10 drugs) for 26 d: Angelicae sinensis radix, Asari herba, Astragali radix, Atractylodis macrocephalae rhizoma, Bupleuri radix, Ephedrae herba, Glycyrrhizae radix, Paeoniae rubrae radix, Poria (Stücke), Scutellariae radix. Total daily dose: 80 g. No co-medication. No alcohol abuse. No adverse event symptoms. ALT at discharge: 234 U/L. First control at 4 wk after discharge: ALT 7 U/L. No hepatitis serology RUCAM-based causality for Bupleuri radix, Ephedrae herba, Glycyrrhizae radix, and Scutellariae radix: Probable (score +7)
Case 4 female 47 yr (1996)	Patient with migraine (ICD-9 346.0) was treated for 28 d with the following herbal TCM decoctions (15 drugs): Angelicae dahuricae radix, Angelicae sinensis radix, Armeniacae amarum semen, Asari herba, Bupleuri radix, Codonopsis pilosulae radix, Evodiae fructus, Forsythiae fructus, Glycyrrhizae radix, Isatidis radix, Ligustici chuanxiong rhizoma, Ligustici rhizoma, Lonicerae flos, Platycodi radix, Prunellae spica. Total daily dose: 130 g. No comedication. No alcohol abuse. No adverse event symptoms. ALT at discharge 168 U/L. At control 4 wk later: 18 U/L; Hepatitis serology post increased ALT detection: anti-HAV (IgM/IgG) negative; HBs-Ag negative; anti-HBs negative; anti-HBc (IgM/IgG) negative RUCAM-based causality for Bupleuri radix and Glycyrrhizae radix: Probable (score +6)
Case 5 male 77 yr (1998)	Patient with post herpes zoster state (ICD-9 053.13), hypertension (ICD-9 401), diabetes mellitus (ICD-9 250) was treated for 12 d with 11 herbal TCM decoctions: Bupleuri radix, Chebulae fructus, Dictamni radicis cortex, Gentianae macrophyllae rhizoma, Margaritifera usta concha (t), Moutan radicis cortex, Myristicae semen, Paeoniae rubrae radix, Rehmanniae radix, Scutellariae radix, Sophorae flavescentis radix. Total daily dose: 110 g. Co-medication with potentially hepatotoxic drugs: zolpidem, anti-factor 10 Xa-activity. No alcohol abuse. Adverse event symptoms: Fever 38.6 °C, erythema, and transient scleral jaundice. At discharge ALT of 330 U/L, at control 12 wk after discharge < 24 U/L. No hepatitis serology RUCAM-based causality for Bupleuri radix, Dictamni radicis cortex, and Scutellariae radix: Excluded (score -1)
Case 6 female 60 yr (1999)	Patient Suffered From Chronic Migraine (ICD-9 346), Cervico-Brachial Pain Syndrome Left Side (ICD-9 723.3), Lower Back Pain Syndrome (ICD-9 724.2), And Diarrhea (ICD-9 787.91) Without Clear Gastrointestinal Diagnosis Since 4 yr. Herbal TCM Medication (22 Drugs) As Decoction For 22 D: Angelicae Dahuricae Radix, Astragali Radix, Atractylodis Rhizoma, Bombyx Batryticatus (T), Chrysanthemi Flos, Cicadae Periostracum (T), Cinnamomi Ramulus, Citri Reticulatae Pericarpium, Codonopsis Pilosulae Radix, Coicis Semen, Euryales Semen, Evodiae Fructus, Ligustici Rhizome, Lycopi Herba, Moutan Radicis Cortex, Myristicae Semen, Pinelliae Praeparatae Rhizome, Poria (Parts), Psoraleae Fructus (Semen), Punicae Granati Pericarpium, Rehmanniae Praeparatae Rhizome, Scutellariae Radix, Uncariae Cum Uncis Ramulus, Viticis Fructus. Total Daily Dose: 150 G. Co-Medication With Potentially Hepatotoxic Drugs: Ibuprofen 800 And Piroxicam 10. Alcohol Consumption 1 Drink Beer Daily. After Treatment For 5 D, Improvement Of Diarrhea, And After 22 D Migraine Attack Treated With Ibuprofen. Directly After Intake Of Ibuprofen, She Noticed Symptoms With Stomach Pain, Nausea, Vomiting. ALT At Discharge 530 U/L, At Control 47 D Later: 14 U/L. Hepatitis Serology Post Increased ALT: Anti-HAV-Igg Positive; Anti-HAV-Igm Negative; Anti-Hbs Negative; Anti-Hbc Negative RUCAM-Based Causality For Bombyx Batryticatus (T), Psoraleae Fructus (Semen), And Scutellariae Radix: Possible (Score +3)
Case 7 female 58 yr (1999)	Patient with lumbosacral plexus syndrome (ICD-9 953.5) and cervico-brachial syndrome (ICD-9 723.3) was treated with 24 drugs for 26 d with decoctions: Angelicae sinensis radix, Asteris radix, Bupleuri radix, Cinnamomi ramulus, Coptidis rhizoma, Dictamni radicis cortex, Ephedrae herba, Farfarae flos, Forsythiae fructus, Ginkgo semen, Glehniae radix, Isatidis folium, Lonicerae flos, Lumbricus (t), Ophiopogonis radix, Paeoniae alba radix, Paeoniae rubra radix, Perillae fructus, Pinelliae praeparatae rhizoma, Platycodi radix, Rehmanniae praeparatae rhizome, Rehmanniae radix, Scutellariae radix. Total daily dose 110 g. Co-medication: theophylline, vitamin E. No alcohol abuse. Adverse event symptoms: Diarrhea, headache, nausea, and vomiting. ALT at discharge: 35 U/L; at first control 5 d later ALT 132 U/L, at second control 4 wk later 8 U/L. No hepatitis serology RUCAM-based causality for Bupleuri radix, Dictamni radices cortex, Ephedrae herba, and Scutellariae radix: Possible (score +5)
Case 8 male 65 yr (2000)	Patient with polyneuropathy (ICD-9 357.2), who was treated with 14 drugs for 22 d with Angelicae pubescentis radix, Astragali radix, Chaenomelis fructus, Cinnamomi ramulus, Coptidis rhizoma, Corydalis rhizoma, Lonicerae caulis, Lumbricus (t), Mori ramulus, Moutan radicis cortex, Paeoniae rubrae radix, Rehmanniae praeparatae rhizoma, Spatholobi caulis, and Trachelospermi caulis. Total daily dose 130 g. Co-medication with potential liver toxicity: allopurinol 300 mg, atorvastatin 10 mg. Alcohol consumption 3-4 drinks beer per day. No adverse events. Safety check after 10 d of treatment: ALT < 24 U/L; at discharge: 193 U/L; first control 16 d later: ALT 24 U/L RUCAM-based causality for all used TCM herbs: Possible (score +3)
Case 9 female 78 yr 2000	Patient with polymyalgia rheumatica (ICD-9 725) and fibromyalgia (ICD-9 74.1), treated with 17 drugs for 23 d with: Astragali radix, Bupleuri radix, Carthami flos, Cinnamomi ramulus, Coptidis rhizoma, Curcumae longae rhizoma, Cyperi rhizoma, Glehniae radix, Ligustri lucidi fructus, Luffae fructus retinervus, Lycopi herba, Mori ramulus, Paeoniae rubrae radix, Rehmanniae praeparatae rhizoma, Scutellariae radix, Sparganii tuber (rhizoma), Trachelospermi caulis. Total daily dose: 84 g. Co-medication with potential liver toxicity: Triamterene, diclofenac. No alcohol abuse. No adverse event symptoms. ALT at discharge 162 U/L; first control 21 d later: 12 U/L. Hepatitis serology post increased ALT: anti-HAV IgG positive: Anti-HAV IgM negative; HBs-antigen negative; anti-HBs 250 U/L, anti-HBc positive RUCAM-based causality for Bupleuri radix and Scutellariae radix: Possible (score +4)

Case 10 female 35 yr (2002)	Patient suffered from migraine (ICD-9 346.0) and tension headache (ICD-9 307) since 20 yr. Treatment with 20 drugs: Albiziae cortex, Amomi cardamomi semen, Angelicae dahuricae radix, Angelicae sinensis radix, Artemisiae argyi folium, Bombyx batryticatus (t), Bupleuri radix, Codonopsis pilosulae radix, Dolichoris album semen, Evodiae fructus, Ligustici rhizoma, Margaritifera usta concha (t), Meliae toosendan fructus, Mori ramulus, Notopterygii rhizoma seu radix, Paeoniae albae radix, Prunellae spica, Puerariae radix, Scutellariae radix, Tribuli fructus as decoctions. Total daily dose: 95 g. Herbal TCM treatment for 28 d. Co-medication with potentially hepatotoxic drug: estradiol. Occasional alcohol use. Adverse event symptoms: abdominal pain and diarrhea. ALT at discharge 195 U/L; at first control 10 d later ALT 56 U/L. No hepatitis serology RUCAM-based causality for Bombyx batryticatus (t), Bupleuri radix, Meliae toosendan fructus, and Scutellariae radix: Possible (score +5)
Case 11 female 74 yr (2003)	Patient with difficulty of walking (ICD-9 719.7), polyneuropathy (ICD-9 357.2), and low back pain (ICD-9 724.2). Herbal TCM treatment with decoctions (25 drugs): Achyranthis bidentatae radix, Albiziae cortex, Amomi cardamomi semen, Angelicae pubescentis radix, Angelicae sinensis radix, Astragali radix, Atractylodis macrocephalae rhizoma, Chaenomelis fructus, Coicis semen, Coptidis rhizoma, Corydalis rhizoma, Glycyrrhizae radix, Margaritifera usta concha (t), Mori ramulus, Moutan radicis cortex, Paeoniae albae radix, Paeoniae rubrae radix, Phellodendri cortex, Rehmanniae praeparatae rhizoma, Salviae miltiorrhizae radix, Sappan lignum, Spatholobi caulis, Trachelospermi caulis, Tribuli fructus, Ziziphi spinosae semen.Total daily dose: 150 g. Co-medication with potentially hepatotoxic drug: candesartan 16 mg/die (regular), enalapril (rare). No alcohol abuse. No adverse event symptoms. Safety check after 14 d: ALT 165 U/L. At discharge: ALT 325 U/L. First control: ALT 61 U/L, Hepatitis serology post increased ALT: hepatitis B and C excluded; anti-HAV (IgG) positive RUCAM-based causality for Glycyrrhizae radix: Excluded (score -1)
Case 12 female 62 yr (2004)	Patient with chronic fatigue (ICD-10 L53), depressive episodes (ICD-10 F32.9), and gastrointestinal symptoms (ICD-10 K59.9) including abdominal pains and flatulence. Treatment with herbal TCM decoctions (18 drugs) for 26 d: Albiziae cortex, Amomi cardamomi semen, Angelicae sinensis radix, Astragali radix, Aurantii fructus, Citri sarcodactylis fructus, Codonopsis pilosulae radix, Coicis semen, Corydalis rhizoma, Curcumae radix, Eriocauli flos, Meliae toosendan fructus, Moutan radicis cortex, Notoginseng radix, Phragmitis rhizoma, Platycodi radix, Poria (parts), Schisandrae fructus. Total daily dose 96 g. Co-medication with clorazepate. No alcohol abuse. ALT at discharge 751 U/L. Seventeen days after cessation of herbal TCM products: ALT 148 U/L. No more subsequent ALT results available. Adverse event symptoms: Directly after discharge from the TCM-hospital, the patient was admitted at another hospital with a department of internal medicine due to deterioration of gastrointestinal symptoms. Serology: EBV-IgG 590 U/L, EBV-IgM negative RUCAM-based causality for Meliae toosendan fructus: probable (score +7)
Case 13 female 57 yr (2004)	Patient with arthralgia (ICD-10 M25.5), lower back pain syndrome (ICD-10 M54.4), and sleeping disorder (ICD-10 G 47.9) was treated with herbal TCM decoctions (14 drugs) for 25 d: Achyranthis bidentatae radix, Albiziae cortex, Angelicae sinensis radix, Astragali radix, Cassiae semen, Cinnamomi ramulus, Curcumae longae rhizoma, Gentianae macrophyllae rhizoma, Loranthi ramulus, Notoginseng radix, Notopterygii rhizoma seu radix, Periploca radicis cortex, Psoraleae fructus (semen), Spatholobi caulis. Total daily dose: 110 g. Co-medication: L-thyroxine, aminophylline. No alcohol abuse. Adverse event symptoms: abdominal pain, loss of appetite, and single vomiting. ALT at discharge 389 U/L; at first control 4 d later: ALT 191 U/L, and at second control 15 d later: ALT 22 U/L. Hepatitis serology post increased ALT: anti-HAV (IgG+IgM) positive; anti-HAV IgM negative; HBs-Antigen negative; anti-HBs positive; anti-HBc negative; HCV ab: negative RUCAM-based causality for Cassiae semen: possible (score +5)
Case 14 Female 52 yr (2006)	Patient with chronic osteomyelitis (ICD-10 M86.99) left leg and a six-year history after open fracture. PMH of hepatitis A 1968. Treatment with herbal TCM decoctions (12 drugs) for 24 d: Achyrantis bidentatae radix, Amomi cardamom semen, Chaenomelis fructus, Citri grandis exocarpium, Coicis semen, Corydalis rhizoma, Mangolie officinalis cortex, Meliae toosendan fructus, Paeoniae rubra radix, Poria (parts), Scutellariae radix, Zingiberis rhizoma. Total daily dose: 155 g. Co-medication with potential hepatotoxicity: use of not overdosed paracetamol (once) and ibuprofen (when needed, but presently no intake). No alcohol abuse. At day 20 after admission, patient showed adverse event symptoms like abdominal pain of the colic type with intestinal cramps, nausea, and mushier diarrhea. No ascites, no splenomegaly, no hyperbilirubinemia. ALT at discharge 1052 U/L. Three days later: 692 U/L and 35 d later: 33 U/L. Normal hepatitis serology with exclusion of hepatitis A, B, and C RUCAM-based causality for Meliae toosendan fructus and Scutellariae radix: Probable (score +7)
Case 15 female 43 yr (2007)	Patient suffered from unclear post-infectious chronic fatigue (ICD-10 G93; 10 F.43) and chronic cephalgia (ICD-10 R51). Known history of EBV infection. Treatment with herbal TCM decoctions containing the following 23 components for 19 d: Albiziae cortex, Anemarrhenae rhizoma, Astragali radix, Bambusae caulis in taeniam, Bupleuri radix, Chaenomelis fructus, Cinnamomi ramulus, Citri reticulatae pericarpium, Curcumae longae rhizoma, Epimedii herba, Leonuri herba, Ligustri lucidi fructus, Lycii fructus, Magnoliae officinalis cortex, Meliae toosendan fructus, Paeoniae rubra radix, Polygalae radix, Poria (parts), Pseudostellariae radix, Pyrrosiae folium, Salviae miltiorrhizae radix, Scutellariae radix, Tribuli fructus. Total daily dose: 69 g. Co-medication: L-thyroxine. No alcohol abuse. Adverse event symptom: abdominal pain. ALT at discharge 290 U/L; 12 d later at first control: ALT 181 U/L. Second control 24 d later: ALT 81 U/L; third control 28 d later: normal ALT values. No hepatitis serology RUCAM-based causality for Bupleuri radix, Meliae toosendan fructus, and Scutellariae radix: Possible (score +4)
Case 16 female 58 yr (2007)	Patient with lichen sclerosus (ICD-10 L90.0) and cervical spondylosis (ICD-10 M47.0) was treated with herbal TCM decoctions (11 drugs) for 25 d: Amomi cardamomi semen, Atractylodis rhizoma, Bambusae caulis in taeniam, Bombyx batryticatus (t), Bupleuri radix, Coicis semen, Kochiae fructus, Phellodendri cortex, Rehmanniae radix, Salviae miltiorrhizae radix, Scutellariae radix. Total daily dose 43 g. No co-medication. No alcohol abuse. No adverse event symptoms. ALT values at discharge normal. Continued use of herbal TCM at home, but at reduced daily dose of 26 g (corresponding to about 60% of the individual hospital dosage). Twenty-one days after hospital stay, safety check: ALT 715 U/L. Cessation of the herb use. Fifteen days after the first control: ALT 113 U/L. Again 14 d thereafter, at a second control: ALT 44 U/L; and at a third control, ALT 23 U/L. Hepatitis serology post first increased ALT values: Hepatitis B and C excluded. Anti-HAV-IgG positive; Anti-HAV-IgM negative RUCAM-based causality for Bombyx batryticatus (t), Bupleuri radix, and Scutellariae radix: Possible (score +5)
Case 17 female 48 yr (2010)	Patient with migraine (ICD-10 G 43.0) and depression (ICD-10 F 32.0) was treated with 11 herbal TCM components as decoctions for 20 d: Angelicae dahuricae radix, Bambusae caulis in taeniam, Bombyx batryticatus (t), Bupleuri radix, Cassiae semen, Curcumae longae rhizoma, Dipsaci radix, Gentianae macrophyllae rhizoma, Scutellariae radix, Siegesbeckiae herba, Trichosanthis fructus. Total daily dose: 60 g. No co-medication. No alcohol, Adverse event symptom: abdominal pain. Safety control: ALT 279 U/L. Cessation of the herb use. Five days after cessation and at discharge: ALT 252 U/L. First control 14 d after hospital discharge: ALT 12 U/L. Hepatitis serology post increased ALT: Hepatitis A, B, C excluded. Anti-EBV-VCA-IgG > 100; EBV-VCA-IgM < 0.9; HBsAb 474 Units; HBc ab negative; HCV ab not reactive; EBV-EBNA1-Ab (IgG) > 100 Units; Anti-HAV (IgM) negative; Anti-HBc (IgM) negative

	RUCAM-based causality for Bombyx baytryticuatus (t), Bupleuri radix, Cassiae semen and Scutellariae radix: Probable (score +6)
Case 18 female 52 yr (2010)	Patient with depression (ICD-10 F32.1), migraine (ICD-10 G43.0), spondylosis cervical (ICD-10 M47.8), and cephalgia (ICD-10 R51) treated with 16 drugs: Achyranthis bidentatae radix, Angelicae dahuricae radix, Bambusae caulis in taeniam, Bombyx batryticatus (t), Bupleuri radix, Citri reticulatae pericarpium, Coicis semen, Curcumae longae rhizoma, Magnoliae officinalis cortex, Paeoniae rubra radix, Polygalae radix, Poria (Stücke), Scutellariae radix, Tribuli fructus, Viticis fructus, Zingiberis rhizoma. Total daily dose: 96g. Duration of treatment for only 7 d because of adverse event symptoms of diarrhea ad deterioration of headache. Co-medication: L-thyroxine. Safety check 14 d after admission: ALT 76 U/L; At first control 20 d after admission: ALT 233 U/L. At discharge: ALT 198 U/L. Control 30 d after discharge: ALT 35 U/L. Serology post increased ALT: Anti-HAV (IgG, IgM) negative; anti-HAV (IgM) negative; HBs-antigen negative, Anti-HBs < 10 IU/L; Anti-HBc (IgG + IgM) negative; Anti-HCV negative
	RUCAM-based causality for Bombyx batryticatus (t), Bupleuri radix, and Scutellariae radix: Probable (score +6)
Case 19 female 60 yr (2011)	In 2011, patient with carcinophobia (ICD-10 F45.2), allergic sensitivity (ICD-10 T78.4) and tinnitus (ICD-10 H93.1) was treated with 28 drugs for 19 d with: Achyranthis bidentatae radix, Angelicae sinensis radix, Armeniacae amarum semen, Astragali radix, Atractylodis macrocephalae rhizoma, Aurantii immaturus fructus, Bambusae caulis in taeniam, Bombyx batryticatus (t), Bupleuri radix, Chrysanthemi flos, Cinnamomi ramulus, Curcumae longae rhizoma, Curcumae radix, Ephedrae herba, Edebouriellae radix, Ligustri lucidi fructus, Liquidambaris fructus, Luffae fructus retinervus, Lycii fructus, Menthae herba, Mori ramulus, Paeoniae albae radix, Paeoniae rubrae radix, Persicae semen, Peucedani radix, Polygoni multiflori caulis, Schisandrae fructus, Scutellariae radix. Daily drug dose 119 g. No co-medication. No alcohol. No adverse event symptoms. ALT at discharge 249 U/L; at first control after 3 d 123 U/L; at second control after 69 d 30 U/L. No hepatitis serology
	RUCAM-based causality in 2011 for Bombyx batryticatus (t), Bupleuri radix, Ephedrae herba, Polygoni multiflori caulis, and Scutellariae radix: Probable (score +6)
63 yr (2014)	In 2014, the patient was treated again with some of the previous herbal components as in 2011, now with 13 drugs: Atractylodis rhizoma; Bupleuri radix; Carthami flos; Clematidis radix; Curcumae longae rhizoma; Curcumae radix; Lycopodii herba; Mori ramulus; Pinelliae praeparatae rhizoma; Poria (parts); Puerariae radix; Sappan lig; Scutellariae radix. Total daily dose: 104 g. No co-medication. No alcohol abuse known. No adverse event symptoms. Because of the previous experience, liver enzyme control already after 7 d: ALT 295 U/L; cessation of all herbal TCM products. Six days later ALT 182 U/L, and 3 d thereafter: ALT 86 U/L. Eleven days later: ALT 34 U/L. No hepatitis serology
	RUCAM-based causality in 2014 for Bupleuri radix, Puerariae radix, and Scutellariae radix: Probable (score +7)
Case 20 female 53 yr (2012)	Patient with depressive episode (ICD-10 F33.1), migraine (ICD-10 G43.1), and low back-pain (ICD-10 M54.1) was treated for 22 d the with following 12 components: Achyranthis bidentatae radix, Angelicae dahuricae radix, Bupleuri radix, Cuscutae semen, Ligustici chuanxiong rhizome, Liquidambaris fructus, Lycii fructus, Rehmanniae radix, Scutellariae radix, Trachelospermi caulis, Tribuli fructus, Uncariae cum uncis ramulus. Total daily dose: 88 g. Additional wind-heat-mixture: Polygoni cuspidate rhizoma, Glycyrrhizae radix for 3 d. Wind-cold-mixture: Ephedra herba and Polygoni cuspidate rhizoma. Total daily dose: No comedication. No alcohol. No adverse effect symptoms. ALT at discharge 207 U/L, 22 d later at first control 30 U/L. No hepatitis serology
	RUCAM-based causality for Bupleuri radix, Ephedra herba, Glycyrrhizae radix, Polygoni cuspidate rhizoma, and Scutellariae radix: Possible (score +4)
Case 21 female 53 yr (2013)	Patient with neurasthenia (ICD-10 F48.0) and fibromyalgia (ICD-10 M79.7) received herbal TCM decoction for 18 d with the following 24 herbs: Achyranthis bidentatae radix, Astragali radix; Atractylodis rhizoma; Aurantii immaturus fructus, Bambusae caulis in taeniam, Bupleuri radix, Carthami flos, Citri reticulatae pericarpium, Citri sarcodactylis fructus, Curcumae longae rhizoma, Dipsaci radix, Glycyrrhizae radix, Inulae flos, Loranthi ramulus, Ophiopogonis radix, Paeoniae albae radix, Paeoniae rubrae radix, Persicae semen, Pinelliae praeparatae rhizoma, Pseudostellariae radix, Rehmanniae radix, Rhei radix et rhizoma, Scutellariae radix, Siegesbeckiae herba. Total daily dose: 78 g. No potentially hepatotoxic co-medication (only L-thyroxine). No alcohol. No adverse event symptoms. ALT at discharge 221 U/L and 41 U/L at control 14 d later. No hepatitis serology
	RUCAM-based causality for Bupleuri radix, Glycyrrhizae radix, Rhei radix et rhizoma, and Scutellariae radix: Possible (score +5)
Case 22 female 52 yr (2013)	Patient with somatoform pain disorder (ICD-10F45.0), drug induced tension headache (ICD-10 G45.2), and polyarthritis (ICD-10 M05.9), who was treated with herbal TCM decoctions for 22 d. The following 35 herbs were applied: Achyranthis bidentatae radix, Anemarrhenae rhizoma, Angelicae pubescentis radix, Astragali radix, Atractylodis rhizoma, Bambusae caulis in taeniam, Chaenomelis fructus, Cinnamomi ramulus, Clematidis radix, Coicis semen, Coptidis rhizoma, Curcumae longae rhizoma, Cyperi rhizoma, Dipsaci radix, Glycyrrhizae radix, Homalomenae rhizoma, Ligustri lucidi fructus, Liquidambaris fructus, Lonicerae caulis, Loranthi ramulus, Luffae fructus retinervus, Magnoliae officinalis cortex, Mori ramulus, Notopterygii rhizoma seu radix, Paeoniae rubrae radix, Pinelliae praeparatae rhizoma, Polygalae radix, Scutellariae radix, Siegesbeckiae herba, Sinomenii caulis, Sparganii tuber (rhizoma), Trachelo-spermi caulis, Tribuli fructus, Trichosanthis fructus, Uncariae cum uncis ramulus. Total daily dose was 78 g. Co-medication with the potentially hepatotoxic drug: omega-3-acidethylester. No alcohol-abuse, Adverse event symptoms: diarrhea, abdominal pain, and flatulence. Hepatitis B serology negative; HBs-Ag negative, Anti-HCV negative. At discharge: ALT 361 U/L and 15 U/L at control 90 d later
	RUCAM-based causality for Glycyrrhizae radix and Scutellariae radix: Possible (score +3)
Case 23 female 46 yr (2014)	Patient with alopecia cranialis totalis (ICD-10 L63.0) and Hashimoto-thyroiditis (ICD-10 E06.3) was treated for 28 d with a decoction containing 15 TCM herbs: Achyranthis bidentatae radix, Angelicae sinensis radix, Atractylodis macrocephalae rhizoma, Bambusae caulis in taeniam, Bupleuri radix, Citri reticulatae pericarpium, Cuscutae semen, Ledebouriellae radix, Lycii fructus, Periploca radicis cortex, Polygonati rhizoma, Polygoni multiflori radix, Poria (parts), Psoraleae fructus (semen), Testudinis plastrum (t). Total daily dose: 72 g. Co-medication with L-thyroxine. No alcohol. Adverse event symptom: flatulence. At discharge ALT 268 U/L, with 210 U/L on day 20 and 62 U/L on day 30
	RUCAM-based causality for Bupleuri radix, Polygoni multiflori radix, and Psoraleae fructus (semen): Possible (score +4)
Case 24 female 51 yr (2014)	Patient with depression (ICD-10 F33.1) and migraine (ICD-10 G43.0) took for 17 d the herbal TCM decoction with the following 18 herbs: Achyranthis bidentatae radix, Angelicae sinensis radix, Bombyx batryticatus (t), Bupleuri radix, Curcumae longae rhizoma, Curcumae radix, Kochiae fructus, Ligustri lucidi fructus, Lycii fructus, Mori radicis cortex, Mori ramulus, Paeoniae rubrae radix, Polygoni multiflori caulis, Salviae miltiorrhizae radix, Scutellariae radix, Sparganii tuber (rhizoma), Spatholobi caulis, Tribuli fructus. Total daily dose was 60 g. Comedication: cimicifuga, zolpidem. No alcohol. Lack of adverse event symptoms. At discharge and control: ALT 210 U/L and 191 U/L. At a subsequent control 19 d later, ALT 36 U/L
	RUCAM-based causality for Bombyx batryticatus (t), Bupleuri radix, Polygoni multiflori caulis, and Scutellariae radix: Probable (score +6)

Case 25 female 53 yr (2015)	Patient with chronic pain syndrome (ICD-10 F 45.41; G43.9) received for 3 wk (twice daily) herbal TCM decoctions containing 20 different herbs: Angelicae sinensis radix, Aurantii fructus, Bambusae caulis in taeniam, Bupleuri radix, Carthami flos, Citri sarcodactylis fructus, Curcumae longae rhizoma, Curcumae radix, Ligustici chuanxiong rhizoma, Loranthi ramulus, Lycopodii herba, Mori ramulus, Persicae semen, Poria (parts), Puerariae radix, Scutellariae radix, Sparganii tuber (rhizoma), Spatholobi caulis, Tribuli fructus, Viticis fructus. Total daily dose: 87 g. Co-medication: intermittent use of sumatriptane and the potentially hepatotoxic drug paracetamol. No alcohol. No adverse effect symptoms. ALT at discharge 359 U/L, at control 18 d later ALT 69 U/L. Hepatitis A and B were excluded serologically RUCAM-based causality for Bupleuri radix and Scutellariae radix: Possible (score +5)
Case 26 female 61 yr -2015	Patient with unspecified somatization (ICD-10 F45.1), who suffered from abdominal symptoms of nausea, diarrhea, and loss of appetite, received herbal TCM decoction therapy for 23 d with 26 herbs: Amomi cardamomi semen, Amomi fructus, Armeniacae amarum semen, Atractylodis macrocephalae rhizoma, Bambusae caulis in taeniam, Bupleuri radix, Cinnamomi ramulus, Citri sarcodactylis fructus, Codonopsis pilosulae radix, Coicis semen, Corydalis rhizoma, Cyperi rhizoma, Forsythiae fructus, Glehniae radix, Glycyrrhizae radix, Ledebouriellae radix, Meliae toosendan fructus, Mori radicis cortex, Ophiopogonis radix, Paeoniae albae radix, Paeoniae albae radix, Peucedani radix, Pinelliae praeparatae rhizoma, Poria (parts), Scutellariae radix, Zingiberis rhizoma. Total daily dose: 87 g. Intermittent co-medication with the potentially hepatotoxic pantoprazole. Alcohol use with 2 drinks a day. During hospital stay, she experienced deterioration of her gastrointestinal symptoms. At discharge, ALT 182 U/L. Two weeks later, normalization of ALT (30 U/L). No hepatitis serology RUCAM-based causality for Bupleuri radix, Glycyrrhizae radix, Meliae toosendan fructus, and Scutellariae radix: Possible (score +3)

All patients showed normal ALT values at admission and experienced liver injury under therapy with herbal TCM. Indication for TCM treatment was based on diagnoses according to ICD classification. Liver injury is defined as ALT > 5 × ULN. TCM herbs with known hepatotoxicity from literature represented in bold[8-10]. Causality for all bold TCM herbs was assessed using the updated RUCAM[25]. Causality levels are as follows: ≤ 0 points, excluded causality; 1-2, unlikely; 3-5, possible; 6-8, probable; and ≥ 9, highly probable[3]. ALT normal ≤ 24 U/L until 2001 for males and females, thereafter ≤ 35 U/L for females and ≤ 50 U/L for males. ab: Antibodies; ag: Antigen; ALT: Alanine aminotransferase; CMV: Cytomegalovirus; EBV: Epstein Barr virus; HAV: Hepatitis A virus; HBc: Hepatitis B core; HBs: Hepatitis B surface; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HEV: Hepatitis E virus; ICD: International Statistical Classification of Diseases and Related Health Problems; PMH: Past medical history; RUCAM: Roussel Uclaf Causality Assessment Method; TCM: Traditional Chinese Medicine.

discontinuation of the herbal TCM therapy. Such excellent investigational conditions rarely exist under normal field conditions, where patients are evaluated in retrospective studies, and often provide cases of limited data quality[2,6,17], mostly with the lack of a robust causality assessment such as RUCAM[25]. Despite these encouraging data under hospital conditions, herbal TCM treatment outside a hospital setting may be associated with higher liver injury risks, requiring a cautionary statement. Consequently and to err on the side of caution, patients who opt for special therapy with herbal TCM should be informed about the low risk of liver injury and its clinical symptoms.

Supportive evidence of causality for TCM herbs in the injury cases was provided by the rapid decline of ALT to nearly normal values shortly following cessation of herbal use in 24/26 patients (Table 5), while two patients escaped final ALT analysis (Table 5). Causality is further supported by the lack of pre-existing liver diseases in the patients in the liver injury study cohort, ruling out that alternative liver diseases could compete with the newly emerging liver injury caused by TCM herbs. Finally, the causality of liver injury for various TCM herbs was established using the updated RUCAM (Table 6), as published in 2016[25]. Causality was excluded in two patients, whereas most cases achieved a possible or even a probable causality level. Using both, RUCAM-based causality assessment and positive tests of unintentional re-exposures, valid causality was provided for numerous TCM herbs by other published analyses of liver injury[9,27,28,43].

It appears that patients with acute liver injury due to TCM herbs commonly have a good prognosis and no transition to chronic liver injury, at least under the treatment conditions of a hospital, and is possibly attributed to the exclusion of prolonged treatment as described in the present study (Table 2). This favorable outcome is in line with a previous RUCAM-based study, which does not report on severe courses[27], but is in contrast to a retrospective study[9] and another analysis[28]: Both publications reported severe clinical courses with the risk of acute liver failure, requirement of liver transplant, and of death[9,28]. In more detail, acute liver failure was reported in 7.8%, a requirement for liver transplant in 0.6%, and a fatality rate in 3.2%, but associated RUCAM-based causality gradings were not published in the study[9]. This was done in another report of 54 patients with an RUCAM-based causality grading of probable for herbal TCM: One patient had used a herbal TCM product for 60 d and required a liver transplantation, while another one died after using TCM herb for 30 d[28]. The difference in outcome between the present study and previous publications[9,28] cannot validly be explained and is certainly open for discussion, especially regarding the duration of herbal TCM exposure, which was 19.5 d in this study (Table 2).

Of note, in addition to the 21470 patients, who were included in the TCM study cohort due to their normal ALT values, 472 patients corresponding to 2.3% had been admitted to the hospital for TCM treatment with increased ALT values on admission and were therefore not included in the TCM study cohort. If included, these patients may have alternative diagnoses as confounders, as initially increased ALT values may reflect already existing liver disease. Similar to the hospital conditions, alternative causes as confounding variables have been described in suspected cases of

Table 6 Causality assessment for cases 1-26 of the liver injury study cohort, using the updated Roussel Uclaf Causality Assessment Method[25]

RUCAM items with attribution of scores (SC)	CASES 1-26																										
	SC	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1 Time to onset from the beginning of the herb																											
5-90 d	+2	+2	+2	+2	+2	+2	+2	+2	+2	+2	+2	+2	+2	+2	+2	+2	+2	+2	+2	+1	+2	+2	+2	+2	+2	+2	+2
< 5 or > 90 d	+1																										
Alternative: Time to onset from cessation of the herb																											
≤ 15 d	+1																										
2 Course of ALT after cessation of the herb																											
Decrease ≥ 50% within 8 d	+3										+3				+3			+3		+1			+3				
Decrease ≥ 50% within 30 d	+2	+2	+2	+2	+2			+2	+2	+2			+2	+2		+2	+2		+2		+2	+2		+2	+2	+2	+2
No information of continued drug use	0					0						0															
Decrease ≥ 50% after the 30th day	0						0																				
Decrease < 50% after the 30th day or recurrent increase	-2																										
3 Risk factors																											
Alcohol use (current drinks/d: > 2 for woman, > 3 for men)	+1																										
Alcohol use (current drinks/d: ≤ 2 for woman, ≤ 3 for men)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/0	0	0	0	0	0	0	0
Age ≥ 55 yr	+1		+1	+1		+1	+1	+1	+1	+1		+1	+1	+1			+1			+1		+1					+1
Age < 55 yr	0	0			0						0				0	0		0	0		0		0	0	0	0	
4 Concomitant drug(s)/herb(s)																											
None or no information	0							0						0	0	0	0	0	0	0/0	0						
Concomitant drug/herb with incompatible time to onset	0			0	0																	0		0			
Concomitant drug/herb with compatible or suggestive time to onset	-1									-1															-1	-1	
Concomitant drug/herb known as hepatotoxin and with compatible or suggestive time to onset	-2		-2			-2	-2		-2		-2	-2											-2				-2
Concomitant drug with evidence for its role in this case (positive rechallenge or validated test)	-3																										
5 Search for alternative causes																											
Group I (7 causes)																											
HAV: Anti-HAV-IgM		Ø	Ø	N	N	Ø	N	Ø	Ø	Ø	Ø		N		N	Ø	Ø	N	N	Ø/Ø	Ø	Ø	Ø	Ø	Ø	N	Ø
HBV: Anti-HBC-IgM, HBV-DNA		Ø	Ø	N	N	Ø	N	Ø	Ø	Ø	Ø	N	N	N	N	Ø	Ø	N	N	Ø/Ø	Ø	Ø	N	Ø	Ø	N	Ø
HCV: Anti-HCV, HCV-RNA		Ø	Ø	N	Ø	Ø	N	N	Ø	Ø	Ø	N	N	N	N	Ø	Ø	N	N	Ø/Ø	Ø	Ø	N	Ø	Ø	Ø	Ø
HEV: Anti-HEV-IgM, anti-HEV-IgG, HEV-RNA		Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø/Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
Hepatobiliary sonography/ colour Doppler sonography of liver vessels/endo-sonography/CT/MRC		Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	N	Ø	Ø	Ø	Ø	Ø/Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
Alcoholism		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N/N	N	N	N	N	N	N	N
Acute recent hypotension history (particularly if underlying heart disease)		N	N	N	N		N	N	N	N	N	N	N	N	N	N	N	N	N	N/N	N	N	N	N	N	N	N
Group Ⅱ (5 causes)																											
Complications of underlying disease(s) such as sepsis, metastatic malignancy, autoimmune hepatitis, chronic hepatitis B or C, primary biliary cholangitis or sclerosing cholangitis, genetic liver diseases																											

Infection suggested by PCR and titre change for CMV (anti-CMV-IgM, anti-CMV-IgG)		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N/N	N	N	N	N	N	N	N
EBV (anti-EBV-IgM, anti-EBV-IgG)		Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø/Ø	Ø	Ø	Ø	Ø Ø	Ø	Ø	Ø
HSV (anti-HSV-IgM, anti-HSV-IgG)		Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø		Ø	Ø	Ø	Ø	+	Ø	Ø/Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
VZV (anti-VZV-IgM, anti-VZV- IgG)		Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø/Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
Evaluation of group I and Ⅱ		Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø/Ø	Ø	Ø	Ø		Ø	Ø	Ø
All causes - groups I and Ⅱ - reasonably ruled out	+2																									
The 7 causes of group I ruled out	+1																									
6 or 5 causes of group I ruled out	0			0	0		0						0		0			0	0						0	
Less than 5 causes of group I ruled out	-2	-2	-2			-2		-2	-2	-2	-2	-2		-2		-2	-2			1	-2	-2	-2	-2	-2		-2
Alternative cause highly probable	-3																									
6 Previous information on hepatotoxicity of the herb																										
Reaction labelled in the product characteristics	+2	+2	+2	+2	+2		+2	+2		+2	+2		+2	+2	+2	+2	+2	+2	+2	+2/+2	+2	+2	+2	+2	+2	+2	+2
Reaction published but unlabelled	+1								+1																	
Reaction unknown	0					0						0														
7 Response to unintentional reexposure																										
Doubling of ALT with the herb alone, provided ALT below 5 ULN before reexposure	+3																									
Doubling of ALT with the herb already given at the time of first reaction	+1																			+1						
Increase of ALT but less than ULN in the same conditions as for the first administration	-2																									
Other situations	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0
Total score for case		4	3	7	6	-1	3	5	3	4	3	-1	7	5	7	4	5	6	6	6/7	4	5	3	4	6	5	3

Total score for causality: ⩽ 0/excluded; 1-2/unlikely; 3-5/possible; 6-8/probable; ⩾ 9/highly probable. Considered were the items for the hepatocellular type of liver injury and the data of 26 patients with normal ALT values at hospital admission, who experienced elevated ALT levels > 5 × ULN after use of herbal TCM. In the above section 4 (part 1 of the tabula) of concomitant drug(s), the following TCM herbs and drugs were considered. In the section 5 (search for alternative causes in part 2 of the tabula), the symbol of (n) denotes that the obtained result was negative, whereas (Ø) indicates that assessment was not performed. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CT: Computed tomography; HAV: Hepatitis A virus; HBc: Hepatitis B core; HBV: Hepatitis B virus; HCV: Hepatitis C virus; CMV: Cytomegalovirus; EBV: Epstein Barr virus; HEV: Hepatitis E virus; HSV: Herpes simplex virus; MRC: Magnetic resonance cholangiography; PCR: Polymerase chain reaction; RUCAM: Roussel Uclaf Causality Assessment Method; ULN: Upper limit normal; VZV: Varicella zoster virus.

DILI[44,45] and HILI[46] including those caused by TCM herbs[9,46], and dietary supplements[46-50].

It is likely that in the real world, some patients seeking therapy with TCM herbs have a pre-existing, initially not recognized liver disease[9]. Uncertainty exists as to whether pre-existing liver disease is a risk factor for liver injury by TCM herbs, in analogy to some chronic liver diseases such as non-alcoholic fatty liver disease or alcoholic liver disease, which are risk factors of drug-induced liver injury (DILI) by some special drugs[51,52]. Our study clearly differentiated liver injury with ALT values of ⩾ 5 × ULN from ALT abnormalities with ALT values of < 5 × ULN (Table 1). Those are considered as an adaptive phenomenon due to the metabolism of the chemicals provided from the herbs to the liver. These adaptive abnormalities are clinically not relevant. The present data do not allow the general clinical recommendations to analyze ALT before therapy with TCM herbs is considered, as liver injury is unpredictable. ALT assessment may be helpful in legal situations to prevent later patient claims.

The rarity of hepatotoxic reactions together with normal dosages of herbal TCM used in this study imply idiosyncrasy as a cause rather than an intrinsic mechanism, which is dose-dependent and can be elucidated by experimental studies. However, pathogenetic steps leading to the dose-independent idiosyncratic liver injury are largely unknown due to lack of appropriate animal models[17]. In analogy with other herbs, TCM herbs including those incriminated as

causes of liver injury in the present study (Tables 3 and 5) contain dozens of known chemicals as ingredients but their specific hepatotoxic potency is difficult to assess and remains largely unknown[28]. Another problem of most TCM therapy regimens is the multiplicity of herbs included as ingredients in herbal mixtures[19,28], such as up to 35 in the present study (Table 5). Multiple plant chemicals of many herbs may lead to an increased risk of liver injury, which were described at least for DILI if several drugs were co-administered[53], and for herb-herb interactions or herb-drug interactions, if concomitantly used with Western drugs[54]. Used as co-medication to Western antipsychotic drugs such as quetiapine, clozapine, and olanzapine, in the case of Bupleuri radix it is known that this is associated with nearly 60% of the risk of adverse outcomes[4,12,55]. Other potential risk factors for liver injury by TCM herbs may include higher dosages and lipophilicity of chemicals and known conditions from DILI cases[56]. Publications on the quality problems of some herbal TCM products[20-24] called for providing excellent quality for our patients as high priority, which is a strength of this study and avoids discussions around product quality as causative for the observed liver injury.

In the present study, Bupleuri radix and Scutellariae radix are the two TCM herbs most implicated in liver injury (Tables 3-5). However, both herbs turned out to be the most frequently prescribed drugs in the TCM hospital in Bad Kötzting in general[57]. Even though liver injury from Polygonum multiflorum has increasingly been reported in recent years[9-11], but convincing evidence for causality is limited in the present analysis (Tables 3-5). Previous regulatory discussions focused on herbal products containing unsaturated pyrrolizidine alkaloids (PAs), and in 2012, EMA stated that herbal medicinal products containing herbal preparations with toxic, unsaturated PAs (even in very low amounts) should not be used orally[58]. Used in high amounts for a prolonged period, PAs can cause HSOS (hepatic sinusoidal obstruction syndrome)[19]. TCM herbs also include Jue Ming Zi (Cassia), but only its leaves and fruits contain PAs and may cause HSOS[19,58]. Cassiae semen lacks PAs that has been used by two patients, who as expected had no signs of HSOS (Table 5).

The use of herbal TCM is widely considered less risky as compared with synthetic drugs, although data on direct comparisons are not available in support of this view. Populations using herbal TCM, drugs, either alone, or combined experience more DILI than HILI, possibly due to a higher use of drugs[27]. Valid data of incidence and prevalence of HILI caused by TCM herbs are lacking[19], and respective data cannot be derived from the present study with a low frequency of liver injury of 0.12% among all 21470 patients treated with herbal TCM. Valid data were published for drugs, showing that idiosyncratic DILI is a rare event, in a population-based French study with an annual estimated incidence of 13.9 ± 2.4 cases per 100000 inhabitants[53]. A good overview of suspicious TCM herbs is provided in several reports[2,13-15,34], which were also used for comparison in our own survey (Table 5). Nevertheless, the list of suspected TCM herbs remains tentative (Tables 3-5).

Limitations of our study: The focus of our investigation was on ALT levels > 5 × ULN, considering thereby real HILI cases. Cases with ALT elevations between 2 and 5 × ULN are per definition not real but milder HILI due to treatment with TCM herbs, not requiring additional causality proof using RUCAM. As all patients with real HILI had a good outcome with ALT normalization during the relatively short follow-up periods, this favorable outcome can be expected also for patients with milder HILI. A single normal pre-treatment ALT value likely excludes pre-existing liver disease, though little uncertainty remains, which would decrease rather than increase the overall frequency of HILI by TCM. By study protocol, patients with increased ALT values were explicitly not included, although it would have been of interest how TCM treatment influences increased pre-treatment ALT values.

In this report, we present liver injury data for the first time derived from a prospective, hospital-based and large-scale study of 21470 patients, who received treatment with TCM herbs and had no liver disease before. ALT was used as a diagnostic biomarker to exclude liver disease prior to therapy initiation and to assess liver integrity during and after the therapy. This study of 21470 patients revealed that herbal TCM products cause rare liver injury in 26 patients corresponding to 0.12%. Liver injury rapidly improved in most patients following cessation of the therapy, also substantiating causality for the suspected TCM herbs. Under the present study conditions, a transition of acute liver injury to a chronic course was not observed. As these encouraging results are based on strict protocol in a hospital setting, it remains to be established whether these data can be transferred to normal field conditions. Indeed, in the real world confounding variables prevail, such as pre-existing chronic liver diseases, complex therapy conditions of co-medication with Western drugs, and possible problems of herbal TCM product quality regarding misidentification of herbs, impurities of heavy metals, pesticides and other toxins, and adulteration by Western drugs to enhance efficacy. To be on the side of caution and for risk minimizing physicians are well advised to inform patients about the low risk and symptoms of liver injury associated with the use of TCM herbs, if patients decide on this special therapy option.

COMMENTS

Background

Herbal Traditional Chinese Medicines (TCMs) are worldwide in common use, which is well documented in the literature. They are highly appreciated, as

they are of natural origin, and mainly based the belief of their efficiency and lack of adverse events, and their preference as valuable alternatives over a conventional treatment with synthetic drugs. However, some criticism emerged regarding the issue of efficiency, and adverse reactions ranging from clinically not relevant events to more severe ones including suspected liver injury.

Research frontiers

In a prospective, hospital-based study, patients with normal values of alanine aminotransferase (ALT) as a diagnostic marker for ruling out pre-existing liver disease were enrolled and reassessed on discharge by routine laboratory within a safety program carried out at the First German Hospital of TCM from 1994 to 2015. Liver injury was detected in 26/21470, patients (0.12%) with normal liver tests prior to treatment initiation. In most of the liver injury cases, the Roussel Uclaf Causality Assessment Method (RUCAM)-based causality for herbal TCM was graded as "possible".

Innovations and breakthroughs

In this report, the authors present for the first time liver injury data derived from a prospective, hospital based and large-scale study of 21470 patients, who received treatment with TCM herbs and had no liver disease before.

Applications

The findings in this study may help to bring more objectification into discussion about TCM herbs and their risks of liver injury. As long as the therapeutic efficacy of TCM herbs is poorly documented in the scientific literature, even low risk of liver injury by TCM herbs has to be communicated to all potential consumers and to the academic public.

Terminology

Liver injury was defined ALT $\geqslant$ 5 × ULN = upper limit of normal as clinically relevant.

Peer-review

This is an interesting and well-written study.

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CASE REPORT

Bone metastases as initial presentation of hepatocellular carcinoma

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Author contributions: Monteserin L, Mesa A and Fernandez-Garcia MS designed the report; Monteserin L, Gadanon-Garcia A and Varela M collected the patient's clinical data; Rodriguez M and Varela M analyzed the data and wrote the paper.

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Abstract

Extra-hepatic spread is present in 5% to 15% of patients with hepatocellular carcinoma (HCC) at the time of diagnosis. The most frequent sites are lung and regional lymph nodes. Here, we report 3 cases of unsuspected HCC with symptoms due to bone lesions as initial presentation. Morphological characteristics and immunohistochemistry from the examined bone were the key data for diagnosis. None of the patients had an already known chronic liver disease. Differential diagnoses with HCC upon ectopic liver disease or hepatoid adenocarcinoma were shown. Therapy with the orally active multikinase inhibitor sorafenib plus symptomatic treatment was indicated.

Key words: Hepatocellular carcinoma; Bone metastases;

Liver cirrhosis; Sorafenib

Core tip: Metastatic hepatocellular carcinoma should be included within the differential diagnoses of bone metastases of unknown origin, even in the absence of already known chronic liver disease.

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INTRODUCTION

Screening programs for early detection of hepatocellular carcinoma (HCC) have been shown to be cost-effective and to improve survival[1]. However, there are a proportion of patients who develop HCC in the presence of an unknown primary chronic liver disease that is diagnosed at the time of first decompensation, generally ascites. In addition, incidence of non-alcoholic fatty liver disease is increasing; it has been shown that these patients develop HCC before cirrhosis was established and, generally, that they are diagnosed out of surveillance as well. In these two last situations, patients can present with extra-hepatic disease and fewer options of effective therapy to prolong survival. We report, herein, 3 cases of HCC that debuted as metastatic bone lesions.

CASE REPORT

Case 1

A 64-year-old male, current smoker (20 cigarettes daily) and drinker (20 g alcohol daily), presented with pain in the lower right limb that had begun several months previous. Initially, the pain was attributed to lumbar degenerative pathology in the fourth and fifth lumbar vertebrae. A multidetector computed tomography (MDCT) scan of thorax-abdomen-pelvis with intravenous and oral contrast showed a large, solid, contrast-enhanced mass affecting psoas, iliac and gluteus minor muscles with iliac bone infiltration. This great lytic lesion affected the iliac paddle wall, thinning the acetabular roof. Pelvic magnetic resonance imaging (MRI) study confirmed the destructive, heterogeneous and highly vascularized tumor (Figure 1A and B), resembling an osteochondroma.

Histologically, a solid neoformation formed by cells of epithelioid habit that showed large eosinophilous cytoplasm compartments and irregular, vesiculous nuclei with patent nucleolus and frequent figures of mitosis was observed, accompanied by a rich vascular network adjacent to a soft tissue. Tumor cells were strongly positive for cytokeratin AE1/AE3, cytokeratin 8 and hepatocytes. Cytokeratin 20, 7, 19 and EMA were completely negative. This was conclusive for HCC. A second biopsy performed later over the same site, just if a mistake had been made in the processing or identification of tissue, showed the presence of a proliferation, composed of cells that mimic hepatocytes, with marked incipient anisopleomorphism, extended in sheets and infiltrating different soft tissues. Immunohistochemical staining was performed, showing hepatocyte, cytokeratin AE 1 and AE 3 positivity; the rest of the requested immunohistochemical tests (alpha-fetoprotein (AFP), prostate antigen) were negative again. Therefore, metastasis from a well-differentiated HCC (Figure 1C) was confirmed.

Tests of peripheral blood showed AFP level of 3.4 ng/mL (upper normal value 8 ng/mL), aspartate aminotransferase (AST) of 133 U/L, alanine aminotransferase (ALT) of 35 U/L, alkaline phosphatase (ALP) of 161 U/L, gamma-glutamyl transpeptidase (GGT) of 95 U/L and bilirubin of 1 mg/dL. Liver stiffness measurement value was 12.5 kPa, suggestive of significant fibrosis. Upper endoscopy revealed no signs of portal hypertension. Hepatitis B and C chronic infection were excluded.

Multiphasic hepatic TCMD depicted a 24-mm, ill-defined area that enhanced in the arterial phase, with washout in delayed phases, in segment IV that was associated with vascular invasion of the left portal vein and with left lobe hypertrophy in a polylobed liver (Figure 1D-F). This imaging impressed the finding of tumor injury meeting non-invasive diagnosis criteria for HCC. Taking into account the symptoms, defined as ECOG PS 2[2], and the results of biopsies and radiological staging, BCLC-C hepatocellular carcinoma was diagnosed.

Treatment with analgesics plus external palliative radiotherapy in the pelvic area was initiated. Once the patient's pain and discomfort were alleviated, sorafenib was started at October 11, 2014. Clinical evolution was good, with progressive recovery of general status to ECOG PS 0 together with radiological regression of intra- and extra-hepatic disease. The bone metastases at initial scan (July 21, 2014) measured 47 mm × 132 mm × 181 mm, and measurement of the same lesion at the last study (September 15, 2016) showed it to be 40 mm × 80 mm × 80 mm.

The patient has been taking sorafenib up to the writing of this report, with minor adverse events at full dose. He has been able to stop morphine-derived and non-steroidal anti-inflammatory drugs. Due to excellent tolerance to sorafenib, in case of radiological progression, the patient will be assessed to switch to regorafenib.

Case 2

This is a 66-year-old male, former drinker of 80 g alcohol daily, with chronic hepatitis C virus infection, secondary iron overload, porphyria cutanea tarda, arterial hypertension and obesity. He frequented Traumatology and Emergency Departments due to left inguinal mechanical pain that distally radiated through the left leg since August of 2011. He presented with spontaneous fracture

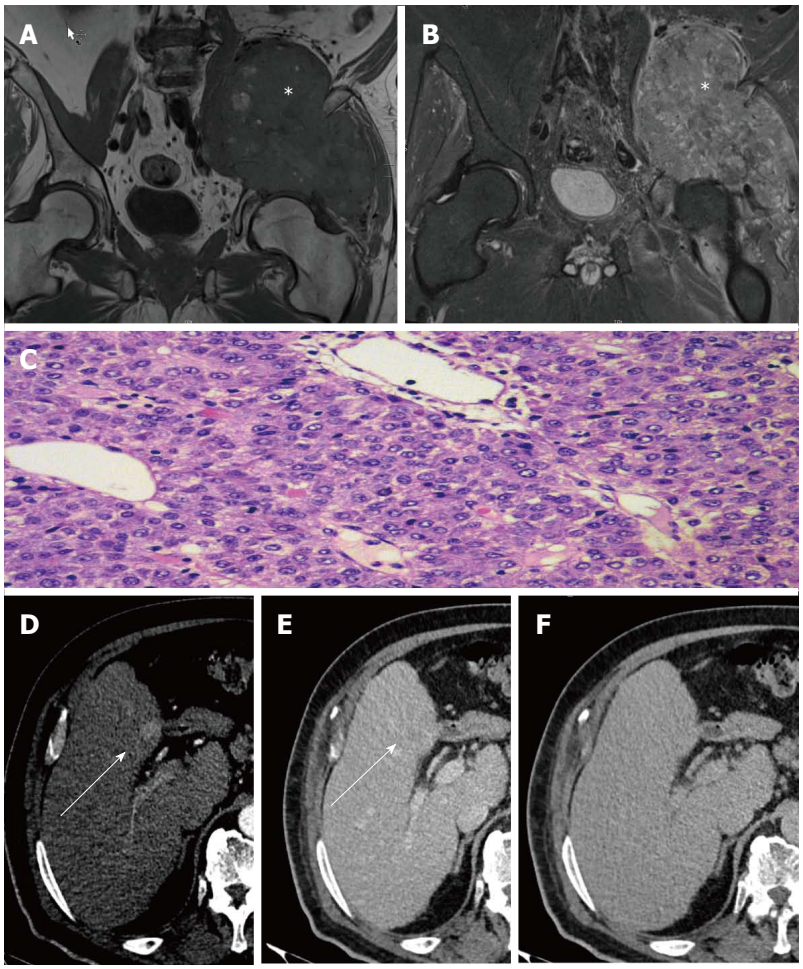

Figure 1 Clinical case 1. A: Axial pelvic MRI T1-weighted imaging showing a large mass in the soft planes with lytic lesion on right iliac blade, markedly hypointense (asterisk); B: Coronal pelvic MRI T1-weighted imaging after administration of intravenous GD-DTPA contrast showing the mass with intense enhancement after administration of contrast (asterisk); C: Biopsy of pelvic mass (hematoxylin-eosin staining, 20 × magnification) showing broad metastatic presentation by well-differentiated HCC; D: Multiphasic liver MDCT showing hepatic focal lesion in segment IV (white arrow) with arterial phase enhancement; E: Multiphasic liver MDCT showing the lesion with slight washout in the portal phase (white arrow); F: Multiphasic liver MDCT showing the lesion as isodense with respect to the parenchyma in the equilibrium phase. HCC: Hepatocellular carcinoma; MDCT: Multidetector computed tomography; MRI: Magnetic resonance imaging.

of the left hip (October 16, 2012) that needed a total hip prosthesis placement (October 22, 2012). Examination of the surgical specimen determined a bone metastasis of undifferentiated carcinoma.

A thorax-abdomen-pelvis MDCT was performed to find the primary tumor. An ill-defined hypodense nodule of less than 1 cm in the right hepatic lobe that cannot be assessed due to its size was seen, together with a small peripancreatic adenopathy and a left femoral neck fracture that did not present sclerotic borders, and represented the location where a hypodense lytic lesion was observed (Figure 2A). A multiphasic liver TCMD informed of normal liver size and morphology.

Upper endoscopy revealed erosive gastritis without esophageal varices. Laboratory values were AFP of 4.2 ng/mL, AST of 227 U/L, ALT of 181 U/L, ALP of 311 U/L, GGT of 629 U/L and bilirubin of 1 mg/dL. A dynamic hepatic MRI was performed and showed multiple small focal lesions, tenuously hypointense in T1 and hyperintense in T2, with intense contrast uptake in the arterial phase and washout in the portal phase in the larger ones (Figure 2B-D). An ultrasonographic-guided biopsy-trucut with an 18-gauge needle was taken over one of the larger liver lesions, and demonstrated a well-differentiated hepatocellular carcinoma, thus identifying the primary focus.

The patient was finally diagnosed with advanced ECOG PS 0 BCLC-C HCC and sorafenib was started on December 18, 2012. Due to radiological progression, he was assessed for second-line clinical trials (February, 2014) but ultimately died due to tumor progression on January 20, 2015.

Case 3

This is a 74-year-old diabetic man, who is a current drinker (40 g alcohol daily) and occasional smoker. In January 2014, he complained of pain in the upper hemiabdomen and pain in the lumbosacral region which radiated to the lateral face of the right thigh. He also presented functional impotence of the right lower limb and dysesthesia. No anorexia, asthenia nor weight lose were present. He was ECOG PS 2.

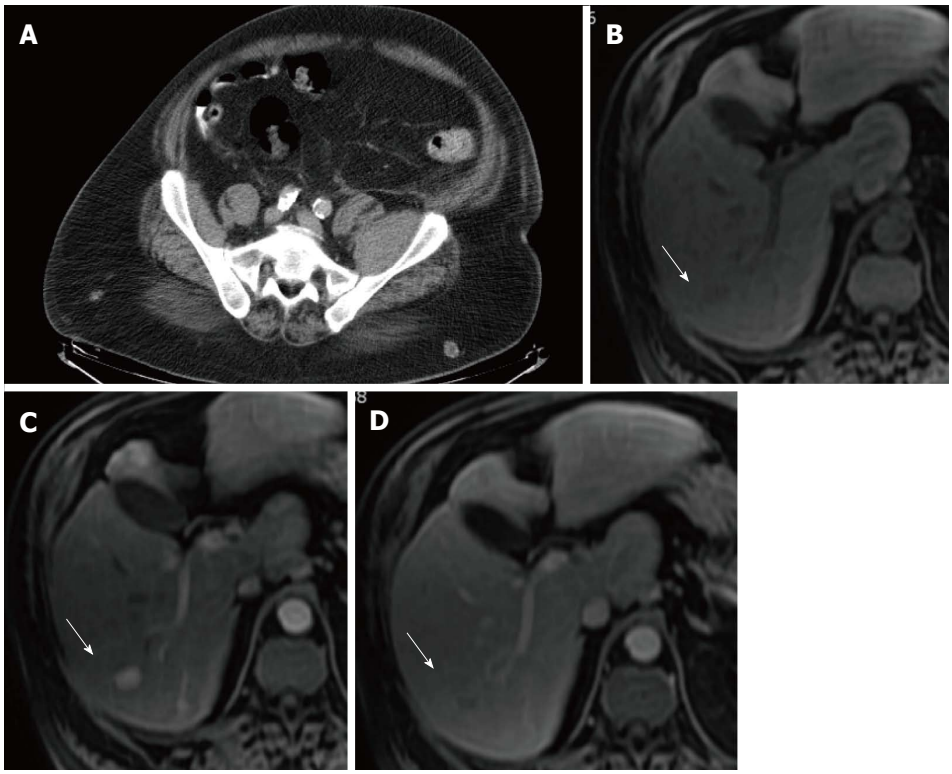

Figure 2 Clinical case 2. A: MDCT showing asymmetry in the psoas-iliac muscles with enlargement of the left iliac muscle; B-D: Dynamic hepatic MRI performed after gadolinium administration. A focal liver lesion with wash in/wash out criteria of hepatocellular carcinoma was depicted in segment VI. MDCT: Multidetector computed tomography; MRI: Magnetic resonance imaging.

Blood count, biochemistry, including liver function tests and AFP, were mostly within the normal range (AFP of 4.2 ng/mL, AST of 128 U/L, ALT of 93 U/L, ALP of 192 U/L, GGT of 459 U/L and bilirubin of 1 mg/dL). Abdominal ultrasound detected a 15-mm hypoecogenic liver focal lesion in the left lobe. The study was completed with a multiphasic abdomen-pelvis MDCT with the finding of a 40 mm × 39 mm focal liver lesion in the right lobe that could correspond with metastases (Figure 3A-C). A pathological fracture with significant stenosis of the central spinal channel provoking compromise of neurological structures was detected in the body of the fourth lumbar vertebrae. Lumbar MRI confirmed this lesion and another very similar one in the second lumbar vertebrae (Figure 3D and E).

The Neurosurgery Team performed a cementation of the fourth lumbar vertebra after an intraoperative biopsy at 14-AUG-2014. The pathological description was compatible with a metastatic focus of HCC, with the following immunophenotypic profile: Cytokeratin AE1/AE3, cytokeratin 8, CD138 and TTF1 positivity, but hepatocyte, cytokeratin 7, CDX, synaptophysin, S100, P40 and P53 negativity (Figure 3F). This patient also presented with several very painful lesions in the dorsal spine.

The patient was administered intrathecal perfusion of fentanyl, intravenous zoledronic bolus and external radiotherapy (total dose of 20 Gy) to ameliorate symptoms. In spite of all efforts, his quality of life was poor and he worsened very fast. He was so fragile that he was not a candidate for sorafenib drug therapy and home-based palliative care provided support during the last 5 mo of his life. He finally deceased on October 26, 2015.

DISCUSSION

HCC is the most common primary tumor in the liver, with the fifth in incidence in men worldwide and occupying the second place in mortality attributed to cancer[3]. It develops mainly in the context of chronic liver disease, especially secondary to chronic infection by hepatitis B and C. Patients who develop HCC generally do not present symptoms; however, it should be suspected in those cases with previously compensated cirrhosis that are complicated by clinical decompensation. Extrahepatic disease is more frequent in advanced tumors (greater than 5 cm, multifocal, with vascular invasion or cancer-related symptoms). Initial manifestation of unsuspected HCC as a bone metastasis is rare according to literature[4-17]. In our referral unit, this is the first sign of unknown HCC in less than 0.9% of incident cases. These cases are usually located in the vertebrae, pelvis, ribs and skull, as shown in Table 1.

In the 3 cases described herein, we have detected both intra- and extra-hepatic disease at once, but sometimes an extra-hepatic HCC without a primary intra-hepatic HCC can be seen. This last situation can

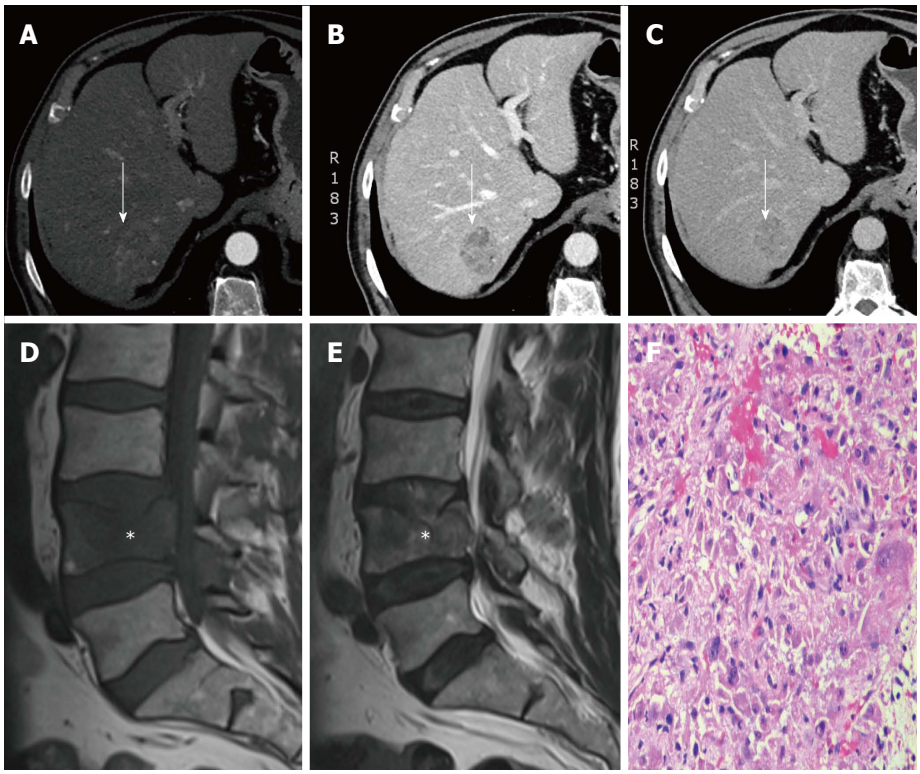

Figure 3 Clinical case 3. A: Multiphasic liver MDCT showing a well-defined hepatic focal lesion in segment VII (white arrow) with heterogeneous enhancement in the arterial phase; B: Multiphasic liver MDCT showing the lesion with clear washout in the portal phase (white arrow); C: Multiphasic liver TCMD showing the lesion as markedly hypodense in the delayed phase; D, E: Lumbar MRI, sagittal T1-weighted and sagittal T2-weighted, showing pathological fracture of the fourth lumbar vertebrae with posterior wall displacement and vertebral channel invasion; F: Surgical specimen, a hemorrhagic biopsy, showing isolated metastatic groups constituted by hepatocytes after staining with hematoxylin-eosin (20 × magnification). MDCT: Multidetector computed tomography; MRI: Magnetic resonance imaging.

Table 1 Cases of bone metastases debuting as unknown hepatocellular carcinoma published in the literature

Ref.	Year	*n*	Location	Survival[1]
Nowak *et al*[4]	1983	1	Rib	NR
Fueyo Margareto *et al*[5]	1986	1	Multiples bones	NR
Raoul *et al*[6]	1995	3	Skull	27 mo (alive)
			Iliac bone	31 mo (alive)
			Femur	31 mo (dead)[2]
Horita *et al*[7]	1996	1	Breastbone	9 mo (alive)[2]
Iosca *et al*[8]	1998	1	Left iliac bone	> 45 mo (alive)[2]
Soto *et al*[9]	2000	1	Rib	28 mo (alive)
Hofmann *et al*[10]	2003	1	Chest wall	12 mo (alive)[2]
Qureshi *et al*[11]	2005	1	Chest wall	NR
Hyun *et al*[12]	2006	1	Rib and third thoracic vertebrae	12 mo (alive)[2]
Rastogi *et al*[13]	2013	1	Scapula and occipital bone	2 mo (alive)[2]
Ruiz-Morales *et al*[14]	2014	2	Vertebrae, ribs, sacrum, scapula	NR
			Cervical vertebrae, right shoulder	NR
Hwang *et al*[15]	2015	1	Vertebral body and iliac bone	8 mo (alive)
Subasinghe *et al*[16]	2015	1	Occipital bone	NR
Alauddin *et al*[17]	2016	1	Left anterior chest wall	NR
Monteserín	2017	3	Iliac bone	42 mo (alive)
			Femur	41 mo (dead)
			Vertebral bodies	20 mo (dead)

[1]From first sign of disease; [2]From diagnosis. NR: Survival not reported.

be explained in different ways.

The first is ectopic liver carcinogenesis[18]. Characteristically, the pathologic examination of HCC arising from ectopic liver reveals normal liver tissue, including portal triads. It may be connected to the liver by a fibrous stalk, which is composed of the portal vein, hepatic artery or bile duct. If no evidence of primary cancer of the liver is present after a long-term follow-up

with various specific liver imaging studies, a malignancy originating from ectopic liver should be suspected. This is a very rare entity with few cases described in literature, but normally a chronic liver disease or cirrhosis is present in the mother liver.

The second possibility is the presence of a hepatoid adenocarcinoma (HAC)[19]. This is a variant form of adenocarcinoma, characterized by vast hepatic differentiation. It generally arises in older patients, produces AFP and originates from the endoderm. The most common primary origin is the gastrointestinal tract and lung. There are some cases of HAC with liver metastases that mimic HCC with extra-hepatic spread. Differential diagnoses between both entities can be facilitated by immunohistochemistry. HAC usually is Hep-Par1-negative, cytokeratin 7-negative, cytokeratin-positive and cytokeratin 19-positive; and, it commonly displays two properties: Canalicular pattern of polyclonal CEA staining and expression of albumin messenger RNA as detected by *in situ* hybridization. The preferred occurrence in the stomach may be explained by the fact that liver and stomach share a common embryologic origin from the primitive foregut.

The third possibility is the presence of a variant of extra-hepatic germ cell tumor, arising either in the ovary or mediastinum, with morphologic as well as immunophenotypic features highly characteristic of HCC. For the most part, the majority of such tumors appear to represent yolk sac tumors with hepatoid differentiation (hepatoid yolk sac tumors), positive for SALL4, glypican 3, and AFP[20,21].

The last possibility, which is very uncommon, is the hazard of metastatic HCC without an intra-hepatic mass due to spontaneous regression of primary HCC in the liver[22].

Metastatic bone lesions of HCC often cause local pain, neurological manifestations, palpable subcutaneous masses and pathological fractures. In the search of the primary tumor, abdominal MDCT can sometimes show a liver with normal morphology and without focal lesions, as in cases 1 and 2, making diagnosis difficult and being necessary to complete the study with contrast-enhanced hepatic MRI, especially in situations of high clinical suspicion. In our cases, the morphology and immunohistochemistry of the bone material were the key data for getting the final diagnosis.

Systemic palliative treatment with sorafenib should be considered at the first line in advanced HCC according to the guidelines[1]. The objectives of concomitant treatment are improvement of pain, preservation of functions and maintenance of bone integrity. Multidisciplinary teams play an essential role in the care of these patients, offering multimodal therapy. In localized lesions, external radiotherapy relieves the pain in 60%-80% of cases, with a complete response described between 15% and 58%[23]. The third patient received additional treatment with intravenous zoledronic acid, which acts by inhibiting osteoclasts and is very efficacious in the case of bone metastases of other tumors, such as prostate or breast[24], contributing to an improvement in the quality of life due to decreased pain. Before starting treatment with zoledronic acid, the levels of hypocalcaemia and vitamin D should be corrected, with a subsequent monitoring of renal function and maintenance of good hydration.

These 3 cases illustrate the spectrum of the metastatic bone HCC debut. Case 1 was treated with local and systemic therapy, and he continues to be alive with minor symptoms. Case 2 presented a regular evolution with shorter survival. Case 3 was too symptomatic from the beginning and he only received supportive care.

Sorafenib has dramatically changed the prognosis of advanced HCC. In the SHARP trial population, the median overall survival was 10.7 mo[25]. Cases 1 and 2 have been taken sorafenib over 26 and 13 consecutive mo respectively, with 26 and 25 mo of survival, in that order. Recently, it has been communicated that the type of radiological progression to sorafenib is an important prognostic factor of postprogression survival[26]. Indeed, postprogression survival is shorter when a new extra-hepatic lesion appears, in comparison with survival after growth of a pre-existing intra-hepatic lesion. This information should be used to switch patients to regorafenib or to second-line trials[27].

To conclude, the appearance of a neurological complaint, such as low back pain or root or motor deficit (regardless of the neurological pathology derived from enolic polyneuropathy, which is so common in these patients), cannot be ignored, because this is sometimes the first manifestation of a metastatic HCC.

COMMENTS

Case characteristics

The 3 middle-aged male patients presented with dissimilar symptoms. Case 1 presented with lower right limb pain from several months. Case 2 presented with left inguinal pain and hip fracture. Case 3 presented with abdominal and lumbosacral pain, together with dysesthesia at right thigh.

Clinical diagnosis

The physical signs of the 3 cases were also dissimilar. Upon physical examination, case 1 presented total functional impotence of the right lower limb. Case 2 had left inguinal mechanical pain distally radiating through the left leg to the knee. Case 3 presented mild abdominal tenderness plus limitation of flexion of dorsolumbar spine.

Differential diagnosis

Malignant tumors: Osteochondroma, soft tissue sarcomas, carcinoma of unknown origin and metastatic tumors.

Laboratory diagnosis

Case 1 had no remarkable findings for the laboratory tests, except mild elevation of aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Case 2 presented higher levels of AST, alanine aminotransferase, ALP and gamma-glutamyl peptidase (GGT), probably related to underlying chronic hepatitis C virus infection. Case 3 presented with mild elevation in AST and GGT. It is remarkable than in all 3 cases AFP remained within normal values.

Imaging diagnosis

For all these cases, computed tomography scan and dynamic magnetic resonance showed the primary tumor located in the liver together with the extra-

hepatic lesions. It is important to say that in the first case the liver cancer had gone unnoticed by the MDCT scan of thorax-abdomen-pelvis with intravenous and oral contrast. It was only detected with a specific multiphasic hepatic TCMD.

Pathological diagnosis

For the 3 cases, histological examination of bone lesions showed a solid neoformation formed by cells of epithelioid habit with large cytoplasmic compartment and irregular, vesiculous nuclei with patent nucleolus and frequent figures of mitosis, accompanied by a rich vascular network adjacent to a soft tissue. In all 3 cases, tumor cells were strongly positive for cytokeratin AE1/AE3 and cytokeratin 8 and negative for cytokeratin 7.

Treatment

All 3 patients received different therapies for pain relief (oral and intravenous analgesics, bisphosphonates, external radiotherapy). In addition, case 2 received a total hip replacement and case 3 received cementation of the fourth lumbar vertebra. Cases 1 and 2 received sorafenib for more than 12 mo as specific therapy for advance hepatocellular carcinoma.

Related reports

Very few cases of metastatic bone presentation of hepatocellular carcinoma have been reported in the literature. The clinical and therapeutic management of these patients is a challenge and several combined therapies can be applied to obtain long survivals with acceptable quality of life.

Term explanation

Multimodal therapy is referred to the combination of local and systemic treatments to relieve symptoms secondary to bone destruction due to metastases of hepatocellular carcinoma. It includes sorafenib but also external radiotherapy, cementation of selected vertebrae, intravenous bisphosphonates, *etc.*

Experiences and lessons

Metastatic hepatocellular carcinoma should be included within the differential diagnoses of bone metastases of unknown origin, even in the absence of already known chronic liver disease. With proper symptomatic and systemic therapies these patients can have a longer survival with preserved quality of life.

Peer-review

The authors have described 3 cases of bone metastases as initial presentation of hepatocellular carcinoma. It is an interesting contribution to the literature on hepatocellular carcinoma and its metastasis.

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WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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REVIEW

Current concepts and future strategies in the antimicrobial therapy of emerging Gram-positive spontaneous bacterial peritonitis

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Author contributions: Fiore M designed the research; Fiore M and Maraolo AE collected the data; Leone S cross-checked results and resolved disagreement; Sansone P, Passavanti MB, Aurilio C and Pace MC contributed to conception of the study; Gentile I and Borgia G supervised the paper; all authors approved the final version of the manuscript.

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Abstract

Spontaneous bacterial peritonitis (SBP) is the most common infection in end-stage liver disease patients. SBP is defined as an ascitic fluid infection with a polymorphonuclear leucocyte count $\geqslant$ 250/mm^3 without an evident intra-abdominal surgically treatable source. Several mechanisms contribute to SBP occurrence, including translocation of gut bacteria and their products, reduced intestinal motility provoking bacterial overgrowth, alteration of the gut's barrier function and local immune responses. Historically, Gram-negative enteric bacteria have been the main causative agents of SBP, thereby guiding the empirical therapeutic choice. However, over the last decade, a worryingly increasing prevalence of Gram-positive and multi-drug resistant (MDR) SBP has been seen. Recently, the microbiological spectrum of SBP seems to have changed in Europe due to a high prevalence of Gram-positive bacteria (48%-62%). The overall proportion of MDR bacteria is up to 22%-73% of cases. Consequently, empirical therapy based on third-generation cephalosporins or amoxicillin/clavulanic acid, can no longer be considered the standard of care, as these drugs are associated with poor outcomes. The

aim of this review is to describe, with an epidemiological focus, the evidence behind this rise in Gram-positive and MDR SBP from 2000 to present, and illustrate potential targeted therapeutic strategies. An appropriate treatment protocol should include daptomycin plus ceftaroline and meropenem, with prompt stepdown to a narrower spectrum when cultures and sensitivity data are available in order to reduce both cost and potential antibiotic resistance development.

Key words: Spontaneous bacterial peritonitis; Multi-drug resistant bacteria; End-stage liver disease; Cirrhosis; Critically ill patient

Core tip: Spontaneous bacterial peritonitis (SBP) is the most common infection in end-stage liver disease cirrhotic patients. Over the last decade, a worryingly increasing prevalence of Gram-positive and multi-drug resistant (MDR) SBP causative bacteria has been seen. Numerous driving factors have been proposed as associated with this epidemiological change. The aim of this review is to describe, with an epidemiological focus, the evidence behind this rise in Gram-positive and MDR SBP from 2000 to present, and illustrate potential targeted therapeutic strategies. Third-generation cephalosporins should be avoided in clinical settings with a high prevalence of MDR. An appropriate treatment protocol should include daptomycin plus ceftaroline and meropenem.

Fiore M, Maraolo AE, Gentile I, Borgia G, Leone S, Sansone P, Passavanti MB, Aurilio C, Pace MC. Current concepts and future strategies in the antimicrobial therapy of emerging Gram-positive spontaneous bacterial peritonitis. *World J Hepatol* 2017; 9(30): 1166-1175 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i30/1166.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i30.1166

INTRODUCTION

The development of abdominal ascites is the most frequent complication in cirrhotic patients[1], and infected ascites, better known as spontaneous bacterial peritonitis (SBP), is the most common infection in these patients, together with urinary tract infections[2]. SBP is defined as a polymorphonuclear (PMN) leucocyte count $\geqslant$ 250/mm^3, with or without positive ascitic culture and the absence of other sources of sepsis in the peritoneum or adjacent tissues[3]. SBP is a distinct clinical entity, as opposed to bacteriascites (positive ascitic culture with PMN < 250/mm^3, not needing therapy in cases of no accompanying symptoms) and secondary bacterial peritonitis, which are usually polymicrobial and linked to the inflammation or perforation of an abdominal organ[3].

Several mechanisms contribute to the occurrence of SBP, including translocation of gut bacteria and their products, reduction of intestinal motility provoking bacterial overgrowth, alteration of the gut's barrier function and local immune responses[4]. These premises explain why historically Gram-negative bacteria (GNB) have been the main causative agents of SBP, thereby guiding the empirical therapeutic choice[5]. However, over the last decade the prevalence of Gram-positive bacteria (GPB) and multidrug resistant (MDR) SBP has increased worryingly[6]. Important driving factors for this epidemiological change have been the extensive use of quinolones, as a prophylactic measure, and the increasing degree of instrumentalization of patients suffering from cirrhosis[7]. Consequently, empirical therapy based on third-generation cephalosporins (3GCs) or amoxicillin/clavulanic acid, especially within a healthcare setting, can no longer be considered the standard of care due to poor outcomes[8].

Bacterial infections are the primary cause of death in patients with end-stage liver disease (ESLD), and require timely and appropriate treatment[9]. Thus, the aim of this review is to describe, with an epidemiological focus, the evidence behind this rise in Gram-positive and MDR SBP from 2000 to present, and illustrate potential targeted therapeutic strategies.

EPIDEMIOLOGY OF SBP: CHANGE OF PARADIGM

Globally, since 2000, there has been an increasing relevance of the role of GPB with respect to SBP (Figure 1). A description of this change follows, according to a geographical criterion.

Asia

Before 2000, GNB were, in consistency with the previous literature[10], the most prevalent etiologic cause of SBP in Asian cohorts of SBP patients. Then, changes occurred, but in a distinct fashion from country to country. In a retrospective study conducted in South Korea, which reviewed records of individuals diagnosed with SBP in 1995, 1998 and 1999, the rate of GPB was just 18.6% (44/237), with just 5 cases of infection by *Staphylococcus aureus* and 3 by *Enterococcus* spp., while the majority of GPB were represented by *Streptococcus* spp.[11]. These data were substantially confirmed by another South Korean retrospective study (episodes referring to the period from October 1998 to August 2003) that showed a proportion of GPB equal to 20.8% (22/106); no strains of *S. aureus* were detected, but there were 16 streptococci and 8 enterococci[12].

In a further South Korean retrospective study, which analysed cases from Janua ry 2002 to December 2004, the prevalence of GPB was also low; the bacterial isolates totalled 204 and *S. aureus*, *Enterococcus* spp. and *Streptococcus* spp. accounted for 3.9% (8/204), 3.9% (8/204) and 8.8% (18/204), respectively[13]. In addition, Heo *et al*[14] found a marginal proportion of GPB in their South Korean retrospective cohort (from June 2005

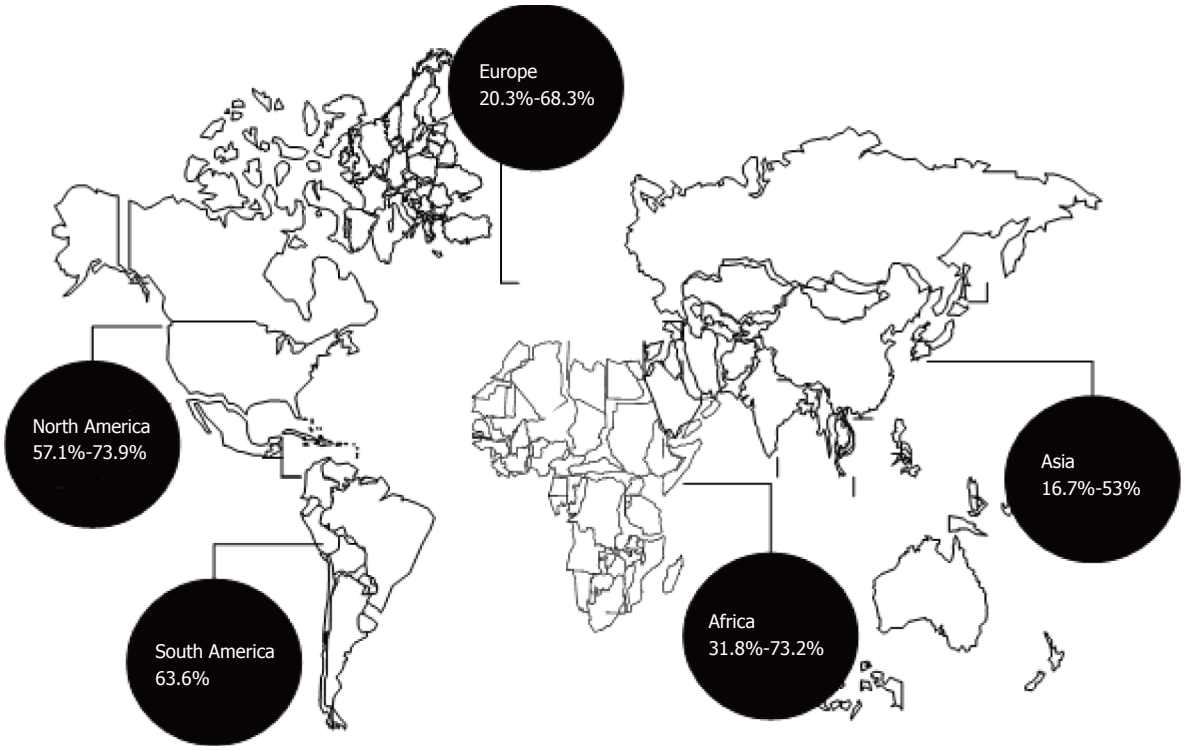

Figure 1 Worldwide prevalence of spontaneous bacterial peritonitis due to Gram-positive bacteria.

to May 2006), namely 16.7% (11/65). Interestingly, when keeping South Korean retrospective cohorts under consideration, the rate of GPB increases when data are split according to the onset of infection. Cheong *et al*[15] reviewing medical records from 1 January 2000 to 30 June 2007, found a relatively low number of GPB (22.9%, 54/236), but among nosocomial SBP (N-SBP; which occurred > 48 h after hospital admission), this rate was equal to 29.3% (37/126). At any rate, South Korea does not seem to be impacted by a remarkable increment of GPB. A recently published retrospective study, referring to a 10-year period (from 2005 to 2014) and comparing cases of culture-positive SBP with cases of culture-negative SBP, showed a rate of GPB equal to 25.5 (66/259), with a low number of *S. aureus* (2.7%, 7/259) and *Enterococcus* spp. (3.5%, 9/259)[16].

On the contrary, in China, the epidemiological shift towards GPB has been more apparent. In a retrospective study of 98 patients, 48 from 1996 to 2002, and 50 from 2003 to 2009, the proportion of GPB passed from 27% (13/48) to 53% (26/49); the rate of staphylococci also increased from 14% (7/48) to 37% (18/49), but with only 1 case of methicillin-resistant *S. aureus* (MRSA)[17]. More importantly, in-hospital mortality was greater among GPB-SBP than GNB-SBP cases (26% *vs* 11%, namely 7 deaths *vs* 2 deaths), although the result was not significant (P = 0.20)[17]. In a subsequent retrospective study conducted in China, which reviewed medical records from 2011 to 2013, Li *et al*[18] found a less prominent rate of GPB, equal to 27.8% (85/306), overlapping between nosocomial (27.3%, 27/99) and non-nosocomial episodes (28.0%, 58/207). Nonetheless, a worrisome percentage of MRSA stood out from this study: 37.5% (6/16) among non-nosocomial infections and, even worse, 85.7% (6/7) among nosocomial cases[18].

More recently in a Chinese study, performed to compare the microbiological profiles of N-SBP and community acquired SBP (CA-SBP), 575 strains were isolated from January 2014 to December 2014. In the CA-SBP cases, the most frequently isolated pathogens were *Escherichia coli* (*E. coli*) (27.4%), coagulase-negative staphylococci (22%), *Klebsiella pneumoniae* (13.7%), *Enterococcus* spp. (9%) and *Streptococcus* (8.2%). In the N-SBP, the most frequently isolated pathogens were *E. coli* (25.9%), coagulase-negative staphylococci (23.4%), *K. pneumoniae* (2.5%), *Enterococcus* spp. (16.6%) and *Streptococcus* (6.2%). In the statistical analysis, there were no significant differences in the distributions of GPB between the CA and N-SBP. In contrast, compared with the CA-SBP, the distribution of enterococci was increased in the N-SBP (9.0% *vs* 16.6%, $P < 0.05$)[19].

Different results have come from studies in other Asian countries. In Iran, a prospective study (from November 2005 to December 2007) showed a proportion of GPB equal to 27.3% (12/44)[20]. A similar result (28.6%, 90/314) was found in another study conducted in Iran (from April 2005 to September 2011)[21]. A small cohort from Pakistan showed a relatively low percentage of GPB: 25% (3/12) in a 2007 prevalence study[22].

Africa

In an Egyptian prospective cohort, the burden of GPB turned out to be as high as 73.2%, namely 30

out of 41 episodes, including 10 cases by *Listeria monocytogenes*[23]. In contrast, a retrospective study conducted in Nigeria, which reviewed medical records from August 2009 to July 2010, showed a much smaller proportion of GBP, which although not marginal was equal to 31.8% (7/22)[24].

South America

In a Brazilian retrospective study referring to a 5-year period (from November 2001 to November 2006), a significant rate of GPB emerged despite the lack of a complete microbiological profile of 63 cases [*Streptococcus* spp. 23.8% (15/63), *S. aureus* 7.9% (5/63)][25]. A more recent and prospective multicentre study conducted in Argentina, from March 2011 to April 2012, showed a clear predominance of GPB over GNB [21/33 (63.6%)]; of note, the study, which aimed at investigating the potential association between proton pump inhibitors (PPIs) and SPB, showed no significant difference with regard to PPIs' consumption and duration between patients with and without SBP (as well as with and without other infections) nor with regard to the type of bacteria[26].

North America

A high number of GPB was found in a United States retrospective study, referring to medical records from July 2009 and November 2010: 80% (8/10), including two vancomycin-resistant enterococci (VRE)[27]. The high impact of GPB in SBP in North America has been further confirmed in a Canadian retrospective cohort that reviewed cases from February 2003 to May 2011; the data indicated that 57.1% (44/77) and 34.1% of these strains (15/44) were resistant to 3GCs (acquired or intrinsic resistance)[28].

Europe

In a prospective French study conducted from January 1996 to March 2001, GPB accounted for 68.3% (125/183) of ascitic fluid infections[29]. GPB cases were mainly explained by enterococci (43/125), streptococci (43/125) and *S. aureus* (36/125); the large majority of the latter (94.4%, 34/36) were MRSA[29]. In that study, the multivariate analysis showed that an infection provoked by *S. aureus* (while taking into account cases of bacteraemia) was independently linked to a higher mortality rate in cirrhotic patients (OR = 2.845, 95%CI: 1.421-5.695, P = 0.031)[29]. In France, Gram-positive cocci are today the predominant ascitic fluid microbes, with isolates ranging from 47.4%[30] to 56.1% of cases[31]. Data from a Spanish study, conducted from April 1998 to April 2000, demonstrated a low proportion of GPB (20.3%, 11/54)[32].

In a retrospective study by Ariza *et al*[33], reviewing medical records related to a subsequent period from 2001 to 2009, GPB rate was again relevant [35.8% (88/246)]. Surprisingly, the lowest percentage was among nosocomial infections (27.3%, 18/66) in comparison with community-acquired (36.5%, 18/85) and healthcare-related infections (41.1%, 39/95); however, the highest rate of MDR-GPB was found among nosocomial cases (27.8%, 5/18)[33]. In Italy, interesting data stem from a recently published randomized clinical trial (RCT), conducted from 2011 to 2014. The aim of that RCT was to compare ceftazidime to the combination of daptomycin plus meropenem, applied as an empirical treatment of N-SBP (in this case, defined if it occurred > 72 h after hospital admission); in particular, 62.5% (10/16) of culture-positive cases were due to GPB (8 enterococci)[34]. Of note, the broad-spectrum regimen proved to be significantly more effective with regard to the primary outcome, namely the resolution of SBP after 7 d of treatment (86.7% *vs* 25%; P < 0.001); that finding did not come as a surprise, in the light of the total rate of MDR bacteria [37.5% (6/16)][34].

In Germany, the growing number of GPB was already a touted issue, more than a decade ago. In a prospective cohort from 2002 to August 2006, Umgelter *et al*[35] found a GPB rate equal to 45.4% (20/44, 10 *E. faecium*). Again, in Germany, a retrospective cohort covering a 12-year period (from January 2001 to November 2011) found a predominance of GPB (53.7%, 65/121), where *Enterococcus* spp. (28 out of 65 GPB) played a highly relevant role[36]. In the multivariate analysis, use of antibiotics (OR = 3.875, 95%CI: 1.189-12.631, P = 0.025) and nosocomial infection (OR = 3.287, 95%CI: 1.311-8.243, P = 0.011) were the independent predictors of enterococcal infections, which were associated with higher mortality (12% probability of 90-d survival *vs* 50% in non-enterococcal cases, P = 0.022 by log-rank test) in case of treatment with a 3GC or a quinolone[36]. Also, in a more recent German prospective cohort, followed from March 2012 to February 2016, and focusing only on nosocomial and healthcare-related SBP, GPB were relevant [*Staphylococcus* spp., *Enterococcus* spp. and *Streptococcus* spp. accounted for 40% of cases (20/50)][37].

Greece was one the first countries to warn about the increasing importance of GPB-SBP. Cholongitas *et al*[38], in a retrospective evaluation, observed that the rate of GPB went from 25% (5/20) to 59.1% (13/22%) in two subsequent periods of time, from 1998 to 1999 and from 2000 to 2002, respectively. This trend in Greece was confirmed by another retrospective study, including cases from 2008 to May 2011, with 26 episodes out of 47 (55%) due to GPB, most of all streptococci (10 isolates), followed by 6 *E. faecalis*, 3 *E. faecium* and 2 *S. aureus*; neither VRE nor MRSA were detected[6].

In Denmark, a retrospective review of medical records from 2000 to 2006 showed a proportion of Gram-positive cocci, without considering other GPB, equal to 45.9% (86/187)[39].

CONTROVERSIES RELATED TO THE DIAGNOSIS OF SBP BY GRAM-POSITIVE BACTERIA

Although some authors have previously considered the isolation of coagulase-negative staphylococci within ascitic

culture as skin contamination[15,40,41], today the clinical significance of such a finding appears relevant in both nosocomial[19,34] and community acquired infections[19]. More than 40 years ago, MacGregor and Beaty[42] proposed guidelines to differentiate contamination from significant positive blood cultures in bacteraemic patients; nowadays guidelines, however, are still lacking in their ability to differentiate contamination from significant positive ascitic cultures.

In our opinion, the absence of recommendations based on solid evidence does not justify concluding isolation of coagulase-negative staphylococci as contamination[15]. Future studies are required to establish the hypothetical difference between the contaminants or pathogens.

CURRENT THERAPEUTIC STRATEGIES FOR SBP BY GPB

The current guidelines rely on outdated epidemiology[43-45] and take into account neither the increasing prevalence of GPB nor the emerging phenomenon of MDR bacteria as aetiological agents of SBP[46]. Opinion leaders recommend 3GCs[47] or piperacillin/tazobactam, meropenem ± glycopeptide[1] for patients at risk of MDR SBP. The role of piperacillin/tazobactam in the treatment of life-threatening infections due to extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* is a controversial issue[47-49]; moreover, meropenem is active against ESBL-producing *Enterobacteriaceae* but is weakly active against Gram-positive cocci[50,51]. Glycopeptides are active against Gram-positive cocci, as well as MDR, but their use is not advisable because of their nephrotoxicity. Acute kidney injury is higher in ESLD patients, it could be related to hemodynamic instability and/or hepatorenal syndrome[52]. Furthermore, the minimum inhibitory concentration (MIC) of vancomycin appears to be shifting upwards in some institutions, a phenomenon known as MIC creep; and, where the MIC increase occurs, treatment failure is common[53,54]. Teicoplanin MIC creep has also been described; but, regardless, when it is administered intravenously it does not achieve therapeutic concentration in the ascitic fluid[55].

Antibiotics active against VRE are linezolid, tigecycline, and daptomycin. Linezolid is not recommended in the majority of ESLD and SBP patients because of high frequency thrombocytopenia[56]. A tigecycline dose adjustment is requested in patients with severe hepatic impairment[57,58]. Daptomycin is a lipopeptide active against MDR GPB, including drug-resistant and drug-susceptible *S. aureus* and VRE[59]. Decreased susceptibility to daptomycin has been reported in drug-resistant *S. aureus*; it is frequently accompanied by a paradoxical decrease in beta-lactam resistance, a process known as the "see-saw" effect. Despite the observed discordance in resistance phenotypes, the combination of daptomycin/beta-lactams has been proven clinically effective for the prevention and treatment of infections due to daptomycin-resistant *S. aureus* strains[60,61]. Therefore, daptomycin monotherapy should not be used for the treatment of SBP due to MRSA, unless the isolate is likely to be fully susceptible[62]. The combination of daptomycin plus ceftaroline is highly active against MRSA, the potent bactericidal activity appears to be sufficiently robust to allow rapid de-escalation to single ceftaroline with daptomycin sparing[63]. Furthermore, ceftaroline in combination with daptomycin restores daptomycin activity against daptomycin-resistant VRE strains[64]. Aminoglycoside antibiotics, especially gentamicin, are used in combination with ampicillin for the treatment of enterococcal systemic infections[65]. Despite rigorous patient monitoring, nephrotoxicity appears in 10%-25% of therapeutic courses[66]. Therefore, their use is not advisable in cirrhotic patients. In recent years, an alternative treatment with ampicillin plus ceftriaxone has proved to be safer than gentamicin in combination with ampicillin[67]. In ESLD patients, the combination of ampicillin plus ceftriaxone should be used for SBP due to enterococci, regardless of aminoglycoside resistance-level status.

FUTURE PERSPECTIVES

The 20th century has been characterized by the dramatic effect of the large-scale use of antibiotics after their discovery, saving millions of lives[68]. Unfortunately, natural selection and misuse of antibiotics, both in human beings and in animals, have led to the development of difficult-to-treat infections by MDR bacteria, also known as superbugs, the nightmare of the new century[69]. Research efforts by pharmaceutical companies are not keeping pace with the worldwide spread of superbugs and this has prompted new strategies to optimize existing resources, such as the reviving of old antibiotics[70], the implementation of antimicrobial stewardship programs[71,72], and the judicious use of new anti-infective agents[73]. However, the epidemiology of bacterial infections has a huge inter-centre variability and the therapeutic approach should be inspired by the principle of "one size does not fit all", which obviously also applies to SBP[74]. In other words, the current challenge is to accurately identify patients with SBP for whom empirical broad-spectrum therapy would be appropriate, with special attention to MDR-GPB in contexts where their prevalence is relevant[74].

Some risk factors are well established. The setting of acquisition (nosocomial or healthcare-related *vs* community-acquired) and the history of exposition to antibiotics, such as beta-lactams and/or quinolones, are probably the main ones[75,76]. Exposure to quinolones, largely used to prevent SBP in cirrhotic patients, is a significant risk factor for MRSA infections[77,78]. Moreover, antibiotics administered within the past 30 d before SBP diagnosis and a lower sepsis-related organ failure assessment (commonly known as SOFA) score proved to be significantly associated with SBP by GPB in a cohort of 77 patients[79]. The impact of MDR-GPB on SBP patient mortality is not well investigated; recently, we performed a systematic review aimed at summarizing the evidence from the literature concerning

Table 1 Characteristics of the studies

Ref.	Journal	Publication year	Observation time span	Study design	Country, clinical setting	Proportion of infections by GPB (%)
Asia - South Korea						
Park *et al*[11]	*J Gastroenterol Hepatol*	2003	1995, 1998, 1999	RC, single centre	South Korea, University Hospital	44/237 (18.6)
Song *et al*[12]	*J Korean Med Sci*	2006	1998 (October) - 2003 (August)	RC, single centre	South Korea, University Hospital	22/106 (20.8)
Cho *et al*[13]	*Scand J Infect Dis*	2007	2002-2004	RC, single centre	South Korea, University Hospital	34/204 (16.6)
Heo *et al*[14]	*Gut Liver*	2009	1998 (June) - 2003 (May)	RC, multicentre	South Korea	11/65 (16.7)
Cheong *et al*[15]	*Clin Infect Dis*	2009	2000 (January) - 2007 (June)	RC, single centre	South Korea, University Hospital	54/236 (22.9)
Na *et al*[16]	*Scand J Infect Dis*	2017	2005-2014	RC, single centre	South Korea, University Hospital	66/259 (25.5)
Asia - China						
Gou *et al*[17]	*Saudi Med J*	2010	1996-2009	RC, single centre	China, University Hospital	39/97 (42.2)
Li *et al*[18]	*World J Gastroenterol*	2015	2011-2013	RC, single centre	China, University Hospital	85/306 (27.8)
Shi *et al*[19]	*Sci Rep*	2017	2014	RC, single centre	China, Tertiary Hospital	293/575 (50.9)
Asia - Other countries						
Kamani *et al*[20]	*BMC Gastroenterol*	2008	2005 (November) - 2007 (December)	PC, single centre	Iran, University Hospital	12/44 (27.3)
Sheikhbahaei *et al*[21]	*Int J Hepatol*	2014	2005 (April) - 2011 (September)	PC, single centre	Iran, University Hospital	90/314 (28.6)
Zaman *et al*[22]	*J Ayub Med Coll Abbottabad*	2011	2007	PC, single centre	Pakistan, University Hospital	3/12 (25)
Africa						
El Sayed Zaki *et al*[23]	*J Infect Public Health*	2011	Not provided	PC, single centre	Egypt, University Hospital	30/41 (73.2)
Oladimeji *et al*[24]	*Pan Afr Med J*	2013	2009 (August) - 2010 (July)	RC, single centre	Nigeria, University Hospital	7/22 (31.8)
South America						
Reginato *et al*[25]	*Sao Paolo Med J*	2011	2001 (November) - 2006 (November)	RC, single centre	Brazil, Tertiary Hospital	20/63 (31.7)
Terg *et al*[26]	*J Hepatol*	2015	2011 (March) - 2012 (April)	PC, multicentre	Argentina	21/33 (63.6)
North America						
Tandon *et al*[27]	*Clin Gastroenterol Hepatol*	2012	2009 (July) - 2010 (November)	RC, single centre	United States, University Hospital	8/10 (80)
Chaulk *et al*[28]	*Can J Gastroenterol Hepatol*	2014	2003 (February) - 2010 (May)	RC, single centre	Canada, Tertiary Hospital	44/77 (57.1)
Europe						
Campillo *et al*[29]	*Clin Infect Dis*	2002	1996 (January) - 2001 (March)	PC, single centre	France, Tertiary Hospital	125/183 (68.3)
Piroth *et al*[31]	*BMC Infect Dis*	2014	2010-2011	PC, multicentre	France, University Hospitals	32/57 (56.1)
Thévenot *et al*[30]	*Am J Gastroenterol*	2016	2014 (March) – 2015 (August)	PC, multicentre	France	40/84 (47.4)
Fernández *et al*[32]	*Hepatology*	2002	1998 (April) - 2000 (April)	PC, single centre	Spain, University Hospital	11/54 (20.3)
Ariza *et al*[33]	*J Hepatol*	2012	2001-2009	RC, single centre	Spain, University Hospital	88/246 (35.8)
Piano *et al*[34]	*Hepatology*	2016	2011-2014	RCT, multicentre	Italy	10/16 (62.5)
Umgelter *et al*[35]	*Infection*	2009	2002 (January) - 2006 (August)	PC, single centre	Germany, University Hospital	20/44 (45.4)
Reuken *et al*[36]	*Aliment Pharmacol Ther*	2009	2002 (January) - 2011 (November)	RC, single centre	Germany, Tertiary Hospital	65/121 (53.7)
Lutz *et al*[37]	*Eur J Clin Invest*	2017	2012 (March) - 2016 (February)	PC, single centre	Germany, University Hospital	20/50 (40)
Cholongitas *et al*[38]	*Liver Int*	2005	1998-200	RC, single centre	Greece, University Hospital	18/42 (42.9)
Novocic *et al*[39]	*Scand J Gastroenterol*	2012	2000-2006	RC, multicentre	Denmark, University Hospitals	86/187 (45.9)

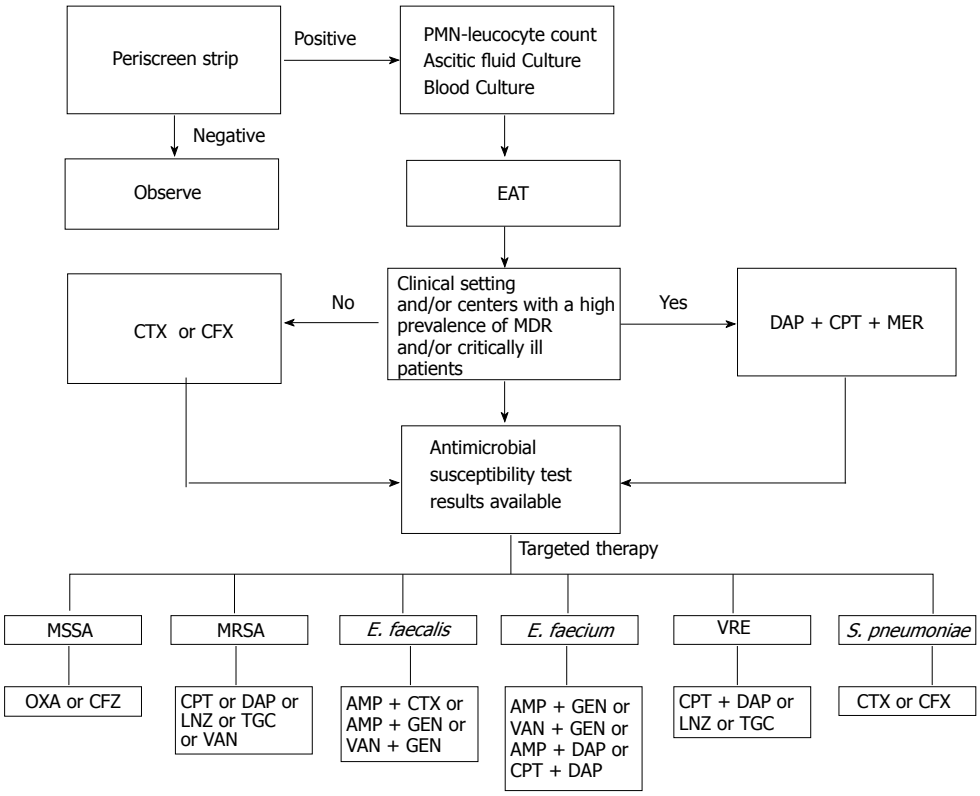

Figure 2 Infection management algorithm of spontaneous peritonitis due to Gram-positive bacteria[45,46,51,54,61,62,65,72,80,82-84]. AMP: Ampicillin; CFX: Cefotaxime; CFZ: Cefazolin; CPT: Ceftaroline; CTX: Ceftriaxone; DAP: Daptomycin; EAT: Empiric antibacterial therapy; GEN: Gentamicin; LNZ: Linezolid; MDR: Multidrug resistant; MER: Meropenem; MRSA: Methicillin-resistant *S. aureus*; MSSA: Methicillin-susceptible *S. aureus*; OXA: Oxacillin; PMN: Polymorphonuclear; TGC: Tigecycline; VAN: Vancomycin; VRE: Vancomycin-resistant enterococci.

the epidemiology of nosocomial cases of SBP, in order to highlight the importance of MDR bacteria outcome; of the initial 2556 manuscripts retrieved, only 9 were included in the qualitative analysis, and a quantitative analysis on mortality was not possible[80].

Risk factors could be integrated into predictive models of mortality in individuals with SBP so as to further help identify patients in need of more aggressive therapeutic strategies from the very start of the infective process[81].

CONCLUSION

GPB are increasingly important as causative agents of SBP. In some contexts, they even supersede GNB as the main cause of this infection (Table 1 describes the main features of included studies). In parallel with this phenomenon, physicians have to face the rise of superbugs, both among GNBs and GPBs. In presence of particularly worrisome epidemiological data and other risk factors for superbug infections, a broad-spectrum empirical approach is required, encompassing antibiotics with well-established activity against pathogens, such MRSA and VRE, pending the results of microbiological tests that would allow a de-escalation strategy whenever possible.

On the basis of the current literature, we propose a treatment algorithm for SBP due to GPB (Figure 2). If an ESLD patient with ascites is "symptomatic" for SBP (temperature above 38 ℃ or below 36.5 ℃, chills, abdominal tenderness, arterial hypotension, developing or worsening hepatic encephalopathy, gastrointestinal bleeding within the previous 15 d) it is necessary to perform a Periscreen strip on the ascitic fluid[30]. If the Periscreen strip is positive the patient requires immediate hospitalization with comparison of this result with cytology and immediate microbiological cultures. A culture of ascitic fluid and blood should systematically be carried out at the bedside[34]. Empiric antibacterial therapy (EAT) should be initiated after obtaining *a*ppropriate cultures. 3GCs should not be used in clinical settings and/or centres with a high prevalence of MDR bacteria. ESLD patients with SBP in clinical settings and/or centres with a high prevalence of VRE, MRSA and ESBL should immediately receive broad-spectrum EAT[82]. An appropriate treatment protocol should include daptomycin plus ceftaroline and meropenem[83]. When the culture is positive and susceptibility data are available, an antibiotic with a narrower spectrum should be promptly initiated (early de-escalation strategy); this strategy limits the selection of antibiotic resistances and saves on costs[83].

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REVIEW

Liver cystic echinococcosis and human host immune and autoimmune follow-up: A review

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Abstract

Cystic echinococcosis (CE) is an infectious disease caused by the larvae of parasite *Echinococcus granulosus* (*E. granulosus*). To successfully establish an infection, parasite release some substances and molecules that can modulate host immune functions, stimulating a strong anti-inflammatory reaction to carry favor to host and to reserve self-survival in the host. The literature was reviewed using MEDLINE, and an open access search for immunology of hydatidosis was performed. Accumulating data from animal experiments and human studies provided us with exciting insights into the mechanisms involved that affect all parts of immunity. In this review we used the existing scientific data and discuss how these findings assisted with a better understanding of the immunology of *E. granulosus* infection in man. The aim of this study is to point the several facts that challenge immune and autoimmune responses to protect *E. granulosus* from elimination and to minimize host severe pathology. Understanding the immune mechanisms of *E. granulosus* infection in an intermediate human host will provide, we believe, a more useful treatment with immunomodulating molecules and possibly better protection from parasitic infections. Besides that, the diagnosis of CE has improved due to the application of a new molecular tool for parasite identification by using of new recombinant antigens and immunogenic peptides. More studies for the better understanding of the mechanisms of parasite immune evasion is necessary. It will enable a novel approach in protection, detection and improving of the host inflammatory responses. In contrast, according to the "hygiene hypothesis", clinical applications that decrease the incidence of infection in developed countries and recently in developing countries are at the origin of the increasing incidence of both allergic

and autoimmune diseases. Thus, an understanding of the immune mechanisms of *E. granulosus* infection is extremely important.

Key words: Lymphocytes; Dendritic cells; Immunity; Autoimmunity; Cytokines; *Echinococcus granulosus*

Core tip: The most common location of a hydatid echinococcal cyst is in the liver. The survival of Echinococcus within host tissues, despite the development of specific antibodies, is possible due to specific immunomodulation induced by parasites. Perpetual survival of parasites indicating multi-level systematic evasion against host protective reaction to persist their growth and spreading. Complement modulation, a metabolic adaptation to the host microenvironment, plentiful thermostable immunogenic antigen B in the cystic fluid, and induction of $CD4^+CD8^+FOXP3^+$ T cells allows the persistence of the parasites. Parasites influence dendritic cell (DC) maturation and impair activation by toll-like receptor. It seems that DC-parasite interaction is pivotal in triggering and regulating parasite induced immunity.

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INTRODUCTION

The dog tapeworm *Echinococcus granulosus* (*E. granulosus*) is the main challenger of a global parasitic zoonosis cystic echinococcosis (CE) in humans caused by the larvae from the infected dog[1]. After incidental ingestion the larvae of parasite, the oncosphere/exacanth larvae is releasing from the keratinized embryophore in the stomach and intestine of the intermediate host (herbivores or humans). Embriophores in the intestine of man *via* hook movements penetrate into the small intestine of host. Its life cycle develops in dogs and other canids (wolves, foxes, coyotes, jakals) that harbor the adult tapeworm. The larval metacestode form develops in different organs of the intermediate host[2]. Hydatid cyst is mainly located in the liver (70%) or lungs (20%), but occasionally they may find their way to other organs (kidney 2%, spleen 2% and brain less than 2%)[3]. In the intermediate host the eggs cross intestinal wall and develop into larvae. The oncosphere is then carried out *via* portal vein flow into the liver and other organs. There oncospheres undergoes a metamorphosis towards the metacestodes. The metacestodes implant into the organ and grow into cysts, with all characteristic layers: Germinal, outer and laminated (Figure 1)[4,5]. Organs may also be reached through the lymphatic system[6]. Echinococcal cysts is surrounded by pericyst (adventia) from the periparasitic host tissue, which surround the larval endocyst (Figure 1A and B). The endocyst is also composed of a cuticular or laminal acellular outer layer and an inner germinal proligerous, which gives rise in a fertile cyst to root capsules and protoscoleces (PSCs)[7]. Some cysts may also harbor daughter cysts of variable sizes (arrows in Figure 1C). Cysts also contain developing PSCs, which constitute an infectious agent. PSC is developing into the adult tapeworm if will be ingested by a suitable definitive host (sheep, cattle, goat). Some vesicles adhere to the walls by means of a peduncle or remain free within the hydatid fluid. A large number of these vesicles (endogenous daughter vesicles) and free protoscoleces float in the hydatid fluid, together forming the co-called "hydatid sand". New offspring vesicles in the hydatid fluid play the same role and have the same constitution of the vesicle mother. The hydatic liquid is clean and clear. It contain all secreted molecules from parasite and host, which is very similar even identical to that of the host's serum containing Na, K, Cl, and CO_2), a density between 1.008 and 1.015, and alkaline pH[8]. Thus, in this way, protoscoleces may develop into either a new cyst or in adult parasite. As the cyst later becomes a successful xenograft in the host, it progressively enlarges until symptoms or complications appear[9,10]. Therefore, the clinical manifestation of infected humans with *E. granulosus*, could be expressed from asymptomatic infection to severe, potentially fatal disease. The parasite die due to dysfunction of germinal membrane (detached, aging or microrta umatisms) but the scolex may transform into vesicle trying to preserve the species[11].

The survival of *Echinococcus* within the host tissues, despite the development of specific antibodies (Abs), is possibly the result of specific immunomodulation induced by the parasite[12]. This phenomenon has been the subject of study by many researchers during the last two decades. They aimed to investigate the host responses to the parasite. The final goal of the present study was to review these modifications of the immune and autoimmune responses induced by *E. granulosus*. For better understanding of host-parasite interactions in this review human clinical study used complementary to animal studies. However, although some of the mutual interactions between parasite and human host in infection have been resolved, essence of protective mechanisms of human host are still unclear.

IMMUNE RESPONSE TO *E. GRANULOSUS* INFECTION OF THE HOST

Effects on innate immunity

Almost exclusively within the intermediate host's liver, parasitic metacestode vesicles grow infiltratively, very similar to that of malignant tumor. The host immune system reacts to these formations. But there is no data of granulosis-induced immune suppression in echinococcosis

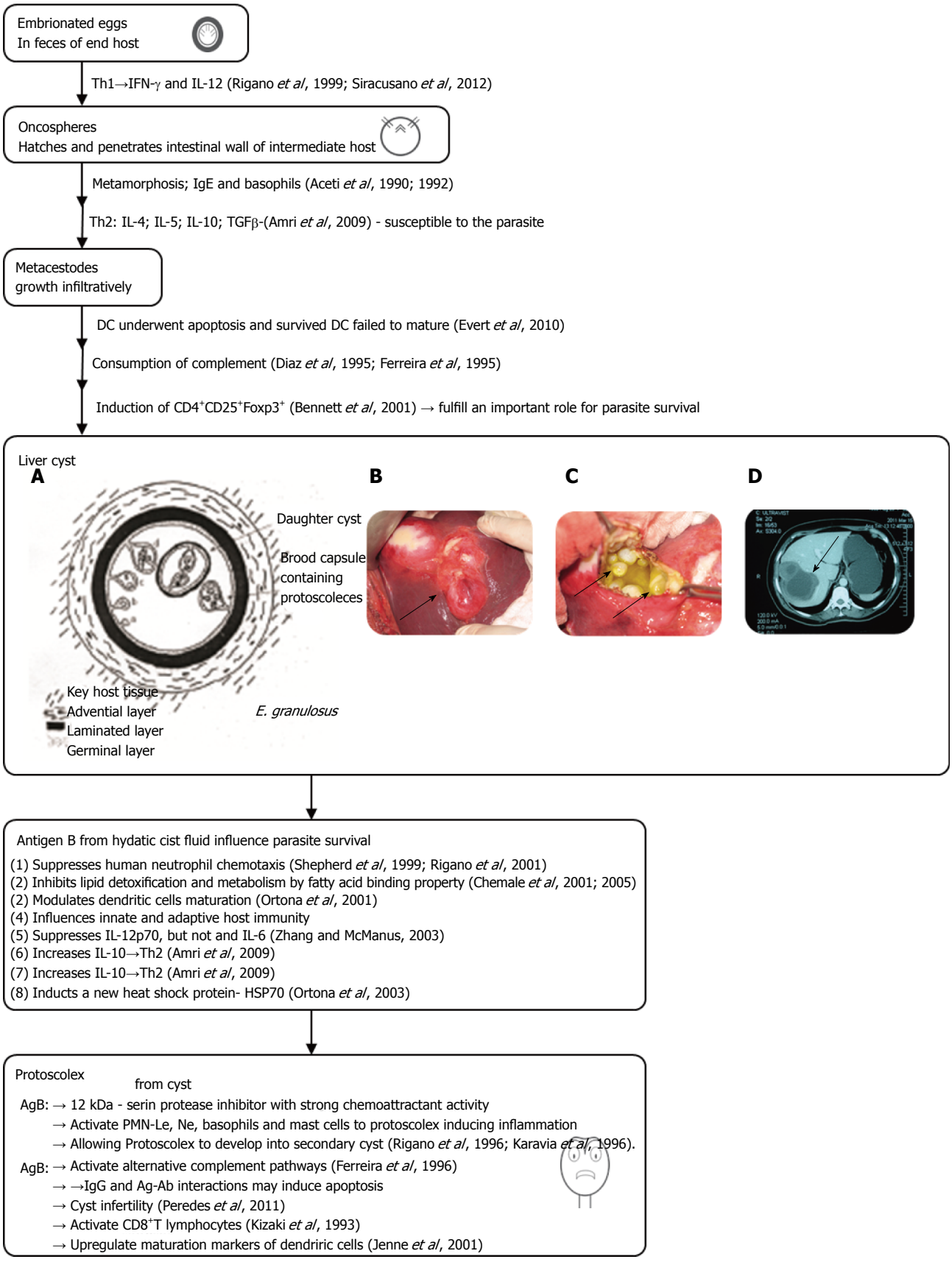

Figure 1 Diagrammatic representation of the development of the *Echinococcus granulosus* liver cyst. A, B: Liver cystic echinococcosis, our surgical material; C: Computed tomography imaging of hepatic cystic echinococcosis; D: Abdominal scan of a female patient with a CE3b cyst in the VI liver segment (adapted from Ref. [120]). IL: Interleukin; IFN: Interferon; TGF: Transforming growth factor; PSC: Protoscolece; PMN: Polymorphonuclear.

on the molecular and cellular level, particularly in the early stages of the infection. On the other hand, there is

no doubt that the defensive immune reaction is missing and parasite survive. Future survival of parasites indicates that they have developed some mechanisms of evasion from host protective immune mechanisms to preserve their expansion[13]. Many studies in humans and mice showed that after parasite infections at the beginning dominates T helper 1 (Th1) immune responses (Figure 2). Th1 immune responses is characterized by the release of interferon-γ (IFN-γ) and after priming by dendritic cells (DCs) with IL-12[11,14]. Both are effective in the elimination of the parasite at an early stage. However, it has become clear that the parasite, probably by its excretory/secretory products, actively influences the host immune response, leading it to the Th2 response and parasite survival. Namely, the Th2 cytokine profile of IL-4, IL-5, immunosuppressive IL-10 and transforming growth factor beta (TGF-β) are generally associated with receptive capability of the parasite that leading to progressive disease (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3283565/ - pntd.0001516-Vuitton1)[15]. On the other hand, the polymorphonuclear (PMN) leukocyte, basophil-mast cell and monocyte participation showed intense local inflammatory reaction to protoscoleces (PSCs)[16]. Significant increases in the chemiluminescence response, superoxide (O_2) production and phagocyte index have been detected in patients with dead cysts compared with healthy subjects, whereas a marked reduction in all the above markers was observed in patients with liver cysts[17]. Functionally and metabolically the PMN leukocytes of infected patients are in an activated state[18]. Regarding basophils, the human basophil degranulation test was found to be positive in 33% of patients with hydatid disease (HD)[19]. Furthermore, evidence of increased histamine release from hydatid patient basophils following a challenge with anti-human IgE has also been obtained[20]. It can be concluded that both the generation of histamine releasing factor (HiRF) and production of IgE, which can bind cytokines, may be involved in this stage of infection[21]. This histamine releasing factor was found to activate basophils through surface-bound IgE, cytokine production and Th2 cell activation[16,21-23]. Method of antibody-dependent cell-mediated cytotoxicity is well established as an important mechanism by which the host can damage a multicellular parasite, but why it is not happening?

Additionally, it is well known that the surface of many parasitic helminths, including *E. granulosus*, is able to activate the alternative pathway of the complement system[24,25]. Although the complement can lyse protoscoleces of *E. granulosus*, parasite with some secretion products could be able to consume the complement, which is an ability that has been proposed as the basis of an invasion mechanism by the parasite[25]. However, the levels of component 3 of complement and chemolitic complement in mice showed no evidence of complement consumption[25]. Moreover, C3 levels were significantly increased in patients with hydatid disease compared to controls[16,26]. Thus, it is possible that local consumption at the site of infection may exist, leading to systemic consumption in the more active cysts. Finally, the existence of several mechanisms of complement modulation was found when comparing complement activation *in vitro* by different *E. granulosus* extracts[27]. These findings further enhanced the possibility of their significant role in the susceptibility of infection and/or maintenance of the disease.

The parasite must be able to adapt metabolically to the host microenvironment, and antigen B (AgB) could be involved in this process. The termostabile AgB (166 kDa) resists boiling for 15 min without losing antigenicity. Thus, AgB proteins as highly immunogenic acts directly to innate and adaptive immunity. Additionally, many antigen B (AgB) molecules in the hydatid cyst fluid possibly guarantee parasite survival. The gene family of AgB comprising at least 10 unique genes in five subclasses differentialy expressed in its life cycle, except EgAgB3 which is expressing predominantly in all cell stages[28,29]. Because of their fatty acid binding property, some of them are involved in lipid detoxification, transport and metabolism[30,31]. Also it is well known that its 12 kDa unit is able to inhibit human neutrophil chemotaxis[32,33]. This unit is a serine protease inhibitor with strong chemoattractant activity. This is a reason for released protoscoleces to develop into secondary cysts[10]. Co-incubated with *Echinococcus* primary cells, AgB functioning similar to invading oncosphere or metacestode vesicles. In these conditions, some dendritic cells (DCs) dead, and the survived fail to mature[34]. But, DCs exposed to protoscoleces up-regulated maturation markers and stay functional**.** Pre-incubation with primary cells and metacestode vesicles, impaired ability of DCs to be activated by the Tall-like receptor ligand LPS. This was not observed in those pre-treated with protoscolex excretory/secretory products[35]. The induction of $CD4^+CD25^+Foxp3^+$ T cells to metacestode E/S-products suggests that these cells play important role for parasite survival. The immunomodulatory products from parasites are therefore of high interest for understanding by infections induced immune pathology and treatment of allergy.

E. GRANULOSUS EVASION MECHANISMS IN THE HOST

Characterization of molecules involved in evasion

In intermediate hosts, protoscoleces develop exclusively in fertile cysts. This formation also consists all of three membranes (inner cellular, other glicin rich and laminated acellular)[36]. Nevertheles, *E. granulosus* cystic form can induce IgG that is able to cross the tegument and plasma membranes between laminar and germinal layers of the cyst. On the other side, method of antibody-dependent cell-mediated cytotoxicity is well established as an important mechanism by which the host can damage a multicellular parasite. There IgG recognize specific cystic antigens, and antigen-antibody complex may inhibit proliferative process of protoscoleces, but why it is not happening? Due to germinal layer of the cyst is a barrier

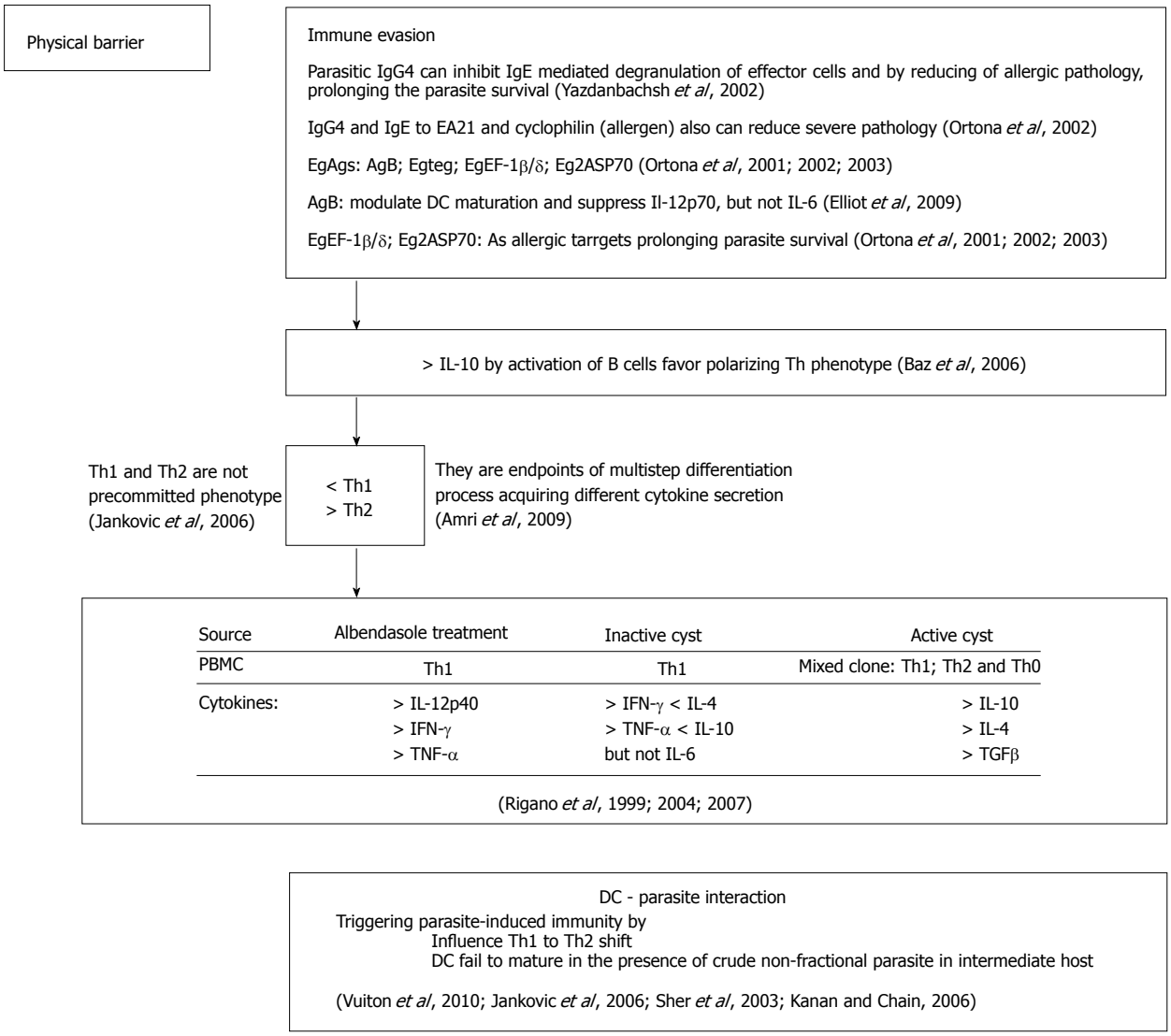

Figure 2 Subversive strategies of *Echinococcus granulosus*. DC: Dendritic cell; IL: Interleukin; IFN: Interferon; TGF: Transforming growth factor.

for immune competent cells of the host[36]. Except this the parasite evolving other immune evasion strategies[37]. *E. granulosus* using two main mechanisms to undermine the host immune response: First is passive escape, in which during the development of hydatid cyst, parasites avoids the damaging effects of host immune reaction; and second is immunomodulation, in which the parasite is actively included in the immune reaction to trigger the host's profit[38,39].

Circulating antibodies as immunological markers in CE

Although patients with hydatidosis releasing amount of circulating IgG, IgM, IgA and IgE antibodies (Abs) to *E. granulosus*, no one is associated with the host protection[40]. IgG4 in echinococcosis is not able to complement fixation neither is cytophilic, also weekly binding to Fc fragment of immunoglobulins, then is not functional. All of these will support the parasite evasion of host immune response[41]. Even, parasite-specific IgG4 antibodies by inhibiting IgE mediated degranulation of effector cells, reducing allergic pathology in the host to prolong parasite survival[42]. In agreement with this study are findings that significantly lower levels of serum IgG4 antibodies is detected in albendazole-treated patients who exhibited a good therapeutic and clinical response compared to that in poor responders or non-responders. Additionally, data obtained in various countries showed reverse trend of IgG1 levels[40,42]. Beside that patients showing differences in IgG1 expression. Those without allergic manifestations releasing IgG4 antibodies specific to EA21, whereas patients with allergic manifestations showed IgE specific to the same antigen. Authors have suggested that CE IgG4 obviously work to block pathogenic processes and to, minimize severe pathology in the host providing parasite survival[42] (Figure 2).

Antigen B and other new antigens in immunomodulation

In CE characterized with Th2 polarized microenvironment, besides AgB, EgTeg and EgEF-1 β/δ, several other parasite molecules can elicit this phenotype. More sophisticated

approach such as proteomic pronounce the presence of a large number of antigenic proteins associated with parasites. Antigen B (AgB) modulates DC maturation and suppresses IL-12p70, but not IL-6 release[43]. As allergic targets in CE in acute cutaneous allergic manifestation, three conserved constitutive proteins at a molecular level have been identified: EgEF-1 β/δ, EA21 and Eg2ASP70[44-47]. At least two of three appear to have immune modulatory properties. EgEF-1 β/δ influences immune modulation and is released after the death or degeneration of protoscoleces[45]. Furthermore, appearance of $CD4^+CD25^+Foxp3^+$ T cells in excretory/secretory products of metacestode suggests that these cells play important role in survival of parasite in chronic echinococcosis[35,48]. The secretory and excretory products of parasite helminthes are therefore extremely important for better understanding of immunopathology of parasite infection and for allergy treatment as well. Future immunological studies will give us opportunity to investigate their role and immunomodulatory effects on parasite infections in humans. However, the long- time (years) development of CE highlights the difficulty in understanding the host-parasite relationship[49,50]. More investigations are necessary for integration of these studies with previous obtained results to recognize and understand well the extreme complexity of the host-parasite interactions. These findings are extremely important for the development and improvement of CE diagnosis and treatment. Finally, it is indispensable for control strategies and vaccine development.

Immunomodulation by cytokine production

Plasticity of both the nature and magnitude of immune host responses depend on infective agents that permit the immune system to tailor its defense strategy. Th1 and Th2 cells are not pre-committed cells with defined phenotype, their phenotype is result of a multistep differentiation process, thus a precursor population acquires secretion of different cytokines profiles[47]. How *E. granulosus* antigens (Ags) encountering the human immune system can influence the differentiation decision in human echinococcosis? First of all it is well known that the immune response established in *E. granulosus* infection is mainly of Th2-phenotype. Also *E. granulosus* antigens modulate polarized T-cells (Figure 2). Furthermore, increased level of IgG4 and IgE antibodies and induced eosinophilia supporting assertion that the immune response established in *E. granulosus* infection is Th2-dominated. Findings from experimental echinococcosis confirm the hypothesis that early IL-10, secreted by B cells in response to mitogens, may favor parasitic survival by established type-2 cytokine response[50]. There are many evidence of molecular studies where reciprocity of IL-4/IL-10 is that impairs the Th1 protective response allowing the parasite survival in human host[51]. In addition, patients responsive to albendazole in peripheral blood monocyte cells (PBMCs) showed high amounts of IFN-γ (Th1-derived), whereas PBMCs from patients who did not respond to albendazol therapy produced a higher level of IL-4 and IL-10 (Th2 derived)[14]. These findings are in coordination with a molecular studies that detected IL-12p40 mRNA in 86% of successfully albendazole-treated patients at the end of chemotherapy who expressed a high level of IFN-γ and TNF-α DNA[15,52]. Finally, patient with an inactive cyst expressing Th1 phenotype, while patients with active and transitional cysts showed mixed Th1/Th2 and Th0 phenotype[53]. No IL-5 and scarce IL-4 and IL-10 is detected in seronegative patients[54]. Seronegativity occurs due to the host or parasite factors or both preclude the possibility of Th2 cell activation that limiting the production of IL-5, crucial for immunoglobulin expression.

It has been shown recently that bone marrow-derived dendritic cells (DCs) from non-lymphoid tissues show capability for antigen presentation and antigen processing[55]. Also there are findings that inflammatory mediators or microbial agents promote the migration of DCs into the lymph nodes and other secondary lymphoid organs. By maturation, DCs lose their ability for antigen presentation and gain an increased capability to prime T-cells[56]. Thus, it is no doubt that DC-parasite interactions are very important for triggering and regulation of parasite-induced immunity. On the other side, *E. granulosus* cystic fluid modulates differentiation and cytokine secretion of dendritic cells[57]. Finally, these cellular findings established that *E. granulosus* except for modulation of DC maturation is included in the polarization of T lymphocytes toward Th2 phenotype[58,59].

ADAPTIVE IMMUNITY

Role of dendritic cells in parasite evasion

Dendritic cells (DCs) as an antigen presenting cells, no doubt represent a link between innate and adaptive immune systems. They are inducing immune responses with Th1, Th2 or Th17-dominated phenotype (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3283565/ - pntd.0001516-Everts1)[60]. After contact with parasite, DCs take up the recognized antigens and undergo maturation in the presence of up regulated MHC/HLA mice/man) complex and co-stimulatory molecules CD86 and CD80[34,60]. Nevertheless, after migration in the lymph nodes, DCs interact with naïve T cells to promote adaptive immune responses with Th1, Th2, and Th17 cell phenotype (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3283565/ - pntd.0001516-Banchereau1)[61,62]. There by activation of T regulatory cells, DCs becoming targets for parasite evasion. Mejri *et al*[63] showed that peritoneal DCs from chronically infected mice, representing the late stage of alveolar echinococcosis. The same authors have reported that DCs in infected mice specifically modulates $CD4^+$ and $CD8^+$ T cell responses, suggesting their immunosuppressive T regulatory function in echinococcosis[63]. In intermediate host parasitic larvae migrate from the intestinal entry site to the liver and late metastasis to other organs (lung, kidney, spleen or brain)[64]. This finding suggest that parasitic larvae encounter DCs *in vivo*[64]. Despite the general importance of DCs in cellular host-parasite interaction, immunomodulatory molecules that are released by

Echinococcus larvae and have an influence on DC function, are still not characterized. Compared to other helminthic infections, immunomodulatory functions of DCs in *E. granulosus* infection in human host provided less attention, although this is an emerging and important field[65]. In two reports, Reyes *et al*[66] and Terrazas *et al*[67] investigated the effects of excretory/secretory products of *Taenia crassiceps cysticerci* on the activation of murine DCs, representing the metacestode larval stage of by *Taenia* infection. DC of susceptible mouse strain when preincubated with parasite excretory/secretory product, authors showed decreased DC maturation because of impaired susceptibility to TLR-dependent stimulus[66,67]. Whether these interactions are relevant *in vivo* is not clear. First of all due to the spectrum of protein products from metacestode excretion and secretion does not necessary overlap the spectrum of proteins in the hydatid cyst fluid[68]. For example, AgB is not detected in excreted or secreted products *in vitro* cultivated metacestode vesicles, but this component is well expressed in hydatic cystic fluid[69]. Second, the intact parasite tissue is showed usually prevents direct contact between hydatid cystic fluid and host immune effector cells. Dendritic cells react with unfractionated helminthic proteins generating anti-parasitic cytotoxic T lymphocyte[70]. Thus, crude methods of *in vitro* preparation of metacestode antigen, insufficient purified which contain vesicle fluid somatic parasite proteins and contaminating host components, tested concerning their effects on DCs, failed to induce maturation as did a purified mucin-type glycoprotein (Em2) that is usually expressing at the surface of LL-containing metacestode vesicles (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3283565/ - pntd.0001516-Hlsmeier1)[71,72]. However, it is well known that extrinsically triggered infectious with viruses, bacteria and parasites, usually results in a bystander effect of induced immunosuppression[73].

Bystander effects of parasite-induced immunosuppression

Dendritic cell apoptosis induction has been reported in nematodes in which their capacity for releasing of pro-inflammatory IL-12 is limited and that prevents activation and proliferation of T cells[74]. Different from others investigator accept the possibility that the diminished function of DCs in metacestode infection is by induction of apoptosis of immature cells rather than due to inhibition of its maturation. It could be a reason for establishing of an immunosuppressive environment around the parasite lesions. Transforming growth factor beta (TBF-β) signaling is involved very early in this process because in animal evolution they are expressed very early in all invertebrate. Therefore, it is understandable that diminished ability of DCs pre-incubated with excretory/secretory-products of primary cells in metacestode is indirectly mediated by the induction of apoptosis of immature DCs, rather than by direct inhibition of DC maturation. Imature DCs secrete TGF-β, which induces differentiation of naïve T cells into FOXP3$^+$ T-regulatory cells, and subsequently immunosuppression around the parasite lesion[73]. TGF-β signaling is involved very early in animal evolution, thus TGF like cytokines are expressed very early in many invertebrate, and in parasites as well[75,76].

Different from DCs incubated with primary cells of metacestode vesicles, those exposed to E/S products of protoscoleces showed up-regulation of surface markers MHC-Ⅱ and CD86, increased secretion of IL-6, but not IL-10 and impaired ability of DCs to produce IL-12 by tall-like receptor lipopolisacharides (LPS) stimulated[74].

Presented phenotypes are similar to that obtained when DCs are incubated with *E. granulosus* hydatid cyst fluid (HCF), and with isolated AgB[55,59].

In contrast to these investigations, DCs incubated with protoscolex compounds as presented in their study Rigano *et al*[55], 2007 and Kanan and Chain[59], 2006, dendritic cells did not release elevated levels of IL-10 or IL-12 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3283565/ - pntd.0001516-Kanan1). Presented phenotypes are similar to the obtained when DCs are incubated with *E. granulossus* hydatid cyst fluid (HCF) and with its isolated AgB. It seems that only protoscoleces weakly expressing AgB[30,77]. Finally, DCs phenotype upon co-incubation with E/S-products of protoscoleces is largely comparable with those incubated with certain *Trypanosoma* antigens, closely associated with the induction of Th2 immune response[78]. The differences obtained between the responses of DCs to E/S-products of early versus late developmental stages of *E. multilocularis* clearly demonstrates that an induction of tolerance of DCs is not a general characteristic of *Echinococcus* material. It is the results of excretory and secretory repertoire of primary cells. Metacestodes are specifically evolved to carry out these purposes[79]. The interpretation of obtained results concerning the immune response in echinococcosis by using of *in vitro* co-incubation-systems of *Echinococcus* protoscoleces with host cells[80-86] or in the mouse model of peritoneal, protoscoleces, should be presented very careful, because of not to provoke suspicious of their verification[87]. The oncosphere that undergo as metamorphosis toward metacestode could induce impaired response of IL-10 secreting DCs *in vitro*[87]. These findings suggests that similar mechanisms might also further investigation by methods of primary cells could resolve the nature of echinococcal products responsible for these effects. But the effects at the begining of infection with E. granulosus and that in chronic phases have not the same nature. They are late reduced, and leading to disappearence of FOXP3$^+$-T-reg from microenvironment and decreased number of immature DCs in protoscolex stage. Beside that the results are concentrated about *in vitro* interactions between parasite larvaes and DCs in response to primary cells. No doubt that similar mechanisms operate in surrounding tissue in response to primary cells. This process might be important for early establishment of the parasite due to higher vulnerability of the host immune system. Later, after production of the laminar layer (LL) and activation of Treg cells, a slightly altered profile of excreted/secreted products could support long-term persistence and growth of the metacestode (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3283565/ - pntd.0001516-Mejri1)[88]. Future investigations by using of genome sequence infor-

mation[5,89], and other genetic manipulation of primary cells[90], will give us opportunity for better understanding of the molecular mechanisms of parasite-host interactions.

CE AND AUTOIMMUNITY

Genetic predisposition in combination to others environmental factors have a decisive role in the induction of autoimmune reactions to *E. granulosus* and others parasitic infections. Genetically predisposed person with defective regulation of immune functions could be more receptive for autoimmune follow-up after parasite infections. A combination of autoimmunity environmental triggers and genetic factors can lead to immune imbalance and could influence the appearance of autoimmune diseases (Figure 3). CE and others parasitic disorders at their source will request the answer on question, why in some persons abnormal immune reactions arise more extensively than in others infected with the same parasite? To answer this question understanding of intrinsic mechanisms responsible for immune suppression need to be resolved. The well-known part of this mechanism is uncontrolled synthesis and elimination of self-reactive lymphocytes. Patients with CE showed increased number of T regulatory (Treg) cells, related cytokines such as IL-17 and IL-23, transcription factor FOXP3 and TGFβ-1 level compared to control healthy subjects[91]. The Th17/Treg balance controls inflammation and thus may play an important role in the pathogenesis of immune evasion[91]. To estimate damage of this disbalance, Rosenblum *et al*[92] (2015) detected Th17/Treg functions at different levels of immune reaction by analyzing the cell frequencies, related cytokine secretions and key transcription factors in CE (Figure 3). Findings showed that a Th17/Treg functional disbalance in patients with chronic CE, suggesting a potential role for a Th17/Treg imbalance in the pathogenesis and immune evasion of *E. granulosus*. Regarding to genes associated with autoimmune diseases, the strongest associations with HLA alleles were detected[93]. But, there are no data how different HLA alleles facilitate any autoimmune disease. Bearing in mind that many HLA alleles are capable to present self-antigens in healthy and in infected subjects, it is not clear how different HLA alleles influences autoimmune diseases. It is unlikely that a disease-associated allele is especially efficient at displaying the autoantigens targeted by self-reactive T cells due to most HLA alleles are capable of presenting self-antigens also in healthy subjects. Yet, most healthy individuals have autoreactive T cells that escape thymic deletion[94,95].

Genetic susceptibility to host autoimmunity

Knowledge of genes that are involved in induction of autoimmune diseases are discouraging, and is in lower level than those for HLA alleles. Completely different, genetic polymorphism of cytokine and cytokine receptors are well examined. Findings indicate that cytokines have been linked to many different autoimmune diseases, and IL-23 and IL-23R augmenting inflammatory capability of Th17 cells[94,95]. In that way, IL-23*R* have been discovered in ankylosing spondylitis, Behcet's disease, Crohn's disease, psoriasis, and ulcerative colitis[96]. Moreover, in all of these diseases IL-17 positive inflammatory cells have also been associated with tissue damage in all mentioned disorders. By using of monoclonal antibodies specific for either p40 (a subunit of IL-23) or IL-17A their efficacy were confirmed in almost all of these disorders[43,97]. Thus, genetic polymorphisms in IL-23R have in some cases been correlated with responses to targeted anti-cytokine therapies. Nevertheless the development of many human autoimmune diseases is result of reaction of multiple genes involved. There is opinion that gene polymorphism is responsible for the most human autoimmune diseases. Only a few examples existing in which genetic alterations in a single gene result in severe autoimmune syndroms. The two best examined monogenetic autoimmune syndroms resulting from the mutations in *AIRE* and *FOXP3* genes are autoimmune polyendocrine syndrome (APS) and immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome[98,99]. These mutations leading to dysfunction in central (APS) and peripheral (IPEX) tolerance. Another example of autoimmune lymphoproliferative disfunction of Fas gene, Fas ligand or of in caspases downstream of Fas signaling, resulting in a defective Fas-mediated apoptotosis and chronic lymphoproliferative causing lymphadenopathy, splenomegaly, and autoimmune cytopenias[100]. Discovering of the single genes responsible for development of aforementioned autoimmune disorders has greatly contribution in our understanding of cellular and subcellular machanisms responsible for the development and follow-up of many by parasite induced autoimmune diseases.

Environmental factors in host autoimmunity

Environmental triggers are factors originating outside of the body, such as parasites, bacteria, viruses, toxins and medications. No doubt that infections could be important triggering factor for autoimmune disfunctions[101,102]. Many theories have been created to explain this connection and excessive innate/pattern recognition receptor activation in autoimmunity. Epitope spreading and antigenic complementary are few between many theories proposed. There is evidence that the presence of *Epstein bar virus* in postmortem brain tissue has been linked with appearance of Multiple sclerosis (MS), but not with other autoimmune inflammatory diseases[103]. Furthermore, peridontal infections and rheumatoid arthritis are also linked with autoimmunity induced by infection[104]. In contrary, ideas existing that infection could protect from some autoimmune disorders. Thus germ-free mice exposed to *Bacteroides fragilis* could be protected from development of experimental autoimmune encephalomyelitis (EAE), by induction of Treg cells[105]. In that way, a higher incidence of MS and type 1 diabetes in developed countries is supposed to correlate with decreased number of infections[106].

Defective regulation as the cause of autoimmunity

The peripheral tolerance to tissue antigens could be induced by the low-level of natural cell death through

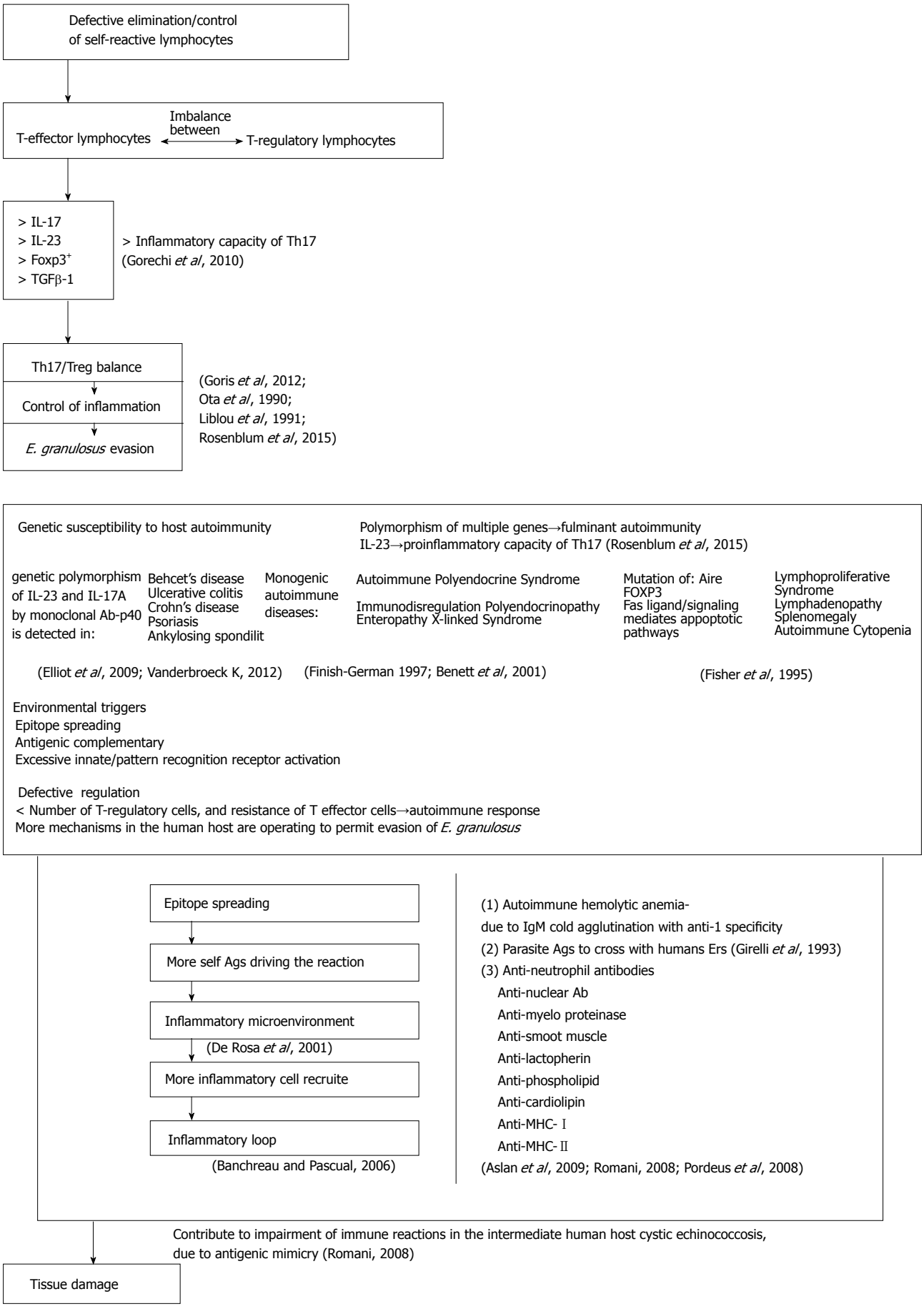

Figure 3 ***Echinococcus granulosus*** **Induced autoimmunity.** Ags: Antigens.

the tolerance of dendritic cell populations[107]. This tolerance of can influence low level of natural cell death in the tissue antigens, respectively[107]. If tolerance is the main abnormality in autoimmune processes, which kind of tolerance is processed in induction of autoimmune diseases? In SLE it is maturation of naïve B cells that can produce autoantibodies even before encountering with antigens. Findings that defects in early B cell tolerance checkpoints possible contribute development of an autoimmune disease[108]. Beside others, deletion cross influences B cell maturation of immature B cells in the bone marrow, receptor editing, and the control of mature B cells in peripheral tissues[108].

Regarding T cell-dependent autoimmunity and inflammatory autoimmune diseases, imbalance between effector and regulatory T cells play a fundamental role in initiation of human autoimmune diseases[109].

Decreased number of functional T regulatory or resistance of effector T cells play decisive role in the initiation of autoimmune diseases in human. Obtained results from patients with autoimmune disorders are often variable and inconsistent, due to the limited assesibility of tissue for examination. Even when an optimal number of cells for analysis is supplied, *in vitro* assays that often cannot be recapitulated, is not reliable for examination of functional capacity *in vivo*. The disease in autoimmunity might be explaned by self-perpetuating ability of autoimmune reactions. The self-antigen that drive autoimmune reaction remain functional and cannot be eliminated from the organism due to the emergency of a new antigenic epitopes in damaged tissue and alterations of self-proteins. This process is known as antigen epitope spreading phenomenon, and a vicious cyrcle is set-up.

Longitudinal studies of effector and Treg cells that are specific for target self-antigens in human disease remain a considerable technical challenge. Newly created antigenic epitopes activate more different lymphocytes in autoimmune reaction, leading to more tissue damage, and more novel epitopes to autoreactive lymphocytes. In that way, potentiated autoimmune reaction creates a new convenient environment for activation of multiple immune cells, cytokines and other mediators that amplify autoimmune reaction with catastrophic results to patient. In that way IFN-I production may induce SLE. Then inflammation in SLE showed is involved in the propagation of the disease[110]. The prolonged survival of *E. granulosus* metacestodes within the human host indicates that some mechanisms are operating to permit evasion of the host immune response. Several authors tried to describe autoimmune phenomena in patients with CE. Autoimmune hemolytic anemia due to IgM cold agglutinin with anti-I specificity was found to be induced in patients with CE. Moreover, the cleavage fragment of C3 has been detected on the erythrocyte membrane of a number of CE patients, suggesting that parasitic antigens may evoke antibodies that cross with human erythrocytes[111]. Furthermore, anti-neutrophil cytoplasmic, anti-myeloperoxidase and anti-lactoferrin antibodies have also been revealed in the sera of CE patients[112]. However, no significant correlations have been observed between CE and anti-nuclear antibodies, tissue specific autoantibodies and rheumatoid factors. In contrast, Aslan and coworkers have measured significant levels of antinuclear antibodies, anti-mitochondrial and anti-smooth muscle antibodies in patients with CE in comparison to age- and sex-matched healthy individuals[113]. Anti-phospholipid antibodies, anti-cardiolipin antibodies and anti-dsDNA have also been shown to be associated with several infectious diseases and some autoimmune diseases such as systemic lupus erythematosus and anti-phospholipid syndrome. Such elevated levels of these antibodies could be explained by the antigenic mimicry between the parasite antigens and host proteins[114]. Since cardiolipin and phospholipids are abundant in most cells of multicellular organisms, the former is an important component of the inner mitochondrial membrane, where it constitutes approximately 20% of the total lipid composition, while phospholipids are a class of lipids and a major component of all cell membranes as they can form a lipid bilayer[115,116]. Moreover, autoantibodies class I and class Ⅱ *MHC* gene products have also been demonstrated in CE patients, which may contribute to impairment of the host immune responses. Chronic and multiple infections with viruses, such as Epstein-Barr virus, cytomegalovirus and bacteria, such as *H. pylori*, may also be involved in the devolvement of an autoimmune disease in susceptible individuals[117].

Finaly, the parasites survive well in the human host and the host attempting to destroy them[118]. From a promotional perspective, knowledge of immune events to response on infection with a helminth parasite could be used to reduce the intensity of undesirable inflammatory reactions. But, poorly characterized cestode extracts cannot help the regulation of human immunocyte function. Yet the impact of these for treatment of autoimmune or allergic diseases is poorly understood. No doubt that helminth parasites are masters for immune evasion and regulation. A likely prerequisite for long-term survival is outwit of their host's attempt to eradicate them[119].

CONCLUSION

E. granulosus is very complex multicellular parasite. As many pathogens is highly immunogenic for human host. Thus, the host immunity play a most important role in host-parasite relationship in human ehinococcosis. The secretory and excretory products from parasite influences immune and immune competent cells in human host and stimulate humoral and proinflammatory cell-mediated immune responses, releasing of significant antibody production, and activate T cells and other antigen-presenting cells in human host. Thus, the understanding of the immune mechanisms is of fundamental importance for revealing of a basic protective processes in human with hydatidosis. No doubt that protective antibodies are also extremely important for development of a new more effective vaccines against *E. granulosus* and other parasites. Knowledge of immune events as a response to

infection with a helminth parasite could be used to reduce the intensity of undesired immune and autoimmune reactions such as a variety of auto-inflammatory diseases and allergy. Relevant findings is accumulating showing that inflammatory reactions that promote a variety of auto-inflammatory disease are dampened as a consequence of infection with helminth parasites *via* either the mobilization of anti-worm spectrum of immune reactions or direct effects of bioactive immunomodulatory molecules and chemical compounds released from the parasite. Also the cestode extracts are poorly characterized and their impact on autoimmune and allergic diseases are not fully examined due to the mechanisms of reaction are not understood. Yet issues related to this topics regarding purification of immunomodulatory molecules, their site effects and action to parasites remains as challenges that need to be addressed.

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ORIGINAL ARTICLE

Retrospective Study

Safety and efficacy of ledipasvir/sofosbuvir on hepatitis C eradication in hepatitis C virus/human immunodeficiency virus co-infected patients

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Abstract

AIM

To evaluate the safety and efficacy of ledipasvir/sofosbuvir on hepatitis C eradication in patients with hepatitis C virus (HCV)/human immunodeficiency virus (HIV) co-infection in an urban HIV clinic.

METHODS

A retrospective cohort study of 40 subjects co-infected with HIV-1 and HCV treated with the fixed-dose combination of ledipasvir and sofosbuvir for 12 wk from 2014 to 2016. All patients included were receiving antiretroviral therapy (ART) with HIV RNA values of 100 copies/mL or fewer regardless of baseline HCV RNA level. The primary end point was a sustained virologic response of HCV at 12 wk (SVR12) after the end of therapy.

RESULTS

Of the 40 patients enrolled, 55% were black, 22.5% had been previously treated for HCV, and 25% had

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Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment.

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cirrhosis. The patients were on a wide range of ART. Overall, 39 patients (97.5%) had a SVR 12 after the end of therapy, including rates of 97.1% in patients with HCV genotype 1a and 100% in those with HCV genotype 1b. One patient with HCV genotype 3a was included and achieved SVR12. Rates of SVR12 were similar regardless of previous treatment or the presence of compensated cirrhosis. Only 1 patient experienced relapse at week 12 following treatment and deep sequencing didn't reveal any resistance associated mutation in the NS5A or NS5B region. Interestingly, 7 (17.5%) patients who were adherent to ART experienced HIV viral breakthrough which resolved after continuing the same ART regimen. Two (5%) patients experienced HIV-1 virologic rebound due to noncompliance with HIV therapy, which resolved after resuming the same ART regimen. No severe adverse events were observed and no patient discontinued treatment because of adverse events. The most common adverse events included headache (12.5%), fatigue (10%), and diarrhea (2.5%).

CONCLUSION

This retrospective study demonstrated the high rates of SVR12 of ledipasvir/sofosbuvir on HCV eradication in patients co-infected with HCV and HIV, regardless of HCV baseline levels, HCV treatment history or cirrhosis condition. The oral combination of ledipasvir/sofosbuvir represents a safe and well tolerated HCV treatment option that does not require modification for many of the common HIV ART. Occasional HIV virologic rebound occurred but later resolved without the need to change ART.

Key words: Hepatitis C; Human immunodeficiency virus; Ledipasvir; Sofosbuvir

Core tip: This is a retrospective study to evaluate the safety and efficacy of ledipasvir/sofosbuvir on hepatitis C eradication in patients with hepatitis C virus (HCV) and human immunodeficiency virus (HIV) co-infection in an urban HIV clinic. It demonstrated the high rates of SVR12 of ledipasvir/sofosbuvir on HCV eradication in patients co-infected with HCV and HIV, regardless of HCV baseline levels, HCV treatment history or cirrhosis condition. The oral combination of ledipasvir/sofosbuvir represents a safe and well tolerated HCV treatment option that does not require modification for many of the common HIV antiretroviral therapy (ART). Occasional HIV virologic rebound occurred but later resolved without the need to change ART.

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INTRODUCTION

More than 185 million people around the world are infected with the hepatitis C virus (HCV), 350000 of whom die each year[1,2]. Human immunodeficiency virus (HIV) and HCV have common routes of transmission, and it is estimated that 4-5 million persons out of the 185 million infected by HCV are also co-infected by HIV. On the other hand, up to 30% of HIV positive patients are infected with HCV[2,3]. There is increasing evidence that HCV coinfection has a harmful effect on the progression of HIV infection with increased risk of mortality[4]. Liver disease has become a major cause of morbidity and mortality in HIV-infected persons.

Sustained viral response (SVR) (equivalent to eradication of HCV) after administering anti-HCV therapy is associated with improved survival and reduced liver decompensation in patients with chronic hepatitis C with HIV infection[5,6]. It may also decrease the progression of HIV infection and mortality not related to liver disease[7]. The mainstay of therapy over the last two decades involved a combination of interferon α and ribavirin (RBV). SVR rates with pegylated interferon and RBV were very low, averaging between 40% and 50% and the treatment duration required is long, ranging from 24 to 48 wk[1]. In addition, peginterferon has many side effects and contraindications. Many patients with HIV infection are unwilling to take interferon. The availability of an effective HCV interferon free regimen is highly needed for the management of hepatitis C in HIV infected patients.

In recent years, the management of chronic hepatitis C has been revolutionized by the development of direct-acting antiviral agents (DAAs) which significantly improved rates of cure in chronic HCV infection. Ledipasvir is an inhibitor of nonstructural protein 5A (NS5A), which has an important role in HCV RNA replication[8]. Sofosbuvir (SOF), a uridine nucleotide analog prodrug, was approved by the US FDA in December 2013. The active metabolite of SOF, is incorporated by the NS5B polymerase into HCV RNA, resulting in chain termination[3]. The fixed-dose combination of ledipasvir and sofosbuvir has demonstrated minimal toxicities and high efficacy, with an overall SVR of over 91%, in patients infected with HCV genotype 1, without the need for either interferon or RBV[8-10]. Osinusi *et al*[11] for the first time, reported that the combination of ledipasvir and sofosbuvir was associated with SVR rate of 98% in patients co-infected with HCV genotype 1 and HIV in a phase 2 study. Later, a larger phase 3 trial (ION-4 study) demonstrated that 12 wk of treatment with ledipasvir/sofosbuvir resulted in a SVR rate of 96% in patients who were co-infected with HIV and HCV genotype 1 or 4[12]. Harvoni, the fixed dose combination of ledipasvir and sofosbuvir, became the first approved once daily Single-tablet-regimen (STR) for treatment of chronic HCV in HIV positive patients in Nov 2015. This combination may have additional mental health benefits in HIV/HCV co-infected patients[13].

Currently, there are few published data on the experience with this newly approved combination of ledi-

Table 1 Demographic characteristics of the patients at baseline *n* (%)

Characteristic	Ledipasvir/sofosbuvir for 12 wk (*n* = 40)
Median age (IQR) - yr	53 (51-57)
Male sex	25 (62.5)
Race[1]	
White	13 (32.5)
Black	22 (55.0)
Asian	1 (2.5)
Other or unknown	4 (10.0)
Mean body-mass index (IQR)[2]	26.2 (22.7-28.7)
Smoking	13 (32.5)
HCV genotype	
1a	34 (85.0)
1b	5 (12.5)
3a	1 (2.5)
Baseline HCV RNA (IQR), log10 IU/mL	6.3 (6.0-6.6)
HCV RNA > 6 million IU/mL	5 (12.5)
Cirrhosis	10 (25.0)
Baseline creatinine, mean (range), mg/dL	0.95 (0.56-1.48)
Baseline eGFR, mean (range), mL/min	90.0 (52-134)
CD4, cells/mm^3	
< 200	1 (2.5)
200-350	7 (17.5)
> 350	32 (80)
Mean CD4$^+$ cell count (IQR), cells/μL	638 (366-857)
Antiviral regimen	40 (100)
HCV treatment history	
No previous treatment	31 (77.5)
Previous treatment	9 (22.5)

[1]Self-reported; [2]Calculated as weight in kilograms divided by height in meters squared. BMI: Body mass index; eGFR: Estimated glomerular filtration rate; HCV: Hepatitis C virus; IQR: Interquartile range.

pasvir/sofosbuvir in HCV/HIV co-infected patients. Here, we reported a single-center, retrospective study evaluating the safety and efficacy of this combination on HCV eradication in the patients co-infected with HCV and HIV with or without previous treatment for HCV.

MATERIALS AND METHODS

Study design and setting

This was a retrospective cohort study. All of the research reviews were conducted under protocols approved by the institutional independent ethics committee and all data were collected and analyzed in a Health Insurance Portability and Accountability Act-compliant manner to ensure patient privacy and data integrity. The study was conducted in Sunshine Care Center (Florida Department of Health in Orange County, Orlando, FL), an urban HIV clinic in Orlando.

Subjects

Patients older than 18 years diagnosed as HIV/HCV co-infection at Sunshine Care Center between 2014 and 2016 and treated with the fixed dose combination of ledipasvir/sofosbuvir for 12 wk were included. Charts were reviewed and data were collected by a trained internal medicine resident. The presence of cirrhosis was determined by liver biopsy with a Metavir score of F4; or a score of more than 12.5 kPa on transient elastography testing (Fibroscan); or Radiological imaging consistent with cirrhosis.

For each patient included in the study, the demographic data were collected through manual chart review, including age, race, sex, body-mass index (BMI), smoking history, HCV genotype, and medical history (Table 1).

Efficacy and safety assessments

The primary efficacy end point was sustained virologic response (HCV RNA level < 15 IU/mL by real-time HCV assay) at 12 wk after treatment completion (SVR12) among all patients enrolled in the study.

Pre-treatment, during treatment, and post-treatment data of the standard laboratory testing (complete blood count (CBC), levels of albumin, bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen, creatinine) and measurements of plasma HCV RNA and HIV-1 RNA levels, along with evaluations of adherence were collected. Plasma HCV RNA levels were measured using the real-time HCV assay (Abbott), with the Lower Limit of Quantitation (LLOQ) of 15 IU/mL. Plasma HIV RNA levels were measured at all points using reverse transcription polymerase chain reaction (real-time HIV assay), with an LLOQ of 40 copies/mL. Adherence to ledipasvir and sofosbuvir was measured by pill counts and patient self-report. All adverse events were recorded and graded according to the NIAID Division of AIDS toxicity table (version 1.0 2009 clarification).

Definitions

Hepatitis C viral relapse was defined as an HCV RNA level higher than the LLOQ at any posttreatment point after having an HCV RNA level lower than the LLOQ at the end of treatment. Hepatitis C viral breakthrough was defined as an HCV RNA level at the LLOQ or higher during treatment after having previously had an HCV RNA level lower than the LLOQ while taking study drugs, confirmed with 2 consecutive values. HIV viral breakthrough was defined as an HIV RNA level at the LLOQ or higher during treatment after having previously had an HIV RNA level lower than the LLOQ while taking ART, confirmed with 2 consecutive values. Patients with plasma HIV-1 RNA levels of 400 copies per milliliter or higher at two or more consecutive post-baseline visits at least 2 wk apart were considered to have HIV-1 virologic rebound.

Deep sequencing

Deep sequencing of the HCV NS5A and NS5B regions was performed only for the patient with virologic failure, from samples collected at the time of virologic failure, using DDL (DDL Diagnostics Laboratory). Variants that were present in at least 1% of the viral population were reported.

Statistical analysis

We calculated the proportion of patients who had a

Table 2 Antiviral regimen

Antiviral regimen	*n* (%)
Efavirenz-emtricitabine-tenofovir DF	1 (2.5)
Tivicay-emtricitabine-tenofovir DF	1 (2.5)
Rilpivirine-emtricitabine-tenofovir DF	5 (12.5)
Raltegravir- Rilpivirine-emtricitabine-tenofovir DF	2 (5)
Raltegravir-emtricitabine-tenofovir DF	6 (15)
Ritonavir- Raltegravir-emtricitabine-tenofovir DF	1 (2.5)
Dolutegravir-emtricitabine-tenofovir DF	1 (2.5)
Raltegravir-telaprevir	4 (10)
Abacavir-dolutegravir-lamivudine	8 (20)
Abacavir-etravirine-lamivudine	1 (2.5)
Darunavir-ritonavir-etravirine-raltegravir	3 (7.5)
Abacavir-lamivudine-darunavir-ritonavir	3 (7.5)
Abacavir-lamivudine-darunavir-ritonavir-etravirine-raltegravir	1 (2.5)
Elvitegravir, cobicistat, emtricitabine, tenofovir alafenamide	3 (7.5)

DF: Disoproxil fumarate.

Table 3 Response during and after therapy

Response	Ledipasvir-Sofosbuvir for 12 wk (*n* = 40), *n* (%)
HCV RNA < LLOQ[1]	
During therapy period	
At wk 4	34 (78.2)
At wk 12	40 (100)
After end of therapy	
At wk 4	40 (100)
At wk 12[2]	39 (97.5)
HCV viral breakthrough	0 (0)
HCV viral relapse	1 (2.5)
HIV viral breakthrough	7 (17.5)
HIV virologic rebound	2 (5)

[1]LLOQ denotes lower limit of quantification (HCV RNA in serum, < 15 copies per milliliter HIV RNA in serum, < 40 copies per milliliter). [2]A sustained virologic response 12 wk after the end of therapy was the primary end point. SVR: Sustained viral response.

sustained virologic response along with exact two-sided 95%CI using the Clopper-Pearson method. Statistical differences were analyzed by χ^2 tests for categorical variables and *t*-test for continuous variables with significance defined as a *P* value less than 0.05.

RESULTS

Study patients

A total of 40 patients were enrolled. Eighty-five percent of patients were infected with HCV genotype 1a, 12.5% with HCV genotype 1b, and 2.5% with HCV genotype 3a (Table 1). Overall, 55% of patients were black, 62.5% were male, 25% had compensated cirrhosis, and 22.5% had received previous unsuccessful treatment for HCV. Among the 10 patients with cirrhosis, the mean baseline albumin level was 4.3 g per deciliter, the mean platelet count was 110940 per microliter, the mean Bilirubin level was 1.0 milligrams per deciliter (mg/dL), the mean ALP level was 101 units per liter (U/L), the mean ALT level was 89 U/L, and the mean AST level was 67 U/L. Nine patients received previous treatments for HCV with pegylated interferon (peginterferon) plus ribavirin. All patients were receiving ART with a wide range of regimen (Table 2).

Efficacy

Among the 40 patients who were enrolled and treated, 39 [97.5%; 95% confidence interval (CI), 90 to 100] had a sustained virologic response 12 wk after the end of therapy (Table 3). The rates of response at 12 wk were similar in patients with genotype 1a (97%) and those with 1b (100%), in men (96%) and women (100%), in black patients (100%) and other races (94.4%), in patients who had undergone previous treatment (100%) and those who had not (96.8%), in patients with cirrhosis (100%) and those without cirrhosis (96.7%).

Only 1 patient did not achieve SVR12 and experienced relapse by week 12 after treatment completion. This was a 53-year-old white male, with HCV genotype 1a infection and stage 1 liver disease. The baseline HCV viral load was 11370594 IU/mL as determined by real-time PCR assay. The medications that he received against HIV infection included raltegravir, etravirine, ritonavir and darunavir. HCV viral suppression was achieved by week 8 with viral load lower than the LLOQ, which was maintained through 12 wk. However, HCV viral load increased to 7043 IU/mL at week 12 after treatment completion and was 7165187 at week 16 after treatment completion. Deep sequencing failed to reveal any mutation was seen in the NS5A or NS5B region.

Changes in liver and renal function

Levels of ALT and AST became normal rapidly with treatment (Figure 1A). There were no significant changes in estimated GFR or serum creatinine levels over time (Figure 1B and C. No participants were identified as having a treatment-emergent eGFR less than 50 mL/min or a decrease in eGFR (mL/min) greater than 25%.

Changes in HIV parameters

The mean $CD4^+$ cell count at baseline was 638 cells per microliter; the $CD4^+$ count was under 200 cells per microliter in 1 patient and under 350 cells per microliter in 7 patients. There were no significant changes in CD4 cell counts with treatment (Figure 1D).

Two patients experienced HIV-1 virologic rebound. One had missed 2 wk ART (emtricitabine/rilpivirine/tenofovir DF) and the other had missed 5 d of ART (emtricitabine/tenofovir DF/raltegravir). They continued the same regimen and the HIV viral load was less than 20 copies/mL by the next visit (4 wk later). Moreover, 7 patients experienced HIV breakthrough, a transient increase in HIV viral load (HIV-1 RNA $\geqslant$ 40 copies/mL) while in the study. All of them denied non-compliance with ART. They continued the same regimen and the HIV viral load was less than 40 copies/mL 4 to 8 wk later. All of these 9 patients achieved

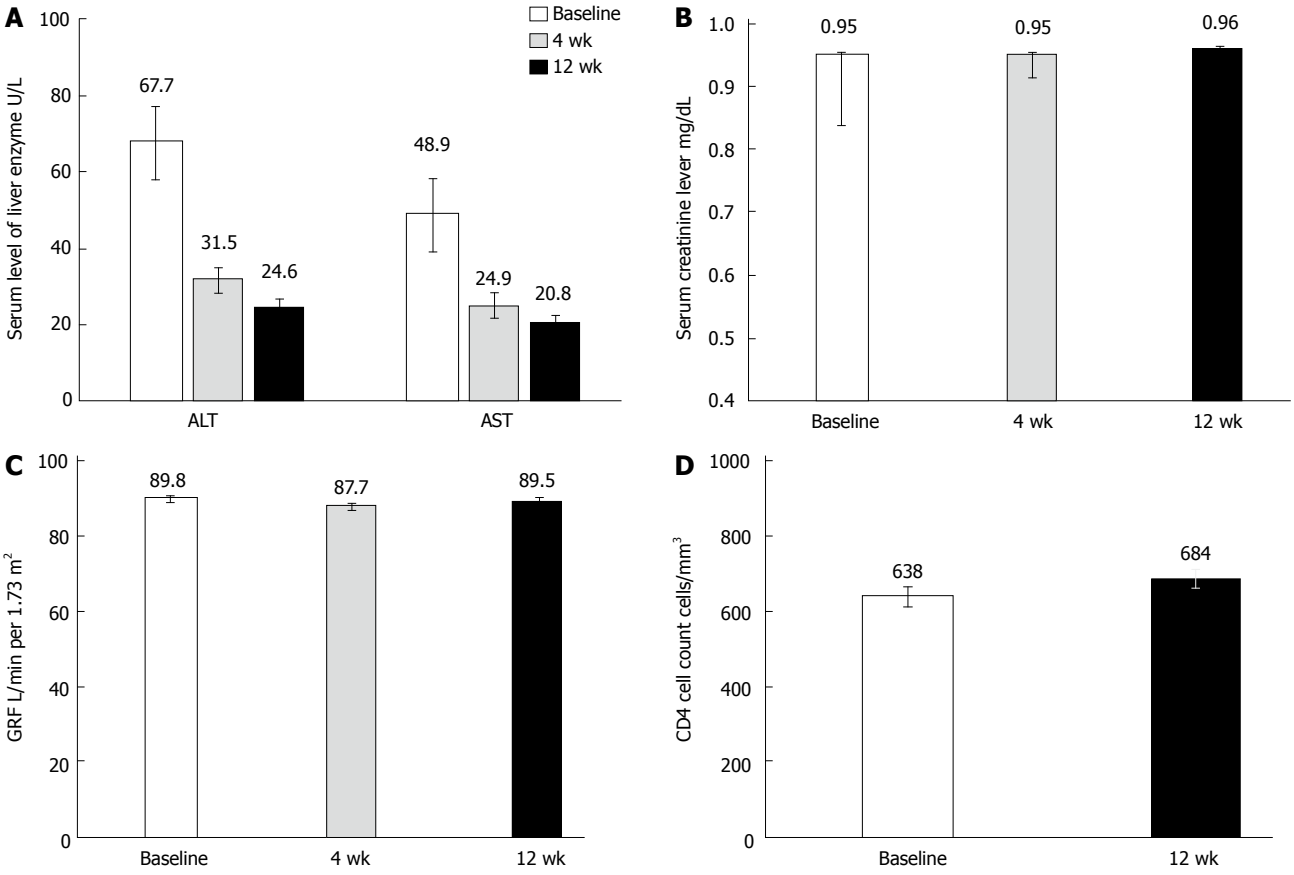

Figure 1 Lab changes during treatment. A: Changes in liver function tests; B: Changes in serum creatinine level; C: Changes in GFR level; D: Changes in CD4 cell count.

SVR12 for HCV treatment.

Adverse events

There were no deaths or serious adverse events observed in this study. The most common adverse events were mild to moderate headache (12.5%), fatigue (10%), and diarrhea (2.5%). Symptoms resolved while the patient was receiving study drug.

Adherence

Adherence to ledipasvir and sofosbuvir, as measured by pill counts, was high over the course of treatment. Ninety-five percent of all participants had no missed doses. Five percent of patients missed 1 to 4 doses of study drug, for an adherence rate greater than 95%. As determined by pill count at the end of study, the participant who experienced HCV viral relapse by week 12 after treatment completion reported no missed doses.

DISCUSSION

In this retrospective study, the combination of ledipasvir and sofosbuvir was associated with a high rate of SVR (97.5%) in HIV and HCV co-infected patients, comparable with SVR rates observed in the previous clinical trials[11-13]. Our study included HCV treatment-naïve (77.5%) and treatment-experienced (22.5%) patients, including patients with compensated cirrhosis (25%). Consistent with the previous reports[11,12], HCV treatment history, baseline HCV RNA levels, and cirrhosis didn't appear to have any effect on SVR12 rates. All 9 treatment experienced patients and 30 of 31 treatment naive patients achieved SVR, regardless of HCV baseline levels. One patient with HCV 3a genotype was also allowed to enroll in the study and successfully achieved SVR12. In the recent ION 4 study, black patients with HCV and HIV co-infection were reported to have lower rates of SVR compared to non-black patients who received 12 wk treatment of ledipasvir/sofosbuvir[12]. However, no differences in efficacy were observed in patients when stratified by race in our study. Using data from the three open-label ION clinical trials, Wilder *et al*[14] evaluated the efficacy of ledipasvir/sofosbuvir in 308 black patients. Consistent with our result, they found that an once daily dosage of ledipasvir/sofosbuvir was similarly effective in black and non-black patients with genotype 1 HCV infection. In the 1 participant who experienced relapse, HCV sequencing data didn't detect any mutation in the NS5A or NS5B region at the time of relapse and the patient was adherent to his medications, suggesting that the underlying mechanism contributing to the resistance to this regimen is unknown and needs future investigation.

All patients enrolled in this study were receiving

an antiretroviral regimen for HIV-1 with evidence of HIV-1 viral suppression to a level less than 100 copies per milliliter. The main strength of this study was that the patients were on a wide range of antiretrovirals, including complex antiretroviral regimens that contained drugs from 3 or more antiretroviral classes. Drug-drug interactions between certain directly acting antiviral agents such as boceprevir, telaprevir, and antiretrovirals could result in adverse events or antiretroviral failures, restricting the wider use of these medications in patients with HIV[15,16]. Although Ledipasvir-sofosbuvir has limited potential for clinically significant drug interactions with most antiretroviral agents[17], the results from the phase 1[17] and 3[12] evaluations suggested potential drug interaction between ledipasvir/sofosbuvir and tenofovir resulting in increased exposure of tenofovir. Four patients developed treatment-emergent worsening of renal function which might be related to increased exposure of tenofovir[12]. In our study, evaluation of renal function didn't reveal significant changes in GFRs and serum creatinine levels throughout this study and no patients taking tenofovir were required to modify HIV treatment due to tenofovir-induced complications. In the previous study, patients taking ritonavir-boosted HIV-1 protease inhibitors or cobicistat-boosted elvitegravir with tenofovir disoproxil fumarate were excluded, so the safety of this HCV combination in patients with HIV-1 infection who are receiving these antiretroviral regimens is unknown[12]. Interestingly, 11 patients enrolled in this study were on ritonavir-boosted or cobicistat-boosted ART and no severe adverse effects were noticed with SVR12 rates of 91.0% (the patient who experienced relapse was receiving the combination of darunavir-ritonavir-etravirine-raltegravir for ART). Thus, ledipasvir/sofosbuvir treatment represents a safe HCV treatment option that does not require modification for many of the common antiretroviral regimens.

In our study, ledipasvir/sofosbuvir treatment in HIV-HCV-coinfected patients did not compromise HIV control. CD4 cell counts remained stable and HIV RNA remained suppressed for the majority of participants throughout the study. Seven patients who were adherent to the medications experienced transient mild HIV viral breakthrough with maximum HIV RNA less than 250 copies. The viral breakthrough resolved spontaneously 4 to 8 wk after the patients were continuing the same ART regimen. HIV viral rebound documented in 2 participants was associated with nonadherence to antiretroviral treatment, which also resolved after resuming the same ART treatment.

In this study, there were no deaths, medication discontinuations, or severe adverse events attributable to ledipasvir/sofosbuvir treatment. Most adverse events associated with combined ledipasvir and sofosbuvir in participants co-infected with HCV and HIV were mild.

In conclusion, excellent treatment outcomes among our cohort of HIV/HCV co-infected patients were achieved with the FDA approved combination of ledipasvir/sofosbuvir for HCV. The main strength of this study was that a broad range of antiretrovirals were included in this study which demonstrated that ledipasvir/sofosbuvir was generally well tolerated when coadministered with a broad range of ART. Larger studies are required to further understand the efficacy and safety of the combination of ledipasvir/sofosbuvir in HIV/HCV co-infected patients.

COMMENTS

Background

Hepatitis C virus (HCV) coinfection has a harmful effect on the progression of human immunodeficiency (HIV) infection with increased risk of mortality. In recent years, the management of chronic hepatitis C has been revolutionized by the development of direct-acting antiviral agents (DAAs) which significantly improved rates of cure in chronic HCV infection.

Research frontiers

The fixed dose combination of ledipasvir and sofosbuvir demonstrated high SVR rate in patients infected with HCV and recently became the first approved once daily Single-tablet-regimen for treatment of chronic HCV in HIV positive patients. Currently, there are few published data on the experience with this newly approved combination of ledipasvir/sofosbuvir in HCV/HIV co-infected patients. The drug interaction is always a safety concern while treating HCV/HIV co-infected patients.

Innovations and breakthroughs

The authors conducted a single-center, retrospective study evaluating the safety and efficacy of the combination of ledipasvir/sofosbuvir on HCV eradication in the patients co-infected with HCV and HIV with or without previous treatment for HCV. Overall, the rate of SVR12 was 97.5% and only 1 (2.5%) patient experienced relapse at week 12 following treatment. No severe adverse events were observed and no patient discontinued treatment because of adverse events.

Applications

The results demonstrated that this combination represents a safe and well tolerated HCV treatment option that does not require modification for many of the common HIV ART.

Terminology

SVR: Sustained viral response. SVR is specific to hepatitis C and is the absence of HCV RNA for 12 wk after the cessation of treatment.

Peer-review

Clearly written and stylish manuscript. The approach is not very original (in the last years several papers regarding the efficacy of DDA in real life settings have been published) but since in this subgroup of patients there are few publications, it is still interesting.

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Abstract

This review considers the modern concepts of pathogenesis, diagnostic methods, and treatment principles of hepatic hydrothorax (HH). HH is the excessive (> 500 mL) accumulation of transudate in the pleural cavity in patients with decompensated liver cirrhosis but without cardiopulmonary and pleural diseases. It causes respiratory failure which aggravates the clinical course of liver cirrhosis, and the emergence of spontaneous bacterial pleural empyema may be the cause of death. The information was collected from the PubMed database, the Google Scholar retrieval system, the Cochrane reviews, and the reference lists from relevant publications for 1994-2016 using the keywords: "liver cirrhosis", "portal hypertension", "hepatic hydrothorax", "pathogenesis", "diagnostics", and "treatment". To limit the scope of this review, only articles dealing with uncomplicated hydrothorax in patients with liver cirrhosis were included. The analysis of the data showed that despite the progress of modern hepatology, the presence of HH is associated with poor prognosis and high mortality. Most patients suffering from it are candidates for orthotopic liver transplantation. In routine clinical practice, stratification of the risk for an adverse outcome and the subsequent determination of individual therapeutic strategies may be the keys to the successful management of the patient's condition. The development of pathogenetic pharmacotherapy and optimization of minimally invasive treatment will improve the quality of life and increase the survival rate among patients with HH.

Key words: Liver cirrhosis; Portal hypertension; Hepatic hydrothorax; Diagnostics; Treatment

Core tip: This review considers the modern concepts of pathogenesis, diagnostic methods, and treatment principles of hepatic hydrothorax (HH). The analysis of the data showed that despite the progress of modern hepatology, the presence of HH is associated with poor prognosis and high mortality. Most patients suffering from it are candidates for orthotopic liver transplantation. In routine clinical practice, stratification of the risk for an adverse outcome and the subsequent determination of individual therapeutic strategies may be the keys to

the successful management of the patient's condition. The development of pathogenetic pharmacotherapy and minimally invasive treatment will improve the quality of life and increase the survival rate among patients with HH.

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INTRODUCTION

Pleural effusion is a syndrome characterized by the accumulation of fluid in the pleural cavity, which has more than 50 causes associated with both pleural and pulmonary diseases, as well as the pathology of other organs and systems. Depending on its nature, it is classified into exudate and transudate. The criteria for their differentiation proposed in 1972, are currently the "gold standard" of differential diagnosis (Table 1)[1]. Hepatic hydrothorax (HH) is the excessive (> 500 mL) accumulation of transudate in the pleural cavity in patients with decompensated liver cirrhosis (LC) but without cardiopulmonary and pleural diseases. Its localization is right-sided in approximately 85% of cases and left-sided in approximately 13%; whereas, only 2% of patients have fluid in the pleural cavity on both sides.

HH is observed infrequently and, depending on the diagnostic method, may be found in almost 5%-10% of patients, constituting 2%-3% of all causes of pleural effusions. However, it causes respiratory failure which aggravates the clinical course of LC. The emergence of spontaneous bacterial pleural empyema may be the cause of death. Median survival in the presence of HH is 8-12 mo[2].

CAUSES AND MECHANISMS OF THE DEVELOPMENT OF HH

Several theories of HH pathogenesis in patients with LC are known, but the most probable one presupposes ascites formation, due to portal hypertension related pathophysiological disorders[3]. Concomitant splanchnic and systemic arterial vasodilation, along with the activation of various neurohormonal signaling pathways, cause kidney dysfunction and hence decrease Na^+ and water excretion, as well as the glomerular filtration rate[4]. Ascitic fluid moves from the peritoneal cavity into the pleural space through small defects located mainly on the right side of the diaphragmatic tendon. The reason for this is negative intrathoracic pressure. Moreover, the liver in this situation may play the role of a piston[5]. Huang *et al*[6] classified diaphragmatic defects into four types: (1) Type 1 - no obvious defects; (2) Type 2 - blebs lying on the diaphragm; (3) Type 3 - broken defects (fenestrations) in the diaphragm; (4) Type 4 - multiple gaps in the diaphragm.

This theory of HH pathogenesis, first proposed in 1966, has been confirmed by numerous studies using air infused intraperitoneally, dyes, or radiolabeled material[7]. It does not contradict the phenomenon of isolated HH, in which the pleural reabsorption rate of ascites is equal to the ascites production in the abdominal cavity[8].

DIAGNOSTICS OF HH

In patients suffering from LC with or without ascites, clinical examination may reveal pleural effusion and allow preliminary diagnosis of HH, which is often right-sided. Its left-sided localization, the presence of fever, and respiratory symptoms require the exclusion of other diseases, as well as spontaneous bacterial pleural empyema. For this purpose, a pleural puncture is performed at the first stage of the diagnosis to obtain and analyze fluid, which is inherently a transudate. This is similar to ascitic fluid, but the different absorption mechanisms from the pleural and abdominal cavities cause some distinctions between the two of them (Table 2)[9]. Radioisotope diagnostic techniques are used to make a decision in doubtful cases. Migration of intraperitoneally infused 99mTc-labeled microspheres of human serum albumin or a sulfur colloid into the pleural cavity confirms the diagnosis of HH, and the rate of isotope movement indicates the size of defects in the diaphragm[10]. Color Doppler and magnetic resonance imaging are used for their direct visualization[11].

TREATMENT OF HH

Currently, there are no evidence-based standards for the management of patients with HH. In usual clinical practice, the aim of therapeutic measures is to treat ascites and to provide thoracentesis if necessary (Figure 1). In case of adequate urine sodium concentration (> 30 mEq/24 h), this means completely eliminating a pleural effusion and thereby removing the signs of respiratory failure[12].

In accordance with modern clinical recommendations, the severity of ascites determines its treatment[13]. Accordingly, patients with LC and mild ascites do not need diuretics and a low-sodium diet. Since sodium excretion is slightly weakened in most patients with moderate ascites, the aim of the treatment is to reduce the intake of Na^+ and stimulate its excretion by using diuretics and maintaining a usual drinking regimen. The consumption of Na^+ should be reduced to 80-120 mmol/d, which corresponds to 4.6-6.9 g of salt per day.

In addition to this diet, patients should take either spironolactone or amiloride at an initial dose of 50-200 mg/d or 5-10 mg/d, respectively. The dose of spironolactone should be gradually increased by 100 mg every seven days. The maximum dose is 400 mg/d. The treatment is effective if there is a body weight reduction

Table 1 Criteria for the differentiation between exudates and transudates in pleural fluid examination

Criteria	Exudate	Transudate
Effusion protein/serum protein ratio	> 0.5	< 0.5
Absolute LDH level in pleural fluid	> 200 IU/L	< 200 IU/L
Effusion LDH/serum LDH ratio	> 0.6	< 0.6

LDH: Lactate dehydrogenase.

Table 2 Diagnostic criteria for uncomplicated hepatic hydrothorax according to pleural fluid analysis

Criteria	Values
The number of white blood cells in pleural fluid	< 250/mm^3
Pleural effusion total protein level	< 25 g/L
Pleural effusion total protein/serum total protein ratio	< 0.5
Pleural effusion LDH/serum LDH ratio	> 0.6
Pleural effusion albumin/serum albumin ratio	> 1.1
Pleural effusion bilirubin /serum bilirubin ratio	< 0.6
PH	> 7.4
Pleural effusion glucose level is equal to serum glucose level	

LDH: Lactate dehydrogenase.

of at least 2 kg/wk. If monotherapy with spironolactone is inefficient or if hyperkalemia develops, furosemide should be taken at a starting dose of 40 mg/d with a gradual increase of 40 mg increments every seven days**,** with a maximum of 160 mg/d. In the case of hyperkalemia, the maximum permissible dose of spironolactone is 400 mg/d. The maximum acceptable weight loss for patients with ascites without peripheral edema is 0.5 kg/d. For patients with ascites and peripheral edema, it is 1 kg/d.

In patients with HH and tense ascites, the recommended procedure is to perform large-volume paracentesis (LVP) and albumin infusions in order to prevent associated circulatory disorders (8 g for each removed liter of ascitic fluid). The prospective study made by Angueira *et al*[14] has shown a statistically significant increase in the total lung capacity and functional residual capacity of the lungs with an improvement in the symptoms of HH within 2 h after LVP. It also contributed to an increase in PaO_2/FiO_2 and lung volume at the end of exhalation without hemodynamic disturbances, as noted in another prospective study of 31 mechanically ventilated patients with LC and acute lung injury[15].

Refractory ascites is defined as ascites that cannot be mobilized to at least first grade using diuretic treatment and dietary sodium restriction or as ascites which early recurrence after LVP cannot be satisfactorily prevented by medical therapy[16]. In this case, the first-line of treatment is LVP repeated every 2-3 wk in combination with albumin infusions and the prescription of diuretics to patients who have the urine sodium concentration > 30 mEq/d[17]. If this is ineffective, transjugular intrahepatic portosystemic shunt (TIPS) is recommended[18]. This operation reduces portal pressure, thereby improving the function of the cardiovascular system, which increases renal blood flow and the glomerular filtration rate[19]. Surgical intervention in refractory ascites may be considered in the case of LVP futility and when TIPS cannot be implemented due to technical, organizational, or anatomical problems. The operation of choice is peritoneovenous shunting[20].

In a multicenter, nonrandomized trial, Bellot *et al*[21] evaluated the efficacy of a device called "Automated Low Flow Pump System" (ALFApump system) in 40 patients with refractory ascites. It moves ascitic fluid from the abdominal cavity to the bladder through a system of catheters connected to a pump implanted beneath the skin. It was noted that 40% of patients had no need for LVP after its implantation, and 70% needed LVP less than once a month. Nevertheless, there is a high percentage of complications, which mainly involve migration and/or blockage of urinary or peritoneal catheters (22.5% and 12.5%, respectively). This fact currently excludes the introduction of this method into clinical practice and requires further study.

Treatment of refractory HH

Refractory HH occurs under significant disturbance of Na^+ excretion (< 10 mEq/d); therefore, measures aimed at treating ascites cannot eliminate it[22].

Pharmacotherapy: Despite the absence of specific pharmacotherapy for refractory HH, it is possible to use drugs recommended for the treatment of portal hypertension[23]. One of the publications demonstrated the good effects of octreotide, a synthetic analog of somatostatin, prescribed after ineffective use of diuretics with a low sodium diet, pleurodesis, and TIPS. It was infused intravenously at a dose of 25 μg/h on the first day, 50 μg/h on the second day, and 100 μg/h for the next five days. Then it was injected subcutaneously. The amount of fluid in the pleural cavity decreased after the fifth day. During a six-month period of observation, there was no relapse of HH[24]. The positive effect of octreotide can be explained by its ability to suppress the activation of the renin-angiotensin-aldosterone system induced by diuretics, as well as to increase the excretion of Na^+ and water[25].

In another case, a positive result was achieved after a five-day course of terlipressin therapy in combination with albumin infusions given to a patient with decompensated LC who had type 1 hepatorenal syndrome along with HH[26].

Therapeutic thoracentesis: Repeated thoracentesis is the routine procedure to remove fluid from the pleural space in refractory HH. This procedure is relatively safe even in patients with an increased risk of bleeding[27]. Nevertheless, it is important to bear in mind the possibility of complications associated with it, such as pneumothorax, pleural empyema, purulent soft tissue infection of the chest wall, and air embolism, among others[28]. In addition, large-volume thoracentesis, which is necessary in a number of cases, may increase microvascular permeability and cause re-expansion pulmonary edema.

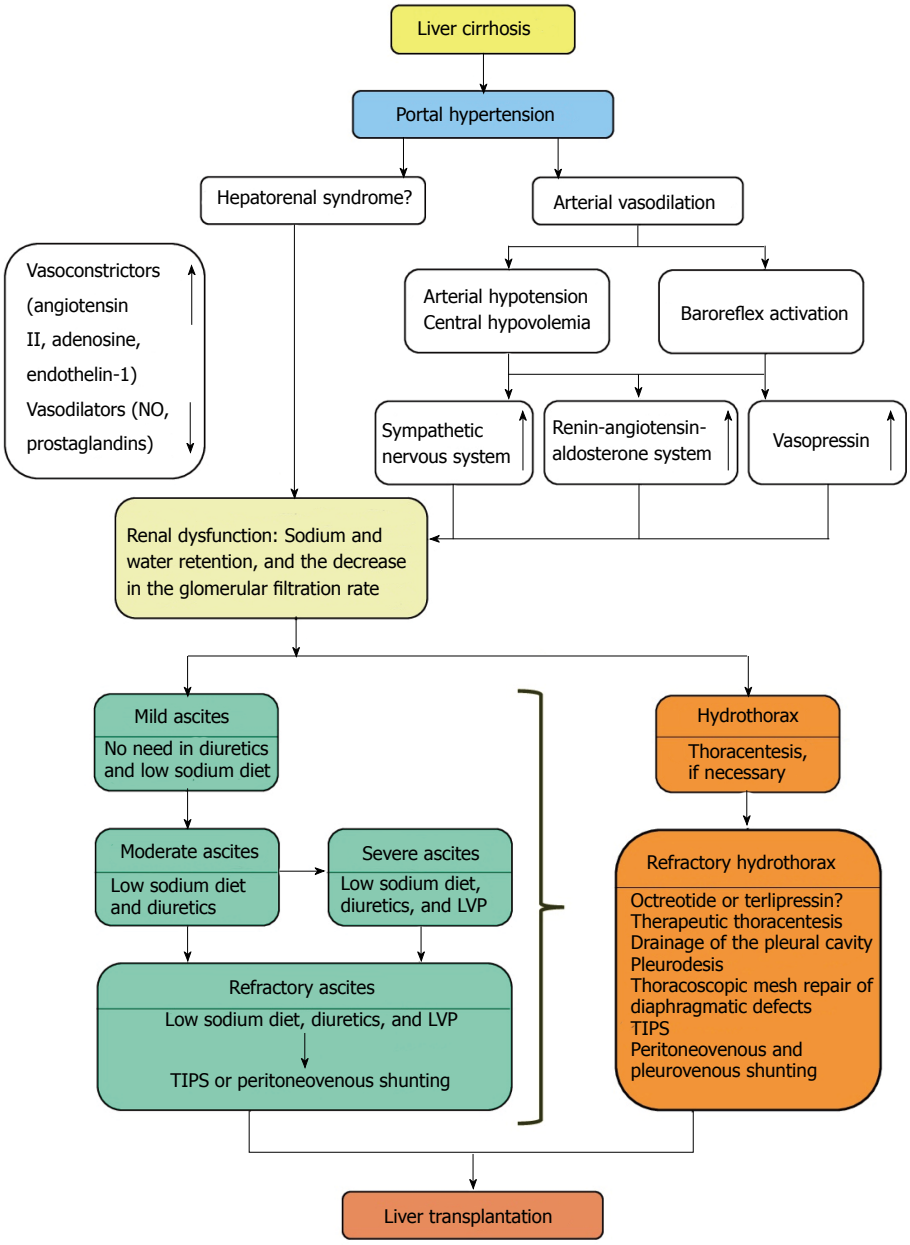

Figure 1 Pathogenetic mechanisms and treatment principles of hepatic hydrothorax. LVP: Large-volume paracentesis; TIPS: Transjugular intrahepatic portosystemic shunt.

It occurs due to inflammatory reaction accompanied by the production of reactive oxygen species and superoxide radicals in response to the rapid expansion of the initially collapsed lung. The key mediators of inflammation in this pathological situation may be interleukin 8, leukotriene B4, monocyte chemotactic and activating factor[29], tumor necrosis factor α, and interleukin 1 β[30] with the participation of Rho/ROCK signaling pathway[31]. Another possible factor is an increase in hydrostatic pressure in pulmonary blood vessels leading to a plasma leakage into the interstitial space[32].

To avoid re-expansion pulmonary edema, it is considered expedient to remove only one liter of transudate at a time. Meanwhile, a study by Feller-Kopman *et al*[33], which included 185 patients who underwent large-volume thoracentesis (from 1 to more than 3 L), did not find clinical and radiological signs of re-expansion pulmonary edema in many of them, and the appearance of this complication did not depend on the amount of removed fluid, pleural pressure, and pleural elasticity. The authors proposed to revise the recommendations for limiting the volume of thoracentesis and assumed that it should be stopped only if there are unpleasant sensations in the chest or a decrease in the pleural pressure to less than -20 mmH_2O at the end of exhalation.

Drainage of the pleural cavity: The installation of tubular drains into the pleural cavity for prolonged aspiration of the contents is undesirable in refractory HH. First, it is fraught with the development of pneumothorax and pleural empyema. Second**,** a large loss of fluid may lead to renal dysfunction and electrolyte imbalance.

Taken together, they significantly worsen the disease prognosis and increase the risk of death[34].

In this respect, catheters such as "Pigtail" or Pleurx® look safer[35]. So, the use of the first was successful in 48 out of 60 patients with refractory HH. The most significant complications were occlusion (3.3%) of the catheters and pain around their location (20%)[36]. The Pleurx® drainage system showed good results in five of the eight patients, who had it installed as a "bridge" before TIPS or liver transplantation. Pleural empyema developed in two patients, which required removal of the catheter in one case[37]. Since these catheters are not widely used**,** their expediency for the treatment of refractory HH cannot be determined, and further research is needed for final conclusions.

Pleurodesis: Pleurodesis may serve as a treatment method for refractory HH in the case of unsuccessful repeated thoracentesis. In most publications devoted to this problem, it was created by using chemical substances acting on visceral and parietal pleura and causing their aseptic inflammation and adhesion. Irritant agents were injected into the pleural cavity through the cannula or during therapeutic thoracoscopy. The most commonly used chemicals were talc, tetracycline, doxycycline, bleomycin, povidone-iodine, and picibanil (OK-432) with or without minocycline[38].

It is better to perform chemical pleurodesis after removal of ascitic fluid and transudate from the pleural cavity. In addition, some authors propose to combine it with constant positive pressure in the airways, which decreases the negative pressure in the pleural cavity. It prevents the ascitic fluid from moving there and leaves it dry for a longer period[39].

In a prospective study, which included 56 patients with refractory HH, 20 mL of a povidone-iodine 10% aqueous solution were administered through a cannula inserted into the pleural cavity under ultrasound control. This procedure was effective in 71.4% of all cases, and the success rate was 66.7% in massive effusion and 80% in moderate effusion. Twenty-eight patients had to undergo a repeat procedure after a week due to refractory HH relapse, of which 12 were successful[40]. Similar results were obtained in another prospective study after administration of 1 g of doxycycline diluted in 100 mL of saline[41].

In a randomized clinical trial, Helmy *et al*[42] evaluated the efficacy of chemical pleurodesis performed in 20 patients during therapeutic thoracoscopy without video assistance. For this purpose, the authors used povidone-iodine 10% aqueous solution (10 mL) in 8 cases, doxycycline (1 g) in 6 cases, and talc (2-3 g) in 6 cases. All drugs were diluted in 50 mL of saline. The observation lasted for three months and showed good results in 15 patients (75%): in 7 when using povidone-iodine (87.5%), in 4 out of 6 from the doxycycline group**,** and in 4 out of 6 from the talc group (66.7%). After the introduction of the talc suspension, one death was due to the development of the hepatic coma as a result of the LC progression.

The introduction of video-assisted thoracoscopic surgery (VATS) expanded the treatment options for patients with refractory HH. This makes it possible to perform not only a chemical, but also a combined pleurodesis with chemical, mechanical, and thermal effects on the pleura, the argon plasma coagulation, and the closure of diaphragmatic defects with fibrin glue, suturing, or synthetic materials[43].

In a systematic review with meta-analysis, Hou *et al*[44] evaluated the efficacy and safety of pleurodesis carried out by using different methods that included various drugs, as well as the closure of diaphragmatic defects with fibrin glue and their suturing during VATS in patients with refractory HH. They summarized the results of 20 clinical observations and 13 series of cases, including 26 and 180 people**,** respectively.

In clinical observations, the severity of hepatic dysfunction was indicated in 10 patients, 3 of whom had Child-Turcotte-Pugh (CTP) class B (30%), and 7 of whom had CTP class C (70%). Of the 26 patients, chemical pleurodesis during a therapeutic thoracoscopy without video assistance was performed in 12 cases (46.2%), and with VATS (19.2%) in 5 patients. The authors mainly used talc at a dose of 2-2.5 g (12/26, 46.2%) and OK-432 at a dose of 10 KE (7/26, 26.9%). The procedure was carried out once in 19 patients (73.1%), twice in 2 patients (7.7%), and thrice in 1 patient (3.9%). Information on the number of operations was missing in 4 cases (15.3%).

Chemical pleurodesis was effective in 17 patients (65.4%). Other methods were successfully applied to 4 patients from the group having negative results. One fatal outcome was associated with bleeding from the upper gastrointestinal tract and liver failure.

In this series of cases, the severity of liver dysfunction was indicated in 113 patients, two of whom had CTP class A (1.8%), 37 of whom had CTP class B (32.7%), and 74 of whom had CTP class C (65.5%). Pleurodesis during therapeutic thoracoscopy without video assistance was performed in 54 patients (30%), and during VATS in 126 (70%). Agents for chemical impact on the pleura were mainly talc at a dose of 2-2.5 g (115/180, 63.9%) and OK-432 at a dose of 10 KE in combination with minocycline or without it (19/180, 10.6%). Nine patients (5%) underwent mechanical pleurodesis (pleural abrasion, electrocautery), which was supplemented with talc applied to the pleural surface in 8 cases (4.4%). Pleurodesis was combined with the closure of diaphragmatic defects by using fibrin glue or suturing in 26 patients (20.6%) during VATS. No more than two sessions of pleurodesis were required to obtain a positive result, and only one procedure was needed in 80%-100% of cases. The complete response rate after pleurodesis was 72% (95%CI: 65%-79%).

The efficacy of pleurodesis performed with different methods was evaluated using meta-analyses of six and two studies including 90 and 16 patients respectively. They showed that the complete response rate was 78%

(95%CI: 68%-87%) when performing therapeutic thoracoscopy without video assistance and 84% (95%CI: 64%-97%) when using VATS.

Meta-analyses of 7 and 2 studies including 114 and 19 patients respectively were performed to evaluate the efficacy of pleurodesis achieved with the use of various drugs. They showed that the complete response rate was 71% (95%CI: 63%-79%) when using talc and 93% (95%CI: 78%-100%) when using OK-432 with or without minocycline.

A meta-analysis of 6 studies including 63 patients showed that the complete complication rate was 82% (95%CI: 66%-94%). They included subfebrile temperature (47.6%), renal insufficiency (17.5%), pneumothorax (15.9%), hepatic encephalopathy (11.1%), pneumonia (9.5%), liver failure (9.5%), pleural empyema (6.4%), pleuro-cutaneous fistulas (4.8%), sepsis (3.2%), intraoperative bleeding (1.6%), and upper gastrointestinal bleeding (1.6%).

Therefore, the presented data suggest that despite a large percentage of complications, pleurodesis may be a promising method for treating refractory HH. Randomized controlled trials with a meta-analysis are necessary to confirm this.

Thoracoscopic mesh repair of diaphragmatic defects: Huang *et al*[45] published the results of thoracoscopic onlay reinforcement with Mersilene mesh to repair diaphragmatic defects in 63 patients with refractory HH (CTP class A - 12, B - 36, C - 15), which in 16 cases was combined with their suturing. The average observation period was 20.5 mo. Of the 4 patients who had a relapse of the disease, the pleural effusion was eliminated using thoracentesis (> 3 times) in 3 patients, and a second operation was required in 1 patient. The 30-d and 3-mo mortality rates were 9.5% and 25.4% respectively, and its main reasons were septic shock (37.5%), acute kidney damage (25%), and gastrointestinal bleeding (25%). The authors concluded that the method is effective in low-risk patients with adequate postoperative management. In their opinion, the most significant factors worsening the prognosis are the preoperative severity of liver and kidney dysfunction assessed according to the Model for End-Stage Liver Disease (MELD) and Acute Kidney Injury Network (AKIN) criteria.

TIPS: In one of the few articles devoted to the use of TIPS in refractory HH, Ditah *et al*[46] provided a systematic review and a cumulative meta-analysis of 6 retrospective studies involving a total of 198 patients suffering from HH (CTP class A - 2, B - 82, C - 114). TIPS successfully eliminated the symptoms of refractory HH in 73% of cases. The early (45-d) and 1-year mortality rates were 18% and 50% respectively, with the most important predictors of an adverse outcome being old age (> 60-65 years), the initial severity of liver disease (CTP class C, MELD $\geqslant$ 15), and increased creatinine level and inefficiency of TIPS. Associated hepatic encephalopathy occurred in about 12% of all cases, and less frequently when expanded polytetrafluoroethylene-covered stents were used. The data obtained by the authors correlated well with the results of TIPS in other complications of portal hypertension[47].

Peritoneovenous and pleurovenous shunting: Pleurovenous shunting is a surgical operation described in 1975 for the treatment of malignant pleural effusion. It is rarely used in refractory HH, and the publications devoted to it are mainly presented as clinical observations and a series of cases. In one of them, Artemiou *et al*[48] performed it on six patients and showed that all the shunts were passable during 1-40 mo and no one needed a pleural puncture to remove transudate. Nevertheless, it is still too early to discuss the prospects of pleurovenous shunting in refractory HH due to the small sample size.

Liver transplantation: Since most of the patients with HH have a terminal stage of LC, they are potential candidates for orthotopic liver transplantation[49]. In the study by Xiol *et al*[50], it was performed in 28 patients with HH (CTP class B - 9, C - 19) without prior TIPS or drainage of the pleural cavity. HH was refractory in 5 cases and combined with ascites in 26 cases. Eleven patients had episodes of spontaneous bacterial pleural empyema. HH was resolved in all transplant patients for three months. At one lethal outcome, the average survival rate was 114 mo.

Sersté *et al*[51] compared the results of orthotopic liver transplantation without previously performed TIPS in three groups of patients at the terminal stage of LC: with refractory HH, tense ascites without HH, and without any of these complications. Patients with HH did not need therapeutic thoracentesis after transplantation. There were no significant differences in the duration of mechanical pulmonary ventilation, the duration of the stay in an intensive care unit and hospital, as well as the frequency of septic complications and early postoperative lethality. The one-year survival rate was similar.

CONCLUSION

Although HH is infrequent, its presence exacerbates the course of LC, and the occurrence of spontaneous bacterial pleural empyema may be the cause of death. Most patients, who suffer from it, are candidates for orthotopic liver transplantation. In routine clinical practice, the key to their successful management may be the stratification of the risk for an adverse outcome and the determination of individual therapeutic strategies. The development of pathogenetic pharmacotherapy and optimization of minimally invasive treatment will improve the quality of life and increase the survival rate among

patients with HH.

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CASE REPORT

Ayurvedic drug induced liver injury

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Author contributions: All authors took care of the patient medically; Dalal KK analyzed and drafted the report; Holdbrook T and Peikin SR made critical appraisals of the report.

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Abstract

Drug induced liver injury is responsible for 50% of acute liver failure in developed countries. Ayurvedic and homeopathic medicine have been linked to liver injury. This case describes the first documented case of Punarnava mandur and Kanchnar guggulu causing drug induced liver injury. Drug induced liver injury may be difficult to diagnosis, but use of multi-modalities tools including the ACG algorithms, causative assessment scales, histological findings, and imaging, is recommended. Advanced imaging, such as magnetic resonance cholangiopancreatography, may possibly have a greater role than previously reported in literature.

Key words: Ayurvedic; Punarnava mandur; Kanchnar guggulu; Drug induced liver injury; T2 heterogeneous hyperintensity; Roussel Uclaf Causality Assessment Method

Core tip: Drug induced liver injury is difficult to diagnose. Certain ayurvedic medications are commonly used without full knowledge of their side effects. This article not only represents the first documented case report of drug induced liver injury secondary to Punarnava mandur and Kanchnar guggulu, but it also demonstrates the possible role for advanced imaging modalities.

Dalal KK, Holdbrook T, Peikin SR. Ayurvedic drug induced liver injury. *World J Hepatol* 2017; 9(31): 1205-1209 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i31/1205.htm
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INTRODUCTION

Complementary and alternative medicine in the form of herbal and homeopathic mediations have been used dating as far back as 2100 BC in ancient China and India. Their use has created a $180 billion market in United States[1]. Though some herbal medications are shown to cause drug induced liver injury (DILI) there are many others that may be implicated. Punarnaya mandur, extract from the Boerhavia diffusa plant, is commonly used in ayurvedic practice for iron deficiency, kidney, and liver pathologies[2]. Kanchnar guggulu, extract from Bauhinia variegate plant, is used for uterine fibroids and BPH. Both these medications have several unlisted uses and properties, but more specifically they can exhibit anti-inflammatory and hepatoprotective properties[3]. These two medications are widely used and have not previously been associated with DILI. DILI is often misdiagnosed or undiagnosed and herbal medications need to considered by healthcare providers as DILI is the most common cause of acute liver failure in the United States[4-6].

CASE REPORT

A 44-year-old female presented to the ED for 2 wk of painless jaundice associated with poor appetite, pale stools, and dark urine. She had a history of cholelithiasis diagnosed in India 6 mo prior to presenting to the ED. She began to use 3 different herbal and homeopathic medications, but reportedly stopped taking them after she noticed the jaundice. She denied any other medication use. She denied any alcohol, acetaminophen use, or a history of infectious hepatitis.

Her physical examination was remarkable for scleral icterus. Her admission labs demonstrated an glutamic-oxalacetic transaminase 1092 U/L, alanine aminotransferase 1185 U/L, total bilirubin 9.0 mg/dL, direct bilirubin 6.2, INR 1.8, and normal alkaline phosphatase of 118. A right upper quadrant ultrasound was ordered demonstrating a shadowing calculus in the gallbladder neck measuring 1.6 cm. There was 0.9 cm gallbladder wall thickening reported to be secondary to acute cholecystitis or a primary liver process. The hepatic ducts were nondilated, and the common bile duct measured 0.4 cm. A HIDA scan was performed revealing hepatic dysfunction. Given the initial 1.6 cm gallstone and hyperbilirubinemia, MRCP was ordered confirming a gallbladder neck stone, but no choledocholelithiasis. There was heterogeneous T2 enhancement of liver with no signs of hepatic steatosis (Figure 1).

Viral serologies (hepatitis A, B, C, and E, HIV, cytomegalovirus) were negative. There was evidence of prior infection with EBV. Antinuclear antibody was weakly positive 1:40 titer in a speckled pattern. Anti-smooth muscle, anti-liver/kidney microsomal, and anti-mitochondrial antibodies were negative. There was an increased iron saturation of 61%, with normal ferritin, TIBC, ceruloplasmin. There was no *HFE* gene mutation detected.

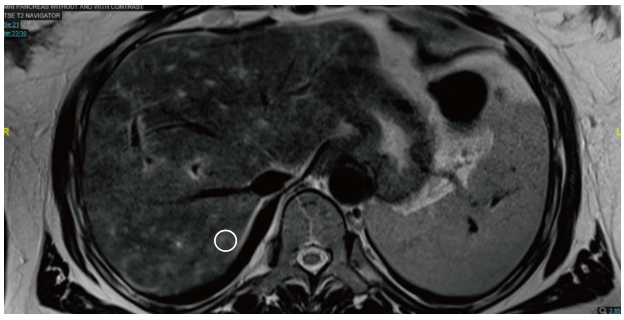

Figure 1 Magnetic resonance imaging pancreas with contrast, T2 imaging. The heterogeneous T2 enhancement (hyperintensities, white patchy areas, one circled) are present throughout the liver parenchyma.

Jaundice and laboratory values began to improve after one week. The patient was diagnosed with DILI secondary to herbal and homeopathic medication. Interestingly, her herbal and homeopathic medications (Punarnava Mandur, Kanchnar guggulu, and one unlabeled) were sent for toxicology analysis and came back negative for a match in their toxin database. Surgical consultation recommendations were outpatient follow up for elective cholecystectomy after the patients lab values normalized. Elective cholecystectomy was completed with a liver biopsy the following month.

Liver biopsy demonstrated mild portal chronic inflammation and interface activity with grade 3 bridging fibrosis. Ceroid-laden Kupffer cells were present in portal tracts. There was ballooning of hepatocytes consistent with injury. Plasma cells were not seen, but eosinophils were conspicuous. Overall, the histologic pattern was suggestive of drug hypersensitivity reaction with resolving hepatitis (Figure 2).

DISCUSSION

To evaluate suspected idiosyncratic drug induced injury there is no established gold standard. We recommend using a 2 step approach with the DILI algorithm recently proposed by ACG in combination with the Roussel Uclaf Causality Assessment Method (RUCAM)[7,8]. DILI is a diagnosis of exclusion and ACG algorithm provides an evidence based approach for a timely diagnosis of DILI due to herbal and dietary supplements. The RUCAM score is a system that assigns points for clinical, biochemical, serologic, and radiologic features of liver injury. This scores gives an overall likelihood that the hepatotoxicity is due to a medication.

After obtaining a thorough history and physical, the algorithm states the diagnostic approach can be tailored to liver injury patterns (hepatocellular, cholestatic, mixed) based off the *R* value. The *R* value is defined as serum ALT/upper limit of normal (ULN) divided by serum Alk *P*/ULN[7]. Our patient was categorized into the hepatocellular injury ($R \geqslant 5.0$) column given the *R* value of 26. Then the first line testing with hepatitis serologies, autoimmune serologies, and imaging (abdominal ultrasound) was

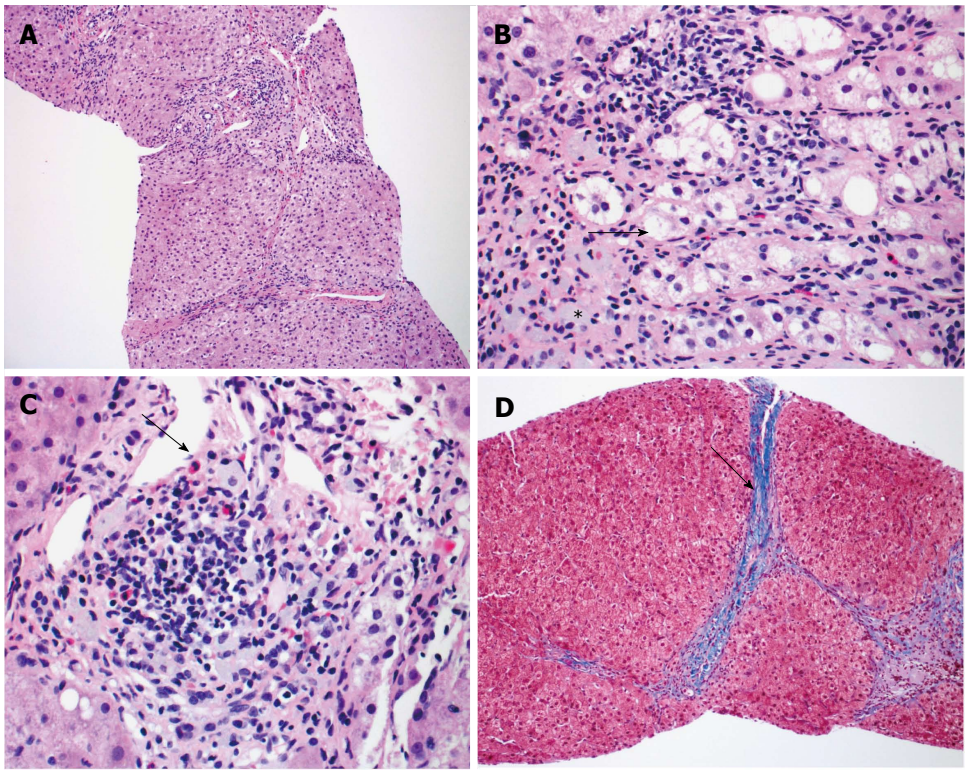

Figure 2 Resolving hepatitis. A: The liver shows a somewhat nodular architecture with increased portal inflammatory cells, HE, × 100; B: Ballooned hepatocytes (arrow) and numerous pigmented Kupffer cells (asterix) are present in portal tracts consistent with injury, HE, × 400; C: Prominent eosinophils (arrow) suggests a drug reaction of the hypersensitivity type, HE, × 400; D: Bridging fibrosis (arrow). Thin fibrous bridges connect portal tracts, Trichrome, × 100.

completed. Then second line testing of ceruloplasmin, less common serology (HEV, CMV, EBV) and liver biopsy was completed. This was combined with complete evaluation to exclude non-DILI causes. Literature review using PubMed and Livertox, and clinical judgement was conducted. This patient's ANA was positive, but the titer was only 1:40, and this feature can be present in DILI. This is proven by a retrospective study that evaluated 29 patients with DILI and autoimmune hepatitis. It concluded that 7/21 DILI patients had positive ANA, but more so all 8/8 patients with autoimmune hepatitis had an ANA titer $\geqslant$ 1:80[9]. The patient's RUCAM score was 5, which indicated a "possible" association with DILI. This helped to establish the diagnosis. The use of the imaging and importantly the histology helped to confirm the diagnosis.

Imaging is recommended for the evaluation of DILI; however the modality will differ based on clinical presentations. This patient, given her cholelithiasis, had a MRCP which demonstrated heterogeneous T2 enhancements of the liver. This finding has not been previously reported in literature for drug induced liver injury. There have been observations of T2 hyperintensities related to certain liver pathologies including hepatocellular carcinoma, cholangiocarcinoma, hepatic adenoma, hemangioma, mesenchymal hamartoma, metastatic disease[10]. These findings are usually more localized, unless the patient has underlying metastatic disease. This heterogeneous pattern T2 enhancement, though relatively nonspecific, may be a unique characteristic that is an underutilized diagnostic clue for DILI secondary to herbal and homeopathic mediations. This will need to be further studied in future DILI cases.

The histology for this patient helped in confirming the diagnosis. The interface activity and hepatocyte ballooning were consistent with liver injury and there was Kupffer cell activation. The Kupffer cells were ceroid laden, which is highly suggestive of DILI[11]. The repeated bouts of active liver injury led to bridging fibrosis over time[12]. Liver fibrosis can be associated with DILI, but interestingly some herbal medications are proposed treatments for fibrosis[13,14]. The prominent eosinophils are associated with DILI. There have been mice models demonstrating eosinophil accumulation during hepatic necrosis after certain medications[15]. After the offending agent was removed, the hepatitis began to resolve. These findings collectively were suggestive of a drug reaction and allowed to confirm a diagnosis of DILI.

The primary treatment for DILI includes withdrawal of the offending agent. If certain drugs are the causal agent, specific therapies such as N-acetylcysteine for acetaminophen toxicity or L-carnitine for valproic overdoses maybe beneficial[16]. For pruritus bile acid sequestrants or antihistamines may be utilized. For herbal and homeopathic medications the early recognition is important with subsequent withdrawal of the medication. The patient should be monitored with serial LFTs until normalization. For patients with acute liver failure early

transfer to a transplant center may be warranted as this indicates a poor prognosis. Overall DILI is difficult to diagnose, the unique findings from our case will aid in future management and awareness of DILI secondary to complementary and alternative medications.

ARTICLE HIGHLIGHTS

Case characteristics

A 44-year-old female had symptoms of painless jaundice, poor appetite, pale stools, and dark urine.

Clinical diagnosis

Patient with history of painless cholelithiasis and 6 mo use of herbal medication had worsening jaundice, scleral icterus.

Differential diagnosis

The differential diagnosis includes viral hepatitis, acetaminophen overdose, autoimmune hepatitis, ischemic hepatopathy, Wilson's disease, acute Budd-Chiari syndrome, obstructive hyperbilirubinemia secondary to cholelithiasis, and the diagnosis of exclusion of drug induced liver injury.

Laboratory diagnosis

Laboratory findings included glutamic-oxalacetic transaminase 1092 U/L, alanine aminotransferase 1185 U/L, total bilirubin 9.0 mg/dL, direct bilirubin 6.2, negative hepatitis serologies, negative autoimmune serologies and negative *HFE* gene mutation.

Imaging diagnosis

Multiple imaging modalities were used, including right upper quadrant ultrasound demonstrating a gallbladder neck calculus, a HIDA scan demonstrating hepatic dysfunction with uptake in the gallbladder, and an magnetic resonance cholangiopancreatography confirming a gallbladder calculus, no choledocholelithiasis, and a unique heterogeneous T2 liver enhancement with no signs of hepatic steatosis.

Pathological diagnosis

Liver biopsy demonstrated grade 3 bridging fibrosis, ceroid-laden Kupffer cells, and eosinophils, which was all suggestive of drug hypersensitivity reaction with resolving hepatitis.

Treatment

After diagnosis of this condition, the major treatment was stopping the offending medication and monitoring with serial LFTs until normalization.

Related reports

There have been case reports of herbal medication causing drug induced liver injury, but this case is unique because it represents the first documented case report of commonly used ayurvedic medications including Punarnava mandur and Kanchnar guggulu that was confirmed with biopsy and demonstrated unique imaging findings.

Term explanation

Drug induced liver injury (DILI) is a diagnosis of exclusion of a rare adverse medication herbal reaction causing jaundice, liver failure, or even death. Roussel Uclaf Causality Assessment Method is a scoring system that assigns points for clinical, biochemical, serologic, and radiologic findings to demonstrate the likelihood of medication induced hepatotoxicity. Magnetic resonance cholangiopancreatography is a magnetic resonance imaging exam that produces detailed images of the hepatobiliary and pancreatic systems *via* a noninvasive manner.

Experiences and lessons

In order to diagnosis drug induced liver injury it is important to get a detailed history while implementing algorithms, causative assessment scales, histological findings, and imaging for all patients with unknown jaundice. DILI needs to be diagnosed early in order to prevent acute liver failure.

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World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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REVIEW

Role of pregnane X-receptor in regulating bacterial translocation in chronic liver diseases

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Abstract

Bacterial translocation (BT) has been impeccably implicated as a driving factor in the pathogenesis of a spectrum of chronic liver diseases (CLD). Scientific evidence accumulated over the last four decades has implied that the disease pathologies in CLD and BT are connected as a loop in the gut-liver axis and exacerbate each other. Pregnane X receptor (PXR) is a ligand-activated transcription factor and nuclear receptor that is expressed ubiquitously along the gut-liver-axis. PXR has been intricately associated with the regulation of various mechanisms attributed in causing BT. The importance of PXR as the mechanistic linker molecule in the gut-liver axis and its role in regulating bacterial interactions with the host in CLD has not been explored. PubMed was used to perform an extensive literature search using the keywords PXR and bacterial translocation, PXR and chronic liver disease including cirrhosis. In an adequate expression state, PXR acts as a sensor for bile acid dysregulation and bacterial derived metabolites, and in response shapes the immune profile beneficial to the host. Activation of PXR could be therapeutic in CLD as it counter-regulates endotoxin mediated inflammation and maintains the integrity of intestinal epithelium. This review mainly focuses PXR function and its regulation in BT in the context of chronic liver diseases.

Key words: Pregnane X receptor; Bacterial translocation; Chronic liver disease; Intestinal permeability; Inflammation; Tight junctions

Core tip: Translocation of bacteria at pathological levels is a major driving factor in the progression of chronic liver diseases (CLD). However, it remains to be known whether it is the CLD condition that triggers leaky gut, or if translocation of bacteria plays an etiological role in the pathogenesis of CLD. Dysregulation of homeostasis in the gut-liver axis is considered as a crucial element that underlies the pathogenesis of BT. The nuclear receptor, pregnane X receptor (PXR) is widely expressed in gut and liver axis and is implicated in maintenance of equilibrium in the gut-liver axis. This

review will summarize the various studies that have highlighted the importance of PXR as the mechanistic linker molecule in the gut-liver axis and its role in regulating bacterial translocation in the pathogenesis of cirrhosis.

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INTRODUCTION

Pregnane X receptor

Pregnane X receptor (PXR) is an adopted orphan nuclear receptor (NR) that is part of a broad nuclear receptor superfamily. Specifically, PXR is encoded by *NR1I2* gene and is categorized as the 2nd member of group I (nuclear receptor subfamily 1) which also comprises VDR (NR1I1) and CAR (NR1I3)[1,2]. Consistent with the majority of class 1 NR's, PXR behaves as a transcription factor that is present in the cytosol and is activated only post ligand binding. After ligand activation, these NR's form complexes with retinoid X receptor (RXR) and bind to DNA response elements of the genes that they regulate[3]. PXR was initially characterized as a xenobiotic receptor that senses and responds only to exogenous toxic substances and prescription drugs. Over the last two decades, PXR is widely recognized for its additional roles in sensing a range of endobiotic compounds such as bilirubin, bile acids, dietary lipids and steroid hormones, and hence is also referred to as steroid and xenobiotic receptor (SXR)[4]. PXR has been shown to be expressed in various tissues including stomach, placenta, kidney, lung, uterus and ovary, but is predominantly expressed in small intestine, colon and liver. Its increased expression in intestinal epithelium and hepatocytes also highlight its vital role in adaptive defense against xenobiotics and endobiotics exposure in intestine and liver[3,5]. A recent study has shown evidence that hepatic stellate cell (HSC) expressed PXR and its activation lead to attenuation of HSC's differentiation and proliferation[6]. A study using mice knock out model also highlighted the expression of PXR in monocyte/macrophage cells and their role in countering inflammatory profile[7]. PXR expression has also been observed in other immune cells including T-cells and dendritic cells[8].

Identical with other NR's, PXR contains a conserved DNA binding domain (DBD) and a flexible ligand-binding domain (LBD). Upon activation by a ligand, PXR binds to its response elements only as heterodimeric complexes that it forms with 9-cis retinoic acid receptor (RXR or NR2B) and others co-activators such as SRC-1[9]. The structural feature that makes PXR stand out from the other NR's is its voluminous and flexible ligand-binding pocket. This enables PXR to bind and be activated by a wide range of hydrophobic ligands. Indeed, its spherical shaped ligand binding pocket has a volume greater than 1150 angstrom, making its ligand cavity one of the largest to be characterized so far[3], and on par with the NR PPAR-γ's ligand pocket[10]. It is also important to highlight the unselective nature of the PXR LBD, enabling it to sense and respond to chemicals within a broad molecular weight range (about 250-850 kDa)[3]. An impressive range of bioactive components from herbal sources, such as Hyperforin, Paclitaxel and Guggul have also been added to the increasing list of naturally occurring PXR ligands[11].

Functions of PXR

The primary and the most conceded function of PXR is to activate genes encoding drug metabolizing and drug transporter enzymes. It acts as sensors that monitor any alteration in the levels of foreign compounds or endobiotics[3,12]. The genes that PXR activate take the responsibility of metabolizing and elimination of exogenous chemicals, and thus form the primary line of defense against toxicity challenge[12,13]. In humans, among drug metabolizing cytochrome P450 (CYP) enzymes, CYP3A is the most copiously expressed isoform in the liver and intestine[14]. Furthermore, transgenic rodent knock out models (KO)[15,16] have established beyond doubt that PXR's are master regulators of *CYP3A* genes[17], which encode proteins responsible for the metabolic oxidation of more than half of the known prescription drugs[3]. Classic PXR activators such as pregnenolone 16 α-carbonitrile (PCN) and Rifampicin have also been used to validate the same. PXR also controls the expression of phase 2 conjugating enzymes such as SulT1a and UGT-1A which are primarily responsible for sulfate conjugation or glucuronidation of steroid hormones, bile acids and bilirubin[13,18-21]. Following conjugation, PXR controlled phase 3 drug transport proteins like P-glycoprotein and MRP-2 are then involved in efflux transport and elimination of the toxic metabolites[13,22].

The function of PXR extends beyond metabolism of drugs and endobiotics, which has made it a considerable area of research over the last decade. NR's, in general, are rising as major targets for drug discovery and the identification of additional roles of PXR has given a new perspective in freshly approaching already known disease pathologies. Apart from its most researched role in inflammatory bowel disorders (IBD), PXR dysregulation has been implicated in CLDs[6,23] various cancers[24] and metabolic disorders like obesity[25]. PXR play a crucial role and aids hepatocytes in uptaking endobiotics or xenobiotics and is further involved in their metabolism and elimination[26,27]. Anti-fibrogenic activity has also been documented, wherein PXR activation by its ligand PCN prevented the transdifferentiation of hepatic stellate cells into myofibroblasts[6]. Similar observations were made when using another established PXR ligand Rifampicin, where PXR was associated with inhibition of major pro-fibrogenic factors such as transforming growth

factor-β (TGF-β) and Alpha smooth muscle actin[6]. Interestingly, a significantly increased expression of PXR was observed in various tumor tissues when compared to non-neoplastic tissues, and a positive correlation was found between cell proliferation and PXR positive cells[24].

PXR has also been attributed to playing a role in energy metabolism and has been linked with diseases such as type 2 diabetes, obesity and hyperglycemia. Activation of PXR has been observed to produce a suppressive effect on hepatic gluconeogenesis. However, it has also been reported to cause hepatic steatosis by increasing lipogenesis and fatty acid uptake[25]. Increased skin inflammation was reported in PXR null mice when challenged with hapten and was associated with increased interferon gamma (INF-γ) and reduced anti-inflammatory cytokine interleukin-10 (IL-10)[28]. The numerous roles of PXR in interaction with microbial metabolites, maintaining innate immunity, epithelial integrity, countering inflammation, bile acid trafficking and detoxification are discussed in detail in the upcoming sections.

GUT MICROBIOTA AND PATHOLOGICAL TRANSLOCATION

Gut microbiome and gut-liver crosstalk

The presence of bacteria in the gut gains significant importance because of the monumental level of interaction that happens between the gut microbiome and the host. In the human body, almost 100 trillion bacteria are in constant communication with the intestinal epithelium, which spans almost 400 m^2 in surface area-which is the largest in humans[29]. It does not come as a surprise that, at such a level of interaction, some of the important physiological functions of the human host including digestion, energy metabolism, maintenance of intestinal integrity and innate immune homeostasis depend largely on the balance of host-microbiome interaction[30]. These physiologic events are dependent on the extensive arsenal of microbial metabolites, which are yet to be characterized completely.

In physiological state, the commensal bacteria have influence beyond the intestine. In this context, Björkholm *et al*[31] have reported that more than 100 genes in the liver are differentially expressed between germ-free mice and their conventionally raised wild counterparts. However, the most gripping evidence put forth by this study was that majority of these genes that varied in expression in GF mice, were in fact related to xenobiotic metabolism. This study highlights the significance of xenobiotic sensors PXR and CAR as mechanistic links between microbes and host[31]. Thus any alterations in the gut microbiome or its sensors will be reflected in the liver functionality. Accordingly, ulcerative colitis patients have been observed to have increased susceptibility to develop primary sclerosing cholangitis[32]. Interestingly, any abnormalities in liver function are also reflected as alterations in the quality and quantity of intestinal bacteria in the gut. Patients with intrahepatic cholestasis have been shown to manifest overgrowth of bacteria in the small intestine[33]. Similar observations were made in NAFLD patients, where fat induced bile acid abnormalities were linked with bacterial dysbiosis[34]. Moreover, studies in NAFLD and chronic alcohol feeding models have observed the manifestation of intestinal inflammation, which indirectly compromises gut integrity[35,36].

Bacterial translocation

Bacterial translocation (BT) is defined as the passage of viable indigenous bacteria and bacterial products, such as endotoxin, from the intestinal lumen through the mucosa into mesenteric lymph nodes (MLNs) and other organs[13,14,37,38]. It is a common physiological event in the healthy individuals and is tightly regulated by various levels of immune and physical barriers[39]. However, BT is seen to happen physiologically at minor levels, where it is considered as a beneficial event to the host, especially in priming the host immune system[38,39]. The mucus and tightly bound intestinal epithelial lining comprise the physical barrier, while gastric acid, antimicrobial peptides (AMPs), IgA antibodies and innate immune cells form the chemical/immune barriers[38]. Physiologically dendritic cells constantly sample bacteria through their processes and help in priming the B-cells to secrete IgA. The DCs also aid in the transport of the translocating microbes to mesenteric lymph node (MLN), which serves as a central hub between the gut and rest of the body. MLN are also the location where the microbes are killed *via* local immune response[38]. However, when the physiologic barriers are compromised, or if the quality or quantity of bacteria is altered in the gut due to other external abnormalities such as alcohol abuse and high fructose diet, the translocation of bacteria or its products is persistent and pathologic. This phenomenon results in chronic induction of both systemic and hepatic inflammation[38,40].

Bacterial infections in patients with cirrhosis are correlated with a poor prognosis and an increased risk of mortality[40]. BT can also exacerbate the hepatic and systemic hemodynamic abnormalities of liver cirrhosis. Pathological BT is an important and emerging mechanism for the pathogenesis of CLDs. Emerging research has also shown clear evidence of increased intestinal permeability, mucosal inflammation and detection of bacteremia and endotoxemia in patients with CLD[41,42]. Indeed, spontaneous bacterial peritonitis (SBP) is considered the most evidenced clinical expression of BT, which responsible for 25%-40% of overall mortality in cirrhotic patients[43]. Pathological BT has been implicated in a range of other complications that arise in CLD, including acute-on-chronic liver failure, hepatorenal syndrome and hepatic encephalopathy (HE)[38]. The exact mechanisms of increased intestinal

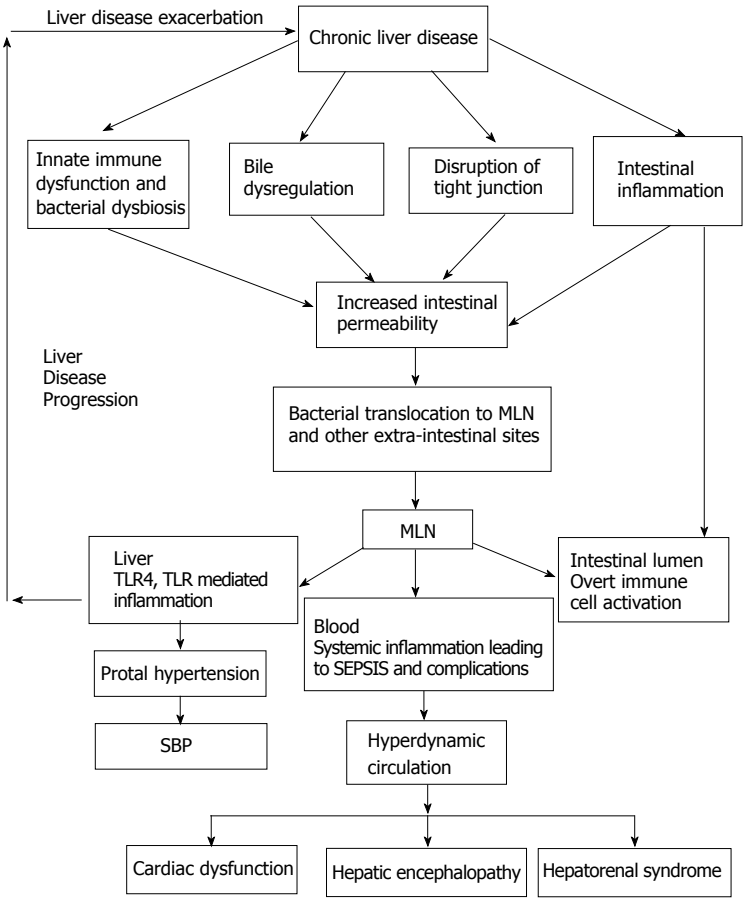

Figure 1 Cyclic cascade of bacterial translocation and associated progression of chronic liver diseases and its complications. The figure enlists the various proposed mechanisms through which chronic liver diseases conditions triggers intestinal permiability and BT. Increased intestinal permeability causes translocation of bacteria across the intestinal epithelium to the MLN and extra-intestinal sites such as liver and blood causing discrete complications in each of the systems. In the intestinal lumen, BT causes overt activation of immune cells and aggravates the pro-inflammatory cytokines in the gut. Translocation of bacteria at pathological levels to MLN is a major risk factor for systemic inflammation which leads to hyperdynamic circulation and is reflected across various organs in the form of complications such as cardiac dysfunction, hepatorenal syndrome and hepatic encephalopathy. Translocation of bacteria to the liver causes TLR4 and TLR9 mediated inflammation which further exacerbates the progression of chronic liver diseases and intestinal permeability. BT to liver also causes portal hypertension, which forms the basis of development of SBP. BT: Bacterial translocation; MLN: Mesenteric lymph node; SBP: Spontaneous bacterial peritonitis.

permeability and BT in CLD remains obscure, and only the probable pathways that might cause the phenomenon have been predicted. The cyclic cascade of events that cause BT and exacerbate CLD have been summarized in Figure 1.

Possible mechanisms of BT in CLD

Compromised Bile in the intestines: Bile flow is altered in liver disease, as the organ is the major producer of bile. Bile acids have been shown to interact with NR's such as FXR in the GI tract and keep bacteria under check. Hence a compromised bile environment may lead to overgrowth of bacteria in liver disease conditions[30,44].

Dysbiosis: It is a phenomenon inclusive of any quantitative or qualitative alterations from the symbiosis maintained between the host and microbiota[41]. Quantitative overgrowth of bacteria also called as small intestinal bacterial overgrowth is usually the first step in the phenomenon of BT. Patients with CLD, especially alcoholic hepatitis have been directly linked with developing bacterial overgrowth, low motility and increased transit time in the intestine[41]. The proportion of beneficial and less beneficial (sometimes pathogenic) bacteria is tightly maintained and is termed symbiosis. In CLD, this balance is disrupted leading to increase in pathogenic bacteria and harmful metabolites that damage the epithelium[41]. Beneficial bacteria such as lactobacillus reduce in numbers and potentially pathogenic bacteria like Enterobacteriaceae increase. Indeed, it was observed that the pathogenic bacteria are more likely to translocate across the intestinal epithelium[41].

Immune dysfunction: The mucosal immune system prevents the exposure of commensal bacteria to systemic circulation through various secretory mechanisms such as mucus and anti-microbial peptides (AMP). In cirrhotic animal models, Paneth cells, have been observed to have diminished expression of AMP's[45].

Intestinal inflammation: Both mucosal and sub-mucosal inflammation have been observed in patients

with CLD. A pro-inflammatory cytokine profile is also actively linked with increased intestinal permeability[43,46-57]. Inflammation and its significance on intestinal permeability are discussed in more detail in the later sections of this review.

Disruption of tight junctions: A single layer of epithelial cells separate 100 trillion gut bacteria from other parts of the body. The epithelial cells are sealed tightly *via* tight junctions (TJ's) that are seen to disassociate in CLD and result in the leaky gut leading to BT[48,49].

PXR AND ITS ROLE IN COUNTERACTING INFLAMMATION

Inflammation is a response mechanism to meet and overcome the challenges an organism faces from an injury or infection. However, when uncontrolled or unregulated, inflammation is often a pathological driving force in various disease conditions. In the context of this review, inflammation is the most prominent player in the pathophysiology of BT and sets the environment that causes increased intestinal permeability. Studies over the last decade have firmly established the role of PXR as a counter-regulator of inflammation[7]. Clinical studies have revealed that, in IBD patients a clear pattern of PXR downregulation was observed in inflamed tissues[50]. In this context, polymorphism of *NR1I2* gene has identified to be associated with more susceptibility to IBD[51-53]. PXR has been described to interact with various components of the immune response signaling cascade to produce an immune regulating effect.

Nuclear factor kappa B (NF-κB) is a transcription factor playing a central role in regulating plethora of genes involved in innate and adaptive immunity[54]. It is well established that drug metabolism is compromised in an inflammatory environment and vice versa[55]. This phenomenon plays an important role in highlighting the counter-regulation that exists between PXR and NF-κB. PXR controls drug metabolism by binding to its response elements as heterodimers. PXR forms heterodimers with RXR-α and studies have revealed that this interaction is inhibited by the binding of p-65 subunit of NF-κB to the RXR unit of PXR heterodimer complex (Figure 2A)[56]. However, of even more interest are the observations that PXR can reciprocally inhibit NF-κB and thus making PXR an excellent target to counteract NK-κB and its associated inflammatory gene kit[57]. Zhou *et al*[58] demonstrated an increased NF-κB activity and inflammatory cytokine profile in PXR null mice when compared to wild type mice that constitutively express PXR. This indicates that in physiological state PXR expression keeps the NF-κB initiated inflammatory response under check. Additionally, when wild type mice were treated with PCN, a specific PXR agonist, majority of the NF-κB target genes were downregulated, an effect that was lost in PXR null mice. This suggests that PCN antagonism of NF-κB happened in a PXR dependent manner[58]. Similar findings were observed in DSS-induced colitis mice with and without PCN treatment[59]. Ultimately, PXR which is responsible for defense against chemicals, and NF-κB which is responsible for mounting an immune defense, counter-regulate each other to maintain physiological state.

Studies by Wallace *et al*[7] employed a SJL/J mice model that is characterized by increased monocyte cell infiltration into the hepatic portal tract, revealed that PXR was expressed in infiltrating monocytes. Further in SJL/J-PXR$^{+/+}$ mice, activation of PXR using PCN, downregulated tumor necrosis factor alpha (TNF-α) and Interleukin-1α (IL-1α), which are cytokines controlled by NF-κB. Consistent with other studies, this effect was lost in SJL/J-PXR$^{-/-}$ mice[7,58]. Thus BT is also associated with considerable recruitment of monocytes cells to the lamina propria, which is accompanied by increased production of TNF-α[60,61]. Similarly, Fiorucci *et al*[60] observed that LPMC's isolated from colitis mice that were treated with Rifaximin, showed complete abrogation of INFγ cytokine production. Unfortunately this study did not consider if activation of PXR by Rifaximin could have produced this effect. However, study by Cheng *et al*[62] using humanized PXR (hPXR) mice with DSS induced colitis showed that Rifaximin indeed works in a PXR-dependent manner and attenuated NF-κB mediated cytokines. Interestingly, TNF-α has also been associated with direct downregulation of epithelial TJ proteins[63]. In this scenario, further studies targeting activation of PXR with an aim to antagonize NF-κB-induced-TNF-α may pose as an exciting therapeutic outcome especially by controlling mononuclear cell infiltration, with an aim to attenuate intestinal epithelial damage, which is a major prequel to BT.

Even though many studies have established the mutual inhibition between PXR and NF-κB, the exact mechanism by which PXR represses NF-κB is not well understood. Indeed, Ye *et al*[64] showed that PXR activation by a natural PXR ligand Ginkgolide-A (GA) repressed NF-κB indirectly by enhancing the expression of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (Iκ-Bα), an inhibitory protein of NF-κB activity. When siRNA was used for silencing of PXR, GA did not increase the expression of Iκ-Bα, showing that the induction happens in a PXR dependent manner[64] (Figure 2B).

Various plant flavanols like chrysin and isorhamnetin have also been shown to inhibit NF-κB activity through a PXR dependent manner[65,66]. Studies using these flavanols in a DSS-induced colitis mouse model showed PXR mediated downregulation of NF-κB target genes including iNOS, ICAM-1 MCP-1, COX-2, TNF-α IL-2 and IL-6. Intriguingly, both studies showed that PXR activation prevented the degradation of Iκ-Bα and thus underlining the possibility that PXR might counteract NF-κB mainly through manipulation of fate of Iκ-Bα[65,66] (Figure 2C).

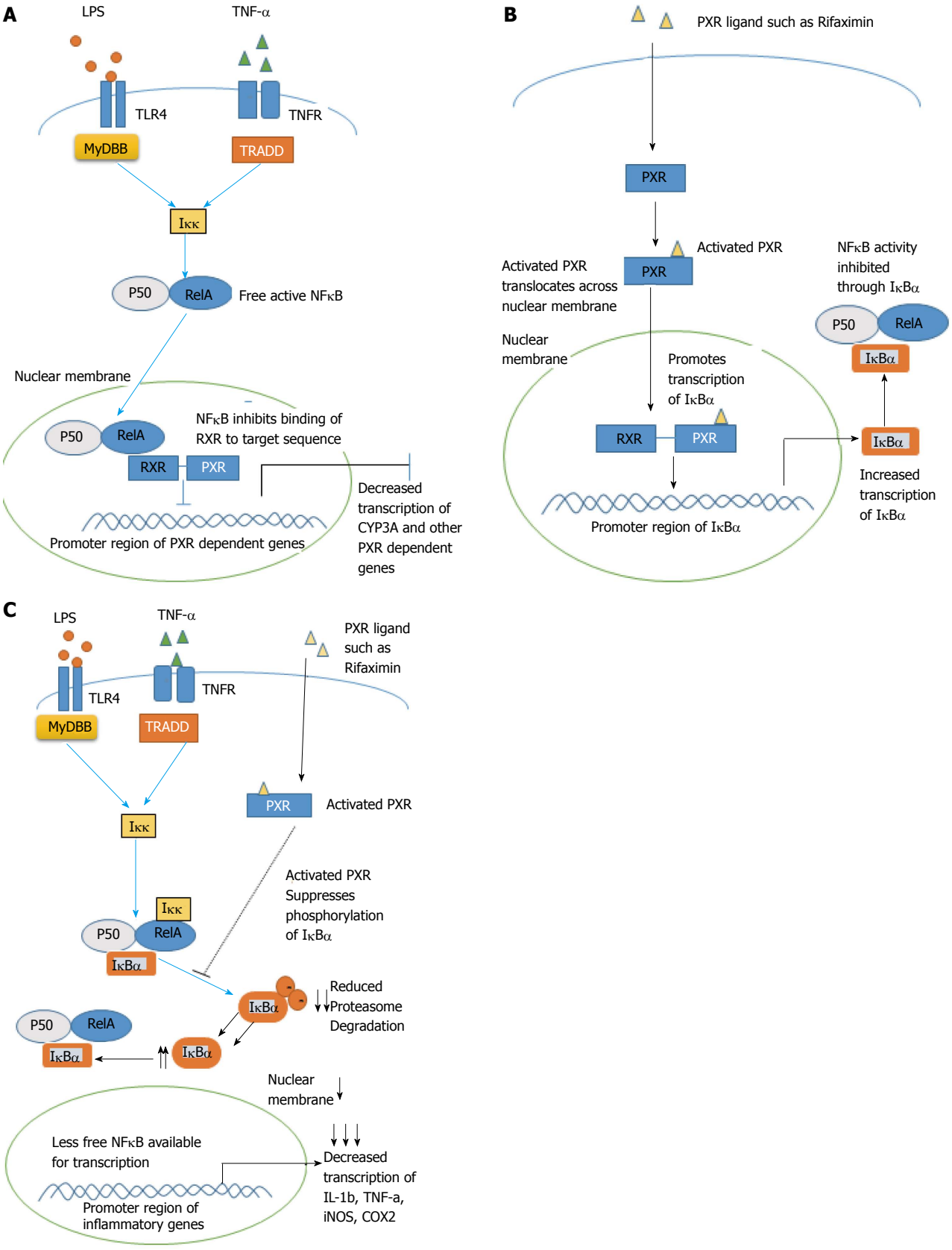

Figure 2 Illustration of counter-regulatory mechanisms existing between nuclear factor kappa B and pregnane X receptor. A: The p-65 subunit of NF-κB binds to the RXR unit of PXR heterodimer complex and represses its transcriptional activity; B: Activated PXR binds to the promoter region of IκBα and increases its transcription leading to indirect repression of NF-κB; C: After activation through its ligands, PXR suppresses the phosphorylation and degradation of IκBα and thus indirectly represses NF-κB activity. NF-κB: Nuclear factor kappa B; PXR: Pregnane X receptor; RXR: Retinoid X receptor; TLR: Toll like receptor.

SUMOYLATION dependent regulation of PXR-NF-κB axis

SUMOYLATION is a post-translational modification process, in which a small ubiquitin-like modifier (SUMO) protein would be added to the ligand-binding domain of PXR protein[67]. A SUMO1 binding site was discovered in the ligand-binding domain of PXR[68]. It was revealed that post SUMOYLATION there is an increase in the transcriptional activity of PXR, marked by increased transcription of PXR target genes (Figure 3A). Also, an increase in interaction between SUMOylated PXR and NR co-repressor (NCOR1) was observed. Thus it is speculated that post-modification, PXR protein might be able to repress NF-κB indirectly by helping to keep the co-repressors (N-COR)/HDAC3 complex intact by preventing their clearance[68,69]. Vice versa, endotoxin stimulus such as LPS would signal the clearance of these repressor complex from the promotor region of pro-inflammatory genes and thus enabling NF-κB to transcribe the inflammatory profile genes[70]. A similar event has already been established to happen in another NR, PPAR-γ, which post SUMOYLATION trans-represses NF-κB by preventing recruitment of Ubc5 protein and initiates the clearance of co-repressors[71]. This mechanism opens attractive opportunities to target the PXR SUMOylation site, particularly in a scenario such as bacterial sepsis, where endotoxin stimulated NF-κB inflammatory response occurs.

Furthermore, Hu *et al*[69] revealed that the PXR SUMOylation occurs as a feedback response to inflammatory stimulus such as TNF-α. They specifically identified SUMO3 chains in the post translationally modified (SUMOylated) PXR protein. An extremely interesting aspect of their discovery is that they found through their in vitro assays that SUMOylated form of PXR played a huge role in diminishing inflammation, but was hardly effective in regulating CYP3A expression. Thus, SUMOylation may shift the functional activity of PXR from a ligand-activated transcriptional inducer which upregulates xenobiotic target genes, towards a ligand-activated transcriptional repressor that brings about an immunosuppressive effect (Figure 3B).

PXR and TNF-α

Dysregulation between the intestinal epithelial cells and the innate immune system is often initiated by endotoxins such as LPS and is one of the established pathological mechanism for gut barrier disruption[73,73]. It has been observed that TNF-α acts as a central mediator of NF-κB in the initiation of mucosal inflammation[74]. Goldman *et al*[74] have reported that an anti-TNF approach which was effective in countering BT. Research conducted by Mencarelli *et al*[75] revealed that IEC on exposure to TNF-α showed significant dampening of PXR mRNA levels. However, TNF-α was completely antagonized when treated with Rifaximin, which showed significant anti-inflammatory effect through PXR activation. Further, when cells cultured from colon biopsies of IBD patients were induced with LPS and followed by Rifaximin treatment, an abrogation of the LPS induced NF-κB target genes such TNF-α, MIP-3α and IL-8 were observed. These evidence clearly indicate that effectively inducing PXR, which inhibits the effects of LPS induced TNF-α and NF-κB, may pose as a desired outcome in the treatment of BT.

PXR and MDR1 puzzle

Multi drug resistance gene (MDR) 1 is categorized under the ABC family of transporters and encodes the transmembrane protein P-glycoprotein (P-gp). MDR1 is one of the primary genes regulated by PXR and is widely expressed in intestinal epithelial cells and the liver[76]. P-gp acts as an ATP-dependent drug efflux system and is responsible for maintaining intestinal homeostasis by pushing out noxious chemicals (from drug or microbial source) from mucosa back into gut lumen[76,77]. Polymorphism in Mdr1a gene that results in a phenotype with reduced P-gp expression was observed in both Ulcerative colitis and Chron's disease subjects[78]. Mdr1a$^{-/-}$ knockout mice models confirmed that, in absence of this efflux pump protein, the animals developed spontaneous colitis resembling human IBD. This effect was ameliorated by treatment with oral antibiotics indicating that reducing the bacterial burden is an effective measure to control inflammation. Reducing the toxin accumulation in the gut might be the probable mechanism for ameliorating inflammation and thus underlining the importance of a xenobiotic clearance system in the gut[77]. Langman *et al*[79] showed that mRNA levels of both PXR and Mdr1a were reduced in UC patients and also assumed that the dampening of PXR expression might be the probable reason for Mdr1a downregulation.

A recent study performed by Toklu *et al*[80] hypothesized that PXR stimulation by antibiotics rifampicin and spironolactone may cause an immunosuppressive effect through induction of *Mdr1a* gene (and thus P-gp protein expression). However, these observations were conflicted by Blokzijl *et al*[81] who showed that PXR protein levels were unchanged between inflamed and uninflamed human colons, in spite of low Mdr1a expression in the same tissues. Thus in such a scenario Mdr1a may be independent of PXR protein concentration. Ros *et al*[82] reported Mdr1a expression to be unaltered in liver of LPS-treated rats. Conflicting evidence regarding reduced Mdr1a levels in intestine was provided by Kalitsky-Szirtes *et al*[83]. Further research using PXR null mice may confirm if PXR-induced-P-gp expression is a valid mechanism in controlling BT, most probably by riding the gut of endotoxins, which may otherwise stimulate mucosal immunity.

PXR and LPS

LPS is a major component of the cell walls of gram-negative bacteria and is considered an endotoxin, which potently stimulates host innate immune response[84,85]. LPS is recognized by its specific receptor toll like receptor 4 (TLR4) and is one of the earliest inflammatory triggers

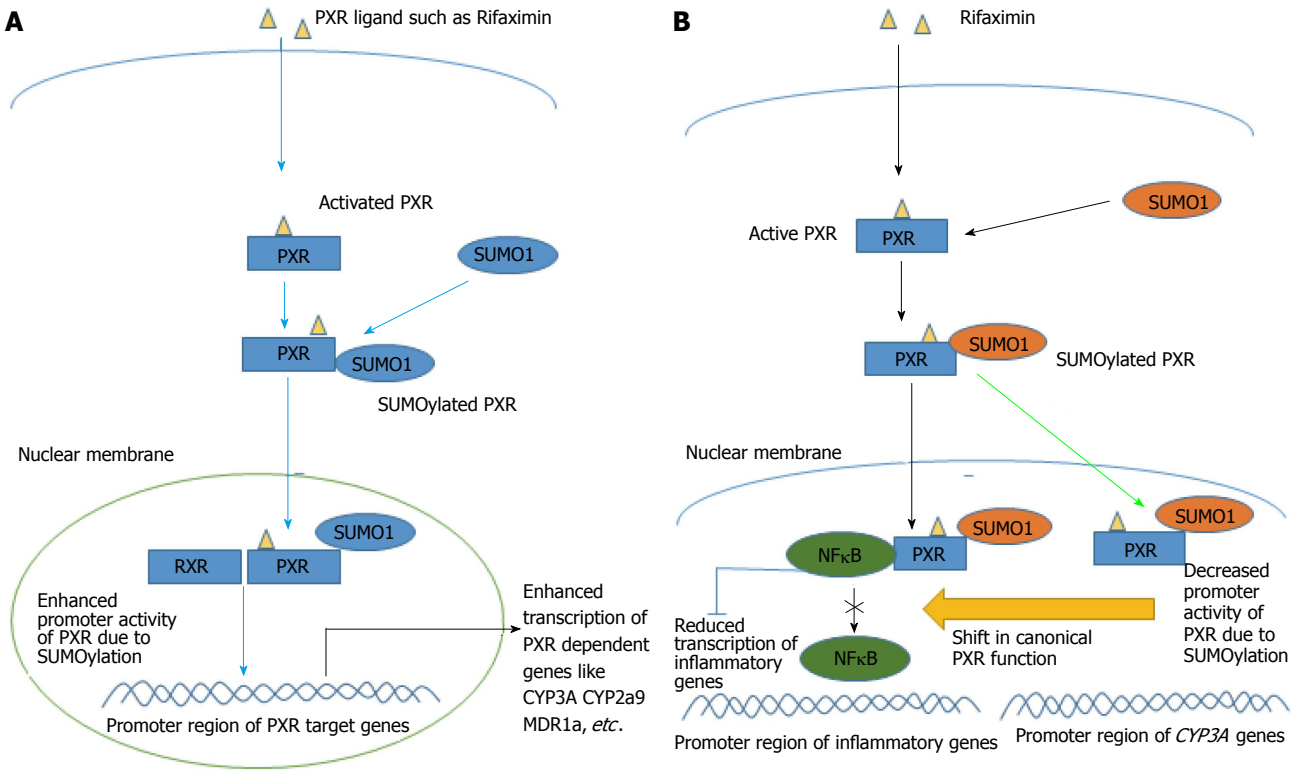

Figure 3 Schematic diagram illustrating the mechanism of modification of pregnane X receptor activity and function after SUMOYLATION. A: SUMO1 binding to PXR has been shown to increase its transcriptional activity; B: Sumo (3) ylation of PXR causes a shift from the canonical transcriptional function of PXR towards the transcriptional repression of NF-κB. NF-κB: Nuclear factor kappa B; PXR: Pregnane X receptor; RXR: Retinoid X receptor.

to induce gut barrier disruption and BT[86]. The pro-inflammatory effects of LPS are mediated primarily through activation of transcription factors like NF-κB, which is present upstream in the inflammatory cascade[56,84]. In humans, a physiological BT state has been defined, where 5%-10% of bacteria, translocate across the intestine with minor exposures of LPS, maintaining a tightly regulated tolerance towards the gut microbes and their toxins[38]. However, when there are alterations in quantity (load) or quality (dysbiosis) of bacteria, the innate immune system is activated overtly. This is often by means of increased exposure to LPS, which stimulate immune cells such as monocytes, neutrophils and lymphocytes. These cells, in turn, produce acute response cytokines including IL-1β, IL-6 and TNF-α, creating an inflammatory profile[87-89].

LPS-induced inflammation models have highlighted the importance of PXR activity in regulating different phases of the immune response. Diminished expression of *CYP3A* gene was identified during infections and has been replicated in LPS-induced animal models[90,91]. Interestingly, mRNA levels of PXR were also seen to be downregulated in such models. Indeed, Moriya *et al*[92] have indicated that LPS treatment significantly reduced both the gene expression and activity of CYP's, even in mice that were pre-stimulated with PXR activator PCN. LPS caused this effect by inducing cytokines, which bring about the inhibition of PXR in an NF-κB dependent manner. A similar observation was made by Gu *et al*[56] where the use of NF-κB suppressor SRIκBα, reversed the LPS induced downregulation of PXR, proving that "NF-κB stimulated PXR inhibition" is central in bringing out the effects of LPS. While IECs are in constant contact with bacterial products like LPS, immune cells are involved in the response to any dysregulation between IEC and microbial products. It should be noted that PXR is expressed in both these cell types and thus play a major role in regulating both the toxin challenge that is faced by IEC, as well as in response to that challenge that is brought about by immune cells. A recent study identified in the primary culture of hepatocytes (PCH) isolated from WT mice that, PCN pretreatment for 24 h alleviated LPS induced an acute response by decreasing cytokines such as IL-1β, TNF-α and IL-6. However, when PCH from PXR-null mice were treated with LPS, enhanced pro-inflammatory cytokine response was documented[93]. When PCH isolated from humanized PXR mice were pre-treated with PXR activators, it led to increased production of IL-1Ra, a natural inhibitor of IL-1β[93]. Thus PXR expression is seen to be important in both dampening endotoxin-stimulated immune response to maintain homeostasis, as well as in resolving the inflammatory state through inducing anti-inflammatory response[93,94].

Relationship between PXR, LPS and ROS

Xu *et al*[95,96] have revealed an interesting pathway in which LPS could suppress the expression of PXR and its associated genes. In their experiment, LPS dose-dependently suppressed PXR mRNA levels in mice and was significantly improved following antioxidants

treatments[95,96]. Furthermore, inhibition of xanthine oxidase and NADPH oxidase that generates ROS, using specific inhibitors allopurinol and diphenylene iodonium respectively, led to attenuation of LPS induced PXR downregulation[95]. In this context, the antioxidant melatonin was also observed to produce a similar effect[97]. Chen *et al*[98] revealed that treatment with a free radical trapping agent alpha-phenyl-N-tbutylnitrone prevented LPS from downregulating PXR. Thus, it is well understood that ROS and oxidative stress have an impact in the LPS induced diminishing of PXR expression. PXR ligands such as Danshen, which have an inherent antioxidant property could be used to further understand the LPS-countering activity of PXR[11]. Research performed using PXR ligands or constitutively active PXR (VP-PXR) have revealed that LPS instigated response is regulated by PXR activation[94,99]. Hence, countering LPS induced ROS promises to be a novel opportunity to counter endotoxin-induced inflammatory response, in BT[94,99].

PXR and TLR4 crosstalk

TLR4 is a transmembrane receptor that recognizes LPS, which is a pathogen associated molecular pattern. LPS can only bind to the TLR4 complex after it has associated itself with LBP (LPS binding protein)[100]. The recognition also involves additional co-receptors such as CD-14 and MD-2 and adaptor protein MyD88[101]. Physiologically TLR4 expression and regulation is of significant importance in the intestine. Hence, TLR4 expression is tightly regulated based on the level of LPS in the gut lumen as it directly correlates with the intensity of immune response at any given time. A pathological state such as small intestinal bacterial overgrowth, may challenge this homeostasis leading to overt activation of TLR4 signaling and trigger NF-κB, which leads to barrier dysfunction and BT[101,102]. Studies have shown that the crosstalk between TLR4 and PXR could determine the homeostasis in the intestine[103]. For instance, when TLR4 was activated using its specific agonist KDO2, increased induction of mucosal TNF-α, followed by intestinal permeability was observed in both WT and PXR null mice[104]. Similarly, when PXR was activated using PCN, a clear reduction in mucosal TNF-α induction was documented. PCN activation did not have any effect in PXR null mice and thus indicating that the TLR4 inhibition was PXR-dependent. This study clearly shows that PXR and TLR4 counter-regulate each other upon their respective activation[104].

A study by Esposito *et al*[105] observed a similar pattern of regulation when Caco2 IECs were induced with Clostridium difficile toxin A (TcdA) to replicate ulceration and inflammation model. They found that the toxin stimulated the expression of TLR4 by 1411% in Caco2 cells. However, this phenomenon was completely reversed by Rifaximin treatment dose dependently, which down-regulated the expression of TLR4, MyD88 and NF-κB. This study also brings to light an additional pathway, where by reducing TLR4 levels following PXR activation, the NF-κB activity might be reciprocally inhibited[105]. Furthermore, PXR and TLR4 double KO mice models have shed more light into PXR's ability to act as a mediator between the microbes and TLR4. Venkatesh *et al*[106] observed that PXR null mice developed leaky gut and showed increased induction of TLR's including TLR4 (1.8 fold increase). However, in TLR4$^{-/-}$ and PXR$^{-/-}$ double KO mice model, the previously observed pathological defects in intestine disappeared. This emphasizes the impact of TLR4 expression, which was particularly high in absence of PXR, in bringing about intestinal inflammation and the gut disruption. In addition, enterocytes isolated from PXR null mice, showed similar results with TLR4 inhibitors. Thus a reciprocal relationship seems to exist between PXR and TLR4 in maintaining homeostasis. They also found that Indole-3 Propionic Acid (IPA), which is an endogenous, microbe derived ligand for PXR, activated PXR and reduced enterocyte TNF-α[106]. This is a clear example of the sensing system: PXR, and the symbiotic bacteria working in coherence to repress overt inflammation. Together, these data suggest that PXR regulates the expression of TLR4, and that PXR activation could have a therapeutic effect in BT by counteracting TLR4 mediated gut disruption.

INTESTINAL INTEGRITY AND PXR: TIGHT JUNCTIONS

PXR and maintenance of intestinal integrity

A single layer of epithelial cells serve as physical barrier that prevent the diverse contents of the gut from entering the systemic circulation and other tissues. The integrity of this barrier is governed by junctional complex proteins including TJ and adherent junctions (AJ) and are involved in sealing the gap between two adjacent cells[107]. The expression of these junctional complexes are tightly controlled and are dynamic in nature, such that allowing passage of only selected molecules across the epithelial barrier[108]. In disease state, the expression of these junctional complexes are highly compromised leading to a leaky gut, which is the major driving factor for BT and its complications[109,110].

PXR is extensively expressed by IECs and have been shown to have a direct impact on the signaling molecules that govern intestinal integrity. Accordingly, studies have shown that in PXR null mice, there is a leaky gut like pathology[106]. Indeed Venkatesh *et al*[106] observed reduced mRNA levels of junctional complexes such as Zonula occludens 1 (ZO1) and E-cad in PXR KO mice. However, they also found an increased expression of Claudin-2, which is associated with promoting paracellular transport of microbes and which in high expression state is linked with hyperpermeability in gut. One of the possible mechanisms through which PXR maintains expression of junctional complexes was revealed through the use of PXR-TLR4 double KO mice[106]. When both PXR and TLR4 were knocked out, the level of TJ expression

was almost relatable to the levels that were found in $PXR^{+/+}$ mice. Hence, PXR may preserve junctional complexes by countering TLR4 and thereby inhibiting the downstream inflammatory cytokines such as TNF-α that are stimulated by TLR4. This shows that PXR knockout state is associated with pattern of upregulation of genes that promote paracellular transport (claudin-2, TNF-α) and downregulation of genes that maintain barrier functions (ZO1) and thereby playing a vital role in maintaining intestinal integrity.

Negative regulation between PXR and MLCK

Several studies that have targeted PXR activation have attributed the preservation of the junctional complexes to PXR's ability to interact with various intercellular signaling mediators. Myosin Light Chain Kinase (MLCK) is associated with regulation of paracellular permeability through its ability to phosphorylate myosin II regulatory light chain (MLC), which underlies the junctional complex arrangement[111]. Hence through phosphorylation of MLC, MLCK is able to stimulate actomyosin contraction and modulate TJ localization[111]. In a pathological state such as infection or inflammation TNF-α induced both the expression of MLCK, and its activity and thus influencing intestinal permeability[112]. He *et al*[113] observed TNF-α induced MLCK expression was increased through the stimulation of NF-κB, which acted upstream of MLCK. Hence PXR, which counter-regulates TNF-α mediated NF-κB could possibly interact with this pathway to preserve TJs. Indeed, study by Garg *et al*[104] reported that TNF-α exposure induced increased relocalization of ZO1 through the upregulation of MLCK expression. However, PXR activation by Rifaximin, countered the MLCK upregulation in Caco2 IEC cells, through its established function of attenuating NF-κB. The same results were reproduced in an *in-vivo* DSS mice model, where PCN treatment attenuated MLCK expression and protected against ZO1 mislocalization[104]. Thus by inhibiting TNF-α induced NF-κB activation, PXR is able to maintain intestinal integrity through indirectly regulating MLCK[104].

Preservation of intestinal integrity by PXR through JNK1/2 interference

PXR activation has been reported to influence on the JNK1/2 pathway. C-jun N-terminal kinase (JNKs) are kinases that are activated in response to stress stimuli including various cytokines like TNF-α and are implicated in apoptosis and inflammation[114-116]. The exact role of JNK1/2 on inflammatory disorders is still unclear. While few studies reported JNK1/2 deletion increased the severity of inflammation in DSS model[117], others showed that JNK1/2 inhibition is protective[114,118]. In this context, Mitsuyama *et al*[119] have reported an increased JNK1/2 expression in IECs of CD patients. Garg *et al*[104] have reported that TNF-α/INFγ stimulation of Caco-2 cells resulted in increased activation of JNK1/2, an effect that was associated with ZO1 mislocalization. However, this phenomenon was completely inhibited using JNK inhibitor SP600125. Most importantly, activation of PXR using Rifaximin was seen to attenuate JNK1/2 activity by inducing the transcription of growth arrest and DNA damage inducible 45β (GADD45β), a protein that is known to block JNK1/2 activity by preventing its phosphorylation. Thus PXR activation was seen to protect intestinal TJ integrity by preventing the activation/phosphorylation of JNK1/2.

PXR and CDX2: An interaction of two transcription factors

Recently a new mechanism of PXR-related immunosuppression was reported by Dou *et al*[120] involving the Caudal related Homeobox transcription factor, CDX2. Interestingly, CDX2 has been implicated in intestinal differentiation and in maintaining intestinal integrity[121,122]. CDX2 is a transcription factor reportedly expressed in the intestine, where it binds to the promotor region of PXR and induces PXR transcription. Further mechanistic studies may establish if CDX2 is a player involved in regulating PXR expression especially in the scenario of mucosal inflammation.

PXR AND BILE ACIDS

Bile acids play a very important physiologic role in the catabolism of cholesterol and are known to regulate bacterial overgrowth owing to their bacteriostatic properties[123,124]. Bile acids are also ligands to NR's such as FXR and VDR[125,126]. On activation these NR's regulate the expression of anti-microbial peptides and innate immunity genes, which keep gut microbiome outgrowth in check[123-126]. However, studies have shown that when bile acids accumulate, they can be potentially toxic, thus highlighting the importance of presence of active bile acid detoxification system to afford protection against their toxicity[127]. Lithocholic acid (LCA) is a secondary bile acid, which is considered to be toxic at higher concentrations than the basal levels and is a byproduct of gut bacterial biotransformation process[128]. Makoto Ishit *et al*[129] have shown that upregulation of PXR and its dependent genes happens as an adaptive response to an increase in LCA, in patients who underwent gastrectomy. They reported that gastrectomy shifted the intestinal PH towards alkaline state due to reduced gastric acid, which led to the increased thriving of LCA-producing bacteria and thus leading to increased accumulation of LCA. This study highlights that PXR is the foremost physiologic and adaptive sensor of LCA especially considering that FXR another important bile acid sensor is unresponsive to LCA[129]. More importantly, this study using the example of gastrectomy sets the precedence that other pathologic events, such as dysbiosis may also shift the balance of gut microbiome composition and ultimately influence the bile acid metabolism and the genes they control.

Bacterial dysbiosis, an established mechanism to cause BT, has been shown to affect the composition of

bile acid pool[130]. Disease states such as Non-Alcoholic Fatty Liver Disease (NAFLD) are associated with both increased bile acids and alteration in bacterial gut microbiome communities[131]. Such states may shift the balance of hydrophobic and hydrophilic secondary bile acids in the overall bile acid pool. In the context of this review, this is of importance, as a linear correlation has been observed between perturbance in gut microbes, disturbance in bile metabolites and disruption of intestinal barrier homeostasis[132,133]. Increased hydrophobic bile acids such as LCA and Deoxycholic acid (DCA) have been shown to be associated with disruption of gut barrier as shown by Stentman *et al*[134] where high fat related concentration of hydrophobic but not hydrophilic bile acids produced barrier disruption. Hughes *et al*[135] made an interesting observation that at physiologic levels, LCA increased paracellular permeability in Caco2 intestinal epithelial cells, which was indicated by a decrease in trans-epithelial resistance and increase in mannitol flux. However, at the same physiologic levels, LCA was seen to increase occludin expression. It would be interesting to see if LCA induced occludin expression happens in a PXR dependent manner, as PXR is the major physiologic sensor of LCA. Also, since this study only focused on acute twelve hour effects of LCA at basal levels[135], future studies at chronic treatment times and higher doses would need to be conducted to illuminate the effect of LCA toxicity on TJ expression.

LCA-feeding has been used a standard *in-vivo* model to induce cholestasis in mice. Fickert *et al*[136], used this model to demonstrate that LCA feeding induced disruption of TJ protein ZO1 in both bile duct epithelial cells and between hepatocytes. Vu *et al*[137], have found the similar observations that TJ permeability was elevated followed by cholestatic dose of LCA.

Role of PXR in LCA detoxification

The role of PXR in LCA detoxification is paramount, evidenced by studies which showed that PXR KO animals were susceptible to bile toxicity and cholestasis[138]. In cholestasis, as the disease progresses the bile acid flow is impeded leading to a compromised bile environment. This is exploited by bacterial outgrowth leading to their uncontrolled translocation[139]. This highlights the fact that, PXR sensing and detoxification may serve as a prophylactic (preventive) setup to maintain homeostasis in bile metabolism and afford protection against cholestasis[140]. The role of PXR in protection against NAFLD may also be critical, as increased LCA has been associated with high-fat consuming population[141,142]. Studies by Staudinger *et al*[143] and Xie *et al*[144] have documented the various mechanisms of PXR-dependent LCA detoxification. PXR has been observed to control the expression of CYP7A1, a rate-limiting enzyme in bile acid production from cholesterol, and is seen to repress CYP7A1 following PXR activation through its ligands[143]. PXR also directly regulates the expression of Na1-independent organic anion transporter 2, a protein that is involved in uptake of bile acids by hepatocytes for metabolism[143,144]. Finally, PXR dependent CYP3A enzymes in the hepatocytes mediate hydroxylation of LCA and prime it for elimination[145]. Additional mechanisms such as sulfonation of LCA by PXR dependent SULT enzymes have also been described[146]. Transgenic mice that constitutively expressed activated PXR (VP-PXR) were seen to be resistant to the toxic effects of LCA[146]. Consequently, PXR plays a very important role prophylactically in affording protection against bile dysregulation and toxicity, as seen in closely related pathologic states such as dysbiosis and NAFLD. The activation of PXR might serve as an attractive option in preventing BT, which might result from direct or indirect effects of bile acid dysregulation.

PXR THE SENSOR OF MICROBIAL METABOLITE CUE

In a physiological state, bacteria have been shown to have an impact on host genes without direct contact[31]. Gut bacteria communicate with the host mainly through the extensive profile of microbial metabolites that they produce, which interact with a range of physiologic sensors such as NR's in the host cells of intestine and liver[14]. The enterohepatic circulation gains major importance in this matter, as the liver and intestine interact with each other through bile and metabolite (nutrient and microbial) profile respectively. Compromised bile availability in disease states like cholestasis has been linked with bacterial dysbiosis in the intestine[147]. Similarly, metabolic disorders such as obesity and Type 1 diabetes have been implicated with alterations in the gut microbiota[148], which indicate alterations in the proportion of the microbial metabolites the microbiota produce. While some of these microbial metabolites have been characterized as essential nutrients, many other metabolites and the NR's that they interact with are yet to be explored. PXR owing to its flexible binding domain and extensive expression pattern in gut and liver has been identified to interact with wide range of bacterial metabolites through which it is seen to maintain homeostasis along the gut-liver axis.

IPA, a bacterial product of the tryptophan metabolism is being recognized as one of the established ligands of PXR[149]. Venkatesh *et al*[106] highlighted the importance of IPA sensing by PXR, where PXR after activation, maintained gut barrier function by downregulating TLR4 and its downstream effector TNF-α. IPA also increased the mRNA levels of junctional proteins. They demonstrated that either loss or reduced expression of PXR (as seen in inflammatory conditions) or the loss of IPA producing bacteria (as documented through commensal depleted organism models) led to worsening of inflammation and increased intestinal permeability[106]. It was also revealed that reintroducing *C. Sporogenes* in the presence of their substrate L-Tryptophan in GF mice led to production of IPA and improved cell-cell junctional

Table 1 Table enlisting the documented effect of various natural occurring ligands of pregnane X Receptor

No	Natural ligand and source	Effective response after binding of ligands with PXR	Ref.
1	Baicalein from roots of *Scutellaria baicalensis Georgi*	Attenuated colonic inflammation in DSS induced colitis mice model through stimulation of CDX2. ↓TNFα and IL-6 mRNA levels in intestinal mucosa	Dou *et al*[122] *Plos One* 2012
2	Forskolin and 1,9 dideoxyforskolin from roots of *Coleus forskohlii*	↑CYP3A expression in primary hepatocytes through activation of Protein Kinase A signaling pathway	Ding and Staudinger[151] *J Pharmacol Exp Ther* 2005
3	Z-guggulsterone from Commiphora mukul (Guggul)	↓*CYP7A1* gene in HepG2 cells	Owsley and Chiang[152] *Biochem Biophys Res Commun* 2003
4	E-guggulsterone from Commiphora mukul (Guggul)	↑CYP3A11 and CYP3A4 mRNA levels only in cultured hepatocytes from PXR +/+ mice and not in PXR KO mice	Brobst *et al*[153] *J Pharmacol Exp Ther* 2004
5	Hyperforin from Hypericum perforatum (St. John's wort)	↑CYP3A4 induction in hepatocytes. Induction of *CYP2C9* gene expression was also reported in humans	Moore *et al*[154] *Proc Natl Acad Sci USA* 2000
6	Colupulone from Humulus lupulus (Hop Extract)	↑*CYP3A4*, *CYP2B6* and *MDR1* gene expression in primary human hepatocytes dose dependently	Teotico *et al*[155] *Mol Pharmacol* 2008
7	Kava Kava (Piper methysticum)	↑CYP3A4 mRNA expression in primary human hepatocytes extensively	Raucy[156] *Drug Metab Dispos* 2003
8	Wu Wei Zi [Dibenzocyclooctene lignans: schisandrol B, schisandrin (A and B)]	↑Transcription of *CYP3A4*, *CYP2C9* and *MRP2* genes in primary hepatocytes	Mu *et al*[157] *J Pharmacol Exp Ther* 2006
9	Ginkgolide A from Gingko Biloba extract	Protection against CCL4 induced acute toxicity model in rats, ↑Iκ-Bα transcription, which in turn inhibited NF-κB	Ye *et al*[64] *Biomol Ther* (Seoul) 2016
10	Ginkgolide B from Gingko Biloba extract	↑Nuclear translocation of PXR, and protected HUVEC cells from drug induced apoptosis. Anti-inflammatory role by reducing VCAM-1 and E-selectin induced by TNFα	Zhou *et al*[158] *Acta Pharmacol Sin* 2016

↑: Upregulation; ↓: Downregulation; PXR: Pregnane X receptor.

complex efficacy. Importantly, the same phenomenon was absent in PXR KO mice. Hence shifting the bacterial composition towards favorable metabolic profile might be an effective method to activate PXR and counteract inflammation[106]. However, it is also important to consider the level of PXR available to sense IPA, which is often compromised during inflammation.

Gut bacteria also play a vital role in the bio-transformation of various natural herbal products into forms that are beneficial to host. β-glucuronidases produced by symbiotic bacteria have been shown to convert the flavonoid baicalin into baicalein[122,150]. Interestingly, Dou *et al*[122] reported that both baicalein and baicalin attenuated gut inflammation induced by DSS *in vivo*. However, baicalin treatment did not have any effect, when the β-glucuronidase inhibitor was used to prevent the bioconversion of baicalin. The study further reported that only baicalein activated PXR to potently produce an anti-inflammatory phenotype in the colitis induced mice. Thus, PXR acts as an important mediator between bacterial derived metabolites and host, and upon activation shapes the immune profile. Table 1 summarizes the various identified natural ligands of PXR along with the effective response they produce after binding with PXR.

CONCLUSION

PXR plays a pivotal role as an endobiotic and xenobiotic sensor scanning for any alterations in the environmental cues and then translates the signals into an epithelial phenotype that is protective to host. Its role in maintaining homeostasis along the gut liver axis is undisputable. The majority of studies in exploration of PXR pathways, have been conducted only in IBD and associated pathologies. However, PXR is expressed copiously along the gut liver axis and has been proved to have a huge functional impact in both liver and intestine, and poses as an excellent target in CLDs with BT. As summarized above, PXR has important functional implications in each of the major pathophysiological mechanisms attributed to causing BT in CLD states. Targeting PXR with naturally occurring herbs or other polyphenolic compounds may potentially cease BT and attenuate the progression of liver disease or the manifestation of the associated fatal complications of CLD. Moreover, even though Rifaximin is a potent agonist for PXR, it can activate only gut PXR and is associated with adverse hepatotoxic side effects. The plethora of bioactive components from natural herbs are being discovered as effective activators for both human and rodent PXR, promising a fertile research ground for future studies. Using a CCL4 induced mouse cirrhotic model, our lab is currently investigating the effect of a naturally identified PXR ligand Ginkgolide-A, to further comprehend the functional impact of PXR activation on regulating BT.

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ORIGINAL ARTICLE

Basic Study

Liver atrophy after percutaneous transhepatic portal embolization occurs in two histological phases: Hepatocellular atrophy followed by apoptosis

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Author contributions: Iwao Y and Ojima H performed the experiments, analyzed the data and wrote the paper; Kobayashi T obtained the pig specimens; Kishi Y, Nara S, Esaki M and Shimada K obtained the human surgical liver specimens and determined the clinical data; Hiraoka N, Tanabe M and Kanai Y coordinated the research.

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Abstract

AIM

To clarify the histological changes associated with liver atrophy after percutaneous transhepatic portal

embolization (PTPE) in pigs and humans.

METHODS

As a preliminary study, we performed pathological examinations of liver specimens from five pigs that had undergone PTPE in a time-dependent model of liver atrophy. In specimens from embolized lobes (EMB) and nonembolized lobes (controls), we measured the portal vein to central vein distance (PV-CV), the area and number of hepatocytes per lobule, and apoptotic activity using the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay. Immunohistochemical reactivities were evaluated for light chain 3 (LC3) and lysosomal-associated membrane protein 2 (LAMP2) as autophagy markers and for glutamine synthetase and cytochrome P450 2E1 (CYP2E1) as metabolic zonation markers. Samples from ten human livers taken 20-36 d after PTPE were similarly examined.

RESULTS

PV-CVs and lobule areas did not differ between EMB and controls at day 0, but were lower in EMB than in controls at weeks 2, 4, and 6 ($P \leqslant 0.001$). Hepatocyte numbers were not significantly reduced in EMB at day 0 and week 2 but were reduced at weeks 4 and 6 ($P \leqslant 0.05$). Apoptotic activity was higher in EMB than in controls at day 0 and week 4. LC3 and LAMP2 staining peaked in EMB at week 2, with no significant difference between EMB and controls at weeks 4 and 6. Glutamine synthetase and CYP2E1 zonation in EMB at weeks 2, 4, and 6 were narrower than those in controls. Human results were consistent with those of porcine specimens.

CONCLUSION

The mechanism of liver atrophy after PTPE has two histological phases: Hepatocellular atrophy is likely caused by autophagy in the first 2 wk and apoptosis thereafter.

Key words: Liver atrophy; Portal vein embolization; Autophagy; Apoptosis; Zonation; Lobule

Core tip: Liver atrophy after percutaneous transhepatic portal embolization (PTPE) in time-independent human studies is associated with hepatocyte shrinkage and apoptosis. In this preliminary study, we performed pathological examinations of liver specimens from five pigs that had undergone PTPE in a time-dependent model of liver atrophy. Two distinct phases of liver atrophy were identified: A hepatocellular atrophic phase, which may relate to autophagy, and an apoptotic phase. Despite liver atrophy appearing to be mostly resolved 2 wk after embolization, the period after PTPE could beneficially be extended to 4 wk to ensure contralateral hypertrophy and to allow the completion of liver atrophy.

Iwao Y, Ojima H, Kobayashi T, Kishi Y, Nara S, Esaki M, Shimada K, Hiraoka N, Tanabe M, Kanai Y. Liver atrophy after percutaneous transhepatic portal embolization occurs in two histological phases: Hepatocellular atrophy followed by apoptosis. *World J Hepatol* 2017; 9(32): 1227-1238 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i32/1227.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i32.1227

INTRODUCTION

The interruption of portal blood flow by portal vein embolization or tumor thrombosis, for example, causes liver atrophy[1]. However, the mechanisms responsible for this effect have not been fully elucidated. Using pig models of percutaneous transhepatic portal vein embolization (PTPE) with absolute ethanol, we previously observed the temporary elevation of serum levels of liver enzymes immediately after ethanol injection. Moreover, in our previous report, macroscopic liver atrophy accompanied by an increased future liver remnant (FLR)/total estimated liver volume ratio was evident 2 wk after PTPE[2]. These observations suggest that the mechanisms responsible for liver atrophy likely commence soon after the disruption of portal blood flow. Consequently, histopathological changes would likely also be observed soon after PTPE.

In pigs that had undergone PTPE using a combination of coils and polyvinyl alcohol particles, the lobule size in the embolized lobe relative to normal liver reportedly decreased gradually to 23% at 12 d; after 12 d, the size of the embolized lobe remained constant[3]. Therefore, to clarify the mechanisms responsible for liver atrophy, pathological analysis should be carried out within this time period. However, to the best of our knowledge, such time-course studies have not yet been carried out.

To assess microscopic changes in liver tissues, it is important to study liver lobules, the smallest functional units of the liver. The observation of clear histological changes would be expected when hepatic blood inflow is disturbed and would be dependent on lobule metabolism, which varies in different zones of the lobule. In particular, we focused on the zonation associated with different levels of metabolism, as illuminated by immunohistochemical (IHC) staining for glutamine synthetase (GS)[4] and cytochrome P450 2E1 (CYP2E1)[5]. Both markers were observed in the pericentral zone of lobules.

Recently, the relationship between apoptosis and autophagy has been extensively reported[6]. The molecular mechanism of autophagy was illuminated by the discoveries of the membrane protein autophagy-related gene 5 in yeast and the microtubule-associated protein 1 light chain 3 (LC3) in mammals[7]. Consequently, IHC staining for these proteins can be used to evaluate levels of autophagy[8,9]. Recent studies have used lysosomal-associated membrane protein 2 (LAMP2) to evaluate autophagy because it is related to autolysosomes for some kinds of autophagy[10]. Autophagy in the liver is reportedly

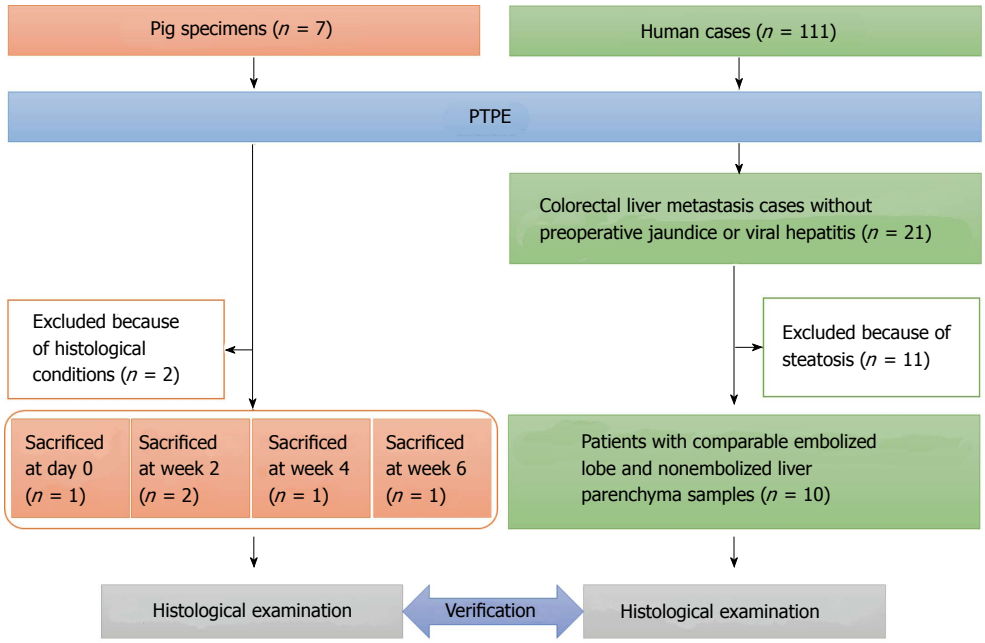

Figure 1 Flow chart of the pig specimens and human cases examined in this study. PTPE: Percutaneous transhepatic portal vein embolization.

caused by starvation and is related to hepatocellular atrophy[11]. The interruption of portal blood flow, which contains a wealth of nutrients[12], is considered a form of starvation. Therefore, autophagy may be related to both cellular shrinking and apoptosis. However, the relationship between portal venous obstruction and autophagy has not been reported.

The aim of this study was to investigate, using specimens from a previously reported porcine PTPE model[2], the microscopic changes associated with apoptosis and autophagy in the days and weeks following portal venous obstruction and to clarify the mechanism by which interrupted portal blood flow causes liver atrophy. Furthermore, we sought to verify the integrity of our pig results by performing the same histopathological investigations in specimens resected from human patients who had undergone PTPE[13-16].

MATERIALS AND METHODS

Animal specimens

Liver specimens obtained from seven female domestic pigs (Saitama Experimental Animal Supply, Saitama, Japan) weighing 30.0-35.0 kg were used in this study. All pigs underwent segmental PTPE under fluoroscopic guidance with injection of 10 mL absolute ethanol, as we described previously[2]. Specimens from two pigs were excluded from this study because their quality was unsuitable for pathological analysis. Finally, specimens from five pigs were selected for analysis; one pig was sacrificed on day 0, two pigs at week 2, one pig at week 4, and one pig at week 6 (Figure 1).

The removed pig livers were observed macroscopically (Figure 2A and B). No pig livers exhibited bleeding, degeneration, or necrosis. To evaluate the pure histological changes of the embolized area compared with those of the nonembolized area without histological regenerative reactions, formalin-fixed paraffin-embedded specimens were produced from samples resected from the embolized segment and a nonembolized lobe (control) far from the lobe containing the embolized segment.

Patients

Formalin-fixed paraffin-embedded specimens obtained from 111 patients who underwent major hepatectomy with preoperative PTPE between 2004 and 2010 were collected at the Hepatobiliary Pancreatic Surgery Division of the National Cancer Center Hospital, Tokyo, Japan. Of these 111 patients, 21 had colorectal liver metastases without preoperative jaundice or viral hepatitis. To facilitate the histological evaluation of liver lobules, 11 patients were excluded because of steatosis. In total, 10 patients with comparable embolized lobe and nonembolized liver parenchyma samples (*e.g.,* the caudate lobe or partial hepatectomy from contralateral lobe of PTPE) were selected (Figure 1). All patients (male-to-female ratio: 4:6, median age: 59 years, range: 43-76 years) underwent hepatectomy with PTPE based on their individual clinical status (Table 1), and samples were collected between 20 and 36 d later (median: 22 d). All patients underwent PTPE *via* the ipsilateral approach using a 21-G needle (Top, Tokyo, Japan) under ultrasonographic guidance. A 5-Fr sheath (introducer set, Medikit, Tokyo, Japan) was introduced into a branch of the portal vein under fluoroscopic guidance and a 5-Fr balloon catheter (Selection Balloon Catheter, Terumo Clinical Supply, Gifu, Japan) was used for the injection of absolute ethanol (99.5% ethanol, Fuso Pharmaceutical Industries, Osaka, Japan). The study was approved by the Ethics Committee of our

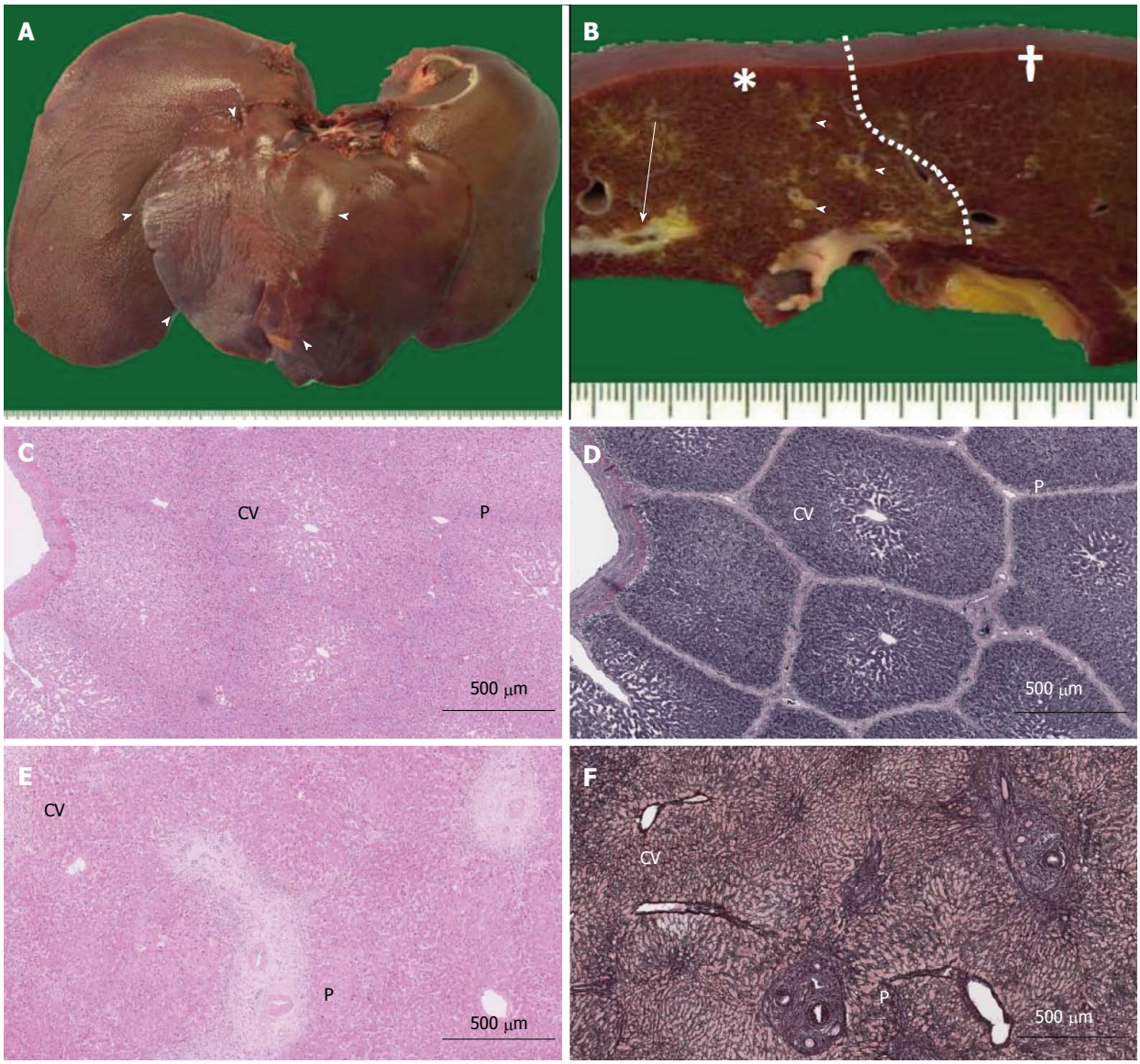

Figure 2 Macroscopic images of pig livers following interruption of portal blood flow and structural comparison of pig and human liver lobules. A: Liver removed from a pig at week 2 after PTPE. Segmental macroscopic atrophy could be seen (arrowheads); B: Portal thrombosis was observed at the cut surface (arrow) in the embolized area (*), and fibrous thickening of the portal areas was observed (arrowheads) compared with nonembolized area (†). A clear border (dotted line) was observed between these areas; C-F: Microscopic views (C, D: Pig; E, F: Human) show central veins (CV) and portal areas (P) in HE-stained sections (C, E), but these features are more clearly observed in silver-stained sections (D, F). Lobule structure was less well defined in human specimens (F).

institution. All patients gave written informed consent for inclusion in this study (ID: 2007-022).

Histological examination

All histological examinations were carried out using digital images scanned by Nanozoomer Digital Pathology (NDP, Hamamatsu Photonics, Hamamatsu, Japan) evaluated by two experienced pathologists (Yasuhito Iwao and Hidenori Ojima) who were blinded to all experimental and clinical data. The pathologists conferred if the original evaluations differed.

Morphological study of the lobule

Sections were stained with hematoxylin and eosin (HE), and the morphological changes in embolized and nonembolized lobules were evaluated at 50 random locations on NDP images. The distance between the endothelium of the portal vein in the portal triad and the associated central vein in the same lobule (PV-CV) and the cross sectional area of the lobule (which has a convex shape around a single central vein) were recorded. After the median lobule size (median, ± 0.100 mm^2) of each group was determined, the number of hepatocytes in each lobule was counted for 20 randomly selected lobules (Figure 2C and D). Hepatocyte density was calculated by dividing the number of hepatocytes by the area of the counted lobule for pig specimens. For human specimens, the hepatocyte density was counted within 20 randomly selected 1-mm-diameter circles.

Evaluation of apoptotic activity

Apoptosis of hepatocytes was quantified by terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay (In situ cell

Table 1 Clinical background of the patients who provided the human specimens

Patient	Gender	Age (yr)	Primary tumor site	Surgery
1	F	74	Rectum	Ex Rt
2	F	57	Rectum	Ex Rt
3	M	76	Cecum	Ex Rt
4	M	43	S/C	Ex Rt
5	M	56	Rectum	Ex Rt
6	M	53	Rectum	Ex Rt + nonAnat S3
7	F	47	S/C	Ex Rt
8	F	61	Rectum	Ex Rt
9	F	64	S/C	Ex Rt
10	F	63	Rectum	Ex Rt + nonAnat S3

All patients underwent right-sided percutaneous transhepatic portal embolization. F: Female; M: Male; S/C: Sigmoid colon; Ex Rt: Extended right hemilobectomy; nonAnat S3: Non-anatomical liver resection of segment 3.

death detection kit, POD, Roche Diagnostic, Mannheim, Germany). The proportion of TUNEL-positive hepatocytes was counted five times in ten random high-power fields.

Immunohistochemical staining

Sections (4-μm thick) were deparaffinized and incubated in an autoclave for 10 min at 121 ℃ and 1.5 bar. IHC staining was performed using a polymer system (Dako, Glostrup, Denmark) with 3,3′-diaminobenzidine (DAB/Tris tablets, Muto Pure Chemicals, Tokyo, Japan) as the chromogen. A mouse monoclonal antibody (1:50, sc-271625, clone G-2, Santa Cruz Biotechnology, Santa Cruz, CA, United States) was used for LC3, a rabbit polyclonal antibody (1:100, bs-2379R, Bioss, Beijing, China) was used for LAMP2, a mouse monoclonal antibody (1:2000, MAB302, clone GS-6, Millipore, Billerica, United States) was used for GS, and a rabbit polyclonal antibody (1:100, bs-4562R, Bioss, Beijing, China) was used for CYP2E1. The sections were incubated for 2 h at room temperature.

After sections stained for LC3 were scanned and captured by NDP, the digital images were analyzed using ImageJ version 1.48 (National Institutes of Health, Bethesda, Maryland, United States). To facilitate comparisons between pig specimens, the IHC intensity of LC3 was evaluated for each lobule and then divided by the IHC intensity of nerve in the same portal area as the positive control.

Electron microscopy

Formalin-fixed pig liver specimens were analyzed using a Hitachi H-7650 (Hitachi, Tokyo, Japan) transmission electron microscope. Magnification at 80 kV achieved a clear depiction of the hepatocyte organelles.

Statistical analysis

Statistical analysis was performed with the Statistical Package for Social Sciences version 22 (SPSS Inc., Chicago, IL, United States). The Mann-Whitney *U* test was used to assess differences between embolized and nonembolized samples at each time point. For nonparametric multiple comparisons, the Kruskal-Wallis test was applied. Differences were considered significant at $P < 0.05$. Data are expressed as medians unless otherwise indicated.

Animal care and use statement

The animal experiment protocols were described in our previous report[2]. All protocols were approved by the Committee for Ethics in Animal Experimentation and were conducted in accordance with the Guidelines for Animal Experiments of our institution (ID: K03-004).

RESULTS

Changes in PV-CV distance, lobule area, and number of hepatocytes per lobule

The PV-CV distance in embolized and control specimens did not differ significantly at day 0 (0.571 mm *vs* 0.485 mm, respectively). However, at weeks 2, 4, and 6, the PV-CV distance was significantly reduced in embolized specimens (week 2: 0.364 mm, week 4: 0.335 mm, and week 6: 0.372 mm, $P < 0.001$, $P = 0.001$, $P = 0.001$, respectively) compared with control specimens. Moreover, at weeks 2, 4, and 6, the PV-CV distance was significantly reduced in embolized specimens compared with embolized specimens at day 0 ($P < 0.001$, $P = 0.001$, $P = 0.001$, respectively) (Figure 3A). The lobule cross sections of embolized specimens at weeks 2, 4, and 6 (week 2: 0.368 mm^2, week 4: 0.532 mm^2, and week 6: 0.462 mm^2) were significantly smaller than those of control specimens and were also smaller than embolized specimens at day 0 (1.096 mm^2) ($P < 0.001$ for all) (Figure 3B). The PV-CV distances and lobule areas in embolized specimens at weeks 2, 4, and 6 did not differ significantly.

The number of hepatocytes in lobules of median size did not differ significantly between embolized and control specimens until 4 wk after PTPE (week 4: 1459 and 2055, respectively, $P = 0.025$; week 6: 1494 and 2642, $P < 0.001$). At weeks 4 and 6, the number of hepatocytes per median-sized lobule in embolized specimens was significantly smaller than those in embolized and in control specimens at day 0 and week 2 ($P < 0.001$ for all) (Figure 3C). Therefore, the hepatocyte density in embolized specimens peaked at week 2 (5878/mm^2) (Figure 3D).

Evaluation of apoptotic activity

The fraction of TUNEL-positive hepatocytes was higher in embolized than in control specimens at day 0 and week 4 (11.1% *vs* 2.37% on day 0 and 5.51% *vs* 0.493% at week 4, $P = 0.018$, $P = 0.009$, respectively; Figure 3E).

Transition of LC3/LAMP2 IHC intensity in the lobule and GS/CYP2E1 zonation

The IHC intensity, as measured by Image J, for LC3

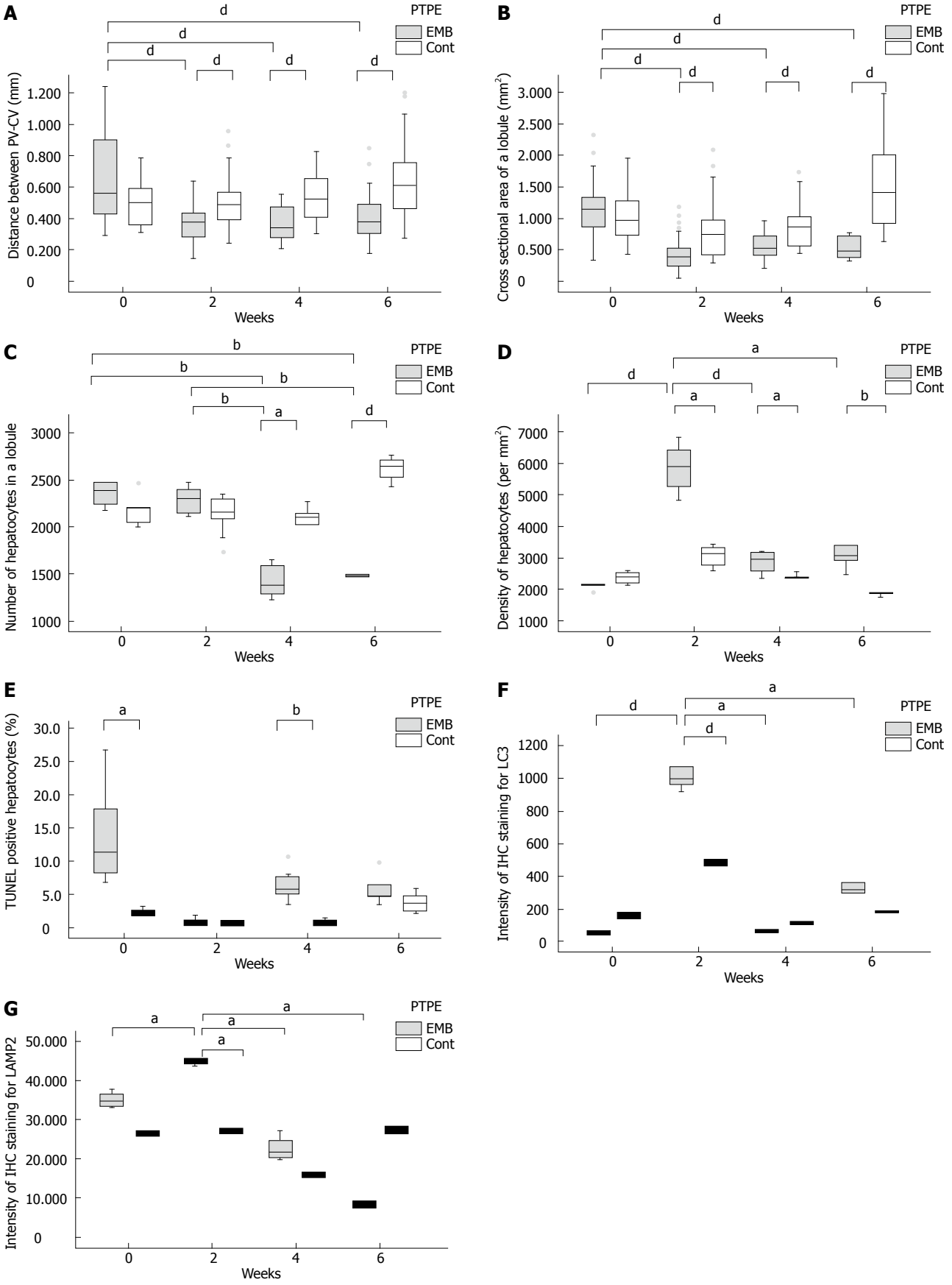

Figure 3 Histological changes in pig livers following the interruption of portal blood flow. A and B: The distance between the portal vein and central vein (A) and the cross sectional area of the lobule (B) of the embolized segment at week 2 after PTPE differed significantly from those of controls; C and D: The number of hepatocytes per lobule (C) of the embolized segment was significantly lower than in control lobes at week 4, and the density of hepatocytes (D) in the embolized area at week 2 was highest; E-G: The fraction of TUNEL-positive hepatocytes (E) in the embolized segment was significantly higher than in control segments at week 4, and the LC3 (F) and LAMP2 (G) intensity was highest in embolized lobes at week 2. $^{a}P \leqslant 0.05$, $^{b}P \leqslant 0.01$, $^{d}P \leqslant 0.001$. EMB: Embolized area; Cont: Control lobe area.

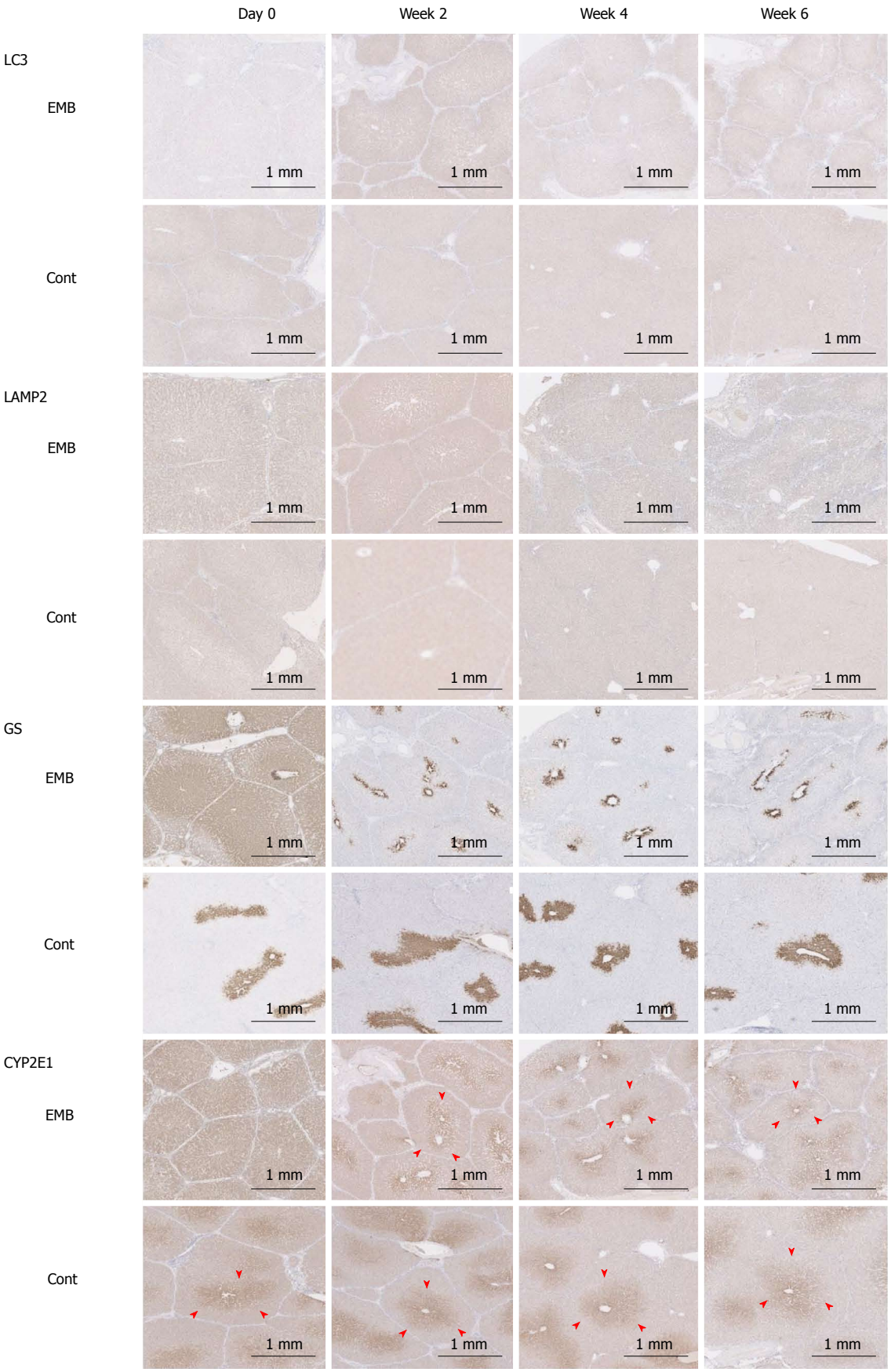

Figure 4 Light chain 3, lysosomal-associated membrane protein 2, glutamine synthetase, and cytochrome P450 2E1 immunohistochemical staining intensities. Expression of LC3 and LAMP2 was highest in the embolized segment of porcine specimens at 2 wk after PTPE. Zonation of GS and CYP2E1 (arrowheads) was expanded in the embolized area immediately after interruption of portal blood flow, but was reduced in the embolized lobe at 2 wk. EMB: Embolized area; Cont: Control lobe area; LC3: Light chain 3; LAMP2: Lysosomal-associated membrane protein 2; GS: Glutamine synthetase; CYP2E1: Cytochrome P450 2E1; IHC: Immunohistochemical.

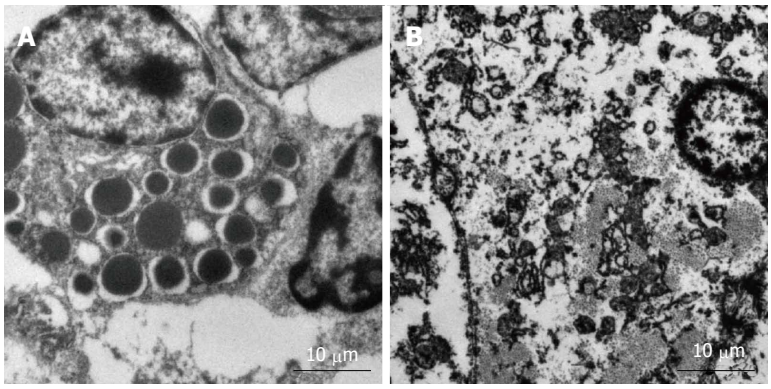

Figure 5 Electron microscopic findings at week 2. A and B: Many more autophagic vacuoles were found in embolized samples (A) compared with control samples (B) at week 2.

and LAMP2 in embolized specimens at week 2 (0.994 and 45.4, respectively) was significantly higher than that in control specimens (0.486, $P = 0.046$ and 27.0, $P = 0.014$, respectively). Moreover, the LC3 and LAMP2 intensities of embolized specimens at week 2 were significantly higher than those in all other specimens ($P \leqslant 0.025$ and $P \leqslant 0.014$ for all; Figure 3F, G and Figure 4). GS and CYP2E1 staining intensities in embolized specimens were not closely associated with those of control specimens at day 0. The extent of the stained zones decreased after 2 wk (Figure 4).

Electron microscopy

Clear findings were hard to establish because of the poor condition of pig liver specimens that had been fixed in formalin some time previously. There was the suggestion of a peak of autophagic vacuoles in embolized samples at week 2 (Figure 5), which was consistent with the IHC staining intensity of LC3.

PV-CV distance and hepatocyte density in human specimens

We sought to validate our findings in porcine samples by repeating the analytical procedures in human liver specimens from patients following PTPE. Because human lobule structures are not as well defined as those in porcine specimens, the PV-CV distance and hepatocyte density were assessed in a morphological study (Figure 2E and F). PV-CV was significantly shorter in embolized specimens than in nonembolized specimens (0.455 mm *vs* 0.563 mm, $P < 0.001$) (Figure 6A), as was also observed in porcine specimens 4 wk after PTPE. The hepatocyte density in embolized specimens was significantly higher than that in nonembolized specimens (2111/mm^2 *vs* 1772/mm^2, $P = 0.038$) (Figure 6B).

Evaluation of apoptotic activity, LC3 intensity, and GS and CYP2E1 zonation in human specimens

A significantly greater fraction of hepatocytes was TUNEL-positive in embolized specimens than in nonembolized specimens (2.804% *vs* 0.559%, $P < 0.001$) (Figure 6C). However, the LC3 intensity did not differ significantly between embolized and nonembolized specimens (Figure 6D, E and H). The extents of GS and CYP2E1 zonation were reduced in embolized specimens compared with nonembolized specimens (Figure 6F, G, I and J); similar results were observed in porcine specimens collected 4 wk after PTPE.

DISCUSSION

Interruption of the portal blood flow causes shrinkage of the embolized lobe and compensatory enlargement of the nonembolized lobes. The effects of portal venous obstruction on hepatocyte volume and apoptosis have been previously reported[13-16]. However, these studies used only human specimens in which the atrophy process was complete. As a result, the process of liver atrophy could not be studied in detail. Our morphological study focused on changes in the lobules over time in porcine samples. We observed two distinct phases of liver atrophy following portal blood flow disruption. The first phase was characterized by lobule shrinkage without a fall in the number of hepatocytes and was accompanied by strong expressions of LC3 and LAMP2 in the first 2 wk after portal venous obstruction. The second phase, which occurred between 2 and 4 wk after portal venous obstruction, was characterized by a reduction in the number of hepatocytes without changes in lobular size. This reduction was accompanied by decreased LC3 and LAMP2 intensity and an increased fraction of TUNEL-positive cells (Figure 7).

Soon after the injection of ethanol, the zonation of GS and CYP2E1 in embolized specimens expanded markedly. Increased GS zonation could represent accelerated ammonia metabolism resulting from the degradation of denatured proteins[17]. Moreover, it has been reported that CYP2E1 is directly associated with ethanol metabolism[18]. Furthermore, in this study, the fraction of TUNEL-positive hepatocytes was observed to increase in embolized specimens at day 0; this finding may reflect damage caused by ethanol.

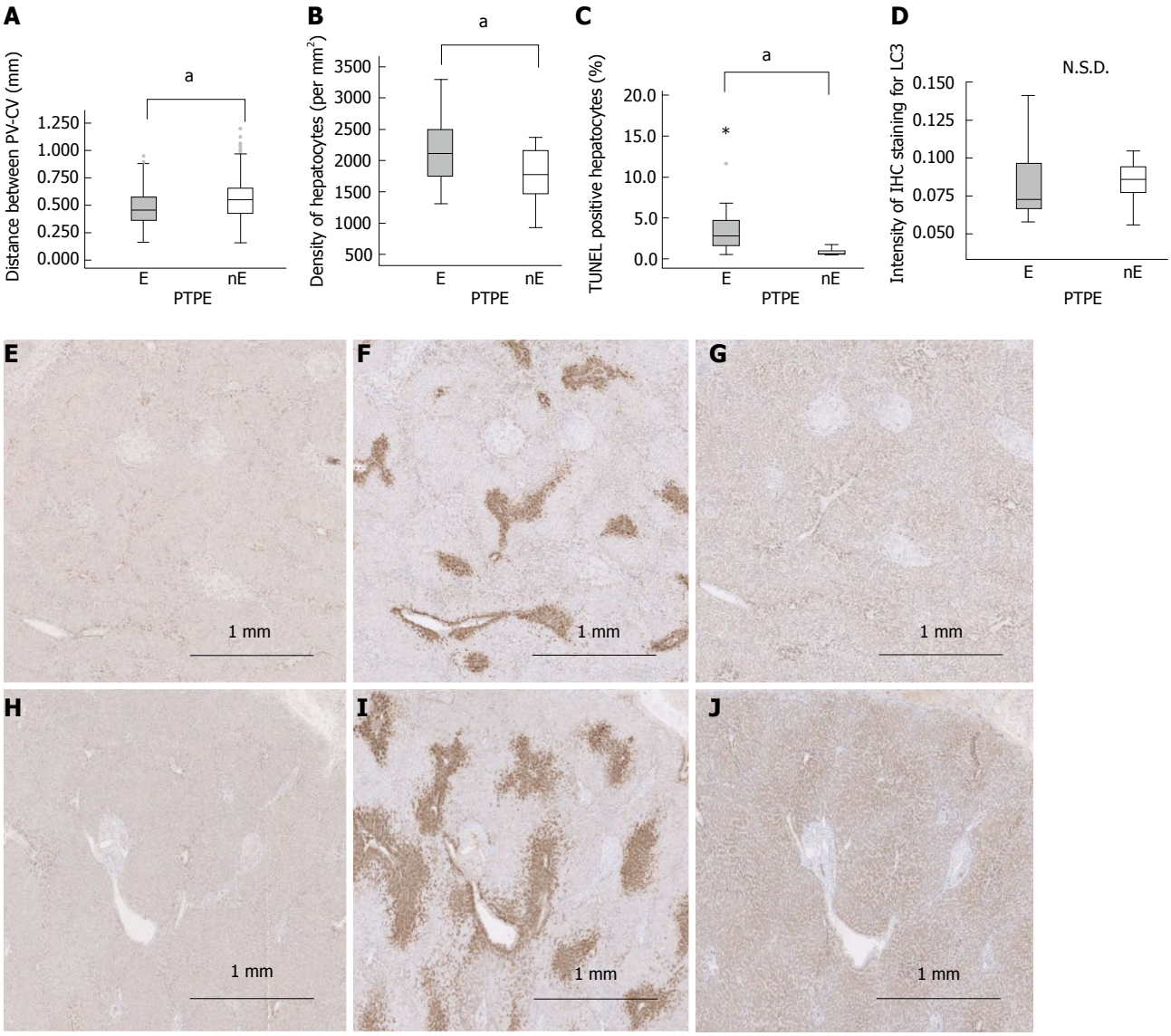

Figure 6 Human specimens. A: The distance between the portal vein and central vein; B: Hepatocyte density; C: Fraction of TUNEL-positive hepatocytes differed significantly between embolized and nonembolized lobes; D: Intensity of IHC staining for LC3; E-J: IHC for LC3 (E, H), GS (F, I), and CYP2E1 (G, J). LC3 expression did not differ significantly (D) between the embolized (E) and the nonembolized area (H), but the zonation of GS and CYP2E1 was narrower in the embolized lobe (F, G) than the nonembolized lobe (I, J). $^{a}P \leq 0.001$. N.S.D: No significant difference; E: Embolized area; nE: Nonembolized area.

Hepatocytes in the embolized lobule may degenerate soon after ethanol injection. These changes are consistent with the clinical observation that circulating levels of transaminases are transiently elevated after ethanol injection to patients undergoing portal vein embolization[19]. In addition, we found that the proportion of TUNEL-positive hepatocytes decreased in the first 2 wk and did not differ between embolized and control specimens at week 2. However, hepatocyte numbers were reported to be restored in 3-4 d after partial hepatectomy[20], and hepatocyte replication in the embolized lobe was reported to be slightly increased approximately 7 d after PTPE with coils and particles[3]. Perhaps the cellular damage observed at day 0 in our study was repaired *via* regeneration within the first few days.

The PV-CV distance and lobule size were reduced without the loss of hepatocytes in embolized specimens at week 2. This first phase could be considered a hepatocellular atrophic phase. Interruption of the portal blood flow (which is rich in nutrients from the gastrointestinal tract) may starve hepatocytes after embolization. Starvation reportedly causes autophagy and hepatocyte atrophy[11]. Interestingly, in our study, LC3 and LAMP2 expression was significantly increased in embolized specimens at week 2. Simultaneously, GS and CYP2E1 zonation were reduced at week 2 as starvation caused a reduction in metabolism. Moreover, we found an increase in the number of autophagic vacuoles in embolized specimens at week 2. Thus, we speculated that disruption of the portal blood flow caused hepatocyte shrinkage by activating autophagy.

Between weeks 2 and 4, the number of hepatocytes in embolized specimens decreased without

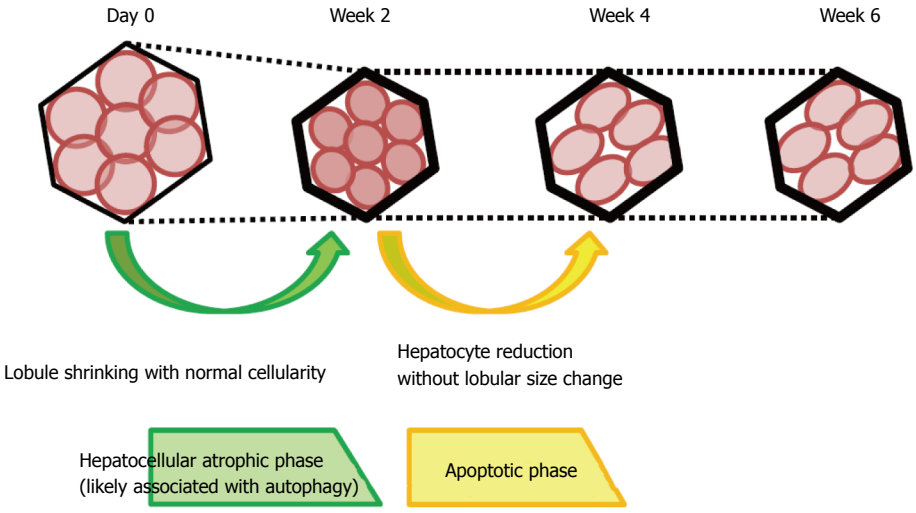

Figure 7 Schema of the histological changes occurring following interruption of portal blood flow. At 2 wk after obstruction of the portal vein, lobular shrinkage was observed without reduction in hepatocyte number, but with strong LC3 and LAMP2 expression. These changes may be associated with autophagy, and this process is termed the hepatocellular atrophic phase. At week 4, hepatocyte numbers fell, without a reduction in lobule size, but with an elevation of TUNEL staining. These secondary changes may be attributed to apoptosis occurring after autophagy, characterizing the hepatocellular atrophic phase. No significant histological changes were observed at week 6 compared with week 4.

significant changes in the lobule size. During the same period, LC3 and LAMP2 expressions fell and a larger proportion of hepatocytes became TUNEL-positive. Consequently, this phase may be regarded as encompassing the deactivation of autophagy and the activation of apoptosis. Recently, autophagy was reported to induce cell death[21]. Therefore, the TUNEL-positive cell death we observed might represent caspase-independent apoptosis, rather than caspase-dependent apoptosis[22]. Because hepatocyte numbers decreased while TUNEL-positive staining increased after the activation of autophagy, we characterized the phase occurring 2-4 wk after PTPE as the "apoptotic phase". During this phase, the zonation of GS and CYP2E1 did not differ significantly from that observed in embolized specimens at week 2. Between weeks 4 and 6, no morphological or IHC changes were observed at the lobular level and no significant difference was observed in the proportion of TUNEL-positive cells between embolized and control specimens at week 6. Therefore, the liver atrophy process likely terminates between weeks 4 and 6. Our results corroborate that FLR hypertrophy usually takes 4 wk to complete after PTPE because the liver atrophy process is not complete until week 4, although it appears at the macro level to have resolved by week 2.

Hepatectomy is usually performed around 4 wk after PTPE. Consequently, we sought to validate our porcine model observations in human specimens. The observations we made concerning the PV-CV distance, hepatocyte density, TUNEL staining, LC3 and LAMP2 expression, and GS and CYP2E1 zonation in embolized and nonembolized specimens at 4 wk after PTPE in porcine samples also applied to human specimens taken between 20 and 36 d after PTPE. Moreover, the TUNEL results supported those already reported for clinical samples[14-17]. Furthermore, the pigs we used underwent the same PTPE protocol that humans undergo clinically. Because the histological observations made using this porcine model did not contradict the human results (Table 2), the mechanism by which the interruption of portal blood flow causes liver atrophy may be similar in pigs and in humans.

The limitations of this preliminary study were that the number of pigs was insufficient for a detailed histopathological study to provide unequivocal evidence of the relationship between hepatocellular atrophy and autophagy. Further we attempted Western blotting for LC3-II, but it was not successful. However, we believe that our results and speculations provide a basis for understanding the mechanism of liver atrophy after interruption of the portal blood flow and will facilitate further study. Future research will hopefully provide a sound theoretical basis for planning treatment strategies for acute portal obstruction-related liver dysfunction or disease and chronic ischemic-related liver diseases with liver atrophy.

In conclusion, to investigate the mechanism by which portal vein obstruction causes liver atrophy, we investigated the histological changes in pig livers following PTPE and observed two distinct phases. The first phase, termed the hepatocellular atrophic phase, is characterized by lobular shrinkage without hepatocyte loss and with high levels of LC3 and LAMP2 expression. This phase lasted for the first 2 wk following PTPE. The second phase, which occurs between weeks 2 and 4, is termed the apoptotic phase and is characterized by a reduction in hepatocyte numbers without a reduction in lobular size. This is accompanied by reduced LC3 and LAMP2 expression and increased TUNEL staining. Human liver specimens resected after PTPE had many similar characteristics

Table 2 Comparison of the results for pigs and humans

	Pig liver specimens at week 4	Human liver specimens resected around week 4
PV-CV distance	EMB < Cont	EMB < nonEMB
Hepatocyte density	EMB > Cont	EMB > nonEMB
TUNEL-positive cells	EMB > Cont	EMB > nonEMB
LC3 Intensity	N.S.D.	N.S.D.
GS zonation	EMB narrower than Cont	EMB narrower than nonEMB
CYP2E1 zonation	EMB narrower than Cont	EMB narrower than nonEMB

PV-CV: Portal vein to central vein; EMB: Embolized lobe; Cont: Control lobe; nonEMB: Nonembolized lobe; N.S.D: No significant difference.

to specimens collected from pigs at week 4. Therefore, our findings suggest that the mechanism by which the interruption of portal blood flow causes liver atrophy may be similar in pigs and in humans.

ARTICLE HIGHLIGHTS

Research background

The interruption of portal blood flow by portal vein embolization or tumor thrombosis, for example, causes liver atrophy. However, the mechanisms responsible for this effect have not been fully elucidated.

Research motivation

The previous study suggested that the mechanisms responsible for liver atrophy likely commence soon after the disruption of portal blood flow. Consequently, histopathological changes would likely also be observed soon after percutaneous transhepatic portal embolization (PTPE). Recently, the relationship between apoptosis and autophagy has been extensively reported. Autophagy in the liver is reportedly caused by starvation and is related to hepatocellular atrophy, and, moreover, interruption of the portal blood flow, which contains a wealth of nutrients, is considered a form of starvation. Therefore, autophagy may be related to both cellular shrinking and apoptosis. However, the relationship between portal venous obstruction and autophagy has not been reported. To clarify the mechanisms responsible for liver atrophy, histopathological analysis should be carried out repeatedly within the first few weeks after PTPE. However, to the best of our knowledge, such time-course studies have not yet been carried out. The results and hypotheses will provide a basis for understanding the mechanism of liver atrophy after interruption of the portal blood flow and will facilitate further study.

Research objectives

The aim of this study was to investigate, using specimens from a previously reported porcine PTPE model, the microscopic changes associated with apoptosis and autophagy in the days and weeks following portal venous obstruction and to clarify the mechanism by which interrupted portal blood flow causes liver atrophy. Furthermore, to understand the mechanism of liver atrophy in humans after PTPE, the authors sought to verify the integrity of the pig results by performing the same histopathological investigations in specimens resected from human patients who had undergone PTPE.

Research methods

The authors performed histopathological examinations of liver specimens from five pigs that had undergone PTPE in a time-dependent model of liver atrophy. In specimens from embolized lobes (EMB) and nonembolized lobes (controls), the authors measured the portal vein to central vein distance (PV-CV), the area and number of hepatocytes per lobule, and apoptotic activity using the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay. Immunohistochemical reactivities were evaluated for light chain 3 (LC3) and lysosomal-associated membrane protein 2 (LAMP2) as autophagy markers and for glutamine synthetase and cytochrome P450 2E1 (CYP2E1) as metabolic zonation markers. Samples from ten human livers taken 20-36 d after PTPE were similarly examined.

Research results

PV-CVs and lobule areas did not differ between EMB and controls at day 0, but were lower in EMB than in controls at weeks 2, 4, and 6. Hepatocyte numbers were not significantly reduced in EMB at day 0 and week 2 but were reduced at weeks 4 and 6. Apoptotic activity was higher in EMB than in controls at day 0 and week 4. LC3 and LAMP2 staining peaked in EMB at week 2, with no significant difference between EMB and controls at weeks 4 and 6. Glutamine synthetase and CYP2E1 zonation in EMB at weeks 2, 4, and 6 were narrower than those in controls. Human results were consistent with those of porcine specimens. However the number of pigs was insufficient for a detailed histopathological study to provide unequivocal evidence of the relationship between hepatocellular atrophy and autophagy.

Research conclusions

To investigate the mechanism by which portal vein obstruction causes liver atrophy, the authors examined the histological changes in pig livers following PTPE and observed two distinct phases. The first phase, termed the hepatocellular atrophic phase, is characterized by lobular shrinkage without hepatocyte loss and with high levels of LC3 and LAMP2 expression. This phase lasted for the first 2 wk following PTPE. The second phase, which occurs between weeks 2 and 4, is termed the apoptotic phase and is characterized by a reduction in hepatocyte numbers without a reduction in lobular size. This is accompanied by reduced LC3 and LAMP2 expression and increased TUNEL staining. Human liver specimens resected after PTPE had many similar characteristics to specimens collected from pigs at week 4. Despite liver atrophy appearing to be mostly resolved 2 wk after embolization, the period after PTPE could beneficially be extended to 4 wk to ensure contralateral hypertrophy and to allow the completion of liver atrophy.

Research perspectives

Histopathological analysis is the best way to clarify the mechanisms responsible for liver atrophy. To assess microscopic changes in liver tissues, it is important to study liver lobules, the smallest functional units of the liver. The observation of clear histological changes would be expected. To clarify the more detailed mechanism of liver atrophy after interruption of the portal blood flow, the authors have to study the histopathological changes using not only the pig model but also small animal models, *e.g.,* mouse models, because such animals are easy to handle. After such detailed studies, future research will hopefully provide a basis for understanding the mechanism of liver atrophy after interruption of the portal blood flow and also give a sound theoretical basis for planning treatment strategies for acute portal obstruction-related liver dysfunction or disease and chronic ischemic-related liver diseases with liver atrophy.

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AIM AND SCOPE

World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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REVIEW

Global elimination of hepatitis C virus infection: Progresses and the remaining challenges

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Abstract

Today, with the introduction of interferon-free direct-acting antivirals and outstanding progresses in the prevention, diagnosis and treatment of hepatitis C virus (HCV) infection, the elimination of HCV infection seems more achievable. A further challenge is continued transmission of HCV infection in high-risk population specially injecting drug users (IDUs) as the major reservoir of HCV infection. Considering the fact that most of these infections remain undiagnosed, unidentified HCV-infected IDUs are potential sources for the rapid spread of HCV in the community. The continuous increase in the number of IDUs along with the rising prevalence of HCV infection among young IDUs is harbinger of a forthcoming public health dilemma, presenting a serious challenge to control transmission of HCV infection. Even the changes in HCV genotype distribution attributed to injecting drug use confirm this issue. These circumstances create a strong demand for timely diagnosis and proper treatment of HCV-infected patients through risk-based screening to mitigate the risk of HCV transmission in the IDUs community and, consequently, in the society. Meanwhile, raising general awareness of HCV infection, diagnosis and treatment through public education should be the core activity of any harm reduction intervention, as the root cause of failure in control of HCV infection has been lack of awareness among young drug takers. In addition, effective prevention, comprehensive screening programs with a specific focus on high-risk population, accessibility to the new anti-HCV treatment regimens and public education should be considered as the top priorities of any health policy decision to eliminate HCV infection.

Key words: Hepatitis C virus; Epidemiology; Elimination; Injecting drug user; Prevention; Vaccine; Diagnosis; Treatment

Core tip: Despite the outstanding progresses in the management of hepatitis C virus (HCV) infection, the elimination of HCV would be difficult due to the emergence of injection drug use as the main source of HCV transmission. Asymptomatic nature of HCV infection,

restricted accessibility to diagnostic approaches and appropriate antiviral treatments in the injecting drug users (IDUs) community are the root cause of failure in control of HCV infection among IDUs. These circumstances create a strong demand for timely diagnosis and proper treatment of HCV-infected patients as well as raising general awareness of HCV infection through public education to mitigate the risk of HCV transmission.

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INTRODUCTION

With a global prevalence rate of 2.8%, equating to over 185 million infections, and more than 350000 deaths annually, hepatitis C virus (HCV) infection is undoubtedly considered a major public health problem[1]. Globally, an estimated 3 million to 4 million new cases of HCV infection emerge every year[1]. Furthermore, the HCV-related mortality is increasing and HCV infection is projected to be the most important leading cause of viral hepatitis-related mortality in the near future[1,2]. Apparently, the management of HCV infection faces several challenges. These challenges merit further attention if elimination of HCV infection is aimed to be achieved.

HCV

HCV is a member of the family *Flaviviridae* and the genus *Hepacivirus*. The HCV genome is a positive-stranded RNA, which encodes a core protein (C), two envelope glycoproteins (E1 and E2), and several non-structural proteins (NS1, NS2, NS3, NS4A, NS4B, NS5A and NS5B)[3,4]. This enveloped positive-stranded RNA virus is usually acquired through exposure to infected blood. This might happen through transfusion of blood and blood products, surgery, organ transplantation, intravenous drug use, tattooing, hemodialysis, unsafe injection practices, mother to fetus, and sexual intercourse[5-8]. However, sexual transmission of HCV is less common and most often observed among men who have sex with men and HIV-infected patients[9,10].

HCV is the causative agents of hepatitis C infection. This infection is characterized by an acute or chronic course in the host. The complications are preliminary asymptomatic, mild or severe, which spontaneously clear or slowly progress to chronic liver disease, cirrhosis and finally hepatocellular carcinoma (HCC) within about 20 years[11,12]. The clinical symptoms of acute HCV infection might include fever, fatigue, malaise, and gastrointestinal symptoms such as anorexia, nausea, vomiting, right upper quadrant pain, dark urine, grey-colored stool, and yellow skin and sclera of the eyes, the well-characterized symptoms of jaundice. These symptoms might appear from 3 to 12 wk after being infected. The clinical symptoms of chronic HCV infection might take decades to develop, and they are usually indicative of an advanced liver disease[13-15].

The long-term chronic HCV infection is capable of causing some extra hepatic manifestations with serious consequences, such as glomerulonephritis, diabetes mellitus, thyroid disorders, porphyria cutaneous tarda, mixed cryoglobulinemia, lichen planus, and B cell lymphoproliferative disorders[16-21]. These extrahepatic complications might outshine the hepatic manifestations of HCV infection, and the presence of HCV infection might be overlooked, paving the way for the silent development of advanced liver disease. Therefore, the possible role of HCV in the development of extrahepatic manifestations merits further attention.

Due to genomic heterogeneity, there are 7 major genotypes and over 67 subtypes of HCV[1,22,23]. HCV genotype distribution varies by the route of transmission and geographical location[24,25]. In addition, pathogenicity, response to antiviral therapy and the duration of treatment can be influenced by different HCV genotypes[5,24,26]. The genotypes 1, 2 and 3 show a widespread distribution in almost all parts of the world. HCV genotype 4 has been traditionally restricted to a few countries in the Middle East and Africa and is more prevalent in Saudi Arabia, Bahrain, Jordan, Egypt and Ethiopia[1,27,28]. HCV genotype 5, 6 and 7 have been reported in South Africa, South East Asia and Central Africa, respectively[11,29,30] (Figure 1).

Genotype 1 is more prevalent among patients with history of blood and blood products transfusion, surgery, and dental procedure[24,25,27]. Infection with HCV genotype 2 is mainly associated with nosocomial transmission and prior dental treatment[1,22]. Genotype 3 is frequently found in the intravenous drug user communities and in those with history of tattooing and piercing[24,31,32]. Genotype 4 is mainly transmitted through high-risk sexual practices, especially among homosexual males, and intravenous drug use[1,22].

Infection with HCV genotype 3 is associated with a more rapid progression of fibrosis, a higher degree of steatosis, and a higher incidence of cirrhosis and hepatocellular carcinoma[1,22,31,33]. Spontaneous clearance is more often observed in infection with HCV genotype 1, while if patients remain HCV RNA positive, the disease progresses in a more aggressive manner than the other genotypes[11]. Genotypes 1 and 4 are associated with lower response rates and higher treatment duration in response to interferon (IFN) and ribavirin (RBV) combination therapy as compared to genotypes 2 and 3[6,24,34].

PROGRESSES IN THE MANAGEMENT OF HCV INFECTION

In addition to IFN-based therapies, the direct-acting antivirals (DAAs) have been developed, which specifically

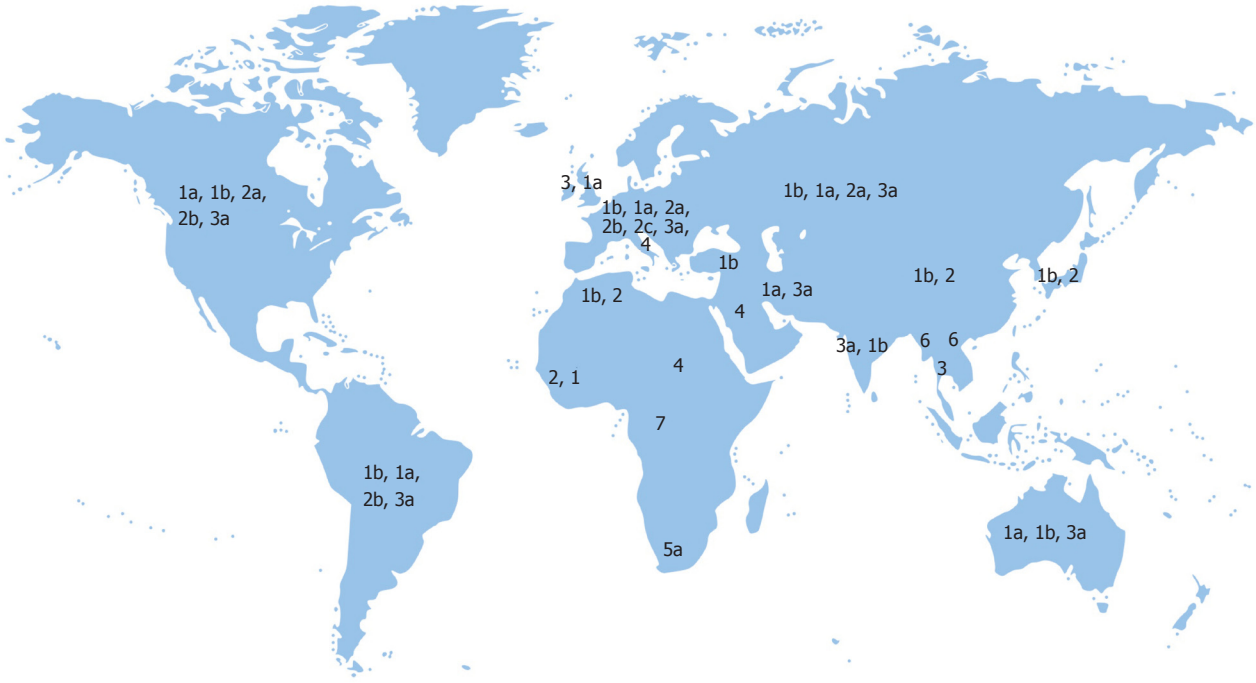

Figure 1 Geographical distribution of hepatitis C virus genotypes. Hepatitis C virus (HCV) genotypes 1, 2 and 3 show a widespread distribution in almost all parts of the world. HCV genotype 4 has been restricted to a few countries in the Middle East and Africa. HCV genotype 5, 6 and 7 have been reported in South Africa, South East Asia and Central Africa, respectively[1,11,30,35,36].

inhibit the function of viral proteins that are essential for viral replication[4,37,38]. These DAAs include NS3/4A protease inhibitors, NS5A replication complex inhibitors, nucleoside NS5B polymerase inhibitors, and non-nucleoside NS5B polymerase inhibitors (Table 1)[39-43]. These novel antiviral drugs, despite having considerable advantages over conventional IFN-based therapy, suffer from the resistance-associated mutations, which occur naturally during the replication of the virus and select under the pressure of DAAs. The emergence of HCV resistance-associated variants (RAVs) decreases the susceptibility to DAAs and finally results in treatment failure[38,44-46]. Assessment of resistance substitutions at pretreatment baseline in patients candidate for DAA therapy seems to be the best option to optimize first-line therapeutic strategies, to avoid the fitness of resistant variants as the predominant viral population and to prevent DAA failure due to baseline resistant variants. In addition, failing DAA-based therapy should be discontinued as soon as possible to avoid an increase in the frequency of RAVs, to preserve HCV re-treatment options. Finally, development of next-generation DAAs with higher resistance barrier is strongly recommended[45,47].

Telaprevir and boceprevir are not recommended by WHO due to the frequent adverse effects and low cure rates[79].

Prior to the treatment, the infected individuals need to be identified. HCV infection is described by the presence of anti-HCV antibodies and HCV-RNA in plasma or serum with either elevated or normal levels of liver enzymes[29]. Anti-HCV antibodies are detected by using serological screening tests, including enzyme linked immunosorbent assay and recombinant immunoblot assay. Detection of anti-HCV antibodies indicates current or past HCV infection. An additional test called HCV RNA test or reverse transcriptase polymerase chain reaction assay (RT-PCR) is needed to determine if a person is currently infected with HCV[17,80-82].

However, those infected individuals with undetectable levels of HCV-RNA in serum or plasma might remain undiagnosed. In this condition, HCV-RNA can be detected in peripheral blood mononuclear cells (PBMCs) specimens, liver biopsies, and ultracentrifugated serum samples[81,83]. Serological screening tests might be negative or positive in these patients. This kind of infection is defined as occult HCV infection, which is a serious threat to blood safety[84,85]. Since, despite having undetectable level of HCV RNA, blood and blood products are potentially infectious[84,86]. In fact, the presence of blood donors with occult HCV infection can increase the risk of HCV transmission through blood transfusion and therefore is a potential source of HCV transmission in the society[87].

Despite having appropriate antiviral treatments and diagnostic approaches, diagnosis rate and access to treatment is considerably low especially in resource-limited settings. Perhaps the most promising strategy to control HCV infection is the development of a prophylactic vaccine[88,89]. Several vaccine candidates against HCV have been developed so far, including recombinant protein vaccine, peptide-based vaccine,

Table 1 Profile of direct-acting antiviral agents[4,37,40,42,44,48-78]

Direct-acting antiviral agent	Generic name (abbreviation)	Code name	Trade name	Active against HCV genotype (based on clinical trial outcomes)	Combination therapy
NS3/4A protease inhibitors (-previr)	Telaprevir (TVR)	VX-950	Incivek/ Incivo	1	TVR + IFN ± RBV
	Boceprevir (BOC)	SCH-503034 EBP-520	Victrelis	1	BOC + IFN ± RBV
	Faldaprevir (FDV)	BI-201335	-	1	FDV + Peg-IFN + RBV
	Simeprevir (SIM)	TMC-435	Olysio	1 and 4	SIM + SOF ± RBV
	Vaniprevir (VNV)	MK-7009	Vanihep	1	VNV + IFN ± RBV
	Asunaprevir (ASV)	BMS-650032	Sunvepra	1 and 4	ASV + DCV
	Paritaprevir (PTV)	ABT-450	Veruprevir	1 and 4	PTV+R+OBV+DAV ± RBV
	Voxilaprevir (VOX)	GS-9857	-	Pan-genotypic antiviral activity	VOX + SOF + VPR
	Sovaprevir	ACH-1625	-	1	Sovaprevir + ODV + RBV
	Grazoprevir (GZP)	MK-5172	-	1a, 1b, 4 and 6	Zepatier (GZP + EBV)
	Danoprevir (DNV)	RG-7227 ITMN-191 ASC08	-	1 and 4	DNV + PEG-IFN + RBV DNV + R + PEG-IFN + RBV
	Deldeprevir (DDV)	ACH-2684	-	1	DDV + ODV
	Neceprevir	ACH-0142684			
	Narlaprevir (NVR)	SCH-900518	Arlansa	1	NVR + R + PEG-IFN ± RBV
	Vedroprevir (VDV)	GS-9451	-	1	VDV + LDV + SOF VDV + LDV + TGV + RBV
	Glecaprevir (GLE)	ABT-493	-	Pan-genotypic antiviral activity	GLE + PIB ± RBV
	-	GS-9256	-	1	GS-9256 + PEG-IFN + RBV GS-9256 + TGV + Peg-IFN ± RBV
NS5A replication complex inhibitors (-Asvir)	Daclatasvir (DCV)	BMS-790052	Daklinza	1, 2 and 3	Sovodak (DCV + SOF) ± RBV DCV + VX-135
	Ledipasvir (LDV)	GS-5885	-	1, 3, 4, 5 and 6	Harvoni (LDV + SOF) ± RBV LDV + SOF ± (VDV or Radalbuvir)
	Ombitasvir (OBV)	ABT-267	-	1 and 4	Viekira Pak (OBV + PTV + R + DSV) ± RBV Technivie (OBV + PTV + R)
	Elbasvir (EBV)	MK-8742	-	1a, 1b, 4 and 6	Zepatier (EBV + GZP) ± RBV
	Velpatasvir (VPR)	GS-5816	-	Pan-genotypic antiviral activity	Epclusa (VPR + SOF) ± RBV
	Odalasvir (ODV)	ACH-3102	-	1	ODV + Sovaprevir + RBV
	Ravidasvir (RVD)	PPI-668 ASC16	-	4	RVD + SOF ± RBV
	-	PPI-461	-	1	-
	-	JNJ-56914845 GSK2336805	-	1	GSK2336805 + PEG-IFN + RBV GSK2336805 + VX-135 + SIM
	Samatasvir	IDX-18719 IDX-719 MK-1894	-	1, 2, 3 and 4	Samatasvir + SIM + RBV
	-	BMS-824393	-	1	BMS-824393 + PEG-IFN + RBV
	Pibrentasvir (PIB)	ABT-530	-	Pan-genotypic antiviral activity	PIB + GLE ± RBV
	Ruzasvir (RZR)	MK-8408	-	Pan-genotypic antiviral activity	RZR + UPR + GZP
Nucleoside NS5B polymerase inhibitors (-Buvir)	Sofosbuvir (SOF)	PSI-7977; GS-7977	Sovaldi; Soforal	Pan-genotypic antiviral activity	SOF + IFN ± RBV Sovodak (DCV + SOF) ± RBV
	Mericitabine (MCB)	RG-7128 RO5024048	-	1 and 4	MCB + PEG-IFN + RBV MCB + DNV MCB + R + DNV ± RBV
	-	VX-135 ALS-2200	-	1	VX-135 + GSK2336805 + SIM VX-135 + TVR + RBV VX-135 + DCV VX-135 + RBV VX-135 + SIM
	Valopicitabine	NM283	-	1	Valopicitabine + Peg-IFN
Non-nucleoside NS5B polymerase inhibitors (-Buvir)	Beclabuvir (BCV)	BMS-791325	-	1	BCV+ ASV+ DCV

	Dasabuvir (DAV)	ABT-333	Exviera	1	DAV + OBV+ PTV + R ± RBV
	Lomibuvir	VX-222 VCH-222	-	1	VX-222 + TVR + RBV VX-222 + Filibuvir
	Filibuvir	PF-00868554, PF-868554	-	1	Filibuvir + Peg-IFN + RBV Filibuvir + VX-222
	Setrobuvir (STV)	ANA-598 RO-5466731 RG-7790	-	1	STV + IFN + RBV STV + R + DNV + RBV ± MCB
	Nesbuvir (NBV)	HCV-796 VB-19796	-	1	NBV +Peg-IFN + RBV
	Tegobuvir (TGV)	GS-9190	-	1	TGV + GS-9256 +Peg-IFN ± RBV TGV + LDV + VDV + RBV
	Deleobuvir (DBV)	BI-207127	-	1	DBV + PEG-IFN + RBV DBV + FDV DBV + FDV + RBV
	Uprifosbuvir (UPR)	MK-3682	-	Pan-genotypic antiviral activity	UPR + RZR UPR + RZR + GZP
	Radalbuvir	GS-9669	-	1	Radalbuvir + LDV + SOF
	AL-335	ALS-335	-	1	AL-335 + ODV + SIM

IFN: Interferon; RBV: Ribavirin; R: Ritonavir; PEG-IFN: Pegylated interferon.

virus-like particles, bacterial-vectored vaccine, viral-vectored vaccine, and DNA vaccine (Table 2)[29,88,90-96]. The currently developed vaccines against HCV, despite inducing strong humoral and cellular immune responses in preclinical animal models or clinical trials in humans, have not been approved for use in human beings[89,90,97]. The reason is high genomic diversity of HCV and viral escape from immune responses[88,90,93,98,99]. Targeting the conserved regions within HCV proteins might help to overcome this genetic variability[100].

In the absence of an approved prophylactic vaccine for hepatitis C, reducing exposure to HCV through prevention seems to be the best option. This can be achieved through routine screening of donated blood for HCV markers, providing safe medical procedures, promoting risk-reduction counseling and services for at risk population, increasing public awareness and offering regular HCV testing to high-risk populations with the goal of breaking the cycle of HCV transmission in the society[7,9,82,133]. Despite the so-called improvements in the management of HCV infection, still a long way is ahead to achieve a world free of HCV infection. Here, the remaining challenges to eliminating HCV infection will be discussed.

REMAINING CHALLENGES TO ELIMINATING HCV INFECTION

For many years, IFN-based therapy, despite having frequent side effects, poor tolerability, suboptimal efficacy and prolonged treatment course, was recommended as the standard treatment for HCV infection[134,135]. Introduction of IFN-free DAAs has solved most of these problems in the treatment course of HCV infection. Switch the HCV treatment regimens from IFN-based therapy to DAA therapy is a desirable approach, yet encounter practical barriers such as high price and the restricted accessibility of DAAs[135-138]. Most of the time, the cost of antivirals rather than their effectiveness is the main driver in the treatment decisions. The use of these DAAs is far beyond the financial means of the most-in-need patients especially those who are IFN-intolerant or non-responder. While, equity in health demands that all patients with every socioeconomic status have equitable access to these treatment regimens. Currently, reducing treatment costs and providing DAAs with a relatively high health insurance coverage seem to be best options to improve access to DAA therapy[139].

Accessibility to DAAs, though, by itself is a superb health achievement, still alone might not be sufficient to mitigate the burden of HCV infection. A further challenge is continued transmission of HCV infection in high-risk population specially injecting drug users (IDUs) as the major reservoir of HCV infection[133,137,139]. Considering the fact that most of these infections remain undiagnosed, unidentified HCV-infected IDUs are potential sources for the spread of HCV infection in the society[133,139-141]. While, silent introduction of HCV infection into the community is a serious threat to the national effort to eliminate HCV infection, a threat that will increase with time. Therefore, timely diagnosis of HCV-infected patients through risk-based screening is of the greatest importance[126,133,137]. Screening of blood donations for hepatitis C initiated in the early 1990s has remarkably reduced the risk of HCV transmission through blood transfusion since then. Blood transfusion before the early 1990s was a major contributor to the HCV transmission, but today this risk has become minute[142]. However, it is far, far more difficult to screen IDUs, those who most need risk assessment. Despite the remarkable advantages, the cultural objections hinder screening progress, resulting in low diagnosis rate and, consequently, persistent silent spread of infection. On the other hand, the stigma of injecting drug use makes recognition of all HCV-infected IDUs impossible or logistically difficult at best[133]. In addition, establishment of HCV screening system with a specific

Table 2 Vaccine candidates against hepatitis C virus in preclinical and clinical trials

Type of vaccine	Vaccine structure/ adjuvant	Stage of development	Outcome	Application	Developer	Year	Current status	Ref.
Recombinant protein vaccine	Recombinant E1 or E2/MF59	7 chimpanzees	Induce strong humoral immune response; complete protection in 5 chimpanzees	Prophylactic vaccine	Chiron/ Novartis	1994	Completed	[101]
	Recombinant E1 or E2/Alum	4 Chimpanzees	Induce antigen-specific T-helper cytokines in either E1 or	Therapeutic vaccine	BPRC	2011	Published	[102]
	Recombinant E1/Alum	Phase Ⅰ 20 healthy volunteers	E2-vaccinated animals; clear HCV infection in only E1-vaccinated animals (neutralizing antibodies) Induce strong cellular and humoral anti-E1 responses	Therapeutic vaccine	Fujirebio Europe	2004	Published	[103]
	Recombinant E1 and E2/MF59	Phase Ⅰ 60 healthy volunteers	Induce humoral and cellular immune responses	Prophylactic vaccine	Novartis	2010	Completed	[104]
	Recombinant E1/Alum	Phase Ⅰ/Ⅱ 20 healthy volunteers and 35 patients with chronic HCV infection/122 HCV-infected patients	Induce HCV specific humoral and cellular immune responses (Th1 type); no change in HCV viral load	Therapeutic vaccine	Innogenetics/ GenImmune	2003/2008	Published	[103,105,106]
	HCV core protein/ ISCOMATRIX	Phase Ⅰ/Ⅱa 30 healthy volunteers	Induce strong humoral immune responses in all except one patients; induce CD8+ T cell responses in 2 of 8 patients receiving the highest dose	Prophylactic vaccine	CSL Ltd	2009	Published	[107]
	GI5005: Inactivated recombinant Saccharomyces cerevisiae expressing NS3-core fusion protein/ GI-5005 plus SOC	Phase Ⅰ/Ⅱ 66 patients with chronic HCV infection/	Improve SVR	Therapeutic vaccine	GlobeImmune	2009/2010	Completed	[108,109]
Peptide-based vaccine	Peptide from core protein (C35-C44)/ ISA51	Phase Ⅰ 26 patients with chronic HCV infection	Induce peptide-specific cellular and humoral immune responses in 15 of 25 patients; decline HCV viral load in 2 of 25 patients	Therapeutic vaccine	Karume University	2009	Published	[110]
	Four peptides from E1, E2, NS3 and NS5A/Freund's adjuvant	Phase Ⅰ 12 nonresponder patients with chronic HCV infection	Induce peptide-specific cellular and humoral immune responses; decline HCV viral load in 3 patients	Therapeutic vaccine	Karume University	2007	Published	[111]
	Autologous dendritic cell delivered six CD8+ T cell epitope peptides from core, NS3 and NS4B	Phase Ⅰ 6 nonresponder patients with chronic HCV infection	Induce transient T-cell response	Therapeutic vaccine	Burnet Institute + others	2010	Completed	[112]

	IC41: Five peptides from core, NS3, and NS4/Poly-L-arginine	Phase Ⅰ / Ⅱ 128 volunteers/60 non-responders with chronic HCV infection	Induce HCV-specific T-cell responses	Therapeutic vaccine	Intercell AG	2006/2008	Published	[113,114]
	IC41/Poly-L-arginine + imiquimod	Phase Ⅰ 54 healthy volunteers	Induce significant T cell responses; low immunogenicity of topical imiquimod	Therapeutic vaccine	Intercell AG	2010	Published	[115]
	IC41 + imiquimod	Phase Ⅱ 50 HCV-infected patients	Decline viral load; induce T cell responses	Therapeutic vaccine	Intercell AG	2012	Completed	[116]
Virus-like particles	Recombinant HCV-like particles (HCV-LPs) containing core, E1, and E2/AS01B	4 chimpanzees	Induce HCV-specific cellular immune responses; viral clearance	Prophylactic vaccine	NIH	2007	Published	[117]
	Recombinant baculovirus containing core, E1 and E2	Mice	Induce high titers of anti-E2 antibodies and strong HCV-specific cellular immune responses (CD8+ T and Th1 cells)	Prophylactic vaccine	NIH	2001	Published	[118]
Bacterial-vectored vaccine	Attenuated Salmonella typhimurium containing *NS3* gene	Mice	Induce long-lasting T-cell responses	Therapeutic vaccine	NIH	2001	Published	[119]
Viral-vectored vaccine	Recombinant adenoviral vectors and plasmid DNA expressing NS3-NS5B	5 chimpanzees	Induce memory HCV-specific T cells; control of viremia	Prophylactic vaccine	NIH/Okairos	2012	Completed	[120]
	Multiple adenoviral vectors (Ad5, Ad6, Ad24, ChAd32 and ChAd33) expressing NS3-NS5B proteins	Mice and rhesus macaque	Induce strong cellular immune responses; long-term maintenance of memory cells	Prophylactic vaccine	Okairos	2006	Published	[121]
	Recombinant vaccinia viruses (rVV) expressing core, E1, E2, P7, NS2 and NS3	4 chimpanzees	Induce cellular immune responses; reduce viral load; resolve HCV infection	Prophylactic vaccine	NYC Blood Center	2008	Published	[122]
	Recombinant adenoviral vectors (Ad6 and ChAd3) expressing NS3-NS5B proteins	Phase Ⅰ 40 healthy volunteers	Induce sustained HCV-specific T cell responses	Prophylactic vaccine	Okairos	2012	Completed	[123]
	Adenovirus vector (Ad6 and ChAd3) expressing NS3-NS5B proteins	Phase Ⅰ 36 healthy volunteers	Highly immunogenic; induce HCV specific T cell responses	Prophylactic vaccine	Okairos and Oxford University	2009	Published	[124]
	TG4040: MVA vector expressing NS3, NS4 and NS5B proteins	Phase Ⅰ 15 patients with chronic HCV infection	Decline HCV viral load in 7 of 15 patients associated with T-cell response	Therapeutic vaccine	Transgene	2009	Withdrawn	[125]
	MVA and ChAd3 vectors expressing NS3, NS4, NS5A and NS5B proteins	Phase Ⅰ / Ⅱ Healthy at risk population (68/472 IDU)	July 28, 2018: Final data collection date	Prophylactic vaccine	NIAID	2017	Ongoing	[126]
	TG4040 + SOC	Phase Ⅱ 153 patients with chronic HCV infection	Induce HCV- and MVA-specific T-cell responses; develop anti-MVA antibodies; increase rate of early virologic response	Therapeutic vaccine	-	2014	Published	[127]
DNA vaccine	Recombinant DNA plasmid encoding E2	2 chimpanzees	Induce humoral and cellular immune responses; resolve the infection; prevent progression to chronicity	Prophylactic vaccine	NIAID/NIH	2000	Published	[128]

Recombinant DNA plasmid and adenovirus vector expressing core, E1, E2 and NS3-5	8 chimpanzees	Induce HCV-specific T-cell and long-lasting E2-specific antibody responses; reduce viral load	Prophylactic vaccine	NIH	2005	Published	[129]
Recombinant DNA plasmids and MVA vector expressing core, E1, E2 and NS3	6 chimpanzees	Induce HCV-specific immune responses; reduce viral load; early control of acute HCV infection; fail to impact on chronicity	Prophylactic vaccine	Transgene	2007	Published	[130]
CIGB-230: Plasmid expressing core/E1/E2 plus recombinant core protein	Phase Ⅰ 15 non-responder patients with chronic HCV infection	Induce humoral and cellular immune responses; no viral clearance	Therapeutic vaccine	University of Montreal + others	2009	Published	[131]
ChronVac-C: Plasmid expressing NS3 and NS4A delivered by in vivo electroporation	Phase Ⅰ / Ⅱa 12 HCV-infected patients	Decline HCV viral load in 4 of 6 patients receiving the highest dose with corresponding HCV-specific T-cell response in 3 patients	Therapeutic vaccine	Tripep AB	2009	Recruiting	[132]

HCV: Hepatitis C virus; SOC: Standard-of-care (PEGylated-IFNalpha and ribavirin); Imiquimod: An activator of the toll-like receptor (TLR) 7; Ad: Human Adenovirus; ChAd: Chimpanzee Adenovirus; MVA: Modified vaccinia Ankara virus; IDU: Injecting drug user.

focus on IDUs imposes high financial burden on the health system. Given the treatment expenses and dependence of these expenses on the stage of liver disease, screening of all at-risk populations seems much more affordable in a long run. Overall, in addition to interrupting unrecognized transmission of HCV, a part of costs expended in the treatment sector will also be saved with the prompt diagnosis and timely treatment of infected but asymptomatic patients[133,143]. While this process would demand allocation of adequate budgets and resources to integrate routine screening of high-risk population into national health programs.

As another solution, the coverage of needle and syringe exchange program should be expanded to increase the daily access to fresh needles and syringes among IDUs[144]. However, this program has not been very successful to control HCV transmission thus far, as the prevalence of HCV infection among IDUs is on the rise[139]. In fact, the overall focus on syringe sharing as the main vehicle for HCV spread has taken focus away from the other risk behaviors of IDUs such as the shared use of drug ampoules or the other injecting paraphernalia, engagement in high-risk sexual practices and the other drug-related harms[145]. These circumstances create a strong demand for precise surveillance of IDUs to obtain a reliable insight into risk behaviors of IDUs community, and subsequently harm reduction interventions should be tailored to the common risk behaviors among IDUs to mitigate the risk of HCV transmission. In addition, raising general awareness of HCV infection, diagnosis and treatment through public education should be the core activity of any harm reduction intervention, as the root cause of failure in control of HCV infection has been lack of awareness among young drug takers[133,141,146]. The growing number of IDUs and the relatively young age distribution of HCV-infected IDUs have evoke huge attention and provided a good opportunity to drive down the increasing trend of HCV-related mortality in near future through timely interventions and appropriate treatment[139,147].

The changes in HCV genotype distribution attributed to injecting drug use is another challenge in eliminating HCV infection. The changes in genotype distribution are so slight as to be unnoticeable but can have a deep impact on the epidemiology of HCV infection in a long run. These changes merit further attention if we want to properly manage the future burden of HCV infection. Globally, the most prevalent genotype is 1 (46%), followed by 3 (22%), 2 (13%) and 4 (13%)[35,137]. Over the last decade, however, a gradual decrease in the prevalence of genotype 1 and an increase in genotype 3 have been reported due to some changes in the route of transmission, risk factors, source of infection, human migration flow, and age distribution[148,149].

Blood transfusion before 1990 was the most important contributor to the spread of HCV, which has been reflected in the predominance of genotype 1 among older individuals[149,150]. In fact, screening for hepatitis C made blood transfusion remarkably safe since 1990s, paving the way for a gradual increase in the prevalence of genotype 3, which is mostly transmitted by IDU[148-150]. In recent years, IDU has become the main source of HCV transmission[35,137,144,145]. Globally, the estimated number of HCV-infected IDUs is up to 10.0 million (6.0-15.2 million), most of whom are young[35,139,144,147,151]. Meanwhile, the most common risk behavior of IDUs, syringe sharing, is more frequent among young drug injectors than in experienced and long-term injectors[152], amplifying the transmission of HCV among young IDUs population and favoring the continuous increase of HCV genotype 3. In

addition to the change in the route of HCV transmission, the ongoing civil strife in the Middle East and the active migration flow from India, Afghanistan and Pakistan, where subtype 3a is endemic, have fuelled the increasing prevalence of genotype 3[148]. On the other hand, death of elderly HCV carriers is slowly driving down the prevalence of HCV genotype 1.

These changes in genotype distribution have profound effects on the prevalence of HCV infection, response to antiviral therapy, cost and duration of treatment, and future burden of HCV infection. Given the higher rates of sustained virological response (SVR) to IFN-based therapy, the first-line therapy in low- and middle-income countries, in patients with HCV genotype 3 as compared to genotype 1[149], an increase in the prevalence of genotype 3 beneficially affects the treatment course both in terms of duration and in terms of cost and brings high benefits on an individual level. However, this increase would impose a greater risk on a population level. In reality the rising prevalence of HCV infection along with the continuous increase in the number of IDUs outweigh this benefit. The disastrous interacting epidemics of HCV infection and IDU are harbinger of a forthcoming public health dilemma, presenting a serious challenge to control transmission of HCV infection. On the other hand, high prevalence of HCV infection among young IDUs is a cause for concern, paving the way for rapid spread of HCV in the community. The old story of hepatitis C has gotten a new scenario. The emergence of IDU as the main risk factor for transmission of HCV is a surrogate in this new scenario. If this scenario is to continue, the emergence of an uncontrollable epidemic of hepatitis C will be expected in the near future.

CONCLUSION

The global community has always been concerned about the future burden of HCV infection. Although action on this concern has started many years ago with great hopes to eliminate HCV infection, the success remains elusive and will become even more elusive if the current HCV management paradigm is to be continued. We believe that it is now time to reconsider the wisdom of the current management strategies, admit failure, and act with all the strength. If we want to succeed in eliminating HCV infection, a more integrated international effort will be required, involving health policy makers, healthcare practitioners, public health organizations, antiviral drug manufacturers, health insurance companies, and all major stakeholders. In addition, effective prevention, comprehensive screening programs with a specific focus on high-risk population, accessibility to the new anti-HCV treatment regimens and public education should be considered as the top priorities of any health policy decision to eliminate HCV infection. While waiting for a solution, prevalence of HCV infection continues to increase. If we do not want to encounter another uncontrollable public health dilemma, the time to act is now, tomorrow will be very late.

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CASE REPORT

Diagnostic and therapeutic challenge of heart failure after liver transplant: Case series

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Abstract

Heart failure (HF) following liver transplant (LT) surgery is a distinct clinical entity with high mortality. It is known to occur in absence of obvious risk factors. No preoperative workup including electrocardiogram, echocardiography at rest and on stress, reasonably prognosticates the risk. In patients of chronic liver disease, cirrhotic cardiomyopathy, alcoholic cardiomyopathy, and stress induced cardiomyopathy have each been implicated as a cause for HF after LT. However distinguishing one etiology from another not only is difficult, several etiologies may possibly coexist in a given patient. Diagnostic dilemma is further compounded by the fact that presentation and management of HF irrespective of the possible underlying cause, remains the same. In this case series, 6 cases are presented and in the light of existing literature modification in the preoperative workup are suggested.

Key words: Liver transplant; Heart failure; Cirrhotic cardiomyopathy; Stress cardiomyopathy; Alcohol cardiomyopathy

Core tip: Heart failure following liver transplant surgery occurs in absence of any obvious risk factors and is associated with high mortality. No preoperative workup including electrocardiogram, echocardiography at rest and on stress, reasonably prognosticates the risk. While cirrhotic cardiomyopathy, alcoholic cardiomyopathy, and stress induced cardiomyopathy each have been

implicated, distinguishing one from another is difficult and several etiologies may possibly coexist. In this case series, 6 cases are presented and in the light of existing literature modification in the preoperative workup are suggested.

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INTRODUCTION

Heart failure (HF) is defined as "Inability of the heart to keep up with the demands on it and, specifically, failure of the heart to pump blood with normal efficiency". HF following liver transplant (LT) surgery is recognized as a distinct clinical entity with a prevalence of 3%-7%[1,2].

On retrospective analysis of 360 recipients who underwent liver transplantation at our tertiary care institute from 2011 to 2016, we identified six patients who developed heart failure in the immediate post-operative period. We diagnosed heart failure by severely decreased left ventricle ejection fraction (LVEF) on echocardiography. Clinical presentation was similar in all six recipients, only two survived. The details of each case are presented with discussion of different etiologies and specific management (Table 1).

CASE REPORT

Case 1

A 38-year-old male with cryptogenic chronic liver disease with a "model for end stage disease" (MELD) score of 16 underwent uneventful live related liver transplantation. No abnormality was detected in preoperative electro-cardiogram (EKG) and resting echocardiography. The Dobutamine stress echocardiography (DSE) was inconclusive due to chronotropic incompetence (failure to achieve target heart rate). Patient was weaned from mechanical ventilation and trachea was extubated six hours after surgery.

Twelve hours after extubation, patient complained of dyspnoea with coarse crepitations suggestive of pulmonary edema unresponsive to diuretics necessitating reintubation with supportive mechanical ventilation. Soon, hemodynamic instability sets in with increasing inotropes requirement to maintain perfusion pressures. Transthoracic Echocardiography (TTE) diagnosed left ventricle (LV) systolic failure with LVEF of 25% and increased systemic vascular resistance (SVR) of 1400 dynes.sec.cm^{-5}. "Troponin T" test was negative but Creatine kinase-MB (CK-MB) was elevated (16.8% of CK).

Pharmacologic intervention was aimed at decreasing pre- and after load using-injection Labetalol, and Nitroglycerine. Low perfusion pressure (mean blood pressure-50 mmHg) was accepted. SVR was maintained between 800-900 dynes.sec.cm^{-5}. Mechanical ventilation was continued for 4 d. LV ejection fraction improved over the period from 25% to 40%. Patient was weaned off mechanical ventilation on POD 5 (postoperative day). Labetalol infusion was continued and was replaced with oral doses from POD 8 onwards. Patient made complete recovery and was discharged from hospital with LVEF of 55% on POD 26.

Case 2

A 53-year-old male with ethanol related CLD, MELD score of 35, chronic smoker with 6 mo abstinence presented for LT. His preoperative TTE at rest showed normal ejection fraction of 65% with absence of in-ducible ischemia on DSE. His ECG was unremarkable but for a prolonged rate corrected QT (QTc) interval of 519 ms. Patient had acute kidney injury (AKI) for which Terlipressin infusion was started in the preoperative period and was continued perioperatively. His portal vein was thrombosed and required thrombectomy.

On POD 1, inotropes requirement increased with a high normal SVR and low stroke volume variation (< 10%). On TTE, LV systolic failure with LV EF of 25% was diagnosed. Hemodynamic parameters were supported using Dobutamine and Nor-adrenaline infusion. "Troponin T" test was inconclusive while Creatine kinase-MB (CK-MB) was elevated (15.1% of CK). Supportive care with mechanical ventilation was continued and LVEF improved over next 10 d. However, sepsis with gram negative infections led to multi-organ dysfunction resulting in patient mortality on the 14th POD.

Case 3

A 55-year-old female with cirrhosis due to extrahepatic portal vein obstruction with intraparenchymal extension with MELD of 9 presented for LT surgery. Her TTE at rest as well as DSE was normal. On her ECG, QTc interval was prolonged (532 ms). Packed red blood cells (15 units) were transfused during the surgery on account of blood loss during dissection of her native liver. In the immediate postoperative period, with progressive increase in inotropes and vasopressors requirement, it became difficult to maintain perfusion pressures. LV failure with EF of 20% was diagnosed on Transesophageal echocardiography (TEE). CK-MB was raised (14.68% of CK). In spite of maximal therapeutic management, hemodynamics deteriorated on second postoperative day leading to multiorgan dysfunction and death.

Case 4

A 26-year-old female with acute liver failure of unknown etiology with normal preoperative TTE, an unremarkable EKG but for a prolonged QTc interval (540 ms) underwent uneventful liver transplantation. She was weaned off respiratory support after overnight mechanical ventilation.

Table 1 Demography, Cardiology workup Pre-Transplant, Clinical course and outcome

(S No.) Demography, age, gender, etiology MELD score	Cardiology workup EKG, QTc, CI, echocardiography EF, DSE	Clinical course: Intraop, post op	CPKMB % of Ck on diagnosis of HF (normal 3%-5%)[25]	Possible underlying cause of heart failure in decreasing order of possibility	Outcome
(1) 38 yr, male, cryptogenic, MELD 16	QTc < 445 ms CI: Present EF: 65% DSE: Inconclusive	Uneventful LDLT; Extubated POD 1; Pul. Edema POD 2; EF: 25%	16.80%	CiCd ABS CAD ALC	EF recovered to 40% on POD 4; EF: 55% on discharge at POD 25; Survived to discharge;
(2) 53 yr, male, ethanol MELD 35	QTc: 519 ms CI: Absent EF: 65% DSE: Negative for inducible ischemia	Uneventful LDLT; Portal vein thrombectomy; Terlipressin infusion preop and intraop; POD 1: EF: 25%; Gram negative sepsis with MOD	15.10%	ALC CiCd CAD ABS	EF recovered to 55% at POD 10; Died
(3) 55 yr, female, EHPVO with intraparenchymal extension, MELD 15	QTc: 532 ms CI: Absent EF: 65% DSE: Negative for inducible ischemia	Turbulent LDLT; Increasing inotrope and vasopressor requirement; EF: 20%; Severe vasoplegia	14.68%	ABS CiCd CAD ALC	EF never recovered; Vasoplegia did not respond; Died
(4) 26 yr female, ALF	QTc: 540 ms CI: Absent EF: 70% DSE: Not done	Uneventful LDLT; Re-exploration POD2 for bleed; SVT; EF: 25%;	14.84%	ABS CiCd CAD ALC	EF recovered to 50% at POD 4; Died
(5) 40 yr, male, ethanol MELD 21	QTc: 550 ms CI: Absent EF: 65% DSE: Negative for inducible ischemia	Uneventful DDLT POD1: EF: 30%	Not done	ALC CiCd ABS CAD	EF recovered to 40% at POD 4; Survived
(6) 38 yr, male, ethanol MELD 32	QTc: 550 ms CI: Absent EF: 65% DSE: Negative for inducible ischemia	Uneventful; POD 1: EF: 20%; Recurrent SVT	39.40%	ALC CiCd ABS CAD	EF never recovered; Died

CI: Chronotopic incompetence; EF: Ejection fraction; DSE: Dobutamine stress echocardiography; CiCd: Cirrhotic cardiomyopathy; HF: Heart failure; ABS: Acute broken heart syndrome; ALC: Alcoholic cardiomyopathy; MOD: Multi-organ dysfunction; QTc: Rate corrected QT interval on ECG.

The postoperative course was complicated with hemoperitoneum on second day necessitating emergency laparotomy. Bleeder was identified and repaired. During this surgery, she had an episode of ventricular tachycardia which responded to lignocaine bolus. Subsequent to VT, LV EF was decreased (25%). The "Troponin T" test was inconclusive while CK-MB was increased (14.84% of CK). Over next four days, LV EF improved to 40%. Hemodyamics were supported during this period using dobutamine infusion which was then tapered and trachea was extubated after successful sponataneous breathing trial. However, on 7th POD, sepsis was diagnosed with positive microbiological cultures which led to multiorgan dysfunction and refractory vasoplegia. She succumbed to septic shock and died on POD 18.

Case 5

A 40-year-old male with ethanol related CLD with MELD score of 21 presented for LT. Preoperative EKG and TTE at rest were normal with LVEF of 60%. DSE was negative for inducible ischemia. After uneventful deceased donor liver transplantation (DDLT), patient was weaned off mechanical ventilation, 5 h after the surgery. On POD 1, patient developed respiratory distress with pulmonary edema, global hypokinesia with LVEF of 30% was diagnosed on TTE. Troponin T card test was negative. Systemic vascular resistance was 1250 dynes.sec.cm^{-5}. Noninvasive mechanical ventilation support was instituted along with preload and after-load reduction with Nitroglycerine infusion and Tablet Amlodepine. Tablet Prazocin was added subsequently. Patient improved symptomatically. LVEF improved to 40% by POD 4. Nitroglycerine infusion was tapered off while Tablet Prazocin and Tablet Amlodepine were continued. Patient was discharged to home with normal LVEF.

Case 6

A 38-year-old male with ethanol related CLD with MELD score of 32 underwent deceased donor liver transplant (DDLT). His preoperative Echocardiography was negative for inducible ischemia with minimal left to right intrapulmonary shunting with prolongation of QTc interval on EKG. Patient was weaned off mechanical ventilation on POD 2. On postoperative day 3, he developed low cardiac output with pulmonary edema with LVEF of 20% on TTE. CK-MB was elevated (39.4% of CK). Mechanical ventilation with tracheal intubation was initiated while

hemodynamic was supported using Nitroglycerine and Levosimendan infusion. Patient developed recurrent tachyarrhythmia in absence of any obvious electrolyte disorder for which Amiodarone was given. Patient was weaned off mechanical ventilation after 2 d but had to be re-intubated very next day on account of repeat episode of supra-ventricular tachycardia (SVT) with pulmonary edema. Subsequently several attempts to wean off mechanical ventilation were not successful. SVT continued to re-occur. Tracheostomy was done and patient was given increasing duration of spontaneous breath trials. However LVEF failed to improve and patient died on POD 29.

DISCUSSION

We observed heart failure after LT even with normal preoperative echocardiography, negative DSE for inducible ischemia and without any obvious cause. Literature suggests a "non-ischemic" cause for the systolic failure with after LT[3,4].

Cardiac risk factors in chronic liver disease

Cardiac risk factors have been identified in patients with chronic liver disease. These include coronary artery disease (CAD) (6%-26%), valvular heart disease (27.5%), asymptomatic foramen ovale (4%), cirrhotic cardiomyopathy (CiCd) (40%-90%), portopulmonary hypertension (2%-14%) and other diseases like amylodosis and hemochromatosis (45%). Cumulative risk of mortality in presence of these risk factors has been calculated to be 50%. Of these, presence of cirrhotic cardiomyopathy alone is associated with 3%-7% risk of severe HF in the post-operative period with 45% risk of mortality[5].

Risk factors associated with heart failure after liver transplantation

General: Presence of diabetes, hypertension, mean arterial pressure $\leqslant$ 65 mmHg, mean pulmonary artery pressure $\geqslant$ 30 mmHg, mean pulmonary capillary wedge pressure $\geqslant$ 15 mmHg, hemodialysis and brain natriuretic peptide (BNP) level (> 50 pg/mL) have been found to be predictive for the development of new-onset systolic heart failure after liver transplantation[1,5].

Etiology specific cirrhotic cardiomyopathy: Cirrhotic cardiomyopathy (CiCd) is defined as a "form of chronic cardiac dysfunction in patients with cirrhosis, characterized by blunted contractile responsiveness to stress, and/or altered diastolic relaxation with electrophysiological abnormalities in the absence of other known cardiac disease"[6].

QTc interval prolongation is a typical feature of CiCD. It is observed more frequently in patients who died after LT than in survivors and QTc interval > 450 ms have been found to be predictive for the development of new-onset systolic heart failure after liver transplantation[7]. We found increased preoperative QTc > 450 ms in 4 out of the 6 recipients with CLD who developed postoperative heart failure. Chronotropic incompetence, another feature of CiCd, was however observed only in one of these 6 recipients.

Alcoholic cardiomyopathy: Alcoholic cardiomyopathy shares pathophysoiology with CiCd. However, the co-existence of liver disease due to cirrhosis may give rise to diagnostic confusion and is therefore a diagnosis of exclusion[8]. Three out of these 6 recipients with postoperative heart failure had ethanol related liver disease and therefore could have had alcoholic cardiomyopathy. Association between Alcoholic cardiomyopathy and Supraventricular arrhythmias is known[9]. Alcoholics with simultaneous cardiomyopathy and cirrhosis are known to have a poor prognosis[10]. Case number 6 discussed in this report had supraventricular arrhythmias in the setting of HF after LT for ethanol related CLD and he did not survive.

Coronary artery disease: The prevalence of CAD in LT candidates over the age of 45-50 years ranges between 6% and 26%. Two of our patients were older than 50-year-old but had no symptoms suggestive of cardiac disease. Even preoperative DSE was negative for inducible ischemia.

Due to presence of ascites, poor nutritional status, cachexia, limited physical activity, it is difficult to diagnosis CAD in patients with CLD. In presence of limited physical activity, presenting signs and symptoms of angina and or angina equivalent are either not present or are not attributable to CAD. DSE has limited usefulness for diagnosing CAD in patients with CLD as it is often inconclusive in such patients due to chronotropic incompetence with resultant failure to achieve target heart rate. Similarly Dipyridamole or Adenosine nuclear myocardial perfusion scan also remain inconclusive as coronary vasculature is already maximally dilated in cirrhotics and therefore like DSE, are relied upon for their negative predictive value only.

Alternative tests like Single-photon emission computed tomography (SPECT) scanning, Cardiac magnetic resonance imaging, Carotid intima-media thickness, and Coronary artery calcification score (CACS) measured by computerized tomography have also been used to investigate presence of coronary artery disease though they have their own limitations[11].

Stress-related cardiomyopathy: Early-onset HF after surgery, directly reflects surgery related stress to the myocardium or hemodynamic changes. Stress related cardiomyopathy therefore cannot be missed as a cause of systolic heart failure in the perioperative period of non-cardiac surgery[12]. Similar conclusion was drawn by Mandell *et al*[1] who concluded that patients having HF after LT, either suffered from stress cardiomyopathy and therefore had no evidence of impaired contraction before the event or the echocardiographic predictors of HF were masked by circulatory changes in patients with cirrhosis.

Stress induced cardiomyopathy, or acute broken heart syndrome (ABS) also known as Takotsubo cardiomyopathy is understood to be caused by catecholamine surge which leads to diffuse microvascular spasm to cause myocardial stunning and HF. ABS and myocardial infarction (MI) share similar clinical and ECG presentation and blood biochemical tests.

To distinguish fromMI and for diagnosing ABS, Mayo clinic has therefore proposed following 4 point criteria[13]: (1) transient LV Systolic dysfunction (hypokinesis, akinesis, dyskinesis): The wall motion abnormalities are typically regional and extend beyond a single epicardial coronary distribution; (2) absence of obstructive coronary disease or angiographic evidence of acute plaque rupture. If coronary disease is found, the diagnosis of stress cardiomyopathy can still be made if the wall motion abnormalities are not in the distribution of the coronary disease; (3) new electrocardiographic abnormalities (either ST-segment elevation and/or T wave inversion) or modest elevation in cardiac troponin; and (4) absence of pheochromocytoma or myocarditis.

Patients in this case series satisfied 3 out of the 4 criteria except for the absence of obstructive coronary lesion or angiographic evidence of acute plaque rupture, which could not be ruled out in absence of coronary angiogram.

Serum cardiac troponin levels and brain natriuretic peptide (BNP) or N-terminal pro-BNP are elevated in most patients with stress cardiomyopathy in the International Takotsubo Registry study[14]. In the patients discussed, while Troponin card test was negative and BNP levels were not done, CPK MB was elevated. These patients therefore could have had ABS manifesting as HF.

In the present case series, only two patients, case No. 2 and 3, aged 53 and 55 years and possibly case No. 5 aged 40 in view of age and lifestyle were at risk of having CAD and these three patients were able to achieve target heart rates on DSE (Otherwise a limitation in patients with cirrhosis of Liver). DSE in these patients was negative for inducible ischemia. Considering this with ongoing hemodynamic instability and presence of global and not regional wall motion abnormality specific to any coronary artery supplied region and the younger age of rest of the patients, decision was taken to not to do coronary angiogram in these patients.

Most patients discussed in this case series had several possible etiologies responsible for the observed heart failure which could not have been definitely identified from one another. In absence of coronary angiogram, evidence against CAD is only circumstantial and therefore cannot be completely ruled out. Diagnosis of ALC and CiCd also cannot be certainly made except on the basis of history of ethanol abuse and presence underlying chronic liver disease. Similarly the diagnosis of ABS in absence of coronary angiogram does not entirely satisfy the Mayo's diagnostic criteria and is also possibly a diagnosis of convenience. It is also possible that several etiologies might be coexisting and therefore the high mortality in these patients.

New preoperative prognostic markers for heart failure after LT

In a prospective study of "myocardial injury after noncardiac surgery" (MINS), troponin elevations, any peak Troponin T (TnT) of 0.03 ng/mL or greater, without a non-ischemic explanation (*e.g.,* sepsis and pulmonary embolus) was diagnostic of MINS[15]. Patients with MINS were at higher risk of congestive heart failure (OR, 10.34; 95%CI: 7.99-13.37, $P < 0.001$) compared with patients who did not suffer MINS. In another study, mortality increased exponentially as a function of peak postoperative troponin concentration[16].

In the setting of surgery for LT, Coss *et al*[17] in a multivariate analysis of 230 transplant recipients found that an abnormal pretransplant troponin I level (> 0.07 ng/mL) predicted postoperative cardiovascular complications in their patients. They concluded that raised Toponin I levels > 50 pg/mL indicate latent cardiac dysfunction that is not recognized by conventional screening methods[17].

While quantitative assay of troponin is not available at our institute, we observed negative test on qualitative analysis using Trop T sensitive test Card test (detects Troponin T $\geqslant$ 0.1 ng/mL in blood). Creatine kinase-MB (CK-MB) was done and was significantly elevated soon after the clinical and echocardiography diagnosis of HF in our patients in absence of any other identifiable cause for the same.

Another novel marker, BNP level and QTc interval > 450 ms were concluded to be predictive for the development of new-onset systolic heart failure after LT in a study by Qureshi *et al*[3].

Management

Therapeutic strategies for addressing this acute and possibly life-threatening complication of heart failure after LT are not well defined. Similarly there is no established treatment for patients suffering MINS. In light of findings of decreased 30 d mortality in POISE trial (PeriOperative Ischemic Evaluation), acetyl-salicylic acid and statin therapy may possibly benefit patients who suffer MINS[18-20].

CiCd, Alcoholic cardiomyopathy, and ABS, all are characterized by inotropic incompetence. After LT, blood pressure is known to rise significantly which may possibly precipitate inotropic incompetence and subsequent HF in such patients[21,22]. Management of heart failure in the post-LT period therefore does not differ from usual heart failure therapies. Diuretics, inotropes, inodilators and vasopressor support form the foundation pillars of treatment.

Heart failure guidelines, such as those adopted by the European Society of Cardiology or the American College of Cardiology/American Heart Association should be followed. Cardio-selective Beta-blockers, ACE inhibitors or angiotensin receptor blockers (ARBs), diuretics and digitalis may be used for management

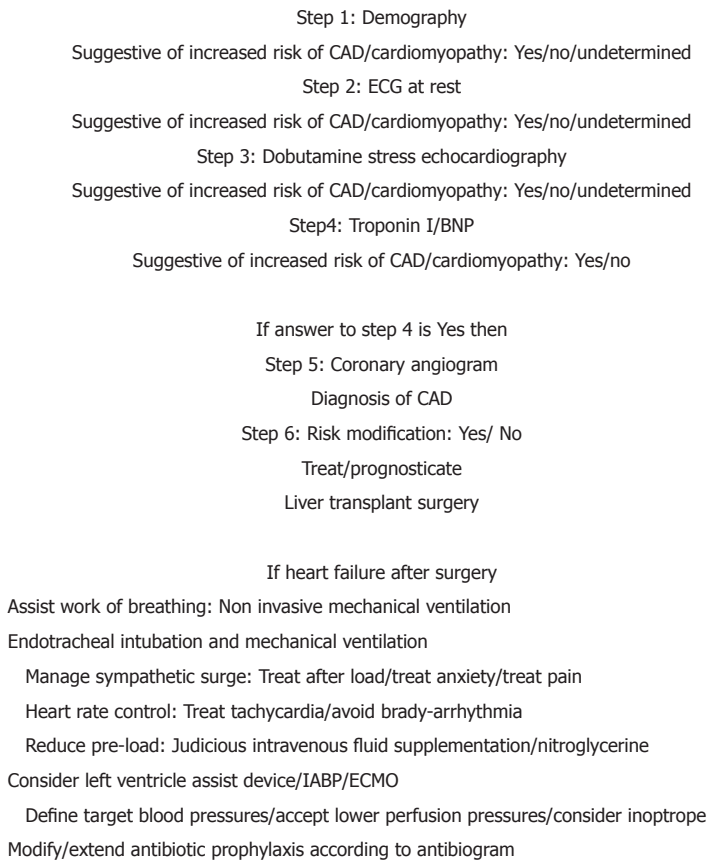

Figure 1 Suggested stepwise approach for diagnosis of patients at risk and for management of heart failure after liver transplant. CAD: Coronary artery disease; ECMO: Extracorporeal membrane oxygenator.

of HF after LT. It is important to treat the adrenergic surge causing raised systemic vascular resistance and also to manage the preload to avoid further worsening of cardiac function. In our case series, LVEF of 4 out of 6 patients recovered with use of therapy aimed at decreasing pre- and after load and adrenergic surge.

Several authors have reported successful outcome after aggressive management of HF following LT using extracorporeal membrane oxygenator (ECMO) and ventricular assist device (VAD), though cost may be a constraint[4,23,24]. Considering advances in clinically applied biomarkers and success of aggressive measures in managing such cases, a stepwise approach to identify patients at risk and for management should be adopted (Figure 1).

In our series, out of 6 patients, only 2 survived despite recovery of EF. Survival therefore is perhaps determined by factors other than myocardial performance like duration and severity of liver disease, presentation either acute or chronic, age of the patient, co-morbid conditions and presence of sepsis. Two patients who survived were relatively younger, aged 38 and 40 years and had chronic and not acute liver disease. Those who did not survive either had ALF or increased severity of CLD as reflected in their MELD scores or additional insult in form of sepsis in the setting of HF.

In conclusion, high MELD, Acute liver failure and sepsis in the setting of Heart failure after LT are probably associated with grave prognosis. While different etiologies may cause HF after LT, combination of several may possibly coexist. It may be prudent to routinely do quantitative Troponin I and/or BNP levels before LT surgery to identify and prognosticate recipients likely to be complicated by heart failure. While Heart Rate control, preload and after-load reduction are the pillars of management, ECMO and VAD may allow sufficient time for recovery of heart failure. In view of the limitations of the commonly used diagnostic modalities and poor outcome, better aides to identify patients at risk are needed which would require greater interdisciplinary interaction involving clinicians and laboratory scientists. Till such time, this entity, Heart failure after Liver transplant continues to remain an enigma.

ARTICLE HIGHLIGHTS

Case characteristics

Patients of acute liver failure and of chronic liver disease, presenting with systolic heart failure within 7 d after the liver transplant surgery in absence of any preoperatively identified and obvious predisposing risk factor.

Clinical diagnosis

Systolic heart failure was diagnosed on basis of clinical presentation and echocardiography with greatly reduced left ventricle ejection fraction.

Differential diagnosis

Liver graft dysfunction and severe sepsis may cause hemodynamic instability

and were ruled out. Underlying cause for the observed systolic heart failure could not be made.

Laboratory diagnosis

Creatine kinase-MB was elevated upon diagnosis of systolic heart failure after liver transplant. Troponin T sensitive card test was negative.

Imaging diagnosis

Severely reduced left ventricle ejection fraction was diagnosed on echo-cardiography.

Pathological diagnosis

Could not be made conclusively.

Treatment

Respiration was assisted. Hemodynamics supported using inotropes and inodilators and beta blockers, aimed at preload and after load reduction. Sedation and analgesia were taken care of to reduce sympathetic adrenergic activity.

Term explanation

Cirrhotic cardiomyopathy and alcohol cardiomyopathy have been described as specific clinical entities that describe cardiomyopathy in setting of underlying chronic liver disease and with history of alcohol indulgence respectively. Acute broken heart syndrome describes the cardiomyopathy typically seen under stressful conditions and not necessarily after surgery and is said to resemble acute myocardial infarction.

Experiences and lessons

In absence of established clinical features and limitations of existing prevalent diagnostic modalities, Bio-chemical makers like BNP and Troponin I may be routinely done as part of preoperative workup of patients posted for liver transplant surgery to help identify patients at greater risk of heart failure after the surgery.

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ABOUT COVER

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WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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ORIGINAL ARTICLE

Basic Study

Morphological alterations and redox changes associated with hepatic warm ischemia-reperfusion injury

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Abstract

AIM

To study the effects of warm ischemia-reperfusion (I/R) injury on hepatic morphology at the ultrastructural level and to analyze the expression of the thioredoxin (TRX)

and glutaredoxin (GRX) systems.

METHODS

Eleven patients undergoing liver resection were subjected to portal triad clamping (PTC). Liver biopsies were collected at three time points; first prior to PTC (baseline), 20 min after PTC (post-ischemia) and 20 min after reperfusion (post-reperfusion). Electron microscopy and morphometry were used to study and quantify ultrastructural changes, respectively. Additionally, gene expression analysis of TRX and GRX isoforms was performed by quantitative PCR. For further validation of redox protein status, immunogold staining was performed for the isoforms GRX1 and TRX1.

RESULTS

Post-ischemia, a significant loss of the liver sinusoidal endothelial cell (LSEC) lining was observed (P = 0.0003) accompanied by a decrease of hepatocyte microvilli in the space of Disse. Hepatocellular morphology was well preserved apart from the appearance of crystalline mitochondrial inclusions in 7 out of 11 patients. Post-reperfusion biopsies had similar features as post-ischemia with the exception of signs of a reactivation of the LSECs. No changes in the expression of redox-regulatory genes could be observed at mRNA level of the isoforms of the TRX family but immunoelectron microscopy indicated a redistribution of TRX1 within the cell.

CONCLUSION

At the ultrastructural level, the major impact of hepatic warm I/R injury after PTC was borne by the LSECs with detachment and reactivation at ischemia and reperfusion, respectively. Hepatocytes morphology were well preserved. Crystalline inclusions in mitochondria were observed in the hepatocyte after ischemia.

Key words: Hepatic ischemia-reperfusion injury; Ischemia reperfusion injury; Warm ischemia-reperfusion injury; Glutaredoxins; Thioredoxins; Electron microscopy; Oxidative stress; Portal triad clamping

Core tip: The complex mechanisms of warm Ischemia reperfusion (I/R) injury in the liver are diverse and have been widely studied but poorly understood. This study aims to investigate the ultrastructural changes at warm I/R injury induced by portal triad clamping. The effects were mainly borne by the liver sinusoidal endothelial cells (LSEC) which detached from the sinusoidal wall after ischemia. Interestingly we found that the LSECs reattached after reperfusion. Hepatocytes were unaffected except for the appearance of crystalline inclusions in the mitochondria. Investigation of redox related proteins showed no changes within our time frame.

Jawad R, D'souza M, Selenius LA, Lundgren MW, Danielsson O, Nowak G, Björnstedt M, Isaksson B. Morphological alterations and redox changes associated with hepatic warm ischemia-reperfusion injury. *World J Hepatol* 2017; 9(34): 1261-1269 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i34/1261.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i34.1261

INTRODUCTION

Ischemia-reperfusion (I/R) injury is a known cause of tissue damage during liver resection and transplantation with direct impact on patients' postoperative morbidity and mortality[1,2]. It is a biphasic phenomenon whereby the initial hypoxic damage is compounded upon restoration of blood supply along with oxygen delivery. The mechanisms of injury are complex and have been widely studied but remain poorly understood. Hepatic I/R injury is classified as warm or cold, where warm ischemia occurs when the blood supply to the liver is interrupted during liver resection, transplantation, trauma, and shock. Cold storage ischemia occurs during organ preservation in cold preservation solutions before transplantation[3]. Although both mechanisms share similarities, there are fundamental differences between warm and cold hepatic I/R injury. Existing knowledge indicates that warm I/R injury inflicts hepatocyte damage, while cold I/R injury is primarily characterized by injury to the sinusoidal endothelial lining[4].

Blood loss is one of the significant determinants of morbidity and tumor recurrence after hepatectomy[5]. Portal triad clamping (PTC), also known as the Pringle maneuver, has been one of the most widely used methods to reduce blood loss during hepatic surgery and involves clamping of the hepatic vascular inflow. PTC causes warm I/R injury in the remnant liver, the consequences of which are determined by the duration of clamping and the underlying health status of the liver parenchyma.

While there is vast literature regarding the biochemical and metabolic alterations associated with hepatic I/R injury, studies investigating the cellular and ultrastructural changes occurring in the liver as a result of I/R injury have mainly involved animal models and data from human studies are limited[6-10].

The ischemic injury occurs as a result of a reduction in blood supply and switching from aerobic to anaerobic metabolism. The initial ischemic insult followed by the sudden oxygen burst upon the reestablishment of vascular flow causes reperfusion injury which to a large extent is ascribed to the production of reactive oxygen species (ROS) and associated cellular injury[11-13]. There are several proteins involved in ROS scavenging and antioxidant defense. Many are regulated at transcriptional level through binding of nuclear factor (erythroid-derived 2)-like 2 (NRF2)[14], to the Antioxidant-Response Element (ARE) localized upstream of the promotor of these genes. By the same mechanism, *Nrf2* regulates glutamate-cysteine ligase (GCLC) and cysteine/glutamate antiporter (xCT), which are essential for glutathione (GSH) synthesis. GSH maintains the cellular redox balance and is considered

as one of the most important cellular antioxidants[15,16]. Thioredoxin (TRX) and glutaredoxin (GRX) are two intricate reduction systems belonging to the thioredoxin superfamily of proteins and are ubiquitously expressed in all cell types[17-19]. There is a lack of information on the involvement of these redox systems in hepatic I/R injury.

The present study aimed at investigating the effects of warm I/R injury induced by PTC in the human liver at the ultrastructural level, determining the degree and character of hepatocyte damage, and the sinusoidal endothelial lining. In addition, the impact of I/R injury on redox proteins was studied, in particular the TRX and GRX systems.

MATERIALS AND METHODS

Patients

Eleven patients (8 men and 3 women), undergoing liver resection for differing indications, but without preoperative clinical or biochemical signs of chronic liver disease, were included in the study. Seven of the patients had colorectal liver metastases and all of them had received preoperative chemotherapy. Two patients had melanoma metastases to the liver and one had metastases from a bowel carcinoid. One patient was operated because of a suspected hepatocellular carcinoma, which on final histopathology turned out to be an inflammatory pseudotumor. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Regional Ethics Committee for human studies, Stockholm, Sweden. All patients were informed orally and in writing and gave written consent.

Study protocol and biopsy acquisition

Laparotomy was performed by a right subcostal incision with an upper midline extension and the falciform ligament then divided. The hepatoduodenal ligament was isolated and a PTC then performed by placing a soft cloth tape around the porta hepatis over which a rubber tubing was then slid. With a hemostat, the rubber tubing was adjusted to constrict the vessels in the porta hepatis. The liver was not manipulated during the experimental time period. One wedge biopsy and two needle biopsies (with a Tru-Cut needle) were taken at three time-points; Baseline (just before the application of PTC), post-ischemia (after 20 min of PTC) and post-reperfusion (after 20 min of reperfusion). The needle biopsies were immediately transferred to the required buffers as detailed below before being stored at 4 ℃ for further analyses. The wedge biopsies were immediately transferred to vials and flash-frozen in liquid nitrogen and stored at -70 ℃ until analysis. The liver resection was then carried out as planned.

Transmission electron microscopy

The needle biopsies were fixed in 2% glutaraldehyde, 1% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4 for 10 min at room temperature and then stored at 4 ℃. The samples were washed with 0.1 mol/L phosphate buffer, pH 7.4 and then postfixed for 2 h in 2% osmium tetroxide, 0.1 mol/L phosphate buffer, pH 7.4 at 4 ℃. Dehydration was performed in ethanol, followed by acetone and the tissue samples were embedded in LX-112. Sections of approximately 70 nm were prepared by an ultramictrotome (Leica EM UC 6). Uranylacetate was added to the sections for contrast followed by lead citrate. A transmission electron microscope (Tecnai 12 Spirit Bio TWIN) was used at 100 kV for examination of the sections and a Veleta® camera used for capturing digital images. Assessment of the electron microscopy (EM) findings included evaluation of the cellular architecture, hepatocyte morphology, sinusoids and bile canaliculi.

Morphometric image analysis

NIS Elements Basic Research software was used for quantification analysis of the sinusoids in the electron micrograph images. Pixel length measurements were applied on the sinusoidal endothelial lining surrounding vessels. The number of pixels was determined on one representative sinusoid for each patient and time point, and the length of the endothelial lining was correlated to the length of the entire sinusoid. The retrieved pixel value was related to actual μm for comparison between different images.

Immunoelectron microscopy

Needle biopsies were fixed in 3% paraformaldehyde in 0.1 mol/L phosphate buffer and rinsed in 0.1 mol/L phosphate buffer with subsequent addition of 2.3 mol/L sucrose and subsequently frozen in liquid nitrogen. An ultramicrotome (Leica EM UC 6) was used for the sectioning on carbon-reinforced formvar coated, 50 mesh Nickel grids. The grids were placed on drops of 0.1 mol/L phosphate buffer, 2% BSA, 2% Fish gelatin. Primary antibodies of TRX1 and GRX1 were applied on the sections (GRX1 1:5, TRX1 1:10, own production as described previously[20], in 0.1 mol/L of phosphate buffer, 0.1% BSA, 0.1% gelatin) overnight in a humidified chamber at room temperature. The sections were rinsed with the same buffer and detection of primary antibodies was achieved by protein A with 10 nm gold at a dilution of 1:100. A second wash of the sections was performed before fixation in 2% glutaraldehyde. Contrast was attained by 0.05% uranylacetate and sections were embedded in 1% methylcellulose. A transmission electron microscope (Tecnai G2 Bio TWIN) was used for examination and digital images captured by a Veleta camera®. Quantification of the staining was performed on 5 hepatocytes in close proximity to vessels for each tissue section. The number of gold particles in the cytosol and the nuclei were recorded.

RNA purification, cDNA synthesis and qPCR

The fresh frozen wedge biopsies (approximately 10

Table 1 Patient demographics

Patient (gender/age)	Diagnosis	Resection performed	Preoperative chemotherapy	Peak AST, μkat/L	Peak ALT, μkat/L	Day of enzyme peak (AST, ALT), (d)	Peak bilirubin, μmol/L, (d)	Fibrosis (stage)	Inflammation	Steatosis
P1 (M/75)	mCRC	Atypical resection	Yes	4.39	2.23	1	24 (1)	1	0	1
P2 (F/71)	mCRC	Multiple resections	Yes	23.05	21.93	2	53 (1)	1	0	2
P3 (M/39)	Inflammatory pseudotumor	Extended right hepatectomy	No	4.19	4.81	1	41 (1)	-	-	1
P4 (M/75)	Malignant melanoma metastasis	Multiple atypical resections	Yes	2.32	1.90	1	12 (2)	1	0	1
P5 (M/63)	mCRC	Bisegmentectomy	Yes	6.25	7.03	2	16 (1)	1	0	1
P6 (F/78)	Carcinoid metastasis	Left hepatectomy	No	3.73	2.89	1	20 (1)	1	0	1
P7 (F/61)	mCRC	Right hepatectomy	Yes	7.14	6.11	1	14 (1)	1	0	0
P8 (M/55)	Malignant melanoma metastasis	Right hepatectomy	No	7.90	8.84	2	48 (2)	0	0	2
P9 (M/68)	mCRC	Right hepatectomy + atypical resection	Yes	3.85	1.91	1	82 (8)	3-4	2	1
P10 (M/37)	mCRC	Right hepatectomy	Yes	6.71	3.96	2	33 (2)	1	0	0
P11 (M/58)	mCRC	Right hepatectomy + atypical resection	Yes	11.16	9.28	1	53 (2)	1	0	3

Steatosis was defined as 1: Mild (< 33%); 2: Moderate (33%-66%); 3: Severe (> 66%). Fibrosis was defined as Stage 1: Portal fibrosis; Stage 2: Periportal fibrosis; Stage 3: Septal fibrosis; and Stage 4: Cirrhosis. mCRC: Metastatic colorectal cancer.

mg) were homogenized and lysed using a TissueLyser LT with RLT plus lysis buffer (Qiagen). RNeasy Plus Mini Kit (Qiagen) was used for RNA purification according to the manufacturer's instructions, and RNA concentration determination was performed on a NanoDrop ND 100 Spectrophotometer (Saveen Werner). In order to validate the purity and quality of the RNA, Experion Automated Electrophoresis System with Experion RNA StdSens Analysis Kit (BIO-RAD) was used according to manufacturer's protocol. The mRNA quality was assessed for all samples and 6 out of 11 patients had good quality of samples from all time points.

For cDNA synthesis, 2 μg RNA was subjected to reverse transcription by Omniscript RT kit (Qiagen) according to manufacturer's instructions. Twenty-50 ng cDNA/reaction was used for qPCR in a BIO-RAD iCycler (BIO-RAD). Forward and reverse primers were designed *via* primer BLAST and Ape and purchased from Invitrogen (primer details in Supplementary Table 1). The genes of interest were isoforms of thioredoxins (*TXN*) and glutaredoxins (*GLRX*) and also the gene of *xCT* and *NRF-2*, which are *SLC7A11* and *NFE2L2* respectively. The operation setting for qPCR instrument was the following: 50 ℃ for 2 min, 95 ℃ for 2 min with 40 amplification cycles of denaturation at 95 ℃ for 15 s. Annealing and elongation temperatures of 60 ℃, 66 ℃ or 55 ℃ were used, depending on the gene of interest, during 30 s. The $2^{-\Delta\Delta CT}$ method was used for quantification by normalizing the CT values against the housekeeping gene β-actin and retrieving fold change relative to the chosen control. A cut off value of 32 cycles was chosen and all primers were optimized for an efficiency of 90%-105%.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 6.0 software. The non-parametric Friedman test followed by Dunn's *post-hoc* test was used for the analysis of endothelial lining, gene expression data, and immunogold staining data. A *P* values of less than 0.05 was considered to be significant.

RESULTS

Patients

The median age of the patients was 68 (range 39-78) years. Light microscopy evaluation of tissue blocks collected for clinical routine diagnostics from macroscopically non-tumorous liver parenchyma revealed no major histological differences in the surgical specimens from the majority of patients. However, one patient had severe fibrosis, suspicious for cirrhosis (stage 3-4) combined with inflammation of grade 1-2 as defined by Batts and Ludwig[21]. Two patients had moderate steatosis (defined as 33%-66% of hepatocytes affected). Furthermore, one patient had pronounced steatosis (> 67% of hepatocytes affected). The relevant patient data has been summarized in Table 1.

Ultrastructural examination

At baseline the biopsies exhibited typical hepatic organization with normal hepatocyte and liver sinusoidal endothelial cell (LSEC) morphology. The hepatocytes had a normal appearance, intact plasma membranes, and large numbers of mitochondria without any discernible morphological aberrations at this point. The presence of lipofuscin lipid lysosomes was observed in most liver sections (Table 2). The morphology of the Space of Disse showed hepatocytes with intact microvilli extensions and normal fenestrated LSECs lining the

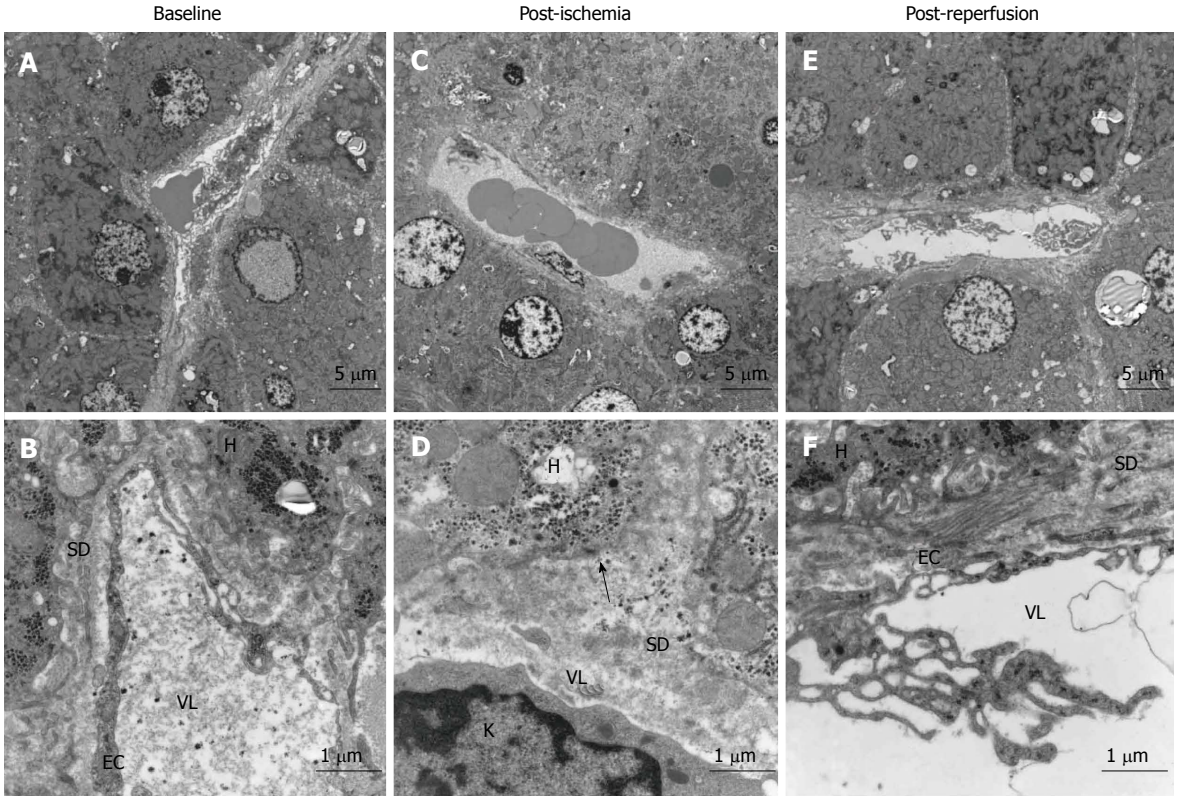

Figure 1 Morphological changes in liver before and after ischemia and reperfusion, transmission electron micrographs of representative images of liver sections from one patient. A: Baseline, before induction of ischemia, shows the normal state of liver morphology at a cellular level; B: Morphology of a sinusoid with neighbouring hepatocytes 20 min after ischemia; C: Representative image of sinusoid and haptocytes 20 min after reperfusion; D: Endothelial lining of a sinusoid with hepatocyte microvilli; E: Morphology of the Space of Disse post-ischemia; F: Morphology of the space of disse post-reperfusion. SD: Space of disse; H: Hepatocyte; EC: Endothelial cells; VL: Vessel lumen; K: Kupffer cell. Arrow shows the absence of hepatocyte microvilli.

Table 2 Quantitative summary of ultrastructural changes studied by endothelial morphology, (*n*) patients out of total (11) patients

	Baseline	Post-ischemia	Post-reperfusion
Disruption of endothelium	2/11	10/11	2/11
Endothelial activation	4/11	3/11	9/11
Mitochondrial inclusions in hepatocytes	1/11	6/11	7/11
Lipids, lipofucsin	10/11	10/11	10/11

sinusoids (Figure 1A and D).

The most noticeable change post-ischemia was a disruption of the LSEC lining (Figure 1B and E) in 10 out of 11 patients (Table 2). Apparent changes were seen in the space of Disse where the hepatocyte microvilli decreased in number and were in some cases undetectable (Figure 1E). There were no signs of hepatocyte plasma membrane rupture in either the ischemic or reperfused states. The hepatocytes exhibited some condensed nuclear chromatin but otherwise preserved hepatocyte morphology (Figure 1). In seven out of eleven patients, the hepatocyte mitochondria exhibited aggregates, so-called crystalline inclusions post-ischemia (Figure 2 and Table 2). These inclusions were accompanied by dilated mitochondria, both round and elongated types.

There was a reactivation of the LSECs with pseudopod-like extensions appearing from the cells' surface (Figure 1F and Table 2) post-reperfusion. The hepatocyte microvilli returned to their normal state within the space of Disse. LSEC apoptosis and phagocytosis by Kupffer cells was noticed in some sections. Hepatocyte morphology remained normal and the mitochondrial crystalline inclusions were persistent.

Morphometric analysis of endothelial cell lining loss

In order to evaluate the apparent loss and re-growth of the LSEC lining, quantitative image analysis was performed. This was expressed as a percentage of intact LSEC lining of the total length of the hepatic sinusoid. There was a quantitatively significant reduction in the lining between baseline and post-ischemia (P = 0.0003) (Figure 3). However, there was no difference between the baseline level and the post-reperfusion level, indicating a recovery of the LSEC lining post-reperfusion.

Gene expression analysis of redox regulating systems

To study the effects on expression of redox proteins during I/R, relative mRNA expression was investigated for genes implicated in the defense against oxidative stress. The gene expression of *NFE2L2* and *SLC7A11*, coding for the redox regulatory proteins NRF-2 and

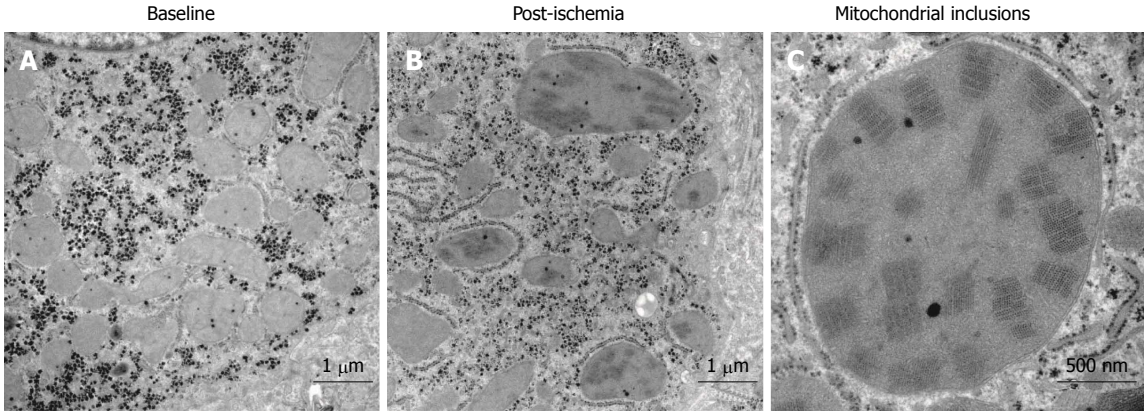

Figure 2 Crystalline mitochondrial inclusions. A: Baseline, before induction of ischemia, shows hepatocyte mitochondria with normal appearance; B: Post-ischemia, showing mitochondria with the crystalline inclusions and a few dilated mitochondria; C: Mitochondrial inclusions, close-up of a single mega-mitochondrion showing the inclusions post-ischemia.

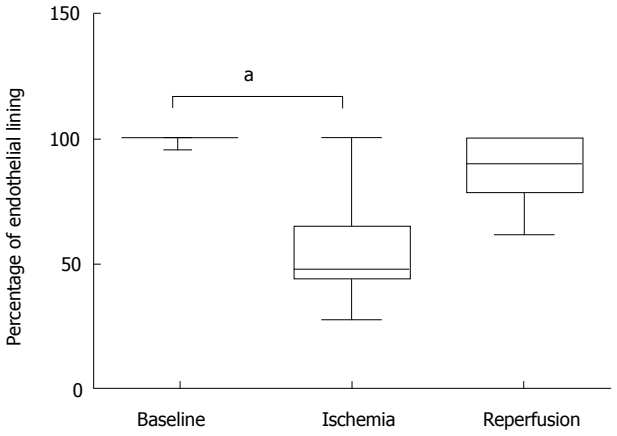

Figure 3 Morphometric analysis of endothelial lining. The percentage of attached endothelial lining along sinusoidal walls was quantified using network information services elements Basic Research Software. Statistical analysis was performed in Graphpad Prism, and differences were determined by the non-parametric Friedman test followed by Dunn's *post-hoc* test ($P < 0.01$). Baseline: Before induction of ischemia; Ischemia: Twenty minutes of ischemia; Reperfusion: Twenty minutes after reperfusion.

xCT, were investigated along with the TRX family of proteins. There were no differences post-ischemia or post-reperfusion compared with baseline (Table 3) of the investigated genes.

Immunoelectron microscopy redox proteins

Immunogold staining for GRX1 and TRX1 was performed in order to study if a translocation of the proteins occurred during I/R. There were no significant changes in the amount of TRX1 either in the nuclei or the cytosol of the hepatocytes during I/R (Figure 4). However, the total amount of TRX differed in the hepatocytes between the time points, and during ischemia the level of TRX decreased in five patients, remained unaffected in three, and increased in two patients (Table 4). The levels of GRX1 did not change in any of the patients during I/R (Figure 4).

DISCUSSION

PTC is an effective method to reduce blood loss during liver resections but is used very selectively in routine clinical practice[22]. In general, the extent of I/R injury depends on the duration and magnitude of the ischemia. This study used PTC to investigate ultrastructural changes associated with warm I/R injury. PTC was carried out for a fixed amount of time in all patients and the liver was not manipulated during the experimental procedure and biopsy acquisition. Thus, controlled experimental conditions were established in order to obtain reliable and comparable data. Since PTC is carried out routinely with a 20 min application time, ethical considerations did not permit a more extensive study. The short ischemia time and defined time points of biopsy acquisition thus limited the experimental scope of this study. The findings, however, provide data from human, eventually facilitating our understanding of the complex pathological alterations associated with warm hepatic I/R injury.

Hepatocytes and the LSECs are the cell types most sensitive to I/R injury. In our study, the most noticeable finding in post-ischemia liver biopsies was the loss of the LSEC lining, which was seen in 10 of 11 patients. This finding was significant as shown by the morphometric analysis. According to the current knowledge, based mainly on animal studies, hepatocytes are more sensitive to warm ischemia and LSECs to cold ischemia[4,23]. Detachment of these specific cells has been previously seen in cold ischemia models in rat[24,25]. On the other hand one animal study reported that LSEC death may precede hepatocyte death in warm I/R injury[26]. Here we show, in the human setting, that the LSECs bore the major impact of warm ischemia, visualized by signs of endothelial cell disruption.

Another striking finding in this study was the formation of pseudopod-like projections from the LSEC surface which has been interpreted as a reactivation or reattachment of the LSECs as a response to reperfusion. This was found in 9 of 11 patients and became evident already after 20 min of reperfusion. This suggests that structural loss of LSEC may be reversible when the duration and magnitude of warm

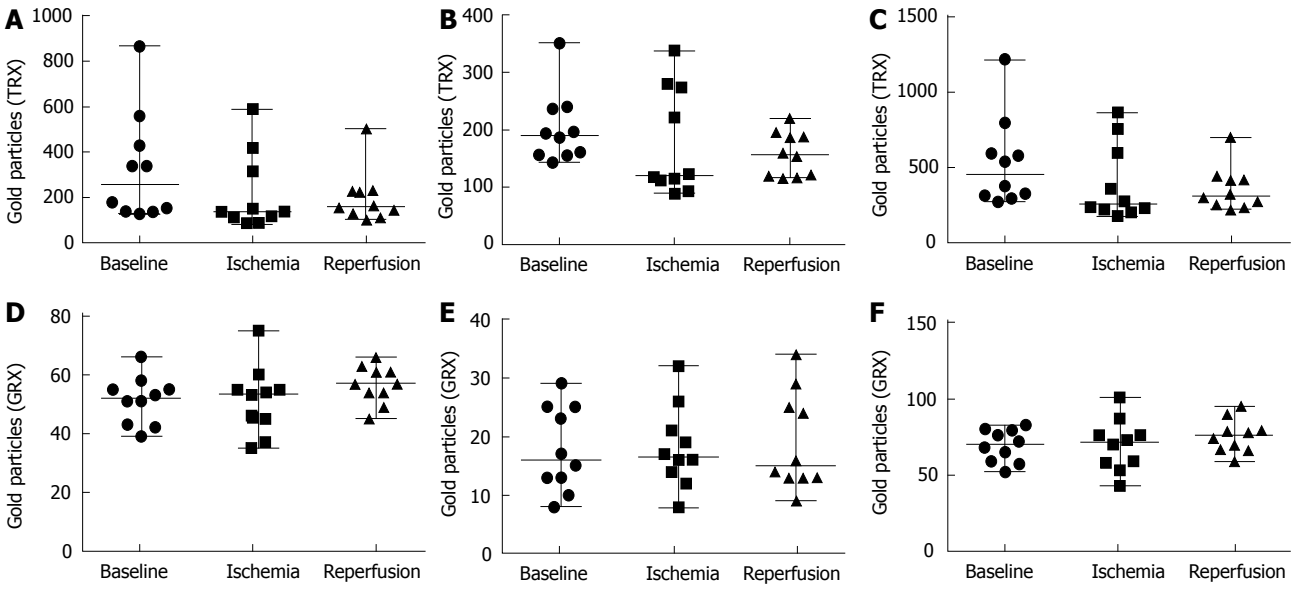

Figure 4 Quantification of immunogold staining. Number of gold particles of 5 hepatocytes for each time point and patient were recorded. A-C: Values from the TRX immunogold staining where A: Quantification of TRX in the cytosol; B: Quantification of TRX in the nuclei; C: Total value from of TRX both in cytosol and nuclei; D-F: Values from the quantification of GRX immunogold staining where D: Quantification of GRX in the cytosol; E: Quantification of GRX in the nuclei; F: Total value of GRX both in cytosol and nuclei. Baseline: Before induction of ischemia; Ischemia: Twenty minute of ischemia; Reperfusion: Twenty minute after reperfusion.

Table 3 Gene expression of redox proteins changes in mRNA expression compared to baseline, calculated using the $2^{-\Delta\Delta CT}$ method

	TNX1	TNX2	TNXR1	TNXR2	GLRX1	GLRX2	GLRX3	GLRX5	NFE2L2	SLC7A11
Ischemia	1.09 ± 0.18	1.14 ± 0.30	1.02 ± 0.28	1.05 ± 0.23	1.19 ± 0.41	1.24 ± 0.46	1.03 ± 0.16	1.20 ± 0.27	0.83 ± 0.15	1.27 ± 1.24
Reperfusion	1.12 ± 0.25	1.31 ± 0.57	0.97 ± 0.33	1.39 ± 0.79	1.12 ± 0.67	1.16 ± 0.43	1.16 ± 0.26	1.18 ± 0.63	1.00 ± 0.44	1.22 ± 0.78

I/R injury is limited. It must be noted, however, that late response to reperfusion was not investigated in this study and secondary phases of injury to the LSECs are therefore unknown, if there are any. In a rat model of liver transplantation cold preservation of donor organs resulted in detachment of LSECs followed by some reattachment after reperfusion[27]. To our knowledge, this phenomenon has never been reported after warm ischemia.

We observed that the hepatocytes lost microvilli in the space of Disse after PTC and some had condensed nuclear chromatin. Furthermore, hepatic mitochondria were dilated and showed the presence of post-ischemic crystalline inclusions which prevailed after reperfusion. Consistent with previous reports, the inclusions almost exclusively appeared in dilated mitochondria[28]. Apart from these findings the overall morphology of hepatocytes was remarkably well preserved. This is in consistency with an earlier report that has shown that the hepatic ultrastructure was unaffected after intermittent PTC, indicating that the tissue might recover from the injury[29].

It is known that early hypoxic changes of the hepatocytes can be detected from the morphological alterations of mitochondria[30]. Mitochondrial crystalline inclusions are commonly seen in early alcohol and non-alcohol related liver diseases and aspirin toxicity[31-33]. These have been previously described as "para"-crystalline inclusions, however, an optical diffraction study showed that they are true crystals. To date, the composition of these inclusions remains unknown[34]. The mitochondria of *E. coli* exhibit crystalline inclusions visually similar to these and may arise from copolymerization of the protein Dps to the bacterial DNA as a protective response against oxidative and nutritional stress[35]. Mitochondrial inclusions could thus be an evolutionarily preserved event and adaptive response to the ischemic state rather than an occurrence secondary to the injury.

Previous studies on oxidative stress in I/R have demonstrated an activation of the transcription factor NRF-2, which regulates a number of redox proteins, including the TRX family of proteins[15,36]. In our study PTC was used as a model of oxidative stress for studying redox proteins from the TRX family and their alterations in I/R injury. However, no changes on the mRNA levels of the redox proteins could be detected during the 20 min of PTC. Immunogold staining for TRX1 and GRX1 showed no changes in the hepatic GRX1 levels, but the levels of TRX1 present in the hepatocytes varied between the time points, suggesting a possible secretion of the protein. Although TRX1 lacks a translocation signal, this protein can be actively secreted through a leaderless

Table 4 Levels of TRX1, evaluated by immunogold staining, total number of gold particles present in five hepatocytes

Patient	Baseline	Post-ischemia	Post-reperfusion
P1	794	357	414
P2	310	202	323
P3	1217	235	219
P4	373	176	298
P5	324	231	232
P6	269	273	443
P7	575	569	252
P8	294	221	699
P9	535	863	417
P10	590	755	272

secretory pathway that is independent of the classical endoplasmic reticulum-Golgi pathway[37]. These findings could support a tentative role for the TRX family of proteins in warm I/R injury, however further studies are needed to elucidate this.

We conclude that in a human experimental model of warm I/R injury the major effect observed at the ultrastructural level was on the non-parenchymal LSECs while hepatocytes morphology remained relatively intact apart from crystalline inclusions in the mitochondria after ischemia. Alterations that arise may be protective adaptations that to some extent seem to be reversible. In situ protein observations were compatible with a tentative role for the thioredoxin family of proteins in I/R injury.

COMMENTS

Background

Portal triad clamping (PTC) is used during liver surgery to reduce blood loss. Limiting the blood supply in a tissue can cause ischemia reperfusion (I/R) injury to the tissue. The ischemic injury occurs initially with a switch from aerobic to anaerobic metabolism. Sudden oxygen burst upon returned vascular flow causes reperfusion injuries related to the production of reactive oxygen species (ROS).

Research frontiers

Primary endpoints of clinical studies concerning the PTC method has involved measurements of liver function test, duration of hospital stay, and post-operative complications. Hepatic tissue injury at the ultrastructural level has been sparsely studied and never has there been a differentiation between the ischemic and reperfusion state due to complexity arising in study design.

Innovations and breakthroughs

This is the first study that investigates ultrastructural changes as a result of warm I/R injury during surgery at each point of tissue insult in the liver.

Applications

Limiting the extent of tissue injury during surgery is important for the post-operative recovery of the patients. Evaluation of morphological changes as a result of PTC can provide insights for clinicians of their preferred methods. The results indicate that the changes on the ultrastructural level upon 20 min of ischemia are mainly localized in the sinusoids with a detachment of liver sinusoidal endothelial cells and a loss of hepatocyte microvilli in the Space of Disse. Upon reperfusion the sinusoids showed a reappearance of some liver sinusoidal endothelial cells. The hepatocytes displayed normal morphology with the exception of crystalline inclusions in mitochondria.

Terminology

Crystalline inclusions are characterized visually by dark lines in the mitochondria in the electron microscope. The true composition of the inclusions remain unknown, however *E.coli* have been recorded to display them as a means of protecting mitochondrial DNA in response to oxidative stress.

Peer-review

The authors present an interesting study on the microstructural alterations of liver in a warm ischemia-reperfusion setting.

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ORIGINAL ARTICLE

Observational Study

Liver decompensation predicts ribavirin overexposure in hepatitis C virus patients treated with direct-acting antivirals

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Author contributions: Guardigni V carried out the statistical analysis and drafting the manuscript; Guardigni V and Badia L developed concept and design of the study; all authors were involved in data collection, interpretation of data and editing of the manuscript.

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Abstract

AIM

To determine whether ribavirin (RBV) concentrations differ according to cirrhosis stage among cirrhotic patients treated with interferon-free regimens.

METHODS

We included patients with hepatitis C virus and cirrhosis [Child-Pugh (CP) A or B], Glomerular Filtration Rate $\geqslant$ 60 mL/min, who started therapy with DAAs and weight-based RBV between October 2014 and February 2016. RBV plasma levels were assessed during the treatment. We focused our analysis on the first 8 wk of therapy.

RESULTS

We studied 68 patients: 54 with compensated (CP-B) and 14 with decompensated (CP-A) cirrhosis. Patients with

decompensated cirrhosis displayed significantly higher RBV concentrations than those with compensated cirrhosis at week 1, 2, 4 and 8 ($P < 0.035$). RBV levels were positively correlated with Hb loss over the treatment ($P < 0.04$). Majority (71%) of CP-B patients required a RBV dosage reduction during the treatment. After adjustment for confounders, Child-Pugh class remained significantly associated (95%CI: 35, 348, $P = 0.017$) to RBV levels, independently from baseline per-Kg RBV dosage.

CONCLUSION

Liver decompensation might affect RBV clearance leading to an overexposure and increased related toxicities in decompensated cirrhosis. Our findings underscore the importance of an early ribavirin therapeutic drug monitoring and suggest that an initial lower RBV dose, rather than weight-based, might be considered in those with advanced liver disease (CP-B) treated with direct-acting antivirals.

Key words: Hepatitis C; Direct-acting antivirals; Ribavirin; Therapeutic drug monitoring; Decompensated cirrhosis

Core tip: In this study, patients with decompensated cirrhosis displayed higher plasma ribavirin concentrations in comparison to compensated patients, when treated with Interferon-free regimens for hepatitis C. Higher ribavirin levels were found to lead to greater rates of related toxicities and Child-Pugh class resulted independently associated with ribavirin plasma levels, in our population. Our findings suggest that ribavirin concentrations should be strictly monitored in subjects with advanced liver disease, during direct-acting antivirals-treatment. An early dosage adjustment of ribavirin should be performed when high levels of this antiviral are detected in patients' plasma, in order to avoid toxicities among these frail individuals.

Guardigni V, Badia L, Conti M, Rinaldi M, Mancini R, Viale P, Verucchi G. Liver decompensation predicts ribavirin overexposure in hepatitis C virus patients treated with direct-acting antivirals. *World J Hepatol* 2017; 9(34): 1270-1277 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i34/1270.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i34.1270

INTRODUCTION

Before the advent of direct-acting antivirals (DAAs), the association of Peg-interferon (Peg-IFN) and ribavirin (RBV) represented the standard of care (SOC) for the treatment of chronic C hepatitis, regardless of hepatitis C virus (HCV) genotype and stage of liver disease[1]. Although HCV-induced cirrhosis represents a major cause of liver-related morbidity and mortality and successful therapy of individuals with advanced fibrosis and liver cirrhosis is associated with better outcomes [*e.g.*, decreased incidence of hepatocellular carcinoma (HCC), decompensation][2], patients with cirrhosis were rarely treated because of the high risk of decompensation due to Peg-IFN administration.

New DAAs-regimens have dramatically changed this scenario, leading to the achievement of high rate of HCV eradication, even in individuals with compensated and decompensated cirrhosis. Although the role of RBV in the era of DAAs will probably decrease, it is currently still recommended for difficult-to-treat patients (*i.e.*, experienced and cirrhotic), to optimize sustained virological response (SVR) in many treatment regimens[3].

Monitoring of ribavirin plasma concentration, given its interindividual variability, was used during combination therapy with Peg-IFN, since RBV exposure had been shown to be associated with treatment efficacy (SVR) and side effects (*i.e.*, anaemia)[4,5]. In particular, trough RBV concentration (C_{trough}) at week 4 and 8 of treatment was commonly used[5-7]. Indeed, steady-state concentrations are reached after the first 4 wk of therapy according to scientific literature[8].

Although higher RBV concentration levels have been associated to higher rates of SVR and therapeutic ranges at week 8 have been established also for first-generation DAAs-based HCV therapies (with telaprevir or boceprevir)[9], role of RBV therapeutic drug monitoring (TDM) in the era of second-generation DAAs regimens has not been investigated and elucidated yet.

Thus far, data on ribavirin TDM among cirrhotic patients with advanced liver disease, treated by the combination of RBV and last generation DAAs are lacking.

Multiple factors are involved in RBV pharmacokinetics consequently affecting RBV plasma concentrations, such as creatinine clearance, gender, age, diet, HIV infection, weight[3,10]. Child-Pugh score, a marker of cirrhosis severity, used to estimate the prognosis in these patients, has never been investigated as a determinant of RBV plasma concentrations during HCV treatment.

The aim of our retrospective study was to determine whether ribavirin plasma levels differ according to cirrhosis stage (defined by Child-Pugh class) in a cohort of subjects with liver cirrhosis, treated for HCV with IFN-free DAA-regimens containing RBV and to define effects of plasma RBV levels.

MATERIALS AND METHODS

We retrospectively included in this study all the patients with chronic C hepatitis and cirrhosis (Child-Pugh A or B), without significant renal impairment [Estimated Glomerular Filtration Rate (eGFR) $\geqslant$ 60 mL/min], who started HCV treatment with DAAs in association with ribavirin (weight-based dose $\geqslant$ 11 mg/kg per die) between October 2014 and February 2016 at Infectious Diseases Department of University Hospital of Bologna (Italy). We excluded from the analysis two subjects

who had less than two available plasma ribavirin level measurements throughout the antiviral therapy. Patients who received Peg-IFN in combination with DAAs and RBV were excluded from the study. All the patients were treated for 12 or 24 wk according to the national and international guidelines and were afterwards monitored for at least 12 wk after completing the therapy to assess SVR (SVR12). Patients were categorized into two groups (compensated or decompensated cirrhosis) according to their Child-Pugh score (< 7 or ⩾ 7, respectively) to perform the analysis of interest.

For each subject, we collected the following data at baseline (corresponding with day of treatment start): Demographics (sex, age, race), medical history on previous HCV treatment (naïve or experienced), on chronic C hepatitis (HCV genotype, HCV RNA quantification) and on liver diseases (fibrosis stage, history of previous HCC, Child-Pugh class), HIV and HBV coinfections, creatinine level, eGFR, haemoglobin (Hb), bilirubin, current anti-HCV regimen (administered DAAs and Ribavirin initial dosage).

Data on RBV dose reduction over the course of treatment, therapy duration and response to the HCV treatment (as SVR12) were also recorded. Liver fibrosis was assessed according to the Metavir score either from elastographic measurements or from liver biopsy prior to HCV treatment. HCV RNA levels were detected by using Roche COBAS AmpliPrep/COBAS TaqMan HCV Test v 2.0 (Roche Molecular Systems, Inc., Pleasanton, CA). eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration formula.

Reasons for RBV dosage adjustment were the following: Occurrence of anaemia (Hb decline of more than 20 g/L or Hb less than 100 g/L), high RBV trough concentration (> 2500 ng/mL), that was considered at high risk for significant Hb decline[11,12], and other adverse effects (*e.g.*, cutaneous rash, itch).

All the subjects' visits and blood samples were standardized according to treatment schedule and performed at baseline and at week 1, 2, 4, 8, 12, 16, 20, 24 over treatment period. For each patient RBV concentrations were quantified using plasma samples that were collected multiple times during the treatment (average of 6.5 samples per subject), although we mainly focused our analysis on the first 8 wk of treatment because steady concentrations are usually reached between week 4 and 8[8] and because changes in RBV concentrations after week 8 (due to dosage adjustment) were expected to occur in our population.

RBV concentrations were measured in plasma samples, collected before taking the next dose of the drug (C_{trough}), using a novel analytical method, validated according to the ISO 15089 predicaments. In brief, Ribavirin is extracted from plasma by means of a methanol-water (20%-80% mL/L) solution containing $ZnSO_4$ (0.1 mol/L in water). The drug is then resolved chromatographically from endogenous and exogenous isobaric interferences by means of a binary chromatographic gradient. Specific signals for the drug are obtained in multiple reaction monitoring mode, recording the 245.1 > 113.2 and 245.1 > 96.2 m/z mass transitions. Ion extraction chromatograms enable drug determination with great specificity and sensitivity (Limit of Quantification = 1 μg/L).

Statistical analysis

Variables were expressed as mean ± SD or median and range interquartile. Continuous variables were compared by *t*-test or by Mann-Whitney-*U* test, when distribution was normal or not normal, respectively. Categorical variables were compared by chi-square test or Fisher's exact as appropriate. Bivariate association between RBV plasma levels and continuous variables of interest were tested by Spearman rank correlations. Linear regression was first performed to evaluate factors associated with RBV plasma levels in the univariate analysis and afterward to determine independent predictors of RBV plasma levels among variables with significant result at the univariate. All statistical analysis were performed using SPSS software version 21. Tests were considered significant for a *P* value < 0.05.

RESULTS

Characteristics of population

We totally evaluated 68 cirrhotic patients who underwent anti-HCV treatment with DAAs in association with RBV in the study period: 54 had a compensated liver cirrhosis and 14 a decompensated cirrhosis (Child-Pugh A, scored < 7 and B, scored ⩾ 7, respectively). Baseline characteristics of our population are shown in Table 1. No differences in demographics were found between the two groups. Fifty-seven point four percent of the population was composed by HIV co-infected patients who showed a greater, but not significantly, prevalence in decompensated subjects (53.7% *vs* 71.4%). Most of the subjects (63.2%) had been previously treated with Peg-IFN-based regimen, unsuccessfully. Genotype 1a was the most common HCV genotype (33.8%), as well as the association of Sofosbuvir (SOF, 400 mg/die) and Daclatasvir (DCV, 60 mg/die, or 30 mg/die in 13 HIV patients taking atazanavir/rtv-containing antiretroviral regimens) was the most frequently used (42.6%) in the overall population, without any difference according to Child-Pugh class. As expected, significant differences in haemoglobin and total bilirubin levels were detected, before starting antiviral therapy between the two groups: Indeed compensated cirrhotic patients showed higher mean Hb (146 ± 16 g/L *vs* 129 ± 17 g/L, *P* = 0.002) and lower median bilirubin levels [0.47, interquartile range (IQR) 2.8-8.2 mg/L *vs* 7.7 mg/L, IQR 5.3-16, *P* < 0.001] compared to decompensated patients. Creatinine values resulted higher in compensated patients (*P* = 0.011), although eGFR was similar in the two groups. 86.8% of subjects was treated for 24 wk, 13.2% for 12 wk.

Table 1 Subjects' baseline characteristics

Characteristics	Overall (*n* = 68)	Child pugh A (*n* = 54)	Child pugh B (*n* = 14)	*P* value
Age (yr), mean ± SD	55 ± 8.6	55.2 ± 9.5	54.6 ± 5	0.8
Male, *n* (%)	52 (76.5)	43 (79.6)	9 (64.3)	0.2
White race, *n* (%)	66 (97.1)	53 (98.1)	13 (92.9)	0.3
HIV infection, *n* (%)	39 (57.4)	29 (53.7)	10 (71.4)	0.2
HBsAg positive, *n* (%)	1 (1.5)	1 (1.9)	0	-
History of HCC, *n* (%)	6 (8.8)	4 (7.4)	2 (14.3)	0.6
HCV treatment experienced, *n* (%)	43 (63.2)	33 (61.1)	10 (71.4)	0.5
Liver stiffness (kPa), median (IQR)	21.4 (15.9-34.4)	21.2 (15.6-33.1)	31.1 (17.8-54.3)	0.1
HCV genotype, *n* (%)				0.7
1a	23 (33.8)	18 (33.3)	5 (35.7)	
1b	11 (16.2)	9 (16.7)	2 (14.3)	
2	6 (8.8)	6 (11.1)	-	
3	22 (32.4)	17 (31.5)	5 (35.7)	
4	6 (8.8)	4 (7.4)	2 (14.3)	
Weight (kg), mean ± SD	73 ± 14.1	73.2 ± 15.3	72.1 ± 8.5	0.7
Hb baseline (g/L), mean ± SD	143 ± 18	146 ± 16	129 ± 17	0.002
Bilirubin (mg/L), median (IQR)	9.5 (5.6-19)	4.7 (2.8-8.2)	7.7 (5.3-16)	< 0.001
Creatinine (mg/L), mean ± SD	8 ± 1.3	8.2 ± 1.5	7.5 ± 0.8	0.011
eGFR, (mL/min per 1.73 m^2), median (IQR)	101 (90-109)	99 (88-105)	102 (98-107)	0.3
HCV-RNA baseline (IU/mL)	933559 (25667-2128936)	1228876 (410822-2940050)	246455 (126308-712307)	0.007
[1]DAAs regimen, *n* (%)				0.2
SOF, *n* (%)	6 (8.8)	6 (11.1)	-	
SOF + DCV, *n* (%)	29 (42.6)	21 (38.9)	8 (57.1)	
SOF + SMP, *n* (%)	1 (1.5)	-	1 (7.1)	
DCV + SMP, *n* (%)	4 (5.9)	3 (5.6)	1 (7.1)	
SOF + LDV, *n* (%)	12 (17.6)	11 (20.4)	1 (7.1)	
OBV/PTV/r + DSV, *n* (%)	12 (17.6)	9 (16.7)	3 (21.4)	
OBV/PTV/r, *n* (%)	4 (5.9)	4 (7.4)	-	

[1]All regimens are intended in association with ribavirin. HCC: Hepatocellular carcinoma; eGFR: Estimated glomerular filtration rate; DAAs: Direct-acting antivirals; SOF: Sofosbuvir; DCV: Daclatasvir; SMP: Simeprevir; LDV: Ledipasvir; OBV: Ombitasvir; PTV: Paritaprevir; R: Ritonavir; DSV: Dasabuvir.

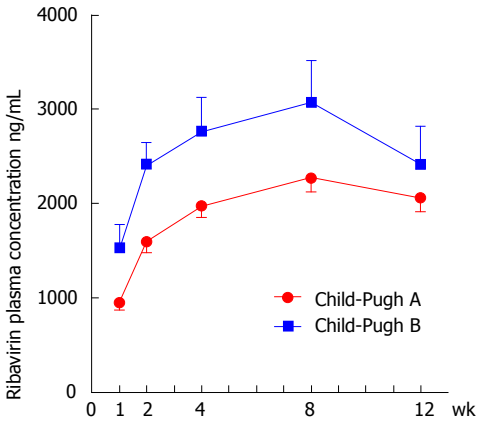

Figure 1 Ribavirin plasma concentrations over the treatment. Error bars represent standard errors. Ribavirin plasma concentrations statistically differ between Child-Pugh A and B patients at week 1, 2, 4 and 8 (all *P* value < 0.025). Legend: Child-Pugh B patients display significantly higher ribavirin concentrations (expressed as C trough) compared to Child-Pugh A patients over the first 8 wk of treatment with direct acting antivirals.

Ribavirin concentrations and dosage

There was no difference in initial mean dose of RBV (mg/kg) between the two groups (*P* = 0.3). Significant differences in RBV plasma concentrations between the two groups were found at each considered time-point (week 1, 2, 4 and 8) with much higher *Ctrough* levels among decompensated (Child-Pugh B) than among compensated (Child-Pugh A) patients. Otherwise, no remarkable difference in overall RBV mean values (measured during all the course of treatment) was revealed (Table 2).

Figure 1 shows the significant different trends of RBV plasma concentration from baseline until week 12 in the two cirrhotic groups.

In almost three-quarters (71.4%) of Child-Pugh B patients a reduction of RBV dosage was required during the treatment period, instead only 50% of Child-Pugh A subjects experienced a dosage adjustment (*P* = 0.15), after an average of 6.8 and 7.1 wk from treatment start among Child-Pugh A and B, respectively. Most common reasons for RBV dosage reduction were onset of anaemia (36.1%) and high RBV concentration revealed by TDM (36.1%), data not shown. Although decompensated patients experienced greater Hb loss (at both week 4 and week 8) than those with compensated cirrhosis, the differences were not significant (Table 2).

Multiple factors potentially associated with RBV concentration were investigated at univariate analysis. The significant predictors of RBV concentrations (as a treatment average between week 1 and 8) were baseline RBV dose per kilogram (β value 205, 95%CI: 77; 332, *P* = 0.002), male gender (β value -539, 95%CI: -975; -103, *P* = 0.016) and Child-Pugh class (β value 232, 95%CI: 66; 399, *P* value= 0.007). After adjusting for all these significant variables, only baseline

Table 2 Ribavirin dosages and plasma levels (C trough) and haemoglobin loss over the course of treatment

Parameters	Overall (*n* = 68)	Child pugh A (*n* = 54)	Child pugh B (*n* = 14)	*P* value
RBV (mg/kg pre die), mean ± SD	14.4 ± 1.7	14.4 ± 1.36	14.9 ± 1.6	0.3
[1]RBV week 1 (ng/L) median (IQR)	9105 (6130-1310)	8750 (5700-11680)	14050 (9010-22280)	0.007
[1]RBV week 2 (ng/L) median (IQR)	15000 (11600-27680)	14150 (11150-20000)	23550 (17200-28930)	0.001
[2]RBV week 4 (ng/L) median (IQR)	18300 (12900-27680)	17850 (12200-26500)	26250 (17700-35130)	0.024
[3]RBV week 8 (ng/L) median (IQR)	23100 (17050-31000)	21500 (16100-27950)	33000 (19800-41380)	0.0034
Mean RBV 1, 2, 4 wk, (ng/L) median (IQR)	14240 (10770-19450)	13280 (10480-18200)	19600 (14670-26870)	0.005
Mean RBV 1, 2, 4, 8 wk, (ng/L) median (IQR)	16490 (12220-21750)	15135 (11850-19830)	21700 (15090-29280)	0.006
Mean RBV, full treatment duration (ng/L), mean ± SD	18079 ± 6609	17528 ± 6020	20327 ± 7700	0.2
RBV dosage reduction, *n* (%)	37 (54.4)	27 (50)	10 (71.4)	0.2
Weeks before reduction, median (IQR)	6.1 (4-9.8)	5 (4-9.7)	7 (4-10)	0.6
Hb loss week 4 (g/L), mean ± SD	19.6 ± 1.8	19 ± 13	21 ± 32	0.5[4]
Hb loss week 8 (g/L), mean ± SD	21 ± 19	20 ± 13	25 ± 35	0.3[4]

[1]Data for 68 subjects; [2]Data for 62 subjects; [3]Data for 95 subjects; [4]Analysis of covariance of Hb at week 4 and haemoglobin (Hb) at week 8, adjusted for baseline Hb levels. RBV: Ribavirin; Hb: Haemoglobin; IQR: Interquartile range.

Table 3 Baseline predictors of average plasmatic Ribavirin levels in the first 8 wk of therapy

	Univariate analysis			Multivariate analysis		
	β value	95%CI	*P* value	β value	95%CI	*P* value
Baseline ribavirin dosage (mg/kg)	205	77; 332	0.002	161	36; 286	0.013
IFN-experienced	97	-303; 497	0.6			
Male gender	-539	-975; -103	0.016	-349	-765; 68	0.099
Age (yr)	13	-9; 34	0.3			
White race	424	-714; 1563	0.5			
Bilirubin (mg/L)	870	-920; 2670	3			
Creatinine (mg/L)	-2890	-16830; 11040	7			
Child-Pugh class	232	66; 399	0.007	192	35; 348	0.017
Liver stiffness (kPa)	-1.6	-14; 11	0.8			
HIV infection	76	-314; 466	0.7			

IFN: Interferon; HIV: Human immunodeficiency virus.

RBV dosage (β value 161.1, 95%CI: 35.6; 286, *P* = 0.013) and Child-Pugh class (β value 191.7, 95%CI: 35; 348, *P* = 0.017) remained significantly associated to RBV levels (Table 3). To investigate whether the association with Child-Pugh class was driven by single items of this composite index, we analysed each of them (*i.e.*, Encephalopathy, Ascites, Bilirubin, INR, Albumin at baseline) in relationship to RBV concentrations, but no significant differences in RBV overexposure were observed. Moreover, clearance of RBV (indirectly assessed by its plasma concentrations) did not seem to be related to portal hypertension signs (*e.g.*, platelet count, esophageal varices), data not shown.

Remarkably, there were no significant differences in RBV concentration (as an average value obtained from the first 8 wk measurements) between subjects who achieved a rapid virological response (RVR, namely HCV undetectability within week 4 of treatment) and those who did not (Figure 2). Furthermore, no correlation between RBV concentrations (at any time-point) and time to reach HCV undetectability was observed in our population (data not shown). The majority of patients included in this study (92.6%) achieved SVR12, without any difference in SVR rate based on Child-Pugh class and RBV levels at week 4 and 8 (data not shown).

Haemoglobin loss

As depicted in Figure 3, Hb loss (g/L) in the first two months of treatment (at the two therapy time-points: Week 4 and 8) was correlated to RBV concentrations measured at the corresponding week (R = + 0.272, *P* value= 0.033 at week 4, R = + 0.279, *P* = 0.025 at week 8), with higher RBV levels associated with greater Hb decline. The relationship between Hb loss at week 8 and RBV levels at that specific week was independent from Child-Pugh class (in a multivariate linear regression, *P* = 0.002).

DISCUSSION

The advent of new IFN-free DAAs regimens has allowed to achieve very high rates of HCV eradication, even in patients with advanced liver disease. Although the role of RBV is now discussed and its relevance will probably decrease over time, this antiviral drug is still recommended in combination with DAAs in several challenging situations: For treatment-experienced but DAAs-naïve patients without cirrhosis or with compensated cirrhosis (Child-Pugh A) to reduce weeks of therapy, for subjects who failed to

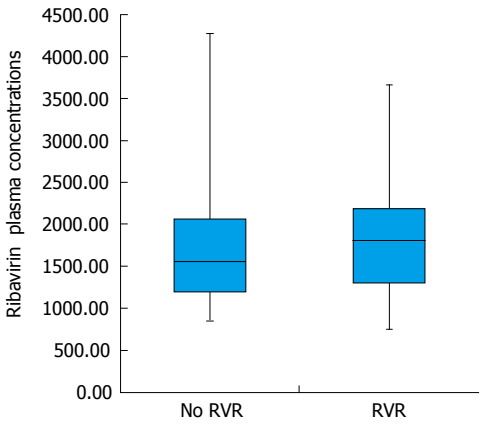

Figure 2 Average of ribavirin plasma concentrations between week 1 and 8 of treatment in association with rapid virological response. Legend: Achievement of Rapid Virological Response (*i.e.*, hepatitis C virus RNA undetectability within week 4 of treatment) was not associated with ribavirin plasma concentrations measured over the first 8 wk of treatment. RVR: Rapid virological response.

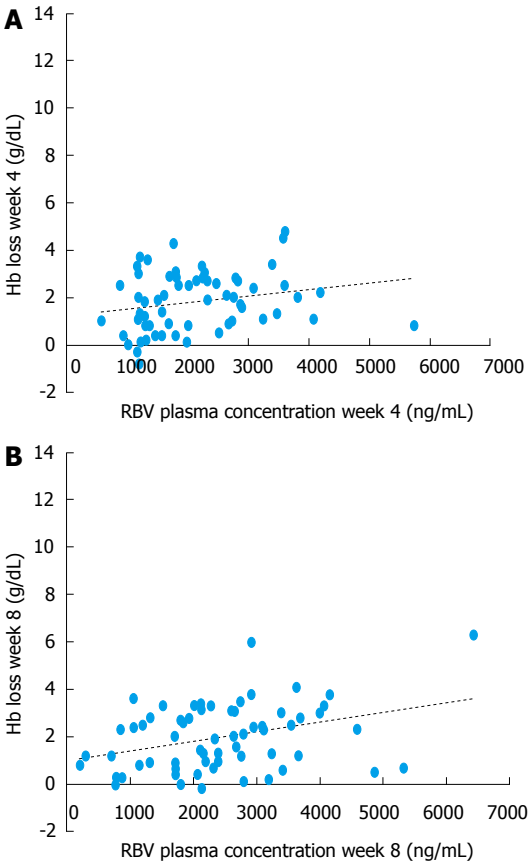

Figure 3 Correlation between ribavirin plasma concentrations and haemoglobin loss at week 4 and 8. Legend: Higher ribavirin plasma concentrations resulted significantly correlated with greater haemoglobin loss at both week 4 (A) and week 8 (B) of therapy in the overall population. RBV: Ribavirin.

achieve SVR on prior antiviral therapy containing DAAs and for those with decompensated cirrhosis (Child-Pugh B or C) to increase SVR rates[13].

To date, specific data regarding ribavirin TDM in cirrhotic patients treated with IFN-free regimens are lacking and daily weight-based ribavirin remains the SOC. In particular, a daily dose of ribavirin higher than 10 mg/kg of body weight had been previously associated to higher rate of SVR among subjects treated with Peg-IFN and RBV[14] and it is still used with DAAs, although the use of RBV according body weight has been occasionally disapproved due to the variability in this antiviral elimination phase[4].

In our study, patients with decompensated cirrhosis (Child-Pugh B) remarkably displayed higher plasma levels of RBV during the first 8 wk of therapy with DAAs compared to those with compensated cirrhosis (Child-Pugh A), although initial weight-dosages were similar. This raises the hypothesis that liver failure might play a meaningful role in RBV clearance, not observed before.

The fact that overall RBV mean plasma concentrations did not differ based on Child-Pugh class in our cohort might be explained by the greater rate of RBV dose reduction (but no discontinuation) and the following RBV exposure decline in those with decompensated cirrhosis, over the course of treatment.

Reduced dosages of ribavirin are currently recommended in patients with moderate or severe renal impairment (Creatinine Clearance $\leqslant$ 60 mL/min), since RBV exposure is predicted to increase up to 17% in this subsets of patients, due to its renal excretion[15]. Also weight, gender, age, fat meals have been shown to clearly affect RBV metabolism[3]. Gastrointestinal tract is another potential site of RBV clearance[16].

To date, no adjustment in ribavirin dosage is required in liver dysfunction, which instead affects metabolism of many drugs[17], since so far liver failure did not seem to impact on ribavirin clearance.

Only Glue[18] assessed the single dose pharmacokinetics of this antiviral in subjects with various degrees of chronic liver disease: In agreement to our results, they observed that C_{max} significantly increased with the severity of liver dysfunction, although there was a remarkable overlap in individual C_{max} values among the groups and they eventually did not propose any dosage reduction in these patients.

Contrarily, we first evaluated RBV pharmacokinetics in patients with liver disease over the course of the treatment in a real-world setting, obtaining then different findings. One could argue that a slower clearance of the drug in our population was due to a cirrhosis-related renal dysfunction (*i.e.*, hepatorenal syndrome), but we included only subjects with eGFR $\geqslant$ 60 mL/min and unexpectedly creatinine was not found to be associated to RBV concentrations over the first 8 wk at univariate analysis in our population.

Conversely, a higher Child-Pugh score determined a RBV overexposure, leading to high rate of dose adjustment in those with Child-Pugh $\geqslant$ 7. To our knowledge, this is the first study exploring ribavirin TDM in decompensated cirrhotic individuals treated with new DAAs regimens and showing an overexposure in this group of patients. Indeed, while few data report RBV plasma levels in therapeutic range in patients with

Child-Pugh A treated with a fixed dose (800 mg daily) of RBV associated with SOF and DCV[19], no information about Child-Pugh B or C patients are currently available. These findings might be useful to clinicians, since an overexposure can be modulated by lowering the initial RBV dosage from the weight-based dosage in certain patients with advanced liver disease, avoiding secondary and detrimental effects.

Furthermore, our data show a positive correlation between RBV concentrations and Hb loss, as already widely reported in literature, even in the context of IFN-free regimens[20]. This finding strengthens the importance of considering a lower RBV dosage in subjects with decompensated cirrhosis, also considering their higher rate of baseline anaemia characterizing this group of patients (confirmed also in our population). Indeed, anaemia occur in about 75% of patients with chronic liver disease and can be linked to different aetiologies (*e.g.*, haemorrhage, splenomegaly)[21], regardless of the use of anti-HCV drugs, such as ribavirin, and represents a condition to be taken into account in the management of cirrhotic patients undergoing anti-HCV treatment.

According to our results, higher RBV concentrations in the first weeks of therapy did not seem to enhance RVR rate in cirrhotic patients treated with IFN-free regimens and no relationship between RBV plasma concentration at week 4 or 8 and rate of SVR was found. This might appear in contrast with prior data regarding Peg-IFN/RBV[22] or, more recently, SOF/RBV treatment (*e.g.*, FISSION, NEUTRINO and FUSION trials)[23,24], in which RBV exposure was one of the factor associated with SVR, with suggested RBV plasma concentration threshold between 2000 and 3000 ng/mL[25,26]. In our cohort, the low rate of patients failing to achieve SVR (7.4%) could explain the lack of relationship between RBV levels and SVR rate: It is in general difficult to identify statistically significant predictive markers with such a high response rate.

This study has some limitation to be considered. The main limitation is the absence of C_{max} and AUC data, due to the observational nature of the study; however C_{trough} can be considered in clinical practice as a surrogate of plasma exposure and its values correlate with drug efficacy and toxicity in other studies[27]. Moreover, this is a retrospective study with a limited population size and this suggests caution prior to extend our results to the all the population with liver cirrhosis treated with RBV and DAAs. The inclusion of patients with mild renal impairment (eGFR 60-89 mL/min) could represent a confounding factor, although baseline creatinine was not found to affect RBV concentrations in our population.

In conclusion, liver failure might affect RBV clearance leading to an overexposure and increased related toxicities (*e.g.*, anaemia) in subjects with decompensated cirrhosis who are now being treated with last-generation DAAs. Our findings underscore the importance of RBV plasma monitoring and early dose adjustments in these patients and suggest that an initial lower dose, rather than weight-based, of RBV might be considered in individuals with advanced liver disease (CP B class) treated with new DAAs in order to reduce toxicities without increasing virological failure rates.

This might improve the knowledge on the tailored use of ribavirin within IFN-free regimens for HCV treatment, in subjects with advanced liver disease. Further studies will be needed to confirm our results in order to determine the optimal dosage of ribavirin in patients with advanced cirrhosis in the era of DAAs-based regimens, since difficult-to-treat patients remain a challenge in HCV treatment and maintaining the benefit of this effective antiviral is still a relevant issue.

COMMENTS

Background

In the era of new direct-acting antivirals (DAAs), ribavirin (RBV) is still recommended for difficult-to-treat hepatitis C virus (HCV) positive patients, but data on RBV therapeutic drug monitoring (TDM) among patients with advanced liver disease treated with RBV and DAAs are lacking.

Research frontiers

TDM represent an innovative technique and is currently widely used approach to personalize and ameliorate anti-infective treatments.

Innovations and breakthroughs

This is the first study showing that patients with decompensated cirrhosis displayed significantly higher RBV concentrations than those with compensated cirrhosis, when treated with new Interferon-free regimens.

Applications

These findings might improve the knowledge on the tailored use of ribavirin within IFN-free regimens for HCV treatment in subjects with advanced liver disease, for whom a lower initial dosage of RBV might be considered, in order to reduce risks related to RBV overexposure.

Terminology

DAAs: Direct-acting antivirals represent the current standard of care for Chronic C Hepatitis; RBV: Ribavirin is an old-fashioned antiviral used in combination with last generation DAAs for difficult-to-treat HCV patients; TDM: Therapeutic drug monitoring by means of blood measurement of a drug concentration; *C trough*: Plasma concentration of a drug measured before taking the next dose of the drug.

Peer-review

This paper offers important approach to the topic and treatment algorithm.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Influence of proton pump inhibitors in the development of spontaneous bacterial peritonitis

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Abstract

AIM

To investigate whether the use of proton pump inhibitors (PPIs) increases the incidence of spontaneous bacterial peritonitis (SBP) in patients with cirrhosis and ascites.

METHODS

An historical cohort study was carried out in cirrhotic outpatients with ascites followed in a specialized clinic at a tertiary hospital in Southern Brazil. Patient charts were reviewed to collect information on the variables of interest as the use of PPIs. Primary outcome was defined as development of SBP during the study period. SBP was diagnosed based on ascitic fluid polymorphonuclear cell count $\geqslant$ 250 cells/mm^3 without evidence of an intra-abdominal, surgically treatable source of infection.

RESULTS

Of 738 cirrhotic patients, 582 (58.2% male) were enrolled, with mean age of 53.6 ± 12 years. Hepatitis C virus infection (36.2%) and alcohol abuse (25.6%) were the main etiologies of cirrhosis. The presence of ascites was detected in 299 (51.4%) patients during the development of the study. Nineteen patients with previous diagnosis of SBP undergoing secondary prophylaxis and 22 patients with insufficient PPI data were further excluded. Of 258

patients with ascites, 151 used PPIs, and 34 developed SBP (22.5%). Among 107 non-users of PPIs, 23 developed SBP (21.5%) (HR = 1.44, 95%CI: 0.85-2.47, *P* = 0.176). The median follow-up time of patients using PPI was 27 mo *vs* 32 mo for non-users. Univariate analysis of the risk factors associated with the development of SBP revealed a significant association of SPB with the severity of liver disease according to the Child-Turcotte-Pugh (CTP) score. Multivariate analysis confirmed that CTP score was the only independent variable influencing the occurrence of SBP. Survival at 60 mo (Kaplan-Meier analysis) was similar in users and non-users of PPI, independently of the presence of SBP (58.4% *vs* 62.7% respectively, *P* = 0.66). For patients with SBP, survival at 60 mo was 55.1%, *vs* 61.7% in patients without SBP (*P* = 0.34).

CONCLUSION

In conclusion, the rate of SBP was not significantly different in users or non-users of PPIs in this cohort of cirrhotic with ascites.

Key words: Cirrhosis; Bacterial infection; Spontaneous bacterial peritonitis; Proton pump inhibitors; Ascites

Core tip: The aim of the present study was to investigate whether the use of proton pump inhibitors (PPIs) increases the incidence of spontaneous bacterial peritonitis (SBP) in patients with cirrhosis and ascites. An historical cohort study was carried out with cirrhotic patients. The primary outcome was development of SBP. Of 258 patients with ascites, 151 used PPIs, and 34 developed SBP (22.5%). Among 107 non-users of PPIs, 23 developed SBP (21.5%) (HR = 1.44, 95%CI: 0.85-2.47, *P* = 0.176). In conclusion, the use of PPIs does not increase the incidence of SBP in patients with cirrhosis and ascites.

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INTRODUCTION

The incidence and severity of bacterial infections have been reported to be greater in cirrhotic patients as compared to the general population[1]. In fact, there is evidence that bacterial infections are the cause of death in up to 25% of patients with cirrhosis[2], leading to a four-fold increase in mortality in this population[3]. Supporting this information, a study conducted in our center analyzed 541 consecutively hospitalized cirrhotic patients, revealing the presence of infection in 25% of the cases. In that study, the mortality of infected patients was also four-fold higher as compared to non-infected patients[4]. In addition, infection may trigger other typical complications associated with increased morbidity and mortality in cirrhosis[5,6].

Spontaneous bacterial peritonitis (SBP) is the most characteristic infection in cirrhosis, and prompt recognition and treatment are required to reduce the associated morbidity and mortality.

Bacterial translocation has been described as a key mechanism in SBP development. Small intestinal bacterial overgrowth potentially promotes bacterial translocation[7,8]. Thus, it has been speculated that chronic acid suppression by proton pump inhibitors (PPIs) - which favors gastric and duodenal bacterial colonization - may contribute to small intestinal bacterial overgrowth and consequently increase the incidence of SBP[9].

Nevertheless, there is some controversy regarding the role of PPIs in SBP. The findings of observational studies suggesting PPIs as a risk factor for SBP[10-12] have been supported by retrospective studies[13-19] and meta-analyses[20,21] providing evidence of increased SBP incidence associated with PPI use; however, recent studies by Mandorfer *et al*[22] and Terg *et al*[23] have not observed this relationship. The present study aimed to investigate the association of PPI treatment with the incidence of SBP in a cohort of outpatients with cirrhosis and ascites.

MATERIALS AND METHODS

This historical cohort study included outpatients with a diagnosis of cirrhosis treated in the Portal Hypertension Clinic at Hospital Santa Casa de Misericórdia de Porto Alegre, a tertiary hospital in the Southern Brazil, between March 2005 and March 2014.

The diagnosis of cirrhosis was confirmed by clinical, laboratory, and imaging data, endoscopy or histologic examination. Outpatient follow-up of at least 1 year was required for inclusion in the study. Primary outcome was defined as development of SBP during the study period.

Patient charts were reviewed to collect information on the variables of interest: Age, sex, etiology of liver disease, Child-Turcotte-Pugh (CTP) score[24] and Model for End-Stage Liver Disease (MELD) score[25], comorbidities, continuous medications (including but not restrict to PPIs), lifetime, hospital admissions, and complications including ascites, SBP, upper gastrointestinal bleeding. At each outpatient visit, serum levels of albumin, creatinine, bilirubin, platelets, and prothrombin time were recorded.

Exclusion criteria were lack of diagnostic confirmation of cirrhosis, co-infection with human immunodeficiency virus (HIV), diagnosis of advanced hepatocellular carcinoma (beyond the Milan criteria)[26] at the first outpatient consultation, and missing clinical data. In addition, in patients with ascites at the moment of enrolment and those undergoing secondary prophylaxis

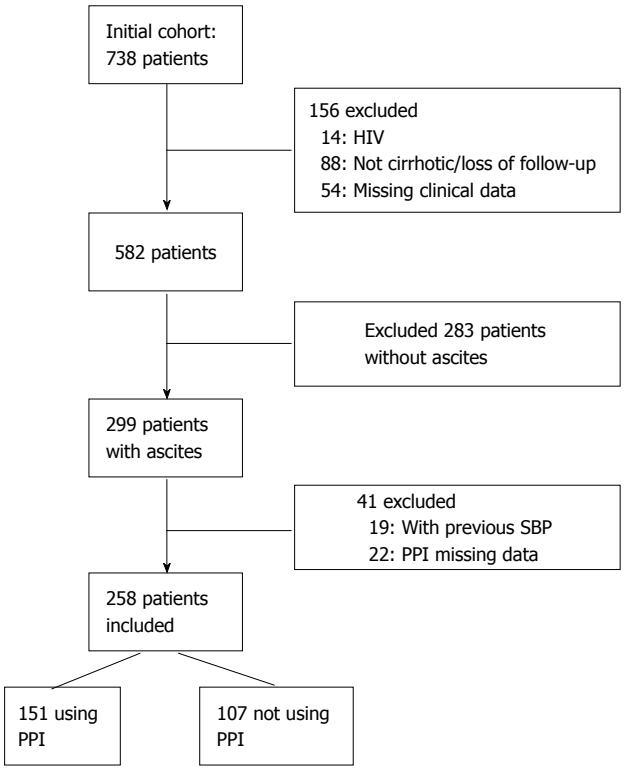

Figure 1 Flow diagram of inclusion. PPI: Proton pump inhibitor; HIV: Human immunodeficiency virus.

due to prior diagnosis of SBP were excluded. PPI treatment was defined as continuous when in use for at least 3 mo. Indications for PPI treatment were determined based on chart review.

The primary outcome, SBP, was diagnosed based on ascitic fluid polymorphonuclear cell count $\geqslant$ 250 cells/mm^3 without evidence of an intra-abdominal, surgically treatable source of infection[7,27,28]. The study was approved by the Research Ethics Committee at Hospital ISCMPA (protocol 3675/11).

Statistical analysis

Continuous data were expressed as means and SD or medians and interquartile range in case of non-Gaussian distribution. Categorical variables were expressed as numbers and percentage. Student's *t* test was used for comparison of means, and Mann-Whitney's *U* test for comparison of medians. Categorical data were compared using the χ^2 test or Fisher's exact test. The incidence of SBP during the follow-up period was estimated using the Kaplan-Meier (KM) method. The comparison of KM curves of users *vs* non-users of PPI was performed using the log-rank test. The magnitude of the association between PPI use and presence of SBP was expressed as hazard ratio (HR) with 95%CI, and calculated using a Cox proportional hazards model adjusted for CTP and MELD scores and the presence of upper gastrointestinal bleeding. Data were processed and analyzed using SPSS v. 22.0 at a significance level of $P = 0.05$.

RESULTS

Of 738 eligible patients, 156 were excluded: 14 patients with HIV, 88 without diagnostic confirmation of cirrhosis or loss of follow-up, and 54 with missing clinical data. The mean age of the 582 patients included in the initial sample was 53.6 ± 12 years, and 58.2% were male. Hepatitis C infection (36.2%) and alcohol abuse (25.6%) were the main etiologies of cirrhosis. Median outpatient follow-up was 5 years.

The presence of ascites was detected in 299 (51.4%) patients during the development of the study. A further 19 patients with a previous diagnosis of SBP undergoing secondary prophylaxis and 22 patients with insufficient PPI data were excluded. Thus, 258 patients with ascites were selected for follow-up (Figure 1). The median follow-up time of patients using PPI was 27.1 (3-60) mo *vs* 32.2 (7-60) mo for non-users of PPI. The patients were using a standard dose of 20 mg qd of omeprazole, the medication available free of charge in the public health system.

Demographic, clinical, and laboratory data of users and non-users of PPI are shown in Table 1. No significant differences were detected between the groups. Of 151 users of PPI, 34 (22.5%) developed SBP *vs* 23 (21.5%) of 107 non-users of PPI. This comparison was not statistically significant (HR = 1.44, 95%CI: 0.85-2.47, $P = 0.176$) (Figure 2).

Univariate analysis of the risk factors associated with the development of SBP revealed a significant association with the severity of liver disease according to the CTP score. Multivariate analysis confirmed that CTP score was the only independent variable influencing the occurrence of SBP. Patients with CTP-B and C had a two-fold and three-fold increase, respectively, in the risk of SBP as compared to patients with CTP-A (HR = 2.16, 95%CI: 1.14-4.09, $P = 0.018$ in CTB B patients and HR 3.77, 95%CI: 1.66-8.59, $P = 0.002$ in CTP C patients) (Table 2). Using the COX model, the events occurred in Child A 18.2%; Child B 35.6%; and Child C 52.7%; $P < 0.001$. Throughout the follow-up period, the Child C patients presented a higher mortality.

Survival at 60 mo (Kaplan-Meier analysis) was similar in users and non-users of PPI, independently of the presence of SPB (58.4% *vs* 62.7% respectively, $P = 0.66$). For patients with SBP, survival at 60 mo was 55.1%, *vs* 61.7% in patients without SBP ($P = 0.34$).

In the group of 151 patients using PPI, 19 patients had a diagnosis of peptic ulcer (12.6%), 20 presented gastric esophageal reflux (13.1%) and 17 used PPI to treat dyspepsia (11.3%). Evidence of formal indication for PPI treatment was not found in the chart of 95 patients (63%).

DISCUSSION

Given the importance of SBP in the context of liver disease, the identification of possible risk factors is crucial to prevent this infection. Among possible risk

Table 1 Sociodemographic and clinical characteristics of patients classified according to the use or not of proton pump inhibitors

Characteristic	Use of PPI		*P*
	Yes (*n* = 151)	No (*n* = 107)	
Age (yr)	54.7 ± 11.2	53.1 ± 11.3	0.26[1]
Male sex (%)	63.30%	62.60%	> 0.99[2]
Etiology of liver disease (%)			0.53[2]
Hepatitis C virus	34.50%	34.00%	
Alcohol	27.00%	34.90%	
Alcohol + hepatitis C virus	24.30%	19.80%	
Other	14.20%	11.30%	
Platelet count, × 10^3/mm^3	126 ± 81	112 ± 56	0.13[1]
Creatinine, mg/dL	1.07 ± 0.69	0.97 ± 0.27	0.15[1]
Albumin, g/dL	3.4 ± 0.6	3.3 ± 0.6	0.70[1]
Total bilirubin, mg/dL	1.30 (0.80-2.60)	1.40 (0.90-2.60)	0.59[3]
Prothrombin time, INR	1.34 ± 0.29	1.41 ± 0.26	0.24[1]
Child-Turcotte-Pugh score (%)			0.37[2]
A	42.40%	36.40%	
B	42.40%	51.40%	
C	15.20%	12.10%	
MELD score	12.5 ± 3.9	12.7 ± 3.8	0.71[1]
Upper gastrointestinal bleeding (%)	21.90%	18.70%	0.64[2]

Data expressed as mean ± SD, median (25-75 interquartile range) or *n* (%). [1]Student's *t* test; [2]Fisher's exact test; [3]Mann-Whitney's *U* test. MELD: Model for end-stage liver disease.

Table 2 Relationship between selected variables and presence of spontaneous bacterial peritonitis

Variable	*n*	Events *n* (%)	Bivariate analysis		Multivariate analysis	
			HR (95%CI)	*P*	HR (95%CI)	*P*
PPI use						
Yes	151	34 (22.5)	1.44 (0.85-2.47)	0.176	1.50 (0.87-2.58)	0.142
No	107	23 (21.5)	1		1	
CTP						
A	103	15 (26.3)	1		1	
B	119	30 (52.6)	2.10 (1.12-3.92)	0.020	2.16 (1.14-4.09)	0.018
C	36	12 (21.1)	3.62 (1.69-7.78)	0.001	3.77 (1.66-8.59)	0.002
MELD						
⩾ 15	78	19 (33.3)	1.41 (0.81-2.45)	0.226	0.95 (0.52-1.72)	0.854
< 15	180	38 (66.7)	1		1	
UGB						
Yes	53	11 (19.3)	0.92 (0.48-1.79)	0.808	0.99 (0.51-1.92)	0.967
No	205	46 (83.7)	1			

PPI: Proton pump inhibitor; CTP: Child-Turcotte-Pugh; MELD: Model for end-stage liver disease; UGB: Upper gastrointestinal bleeding.

factors, the role of PPIs has been recently discussed. To our knowledge, this is the first study conducted in Brazil in a cohort representing a population with typical environment and demographic characteristics as racial heterogeneity, probably traducing a differentiation in the gut microbiota.

The gastric acidity exerts a defense of the host against enteric pathogens, supporting the hypothesis of an influence of acid suppression on the development of secondary infections resulting from increased bacterial populations in the gastrointestinal tract. As in the pathogenesis of other bacterial infections in patients with cirrhosis, bacterial translocation plays a key role in the genesis of SBP, and has been described as the main trigger of SBP development[29-31]. The increased prevalence of bacterial overgrowth and intestinal dysmotility in cirrhotic patients with SBP when compared to cirrhotic patients without SBP underscores the role of intestinal microbiota in the pathogenesis of this infection[32]. A prospective study with 70 patients with cirrhosis analyzed jejunal secretion cultures and observed an association of bacterial overgrowth with acid-suppressive therapy ($P = 0.01$) and hypochlorhydria ($P < 0.001$); nevertheless, no statistical association was detected between the presence of SBP and bacterial overgrowth or acid-suppressive therapy[8]. With regard to the microbiota, few studies[33-35] were carried out in Brazil, making interesting the pioneer knowledge of the impact of the PPIs in cirrhosis.

In the present study, a cohort of patients with cirrhosis was followed-up, allowing the estimation of

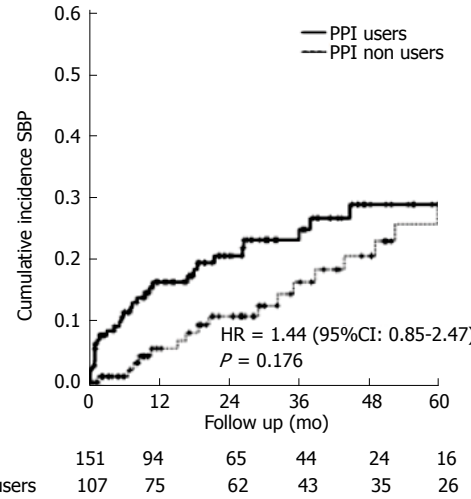

Figure 2 Kaplan-Meier curves of the cumulative incidence of spontaneous bacterial peritonitis events in patients with ascites using or not proton pump inhibitors. PPI: Proton pump inhibitor; SBP: Spontaneous bacterial peritonitis.

the incidence of SBP in users or non-users of PPI. We did not observe an association between the use of PPI and the incidence of SBP. However, the degree of liver dysfunction expressed as CTP score was strongly related to incidence of SBP, with a three-fold increase in risk of SBP in patients with more severe disease (CTP C), as reported in other studies[22,36]. This association is also emphasized by previous observations showing that liver dysfunction is related to increased bacterial translocation[7,37].

It should be noted that some studies suggesting an association between PPIs and SBP did not achieve statistically significant results[8,13], or were unable to confirm this association in multivariate analyses[17]. It is important to emphasize that the studies linking the use of anti-secretory therapy to increased frequency of SBP are mostly retrospective or case-control in design[13-19,38].

Bajaj *et al*[38] have not observed significant associations between the use of PPI and the rate of severe infections (HR = 1.08, 95%CI: 0.90-1.30) or infections related to acid-suppressive therapies (HR = 1.22, 95%CI: 0.97-1.52), except when the duration of PPI treatment was taken into account. In this study the authors do not describe the severity of liver disease of patients.

Min *et al*[39] reported an association between PPIs and SBP based on results from 1554 patients with cirrhosis and ascites. There were 90 cases of SBP among 512 users of PPI (10.6%) and 146 cases of SBP among 1042 non-users (5.8%). The annual incidence rate of SBP was higher in those using PPIs (HR 1.396, 95%CI: 1.057-1.843, $P = 0.019$).

Regarding the influence of acid-suppressive therapies on the development of SBP, some works have described different results for PPI and histamine-2 receptor antagonists (H2RA)[15,21,38], with no reported influence of H2RA. This has prompted a discussion regarding whether the difference between these acid-suppressive therapies results from a stronger acid-suppressive effect and greater delay in gastric emptying with PPIs[40,41] or from weaknesses in the hypothesis of acid-suppressive therapy as an independent risk factor for SBP. In the present study, all patients received omeprazole 20 mg qd, since this is the medication available free of charge in the Public Health System.

Meta-analyses[20,21,42] carried out to evaluate the association between acid-suppressive therapies and SBP have confirmed a relationship. The first of these[20] meta-analyzed case-control and retrospective studies with hospitalized patients. The meta-analyzed studies involved 772 individuals with cirrhosis using PPIs, for and odds ratio (OR) of 2.77 (95%CI: 1.82-4.23). A second meta-analysis[21] involved 3815 patients with cirrhosis, and showed significantly higher risk of SBP in users of PPIs *vs* non-users (OR = 3.15, 95%CI: 2.09-4.74, $P < 0.00001$); however, once again that study included mostly retrospective, case-control studies of hospitalized patients. Other limitations included the lack of information regarding dose and duration of PPI and H2RA treatment. The more recent meta-analysis[42] evaluated 7822 patients from 14 studies (6 case-control studies with 817 patients and 8 cohort studies with 7005 patients). The authors found statistically significant but quantitatively small associations between SBP and the use of PPIs. After adjustment for publication bias, there was very low-quality evidence per the GRADE approach in favor of this association. Therefore, they suggest that patients with cirrhosis who have indications for the use of PPI should not be denied because of concern for precipitating SBP.

In the same way, van Vlerken *et al*[36] did not observe an influence of PPIs on bacterial infection in a prospective analysis of cirrhotic patients receiving outpatient follow-up (HR = 1.2, 95%CI: 0.5-3.0, $P = 0.72$). It should be noted, however, that those authors had only a small number of cases of SBP. More recently, Mandorfer *et al*[22] carried out a retrospective cohort analysis of 607 patients submitted to paracentesis and did not identify PPIs as a risk factor for SBP. Similarly, in a multicenter study with 521 cirrhotic patients, Terg *et al*[23] reported similar SBP rates in patients at increased risk of SBP infection - 79.5% in users and 78.7% in non-users of PPIs.

The low mortality observed in patients with SBP in relation to the group without this infection is probably related to the fact that these infections are community-acquired, which results in a lower severity. We recently published a study showing the relevance of multiresistant bacteria in patients with nosocomial SBP, which certainly worsens the prognosis of these patients[43]. However, when patients with a greater impairment of hepatocellular function were evaluated (Child C), mortality was higher.

One aspect that deserves attention is the high prevalence of PPI use (58%) in our patients, and the fact that 63% of those using PPI did not have evidence of formal indication for PPI therapy. Similar data have been previously described, with PPI used by as many as 86% of patients[23] and used by as many as 63% patients without documented indications[16,19,23,36,44-46]. PPIs have been used to prevent gastroesophageal reflux and worsening of inflammation and esophageal ulceration following band ligation and sclerotherapy in cirrhotic patients; however, this practice is questionable[45-47]. As possible limitations of the present study we should note that most of the data were obtained from reviewing the charts, which is important to remark thus we are aware of the potential biases.

In conclusion, considering the current uncertainty regarding PPIs as a risk factor for SBP in patients with cirrhosis, the present study evaluated an historical cohort of cirrhotic outpatients with ascites and did not find evidence of increased incidence of SBP with the use of PPIs. In addition, the CTP score was strongly related to incidence of SBP.

COMMENTS

Background

Spontaneous bacterial peritonitis (SBP) is the most characteristic infection in cirrhosis, and has been associated to morbidity and mortality. Small intestinal bacterial overgrowth potentially promotes bacterial translocation. Thus, it has been speculated that chronic acid suppression by proton pump inhibitors (PPIs) - which favors gastric and duodenal bacterial colonization - may contribute to small intestinal bacterial overgrowth and consequently increase the incidence of SBP. Nevertheless, there is some controversy regarding the role of PPIs in SBP.

Research frontiers

The increased prevalence of bacterial overgrowth and intestinal dysmotility in cirrhotic patients with SBP when compared to cirrhotic patients without SBP underscores the role of intestinal microbiota in the pathogenesis of this infection. However, few studies evaluating the gut microbiota were carried out in cirrhotic patients, mainly in Brazil, making interesting the pioneer knowledge of the impact of the PPIs in cirrhosis.

Innovations and breakthroughs

To the knowledge, this is the first study conducted in Brazil in a cohort representing a population with typical environment and demographic characteristics as racial heterogeneity, probably traducing a differentiation in the gut microbiota. Considering the current uncertainty regarding PPIs as a risk factor for SBP in patients with cirrhosis, the present study evaluated an historical cohort of cirrhotic outpatients with ascites and did not find evidence of increased incidence of SBP with the use of PPIs.

Applications

One aspect that deserves attention is the high prevalence of PPI use (58%) in the patients, and the fact that 63% of those using PPI did not have evidence of formal indication for PPI therapy. Similar data have been previously described in the literature, with PPI used by as many as 86% of patients and used by as many as 63% patients without documented indications. So, it is possible that these results may alert and promote the correct use of PPI in cirrhotics.

Peer-review

The study is well conducted and statistical methods are sound.

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ORIGINAL ARTICLE

Retrospective Study

Occult hepatitis B virus infection and surgical outcomes in non-B, non-C patients with curative resection for hepatocellular carcinoma

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Author contributions: Koga H is the main author of this article and performed the data correction and statistical analyzes; Kai K designed the study and conducted the data collection and contributed to the statistical analyses and the writing of the manuscript; Aishima S performed the pathological work and reviewed the manuscript; Kawaguchi A supervised the statistical analyses; Yamaji K, Ide T and Ueda J contributed to the data collection; Noshiro H contributed to the data collection and review the manuscript; all authors have read and approved the final manuscript.

Institutional review board statement: The study protocol was approved by the Ethics Committee of the Faculty of Medicine at Saga University.

Informed consent statement: Informed consent for the use of resected tissue and medical information was obtained from all patients.

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Abstract

AIM

To investigate the prevalence, clinicopathological characteristics and surgical outcomes of occult hepatitis B virus (HBV) infection (OBI) in patients with non-B, non-C (NBNC) hepatocellular carcinoma (HCC).

METHODS

This study retrospectively examined the cases of 78 NBNC

patients with curative resection for HCC for whom DNA could be extracted from formalin-fixed paraffin-embedded tissue. OBI was determined by the HBV-DNA amplification of at least two different sets of primers by TaqMan real-time polymerase chain reaction. Possibly carcinogenetic factors such as alcohol abuse, diabetes mellitus, obesity and non-alcoholic steatohepatitis (NASH) were examined. Surgical outcomes were evaluated according to disease-free survival (DFS), overall survival (OS) and disease-specific survival (DSS).

RESULTS

OBI was found in 27/78 patients (34.6%) with NBNC HCC. The OBI patients were significantly younger than the non-OBI cases at the time of surgery (average age 63.0 *vs* 68.1, P = 0.0334) and the OBI cases overlapped with other etiologies significantly more frequently compared to the non-OBI cases (P = 0.0057). OBI had no impact on the DFS, OS or DSS. Only tumor-related factors affected these surgical outcomes.

CONCLUSION

Our findings indicate that OBI had no impact on surgical outcomes. The surgical outcomes of NBNC HCC depend on early tumor detection; this reconfirms the importance of a periodic medical examination for individuals who have NBNC HCC risk factors.

Key words: Hepatocellular carcinoma; Non-B non-C; Occult hepatitis B virus infection; Surgery; Surgical outcome

Core tip: We analyzed the occult hepatitis B virus infection (OBI) status of 78 cases of non-B, non-C hepatocellular carcinoma (NBNC HCC). OBI was found in 27/78 patients (34.6%). The OBI patients were significantly younger than the non-OBI patients at the time of surgery, and the OBI cases were frequently overlapped with other etiologies. OBI had no impact on surgical outcomes. Only tumor-related factors affected the surgical outcomes. The surgical outcomes of NBNC HCC thus depend in part on the early detection of the tumor.

Koga H, Kai K, Aishima S, Kawaguchi A, Yamaji K, Ide T, Ueda J, Noshiro H. Occult hepatitis B virus infection and surgical outcomes in non-B, non-C patients with curative resection for hepatocellular carcinoma. *World J Hepatol* 2017; 9(35): 1286-1295 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i35/1286.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i35.1286

INTRODUCTION

Although the most major risk factors for hepatocellular carcinoma (HCC) are hepatitis C virus (HCV) infection and hepatitis B virus (HBV) infection, the prevalence of non-B, non-C (NBNC) HCC patients who are negative for both hepatitis C antibody (HCVAb) and hepatitis B surface antigen (HBsAg) has gradually increasing. In a 2010 Japanese survey, the prevalence of NBNC HCC were 24.1% of all HCC patients[1].

Alcoholic liver disease (ALD)[2] and non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH)[3,4] are well-known etiologies of NBNC HCC. Other known etiologies of NBNC HCC include hemochromatosis[5], Budd-Chiari syndrome[6], metabolic disease, autoimmune hepatitis, primary biliary cirrhosis, parasitic disease, congestive disease and unknown etiology[7]. Occult HBV infection (OBI) was also recognized as one of the risk factors for the development of HCC[8,9]. OBI is considered one of the possible phases in the natural history of chronic HBV infection[10], and it reflects the persistence of HBV genomes in the hepatocytes of individuals who test negative for HBsAg[11]. The gold standard to diagnose OBI is the detection of HBV DNA in the hepatocytes by highly sensitive and specific techniques such as real-time polymerase chain reaction (PCR) using the sets of specific primers for different HBV genomic regions[11-14].

The virology and pathogenesis of OBI have been well investigated[15,16], and many epidemiological and molecular biological studies have addressed that OBI is an important risk factor for developing HCC[9]. However, the clinical characteristics and surgical outcomes of OBI-associated HCC have not been well-investigated. We could not find any study that investigated in detail a surgical series of OBI-associated HCC. It is quite important to determine the clinical characteristics and surgical outcomes of OBI-associated HCC among cases of NBNC HCC or HCV-associated HCC because different etiologies of HCC may modulate the clinical characteristics and outcomes, thereby requiring different preventive and therapeutic strategies.

Our aim in the present study was to clarify the prevalence, clinicopathological characteristics and surgical outcomes in patients with OBI-associated HCC in our surgical series of NBNC HCC patients. To the best of our knowledge, this is the first study investigating the surgical outcomes in OBI-associated NBNC HCC.

MATERIALS AND METHODS

Patients

Initially, 477 patients with HCC who underwent curative surgical resection for the primary lesion at Saga University Hospital between 1984 and 2012 were enrolled the study. All patients enrolled in this study had no lymph node metastasis or distant metastasis at the time of surgery. Of these, 83 cases of NBNC HCC were identified and subjected to DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue blocks. These 83 NBNC HCC cases were same population of previous our study[17]. We retrospectively examined a

final total of 78 cases of NBNC HCC (in the other five cases, DNA was unavailable). Written informed consent for the use of their liver tissues and clinical information was obtained from all patients. The study protocol was reviewed and approved by the Ethics Committee of the Faculty of Medicine at Saga University (Approval No. 27-18).

Nucleic acid extraction from liver tissues

Sections cut from FFPE tissue blocks of noncancerous liver tissue were used. The NucleoSpin® DNA FFPE system (Takara Bio, Shiga, Japan) was used to extract the nucleic acid from liver tissues (< 10 mg) per the manufacturer's instructions. The DNA was eluted in 20 μL of Tris Borate EDTA (TBE) buffer. The amount and quality of extracted DNA was confirmed by NanoDrop® (Thermo Fisher Scientific, Yokohama, Japan).

Detection of HBV DNA and definition of occult HBV infection

The regions of HBs, hepatitis B core (HBc), and hepatitis B x (HBx) in the HBV DNA were analyzed by TaqMan real-time PCR per the manufacturer's guidelines (TaqMan Fast Universal PCR Master Mix; Applied Biosystems, Foster City, CA). The oligonucleotide primers and probes which were specific for the S, X and C regions of HBV were as described by Kondo *et al*[18]. Plasmid pBRHBadr72 (full-length HBV DNA) was used as an internal standard. The detection limit of our TaqMan real-time PCR was 100 copies/mL. Only the cases in which HBV DNA was detected by the TaqMan real-time PCR using at least two different sets of primers were considered to exhibit OBI[11].

Analyses of alcohol abuse, obesity, diabetes mellitus

To analyze the relationships between OBI and other etiologies of NBNC HCC, we also investigated the patients' alcohol consumption status and metabolic factors such as diabetes mellitus, obesity and NASH. The patients who were clinically diagnosed as having diabetes mellitus were categorized as diabetes mellitus group. A body mass index (BMI) > 25 kg/m^2 in both genders was defined as obesity. We defined an alcohol abuse as a daily ethanol consumption of > 40 g for men and > 20 g for women.

Histopathological analysis

To pathologically assess the degree of fibrosis in noncancerous liver tissues, we used the new Inuyama classification system which is widely used in Japan: F0, no fibrosis; F1, portal fibrosis widening; F2, portal fibrosis widening with bridging fibrosis; F3, bridging fibrosis plus lobular distortion; and F4, cirrhosis[19]. The diagnoses of NASH were pathologically confirmed. These histopathological analysis and classification were performed by two pathologists (Keita Kai and Shinichi Aishima).

Table 1 Status of occult hepatitis B virus infection (n = 78)

Occult HBV infection (%)	
(+)	27 (34.6)
(−)	51 (65.4)
Details of HBV amplification	
HBc lesion (%)	23 (29.4)
HBs lesion (%)	50 (64.1)
HBx lesion (%)	32 (41.0)
Amplification of at least one lesion (%)	64 (82.1)

HBV: Hepatitis B virus.

Statistical analysis

All statistical analyses were supervised by a statistician (Atsushi Kawaguchi). The statistical analysis was performed using JMP ver. 12 software (SAS Institute, Cary, NC) and SAS software ver. 9.4 (SAS Institute, Cary, NC). Continuous variables are expressed as the mean ± SD and were compared using the Student's t test. Categorical variables were compared using the χ^2 test and Fisher's exact test, as appropriate. Disease-free survival (DFS), overall survival (OS) and disease-specific survival (DSS) was determined according to our previous report[17]. The uni- and multi-variate analyses were performed using a Cox proportional hazards model. To adjust the potential covariates for the comparison of OBI status in the multivariate analysis, age, gender and OBI status were always kept in the model and other parameters were selected by the stepwise procedure with the P-value threshold of 0.2. P-values < 0.05 were considered as statistically significant.

RESULTS

Status of OBI

The OBI status of the patients is summarized in Table 1. Twenty-seven patients (34.6%) were categorized as having an OBI in this study. The details of HBV-DNA amplification were HBc lesion, 23 cases (29.4%); HBs lesion, 50 cases (64.1%); and HBx lesion, 32 cases (41.0%). The number of cases with amplification of at least one lesion was 64 cases (82.1%).

Clinicopathological features of NBNC HCC according to OBI status

Table 2 demonstrates the summary of the clinico-pathological features. The 78 patients with NBNC HCC were consisted of 61 men (78.2%) and 17 women (21.8%). The mean age at the time of surgery was 66.3 years. Alcohol abuse was identified in 19 patients (24.4%). Twenty-seven patients (34.6%) had diabetes mellitus and obese was found in 24 patients (30.8%). NASH was pathologically confirmed in eight patients (10.3%).

We compared the OBI cases (n = 27) with the non-OBI cases (n = 51) regarding clinicopathologic

Table 2 Clinicopathologic features of the patients with non-B, non-C hepatocellular carcinoma (*n* = 78) according to occult hepatitis B virus infection status

	Total cases (*n* = 78)	OBI (*n* = 27)	Non-OBI (*n* = 51)	*P*[1]
Age (mean ± SD)	66.3 ± 11.9	63.0 ± 17.0	68.1 ± 7.6	0.0334
Gender (%)				0.6066
Male	61 (78.2)	22 (81.5)	39 (79.5)	
Female	17 (21.8)	5 (18.5)	12 (20.5)	
Alcohol abuse (%)				0.8151
(+)	19 (24.4)	7 (25.9)	12 (23.5)	
(-)	59 (75.6)	20 (74.1)	39 (77.1)	
Diabetes mellitus (%)				0.106
(+)	27 (34.6)	10 (37.0)	17 (33.3)	
(-)	51 (65.4)	17 (63.0)	34 (66.7)	
Obesity (%)				0.4966
(+)	24 (30.8)	7 (25.9)	17 (33.3)	
(-)	54 (69.2)	20 (74.1)	34 (66.7)	
BMI (mean ± SD)	22.7 ± 4.56	22.1 ± 3.67	23.1 ± 4.97	0.3537
Size (mean ± SD), mm	64.2 ± 41.8	72.7 ± 45.6	59.8 ± 39.4	0.1955
Solitary/multiple (%)				0.8959
Solitary	47 (60.3)	16 (59.3)	31 (60.8)	
Multiple	31 (39.7)	11 (40.7)	20 (39.2)	
Vp (%)				0.7217
(+)	31 (39.7)	10 (37.0)	21 (41.2)	
(-)	47 (60.2)	17 (60.3)	30 (58.8)	
Liver fibrosis (%)				0.2851
F0-2	44 (56.4)	13 (48.2)	31 (60.8)	
F3-4	34 (43.6)	14 (51.8)	20 (39.2)	
NASH (%)				0.007
(+)	8 (10.3)	0	8 (15.7)	
(−)	70 (89.7)	27	43 (84.3)	
No. of etiologies				0.0057[2]
Single	38 (48.7)	11 (40.7)	27 (52.9)	
Multiple	25 (32.1)	16 (59.3)	9 (17.7)	
Unknown	15 (19.2)	0	15 (29.4)	

[1]Comparison between OBI and non-OBI cases; [2]Analysis excluding unknown cases. OBI: Occult HBV infection; Vp: Portal vein invasion; NASH: Non-alcoholic steatohepatitis; BMI: Body mass index.

factors (age, gender, alcohol abuse, diabetes mellitus, obesity, BMI, tumor size, solitary/multiple, portal vein invasion, degree of background liver fibrosis, NASH and number of etiologies). Significant differences were observed in age, NASH and the number of etiologies. The OBI patients were significantly younger than the non-OBI patients at the time of surgery (P = 0.0334): 63.0 ± 17.0 and 68.1 ± 7.6 years (mean age ± SD), respectively. All eight NASH cases were non-OBI cases (P = 0.007). The OBI patients had multiple etiologies for HCC significantly more frequently compared to the non-OBI patients, and high significance was observed even in the analysis excluding etiology-unknown cases (P = 0.0057).

Etiologies for NBNC HCC

As shown in Table 2, the etiologies of our NBNC HCC cases consisted of 38 (48.7%) single-etiology cases, 25 (32.1%) multiple-etiology cases, and 15 (19.2%) unknown-etiology cases. The Venn diagram for the etiologies of NBNC HCC is given as Figure 1. OBI and alcohol abuse were frequently associated with other etiologies. The Venn diagram for the metabolic factors (obesity, diabetes mellitus and NASH) is given as Figure 2. NASH was frequently associated with other metabolic factors.

Univariate and multivariate analyses for DFS, OS and DSS

Table 3 demonstrates the results of the uni- and multivariate analyses for DFS by Cox's proportional hazards model. The significant factors which correlated with DFS by the univariate analyses were portal vein invasion, T factor of TMN classification, and multiple tumors at the time of surgery (P = 0.0013, P = 0.0006 and P = 0.0002, respectively). The factors significantly correlated with DFS by the multivariate analysis were portal vein invasion (P = 0.0217) and multiple tumor (P = 0.0499). No patient had undergone adjuvant therapy after curative surgery until recurrence.

The results of the univariate and multivariate analyses for OS are summarized in Table 4. Only the factors of portal vein invasion (P = 0.022) and multiple tumors (P = 0.0334) correlated with OS by the univariate analyses. The multivariate analysis for OS indicated only one significant correlation of portal vein invasion (P = 0.0378). Table 5 demonstrates the results of the uni- and multi-variate analyses for DSS. In the univariate analysis, only the factor "multiple

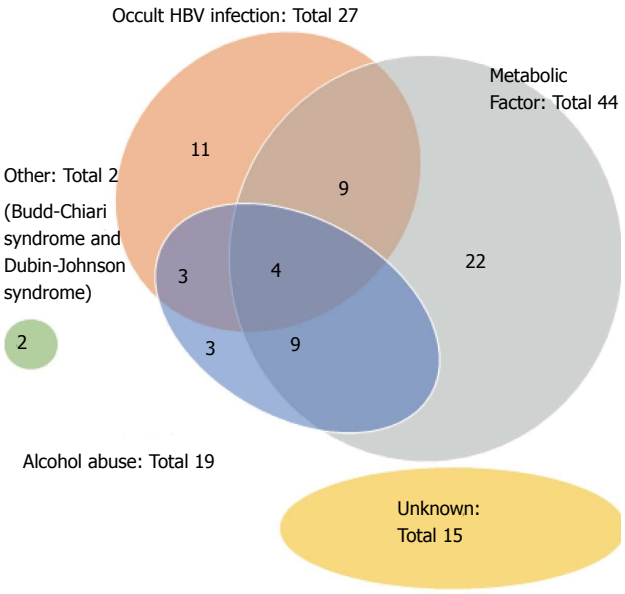

Figure 1 Venn diagram of the etiologies of non-B, non-C hepatocellular carcinoma. Occult hepatitis B virus infection and alcohol abuse were frequently associated with other etiologies.

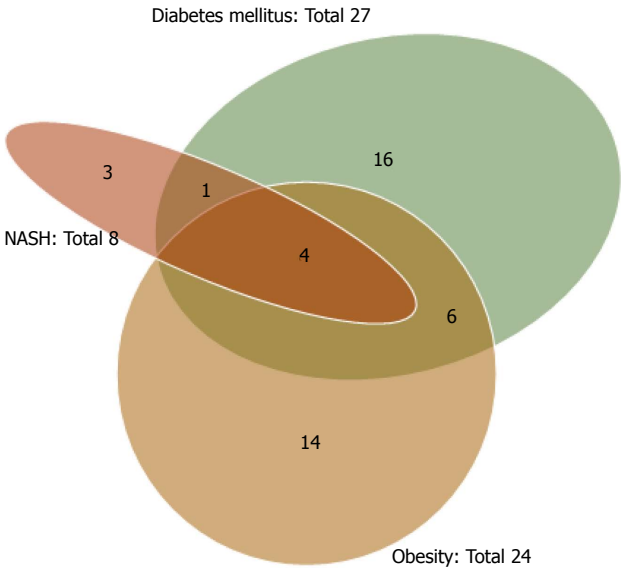

Figure 2 Venn diagram of metabolic factors (obesity, diabetes mellitus and non-alcoholic steatohepatitis). Non-alcoholic steatohepatitis (NASH) was frequently associated with other metabolic factors.

Table 3 Uni- and multi-variate analyses for disease-free survival after hepatic resection

Characteristic	*n*	Univariate analysis		Multivariate analysis	
		HR (95%CI)	*P*-value	HR (95%CI)	*P*-value
Age			0.316		0.1007
≤ 69	39	1		1	
> 69	39	0.74 (0.40-1.33)		0.58 (0.30-1.11)	
Gender			0.4847		0.298
Female	17	1		1	
Male	61	0.78 (0.41-1.60)		0.66 (0.31-1.41)	
Occult HBV infection			0.8739		0.7096
Absent	51	1		1	
Present	27	1.05 (0.55-1.93)		1.13 (0.59-2.17)	
Alcohol abuse			0.2752		
Absent	59	1			
Present	19	0.66 (0.28-1.35)			
Diabetes mellitus			0.8853		
Absent	51	1			
Present	27	0.95 (0.49-1.78)			
NASH			0.6226		
Absent	70	1			
Present	8	1.25 (0.47-2.75)			
Obesity			0.7641		
Absent	54	1			
Present	24	1.10 (0.57-2.02)			
Fibrosis			0.1477		0.2273
F0-2	44	1		1	
F3, 4	34	1.54 (0.86-2.80)		1.54 (0.78-2.81)	
Vp			0.0013		0.0217
Absent	47	1		1	
Present	31	2.90 (1.53-5.50)		2.52 (1.15-5.50)	
T12/T34			0.0006		0.4074
T12	38	1		1	
T34	40	3.14 (1.62-6.43)		1.57 (0.53-4.67)	
Solitary/multiple			0.0002		0.0499
Solitary	47	1		1	
Multiple	31	3.23 (1.73-6.14)		2.32 (0.99-5.42)	

HR: Hazard ratio; CI: Confidence interval; Vp: Portal vein invasion; NASH: Non-alcoholic steatohepatitis; HBV: Hepatitis B virus.

Table 4 Uni- and multi-variate analyses for overall survival after hepatic resection

Characteristic	*n*	Univariate analysis		Multivariate analysis	
		HR (95%CI)	*P*-value	HR (95%CI)	*P*-value
Age			0.8321		0.6843
⩽ 69	39	1		1	
> 69	39	0.94 (0.50-1.73)		0.87 (0.45-1.67)	
Gender			0.2713		0.342
Female	17	1		1	
Male	61	1.54 (0.73-3.80)		1.51 (0.68-3.85)	
Occult HBV infection			0.6039		0.5263
Absent	51	1		1	
Present	27	1.18 (0.61-2.20)		1.23 (0.63-2.31)	
Alcohol abuse			0.3061		
Absent	59	1			
Present	19	1.45 (0.69-2.82)			
Diabetes mellitus			0.2441		
Absent	51	1			
Present	27	1.45 (0.76-2.67)			
NASH			0.7366		
Absent	70	1			
Present	8	0.84 (0.25-2.10)			
Obesity			0.9432		
Absent	54	1			
Present	24	1.02 (0.51-1.93)			
Fibrosis			0.7084		
F0-2	44	1			
F3,4	34	1.12 (0.60-2.06)			
Vp			0.022		0.0378
Absent	47	1		1	
Present	31	2.06 (1.11-3.81)		2.34 (1.05-5.24)	
T12/T34			0.0767		0.3344
T12	38	1		1	
T34	40	1.73 (0.94-3.27)		0.58 (0.20-1.73)	
Solitary/multiple			0.0334		0.0809
Solitary	47	1		1	
Multiple	31	1.94 (1.05-3.58)		2.17 (0.92-5.25)	

HR: Hazard ratio; CI: Confidence interval; Vp: Portal vein invasion; NASH: Non-alcoholic steatohepatitis; HBV: Hepatitis B virus.

tumors" was significantly correlated with DSS (P = 0.0173). No significant factor was determined by the multivariate analysis.

DISCUSSION

Many studies have reported an association between OBI and HCC[9]. A meta-analysis in 2012 demonstrated that OBI increases the risk of developing HCC in both HCV- and non-HCV-infected patients[20]. However, other studies did not find such an association[21,22]. Although the debate remains, OBI has been recognized as a possible etiology in the development of HCC. Pathogenetic mechanisms of HCC development *via* OBI would be implicated in HBV-induced hepatocarcinogenesis, namely chronically sustained inflammation and direct oncogenic effect through integration into the host genome[9,23].

If OBI is an important etiology of HCC, it is quite important to clarify the clinical characteristics and outcomes of OBI-related HCC because different etiologies of HCC may modulate the clinical characteristics and outcomes, thereby requiring different preventive and therapeutic strategies. We therefore focused on NBNC HCC cases which were not influenced by HCV or overt HBV infection.

The prevalence of OBI in this study was 27/78 patients (34.6%). The prevalence of OBI has varied widely among the reported case series[8,24]. The difference in the prevalence of OBI may be due to the lack of methodological uniformity among the different studies[11]. Although the gold standard to diagnose OBI is the detection of HBV DNA in hepatocytes, studies testing OBI by using serum samples have been reported, and the methods of DNA detection varied widely. In addition, the detection of HBc antibody in the serum of HBsAg-negative patients has been used as a surrogate serum marker of OBI[12]. The previous reported prevalence of OBI varied from 12.1% to 78.0% in an anti-HBc positive patient series and from 5.7% to 50.0% in a series in which HBV-DNA was detected in hepatocytes or serum samples[12].

In the present study, OBI was determined by the HBV-DNA amplification of at least two different sets of primers by TaqMan real-time PCR using DNA extracted from FFPE tissues. It has been stated that the DNA extraction from frozen tissues was better than that

Table 5 Uni- and multi-variate analyses for disease-specific survival after hepatic resection

Characteristic	*n*	Univariate analysis		Multivariate analysis	
		HR (95%CI)	*P*-value	HR (95%CI)	*P*-value
Age			0.4181		0.2941
⩽ 69	39	1		1	
> 69	39	0.74 (0.34-1.54)		0.65 (0.27-1.44)	
Gender			0.3814		0.4598
Female	17	1		1	
Male	61	1.51 (0.62-4.48)		1.45 (0.56-4.56)	
Occult HBV infection			0.4661		0.4693
Absent	51	1		1	
Present	27	1.32 (0.60-2.78)		1.33 (0.60-2.88)	
Alcohol abuse			0.5064		
Absent	59	1			
Present	19	1.34 (0.52-3.02)			
Diabetes mellitus			0.4775		
Absent	51	1			
Present	27	1.31 (0.60-2.76)			
NASH			0.6755		
Absent	70	1			
Present	8	1.26 (0.37-3.27)			
Obesity			0.6466		
Absent	54	1			
Present	24	1.20 (0.53-2.53)			
Fibrosis			0.1392		0.2147
F0-2	44	1		1	
F3,4	34	1.74 (0.84-3.73)		1.62 (0.75-3.58)	
Vp			0.0806		0.1478
Absent	47	1		1	
Present	31	1.92 (0.91-4.03)		2.00 (0.78-5.08)	
T12/T34			0.0824		0.6238
T12	38	1		1	
T34	40	1.92 (0.92-4.15)		0.72 (0.19-2.75)	
Solitary/multiple			0.0173		0.0984
Solitary	47	1		1	
Multiple	31	2.44 (1.17-5.19)		2.55 (0.84-7.96)	

HR: Hazard ratio; CI: Confidence interval; Vp: Portal vein invasion; NASH: Non-alcoholic steatohepatitis; HBV: Hepatitis B virus.

from FFPE[11]. In addition, all previous OBI studies using liver tissue were based on the frozen or raw liver tissue. Therefore, it was challenging to analyze the OBI status from FFPE samples. We have performed a pilot study using DNA extracted from FFPE tissues of overt HBV infection cases, and the results confirmed good HBV amplification in each primer set. Nevertheless, the possibility cannot be denied that the prevalence of OBI in this study would have been higher if frozen tissues were available. HBV covalently closed circular DNA (cccDNA) is harbored in the nucleus of HBV-infected hepatocytes, and therefore the results might have been affected if we had examined ccc DNA.

Our comparison of the OBI and non-OBI groups revealed that the patients with OBI were significantly younger than the patients without OBI at the time of surgery. This finding seems to support hepatocarcinogenesis of OBI. Recently, similar result has been reported. Coppola *et al*[25] analysed OBI in 68 consecutive HBsAg-negative patients with HCC by the presence of HBV DNA in at least two different PCRs and found that patients with OBI were significantly younger than the patients without OBI (mean age: 65.7 *vs* 71.2, $P = 0.03$). However, these results involving our series are not conclusive because the infection period of OBI was unknown. Additional studies are thus needed before a conclusion can be made regarding whether NBNC HCC develops more often in younger individuals with OBI compared to non-OBI patients.

The impact of OBI on liver fibrosis remains controversial. Several studies suggest an impact of OBI on the progression of liver fibrosis[25-29], whereas other studies found no association between OBI and liver fibrosis[29-32]. In the present study, we compared the degree of background liver fibrosis between the OBI and non-OBI cases, and we observed that the OBI group had a higher proportion (51.8%) of severe fibrosis cases (F3-4) compared to the non-OBI group (39.2%), although the difference was not significant.

Analyses of the surgical outcomes and clinicopathologic features according to OBI status were the main purpose of this study. Surgical outcomes according to known NBNC HCC etiologies such as alcohol, NAFLD/NASH, diabetes mellitus and obesity had been well investigated[1,33-36]. However, to the best of our knowledge, no previous study investigated the

association of OBI status and surgical outcomes in patients with NBNC HCC. Previous studies regarding OBI have been focused on the prevalence, the risk of developing HCC, and the prevalence of OBI in HCC cases[9,37,38].

Our present analyses of surgical outcome (DFS, OS and DSS) revealed that OBI status did not affect the surgical outcomes of NBNC HCC patients. The other analyzed etiologies also did not affect the surgical outcomes. Only tumor-related factors (*i.e.*, portal vein invasion, T-stage and multiple tumor) were associated with surgical outcomes of NBNC HCC. These findings indicate that the surgical outcome of NBNC HCC does not depend on the type of etiologies but that it does depend on the early detection of HCC. Therefore, a periodical screening of HCC using the abdominal echo and/or serum tumor markers is quite important for individuals who have one or more risk factors for NBNC HCC. For the early detection of NBNC HCC, the efficacy of the OBI screening using clinical samples (such as peripheral blood or liver biopsy specimen) should be discussed by accumulation of studies regarding OBI using clinical samples.

The limitations of our study were its retrospective nature, the long study period and the small number of patients. Information of actual number of tumors, viral serological markers except for HBsAg and HCVAb, and status of neoadjuvant treatments were not available. Diagnostic and therapeutic modalities also have changed in the recent decades. Our patients with NBNC HCC showed frequent overlapping in their etiology. Therefore, it is not an ideal method to compare OBI-associated patients to all the other NBNC patients. Association between metabolic factors (diabetes mellitus, NASH, and obesity) and HCC is considered much weaker than that of those of HBV and/or HCV. Therefore, it is doubtful these metabolic factors truly affected development of HCC.

In conclusion, the results of our study indicate that OBI was found in 34.6% of our series of patients with NBNC HCC. The patients with OBI were younger those without OBI at the time of surgery, and the OBI cases were frequently overlapped with other etiologies. The patients' surgical outcomes were not affected by the OBI status but were affected by only tumor-related factors, and thus the importance of the early detection of the tumors was reconfirmed. We hope to conduct larger retrospective or prospective studies to test our present findings.

COMMENTS

Research frontiers

Although many epidemiological and virological studies regarding occult HBV infection (OBI) have accumulated, the surgical outcomes of OBI-associated non-B, non-C (NBNC) hepatocellular carcinoma (HCC) have not been focused.

Innovations and breakthroughs

OBI was found in 27/78 (34.6%) patients with NBNC HCC. The OBI patients were significantly younger than the non-OBI patients at the time of surgery, and the OBI cases were frequently overlapped with other etiologies. OBI had no impact on surgical outcomes. Only tumor-related factors affected the surgical outcomes.

Applications

The results of present study indicated the possibility of OBI screening from formalin-fixed paraffin-embedded tissue. The importance of the early detection of HCC by a periodical checkup for individuals who have one or more risk factors for NBNC HCC was reconfirmed.

Terminology

In this study, OBI was determined by the HBV-DNA amplification of at least two different sets of primers by TaqMan real-time PCR using DNA extracted from FFPE tissues. NBNC-HCC is defined as hepatocellular carcinoma that has arisen in an individual who is negative for both hepatitis B surface antigen and hepatitis C antibody. Disease-free survival (DFS) was determined as the length of time after surgery that the patient survived without new lesions of HCC. Overall survival (OS) was determined from the time of surgery to the time of death or the most recent follow-up. Disease-specific survival (DSS) was determined from the time of surgery to the time of cancer-related death or the most recent follow-up.

Peer-review

It is a very interesting retrospective study in which they were able to show from the formalin-fixed paraffin-embedded tissue DNA of 78 patients that OBI had no impact on the surgical outcome and surgical outcomes of NBNC HCC depend on early tumor detection. This finding indicates that the importance of a periodic medical examination for individuals who have NBNC HCC risk factors. It is well-written, and presented.

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MINIREVIEWS

Hepatectomy for hepatocellular carcinoma with portal vein tumor thrombus

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Abstract

Despite surgical removal of tumors with portal vein tumor thrombus (PVTT) in hepatocellular carcinoma (HCC) patients, early recurrence tends to occur, and overall survival (OS) periods remain extremely short. The role that hepatectomy may play in long-term survival for HCC with PVTT has not been established. The operative mortality of hepatectomy for HCC with PVTT has also not been reviewed. Hence, we reviewed recent literature to assess these parameters. The OS of patients who received hepatectomy in conjunction with multidisciplinary treatment tended to be superior to that of patients who did not. Multidisciplinary treatments included the following: preoperative radiotherapy on PVTT; preoperative transarterial chemoembolization (TACE); subcutaneous administration of interferon-alpha (IFN-α) and intra-arterial infusion of 5-fluorouracil (5-FU) with infusion chemotherapy in the affected hepatic artery; cisplatin, doxorubicin and 5-FU locally administered in the portal vein; and subcutaneous injection of IFN-α, adjuvant chemotherapy (5-FU + Adriamycin) administration *via* the portal vein with postoperative TACE, percutaneous isolated hepatic perfusion and hepatic artery infusion and/or portal vein chemotherapy. The highest reported rate of operative mortality was 9.3%. In conclusion, hepatectomy for patients affected by HCC with PVTT is safe, has low mortality and might prolong survival in conjunction with multidisciplinary treatment.

Key words: Hepatocellular carcinoma; Portal vein tumor thrombus; Hepatectomy; Multidisciplinary treatment; Operative mortality

Core tip: Hepatocellular carcinoma (HCC) is characterized by early formation of portal vein tumor thrombus (PVTT). Even after surgical removal of the tumors with PVTT in HCC patients, early recurrence has been frequently

reported due to intrahepatic metastasis from PVTT. There have been reports of long-term survival after hepatectomy in patients with macroscopic PVTT. The operative mortality of major hepatectomy for HCC patients with macroscopic PVTT has not been well documented or discussed. To this end, we reviewed recent literature on the significance of hepatectomy in HCC with macroscopic PVTT with respect to the long-term survival and mortality.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is characterized by early formation of portal vein tumor thrombus (PVTT)[1]. An important prognostic factor and predictor for HCC recurrence is PVTT[2,3]. The effectiveness of transarterial chemoembolization (TACE) for HCC with PVTT remains unclear[4,5] though TACE is included in the treatment for HCC with tumor thrombus in the main portal branch[6]. However, it was suggested that hepatic arterial infusion chemotherapy might be a hopeful approach[7,8]. Because the median survival for untreated patients with PVTT is only 2.7 mo, this suggestion is especially relevant[9]. Hepatectomy for advanced HCC with removal of PVTT might also warrant consideration as an adjuvant treatment though it is usually performed as an emergency operation to avoid lethal complications[10]. Early recurrence has been reported in many cases due to intrahepatic metastasis from PVTT[11] even after tumors with PVTT in HCC patients was surgically removed. On the other hand, there have been reports of long-term survival after hepatectomy in patients with macroscopic portal invasion[12,13], but whether this treatment is optimal for patients with major PVTT remains controversial. Moreover, the operative mortality of major hepatectomy for HCC with macroscopic PVTT has not been well documented and reviewed. Therefore, we review literature published after January 2000 about the significance of hepatectomy in HCC with macroscopic PVTT with respect to long-term survival and mortality.

SURGICAL GUIDELINES FOR RESECTION IN HCC WITH PVTT

Because the cancer has already disseminated at this stage, leading to high rates of recurrence, hepatectomy for HCC with portal invasion is not recommended in the barcelona clinic liver cancer (BCLC) staging and treatment strategy. Portal invasion is associated with the development of metastatic nests, with higher incidence in tumors exhibiting microvascular invasion and/or satellite lesions[14].

According to the BCLC staging classification, sorafenib is the treatment of choice for HCC with macroscopic portal invasion (BCLC stage C). The efficacy of sorafenib in the treatment of advanced HCC was recently confirmed by the Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) trial. In that report, the median overall survival was 7.9 mo in the placebo group compared to 10.7 mo in the sorafenib group. The benefit of sorafenib was consistent in the pre-specified stratification groups that included patients with the worst prognosis, such as those with macroscopic vascular invasion[15]. According to the BCLC staging classification, hepatectomy is contraindicated in HCC with Vp3 (tumor thrombus in the first branch of the portal vein) or Vp4 (tumor thrombus extension to the trunk or to the opposite side branch of the portal vein) and should only be performed in patients with small single tumors without signs of portal hypertension or hyperbilirubinemia. On the other hand, the proposed treatment for HCC with minimal portal invasion, such as Vp1 (tumor thrombus distal to the second branches of the portal vein) and Vp2 (tumor thrombus in the second branches of the portal vein), is hepatectmy combined with TACE in the Japan Society of Hepatology (JSH) algorithm[16]. Indeed, when hepatectomy was performed in selected patients affected by HCC with macroscopic PVTT, in combination with either postoperative arterial infusion therapy or preoperative TACE, long-term survival was achieved[13,17].

In the 17th Nationwide Follow-up Survey of Primary Liver Cancer report in Japan, it was stated that the survival rates of 976 hepatectomized HCC patients with Vp3 or Vp4 were 50.4%, 25.8% and 18.4% at 1, 3 and 5 years, respectively[1].

HEPATECTOMY WITH MULTIDISCIPLINARY TREATMENT FOR PVTT

The prognosis of the HCC patients with PVTT in the first branch or main trunk is very poor, with a median survival of only 2.7 mo if appropriate treatments are not employed[9]. However, in the present literature search, we identified instances of long-term survival after hepatectomy. The range of overall survival (OS) rates for patients who received hepatectomy without multidisciplinary treatment were from 14.2% to 86.5% at 1 year, 0% to 60.4% at 3 years and 0 to 33.3% at 5 years (Table 1). On the other hand, the range of the OS rates for patients who received hepatectomy with multidisciplinary treatment were from 0% to 100% at 1 year, 14.0% to 74.0% at 3 years and 21.5% to 42.0% at 5 years (Table 1). From these data, we can see that the OS rates of patients who received hepatectomy with multidisciplinary treatment tended to be superior to those of patients who did not receive multidisciplinary treatment. This favorable

Table 1 Surgical outcome of hepatectomy for hepatocellular carcinoma patients with portal vein thrombus

Ref.	Vp type	Patients	Child-Pugh A (%)	HBV (%)	OS, 1 yr (%)	OS, 3 yr (%)	OS, 5 yr (%)	Treatment
Ohkubo *et al*[11] 2000	Vp234	47	91.5	42.6	53.9	33.2	23.9	
Minagawa *et al*[13] 2001	Vp234	18	NS	44.4	82	42	42	Preop TACE
Fan *et al*[26] 2003	Vp34	19	78.9	NS	14.2	0	0	
	Vp34	64	78.1	NS	37.6	14	NS	Postop PVI HAI
Capussotti *et al*[42] 2004	Vp234	13	NS	NS	NS	18.5	18.5	
Zhou *et al*[19] 2006	Vp234	381	NS	90	47	16	12	
Ikai *et al*[27] 2006	Vp34	78	NS	30.8	45.7	21.7	10.9	
Chen *et al*[49] 2006	Vp234	438	NS	NS	52.1	16	11.8	
	Vp23	286	13.3	60.1	58.7	22.7	18.1	
	Vp34	152	NS	62.5	39.5	5.7	0	
Nagano *et al*[17] 2007	Vp3	15	NS	66.7	100	74	NS	Postop 5-FU/IFN
	Vp3	15	NS	40	41	22	NS	
Kamiyama *et al*[12] 2007	Vp34	15	100	66.7	86.2	43.5	34.8	Preop radiation
	Vp34	28	85.7	64.3	39	13.1	13.1	
Liang *et al*[20] 2008	Vp34	33	54.5	93.9	46.8	14.4	NS	Postop PIAF
	Vp34	53	69.8	92.5	23.4	5.8	NS	
Peng *et al*[22] 2009	Vp34	51	86.3	NS	50.9	33.8	21.5	Postop TACE
	Vp34	53	86.8	NS	33.3	17	8.5	
Ban *et al*[57] 2009	Vp34	45	66.7	42.2	69.6	37.4	22.4	
Shi *et al*[48] 2010	Vp3	169	99.4	81.1	38.2	17.7	NS	
	Vp4	78	97.4	87.2	24.7	3.6	NS	
	Vp4[1]	20	95	90	18.3	0	NS	
Zhou *et al*[21] 2011	Vp34	21	NS	NS	47	22	NS	
	Vp34	38	NS	NS	70	20	NS	Adjuvant chemotherapy *via* portal vein
Matono *et al*[45] 2012	Vp34	19	NS	55.2	62.1	24.1	17.2	
	Vp34[2]	10	NS	NS	38.5	0	0	
Chen *et al*[50] 2012	Vp34	88	84.1	89.8	31.1	18.3	15.2	
Peng *et al*[46] 2012	Vp3	68	NS	NS	46.3	17.2	17.2	
	Vp4	83	NS	NS	32.5	3.6	3.6	
	Vp4[1]	23	NS	NS	21.7	0	0	
Tang *et al*[43] 2013	Vp234	186	91.9	85.5	40.1	17	13.6	
Li *et al*[58] 2013	Vp3	10	100	100	43	16	NS	
	Vp3[3]	20	100	90	32	11	NS	
Chok *et al*[47] 2014	Vp3	71	95.8	90.1	45.8	22.7	11.2	
	Vp3[4]	10	90	100	50	12.5	12.5	
	Vp34[5]	7	85.7	100	28.6	14.3	14.3	
Fukumoto *et al*[25] 2014	Vp234	41	NS	NS	80.5	32.4	NS	Postop PIHP
Yamamoto *et al*[44] 2015	Vp34	10	NS	NS	NS	NS	30	
Pesi *et al*[59] 2015	Vp3	21	NS	NS	60	39	10	
Kojima *et al*[18] 2015	Vp34	27	92.6	33.3	77.8	48.2	25.9	Postop HAIC (FP, epi-ADM)
	Vp34	25	88	32	68	32	12	
Xiao *et al*[60] 2015	Vp2	28	NS	NS	53.6	25	25	
	Vp3	38	NS	NS	39.5	15.8	5.3	
Bai *et al*[23] 2016	Vp23	51	92.2	22	19.6	NS	NS	
	Vp23	31	96.8	19	53.3	NS	NS	Postop TACE
	Vp23	10	100	30	71.1	NS	NS	Postop radiation
Zheng *et al*[51] 2016	Vp234	96	78.1	58.3	86.5	60.4	33.3	
Li *et al*[30] 2016	Vp4	39	88.9	82.2	69	NS	NS	Preop radiation
	Vp23	50	84	88	35.6	NS	NS	
Ye *et al*[24] 2016	Vp4	54	NS	85.2	0	NS	NS	Postop TACE
Hamaoka *et al*[33] 2017	Vp34	7	100	NS	100	71	NS	Preop radiation, HAIC (FP, IFN/5-FU)

[1]Tumor thrombi involving the superior mesenteric vein; [2]Non-curative resection; [3]Hepatectomy with caudate lobe; [4]PVTT extending to or beyond the portal vein bifurcation, treated by en bloc resection followed by portal vein reconstruction; [5]PVTT extending to or beyond the portal vein bifurcation, treated by thrombectomy. OS: Overall survival; HBV: Hepatitis B virus; Vp2: Tumor thrombus in the second branches of the portal vein; Vp3: Tumor thrombus in the first branch of the portal vein; Vp4: Tumor thrombus extension to the trunk or the opposite-side branch of the portal vein; TACE: Transarterial chemoembolization; PVI: Portal vein infusion; HAI: Hepatic arterial infusion; PIAF: Cisplatin, doxorubicin and 5-fluorouridine (5-FU) locally administered in the portal vein with subcutaneous injection of interferon-α; PIHP: Percutaneous isolated hepatic perfusion; FAIT: FU arterial infusion and interferon therapy; HAIC: Hepatic arterial infusion chemotherapy; FP: Cisplatin+5-FU; ADM: Adriamycin; NS: Not stated.

outcome was achieved when hepatectomy was pre- or postoperatively combined with multidisciplinary treatment. The multidisciplinary treatments included the following: Preoperative radiotherapy (RT) on PVTT in the main trunk or first branch[12]; preoperative TACE[13]; subcutaneous administration of interferon-alpha (IFN-α)

and intra-arterial infusion of 5-fluorouracil (5-FU)[17]; Epi-Adriamycin/cisplatin+5-FU[18]; cisplatin+5-FU infused in the portal vein or in the proper hepatic artery[19]; PIAF regimen (cisplatin, doxorubicin and 5-FU locally administered in the portal vein with subcutaneous injection of IFN-α)[20]; adjuvant chemotherapy (5-FU and Adriamycin) *via* the portal vein[21]; postoperative TACE[22-24]; percutaneous isolated hepatic perfusion (PIHP)[25]; and hepatic artery infusion and/or portal vein chemotherapy[26].

It was reported that the survival periods of approximately 10% of patients with tumor thrombi in the first branch and the portal trunk is more than 5 years following hepatectomy and that postoperative multidisciplinary treatments, including local and systemic adjuvant chemotherapy, are required in addition to hepatectomy to prevent intrahepatic metastasis[27]. Fukumoto *et al*[25] described that the efficacy of PIHP for hepatectomized patients with macroscopic PVTT had a median OS of 23 mo compared with a 6.5 mo median survival for patients treated with sorafenib[15]. However, PIHP treatment requires special equipment/expertise that is not currently available outside of Kobe University. On the other hand, Minagawa *et al*[13] reported the survival rate of 42% at 5 years for patients who underwent hepatectomy with preoperative TACE, which can be easily performed in any center, with only 9 cases exhibiting portal vein invasion in the second-order branches. It was reported that treating 15 cases of HCC with PVTT using FU arterial infusion and interferon therapy (FAIT) in addition to surgery, and 100% of the patients survived more than 1 year. In contrast, 10 patients (67%) died within 1 year without FAIT and surgery[17]. Peng *et al*[22] conducted a randomized controlled trial and showed that postoperative TACE enhances the effect of liver resection combined with PVTT removal. Estimated 1-, 3- and 5-years survival rates were better in the TACE group (50.9%, 33.8%, and 21.5%, respectively) than in the control group (33.3%, 17.0%, and 8.5%, respectively).

Liang *et al*[20] reported that the efficacy of intra-portal infusion chemotherapy using the PIAF regimen. They describe their procedure for administration of chemotherapeutic agents into portal vein as an attempt to kill the residual cancer cells in the portal venous system and subsequently curtail postoperative cancer recurrence. Moreover, another randomized controlled trial reported that postoperative TACE combined with portal vein chemotherapy is beneficial for patients with HCC complicated by PVTT but that the long-term efficacy of this approach is uncertain[28]. A combination of hepatectomy and preoperative RT has been reported to be effective for PVTT in the first branch or main trunk[12]. The survival rates at 1-, 3-, and 5-year were 100%, 53.3%, and 40.0%, respectively. Therefore, one of the abovementioned perioperative treatments combined with hepatectomy for HCC with PVTT might be necessary to prolong patient survival, though which of these options are superior cannot be concluded from this review. More appropriate regimens of perioperative treatment continue to be developed. Because Ando *et al*[7] reported that hepatic arterial infusion chemotherapy (HAIC) with 5-FU and low-dose cisplatin may be a beneficial therapeutic option for patients with HCC with PVTT in the main portal trunk or in the first portal branch or in the second portal branch; this regimen may be promising as adjuvant therapy for hepatectomy in HCC with macroscopic PVTT.

As a curative treatment for HCC with PVTT, only hepatectomy might be insufficient, and multidisciplinary treatments must be required because portal invasion is associated with the development of metastatic nests.

Significance of local treatment for PVTT

What about targeting PVTT for local treatment? Yamanaka *et al*[29] reported that the portal pedicles should be divided before liver parenchymal dissection during segmentectomy and lobectomy to decrease the chance of dissemination of the intravasated cancer cells because the cancer cells can dislodge into the portal venous stream during hepatectomy for HCC. From this point of view, targeting the PVTT to prevent cancer cell dissemination is a desirable approach. It was reported that preoperative radiation on the PVTT caused the tumor thrombus to become completely necrotic based on pathological examination, and 5 (83.3%) of the 6 patients survived for over 2 years after treatment[12]. Li *et al*[30] also demonstrated that better postoperative survival outcomes were provided by neoadjuvant radiotherapy before partial hepatectomy than partial hepatectomy alone for patients with HCC containing the main portal tumor thrombus. In 12 of 45 patients, the extent of PVTT after radiotherapy was significantly reduced, with the remaining 31 showing partial response (PR) and stable disease (SD) or two with progressive disease (PD)[30]. Because the tolerance of the liver for RT is low, RT for HCC has been limited to palliative treatment[31,32]. However, for the treatment of HCC, the effects of a high dose of local RT have been investigated[12,30]. Minagawa *et al*[13] described that radiation hepatitis did not occur in any of their patients and no apparent late radiation-induced complications were noted in any patients. For this reason, preoperative external RT was targeting the PVTT, not the whole tumor. By their method of RT, the irradiation in the normal liver tissue was minimized and the RT dose was increased without significantly increasing toxicity. Good survival outcome of hepatectomy with preoperative TACE for HCC patients with PVTT was reported[13]. Pathological examination detected necrosis of the PVTT in these patients. Therefore, the dissemination of HCC cells in the portal vein decrease because preoperative TACE or RT in PVTT induces necrosis. Consequently, these preoperative treatments might prevent HCC recurrence. Moreover, Hamaoka *et al*[33] reported that hepatectomy after down-staging with 3D-CRT for PVTT combined with HAIC for advanced HCC is safe and results in long-term survival outcomes. Hepatectomy for patients affected

by HCC with PVTT might prolong survival in conjunction with local treatment targeting PVTT: RT or TACE.

Operative mortality of hepatectomy for PVTT

Hepatectomy was indicated for living donor liver transplantation and the following liver tumors: Metastatic liver tumor, HCC, biliary malignancy. Of all the local treatments, hepatectomy for HCC had the highest local controllability and yielded a good survival outcome[34,35]. The liver functional reserve was decreased in almost all the patients with HCC because almost of patients with HCC had hepatitis B and/or hepatitis C viral infection and therefore had chronic hepatitis or cirrhosis[1]. Patients with liver cirrhosis decreased reticuloendothelial system functions, had elevated portal venous pressures, and impaired liver regeneration and coagulopathy[36]. Therefore, the mortality rates of hepatectomy in patients with liver cirrhosis was high form 8.9% to 19.6%[37]. On the other hand, recent advances in pre- and postoperative care, the decision criteria for hepatectomy and indications for hepatectomy and surgical techniques have been applied to extended hepatectomy[38-40]. Although operative mortality is not avoided even in donor hepatectomy for living donor liver transplantation, major hepatectomy become more safe by these preoperative evaluations[41].

There were 6 papers that reported mortality within 30 d. In Ohkubo *et al*[11]'s series, one patient died within 1 mo of the operation due to liver failure. In Capussotti *et al*[42]'s report, two patients died within 30 d due to postoperative bleeding and liver failure. In Ikai *et al*[27]'s report, patients who died within 30 d were 3, two from growth of the extrahepatic metastases and another because of pulmonary bleeding. There were 2 deaths (2.3%) recorded within 30 d after the operation due to operative mortality in Liang *et al*[20]'s paper. Two patients died within 30 d of surgery due to hepatic decompensation in Tang *et al*[43]'s study. Yamamoto *et al*[44] described one patient dying within 30 d of the operation due to acute renal failure. Thirteen papers mentioned mortality or operative mortality without reporting times. No mortality was described in six of these papers. The other 7 papers showed the number of patients or percentage of mortality. Fan *et al*[26] reported an operative mortality of 4.8%. In the control group, two patients died from operative complications in Peng *et al*[22]'s study. One patient in Matono *et al*[45]'s report died of operative morbidity. In Peng *et al*[46]'s report, there was one in-hospital postoperative death due to liver failure. The overall hospital mortality was 3.4% (n = 3) in Chock *et al*[47]'s study. There was one in-hospital death after the operation caused by postoperative bleeding (n = 169) in Shi *et al*[48]'s series. Operative mortality of Group A was 0% and that of Group B was 2.6% in Chen *et al*[49]'s paper. Chen *et al*[50] reported a mortality of 4.5%. Ye *et al*[24] reported that 5 patients died (9.3%) (liver failure: 3, serious infection: 1, and heart failure: 1). Zheng *et al*[51] reported that 1 in-hospital postoperative death (1.0%) occurred in the hepatic resection group, caused by a serious postoperative infection. Mortality on postoperative day 38 and 58 was described in studies by Capussotti *et al*[42] and Minagawa *et al*[13], respectively (Table 2).

When an HCC with major PVTT is surgically resected, a major hepatectomy should be performed with removal of the parenchyma fed by the portal vein obstructed by the PVTT vein. This operative procedure is quite technically complicated. In Asiyanbola *et al*[52]'s report, the type of operative procedure (more than or equal to hemi-hepatectomy *vs* less than hemi-hepatectomy) was related with in-hospital mortality and, specifically, patients who underwent more than or equal to a hemi-hepatectomy had a mortality rate of 6.5% compared with 4.1% for patients who underwent less than a hemi-hepatectomy. Therefore, major hepatectomy requires a very refined technique[52]. However, the operative mortality for hepatectomy in HCC with macroscopic PVTT has not been discussed. In the present review, the highest rate of operative mortality found in the literature was 9.3%[24]. The rest were not as high compared to the rates reported by Asiyanbola *et al*[52], though the mortality data were represented in a variety of ways. Major hepatectomy for HCC with macroscopic PVTT has been safely performed in many cases. The estimated cause was that the majority of patients described in this review paper had a Child-Pugh A and were infected with HBV (Table 1) and thus had a good liver function reserve. Therefore, we propose that the indication for hepatectomy in HCC with major PVTT should be expanded.

LONG-TERM SURVIVAL-RELATED FACTORS: THE EXTENT OF THE TUMOR THROMBUS

What are the long-term survival-related factors? Shi *et al*[48] previously classified PVTT into 4 groups by the extent of the tumor thrombus. Patients of types I and II: PVTT located in the segmental, sectoral, or right and/or left portal veins showed significantly better survival than those of types III and IV: PVTT extended to the main trunk of the portal vein or the superior mesenteric vein. Therefore, they concluded that hepatectomy with thrombectomy is justified in selected patients with HCC and PVTT located in the first, second, or lower branch of the portal vein. Zheng *et al*[51] also reported that the long-term survival in patients with type I and II PVTT was remarkably improved compared with in patients with type III and IV PVTT. Moreover, Kokudo *et al*[53] reported data from the nationwide survey of patients with primary liver cancer performed by the Liver Cancer Study Group of Japan, which stated that the survival benefit of liver resection was statistically significant only in patients with PVTT invading the main trunk or contralateral branch. From these data, while HCC with PVTT located in the first or second branch of the portal vein might be a relatively

Table 2 Patient mortality

	Ref.	Patient number or percent	Cause of death
Within 30 d			
	Ikai *et al*[27]	3	Pulmonary bleeding
			Extrahepatic growth
	Yamamoto *et al*[44]	1	Renal failure
	Ohkubo *et al*[11]	1	Liver failure
	Capussotti *et al*[42]	2	Postoperative bleeding
			Liver failure
	Liang *et al*[20]	2	
	Tang *et al*[43]	2	Decompensation
38 d			
	Capussotti *et al*[42]	1	Sepsis
58 d			
	Minagawa *et al*[13]	1	Liver failure
Operative mortality			
	Fan *et al*[26]	4.80%	
	Peng *et al*[22]	2	Operative complication
	Matono *et al*[45]	1	
	Peng *et al*[46]	1	Liver failure
	Chock *et al*[47]	3.40%	
	Shi *et al*[48]	1	Postoperative bleeding
	Zheng *et al*[51]	1	Serious postoperative infection
	Ye *et al*[24]	9.30%	Liver failure, serious infection, heart failure
Mortality			
	Chen *et al*[49]	0%	
		2.60%	
	Nagano *et al*[17]	0%	
	Kamiyama *et al*[12]	0%	
	Ban *et al*[57]	0%	
	Chen *et al*[50]	4.50%	
	Fukumoto *et al*[25]	0%	
	Li *et al*[30]	0%	

good indication for hepatectomy, hepatectomy for HCC combined with PVTT in the contralateral branch or main trunk should be performed after careful consideration.

Long-term survival-related factors: Liver function

Ikai *et al*[27] reported that the absence of ascites, prothrombin activity, and tumor diameter are independent prognostic factors reflecting portal hypertension, liver function and tumor status, respectively. Kondo *et al*[54] reported negative prognostic factors of hepatectomized patients with PVTT, including age < 60 years and factors related to liver function: Total serum bilirubin > 0.8 mg/dL and serum alkaline phosphatase > 300 IU/mL. Pawlik *et al*[55] concluded that patients with HCC and the major vascular invasion of the main portal or hepatic vein branches derive long-term resection benefits if they have no, or minimal, underlying fibrosis. In another report, the presence of fibrosis: Moderate to severe was the individual significant predictive factor on multivariate analysis that was related with worse short-term (≤ 6 mo) and long-term (> 6 mo) survival. In this paper, the authors argued that this result is due to postresection hepatic decompensation and to a "field cancerization"[56] effect in the cirrhotic liver, which places these patients at a higher risk for metachronous or synchronous disease. Most of the patients described in the studies we reviewed had a Child-Pugh A and were infected HBV (Table 1). This status might be a requirement for adaptation to hepatectomy to prevent postoperative hepatic decompensation. Moreover, increased liver function reserve might lead to a better prognosis for patients with HCC complicated by PVTT after hepatectomy due to the prevention of synchronous or metachronous tumors. Of the HCC patients with macroscopic PVTT, the indication of hepatectomy should be restricted within good liver function reserve.

A limitation of this review is that most of the articles selected were published from Eastern Asian countries, and the findings may not be applicable to other regions of the world. A more comprehensive review of the global literature would be very valuable in the future.

CONCLUSION

Hepatectomy might prolong the survival of patients with HCC with PVTT when the liver function reserve is preserved, such as in Child-Pugh score A cases. Effective multidisciplinary treatments may improve the prognosis and prevent recurrence due to disseminated cancer cells in these patients. Moreover, hepatectomy may be a feasible adjunct treatment for HCC with PVTT due to the current mortality rates after hepatectomy being quite low.

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MINIREVIEWS

Molecular basis of hepatocellular carcinoma induced by hepatitis C virus infection

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Abstract

Present study outlines a comprehensive view of published information about the underlying mechanisms operational for progression of chronic hepatitis C virus (HCV) infection to development of hepatocellular carcinoma (HCC). These reports are based on the results of animal experiments and human based studies. Although, the exact delineated mechanism is not yet established, there are evidences available to emphasize the involvement of HCV induced chronic inflammation, oxidative stress, insulin resistance, endoplasmic reticulum stress, hepato steatosis and liver fibrosis in the progression of HCV chronic disease to HCC. Persistent infection with replicating HCV not only initiates several liver alterations but also creates an environment for development of liver cancer. Various studies have reported that HCV acts both directly as well as indirectly in promoting this process. Whereas HCV related proteins, like HCV core, E1, E2, NS3 and NS5A, modulate signal pathways dysregulating cell cycle and cell metabolism, the chronic infection produces similar changes in an indirect way. HCV is an RNA virus and does not integrate with host genome and therefore, HCV induced hepatocarcinogenesis pursues a totally different mechanism causing imbalance between suppressors and proto-oncogenes and genomic integrity. However, the exact mechanism of HCC inducement still needs a full understanding of various steps involved in this process.

Key words: Hepatitis C virus; Hepatocellular carcinoma; Fibrosis; Core; NS5A; Inflammation

Core tip: Hepatocellular carcinoma (HCC) is one of the most common cancer occurring in human population all over the world. Chronic hepatitis C virus (HCV) infection is considered as a major cause of producing

HCC in developed countries. HCV infection induces chronic inflammation in liver, which initiates several changes including production of oxidative stress, steatosis, progressive fibrosis, cirrhosis and finally HCC. HCV related proteins also interact directly with cellular proteins at various steps of cell signaling disturbing cell cycle and regeneration process. HCC is supposed, now a days, to be the foremost indication for liver transplant.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a global health problem reported from all parts of the world. HCV was characterised by Choo *et al*[1] and Kuo *et al*[2] in 1989. As per World Health Organization report, about 3% world population is having HCV infection with 170 million people becoming as chronic carriers of HCV[3]. These people always remain at high risk of developing cirrhosis of liver and hepatocellular carcinoma (HCC) in later years. There is an increase in the cases of HCC with 1%-7% chronic HCV infected patients developing HCC after establishment of cirrhosis[4,5]. HCC caused by HCV infection is a prominent indication for liver transplant[6].

HCV is an enveloped RNA virus included under Flaviviridae family[7]. It has 9.6 kb single stranded RNA with positive polarity. HCV genome encodes a long protein of 3000 amino acids which undergoes proteolysis to yield structural proteins (Envelop E1, E2 and Core) and nonstructural proteins (P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B)[8]. Whereas structural proteins play important role in its morphological features and entry into the host cell, nonstructural proteins are involved mainly in viral replication, assembly and pathogenesis of diseases caused. HCV genome is highly heterogeneous with 32%-35% variations in different HCV genotypes[9]. Based on current reports at least seven genotypes and several subtypes of HCV have been reported till date[10]. Although, variability of genomic sequence has been reported throughout the viral genome, the E1 and E2 regions have been reported to be maximally variable[10].

HCC develops more frequently in cirrhotic patients in comparison to those having mild fibrosis[11]. In addition, hepatitis B virus infection, insulin resistance, obesity and steatohepatitis also promote HCV related HCC[12]. HCC may result from a combined effect of host, environment and viral factors[13]. Immune mediated chronic inflammation during HCV infection is supposed to facilitate the development of HCC. Simultaneously, it may induce HCC by altering many cell pathways involved in cell proliferation, energy metabolism, and apoptosis[14].

As such, HCV is a non-cytopathic virus and initiates hepatic injury by immune mediated reaction-cascade. Although, it is not fully established, however, on the basis of animal experiments and human studies, it is assumed that HCV plays both direct as well as indirect role in inducing HCC[15,16]. Current literature demonstrates that cell death, regeneration, inflammation, oxidative stress and steatosis noted during chronic HCV infection are some of the main reasons responsible for hepatocarcinogenesis[13,17]. Similarly, dysregulation of cell cycle by altered intracellular signaling cascade arising during chronic HCV infection is an important phenomenon in the direction of HCC development. In fact, mechanism of hepatocarcinogenesis during chronic HCV infection is slightly distinct from those responsible for causing other types of cancers. HCV core protein was found to induce HCC in absence of genetic aberrations and so, this was named as "non- Vogelstein- type" carcinogenesis in some reports[18]. This may explain a high incidence and multicentric nature of HCC developed during HCV infection. Present review describes a compilation of informations on the mechanisms of HCC development during chronic hepatitis C virus (HCV) infection.

MECHANISMS OF HCV INDUCED HCC

HCV is a hepatotropic virus and enters host cell *via* a complex sets of molecules present on cell surface including CD81 (receptor molecule), SRB-1 (scavenger receptor) and Occludin-1 and Claudin (tight junction proteins)[19-21]. After its entry, HCV replicates in hepatocytes and leads to different types of cellular and immune mediated changes. A majority of patients infected with HCV fail to clear the virus. In these patients HCV persists for longer duration causing chronic HCV infection and a high risk for progressive hepatic fibrosis, cirrhosis and HCC[22]. Simultaneously, the ensuing chronic inflammation associated with oxidative stress and emerging cellular DNA damage, also contribute to development of HCV associated HCC. The question whether cancer develops in infected hepatocytes or in uninfected hepatocytes still needs to be answered. Based on some experimental studies it was reported that Ki67 proliferation marker is raised in advanced HCV infected hepatocytes pointing towards HCV infected cells at higher risk for HCC as compared to uninfected cells[23,24]. Several studies suggest that liver cancer develops by an interplay of host, viral and environmental factors. All these finally bring some epigenetic changes in HCV infected hepatocytes leading to development of HCC[13,25].

Chronic HCV infection is often accompanied by several disturbances including inflammation, steatosis and progressive fibrosis in the liver[25]. All these changes ultimately progress to cirrhosis and hepatocarcinogenesis. Therefore, it is suggested that HCC is caused by an interplay of chronic inflammation, insulin resistance

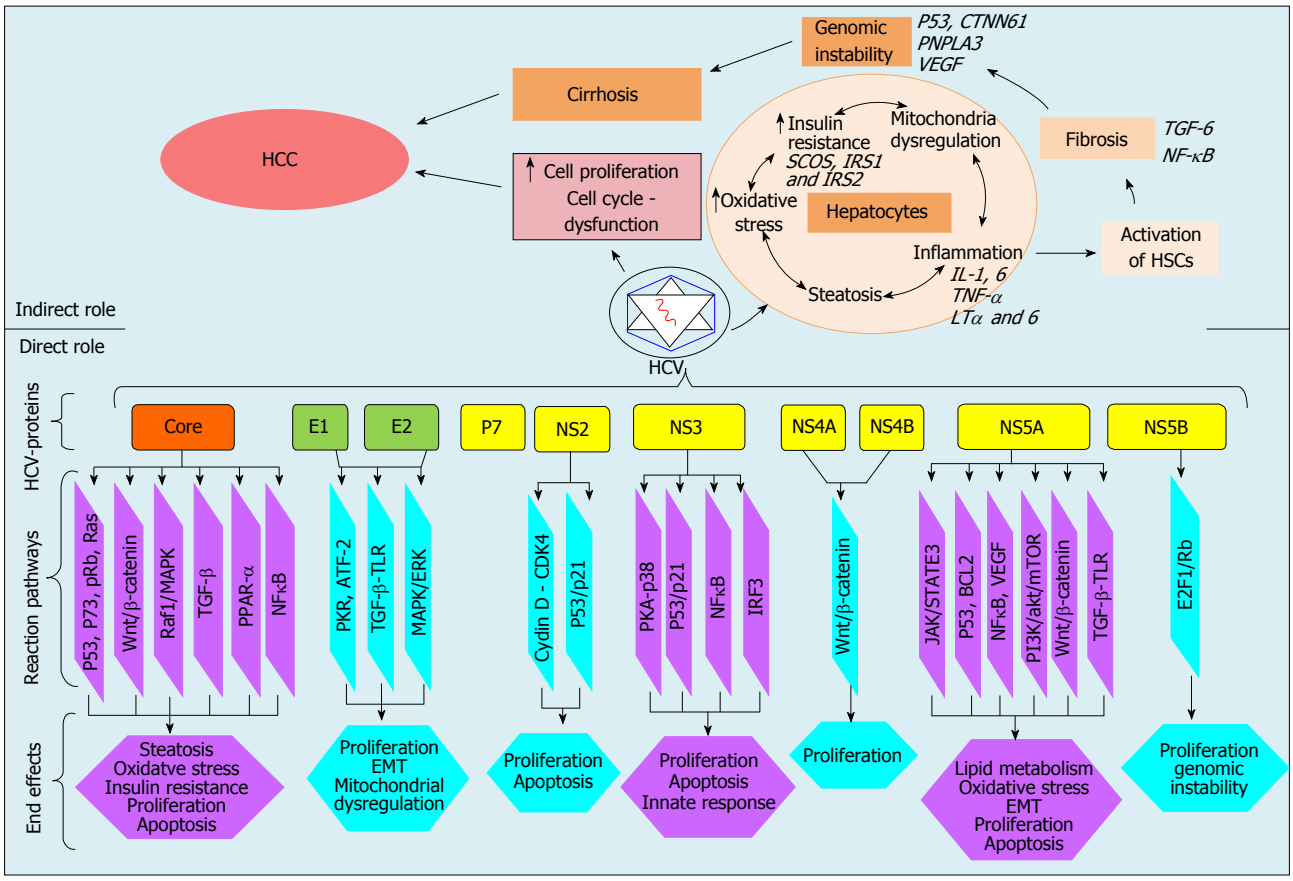

Figure 1 Direct and indirect role of hepatitis C virus in causing hepatocellular carcinoma. Role of hepatitis C virus (HCV) and its structural and non-structural proteins in inducement of hepatocellular carcinoma (HCC) during chronic HCV infection. Viral onset causes various cellular alterations leading to activation of hepatic stellate cells which in turn, produce progressive fibrosis leading to cirrhosis of liver. Simultaneously, HCV also dysregulates cell cycle causing cell proliferation. Both cirrhosis and cell proliferation induce development of HCC. In this figure, the top half portion shows an indirect role of HCV *via* cellular alterations and causing cirrhosis by inter-related mechanisms and cell dysregulation leading to cell proliferation. The lower half shows a direct role of HCV by interaction of its proteins with various cellular pathways producing different effects as preconditions for inducement of HCC. The link bars show the underlying pathways and the bottom boxes show the end effects. EMT: Epithelial to mesenchymal trans-differentiation; HSCs: Hepatic stellate cells; TGF: Transforming growth factor; PKR: Protein kinase; VEGF: Vascular endothelial growth factor; TNF-α: Tumor necrosis factor-α; PPAR-α: Peroxisome proliferator-activated receptor alpha; ERK: Extracellular signal regulated protein kinase; PKA: Protein kinase A; NF-κB: Nuclear factor-κB.

(IR), hepatosteotasis, oxidative stress, fibrosis, and the resulting liver damages by chronic HCV infection. This interplay produces a pro-oncogenic microenvironment which promotes fibrogenesis and genetic instability[26]. Simultaneous with a direct transforming role of HCV, the liver microenvironment is supposed to have a modulating effect on cell transforming process during HCC development. Several HCV proteins have direct oncogenic effects and use liver changes in upregulating mitogenic process[27]. At the same time, increasing cell proliferation in this environment also results in DNA damage causing genomic disturbances. This becomes another basis for malignant transformation of hepatocytes. In view of all these available reports[25-27], the mechanism of HCV induced HCC may be illustrated by a direct and indirect role of HCV in relation to the microenvironment produced by chronic HCV infection (Figure 1).

HOST FACTORS

Inflammation and oxidative stress in HCV induced HCC

Immune mediated inflammation caused during chronic HCV infection indirectly triggers hepatocarcinogenesis. Simultaneous with a direct role of HCV in inducement of HCC by altering several cellular pathways involved in metabolism, DNA repair and apoptosis[14], chronic HCV infection enhances the reactive oxygen species (ROS) which damages the liver cells. At the same time, HCV also induces inflammation by activating hepatic stellate cells (HSCs)[28]. These HSCs get activated by ROS, growth factors, cytokines, adipokines and chemokines secreted by hepatocytes, Kuffer cells and inflammatory cells[29]. The progress of disease is increased by cumulative effect of inflammation, ROS, steatosis and IR caused during chronic HCV infection. The activated HSCs, under the effect of fibrogenic cytokines undergo epithelial to mesenchymal trans-differentiation (EMT) into myofibroblast like cells which cause liver fibrosis[14]. Transforming growth factor beta (TGF-β) cytokine regulates EMT demonstrating its pro-oncogenic functions[30]. Hepatic fibrosis is closely associated with HCC development. EMT pathway plays a major role in transition of hepatocyte to cancerous cell and process of metastasis known with expression of E-cadherin and

Vimentin[31]. The IR stimulates HSCs and links fibrosis with steatosis. The process of fibrogenesis is regulated by a number of signaling pathways including SMADs, phosphatidylinositide 3-kinases (PI3K), protein kinase (Akt), mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinases (JNKs) pathways. JNK activation by IL1-β cytokine increases fibrogenesis, oncogenesis and cell motility[32,33]. Thus all these liver alterations finally produce a suitable environment for development of HCC in chronic HCV infection.

Insulin resistance and hepato steatosis in HCV induced HCC

It has been observed that HCV *genotype-3* induces steatosis in patients with chronic HCV infection[34]. HCV induces steatosis by increasing lipid synthesis and reducing its secretion and degradation. The structural and nonstructural proteins of HCV directly interfere in lipid synthesis[35] and very-low-density lipoprotein secretion[36,37]. These HCV related proteins also inhibit fatty acid oxidation[38,39] and enhance fatty acid release from adipocytes[34]. All this finally results in hepatic steatosis. The HCV related proteins are also involved in producing ROS[40] and glucose homeostasis. HCV interferes with insulin signaling by proteosomal degradation of insulin receptor substrate 1 (IRS-1) and IRS-2 by suppressor of cytokine signaling (SOCS) protein or PI3K/Akt/mTOR pathway. IRS-1 is reported to be inactivated by TGF-α and PI3K/Akt also[41]. In this manner, the early stage of chronic HCV infection with increasing steatosis and IR creates an environment to help in hepatocarcinogenesis leading to development of HCC.

Immune mediated liver alteration in HCV induced HCC

HCV influences both innate and adaptive immunity. This virus inhibits type 1 Interferon production and $CD4^+$ T-cell transformation to Th2, Th17 and regulatory T-cell. This disturbs the function of cytotoxic $CD8^+$ T-cells and natural killer (NK) cells[42-48]. It results in chronic liver inflammation which disturbs tissue homeostasis and promotes pro-carcinogenic environment. Simultaneously, there is an increase in the release of ROS, nitric oxide (NO), cytotoxic cytokines and lipid peroxidation. It also helps in immune escape of neoplastic transformed cells facilitating the development of HCC[49]. During chronic HCV infection, the inflammatory cytokine like tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-23, IL-6 and lymphotoxins-alpha and beta (LT-α and β) are also increased causing chronic liver inflammation and HCC progression[49-51]. There is already a report demonstrating an important role of LT-α and LT-β in the development of HCC[51]. In fact, activation of NF-κB pathway by LTs triggers the hepatocarcinogenesis by increasing production of chemokines and cytokines. In patients with chronic HCV infection, the liver infiltrating T and B-cells not only fail viral clearances but also increase chronic inflammation[51,52]. Also, an increased number of $CD8^+$ is accompanied by reduction in NK and NKT cells which are involved in cancer immune surveillance[52]. These informations indicate that during chronic HCV infection there is a regular tumor promoting inflammation and impaired anticancer immune scanning, which ultimately facilitates towards HCC.

Hepatic fibrosis in HCV induced HCC

As described earlier there is high occurrence of steato-hepatitis in patients with chronic HCV infection. The accumulation of free fatty acid induces production of ROS and mitochondrial dysfunction and Endoplasmic reticulum (ER) stress. In turn, oxidative stress stimulates lipid peroxidation and increases inflammation in liver tissue. Increased ROS levels have direct effect on fibrosis by increasing collagen 1 expression[50]. The HCV induced steatosis changes the liver T-cell function. HCV related proteins in the liver develop extensive steatosis which is accompanied by an infiltrate of $CD8^+$ T-cell secreting Th2 type cytokine[53]. A massive liver infiltration by $CD8^+$ and NKT cells induces steatosis, inflammation and carcinogenesis[54]. In HCV infected patients, the risk of HCC development may also be linked with the severity of liver fibrosis. TGF-β is an important cytokine involved in fibrogenesis. Its expression is directly affected by HCV related proteins or oxidative/ER stress and NF-κB pathway activation[55-58]. This concludes that hepatic fibrosis caused by various mechanisms is a big inducer promoting hepatocarcinogenesis.

Genetic factors in HCV induced HCC

There are a number of genes associated with HCV induced HCC. The tumor suppressor gene *P53* was the first one noted for its association with development of HCC. Recent studies have shown a subset of genes frequently mutated in HCV patients[59,60]. Oncogene CTNNB1 which encodes β-catenin protein of WNT-pathway shows a mutation of 30%. WNT ligands activate signal transduction cascade resulting in inhibition of beta-catenin degradation complex. It has been observed that WNT pathway gets mutated in HCC, which stabilizes β-catenin, this β-catenin translocates to the nucleus and regulate genes responsible for cell survival and proliferation. NS5A indirectly regulate the WNT pathway through PI3K and activate Akt. Increased β-catenin has been observed in HCV infected cells. The significance of β-catenin with is HCV infected cells is still uncertain[61]. However, its level is increased mostly in HCC patients. Similarly, reduction in the size of telomere triggers cellular senescence. Activation mutation in the telomerase reverse transcriptase (TERT) promoter gene has been detected in HCC induced by HCV infection in addition to other etiologies[62-64]. HCV core protein downregulates CDKN2A expression to overcome hepatocyte senescence. Increased telomerase activity, a characteristic of transforming or transformation prone cells was observed in HCV core- transfected primary human hepatocytes that acquired an immortalized phenotype. In line with this observation, somatic mutation in the

TERT promoter that enhance TERT expression were shown to be among the earliest and most prevalent neoplastic events associated with all major etiologies including HCV. Host genetic variants are also associated with a high risk of HCC[65]. *PNPLA3* gene (patatin-like phospholipase domain-containing protein-2) shows a significant association with fatty liver disease in HCV patients having a higher risk of HCC[66-68]. On a similar pattern, polymorphisms in several other cytokines/receptors genes have been found to be associated with HCC. These are cytokines *TNF-α*, *IL-10*, *IL-23R* and vascular endothelial growth factor (*VEGF*) *etc.* genes. Host respond differently to variation in viral genome for example HCV genotype 1a and 1b reported to be associated with HCC[69].

Epigenetic alterations in HCV induced HCC

Various studies have demonstrated a dysregulation of epigenetic regulatory genes in HCC[70]. Histone-lysine N-Methyltransferase enzyme (EZH2) is one such an example which is aberrantly expressed in HCC[71] and this also targets expression of tumor suppressor miRNAs[72]. The changes in gene methylation were also related with virus induced tumors[73]. Various tumor suppressor genes including CDKN2A, GSTP1, RUNX3, APC, SOCS-1 and RASSF1A are highly methylated in HCC caused by HBV and HCV infection[74]. Epigenetic alterations in HCC may be mediated by changes in miRNAs and long noncoding RNAs. There are several miRNAs which modulate HCV replication in a positive and negative manner[75].

Neoangiogenesis in HCV induced HCC

Structural and nonstructural HCV proteins have a direct role in inducing neoangiogenesis. HCV core promotes angiogenesis by upregulating hypoxia inducible factor 1-alpha which regulates VEGF and cyclooxygenase 2. VEGF is an important endothelium specific growth factor in HCC and for this reason, VEGF level in serum is used as a prognostic factor in HCC[76]. Angiopoietin-2 is also upregulated by HCV infection[77].

VIRAL FACTORS

HCV replicates and releases its protein component in cytosol. HCV related proteins which have a major role in regulating viral replication and HCV particle assembly, have been demonstrated to influence several cell signal pathways and metabolic mechanisms indicating their role in cell cycle and cell transformation. Both structural and nonstructural proteins interact with different host cellular proteins to promote malignant transformation of hepatocytes. Based on these studies we describe here the role of each individual HCV protein in the process of cell transformation to malignant liver cell.

Core protein

HCV core protein, which regulates HCV RNA translation and its replication, interacts with component proteins of various cell-signaling pathways. In addition, this protein modulates host immune response, oxidative stress, lipid metabolism and also apoptosis[78]. In some recent studies on HCV infected patients, core gene has been found to undergo frequent mutations[79]. The role of core protein in the development of HCC was studied in transgenic mouse model. The information collected from these studies indicate that core gene overexpression results in steatosis in early life with development of adenoma and HCC in later years[80]. In few other studies, the presence of steatosis in liver induced by core protein could not be related to HCC development[29,81]. According to recent reports, core protein shows interference with cellular proteins and it is considered as a major risk factor for the progression of HCC[82]. Of course, the presence of core protein has been associated with its activation of lipogenic pathway in HCC cases[83]. Core protein often remains associated with lipid droplets in CHC cases and possibly causes steatosis through several mechanisms including peroxisome proliferator-activated receptor alpha and sterol-regulatory element binding protein-1 pathways[46,81,84].

Similarly, core protein also interacts with ER or mitochondria and induces ER stress by accumulation of ROS[85]. ROS causes DNA damage and accelerates hepatocarcinogenesis. The effect of HCV core have also been demonstrated on signaling pathways responsible for cell cycle like stimulation of G_1/S transition by increasing the levels of cyclin E/Cdk2[86] and apoptosis. Core protein interacts with tumor suppressor including P53, P73 and P21[87] as well as regulator of apoptosis like TNF-α signaling or Bcl-2 members. Core proteins also effects growth and proliferation of cells through activation of signaling pathways like RAF/MAPK (Mitogen activated protein kinase)[88], Wnt/β-catenin and TGF-β[39,89]. All these pathways have been reported to be active in HCC. Therefore, these findings about HCV core indicate that this protein has a potential role in cell proliferation and reduction of apoptosis during development of HCC.

E1/E2 protein

The effect of structural proteins E1/E2 was also studied on malignant transformation of hepatocytes. The results indicated these proteins to interfere with Interferon actions by inhibiting dsRNA protein kinase (PKR)[90,91]. In addition, E2 protein also inhibits activation of T and NK cells[91] and MAPK/extracellular signal regulated protein kinase pathway including the transcription factor ATF-2 and promotes cell proliferation and cell survival[92].

NS2 protein

NS2 activates cyclin D/CDK4 and induces expression of Cyclin E[93]. Some studies also supported its role in the inhibition of apoptosis by interference with p53 pathway.

NS3 protein

The NS3 transforms mammalian cells but its role in

HCC is less clear[94,95]. This protein interacts with tumor suppressor p53. NS3 protein modulates various signal transduction pathways having transformation potential. NS3 interacts with protein kinase A and inhibits its translocation to nucleus. NS3 also inhibits interferon response factor (IRF-3) mediated induction of type-1 interferon, necessary to escape immune surveillance. NS3/4A interacts with ATM, Check point kinase, preventing DNA repair. This also disturbs endoplasmic reticulum leading to cell death[96]. Similarly, NS3/4A target adaptor molecules in TLR3 and RIG-1 signal pathway, thereby interfering with activation of IRF-3 transcription factor and promoting proliferation[97-99]. All these reactions contribute to cancer promoting effect of HCV.

NS5A protein

This protein is needed for replication of HCV genome. It forms part of viral replicates complex. Inside the nucleus, NS5A acts as transcription factor activator[100] and interacts with various signaling pathways including cell cycle/apoptosis, lipid metabolism[46,101] and also shares some signaling targets with core. It has been reported to interfere with PKR-p38 signaling pathway and inducing aberrant mitosis and chromosomal instability leading to HCC[102]. NS5A inhibits TGF-β signaling by preventing nuclear translocation of SMAD proteins down regulating tumor suppressor CDKN1A[103]. On a similar pattern, NS5A inhibits tumor necrosis factor-α (TNF-α) mediated apoptosis[104]. NS5A acts a transcriptional activator for many genes including p53. NS5A also interacts with pathways like Bcl-2, PI3K, Wnt/β-catenin signal and mTOR for proliferation of cells and inhibition of apoptosis. It has been found that HCV NS5A influences EMT pathway and helps in transition process of epithelial cells to mesenchymal stem cells. NS5A work in cooperation to TGF-β to activate stellate cell causing fibrosis. Also HCV core protein was found to induce EMT in primary hepatocyte by suppressing cytostatic effect *via* SMAD3[105,106]. Thus NS5A and core produce cells in tumor mass that are not differentiated and mobile *via* EMT pathway EMT contributes to liver fibrosis on the line as in lungs, kidney and intestine.

NS5B protein

NS5B binds with Rb and promotes its cytoplasmic relocalization and proteasomal degradation[107,108]. This finally activates E2F responsive genes, which in turn stimulates cell cycle progression[108].

Above reports demonstrate a clear effect of HCV-related proteins on various pathways engaged in progression of infected cells to malignant cells. These proteins enhance the level of underlying inflammation, oxidative stress, ER stress, steatosis, fibrogenesis and finally cell proliferation. Although it is not possible to emphasis their direct effect in exclusion either on initiation or progression, but there is no doubt that involvement of these proteins at various steps of complex mechanism, helps in progression of carcinogenesis resulting in development of HCC.

CONCLUSION

This update on the development of HCC following chronic HCV infection demonstrates that HCV infection is a serious health problem recorded globally. A majority of patients progress to end stage liver diseases including liver cirrhosis and HCC. Once established, the chronic HCV infection produces several changes in the liver including chronic inflammation, insulin resistance, oxidative stress, steatosis and continuing liver fibrosis. These changes are caused by the mechanism influenced either directly or indirectly by HCV particles. HCV related proteins interact with several cellular proteins thereby modulating cell signaling. Similarly, chronic inflammation caused by HCV inflammation also promotes all above liver changes. During this interplay of various reaction cascade there is possibility of genomic imbalance disturbing the normal reactions leading to abnormal cell cycle and apoptosis. The cumulative effect of all these finally facilitates the tumorigenesis in liver causing HCC. Although several lines of information are available, however, much more still needs to be answered to extricate this mystery.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Recent trends in liver transplantation for alcoholic liver disease in the United States

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Abstract

AIM

To examine temporal changes in the indications for liver transplantation (LT) and characteristics of patients transplanted for alcoholic liver disease (ALD).

METHODS

We performed a retrospective cohort analysis of trends in the indication for LT using the United Network for Organ Sharing (UNOS) database between 2002 and 2015. Patients were grouped by etiology of the liver disease and characteristics were compared using χ^2 and *t*-tests. Time series analysis was used identifying any year with a significant change in the number of transplants per year for ALD, and before and after eras were modeled using a general linear model. Subgroup analysis of recipients with ALD was performed by age group, gender, UNOS region and etiology (alcoholic cirrhosis, alcoholic hepatitis and hepatitis C - alcoholic cirrhosis dual listing).

RESULTS

Of 74216 liver transplant recipients, ALD (n = 9400, 12.7%) was the third leading indication for transplant after hepatitis C and hepatocellular carcinoma. Transplants for ALD, increased from 12.8% (553) in 2002 to 16.5% (1020) in 2015. Time series analysis indicated a significant increase in the number of transplants per year for ALD in 2013 (P = 0.03). There were a stable number of transplants per year between 2002 and 2012 (linear coefficient 3, 95%CI: -4.6, 11.2) an increase of 177 per year between 2013 and 2015 (95%CI: 119, 234). This increase was significant for all age groups except those 71-83 years old, was observed for both genders, and was incompletely explained by a decrease in transplants for hepatitis C and ALD dual listing. All UNOS regions except region 9 saw an increase in the mean number of transplants per year when comparing eras, and this increase was significant in regions 2, 3, 4, 5, 6, 8, 10 and 11.

CONCLUSION

There has been a dramatic increase in the number of transplants for ALD starting in 2013.

Key words: Alcoholic liver disease; Liver transplantation; Cirrhosis; Epidemiology; Hepatitis C

Core tip: Although the number of liver transplants done for alcoholic liver disease (ALD) has been stable been 2002 and 2012, since 2013 there has been a significant increase. This increase is seen across all age groups, although the proportional increases are higher for younger patients than older ones. The increase corresponds, but is incompletely explained, by a decrease in transplants for hepatitis C - ALD dual listing. The increase was also seen in most, but not all UNOS regions.

Kling CE, Perkins JD, Carithers RL, Donovan DM, Sibulesky L. Recent trends in liver transplantation for alcoholic liver disease in the United States. *World J Hepatol* 2017; 9(36): 1315-1321 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i36/1315.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i36.1315

INTRODUCTION

Liver transplantation (LT) has become a life-saving procedure for patients with irreversible liver diseases. A total of 7841 liver transplants were performed in 2016 in the United States with 14389 potential recipients on the waiting list[1]. One of the common causes of chronic liver disease for which LT is potentially life saving is alcoholic liver disease (ALD). Progression of ALD is dependent on patient characteristics (sex, race, ethnicity, malnutrition), genetic factors, coexisting liver pathology [*e.g.,* hepatitis C virus (HCV) or non-alcoholic steatohepatitis (NASH)] as well as drinking patterns (volume consumed, drinking outside meal times, binge drinking, and duration of consumption). The risk of developing cirrhosis is increased with consumption of > 60-80 g/d of alcohol for ⩾ 10 years for men and > 20-40 g/d in women[2,3]. However, despite drinking at these levels, only 6%-41% of people develop cirrhosis[2,4].

Population-based studies have shown that although the proportion of the population who drink any alcohol is not increasing, there has been an increase in the prevalence of both heavy drinking (defined as more than 1 drink per day for women or 2 drinks per day for men, on average) and binge drinking (defined as at least 4 drinks for women or 5 for men in the last thirty days)[5]. Heavy drinking has been shown to increase the risk of ALD and all-cause mortality[6]. Because we have noticed a recent increase in the number of referrals to our transplant center for ALD, we decided to critically review the temporal and geographic trends in the LT for ALD and examine characteristics of patients transplanted for ALD.

MATERIALS AND METHODS

Data source

We conducted a retrospective cohort analysis of transplant recipients in the United Network for Organ Sharing (UNOS) Standard Transplant Analysis and Research file. United States donor data for this analysis is Organ Procurement and Transplantation Network data released 2016-06-17 based on data collected through 2016-03-31. UNOS as the contractor for the Organ Procurement and Transplantation Network supplied this data. The interpretation and reporting of these data are the responsibility of the authors and in no way should be seen as an official policy of or interpretation by the Organ Procurement and Transplantation Network or the United States Government. The statistical methods of this study were reviewed by Dr. James Perkins from the University of Washington. This study met expedited review criteria as approved by the University of Washington Institutional Review Board.

Study population and temporal trends

We identified all liver transplant recipients in the UNOS database from 2002 to 2015 and characterized them according to the etiology of their liver disease. The category ALD was defined as recipients with a diagnosis of alcoholic cirrhosis or acute alcoholic hepatitis. However, in order to minimize the effect of concomitant liver disease, we categorized those with a listing diagnosis of both HCV and alcoholic cirrhosis (HCV/ALD) as HCV.

Recipient characteristics were compared among the leading four etiologies of cirrhosis using χ^2 test for categorical values and student's t-test used for continuous variables. The number of transplants per year by liver disease was graphed to illustrate changes

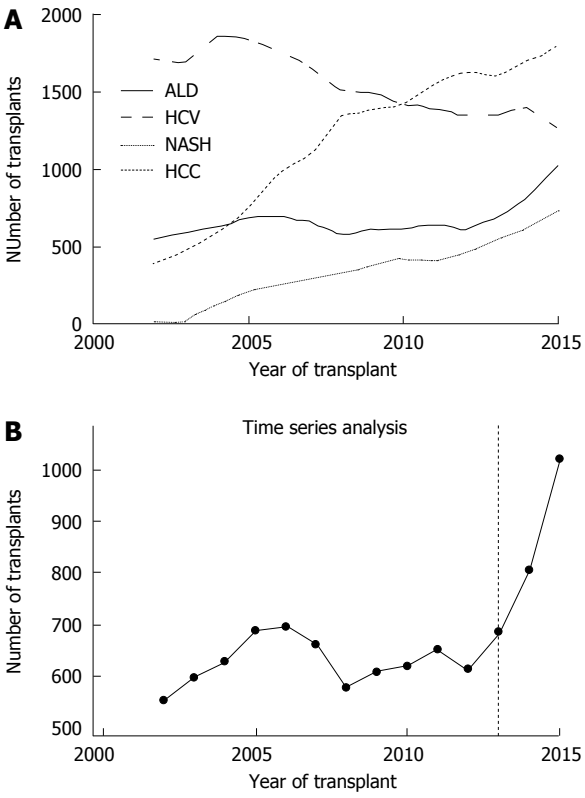

Figure 1 Time series analysis demonstrated a significant increase in the number of transplants for alcoholic liver disease starting in 2013. A: Number of transplants per year by etiology of liver disease; B: Time series analysis of alcoholic liver disease liver transplant recipients demonstrating a significant change in the number of transplants starting in 2013 (P = 0.03). ALD: Alcoholic liver disease; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis; HCC: Hepatocellular carcinoma.

over time.

ALD subgroup analysis

We performed a subgroup analysis of recipients transplanted for ALD. Temporal trends in recipient characteristics were studied and compared using χ^2 test for categorical values and student's t-test used for continuous variables. We then used time series analysis to identify any year with a significant change in the number of transplants per year, and then compared transplant rates in the "before" and "after" eras. To model transplant growth in each era, we used a spline linear regression model with the cut point at the year predicted by the time series analysis.

To determine if age or gender had any affect on change in transplant rates, we also compared mean transplants per year in the before and after eras for categorical age groups (18-30, 31-40, 41-50, 51-60, 61-70 and 71-83 years old) and gender using student's t-test. We also used this method to evaluate the contribution of transplants for acute alcoholic hepatitis, separating the ALD population into acute alcoholic hepatitis from alcoholic cirrhosis subgroups. We hypothesized that the increasing use of curative treatment for HCV cirrhosis could lead to a change in the classification of cirrhosis etiology, such that patients previously listed as HCV/ALD were subsequently listed as alcoholic cirrhosis alone. Hence, we analyzed the change in time for the HCV/ALD population using the same approach as above.

Analysis of transplant changes by region

UNOS is an organization involved in many aspects of the organ transplant and donation process and operates by grouping states into several different regions throughout the country. To facilitate transplantation, the US is divided into 11 geographic regions. Liver transplant recipients were grouped by UNOS region and the mean number of transplants per region per year for the before and after eras was calculated.

Statistical analysis

Analyses were conducted using JMP Pro 13.0.0 (SAS Institute Inc. Cary, NC) statistical software, graphics were made in Stata 12.1 (College Station, TX, United States).

RESULTS

Study population

Of 74216 liver transplant recipients, ALD (n = 9400, 12.7%) was the third leading indication for transplant after HCV (n = 21707, 29.2%) and hepatocellular carcinoma (HCC) (n = 16627, 22.4%) (Figure 1A). Recipients with ALD were younger, more likely to be non-black and have a higher model for end-stage liver disease (MELD) at transplant than recipients with HCV, HCC or NASH cirrhosis (Table 1). Time series analysis demonstrated a significant increase in the number of transplants for ALD starting in 2013 (P = 0.03) (Figure 1B).

ALD subgroup analysis

The total number of transplants performed for ALD increased from 553 (12.8% of the annual total) in 2002 to 1020 (16.5%) in 2015 (Table 2). Age and BMI remained unchanged over the study period, but there was a significant increase in the proportion of female recipients (from 22.4% in 2002 to 27.5% in 2015, P = 0.001) and an increase in MELD (20.6 ± 8.4 in 2002 to 28.9 ± 10.4 in 2015, P < 0.001). In the before era, the number of transplants per year was stable as predicted by the linear spline model (coefficient 3.3, 95%CI: -4.6, 11.2). In the after era, there were approximately 177 more transplants per year for ALD (coefficient 176.7, 95%CI: 119.4, 234.0) (Figure 2).

All age groups except those 71-83 years old showed a significant increase in the mean number of transplants per year for ALD when comparing before and after eras, but the greatest proportional increase was seen in the youngest recipients (Table 3). The proportional increase in mean transplants per year was greater in females than males, and was significant for both genders (P

Table 1 Recipient characteristics by etiology of liver disease

	HCV 21707 (29.2%)	HCC 16627 (22.4%)	ALD 9400 (12.7%)	NASH 4745 (6.4%)	*P* value
Age	54.1 ± 7.19	57.9 ± 7.8	53.5 ± 9.02	56.7 ± 10.2	< 0.001
Female	5799 (26.7%)	3928 (23.6%)	2210 (23.5%)	2237 (47.1%)	< 0.001
Race					< 0.001
White	15408 (71.0%)	11133 (67.0%)	7533 (80.1%)	4006 (84.4%)	
Hispanic	3071 (14.2%)	2496 (15.0%)	1285 (13.7%)	524 (11%)	
Black	2504 (11.5%)	1542 (9.3%)	371 (4.0%)	95 (2%)	
Other	724 (3.3%)	1456 (8.8%)	211 (2.2%)	120 (2.5%)	
BMI	28.4 ± 5.3	28.3 ± 5.3	27.9 ± 5.41	32 ± 6.1	< 0.001
Diabetes					< 0.001
None	16912 (77.9%)	11741 (70.6%)	7440 (79.2%)	2139 (45.1%)	
Any	4430 (20.4%)	4733 (28.5%)	1828 (19.5%)	2545 (53.6%)	
Unknown	365 (1.7%)	153 (0.9%)	132 (1.4%)	61 (1.3%)	
MELD at transplant	22 ± 10	15 ± 8.3	25.1 ± 9.6	23.8 ± 9.2	< 0.001
HCC in explant	3919 (18.1%)	11034 (66.4%)	482 (5.1%)	260 (5.5%)	< 0.001

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ALD: Alcoholic liver disease; NASH: Non-alcoholic steatohepatitis; BMI: Body mass index.

= 0.001, 0.005, respectively). Although there was a 1.4 fold increase in transplants for alcoholic hepatitis, this was not statistically significant (P = 0.58), only represented an increase of approximately 3 transplants per year, and did not explain the overall increase in transplants for ALD. As expected, there was a decrease in transplants for HCV/ALD, however this decrease (90.7 transplants per year) was much less than the per year increase for ALD (210.3 transplants per year).

Analysis of transplant and alcohol use by region

All regions except region 9 saw an increase in the mean number of transplants per year when comparing eras, and this increase was significant in regions 2, 3, 4, 5, 6, 8, 10 and 11 (Table 4, Figure 3).

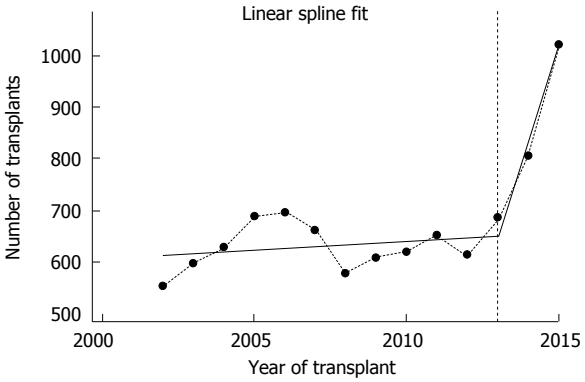

Figure 2 Linear spline fit for number of transplants for year for alcoholic liver disease in the before and after eras.

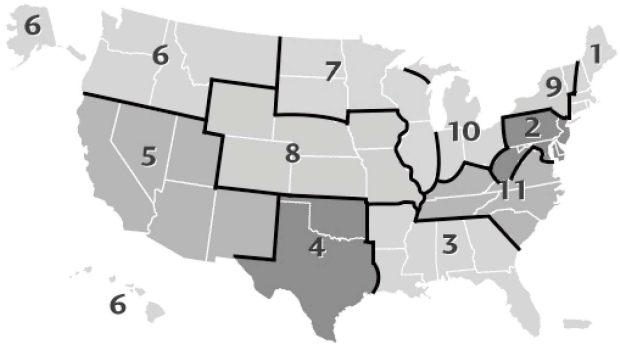

Figure 3 UNOS regions in the United States[29].

DISCUSSION

In a nationwide cohort of liver recipients, we found that the number of transplants for ALD was stable between 2002 and 2012, but rose by approximately 177 transplants per year between 2013 and 2015. This increase was observed more in young recipients and in females and was incompletely explained by a decrease in transplants for HCV/ALD. There was a significant increase in 8 out 11 UNOS regions, and a decrease only in region 9. This increase in transplants for ALD has not been previously described.

Prior epidemiologic studies on the indication for liver transplant have shown stable to decreasing rates of transplants for ALD, but these studies were based on data collected before 2013[7,8]. However, a more recent study noted an increase in transplants for ALD in recent years, which is more rapid than that for NASH[9]. Population-based studies have shown an increase in heavy alcohol use[5], binge drinking[5] and per capita alcohol use[10] since the early 2000s. During the same time period, there was an increase in hospitalization for alcohol-related diagnosis and an increase in age-adjusted death rates from ALD[11,12]. Furthermore, the proportion of cirrhosis-related deaths attributable to alcohol have increased in young patients (25-54 years old)[12]. However, other data suggest decreasing overall prevalence of ALD in the population[9].

The reason for this increase in transplants for ALD starting in 2013 is uncertain. Our data suggest that the surge is not due to an increasing BMI in this population or an increase in transplants for acute alcoholic hepatitis,

Table 2 Temporal trends in characteristics of alcoholic liver disease liver transplant recipients

	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	*P* value
n (% annual)	553 (12.8%)	597 (12.8%)	628 (12.4%)	688 (13.0%)	695 (12.7%)	660 (12.3%)	578 (11.1%)	608 (11.6%)	619 (11.7%)	651 (12.1%)	613 (11.4%)	685 (12.4%)	805 (13.9%)	1020 (16.5%)	
Age	53.0 ± 8.2	52.9 ± 8.5	54.0 ± 8.8	53.4 ± 8.6	53.7 ± 8.6	54.5 ± 8.7	53.6 ± 8.9	54.3 ± 8.5	54.1 ± 8.9	54.0 ± 8.9	53.6 ± 8.9	52.8 ± 9.4	53.7 ± 9.7	52.5 ± 10.2	0.1
Female	124 (22.4%)	126 (21.1%)	125 (19.9%)	134 (19.5%)	156 (22.4%)	133 (20.2%)	127 (22.0%)	146 (24.0%)	164 (26.5%)	170 (26.1%)	153 (25.0%)	172 (25.1%)	200 (24.8%)	280 (27.5%)	0.001
Race															0.03
Black	15 (2.7%)	16 (2.7%)	23 (3.7%)	24 (3.5%)	32 (4.6%)	19 (2.9%)	21 (3.6%)	22 (3.6%)	18 (2.9%)	37 (5.7%)	23 (3.8%)	35 (5.1%)	38 (4.7%)	48 (4.7%)	
Hispanic	71 (12.8%)	76 (12.7%)	76 (12.1%)	77 (11.2%)	98 (14.1%)	98 (14.8%)	95 (16.4%)	79 (13.0%)	85 (13.7%)	88 (13.5%)	93 (15.2%)	96 (14.0%)	103 (12.8%)	150 (14.7%)	
Other	6 (1.1%)	12 (2.0%)	13 (2.1%)	12 (1.7%)	9 (1.3%)	12 (1.8%)	16 (2.8%)	9 (1.5%)	20 (3.2%)	16 (2.5%)	13 (2.1%)	18 (2.6%)	17 (2.1%)	38 (3.7%)	
White	461 (83.4%)	493 (82.6%)	516 (82.2%)	575 (83.6%)	556 (80.0%)	531 (80.5%)	446 (77.2%)	498 (81.9%)	496 (80.1%)	510 (78.3%)	484 (79.0%)	536 (78.2%)	647 (80.4%)	784 (76.9%)	
BMI	27.7 ± 5.3	27.8 ± 5.2	27.7 ± 5.5	27.9 ± 5.4	27.7 ± 5.4	28.4 ± 5.5	27.9 ± 5.4	27.9 ± 5.4	28.0 ± 5.6	27.9 ± 5.5	28.1 ± 5.4	28.0 ± 5.3	27.8 ± 5.4	28.0 ± 5.5	0.3
Diabetes															< 0.001
None	433 (78.3%)	458 (76.7%)	493 (78.5%)	538 (78.2%)	548 (78.8%)	484 (73.3%)	440 (76.1%)	485 (79.8%)	487 (78.7%)	518 (79.6%)	509 (83.0%)	560 (81.8%)	659 (81.9%)	828 (81.2%)	
Any	103 (18.6%)	122 (20.4%)	116 (18.5%)	138 (20.1%)	134 (19.3%)	167 (25.3%)	131 (22.7%)	109 (17.9%)	127 (20.5%)	128 (19.7%)	100 (16.3%)	122 (17.8%)	144 (17.9%)	187 (18.3%)	
Unknown	17 (3.1%)	17 (2.8%)	19 (3.0%)	12 (1.7%)	13 (1.9%)	9 (1.4%)	7 (1.2%)	14 (2.3%)	5 (0.8%)	5 (0.8%)	4 (0.7%)	3 (0.4%)	2 (0.2%)	5 (0.5%)	
MELD	20.6 ± 8.4	21.6 ± 9.2	22.6 ± 9.5	22.6 ± 8.7	22.8 ± 8.5	23.9 ± 8.9	24.8 ± 9.0	25.1 ± 8.7	25.8 ± 9.4	26.1 ± 9.5	27.0 ± 9.3	27.5 ± 9.7	28.1 ± 9.6	28.9 ± 10.4	< 0.001
HCC in explant	58 (10.5%)	41 (6.9%)	51 (8.1%)	37 (5.4%)	44 (6.3%)	39 (5.9%)	30 (5.2%)	23 (3.8%)	18 (2.9%)	27 (4.1%)	24 (3.9%)	28 (4.1%)	23 (2.9%)	39 (3.8%)	< 0.001

BMI: Body mass index; HCC: Hepatocellular carcinoma.

and it is not solely due to reclassification of HCV/ALD transplants as ALD. There has been a steady increase in the number of new waitlistings for ALD, but the rate of rise of transplants since 2013 seems to exceed the rate of rise of listings[9]. Perhaps there has been a recent improvement in both the referral for transplant and wait-listing for patients with ALD, who have historically have lower rates of both referral[13,14] and waitlist[15]. The American Association for the Study of Liver disease revised the "Evaluation for Liver Transplantation in Adults Practice Guidelines" in 2005[16] and again 2013[17]. The 2005 Guidelines recommended "it is prudent to delay transplantation for a minimum of 3-6 mo of abstinence from alcohol." However, in the 2013 guidelines it was acknowledged that 6 mo of sobriety before referral "may result in deterioration of the patient's medical condition so that psychosocial or addiction requirements determined from the initial evaluation may not be achievable." While there is not a temporal relationship between this publication (March 2014) and our observed increased in transplants for ALD (start of 2013), the 2013 Guidelines may reflect a developing leniency of the abstinence requirement amongst transplant programs.

ALD is historically the second most common etiology for LT in the European Liver Transplant Registry at 33.6%, trailing only virus related cirrhosis. However, in the setting of treatment for HCV, ALD has become the leading indication for LT[18]. There has been a sustained increase in the proportion of transplants performed for ALD since the late-1980s, including 2013-2015. In a Nordic paper, the proportion of transplants for ALD remained relatively constant between 1994 and 2013[19].

Early identification of problematic alcohol use and reduction in drinking has the potential to change the pattern we have described. Only 10% of patients with drinking problems are identified by primary care providers, and under-diagnosis is common in teenagers[20]. Brief interventions in the primary care setting can result in reduced consumption and may subsequently reduce alcohol-related harm and mortality[21,22]. After a single course of treatment by a qualified alcohol counselor, abstinent rates are 17 to 33% and an additional 7% to 12% reduce their intake[23].

There are several simple screening tools for alcohol use that are designed to be highly sensitive and easy to use in the primary care setting. The CAGE questionnaire is a 4-question test with binary answers; two "yes" responses are considered a positive test and should prompt additional testing[24,25]. Alternatively, the more extensive alcohol use disorders identification test was developed by the World Health Organization and consists of ten questions with five possible answers and a focus on identification of heavy drinkers[26,27]. Another option is a single screening question "How many times in the past year have you had 5 (males) or 4 (females) or more drinks in a day?" with a cutoff of 8 times, and can also be used to accurately identify patients with unhealthy alcohol use with good discrimination[28]. The widespread use of electronic medical records make systematic implementation of well validated tools inexpensive and quite practical. This would follow the approach for identification of smoking using the electronic medical

Table 3 Changes in number of transplants per year for alcoholic liver disease by age group, gender and etiology

	Mean per year 2002-2012	Mean per year 2013-2015	Difference	Change	*P* value
Total	626.4	836.7	210.3	1.34	0.002
Age group (yr)					
18-30	4.3	14.3	10.1	3.35	0.003
31-40	40.7	84.0	43.3	2.06	0.001
41-50	170.5	219.7	49.1	1.29	0.005
51-60	264.5	314.3	49.8	1.19	0.040
61-70	138.4	195.0	56.6	1.41	0.010
71-83	7.9	9.3	1.4	1.18	0.500
Gender					
Female	141.6	217.3	75.7	1.53	0.001
Male	484.7	619.3	134.6	1.28	0.005
Etiology					
Alcoholic cirrhosis	619.5	827.0	207.5	1.33	0.002
Alcoholic hepatitis	6.8	9.7	2.8	1.42	0.580
HCV/ALD	274.4	183.7	-90.7	0.67	0.050

HCV: hepatitis C virus; ALD: Alcoholic liver disease.

Table 4 Changes in number of transplants per year for alcoholic liver disease by UNOS region

UNOS region	Mean per year 2002-2012	Mean per year 2013-2015	Difference	Change	*P* value
1	28.5	37.3	8.8	1.31	0.09
2	86.0	120.3	34.3	1.40	0.01
3	103.4	142.7	39.3	1.38	0.02
4	54.1	75.7	21.6	1.40	0.05
5	75.5	117.3	41.8	1.55	0.003
6	13.0	24.7	11.7	1.90	0.001
7	83.1	89.0	5.9	1.07	0.32
8	33.6	52.3	18.7	1.56	0.002
9	43.4	28.3	-15.0	0.65	0.23
10	52.3	71.0	18.7	1.36	0.03
11	53.5	78.0	24.5	1.46	0.005

record. The potential for this approach on the prognosis of patients with ALD could be profound.

There were several limitations to our study. We examined only patients transplanted for ALD, not those listed for transplantation, so we are unable to determine whether the increase observed is due to an increasing listing for ALD or an increase in the proportion of waitlisted patients with ALD undergoing transplant. However, Goldberg *et al*[29] recently showed a steeper rate of rise for LTs for ALD than absolute number of new waitlistings, although both are increasing. Additionally, we were unable to further explore why all but three UNOS regions demonstrated an increase in transplants for ALD.

In conclusion, in this study we demonstrate a nationwide increase in the number of transplants per year for ALD beginning in 2013, particularly in young and female patients. The reason for this increase is unknown, but comes in the setting of widespread and increasing alcohol use and hospital admissions for ALD. Consideration should be given to the use of screening tools aimed at detecting alcohol use in the primary care setting to identify patients with problematic alcohol use and promote reduction in consumption in order to avoid harm.

ARTICLE HIGHLIGHTS

Research background

Liver transplantation (LT) has become a life-saving procedure for patients with irreversible liver diseases. One of the common causes of chronic liver disease for which LT is potentially life-saving is alcoholic liver disease (ALD).

Research motivation

Population-based studies have shown that there has been an increase in the prevalence of both heavy drinking and binge drinking.

Research methods

Authors conducted a retrospective cohort analysis of transplant recipients in the United Network for Organ Sharing Standard Transplant Analysis and Research file.

Research results

Between 2002 and 2015, ALD was the third leading indication for transplant after HCV and hepatocellular carcinoma. The total number of transplants performed for ALD increased from 553 (12.8% of the annual total) in 2002 to 1020 (16.5%) in 2015.

Research conclusions

A nationwide increase was noted in the number of transplants per year for ALD beginning in 2013, particularly in young and female patients. This comes in the setting of widespread and increasing alcohol use and hospital admissions for ALD.

Research perspectives

Consideration should be given to the use of screening tools aimed at detecting alcohol use in the primary care setting to identify patients with problematic alcohol use and promote reduction in consumption in order to avoid harm.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Reverse time-dependent effect of alphafetoprotein and disease control on survival of patients with Barcelona Clinic Liver Cancer stage C hepatocellular carcinoma

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Abstract

AIM

To characterize the survival of cirrhotic patients with Barcelona Clinic Liver Cancer (BCLC) stage C hepatocellular carcinoma (HCC) and to ascertain the factors predicting the achievement of disease control (DC).

METHODS

The cirrhotic patients with BCLC stage C HCC evaluated by the Hepatocatt multidisciplinary group were subjected to the investigation. Demographic, clinical and tumor features, along with the best tumor response and overall survival were recorded.

RESULTS

One hundred and ten BCLC stage C patients were included in the analysis; the median overall survival was 13.4 mo (95%CI: 10.6-17.0). Only alphafetoprotein (AFP) serum level > 200 ng/mL and DC could independently predict survival but in a time dependent manner, the former was significantly associated with increased risk of mortality within the first 6 mo of follow-up (HR = 5.073, 95%CI: 2.159-11.916, P = 0.0002), whereas the latter showed a protective effect against death after one year (HR = 0.110, 95%CI: 0.038-0.314, P < 0.0001). Only patients showing microvascular invasion and/or extrahepatic spread recorded lower chances of achieving DC (OR = 0.263, 95%CI: 0.111-0.622, P = 0.002).

CONCLUSION

The BCLC stage C HCC includes a wide heterogeneous group of cirrhotic patients suitable for potentially curative treatments. The reverse and time dependent effect of AFP serum level and DC on patients' survival confers them as useful predictive tools for treatment management and clinical decisions.

Key words: Hepatocellular carcinoma; Cirrhosis; Barcelona Clinic Liver Cancer stage C; Alphafetoprotein; Disease control; Performance status; Survival

Core tip: Refining the prognosis of Barcelona Clinic Liver Cancer (BCLC) stage C hepatocellular carcinoma (HCC) is crucial to select patients that can get benefit from and be suitable for locoregional or surgical treatments. This study confirms that high alphafetoprotein serum level and DC are the best predictors of mortality for BCLC C patients, highlighting that the effect of these two variables is reverse and dynamic, in a time dependent manner. Outstandingly, performance status has not been found to be a strong predictor of mortality. According to our results, curative treatments should not be "a priori" excluded in a subset of BCLC stage C patients with favorable prognostic factors.

Ponziani FR, Spinelli I, Rinninella E, Cerrito L, Saviano A, Avolio AW, Basso M, Miele L, Riccardi L, Zocco MA, Annicchiarico BE, Garcovich M, Biolato M, Marrone G, De Gaetano AM, Iezzi R, Giuliante F, Vecchio FM, Agnes S, Addolorato G, Siciliano M, Rapaccini GL, Grieco A, Gasbarrini A, Pompili M. Reverse time-dependent effect of alphafetoprotein and disease control on survival of patients with Barcelona Clinic Liver Cancer stage C hepatocellular carcinoma. *World J Hepatol* 2017; 9(36): 1322-1331 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i36/1322.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i36.1322

INTRODUCTION

Hepatocellular carcinoma (HCC) has been recognized as a major health problem, as it ranks third among the leading causes of death due to cancer and is the sixth most common tumor with a worldwide occurrence[1].

While there are several options available for the treatment of HCC, their choice most likely depends on tumor stage, impairment of normal liver function, patient's performance status (PS) and comorbidities. The most widely accepted staging system for HCC is the Barcelona Clinic Liver Cancer (BCLC), which was based on the patients clinical features along with tumor-related variables and therefore categorized five different stages with progressively worsening prognosis and different treatment options[1,2].

The patients with an advanced HCC belong to the BCLC stage C, which includes tumors with macrovascular invasion, and/or extrahepatic spread

and/or mild cancer-related symptoms, PS 1-2 (Eastern Cooperative Oncology Group), and mild to moderate liver function impairment (Child-Pugh stage A-B). The only therapeutic option recommended for BCLC stage C HCC is the drug sorafenib, a multikinase inhibitor that has been reported to extend the overall survival of patients up to nearly 3 mo[3].

Given the higher number of heterogeneous and complex cases encountered in the field-practice, the BCLC classification is often not exhaustive, and the increasing number of new therapeutic options and their combinations makes difficult to strictly adhere to BCLC suggestions. This has been largely demonstrated in other categories of patients such as those belonging to the BCLC stage B group, who had not been subjected to transarterial chemoembolization (TACE), the treatment recommended by the BCLC algorithm, in more than one third of cases[4-6].

The BCLC stage C HCC encompasses a wide spectrum of tumors and patients' with different characteristics that may get benefit from and be suitable for locoregional or surgical treatments[7-9]. Nonetheless, in this stage too, the universal administration of sorafenib to the patients following the BCLC algorithm may sometimes be arguable and other therapeutic options could be explored according to patient's individual conditions.

The current study is principally aimed at characterizing the prognosis of cirrhotic patients with BCLC stage C HCC as assessed by a multidisciplinary team in an Italian tertiary care center. In addition to this, the other objective is the identification of the factors predicting the achievement of disease control (DC).

MATERIALS AND METHODS

The present study was performed at the Agostino Gemelli University Hospital, Rome, Italy. The prospective database of the Hepatocatt multidisciplinary group, containing clinical, tumor and outcome data of all liver cancer subjects evaluated in the seven years at our Institute was reviewed, and the cohort of cirrhotic patients with BCLC stage C HCC were selected as the prime object of the investigation.

The following criteria were adopted for the selection of patients: PS grade $\leqslant$ 2; Child-Pugh class A or B; tumor macrovascular invasion (mainly portal vein and/or hepatic veins and/or inferior vena cava); and/or extrahepatic spread. The HCC was diagnosed by multiphasic contrast-enhanced computed tomography (CT), gadolinium-enhanced magnetic resonance imaging (MRI) and/or by ultrasound-guided biopsy, as per the guidelines of European Association for the Study of the Liver and the American Association for the Study of Liver Diseases[1,2]. Based on the liver function and patients' characteristics, the modalities of HCC treatment were decided by the Hepatocatt multidisciplinary board, comprising of hepatologists, hepatobiliary and transplant surgeons, oncologists, radiologists, and pathologists. The imaging criteria (CT and/or MRI) for assessing the tumor response established by mRECIST were followed[10]. For individual patient, the treatment outcome was documented; DC was achieved in those patients who acquired a stable disease (SD), partial response (PR) or complete response (CR) as the best treatment outcome.

The patients' survival was the measure of success as primary outcome. The follow-up time was defined as the number of months from the entry in the BCLC stage C till their death or last visit. The factors that could predict the achievement of DC were also investigated as secondary endpoint.

Statistical analysis

Statistical analysis was performed using non-parametric tests due to the non normal distribution of data. The continuous variables were expressed as median and range, while the categorical variables as frequencies and percentages.

Pre-treatment variables [Child-Pugh score, PS, number and maximum size of HCC lesions, presence of macrovascular invasion or extrahepatic spread, alphafetoprotein (AFP) serum level, NIACE score value[11], and diabetes] and post-treatment variables (the number of treatments received after entry in the BCLC stage C and the achievement of DC) were considered as prognostic factors of patients' survival. The univariate analysis of survival estimates was performed using the Kaplan-Meier curve and the *log-rank* test was applied to check the differences between the groups. The variables with a $P < 0.100$ were included in the Cox proportional hazard regression model for the multivariate survival analysis, adjusting for gender and age.

The assumption of proportionality was confirmed by plotting the scaled Schoenfeld residuals over the time [log hazard ratio (beta) over time] and by performing a non-proportionality test (Pearson correlation test) for the overall model and for each covariate of the model. Interaction terms were subsequently introduced in the analysis for that factors that varied significantly over time. Fisher's exact test and binomial logistic regression were performed to identify the predictors of DC among pre- and post-treatment variables.

Statistical analysis was carried out using the R statistics program version 3.1.2. All statistical tests were two-sided and differences were considered significant at $P < 0.05$.

RESULTS

A total of 1030 records of liver cancer patients evaluated between May 2008 and May 2015 were reviewed, of which, 146 non-HCC liver tumors and 774 HCC in BCLC stage other than C (0, A, B or D) were disqualified from the study. Therefore, finally, 110 patients classified as BCLC stage C were included in the investigation. Clinical data and tumor characteristics of the study population are given in Table 1.

Table 1 Clinical and tumor characteristics of patients included in the study

Variable	Overall (110)
Age (yr)	67.5 (41-80)
Gender	
Male	91 (82.7)
Female	19 (17.3)
Etiology of liver disease	
Viral (HBV/HCV/HBV and HCV)	70 (63.6)
Alcohol	17 (15.5)
NASH/NAFLD	14 (12.7)
Viral and alcohol	9 (8.2)
PS	
0	33 (30)
1	64 (58.2)
2	13 (11.8)
Diabetes	
No	87 (79.1)
Yes	23 (20.9)
Child-Pugh score	
A	82 (74.5)
B	28 (25.5)
N nodules	
Single	35 (31.8)
2-3	20 (18.2)
> 3 or infiltrating	55 (50)
Maximum size	
⩽ 5 cm	56 (50.9)
> 5 cm	54 (49.1)
Macrovascular invasion	
No	60 (54.5)
Yes	50 (45.5)
Extrahepatic spread	
No	91 (82.7)
Yes	19 (17.3)
Macrovascular invasion and/or extrahepatic spread	
No	49 (44.5)
Yes	61 (55.5)
NIACE	
⩽ 3	84 (76.4)
> 3	26 (23.6)
AFP	
⩽ 200 ng/mL	74 (67.3)
> 200 ng/mL	36 (32.7)
Treatment before BCLC C diagnosis	
No	53 (48.2)
Yes	57 (51.8)
Type of treatment before BCLC C diagnosis (one or more per patient)	
TACE	35
Surgical resection	20
RFA	18
Sorafenib	13
PEI	11
TACE + RFA	8
TARE	4
DSM-TACE	1
Number of treatments after BCLC C diagnosis	
None	22 (20)
Single	32 (29.1)
Multiple	56 (50.9)
Type of treatment after BCLC C diagnosis (one or more per patient)	
Sorafenib	53
TACE	25
TARE	18
Second line systemic agent	15
PEI	12
DSM-TACE	5
LT	3
RFA	1
Best tumor response	
CR	10 (9.1)
PR	21 (19.1)
SD	12 (10.9)
PD	67 (60.9)
DC	
No	67 (60.9)
Yes	43 (39.1)

Continuous variables are reported as median value and range, categorical variables as frequencies and percentage. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Nonalcoholic Steatohepatitis; NAFLD: Nonalcoholic fatty liver disease; PS: Performance status; AFP: Alphafetoprotein; DC: Disease control; TACE: Transarterial chemoembolization; TARE: Transarterial radioembolization; DSM-TACE: Degradable starch microspheres transarterial chemoembolization; PEI: Percutaneous ethanol injection; RFA: Radiofrequency ablation; LT: Liver transplant; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; DC: Disease control.

Primary endpoint: Patients' survival

Out of 110 BCLC stage C patients included in the investigation, only 32 received a single treatment and 56 more than once, whereas 22 of them received only best supportive care due to the inadequate liver function. Sorafenib was the most common choice of treatment, followed by TACE, TARE, and second-line systemic agents in patients who were either intolerant to sorafenib or sorafenib failed for them (Table 1). In selected cases, PEI or RFA in combination with other treatments and DSM-TACE were also performed; three PS 1 patients without macrovascular invasion or extrahepatic spread and with tumors complying the Milan criteria after effective downstaging (when needed) underwent liver transplant (LT). The best-succeeded response was CR in 9.1% of cases, PR in 19.1%, SD in 10.9%, and PD in 60.9% of cases; overall, 43 (39.1%) patients obtained DC.

After a median follow-up of 22.9 mo (95%CI: 17.3-38.1), the cumulative median survival of the overall population was 13.4 mo (95%CI: 10.6-17.0, Figure 1). A total of 66 patients died and the most prevailing cause of death was attributed to tumor progression (50/66; 75.7%), followed by liver function failure (13/66; 19.7%), while in the remaining 3 patients, the death was caused by sepsis, post LT complications and bone fracture.

At univariate analysis, AFP serum level > 200 ng/mL, tumor size > 5 cm, the presence of macrovascular invasion, the presence of macrovascular invasion and/or extrahepatic spread as pre-treatment factors and the absence of DC as post-treatment factor were considered to be correlated with a worse outcome (Table 2). However, at the multivariate Cox regression, only AFP serum level > 200 ng/mL and DC were independent predictors of mortality (HR = 2.194, 95%CI: 1.249-3.855, P = 0.006 and HR = 0.190, 95%CI: 0.098-0.367, P < 0.0001, respectively). In particular, the effect of these two variables was reverse in a time dependent

Table 2 Univariate (Kaplan-Meier) and multivariate (Cox proportional hazard regression) survival analysis of patients with Barcelona Clinic Liver Cancer C hepatocellular carcinoma according to clinical and tumor variables

Variable	Univariate analysis		Multivariate analysis	
	Survival time (mo)	*P* value	Hazard ratio (95%CI)	*P* value
Age				
< 65 yr	13.9	0.903	-	-
⩾ 65 yr	13.8			
Gender				
Male	13	0.900	-	-
Female	14.2			
PS				
0	10.3	0.128	-	-
1/2	13.9			
Diabetes				
No	13	0.813	-	-
Yes	13.8			
Child-Pugh score				
A	13.4	0.957	-	-
B	12.8			
N nodules				
Single	13.8	0.776	-	-
2-3	13.4			
Multinodular/infiltrating	13			
Tumor size				
⩽ 5 cm	13.9	0.022[1]	1	0.275
> 5 cm	9.9		1.357 (0.784-2.349)	
Macrovascular invasion				
No	15.8	0.014[1]	1	0.866
Yes	9.5		1.095 (0.379-3.162)	
Extrahepatic spread				
No	11.2	0.274	-	-
Yes	6.7			
Macrovascular invasion and/or extrahepatic spread				
No	13.8	0.008[1]	1	0.429
Yes	6.3		1.547 (0.523-4.571)	
AFP				
⩽ 200 ng/mL	15.8	0.0002[1]	1	0.006[1]
> 200 ng/mL	6.3		2.194 (1.249-3.855)	
DC				
No	7.6	< 0.0001[1]	1	< 0.0001[1]
Yes	15.8		0.190 (0.098-0.367)	
NIACE score				
⩽ 3	13.8	0.515	-	-
> 3	6.7			

[1]Statistically significant results. PS: Performance status; AFP: Alpha-fetoprotein; DC: Disease control.

manner, as depicted by plotting the log hazard ratios (beta) over time (Figure 2). In the first 6 mo of follow-up, serum AFP > 200 ng/mL was directly associated with lower chances of survival, but the effect declined subsequently. Conversely, the favorable prognostic impact of DC curtailed in the early-intermediate period and became noticeable after 1 year of follow-up.

A term of interaction of these two covariates with time was then introduced in the Cox model and hazard

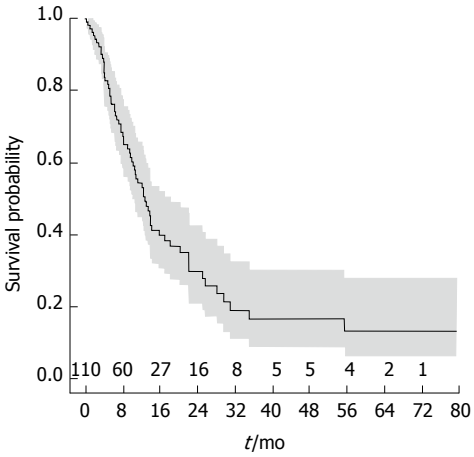

Figure 1 Cumulative survival of the overall cirrhotic patients with Barcelona Clinic Liver Cancer C stage hepatocellular carcinoma included in the study. The solid line shows the overall survival and the dotted lines the 95%CIs.

ratios were reported by each time interval (⩽ 6 mo, 7-12 mo, > 12 mo; Table 3). The AFP serum level > 200 ng/mL was significantly associated with higher risk of mortality within the first 6 mo of patients' entry into the BCLC stage C (⩽ 6 mo, HR = 5.073, 95%CI: 2.159-11.916, *P* = 0.0002). Conversely, DC exercised a significant protective effect in long-term phase (> 12 mo, HR = 0.110, 95%CI: 0.038-0.314, *P* < 0.0001).

There were also identified 5 patients who had unexpectedly longer survival (above the 95th percentile; median 63.3 mo). The characteristics of those subjects have been described in Table 4; outstandingly, in most of the cases (3/5) PS 1-2 was the major cause for categorizing them in BCLC stage C. Pre-treatment AFP serum level was ⩽ 200 ng/mL in all these patients; and two of them showed tumor macrovascular invasion without any extrahepatic spread. In one case Sorafenib, and in another TARE was prescribed; whereas, in the remaining three patients, curative treatments (LT), DSM-TACE or second-line systemic therapies were administered. Remarkably, DC was achieved in all these long-term survivors.

Secondary endpoint: DC

The examination of factors associated with DC was the second landmark of the study (Table 5). The patients who achieved DC (43/110; 39.1%) were illustrated by small-size tumors (> 5 cm: 13/43, 30.2% *vs* 41/67, 61.2%; *P* = 0.002), a lower frequency of macrovascular invasion (11/43, 25.6% *vs* 39/67, 58.2%; *P* = 0.0009), extrahepatic spread (3/43, 7% *vs* 16/67, 23.9%; *P* = 0.036) and of macrovascular invasion and/or extrahepatic spread (14/43, 32.6% *vs* 47/67, 70.1%; *P* = 0.0001), lower AFP serum level (> 200 ng/mL: 8/43, 18.6% *vs* 28/67, 41.8%; *P* = 0.013) and more frequently received at least one treatment (39/43, 90.7% *vs* 49/67, 73.1%; *P* = 0.029). However, only the presence of macrovascular

Table 3 Multivariate Cox regression model including alpha-fetoprotein and disease control as time dependent covariates

Variable	Multivariate analysis	
	Hazard ratio (95%CI)	*P* value
Macrovascular invasion		
No	1	0.917
Yes	1.066 (0.412-2.762)	
Macrovascular invasion and/or extrahepatic spread		
No	1	0.366
Yes	1.552 (0.584-4.124)	
Tumor size		
⩽ 5 cm	1	0.266
> 5 cm	1.369 (0.786-2.382)	
AFP (> 200 ng/mL *vs* ⩽ 200 ng/mL)		
< 6 mo	5.073 (2.159-11.916)	0.0002[1]
7-12 mo	0.948 (0.275-3.267)	0.932
> 12 mo	1.698 (0.620-4.648)	0.303
DC (Yes *vs* No)		
< 6 mo	0.220 (0.075-0.650)	0.096
7-12 mo	0.463 (0.181-1.189)	0.109
> 12 mo	0.110 (0.038-0.314)	< 0.0001[1]

For all other variables single hazard ratios were reported. [1]Statistically significant results. AFP: Alphafetoprotein; DC: Disease control.

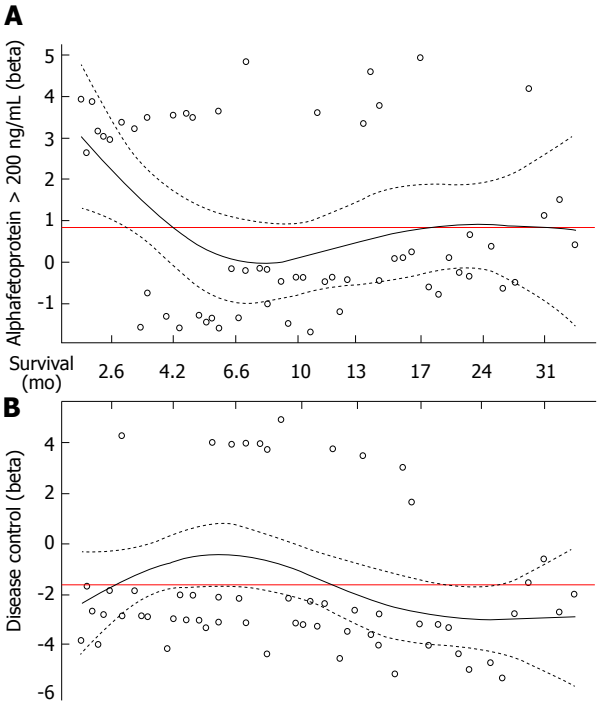

Figure 2 Plot of the scaled Schoenfeld residuals over time for alpha-fetoprotein serum level > 200 ng/mL (A) and disease control (B). The solid line shows the log of hazard ratio (beta) as a function of survival time with the 95%CI (dotted lines). The average beta value obtained at the Cox model without any time-adjustment is also reported (solid red line).

invasion and/or extrahepatic spread was independently associated with reduced likelihoods of achieving DC (OR 0.263, 95%CI: 0.111-0.622, *P* = 0.002). It is important to mention that among the 61 patients who showed macrovascular invasion and/or metastases, 44 (72.1%) received treatment and this proportion was significantly lower than that of patients showing intrahepatic disease without vascular involvement (44/49, 89.8%, *P* = 0.029).

DISCUSSION

The BCLC staging system is the most widely used approach for the therapeutic and prognostic classification of cirrhotic patients with HCC. While exploring the implementation of biomarker research in clinical practice to stratify tumors based on their biological aggressiveness[11], several sub-classifications of the BCLC stages consistent with prognostic factors and new scores have been proposed to improve the predictive power of this algorithm[12-14]. A more detailed stratification system based on the life expectancy may avoid offering treatments having a poor impact on patients' prognosis and often impairing the quality of life. These considerations are extremely important with regard to the selection of patients for the clinical trials of first or second line novel systemic agents.

The present study was aimed at investigating the predictors of survival in cirrhotic patients with BCLC stage C HCC and at assessing their effect in a time dependent manner. At the preliminary survival analysis, AFP serum level > 200 ng/mL and DC were found to be independent predictors of mortality (HR = 2.194, *P* = 0.006 and HR = 0.190, *P* < 0.0001, respectively).

Hence, the first finding of our report confirms high AFP serum level as a negative predictive marker in patients with advanced HCC and its impact on survival irrespective of the tumor stage at the time of diagnosis[15-20]. Furthermore, although this category of patients is classified as "advanced stage", we demonstrated a promising impact of the response to treatment, as shown by DC, on prognosis. Based on these findings, curative and locoregional treatments should not be "a priori" excluded in a subset of BCLC stage C patients with favorable predictive factors. As reported previously, surgical resection and LT can extend patients' survival in the BCLC stage C also[4-6,8,9,21], which supports the need of a novel method of prediction more customized to the specific patient. The identification of 5 long-term survivors (median 63.3 mo), where 3 were included in this stage only at impaired PS (1 or 2) in absence of vascular invasion or extrahepatic tumor spread, confirms the heterogeneity of patients included in the BCLC stage C and the benefits they got in terms of DC. In four patients, locoregional treatments were feasible and two of them were subjected to LT successfully. As already reported[22], the provision based on PS used in the BCLC algorithm is questionable. Furthermore, PS scores are subjective measures with high inter-observer variability, and it is often difficult to correctly evaluate tumor-related symptoms in patients already presenting compromised general conditions. In the current study, PS has not been found to be an independent predictor of survival, and this supports the hypothesis that alone it cannot be considered as an

Table 4 Characteristics of the 5 patients with long-survival (median 63.3 mo)

PT	Gender	Age	Etiology	PS	Child-Pugh	AFP > 200 ng/mL	No. of nodules	Maximum size	Macrovascular invasion	Extrahepatic spread	Diabetes	Pre-BCLC C treatments	Post-BCLC C treatments	Best response	DC	Survival (mo)	Status
PT3	M	65	HBV	1	A	No	Infiltrating	Infiltrating	Yes	No	No	None	Sorafenib	CR	Yes	79.4	Alive
PT10	M	73	HCV	1	A	No	> 3	18	No	No	No	TACE, resection, sorafenib	Second line systemic agent, DSM-TACE (2)	SD	Yes	63.3	Alive
PT27	M	58	HBV	2	B	No	2	19	No	No	No	RFA, TACE	LT	CR	Yes	67.1	Alive
PT53	M	63	Alcohol	1	B	No	> 3	50	No	No	No	None	TACE (4), TACE + RFA (1), LT	CR	Yes	58.9	AliVe
PT54	M	65	HCV	0	A	No	Single	40	Yes	No	Yes	None	TARE (2)	SD	Yes	38.1	Alive

HBV: Hepatitis B virus; HCV: Hepatitis C virus; PS: Performance status; BCLC: Barcelona Clinic Liver Cancer; AFP: Alphafetoprotein; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation; DSM-TACE: Degradable starch microspheres transarterial chemoembolization; LT: Liver transplant; TARE: Transarterial radioembolization; CR: Complete response; SD: Stable disease; DC: Disease control.

exclusion criterion for curative treatments. Outstandingly, the majority of the patients (88.2%) in our series showed a PS 0 or 1, and therefore, only a small subgroup of patients (11.7%) fell in PS 2 class, and that may have influenced the overall survival insignificantly. However, the non-homogeneity of PS stages among BCLC C patients may be attributed to the sequential enrollment of the subjects included in the analysis rather than a selection-bias, and gives a better understanding of what happens in the real field practice.

The novel finding surfaced out from our study is the dynamic influence of AFP serum level and DC on survival period (Figure 2). In particular, the log curve of the hazard ratio for AFP serum level > 200 ng/mL elevated at high beta points implying a direct correlation with mortality, but declined steadily over time. This was more evident during the early follow-up (within 6 mo), which reached the zero point and then increased slightly afterwards, and finally became constant in the later stage. The DC beta value showed an inverse tendency, being constantly negative and increasing towards the zero point at about 6 mo of follow-up; however, it decreased significantly after the first year. At the Cox regression model including time-dependent coefficients, the most noticeable prognostic effect of AFP appeared in the early follow-up period, with 83.5% probability of mortality during the first 6 mo of follow-up of patients with AFP serum level > 200 ng/mL compared to those with a lower value (HR 5.073, 95%CI: 2.159-11.916, $P = 0.0002$). On the other hand, the DC was found to be defensive against death, as evident especially in the long-term follow-up (> 12 mo, HR 0.110, $P < 0.0001$). This type of dynamic behavior of prognostic factors has not been documented earlier during the establishment of HCC, while for other malignancies, such as breast, lung, and colorectal cancer, it has already been described. Time-dependent analysis has allowed to model patients' survival more precisely, considering the dynamic behavior of mortality risk factors and pointing out the reverse effect of AFP serum level and DC on prognosis temporally. Our findings, therefore, emphasize that tumor biological aggressiveness remains the most important short time prognostic indicator whereas in the long term, the achievement of DC is very decisive to ameliorate patients' survival expectancy. This further supports the efforts towards improving the therapy management and also implementing the treatment options in the BCLC algorithm for stage C patients. Nevertheless, since high AFP serum level is associated with an increased risk of early mortality, the trials assaying new systemic agents or second line therapies should be very careful in selecting the patients, and consequences should be evaluated optimally based on the stratification of the biological aggressiveness.

The second milestone of our study was to identify predictive factors of DC. The presence of macrovascular invasion and/or extrahepatic spread was found to be independently associated with a reduced likelihood of achieving DC (OR = 0.263, $P = 0.002$). The negative effect of tumor diffusion outside the liver or into the bloodstream on patients' prognosis is well known, as thoroughly discussed in previous reports[23-25], and this could be indirectly due to the inadequacy of the currently available treatments to control an aggressive disease in an effective and systemic manner. Nevertheless, in our study the 61 patients showing macrovascular invasion and/or extrahepatic spread received treatment with a lower frequency as compared to those with non-invasive tumors (44/61, 72.1% *vs* 44/49, 89.8%, $P = 0.029$). Due to the extensive tumor burden, in this subgroup of patients supportive care was taken more often and this may also be the reason for the reduced DC rates to some extent.

Table 5 Univariate (Fisher's exact test) and multivariate (binomial logistic regression) analysis of factors associated with the achievement of disease control in patients with Barcelona Clinic Liver Cancer C hepatocellular carcinoma

Variable	DC (43)	No DC (67)	Univariate analysis	Multivariate analysis	
			P value	Odds ratio (95%CI)	*P* value
Age					
< 65 yr	15	27	0.229	-	
⩾ 65 yr	28	40			
Gender					
Male	35	56	0.471	-	
Female	8	11			
PS					
0	37	60	0.06	-	
1/2	6	7			
Diabetes					
No	34	53	0.653	-	
Yes	9	14			
Child-Pugh score					
A	31	51	0.524	-	
B	12	16			
N nodules					
Single	13	22	0.078		
2-3	11	9		-	
Multinodular/infiltrating	19	36			
Tumor size					
⩽ 5 cm	30	26	0.006[1]	1	0.298
> 5 cm	13	41		0.617 (0.236-1.610)	
Macrovascular invasion					
No	32	28	0.0003[1]	-	-
Yes	11	39			
Extrahepatic spread					
No	40	51	0.02[1]	-	-
Yes	3	16			
Macrovascular invasion and/or extrahepatic spread					
No	29	20	< 0.0001[1]	1	0.002[1]
Yes	14	47		0.263 (0.111-0.622)	
AFP					
⩽ 200 ng/mL	35	39	0.008[1]	1	0.179
> 200 ng/mL	8	28		0.461 (0.169-1.258)	
NIACE score					
⩽ 3	34	50	0.502	-	
> 3	9	17			
Treatment after BCLC C diagnosis					
No	4	18	0.04[1]	1	0.270
Yes	39	49		0.531 (0.147-1.917)	

[1]Statistically significant results. PS: Performance status; AFP: Alpha-fetoprotein; DC: Disease control.

Recently, the NIACE score has been proposed as a useful tool for the prognostic sub-staging of BCLC stage C patients, as well as for the management of treatment and for the selection of patients in clinical trials[14]. Probably, the different biological characters of tumors encompassed in our investigation could have negatively affected the prognostic ability of the NIACE score. Indeed, only 14% of the patients in the NIACE study cohort had previously undergone a treatment for HCC, as compared to 51.8% of the patients in our series, and the prevalence of alcohol related liver disease was higher than in our series of patients (30% *vs* 15.5%).

A possible limitation of this study could be its retrospective nature, although this was partially overcome by the rigorous and prospective collection of clinical records by the multidisciplinary group. Despite of having limited the number of records included in the analysis, the inclusion of patients treated only at our Center has reduced biasness related to diverse modalities of treatment or imaging interpretation by radiologists at different Centers.

The liver function did not appear to have a significant impact on patients' prognosis in our analysis; probably, a high tumor-related mortality has overcome the impact of hepatic impairment on survival. However, this cannot be absolutely confirmed, as the number of patients with conserved liver function largely exceeded that of patients with more severe liver impairment (74.5% Child A *vs* 25.5% Child B class).

In conclusion, our data confirm that the BCLC stage C comprises a huge heterogeneous group of cirrhotic patients suitable for locoregional and potentially curative treatments. This is the first report

highlighting the reverse and time-dependent effect of AFP serum level and DC as prognostic factors in cirrhotic patients with advanced stage HCC. In the patients with pre-treatment AFP serum level > 200 ng/mL the risk of early death increases up to 80%, while the achievement of post-treatment DC, which is less likely in the presence of macrovascular invasion and/or extrahepatic tumor spread, suggests higher chances of long-term survival. The combination of these predictive factors may be helpful in the sophistication of patients' prognosis, thereby being valuable in the selection of patients suitable for clinical trials and in designing the therapeutic strategy.

ARTICLE HIGHLIGHTS

Research background

Barcelona Clinic Liver Cancer (BCLC) stage C hepatocellular carcinoma (HCC) includes a heterogeneous group of patients with different clinical and tumor characteristics and survival expectancy, for whom sorafenib is the only recommended treatment option. The present study investigates the outcome of BCLC C patients who underwent different locoregional, surgical or systemic treatments.

Research motivation

To better stratify the prognosis of patients with BCLC C stage HCC.

Research objectives

To characterize the prognosis of cirrhotic patients with BCLC stage C HCC as assessed by a multidisciplinary team in an Italian tertiary care center and to identify those factors predicting the achievement of disease control (DC).

Research methods

The prospective database of the Hepatocatt multidisciplinary group, containing clinical, tumor and outcome data of all liver cancer subjects evaluated in the seven years at our Institute was reviewed.

Research results

The study confirms that the BCLC stage C comprises a huge heterogeneous group of cirrhotic patients suitable for locoregional and potentially curative treatments. Moreover, this is the first report highlighting the reverse and time-dependent effect of alphafetoprotein (AFP) serum level and DC as prognostic factors in cirrhotic patients with advanced stage HCC.

Research conclusions

The novel finding surfaced out from our study is the dynamic influence of AFP serum level and DC on survival period. In particular, the AFP serum level > 200 ng/mL was significantly associated with higher risk of mortality within the first 6 mo of patients' entry into the BCLC stage C; conversely, DC exercised a significant protective effect in long-term phase. Our report also highlight that the presence of macrovascular invasion and/or extrahepatic spread is independently associated with a reduced likelihood of achieving DC. Based on these findings, curative and locoregional treatments should not be "a priori" excluded in a subset of BCLC stage C patients. Indeed, predictive factors may be helpful in the sophistication of patients' prognosis, thereby being valuable in the selection of patients suitable for clinical trials and in designing the therapeutic strategy.

Research perspectives

Given the higher number of heterogeneous and complex cases encountered in the field-practice, the BCLC classification is often not exhaustive, and the increasing number of new therapeutic options and their combinations makes difficult to strictly adhere to BCLC suggestions. New algorithms for the stratification of patients' prognosis are needed to improve clinical practice.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Hospital contacts with alcohol problems prior to liver cirrhosis or pancreatitis diagnosis

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Abstract

AIM

To evaluate prior hospital contacts with alcohol problems in patients with alcoholic liver cirrhosis and pancreatitis.

METHODS

This was a register-based study of all patients diagnosed with alcoholic liver cirrhosis or pancreatitis during 2008-2012 in Denmark. Hospital contacts with alcohol problems (intoxication, harmful use, or dependence) in the 10-year period preceding the diagnosis of alcoholic liver cirrhosis and pancreatitis were identified.

RESULTS

In the 10 years prior to diagnosis, 40% of the 7719 alcoholic liver cirrhosis patients and 40% of the 1811

alcoholic pancreatitis patients had at least one prior hospital contact with alcohol problems. Every sixth patient (15%-16%) had more than five contacts. A similar pattern of prior hospital contacts was observed for alcoholic liver cirrhosis and pancreatitis. Around 30% were diagnosed with alcohol dependence and 10% with less severe alcohol diagnoses. For the majority, admission to somatic wards was the most common type of hospital care with alcohol problems. Most had their first contact with alcohol problems more than five years prior to diagnosis.

CONCLUSION

There may be opportunities to reach some of the patients who later develop alcoholic liver cirrhosis or pancreatitis with preventive interventions in the hospital setting.

Key words: Alcoholic liver disease; Alcoholic pancreatic disease; Nationwide; Prevention; Hospital contacts

Core tip: Alcohol-related liver and pancreatic disease are preceded by many years of heavy drinking. Hospital contacts with obvious alcohol problems prior to development of alcohol-related liver or pancreatic disease may constitute opportunities for prevention if alcohol problems were to be consistently managed. In this study of all Danish alcoholic liver cirrhosis and alcoholic pancreatitis patients, forty percent had at least one previous hospital contact with obvious alcohol problems in the 10 years prior to diagnosis. Most of these patients had their first contact with alcohol problems more than five years prior to diagnosis.

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INTRODUCTION

Alcohol is the single most important cause of liver and pancreatic disease in Western countries[1,2]. Alcohol-related liver and pancreatic disease are associated with a considerably mortality risk[3,4], preceded by years of heavy drinking[5,6]. However, among hazardous drinkers reducing or abandoning alcohol consumption can attenuate the risk of full blown disease or death due to alcohol-related liver and pancreatic disease[2,7,8]. Since these diseases develop over many years prior to diagnosis, this offers a window of opportunity in which preventive interventions could be implemented.

Hospital contacts with alcohol problems in the period before disease may constitute opportunities for offering alcohol treatment[9,10]. Such hospital contacts include those involving alcohol intoxication (a marker of excessive drinking), harmful alcohol use (a diagnosis used for mild cases of alcohol dependence or when the alcohol use has caused physical or mental disease), and alcohol dependence in more severe cases of alcohol problems[11,12]. In Denmark[13], as in many other countries[14-16], formalised hospital-based alcohol treatment is not available. For example, patients admitted with alcohol withdrawal will be discharged when acute symptoms have been alleviated, without the development of further treatment for underlying alcohol misuse or dependence.

We recently found that patients with hospital contacts with alcohol problems had a more than 10-fold greater rate of alcoholic liver cirrhosis compared to the general population[17]. In the present study, the reverse situation was evaluated; the extent to which patients with alcoholic liver cirrhosis or alcoholic pancreatitis have prior hospital contacts with alcohol problems. Earlier studies found that 33%-58% of liver cirrhosis patients had prior hospital contacts indicated by disorders that are sometimes, though not always, associated with alcohol problems such as injuries, non-variceal upper gastrointestinal bleeding, and epilepsy[18-20]. Hospital contacts with a more specific set of alcohol problems, however, might represent a more feasible opportunity to offer alcohol treatment.

We conducted a nationwide study of all patients who were diagnosed with alcoholic liver cirrhosis and alcoholic pancreatitis 2008 to 2012 in Denmark. In these patients, we evaluated the extent of prior hospital contacts with alcohol problems in the 10 years prior to their diagnosis of alcoholic liver cirrhosis or alcoholic pancreatitis.

MATERIALS AND METHODS

Data sources

The study was based on Danish nationwide registries. All Danish citizens have access to free healthcare. The National Patient Register contains data on all somatic hospital admissions since 1977[21]. From 1995 contacts with emergency rooms, outpatient clinics, and psychiatric hospital were recorded. The Danish Register of Causes of Death has recorded causes of death among all Danish citizens since 1970[22]. In all registries, diagnoses are recorded according to the 8th (1971-1993) and 10th (1994-present) revision of the international classification of diseases (ICD)[21].

Information on vital status, civil status, and migration to and from Denmark was obtained from the Danish Civil Registration System and education from Statistics Denmark[23]. The registries were linked by a personal identification number, a identifier assigned to all Danish residents at birth since 1968[23].

Study population

The study population consisted of all patients with a first diagnosis of alcoholic liver cirrhosis or alcoholic pancreatitis in Denmark from 2008 to 2012 (alcoholic liver cirrhosis; ICD-8: 571.0 and ICD-10: K70.3, K70.4 and alcoholic pancreatitis; ICD-10: K85.2, K86.0). Patients diagnosed with alcoholic liver cirrhosis or alcoholic pancreatitis from 1977, when The National Patient Register was initiated, to 2008 were therefore excluded. We combined acute and chronic alcoholic pancreatitis since they are often found together and are both preceded by years of heavy drinking[2,7]. In Denmark, there are restrictions on alcohol sale for young people less than 16-18 years. To ensure 10 years of follow-back before the diagnosis, we excluded patients less than 28 years of age at diagnosis (n = 27). Information from The National Patient Register and Danish Register of Causes of Death were combined. The patients not diagnosed during life but with alcoholic liver cirrhosis or alcoholic pancreatitis as their cause of dead were included. Patients diagnosed with alcoholic liver cirrhosis and alcoholic pancreatitis on the same day (n = 65) were assigned to the alcoholic liver cirrhosis group due to the higher mortality associated with this disease[3,24].

Comorbidity was assessed according to the Charlson Comorbidity Index score based on diagnoses made in the course of hospital contacts in the 10 years prior to diagnosis[25]. Psychiatric comorbidity was measured as the number of the following psychiatric diseases (ICD-10 codes): Dementia and organic disorders not caused by alcohol (F00-09), schizophrenia (F20-29), mood disorders (F30-39), neurotic and stress-related (F40-49), behavioural syndromes associated with physiological disturbances (F50-59), personality disorders (F60-69), mental retardation (F70-79), disorders of psychological development (F80-89), and behavioural and emotional disorders (F90-99)[11].

Prior hospital contacts with alcohol problems

A prior hospital contact with alcohol problems [alcohol intoxication (ICD-10: F10.0), harmful alcohol use (ICD-10: F10.1), or alcohol dependence (ICD-10: F10.2, F10.3, F10.4, F10.5)] was restricted to those occurring in the 10 years before the diagnosis of alcoholic liver cirrhosis or alcoholic pancreatitis. However, contacts occurring in the three months prior to diagnosis were excluded to avoid including hospital contacts that might have been precipitated by symptoms of liver or pancreatic disease that were not immediately recognised. A maximum of one hospital contact with alcohol problems per day was included.

Statistical analysis

Analyses were carried out separately for patients diagnosed with alcoholic liver cirrhosis and alcoholic pancreatitis. We did not calculate confidence limits since we had nationwide data[26]. Assessment of comparability of demographic and medical characteristics between patients with and without prior hospital contacts with alcohol problems were performed using χ^2 test for categorical data and t-test for continuous data on age, which followed a normal distribution. Alcohol diagnoses (alcohol intoxication, harmful alcohol use, and alcohol dependence) were assessed as an indicator of the severity of alcohol problems among patients with alcoholic liver cirrhosis and alcoholic pancreatitis[12]. We also estimated the type of hospital care of the prior hospital contacts with alcohol problems (somatic, psychiatric, inpatient, emergency room, or outpatient clinic). Finally, we estimated the time in years that had passed from the initial hospital contact with alcohol problems to alcoholic liver cirrhosis or pancreatitis diagnosis. All analyses were carried out in SAS version 9.4.

RESULTS

From 2008 to 2012, 7719 patients were diagnosed with alcoholic liver cirrhosis and 1811 were diagnosed with alcoholic pancreatitis in Denmark. Of patients with alcoholic liver cirrhosis, 3058 (40%) had at least one hospital contact with alcohol problems within the prior 10 years excluding the three months prior to diagnosis (Table 1). The equivalent number was 719 (40%) for patients with alcoholic pancreatitis. In both patient groups, those with prior hospital contacts with alcohol problems were younger, more often men, more often married, and more often had somatic and psychiatric disease compared to those with no such contacts. For example, of patients with alcoholic liver cirrhosis with prior hospital contacts with alcohol problems, 2217 (72%) had no psychiatric comorbidity, 522 (17%) one, and 319 (10%) had two or more. In alcoholic liver cirrhosis patients without prior hospital contacts with alcohol problems, these numbers were 4394 (94%), 203 (4.6%) and 64 (1.4%).

The number of patients not diagnosed during life but having alcoholic liver cirrhosis and alcoholic pancreatitis as their cause of death were 875 (11%) and 106 (5.9%).

Number of prior hospital contacts with alcohol problems

The 7719 patients with alcoholic liver cirrhosis had a total of 38227 hospital contacts with alcohol problems in the prior 10-years (mean of 5.0 contacts). The median number (5^{th}-95^{th} percentiles) of prior contacts was 0 (0-19). The 1811 patients with alcoholic pancreatitis had 8997 prior hospital contacts with alcohol problems in the prior 10 years (mean of 5.0 contacts). The median number (5^{th}-95^{th} percentiles) of prior contacts was also 0 (0-19) in these patients.

Whereas 60% of the alcoholic liver cirrhosis patients had no prior hospital contacts with alcohol problems in the prior 10 years, 902 (12%) had one, 992 (13%) had two to four, 509 (7.0%) had five to nine, and 650 (8.0%) had ten or more (Figure 1). The percentages were similar in patients with alcoholic pancreatitis.

Table 1 Demographic and medical characteristics among patients newly diagnosed with alcoholic liver cirrhosis or alcoholic pancreatitis 2008-2012 in Denmark according to a prior hospital contact with alcohol problems within 10 years *n* (%)

Characteristic	Alcoholic liver cirrhosis (*n* = 7719)			Alcoholic pancreatitis (*n* = 1811)		
	Yes	No	*P* value	Yes	No	*P* value
Cohort, *n*	3058 (40)	4661 (60)		719 (40)	1092 (60)	
Age, mean (range)	57 (29-92)	61 (28-93)	< 0.0001	53 (28-90)	58 (28-92)	< 0.0001
Sex, men	2144 (70)	3159 (68)	0.03	562 (78)	810 (74)	0.05
Civil status, married	1338 (44)	1681 (36)	< 0.0001	289 (40)	345 (32)	0.000
Education (yr)			0.001			0.38
⩽ 9	1479 (48)	2062 (44)		342 (48)	483 (44)	
9-11	1170 (38)	1950 (42)		297 (41)	481 (44)	
⩾ 12	409 (14)	649 (14)		80 (11)	128 (12)	
Charlson comorbidity index			< 0.0001			0.02
0	938 (31)	1890 (41)		263 (36)	472 (43)	
1-2	1201 (39)	1402 (30)		285 (40)	391 (36)	
⩾ 3	919 (30)	1369 (29)		171 (24)	229 (21)	
Number of psychiatric comorbidities			< 0.0001			< 0.0001
0	2217 (73)	4394 (94)		478 (67)	990 (91)	
1	522 (17)	203 (4.6)		139 (19)	68 (6.0)	
⩾ 2	319 (10)	64 (1.4)		102 (14)	34 (3.0)	

Values are numbers (percentages) unless otherwise stated, *n* = 9530.

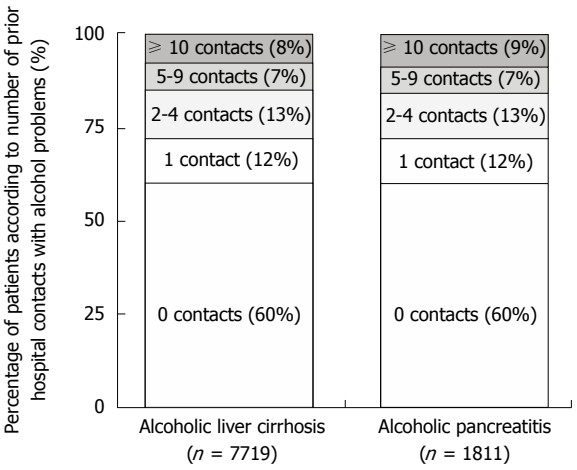

Figure 1 Number of hospital contacts with alcohol problems in the prior 10 years among patients newly diagnosed with alcoholic liver cirrhosis or alcoholic pancreatitis 2008-2012 in Denmark. Values are percentages of patients (*n* = 9530).

Alcohol diagnoses and type of hospital care of prior hospital contacts with alcohol problems

Nearly a third of patients with alcoholic liver cirrhosis and alcoholic pancreatitis had a diagnosis of alcohol dependence when hospitalized with alcohol problems in the prior 10 years (Table 2). Only 10% had less severe alcohol diagnoses of harmful alcohol use (6.7%-7.5%) or alcohol intoxication (2.3%-2.5%).

More patients had been admitted to a somatic hospital (36%) with alcohol problems than to a psychiatric hospital (15%-16%) in the 10 years prior to diagnosis. Admission to somatic wards with alcohol problems was the most common type of hospital care, which accounted to 2051 (27%) of patients with alcoholic liver cirrhosis and 509 (28%) of patients with alcoholic pancreatitis.

Time between the initial hospital contact with alcohol problems and diagnosis

In those patients diagnosed with alcoholic liver cirrhosis who had a prior hospital contact with alcohol problem, more than half had it at least five years before their diagnosis (Figure 2). Only 340 (4%) of all alcoholic liver cirrhosis patients had an initial contact with alcohol problems in the year before diagnosis whereas 980 (14%) had it one two to four years before, 1312 (16%) five to nine years before, and in 426 (6%) ten years before. A similar pattern was seen for those diagnosed with alcoholic pancreatitis.

DISCUSSION

In the present study, 40% of all Danish patients with alcoholic liver cirrhosis and alcoholic pancreatitis diagnosed from 2008 to 2012 had at least one hospital contact with alcohol problems in the prior 10 years before diagnosis. Every sixth patient (15%-16%) had more than five contacts. The pattern of prior hospital contacts with alcohol problems was similar for patients diagnosed with alcoholic liver cirrhosis and alcoholic pancreatitis. Roughly 30% had been given a prior diagnosis of alcohol dependence and 10% had less severe alcohol diagnoses (harmful use and intoxication). Inpatient admission to a somatic ward was the type of hospital care most patients have had with prior alcohol problems. More than half of cases with a prior hospital contact in the preceding 10 years had had their initial alcohol-related contact five or more years prior to diagnosis.

This study has a number of strengths. It covers

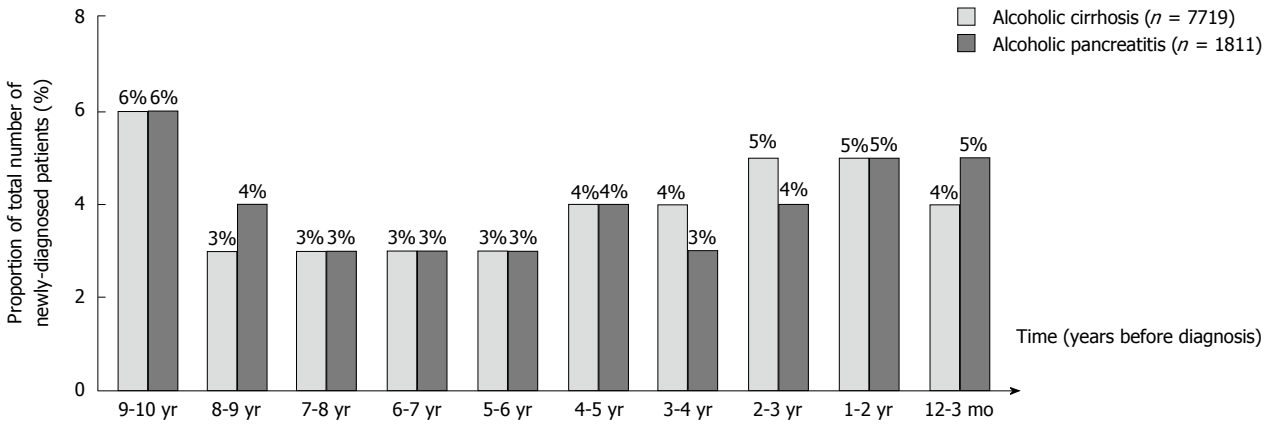

Figure 2 Years between initial hospital contact with alcohol problems and alcoholic liver cirrhosis or pancreatitis diagnosis among patients newly diagnosed with alcoholic liver cirrhosis or alcoholic pancreatitis 2008-2012 in Denmark. Values are percentages of patients (*n* = 9530).

Table 2 Most severe alcohol diagnosis recorded and types of hospital care of prior hospital contacts with alcohol problems within 10 years, among patients newly diagnosed with alcoholic liver cirrhosis or alcoholic pancreatitis 2008-2012 in Denmark

	Alcoholic liver cirrhosis (*n* = 7719)	Alcoholic pancreatitis (*n* = 1811)
Prior hospital contact with alcohol problems		
No	4661 (60)	1092 (60)
Yes	3058 (40)	719 (40)
If yes, the most severe alcohol problem diagnosis recorded		
Intoxication	184 (2.3)	46 (2.5)
Harmful use	527 (6.7)	141 (7.8)
Dependence	2347 (31)	532 (30)
If yes, types of hospital care[1]		
Somatic hospital	2743 (36)	644 (36)
Somatic ward	2051 (27)	509 (28)
Somatic emergency room	970 (13)	226 (12)
Somatic outpatient clinic	1150 (15)	250 (14)
Psychiatric hospital	1157 (15)	294 (16)
Psychiatric ward	454 (5.9)	126 (7.0)
Psychiatric emergency room	775 (10)	192 (11)
Psychiatric outpatient clinic	431 (5.6)	125 (6.9)

[1]Patients counted in several categories if they had more than one prior hospital contact with alcohol problems with different types of hospital care. Values are numbers (percentages) of patients (*n* = 9530).

the entire Danish population for which there is almost complete data on hospital care[21], and cause of death[22]. The alcoholic liver cirrhosis diagnosis in the registry has a high positive predictive value of correctly specifying liver cirrhosis: 78%-92% when compared to information from liver biopsies or clinical evaluation[27,28]. The validity of the alcoholic pancreatitis diagnosis has not been evaluated, but since this diagnosis is managed by gastroenterology specialists in Denmark, we expect the validity to be high[4]. A potential limitation is the validity of the classification of hospital contacts with alcohol problems. These diagnoses are most likely underreported leading to an underestimation of prior hospital contacts with alcohol problems[29].

Prior studies of cirrhosis patients found that 33%-58% had prior health care attendances with disorders that are sometimes associated with alcohol problems[18,20]. This is in accordance with our study where 40% of alcoholic cirrhosis patients had prior hospital contacts with alcohol problems. Our finding that some of the patients with alcohol problems have a considerably high number of repeated contacts due to alcohol has been reported before[12]. To our knowledge, no other study has assessed alcohol problems in patients with alcoholic pancreatitis.

The proportion of alcoholic liver cirrhosis patients with alcohol problems and the severity of these problems found in our study are in line with results from questionnaire-based studies[30-32]. These studies found roughly one third of patients to be moderate or severely alcohol dependent, one third mildly dependent and one third not dependent[30-32]. This underscores the observation that alcoholic liver cirrhosis patients in general have a lower degree of alcohol problems than people seeking treatment for alcohol problems[31,33].

The majority of prior hospital contacts with alcohol problems were with somatic, not psychiatric hospitals.

This is likely to reflect the fact that the majority of these cases were precipitated by injuries or non-psychiatric comorbidity[19,20]. That most contacts were as ward admissions rather than emergency room indicate a higher level of disease severity needing longer observation or more complex treatment than could be offered in the emergency room. The relatively few outpatient contacts with alcohol problems might indicate a lower utilization of routine or preventive care in favour of acute hospital admissions when health problems have become more severe, which was observed in heavy drinkers of old age[34].

Finally, in agreement with the long period of heavy drinking that commonly precedes the development of alcoholic liver cirrhosis and alcoholic pancreatitis[5,6,8], for the majority of patients in our study with prior alcohol contacts, more than five years had passed between their initial contact and diagnosis of alcoholic liver cirrhosis and pancreatitis.

The implication of our study is that there are opportunities to reach around half of patients who later develop alcoholic liver cirrhosis or alcoholic pancreatitis with preventive interventions in the hospital setting[9]. Suggested preventive interventions for liver disease involve implementation of hospital-based alcohol care teams which was shown to reduce alcohol-related admissions[9,35]. It may also involve non-invasive assessment of liver disease[36,37]. Hospital patients with alcohol problems and somatic disease or injury are in particular motivated for alcohol treatment[38-41].

Future studies should assess contacts with obvious alcohol problems in primary care in addition to hospital contacts to compare where patients are most frequently seen with alcohol problems prior to diagnosis of alcoholic liver cirrhosis or alcoholic pancreatitis[18,20,42].

About half of alcoholic liver cirrhosis and pancreatitis patients had hospital contacts with alcohol problems prior to diagnosis. There seems to be opportunities to reach some of the patients who later develop alcoholic liver cirrhosis or pancreatitis with preventive interventions in the hospital setting.

ARTICLE HIGHLIGHTS

Research background

Alcoholic liver cirrhosis and alcoholic pancreatitis develop over many years prior to diagnosis, which offers a window of opportunity in which preventive interventions could be implemented. Hospital contacts with alcohol problems in the period before disease may constitute opportunities for offering alcohol treatment. Earlier studies found that 33%-58% of liver cirrhosis patients had prior hospital contacts indicated by disorders that are sometimes, though not always, associated with alcohol problems such as injuries, non-variceal upper gastrointestinal bleeding, and epilepsy. Hospital contacts with a specific set of alcohol problems (alcohol intoxication, harmful alcohol use, and alcohol dependence) might represent a more feasible opportunity to offer alcohol treatment than disorders associated with alcohol problems. No prior studies evaluated hospital contacts with alcohol problems in patients with alcoholic pancreatitis.

Research motivation

In Denmark, as in many other countries, formalised hospital-based alcohol treatment is not available. Hospitalization with alcohol problems prior to alcoholic liver cirrhosis or pancreatitis diagnosis may represent an opportunity to offer preventive interventions. In a nationwide study, we evaluated previous hospital contacts with alcohol problems in patients with incident alcoholic liver cirrhosis and alcoholic pancreatitis diagnosis.

Research objectives

The objective was to conduct a nationwide study of all patients diagnosed with alcoholic liver cirrhosis and alcoholic pancreatitis 2008 to 2012 in Denmark. In these patients, the extent of prior hospital contacts with alcohol problems in the 10 years prior to their diagnosis of alcoholic liver cirrhosis or alcoholic pancreatitis were evaluated.

Research methods

This was a nationwide, register-based study of all patients diagnosed with alcoholic liver cirrhosis or pancreatitis during 2008-2012 in Denmark. Hospital contacts with alcohol problems (intoxication, harmful use, or dependence) in the 10-year period preceding the diagnosis of alcoholic liver cirrhosis or pancreatitis were identified. Data was obtained from nationwide registries on hospital contacts and causes of death. This is the first study to evaluate prior hospital contacts with alcohol problems in a nationwide design. Furthermore, no prior studies included psychiatric hospital contacts with alcohol problems. Hospital contacts with alcohol problems occurring in the three months prior to diagnosis of alcoholic liver cirrhosis and pancreatitis were excluded to avoid including hospital contacts that might have been precipitated by symptoms of liver or pancreatic disease that were not immediately recognised. Alcohol diagnoses (alcohol intoxication, harmful alcohol use, and alcohol dependence) were assessed as an indicator of the severity of alcohol problems among patients with alcoholic liver cirrhosis and alcoholic pancreatitis. We also estimated the type of hospital care of the prior hospital contacts with alcohol problems (somatic, psychiatric, inpatient, emergency room, or outpatient clinic). Finally, we estimated the time in years that had passed from the initial hospital contact with alcohol problems to alcoholic liver cirrhosis or pancreatitis diagnosis.

Research results

In the 10 years prior to diagnosis, 40% of the 7719 alcoholic liver cirrhosis patients and 40% of the 1811 alcoholic pancreatitis patients had at least one prior hospital contact with alcohol problems. Every sixth patient (15%-16%) had more than five contacts. The 7719 patients with alcoholic liver cirrhosis had a total of 38227 hospital contacts with alcohol problems in the prior 10-years (mean of 5.0 contacts). The median number (5^{th}-95^{th} percentiles) of prior contacts was 0 (0-19). The 1811 patients with alcoholic pancreatitis had 8997 prior hospital contacts with alcohol problems in the prior 10 years (mean of 5.0 contacts). The median number (5^{th}-95^{th} percentiles) of prior contacts was also 0 (0-19) in these patients. A similar pattern of prior hospital contacts was observed for alcoholic liver cirrhosis and pancreatitis. Around 30% were diagnosed with alcohol dependence and 10% with less severe alcohol diagnoses. For the majority, admission to somatic wards was the most common type of hospital care with alcohol problems. Most had their first contact with alcohol problems more than five years prior to diagnosis.

Research conclusions

In the present study, 40% of all Danish patients with alcoholic liver cirrhosis and alcoholic pancreatitis diagnosed from 2008 to 2012 had at least one hospital contact with alcohol problems in the prior 10 years before diagnosis. Every sixth patient (15%-16%) had more than five contacts. The pattern of prior hospital contacts with alcohol problems was similar for patients diagnosed with alcoholic liver cirrhosis and alcoholic pancreatitis. Roughly 30% had been given a prior diagnosis of alcohol dependence and 10% had less severe alcohol diagnoses (harmful use and intoxication). Inpatient admission to a somatic ward was the type of hospital care most patients have had with prior alcohol problems. More than half of cases with a prior hospital contact in the preceding 10 years had had their initial alcohol-related contact five or more years prior to diagnosis. The implication of our study is that there are opportunities to reach around half of patients who later develop alcoholic liver cirrhosis or alcoholic pancreatitis with preventive interventions in the hospital setting. Suggested preventive interventions for liver disease involve implementation of hospital-based alcohol care teams which was shown to reduce alcohol-related admissions. It may also

involve non-invasive assessment of liver disease. Hospital patients with alcohol problems and somatic disease or injury are in particular motivated for alcohol treatment.

Research perspectives

Future studies should assess contacts with obvious alcohol problems in primary care in addition to hospital contacts to compare where patients are most frequently seen with alcohol problems prior to diagnosis of alcoholic liver cirrhosis or alcoholic pancreatitis. In particular, randomized controlled trials are needed to evaluate if alcohol treatment in the hospital setting can decrease the incidence of alcoholic liver cirrhosis and alcoholic pancreatitis.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Efficacy and safety of sofosbuvir and ledipasvir in japanese patients aged 75 years or over with hepatitis C genotype 1

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Abstract

AIM

To evaluate the efficacy and safety of a regimen containing sofosbuvir (SOF) and ledipasvir (LDV) in Japanese patients aged ⩾ 75 years with hepatitis C genotype 1.

METHODS

This multicenter, retrospective study consisted of 246 Japanese patients with HCV genotype 1 at nine centers in Miyazaki prefecture in Japan. Demographic, clinical, virological, and adverse effects (AE)-related data obtained during and after SOF/LDV therapy were collected from medical records. These patients were divided into two groups, younger (aged < 75 years) and elderly (aged ⩾ 75 years). Virological data and AEs were analyzed by age group.

RESULTS

The sustained virological response (SVR) rates at 12 wk after treatment were 99.2%, 99.4%, and 98.7% in the overall population and in patients aged < 75 and ⩾ 75 years, respectively. Common AEs during therapy were headache, pruritus, constipation, and insomnia. These occurred in fewer than 10% of patients, and their incidence was not significantly different between the younger and elderly groups. Two patients discontinued treatment, one due to a skin eruption and the other due to cerebral bleeding.

CONCLUSION

Compared with younger patients, elderly patients had a similar virological response and tolerance to SOF/LDV therapy.

Key words: Chronic hepatitis C; Sofosbuvir; Ledipasvir; Sustained virological response; Direct acting antivirals

Core tip: Most Japanese patients with hepatitis C are elderly, and those aged ⩾ 75 years account for more than 50%. However there are few reports regarding sofosbuvir (SOF) and ledipasvir (LDV) therapy in patients aged ⩾ 75 years in the real-world. The present study demonstrated that patients aged ⩾ 75 years had a similar virological response and tolerance to SOF/LDV therapy compared with patients aged < 75 years in the real-world cohorts. Therefore, SOF/LDV therapy might be effective and safe in elderly patients.

INTRODUCTION

Hepatitis C virus (HCV) infection is one of the major global causes of liver-related diseases such as chronic hepatitis, liver cirrhosis, liver failure, and hepatocellular carcinoma (HCC)[1,2]. In Japan, the prevalence of anti-HCV antibodies in the general population was estimated to be 0.9%[3], and significantly increased with age[3,4]. In fact, most Japanese patients with hepatitis C are elderly, and those aged ⩾ 75 years account for more than 50%[5]. However, elderly patients (⩾ 75 years) treated with interferon-based therapies have poor sustained virological response (SVR) rates and high discontinuation rates due to adverse effects (AEs)[6]. Moreover, in Japan the proportion of patients with HCV genotype 1 infection was found to 70%; most were reported to be infected with subgenotype 1b, compared to only approximately 1% with subgenotype 1a[7]. These population was known to exhibit treatment resistance with interferon (IFN) therapy[8], therefore novel anti-viral therapies for this population are urgently needed.

In 2014, the combination of daclatasvir (DCV), an NS5A inhibitor, and asunaprevir (ASV), an NS3/4A protease inhibitor, was the first interferon-free regimen to be approved for Japanese patients with HCV genotype 1[9]. Moreover, in 2015, the HCV NS5A inhibitor ledipasvir (LDV) and the HCV polymerase inhibitor sofosbuvir (SOF) were approved for this same population[10]. These regimens have demonstrated high efficacy with an improved safety profile and shorter treatment duration than interferon-based therapies[9,10]. However, patients aged ⩾ 75 years were excluded from these clinical trials[9,10], and therefore no data have been reported regarding the efficacy and safety of these regimens in this population. Recently, with respect to DCV/ASV therapy, several real-world studies showed that the SVR rate and discontinuation rate due to AEs were comparable in patients aged ⩾ 75 and < 75 years[11-13]. On the other hand, there are few reports regarding SOF/LDV therapy in patients aged ⩾ 75 years. Therefore, in the present study, we assessed the efficacy and safety of SOF/LDV therapy in Japanese patients aged ⩾ 75 years with hepatitis C genotype 1.

MATERIALS AND METHODS

Patients and therapy regimens

Between September 2015 and December 2016, 246 patients infected with HCV genotype 1 were treated with SOF/LDV at nine centers in Miyazaki prefecture in Japan. Demographic, clinical, virological and AE-related data obtained during and after therapy were retrospectively collected from medical records. Patients who had already received DCV/ASV therapy were excluded. Cirrhotic patients with Child-Pugh class B and C were excluded. Patients received 12 wk of treatment with a fixed-dose combination tablet containing 90 mg of LDV and 400 mg of SOF, administered orally once daily. In a phase 3 clinical trial in Japan, the addition of ribavirin to SOF/LDV did not improve the SVR12 rate, but did increase the number of AEs[10]. Thus, the combination of ribavirin and SOF/LDV is not approved in Japan for the treatment of chronic HCV infection, including in cirrhotic or treatment-experienced patients. Patients were divided into younger (< 75 years) and elderly (⩾ 75 years) groups, and clinical data were analyzed by group. This study was approved by the Research Ethics Committee of the University of Miyazaki.

Laboratory and virological assessments

Laboratory tests were performed at baseline, at weeks 4, 8, and 12 during therapy, and at 4, 8, and 12 wk after therapy. HCV RNA was measured using the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). The dynamic range was 1.2-7.8 log IU/mL. HCV RNA levels were measured at weeks 4, 8, and 12 during therapy, and at weeks 4, 8, and 12 after therapy. Liver cirrhosis was diagnosed clinically based on laboratory tests and imaging findings, including portosystemic shunt, splenomegaly, or esophageal/gastric varices. The fibrosis-4 index (Fib-4) was calculated before the initiation of SOF/LFV therapy. NS5A resistance-associated variants (RAVs) (Y93C/H/N/S or L31I/F/M/V) of HCV were tested by direct sequencing in some patients. In this study, virological responses were categorized as follows: Undetectable HCV RNA at 4 wk after the initiation of therapy was defined as rapid virological response (RVR), and that at 12 wk after the end of the therapy was defined as sustained virological response (SVR12). Relapse was defined as undetectable HCV RNA levels by the end of therapy and detectable levels during the follow-up period.

Statistical analysis

Statistical analyses were performed with SPSS software (IBM SPSS Statistics for Windows, version 20.0). Baseline continuous data are expressed as median, and categorical data are expressed as number and percentage. The effectiveness of SOF/LDV therapy was evaluated using intention-to-treat analysis. Univariate analyses were performed using the χ^2, Fisher's exact, or Mann-Whitney *U* tests. *P* values < 0.05 were considered statistically significant in all analyses.

RESULTS

Patient characteristics

Patient characteristics are shown in Table 1. The median age was 69 years (range, 29-88 years), and 79 (32%) patients were aged ⩾ 75 years (elderly group). Of the 246 patients, 103 (42%) were male. Fifty-one patients (21%) had cirrhosis, and all were Child-Pugh class A. Sixteen patients (7%) were previously treated for HCC. Fifty-two patients (21%) previously received interferon-based therapy. Of the 75 patients who were tested for HCV NS5A-RAVs before therapy, 22 (29%) were positive at baseline. Of these, only five had both NS5A Y93 and L31. Before therapy, the median HCV viral load was 6.1 log IU/mL (range 1.6-7.3 log IU/mL). Baseline platelet count and glomerular filtration rate were lower and FIB4 was higher in the elderly.

Effectiveness

The overall RVR rate was 86.9%. All patients had undetectable HCV RNA at 8 wk of therapy, and none exhibited viral breakthrough during treatment. The SVR12 rates were 99.2%, 99.4%, and 98.7% in the overall population and in patients aged < 75 and ⩾ 75 years, respectively. Table 2 shows the SVR12 rates according to various clinical and demographic factors. There was no difference between the two groups in any parameter. Two patients experienced virological relapse, one after 4 wk (elderly patient) and the other after 8 weeks (younger patient), and one of these had an NS5A RAV (L31M) at baseline.

Safety and adverse events

The safety profile for SOF/LDV is shown in Table 3. Common AEs during therapy were headache, pruritus, constipation, and insomnia. All were found in fewer than 10% of patients, at similar rates in the elderly and younger groups. Serious AEs, including hematological and laboratory abnormalities, were rare. None of the patients had decreased hemoglobin levels or platelet counts, and none had elevated total bilirubin levels over 3.0 mg/dL, alanine aminotransferase levels over five times the upper limit of normal, or creatinine levels over 1.5 times baseline values. Two patients (0.8%) discontinued therapy prematurely, one due to cerebral hemorrhage (pontine hemorrhage) at 7 wk after initiation of therapy, and one due to a skin eruption after 10 wk. The former was a 62-year-old man, while the latter was a 72-year-old woman. Both patients were treatment naïve, and eventually achieved SVR12.

DISCUSSION

Recently, a number of oral direct-acting antivirals (DAAs)

Table 1 Baseline characteristics

Characteristics	Total (n = 246)	< 75 yr (n = 167)	⩾ 75 yr (n = 79)	P value
Sex (male)	103 (42)	65 (39)	37 (47)	0.239
Age (yr)	69 (29-88)	65 (29-74)	78 (75-88)	< 0.001
Body weight (kg)	53 (35-91)	53 (38-91)	53 (35-78)	0.527
Cirrhosis	51 (21)	30 (18)	21 (26)	0.120
HCV RNA (log_{10}IU/mL)	6.1 (1.6-7.3)	6.1 (1.6-7.3)	6.1 (4.0-6.8)	0.337
Hemoglobin (g/dL)	13.6 (9.0-16.8)	13.6 (9.5-16.8)	13.3 (9.0-15.9)	0.163
Platelets (× 10^9/L)	156 (26-340)	167 (26-340)	132 (57-278)	0.001
Aspartate aminotransaminase (U/L)	42 (17-191)	40 (17-191)	45 (20-155)	0.140
Alanine aminotransaminase (U/L)	38 (11-319)	38 (12-319)	37 (11-167)	0.341
eGFR (mL/min per 1.73 m^2)	72 (36-132)	76 (38-132)	63 (36-98)	< 0.001
α-fetoprotein (ng/mL)	4 (1-382)	4 (1-382)	4 (1-74)	0.525
Fib-4 index	3.3 (0.5-23.2)	2.5 (0.5-23.2)	4.4 (1.5-10.7)	< 0.001
NS5A RAVs				
Y93	22 (29)	10 (21)	12 (43)	0.146
L31	6 (8)	3 (6)	3 (11)	0.798
Y93/L31	5 (7)	4 (9)	1 (4)	0.645
Treatment experienced	52 (21)	41 (25)	11 (14)	0.064
Previous HCC treatment	16 (7)	11 (7)	5 (6)	0.841

Data are expressed as n (%) or median (range). eGFR: Estimated glomerular filtration rate; RAVs: Resistance-associated variants; HCC: Hepatocellular carcinoma.

Table 2 Sustained virological response 12 rates according to clinical and demographical factors

Parameters	n	SVR 12 (%)	P value
Sex			0.6272
Male	103	100.0	
Female	143	98.6	
Age (yr)			0.8287
< 75	167	99.4	
⩾ 75	79	98.7	
HCV RNA (log10IU/mL)			0.7076
< 6.0	93	100.0	
⩾ 6.0	153	98.7	
Liver fibrosis			0.8811
No cirrhosis	195	99.5	
Cirrhosis	51	98.0	
Fib-4 index			0.4634
< 3.25	125	100.0	
⩾ 3.25	121	98.3	
Prior treatment			0.8931
Treatment naïve	194	99.0	
Treatment experienced	52	100.0	
Previous HCC treatment			0.2868
No	230	99.6	
Yes	16	93.8	
NS5A RAVs			0.5471
None	48	97.9	
Y93	22	100.0	
L31	6	83.3	
Y93/L31	5	100.0	

RAVs: Resistance-associated variants; HCC: Hepatocellular carcinoma; SVR: Sustained virological response.

Table 3 Safety profile

	Total (n = 246)	< 75 yr (n = 167)	⩾ 75 yr (n = 79)
Common adverse effects			
Headache	6 (2.4)	4 (2.4)	2 (2.5)
Pruritus	2 (0.8)	0	2 (2.5)
Constipation	2 (0.8)	2 (1.2)	0
Stomatitis	2 (0.8)	2 (1.2)	0
Skin eruption	1 (0.4)	1 (0.6)	0
Chill	1 (0.4)	1 (0.6)	0
Nausea	1 (0.4)	1 (0.6)	0
Fever	1 (0.4)	1 (0.6)	0
Insomnia	1 (0.4)	1 (0.6)	0
Hematological abnormalities			
Hemoglobin < 10.0 g/dL	0	0	0
Platelet count < 50 × 10^9/L	0	0	0
Laboratory abnormalities			
Total bilirubin > 3.0 mg/dL	0	0	0
Alanine aminotransferase > 5 × ULN	0	0	0
Serum creatinine > 1.5 × baseline	0	0	0
Death	0	0	0
Discontinuation due to adverse effects	2 (0.8)	2 (1.2)	0
Cerebral hemorrhage	1 (0.4)	1 (0.6)	0
Skin eruption	1 (0.4)	1 (0.6)	0

Data are expressed as n (%).

for HCV treatment were introduced worldwide, and have been reported to be more effective and safer compared with IFN-based therapies. In 2015, the combination of the NS5B polymerase inhibitor SOF and the NS5A inhibitor LDV was approved in Japan[10]. This regimen have demonstrated high efficacy with an improved safety profile and shorter therapy duration than interferon-based therapies, however, patients aged ⩾ 75 years were excluded from this clinical trials[10]. Moreover, the majority of Japanese patients with hepatitis C are elderly, and in particular, those aged ⩾ 75 years account for more than 50% of this population[5]. In our study, patients aged ⩾ 75 years showed a high SVR rate (98.7%) and none discontinued treatment due to AEs. Moreover, both the SVR rate and rate of discontinuation secondary to AEs were nearly equal in elderly (⩾ 75 years) and younger (< 75 years) patients. Although real-world cohort studies demonstrating the effectiveness of several SOF-containing regimens in elderly patients

have been published worldwide[14-16], to the best of our knowledge, this is the first real-world study focusing on a high SVR rate and low discontinuation rate due to AEs in Japanese HCV genotype 1 patients aged ⩾ 75 years following SOF/LDV therapy.

Elderly patients in the present study were more likely to have advanced liver fibrosis than younger patients because of their lower platelet counts and higher Fib-4 index. This is consistent with a previous report showing that the prevalence of advanced fibrosis was higher in the elderly than in a younger population[17]. Only 32% of the HCV patients in our sample were over 75 years old, while Karino[5] found that over 50% of people with HCV in Japan are age 75 years or older, as mentioned above. Elderly patients accounts for the majority of those with advanced cirrhosis (Child-Pugh class B or C), and patients with this condition were excluded from the present analysis. It is suggested that this is the reason for the relatively low proportion of elderly patients (⩾ 75 years) compared with younger patients (< 75 years) in our study. Although advanced fibrosis was found to lower the SVR rate achieved by interferon-based therapy in patients with HCV genotype 1[18], SOF/LDV therapy resulted in similarly high SVR rates in cirrhotic and non-cirrhotic patients, both in a clinical trial[10] and in the real world[19-21]. Likewise, in our study the SVR rate was high irrespective of liver status.

Two of 246 patients in our study experienced virological relapse, one of whom had an NS5A RAV (L31M) at baseline. Although pre-existing NS5A and NS5B RAVs for HCV genotype 1b were shown to have a minimal influence on SVR rates following SOF/LDV therapy[22,23], Ogawa *et al*[24] reported that cirrhotic patients with pre-existing NS5A RAVs had significantly lower SVR12 rates than those without these RAVs at baseline. In the present study, one of the two relapsed patients had an NS5A RAV (L31M) and liver cirrhosis, which may have prevented the achievement of SVR12. However, the other had no NS5A RAVs or cirrhosis at baseline, so there were no common factors that were obviously associated with therapy failure.

Our study has several limitations. First, it used a retrospective design. Second, NS5A RAVs could not be tested in all patients and few patients failed to achieve SVR12, therefore we could not correlate NS5A RAVs with therapy failure. Further research including a large number of patients is necessary.

In conclusion, SOF/LDV therapy resulted in similarly high virological response and good tolerance in elderly and younger patients, and may therefore be effective and safe in patients aged ⩾ 75 years.

ARTICLE HIGHLIGHTS

Research background

The majority of Japanese patients with hepatitis C are elderly, however, elderly patients (⩾ 75 years) treated with interferon (IFN)-based therapies have poor sustained virological response (SVR) rates and high discontinuation rates due to AEs. As a result, it is critical that new anti-viral therapies be developed for elderly patients. The combination of sofosbuvir (SOF) and ledipasvir (LDV) was approved in Japan, and though this regimen has demonstrated high efficacy with an improved safety profile and shorter therapy duration than IFN-based therapies, there are few real-world studies of Japanese patients aged ⩾ 75 years.

Research motivation

Evaluating the efficacy and safety of SOF and LDV in elderly patients with hepatitis C genotype 1 will help clinicians assess whether they can treat these patients similarly to younger patients in the real-world.

Research objectives

To evaluate the efficacy and safety of SOF and LDV in Japanese elderly patients with hepatitis C genotype 1.

Research methods

Demographic, clinical, virological, and AE-related data obtained during and after SOF/LDV therapy were retrospectively collected from medical records.

Research results

The SVR rates at 12 wk after treatment were 99.2%, 99.4%, and 98.7% in the overall population and in patients aged < 75 and ⩾ 75 years, respectively. Common AEs occurred in fewer than 10% of patients, and their incidence was not significantly different between the younger and elderly groups.

Research conclusions

The present study demonstrated that patients aged ⩾ 75 years had a similar virological response and tolerance to SOF/LDV therapy compared with patients aged < 75 years in a real-world cohort. Therefore, SOF/LDV therapy might be effective and safe in elderly patients.

Research perspectives

Further prospective studies with large sample sizes are necessary.

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ORIGINAL ARTICLE

Retrospective Study

Women receive more inpatient resections and ablations for hepatocellular carcinoma than men

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Abstract

AIM

To evaluate disparities in the treatment of hepatocellular carcinoma (HCC) based on gender.

METHODS

A retrospective database analysis using the Nationwide Inpatient Sample (NIS) was performed between 2010 and 2013. Adult patients with a primary diagnosis of hepatocellular carcinoma determined by International Classification of Disease 9 (ICD-9) codes were included. Univariate analysis and multivariate logistic regressions were performed to analyze differences in treatment, mortality, features of decompensation, and metastatic disease based on the patient's gender.

RESULTS

The analysis included 62582 patients with 45908 men

and 16674 women. Women were less likely to present with decompensated liver disease (OR = 0.84, P < 0.001) and had less risk of inpatient mortality when compared to men (OR = 0.75, P < 0.001). Women were more likely to receive inpatient resection (OR = 1.31, P < 0.001) or an ablation (OR = 1.22, P = 0.028) than men. There was no significant difference between men and women in regard to liver transplantation and transcatheter arterial chemoembolization (TACE).

CONCLUSION

Gender impacts treatment for hepatocellular carcinoma. Women are more likely to undergo an ablation or resection then men. Gender disparities in transplantation have resolved.

Key words: Hepatocellular carcinoma; Gender disparities; Liver transplantation; Liver resection; Ablation

Core tip: Previous studies have evaluated treatment disparities in the treatment of hepatocellular carcinoma (HCC) based on gender. Despite recent emphasis to ensure equal care for all patients this study continues to show disparities in the treatment of HCC, specifically in resection and ablation. Gender disparities in the treatment of HCC with transplantation have resolved.

Sobotka L, Hinton A, Conteh L. Women receive more inpatient resections and ablations for hepatocellular carcinoma than men. *World J Hepatol* 2017; 9(36): 1346-1351 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i36/1346.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i36.1346

INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) in the United States is increasing. In 2016, it is estimated that more than 35000 people in the United States will be diagnosed[1]. The diagnosis has tripled since the 1980s. Men are three times as likely to be diagnosed with HCC as women[2]. Once diagnosed with HCC, survival rates are dependent on the stage with a 5 year survival of approximately 30.5 and metastatic HCC survival of 3.1[2].

There has been an emphasis on evaluating gender disparities in healthcare; HCC is not an exception. Gender disparities in the treatment for HCC have been noted in the past, specifically in transplantation. Studies reveal that men were more likely to receive a liver transplantation during pre-Model for End Stage Liver Disease (MELD) organ allotment, while women were more likely to die while waiting for organ transplantation[3]. Other studies have concluded that women were more likely to receive resection for earlier stage disease[4].

The aim of this study is to use the Nationwide Inpatient Sample (NIS) to determine if gender disparities still exist in the inpatient treatment for HCC.

We hypothesize that gender disparities continue to exist and seek to identify potential factors associated with this disparity.

MATERIALS AND METHODS

Data source

Data was obtained from the NIS, which is a component of the Healthcare Cost and Utilization Project (HCUP). This is the largest publically available database in the United States specifically designed to analyze data regarding hospital inpatient stays. Data is collected from over 1000 hospitals and represents more than 35 million discharges annually. The database contains clinical and research use information regarding primary and secondary diagnoses and procedures, patient demographics, length of stay, severity, and comorbidity measures[5].

Data was obtained between 2010 and 2013 and included patients 18 and older with a primary diagnosis of HCC using ICD-9 code of 155.0. This ICD-9 code has been utilized in other peer reviewed manuscripts[6].

Demographic information collected included age, gender, and race. Other evaluated information included risk factors of HCC, comorbidities, metastasis, and features of liver decompensation.

Degree of decompensation was characterized by the number of complications, including ascites, coagulopathy, esophageal varices, portal hypertension, encephalopathy, edema, and hepatorenal syndrome. Metastases were categorized as none, single, and greater than two sites. Comorbidities were evaluated using the Elixhauser Comorbidity Score which was modified to exclude liver disease and metastatic cancer[7].

Treatment was identified using ICD-9 codes and included transplantation, resection, ablation, and transarterial chemoemolization (TACE). If a patient did not receive treatment, the patient was listed as "noninvasive therapy." If a patient had multiple admissions in which treatment was performed, they were assigned to treatment group by their most invasive treatment.

The Ohio State University Data and Specimen Policy and Human Subjects Research Policy does not require Institutional Review Board approval for population-based public data sets. Per 45 Code of Federal Regulations (CFR 46.101), research using certain publicly available data sets does not involve "human subjects".

Statistical analysis

Associations between gender and factors of interest were evaluated using χ^2 tests. Multivariate regression models were fit for the presence of metastatic HCC, liver decompensation, mortality, and treatment. Terms included in each model were determined through backwards selection where hepatitis C, hepatitis B, alcohol, NASH, primary biliary cirrhosis, primary sclerosing cholangitis, autoimmune liver disease, features of liver

Table 1 Demographic and clinical parameters in patients with hepatocellular carcinoma grouped by gender between 2010 and 2013

	Male (*n* = 45908)		Female (*n* = 16674)		*P* value
	n	%	*n*	%	
Age (yr)					< 0.001
⩽ 64	28784	62.70	7847	47.06	
65-79	13683	29.81	6226	37.34	
⩾ 80	3441	7.50	2602	15.60	
Race					0.865
Caucasian	23845	51.94	8583	51.47	
African-American	7172	15.62	2554	15.32	
Hispanic	6572	14.32	2416	14.49	
Asian	3660	7.97	1316	7.89	
Others/unknown	4658	10.15	1806	10.83	
Primary payer					< 0.001
Medicare	18592	40.50	8803	52.79	
Medicaid	9198	20.04	2426	14.55	
Private insurance	12757	27.79	4139	24.82	
Self-pay	2771	6.04	695	4.17	
No charges	319	0.70	76	0.46	
Unknown/other	2270	4.95	535	3.21	
Geographic region					0.006
Northeast	10910	23.77	3643	21.85	
Midwest	7929	17.27	3311	19.86	
South	16808	36.61	5961	35.75	
West	10261	22.35	3759	22.54	
Hepatitis C	8449	18.40	2359	14.15	< 0.001
Hepatitis B	2839	6.18	580	3.48	< 0.001
Alcohol	9102	19.83	923	5.53	< 0.001
NASH	15935	34.71	6044	36.24	0.126
Primary sclerosing cholangitis	394	0.86	188	1.13	0.171
Primary biliary cirrhosis	51	0.11	123	0.74	< 0.001
Autoimmune	50	0.11	136	0.81	< 0.001
Other	17124	37.30	8542	51.23	< 0.001
Liver decompensation Features					< 0.001
Zero	24826	54.08	10538	63.20	
One	13348	29.08	4308	25.84	
Two	6126	13.34	1539	9.23	
Three or greater	1608	3.50	289	1.73	
Metastasis					0.627
None	38219	83.25	13992	83.92	
Single site	5954	12.97	2052	12.30	
Two or more sites	1735	3.78	630	3.78	
Elixhauser comorbidity Score					
< 3	22662	49.36	7982	47.87	0.141
⩾ 3	23246	50.64	8692	52.13	
Treatment options					
Transplant	1553	3.38	492	2.95	
Resection	4945	10.77	2551	15.30	
Ablation	2702	5.89	1103	6.62	
TACE	3701	8.06	1241	7.44	
Noninvasive treatment	33007	71.90	11288	67.70	

NASH: Non-alcoholic steatohepatitis; TACE: Transcatheter arterial chemoembolization.

decompensation, metastasis, treatment, and Elixhauser comorbidity were all eligible for inclusion, where appropriate. Analyses were performed using weighted data employing appropriate survey procedures to produce national estimates. Data was analyzed using SAS software (version 9.4 SAS Institute Inc. Cary, NC, United States).

RESULTS

Demographics

There were 62582 patients with a primary diagnosis of HCC included in the study (Table 1). The majority (45908; 73) of patients was male and Caucasian (52). The major identifiable insurance payer was Medicare (44).

Liver severity, evidence of metastasis of HCC and inpatient mortality

Women were more likely to present without evidence of decompensated disease than men ($P < 0.001$). There was no difference between genders in patients with metastatic disease. Women had a lower rate of inpatient mortality ($P < 0.001$) (Table 1).

On multivariate analysis, there was no significant

Table 2 Multivariate logistic regression comparing outcomes of hepatocellular carcinoma by gender

Outcome	Gender	OR	95%CI	*P* value
Metastatic hepatocellular carcinoma[1]	Male	1.00	0.84, 1.05	0.303
	Female	0.94		
Liver decompensation[2]	Male	1.00	0.77, 0.92	< 0.001
	Female	0.84		
Inpatient mortality[3]	Male	1.00	0.65, 0.87	< 0.001
	Female	0.75		

[1]Model is adjusted for age, primary payer, hepatitis C, alcohol, non-alcoholic steatohepatitis (NASH), liver decompensation features, and Elixhauser comorbidity score; [2]Model is adjusted for age, race, primary payer, geographic region, hepatitis C, alcohol, NASH, primary biliary cirrhosis, metastasis, and Elixhauser comorbidity score; [3]Model is adjusted for age, race, primary payer, hepatitis C, hepatitis B, alcohol, NASH, liver decompensation features, metastasis, and treatment.

Table 3 Multinomial logistic regression to evaluate gender disparities in treatment for hepatocellular carcinoma[1,2]

Treatment	Gender	OR	95%CI	*P* value
Liver transplant	Male	1.00	0.95, 1.50	0.132
	Female	1.19		
Resection	Male	1.00	1.15, 1.48	< 0.001
	Female	1.31		
Ablation	Male	1.00	1.02, 1.45	0.028
	Female	1.22		
TACE	Male	1.00	0.84, 1.16	0.841
	Female	0.98		

[1]Noninvasive treatment is treated as the reference category; [2]Model adjusts for age, race, primary payer, hepatitis C, hepatitis B, alcohol, NASH, primary sclerosing cholangitis, primary biliary cirrhosis, liver decompensation features, metastasis, and Elixhauser comorbidity score. NASH: Non-alcoholic steatohepatitis; TACE: Transcatheter arterial chemoembolization.

difference between rates of metastatic HCC in men *vs* women. Women were less likely to present with evidence of decompensated disease (OR = 0.84, $P < 0.001$). Women had a significantly smaller risk of inpatient mortality (OR = 0.75, $P < 0.001$) (Table 2).

Inpatient treatment of HCC

Women were more likely to receive a resection with 15 of women receiving this treatment compared to 11 of men. The gender disparity rate was to a lesser extent for the other treatments. However, 71 of the patients included in this study are listed as "noninvasive treatment" which includes patients that did not undergo transplant, resection, ablation, or TACE (Table 1).

On multivariate analysis, women were more like to have a resection (OR = 1.31, $P < 0.001$) and an ablation (OR = 1.22, $P = 0.028$). There were no significant differences between the rates of transplantation or TACE (Table 3).

DISCUSSION

This study shows gender differences for the inpatient management of HCC. Women are still more likely to undergo resection which is consistent with prior publications. This study also determined that women are more likely to undergo ablation. Women may be more likely to undergo these procedures because of functional status, compensated disease, and increased likelihood of undergoing screening exams that allow them to be diagnosed earlier. Despite advances in treatment of HCC, females are more likely to receive curative treatment with resection and ablation. It is important to recognize this difference and find ways to reduce it given that ablations and resections are associated with lower costs and decreased 30-d mortality.

Multiple factors predispose a patient to develop HCC, including cirrhosis of the liver, hepatitis B and C[8]. Screening for HCC consists of a liver ultrasound and serum alpha fetoprotein (AFP) every 6 mo. Once an abnormal screening exam is found, patients will undergo triple phase CT or MRI of the liver. If a nodule has imaging characteristics that are stereotypical for HCC, a diagnosis of HCC can be made and biopsy is not necessary. If the nodule is smaller, a biopsy can be performed to confirm diagnosis[9]. Once diagnosed with HCC, staging and treatment are determined. The Barcelona Liver Clinic Staging Classifications is widely used to determine treatment based on the size of the lesion. Early stage disease is defined as 1 to 3 nodules less than 3 cm; therefore, treatment with resection, liver transplantation, ablation, or TACE are more viable options and could be considered curative[10].

Women continue to receive certain curative treatments for HCC more frequently than men and there are multiple factors that likely contribute to this. Studies show that patients are more likely to undergo curative treatment if they present with compensated disease and good functional status. This study and previous studies have shown that women are more likely to present with

compensated liver disease than men. Previous studies have shown slower progression of disease making women more likely to receive curative treatment. Multiple theories support these findings including studies that show estrogen can prevent stellate cell activation which plays a major factor in developing underlying liver fibrinogenesis and women are less likely to have complications such as portal vein thrombosis and renal dysfunction that may prohibit them from undergoing curative treatment[11].

Patients who undergo regular screening for HCC are also more likely to be diagnosed with early stage disease *vs* metastatic disease and would be a better candidate for curative treatment. Studies have shown that women are more likely to follow stricter screening protocols than men which may allow earlier diagnosis of HCC when it is still at a size that is amendable to treatment with ablation or resection[12].

It is important to understand why this is relevant in daily practice. This difference in treatment can have a profound effect on healthcare costs, mortality, and rates of metastatic disease, which is crucial to recognize in a time of rapid increase in healthcare expenditures and increasing mortality rates in patients with HCC.

Women are presenting with more compensated disease and tumor size that is amendable to resection and ablation and are able to receive these interventions in a timelier manner compared to liver transplant. This could theoretically decrease the chances of developing metastatic disease, though this is not reflected in the data from this study.

Ablation and resection are curative treatments like a liver transplant; however, they have less of a financial burden on the medical system. United Network for Organ Sharing (UNOS) estimated that the average cost for a liver transplant in 2011 was $577100 with all other forms of treatment being less expensive[13]. It is important to recognize the factors that make women more likely to undergo these procedures and apply these across both genders in order to facilitate a quality driven and fiscally responsible healthcare system.

Mortality must also be considered a crucial factor when analyzing the importance of women receiving more ablation and resections than men. This study shows that women have a smaller risk of inpatient mortality; this may be partially due to women undergoing these less invasive procedures more frequently than men. The mortality rate is around 4[14] for liver resection and 1.5 for ablation[15]. For patients undergoing liver transplant, the mortality rate is greater and is estimated to be 7 to 17 30-d mortality rate[16]. Ablation and resection can also be curative; however, they have a decreased risk of 30-d mortality compared to transplant, and therefore should be considered an ideal for of treatment for both men and women.

This study does have limitations: the most important being the use of administrative data and the accuracy of ICD-9 CM coding. These codes could not be verified by medical chart given privacy issues and are susceptible to error. This study was completed using data obtained from an inpatient database and therefore does not include patients that may have received procedures as an outpatient. Size of tumor effects treatment, however the effect of tumor size on treatment could not be determined with the use of the NIS. Given this study uses administrative data, we are unable to determine MELD score or Childs Pugh Score and therefore used factors of liver decompensated to determine disease severity.

In conclusion, this study shows that a gender difference in the treatment of HCC continues to exist, specifically with resection and ablation. It is important to recognize this disparity and make an effort to reduce this given that interventions are associated with decreased financial burden and lower 30-d mortality rate. It is unclear why this disparity continues to exist, and further research should be completed to determine the cause and ways to reduce this difference between genders.

ARTICLE HIGHLIGHTS

Research background

Gender disparities have been noted in the treatment of hepatocellular carcinoma (HCC), specifically with liver transplantation.

Research motivation

There has been an emphasis on evaluating gender disparities in healthcare; HCC is not an exception. Gender disparities in the treatment for HCC have been noted in the past, specifically in transplantation. Studies reveal that men were more likely to receive a liver transplantation during pre-model for end stage liver disease (MELD) organ allotment, while women were more likely to die while waiting for organ transplantation. Other studies have concluded that women were more likely to receive resection for earlier stage disease.

Research objectives

The aim of this study is to determine if disparities continue to exist despite an emphasis to reduce disparities in healthcare.

Research methods

A retrospective database analysis utilizing the NIS was performed.

Research results

The authors determined that women are more likely to undergo an ablation or resection then men. Disparities in liver transplantation have resolved. Further research should be completed to determine ways to reduce gender disparities in hepatocellular carcinoma given the effect this has on patient mortality and healthcare cost.

Research conclusions

This study shows that a gender difference in the treatment of HCC continues to exist, specifically with resection and ablation. It is important to recognize this disparity and make an effort to reduce this given that interventions are associated with decreased financial burden and lower 30-d mortality rate.

Research perspectives

It is unclear why the previous disparity continues to exist, and further research should be completed to determine the cause and ways to reduce this difference between genders.

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ORIGINAL ARTICLE

Retrospective Study

Impact of sustained virologic response on chronic kidney disease progression in hepatitis C

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Abstract

AIM

To determine how sustained virological response at 12 wk (SVR12) with direct acting antivirals (DAAs) for the treatment of hepatitis C virus (HCV) infection affects chronic kidney disease (CKD) progression.

METHODS

A retrospective analysis was performed in patients aged ⩾ 18 years treated for HCV with DAAs at the VA Greater Los Angeles Healthcare System from

2014-2016. The treatment group was compared to patients with HCV from 2011-2013 who did not undergo HCV treatment, prior to the introduction of DAAs; the control group was matched to the study group in terms of age, gender, and ethnicity. Analysis of variance and co-variance was performed to compare means between SVR12 subgroups adjusting for co-variates.

RESULTS

Five hundred and twenty-three patients were evaluated. When comparing the rate of change in estimated glomerular filtration rate (eGFR) one-year after HCV treatment to one-year before treatment, patients who achieved SVR12 had a decline in GFR of 3.1 mL/min ± 0.75 mL/min per 1.73 m^2 compared to a decline in eGFR 11.0 mL/min ± 2.81 mL/min per 1.73 m^2 in patients who did not achieve SVR12 (P = 0.002). There were no significant clinical differences between patients who achieved SVR12 compared to those who did not in terms of cirrhosis, treatment course, treatment experience, CKD stage prior to treatment, diuretic use or other co-morbidities. The decline in eGFR in those with untreated HCV over 2 years was 2.8 mL/min ± 1.0 mL/min per 1.73 m^2, which was not significantly different from the eGFR decline noted in HCV-treated patients who achieved SVR12 (P = 0.43).

CONCLUSION

Patients who achieve SVR12 have a lesser decline in renal function, but viral eradication in itself may not be associated improvement in renal disease progression.

Key words: Hepatitis C; Direct-acting antivirals; Chronic kidney disease; End stage renal disease; Sustained virological response

Core tip: In hepatitis C patients treated with direct acting antivirals, there is a lesser decline in renal function in those who are treated and achieved sustained virological response at 12 wk (SVR12) compared to those who do not achieve SVR12. However, the decline in renal function is no different between those who achieve SVR12 and those who are never treated. This suggests that viral eradication may not be associated improvement in the progression of renal disease and other factors, such as cryoglobulinemia, may be implicated in renal disease progression.

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INTRODUCTION

Hepatitis C virus (HCV) is a significant public health issue that affects around 3 million individuals in the United States[1]. The prevalence of chronic HCV infection in veterans affairs (VA) healthcare users is more than 2-fold higher than the general United States population, thus being the nation's largest provider for HCV care[2,3].

The consequences of HCV infection extend beyond the liver, including renal complications such as membranoproliferative glomerulonephritis (MPGN) in the setting of cryoglobulinemia[4]. Patients with HCV were found to have a five-fold increase in the odds of developing MPGN compared with individuals who were not infected[5]. Chronic HCV infection has also been associated with reductions in glomerular filtration rate (GFR) < 60 mL/min per 1.73 m^2, development of end-stage renal disease (ESRD), and a rapid decline in renal function[4,6-9]. Interestingly, the duration of chronic HCV infection influences the risk of developing chronic kidney disease (CKD)[10]. Previous systematic reviews suggest a relationship between HCV infection and higher incidence of low estimated GFR (eGFR)[11]. In a meta-analysis of nearly 3 million individuals, chronic HCV infection predicted a 51% increase in the risk of proteinuria and a 43% increase in the incidence of CKD[11]. CKD is an important public health problem as it increases the likelihood of adverse outcomes and is associated with high healthcare costs[12].

Given that HCV infection is associated with CKD progression, the aim of our study was to determine if the achievement of sustained virological response at 12 wk (SVR12) with interferon-free, direct acting antivirals (DAAs) impacts the progression of CKD. We hypothesize that viral eradication would result in a reduction in CKD progression. No previous study has rigorously investigated whether eradication of HCV infection with newer DAA therapies is associated with improved renal function.

MATERIALS AND METHODS

Data source and study population: The VA Greater Los Angeles Healthcare System (VAGLAHS) institutional review board approved this study. Data were abstracted using the Corporate Data Warehouse, a national repository of patient data, for all patients evaluated at the VAGLAHS. A retrospective medical records review was performed by reviewing those patients over 18 years of age who initiated hepatitis C treatment with interferon-free DAAs from January 1st, 2014 to June 1st, 2016. The control group consisted of patients over 18 years of age who did not undergo hepatitis C treatment from January 1st, 2011 to January 1st, 2013, prior to the introduction of DAAs; the control group was matched to the study group in terms of age, gender and ethnicity.

Baseline patient characteristics

Demographic data, including age, gender, body mass index (BMI) and ethnicity, were obtained at the initial visit. Baseline laboratory data were collected at the time of initial visit. Serum creatinine and estimated

GFR was collected yearly for two consecutive years before and one year after treatment. Patients were excluded if there was incomplete kidney function data one year after treatment. Patients were also excluded if they were lost to follow-up or died within 1 year of treatment. The diagnoses of comorbidities were based on International Classification of Disease, Ninth Revision and/or Tenth Revision, Clinical Modification (ICD-9 CM/ICD-10 CM) and use of anti-hypertensive or diabetes medications. The ICD-9/ICD-10 codes that were used were 250.00-250.93/E08-E13 for diabetes mellitus and 401.0, 401.1 and 401.9/I10 for essential hypertension. Cirrhosis and diuretic use were determined through chart review of hepatology provider notes. Patients receiving hemodialysis therapy were excluded. HCV patients were identified by ICD-9/ICD-10 coding, 070.0-0.70.1/B18.2 and B19.2. The primary outcome of our study was SVR12, which was defined as an undetectable HCV RNA (< 15 IU/mL) 8, 12 wk or beyond the conclusion of treatment[13].

Statistical analysis

Patient characteristics were measured by continuous and categorical variables. The baseline stage of kidney disease was measured with the GFR at the time of treatment as defined by the National Kidney Foundation Kidney Disease Outcomes Quality Initiative[14]. The mean values of baseline characteristic were analyzed using student's *t*-test. Proportions were compared using χ^2 test. Medians were compared using the Wilcoxon rank-sum test. The mean GFR of the control group and the treatment group was tested for a normal distribution by using a kernel density estimation. Mean GFR between groups were compared using analysis of variance. Analyzed covariates included gender, age by tertile, ethnicity, treatment experience, HCV genotype, treatment regimen, baseline kidney disease, diuretic use, and the presence of such comorbidities as obesity, hypertension, diabetes, heart failure (CHF), coronary artery disease (CAD), and peripheral artery disease (PAD). In addition, the change in eGFR from 1-year prior to DAA initiation was calculated and compared to the change in eGFR between DAA initiation and 1-year post-DAAs; a paired *t*-test was performed. A *P* value of < 0.05 was considered as significant. Data analysis was done using STATA® v14.2.

RESULTS

A total of 523 patients met inclusion criteria for the study. Baseline characteristics of the cohort are presented in Table 1. The majority of patients were white males with a mean age of 62.7 (SE ± 0.3) years. A total of 48.6% had cirrhosis and 22.4% were treatment-experienced. Thirty-two percent had diabetes, 68.5% had hypertension, 10.1% had CAD, 4.2% had CHF and 2.9% had been diagnosed with PAD. The most common genotype was genotype 1a (53.2%) followed by genotype 1b (28.1%). The most common HCV treatment regimen was a combination of ledipasvir with sofosbuvir followed by sofosbuvir plus ribavirin. The majority of patients were CKD stages 1 or 2 prior to HCV treatment.

Within the treated groups, there was a significant difference in age between patients who achieved SVR12 compared to those who did not, with the group who achieved SVR12 being slightly older (*P* = 0.02). There were no other significant clinical differences between patients who achieved SVR12 compared to those who did not in terms of gender, ethnicity, cirrhosis, treatment course, treatment experience, CKD stage prior to treatment, diuretic use or other co-morbidities.

The control group consisted of 439 patients who were not treated for HCV and followed from January 1st, 2011 to January 1st, 2013. These patients were not treated for HCV given that DAAs were not available at VAGALHS during that time period. Baseline characteristics of the study population and control groups are shown in Table 2. The control group was matched to the treatment group by age, gender, and ethnicity. The control group was not statistically different from the cohort of HCV treated patients in terms of age, gender, ethnicity, HCV genotype, diabetes, CAD, PAD, CKD stage prior to treatment, and diuretic use. The median MELD score (interquartile range) for the cirrhotic patients at baseline was 8.4 (7.49-9.72) in the treatment group compared to 7.7 (6.43-9.16) in the control group that did not undergo treatment; there were no significant differences in MELD score between groups (*P* = 0.19). There were significantly more patients with cirrhosis and obesity (BMI > 30 kg/m^2) in the cohort who underwent HCV treatment compared to the control group (*P* = 0.001, 0.005 respectively). The control group, however, had significantly more patients with hypertension and CHF compared to the cohort who underwent HCV treatment (*P* = 0.02, 0.001 respectively).

When comparing the rate of change in eGFR one-year after HCV treatment compared to one-year before treatment, patients who achieved SVR12 had a decline in GFR of 3.1 mL/min ± 0.75 mL/min per 1.73 m^2 compared to a decline in eGFR 11.0 mL/min ± 2.81 mL/min per 1.73 m^2 in patients who did not achieve SVR12 (*P* = 0.002; Figure 1). In those who achieved SVR12, the change in eGFR 1-year prior to treatment was -6.2 mL/min ± 1.06 mL/min per 1.73 m^2 compared to -1.8 mL/min ± 0.75 mL/min per 1.73 m^2 in the year following DAA therapy; those who achieved SVR12 had a lesser decline in renal function following DAA treatment (*P* = 0.002). In those who were treated with DAAs but did not achieve SVR12, the change in eGFR 1-year prior to treatment was -5.4 mL/min ± 2.79 mL/min per 1.73 m^2 compared to -7.42 mL/min ± 2.2 mL/min per 1.73 m^2 in the year following DAA therapy (*P* = 0.62). In the control group, the decline in eGFR over two years was 2.8 mL/min ± 1.0 mL/min per 1.73 m^2. This decline in eGFR in untreated patients over two years was not significantly different from the eGFR decline noted in patients who achieved SVR12 after

Table 1 Baseline characteristics for patients undergoing treatment for hepatitis C virus at the West Los Angeles Veterans Administration

	All patients (*n* = 523)	SVR12 not achieved (*n* = 38)	SVR12 achieved (*n* = 485)	*P* value
Age (mean, yr) (SE)	62.7 (0.3)	60.5 (1.1)	63.0 (0.3)	0.02[a]
Gender (%)				
Male (*n* = 512)	97.9	94.7	97.9	0.81
Female (*n* = 11)	2.1	5.3	2.1	
Ethnicity (%)				
White (*n* = 278)	53.2	52.6	56.3	0.78
Black or African American (*n* = 174)	33.3	34.3	33.2	
American Indian or Alaska Native (*n* = 11)	2.1	2.6	2.1	
Asian (*n* = 4)	0.8	2.6	0.6	
Native Hawaiian or other pacific islander (*n* = 5)	1.0	0.0	1.0	
Unknown/declined to answer (*n* = 51)	9.8	7.9	9.9	
Cirrhosis (%)				
Non-cirrhotic (*n* = 269)	51.4	47.2	51.7	0.31
Cirrhosis (*n* = 254)	48.6	52.8	48.3	
Treatment experience (%)				
Treatment naive (*n* = 406)	77.6	73.7	77.9	0.54
Treatment experienced (*n* = 117)	22.4	26.3	22.1	
HCV genotype (%)				
HCV genotype 1a (*n* = 278)	53.2	50.7	53.3	0.44
HCV genotype 1b (*n* = 147)	28.1	22.2	28.6	
HCV genotype 2 (*n* = 48)	9.2	12.6	8.9	
HCV genotype 3 (*n* = 40)	7.6	17.1	6.9	
HCV genotype 4 (*n* = 6)	1.1	0.0	1.2	
HCV genotype 6 (*n* = 1)	0.2	0.0	0.2	
Combination (*n* = 3)	0.6	0.0	0.6	
Treatment (%)				
Dasabuvir, ombitasvir, paritaprevir and ritonavir (*n* = 104)	19.9	23.7	19.6	0.68
Ledipasvir and sofosbuvir (*n* = 200)	38.2	44.7	37.7	
Simeprevir (*n* = 55)	10.5	5.3	10.9	
Sofosbuvir + Ribavirin (*n* = 164)	31.4	26.3	31.8	
Obesity (%)				
BMI < 30 (*n* = 284)	54.3	63.5	53.6	0.25
Obese (*n* = 239)	45.7	36.5	46.4	
Hypertension (%)				
No hypertension (*n* = 177)	33.8	47.5	32.8	0.07
Hypertension (*n* = 346)	68.5	52.5	67.2	
Diabetes (%)				
No diabetes (*n* = 358)	31.5	73.5	68.1	0.47
Diabetes (*n* = 165)	30.9	26.5	31.9	
Congestive heart failure (%)				
No congestive heart failure (*n* = 501)	95.8	97.4	95.7	0.2
Congestive heart failure (*n* = 22)	4.2	2.6	4.3	
Coronary artery disease (%)				
No coronary artery disease (*n* = 469)	89.7	94.7	89.3	0.12
Coronary artery disease (*n* = 53)	10.1	2.6	10.7	
Peripheral arterial disease (%)				
No peripheral arterial disease (*n* = 508)	97.1	94.7	97.2	0.99
Peripheral arterial disease (*n* = 15)	2.9	5.3	2.7	
Baseline CKD before treatment (%)				
Stage 1 CKD (*n* = 263)	50.3	60.5	49.5	0.24
Stage 2 CKD (*n* = 218)	41.7	39.5	41.9	
Stage 3 CKD (*n* = 41)	7.8	0.0	8.5	
Stage 4 CKD (*n* = 1)	0.2	0.0	0.2	
Diuretic use (%)				
No diuretic use (*n* = 367)	70.2	71.1	70.1	0.9
Diuretic use (*n* = 156)	29.8	28.9	29.9	

[a]Significant *P*-value, $P < 0.05$. CKD: Chronic kidney disease; HCV: Hepatitis C virus; SVR12: Sustained virological response at 12 wk following therapy.

HCV treatment ($P = 0.43$).

Figure 2 demonstrates the rate of change in eGFR one-year after HCV treatment compared to one-year before treatment stratified by genotype. In patients with genotype 1a and 1b, there was less of a decline in eGFR between one-year before HCV treatment compared to one-year after treatment in patients who achieved SVR12 compared to those who did not ($P = 0.02$). There was no significant difference in eGFR decline between patients who achieved SVR12 and

Table 2 Baseline characteristics for patients undergoing treatment for hepatitis C virus at the West Los Angeles Veterans Administration compared to patients with hepatitis C virus who did not undergo treatment

	All patients (n = 523)	Control patients (n = 439)	P value
Age (mean, yr) (SE)	62.8 (0.3)	63.2 (0.3)	0.13
Gender (%)			
Male	97.9	98.1	0.75
Female	2.1	1.9	
Ethnicity (%)			
White	53.2	55.4	0.3
Black or African American	33.3	35.1	
American Indian or Alaska Native	2.1	2.5	
Asian	0.8	0.5	
Native Hawaiian or other pacific islander	1.0	0.9	
Unknown/declined to answer	9.8	5.7	
Cirrhosis (%)			
Non-cirrhotic	51.4	72.0	0.001[a]
Cirrhosis	48.6	28.0	
HCV genotype (%)			
HCV genotype 1a	53.2	55.9	0.31
HCV genotype 1b	28.1	22.8	
HCV genotype 2	9.2	11.8	
HCV genotype 3	7.6	6.7	
HCV genotype 4	1.1	1.7	
HCV genotype 6	0.2	0.0	
Obesity (%)			
BMI < 30	54.3	63.3	0.005[a]
Obese	45.7	36.7	
Hypertension (%)			
No hypertension	33.8	26.7	0.02[a]
Hypertension	68.5	73.3	
Diabetes (%)			
No diabetes	68.5	64.0	0.15
Diabetes	31.5	36.0	
Congestive heart failure (%)			
No congestive heart failure	95.8	90.4	0.001[a]
Congestive heart failure	4.2	9.6	
Coronary artery disease (%)			
No coronary artery disease	89.7	88.4	0.46
Coronary artery disease	10.1	11.6	
Peripheral arterial disease (%)			
No peripheral arterial disease	97.1	95.2	0.09
Peripheral arterial disease	2.9	4.8	
Baseline CKD before treatment (%)			
Stage 1 CKD	50.3	47.1	0.56
Stage 2 CKD	41.7	37.8	
Stage 3 CKD	7.8	5.4	
Stage 4 CKD	0.2	0.4	
Diuretic use (%)			
No diuretic use	70.2	67.2	0.3
Diuretic use	29.8	32.8	

[a]Significant P-value, $P < 0.05$. CKD: Chronic kidney disease; HCV: Hepatitis C virus; BMI: Body mass index.

those who did not in genotypes 2 (n = 48) and 3 (n = 40)

Figure 3 shows the rate of change in eGFR one-year after HCV treatment compared to one-year before treatment separated out by treatment type. In patients treated with dasabuvir, ombitasvir, paritaprevir, and ritonavir and ledipasvir/sofosbuvir, there was less of a decline in eGFR between one-year before HCV treatment compared to one-year after treatment in patients who achieved SVR12 compared to those who did not (P = 0.005). In patients treated with sofosbuvir, there was not statistically significant difference in the rate of change in eGFR between those achieved SVR12 compared to those who did not (P = 0.68) although the decline in eGFR was less in those who achieved SVR12.

DISCUSSION

In this single-center cohort of Veterans, we demonstrate that patients who achieved SVR12 with interferon-free DAAs had a reduced progression of renal disease that was statistically significant compared to patients who did not achieve SVR12. However, there were no significant differences in renal function decline between patients who were not treated with DAAs compared to

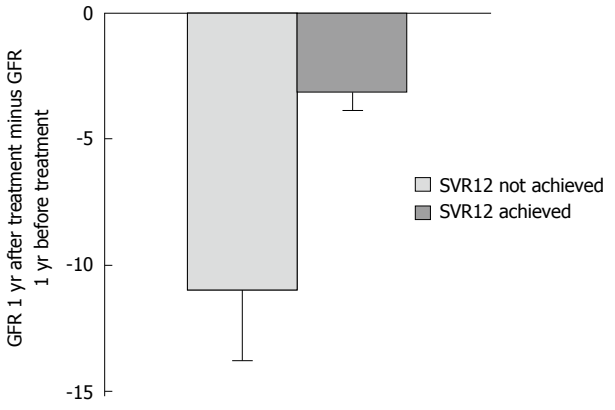

Figure 1 Rate of change in glomerular filtration rate one-year after hepatitis C virus treatment compared to one-year before treatment in relation to achievement of sustained virological response at 12 wk (*P* = 0.002). GFR: Glomerular filtration rate; SVR12: Sustained virological response at 12 wk following therapy.

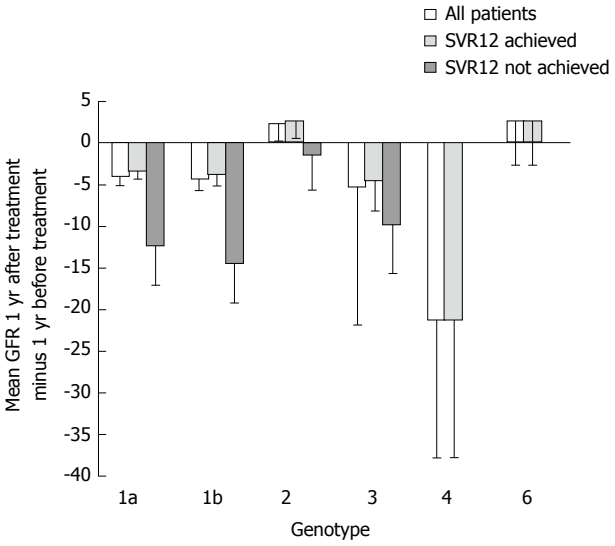

Figure 2 Rate of change in glomerular filtration rate one-year after hepatitis C virus treatment compared to one-year before treatment in relation to achievement of sustained virological response at 12 wk separated by genotype. GFR: Glomerular filtration rate; SVR12: Sustained virological response at 12 wk following therapy.

those who were treated and achieved SVR12.

While there appears to be an association between HCV infection and progression CKD, the mechanism of HCV-induced kidney injury continues to be debated. One hypothesis is that HCV triggers immune and inflammatory responses locally, within vascular tissues, or potentially systemically through inflammatory mediators, causing atherothrombosis and thus progression of CKD[11]. Immune complex deposition with HCV proteins and anti-HCV antibodies may provoke kidney injury[15]. HCV RNA and related proteins have been found in mesangial cells and the existence of these HCV-related proteins in the mesangium is associated higher proteinuria, which may suggest HCV infection causes direct mesangial injury[16]. Another thought is that HCV seropositive status induces accelerated

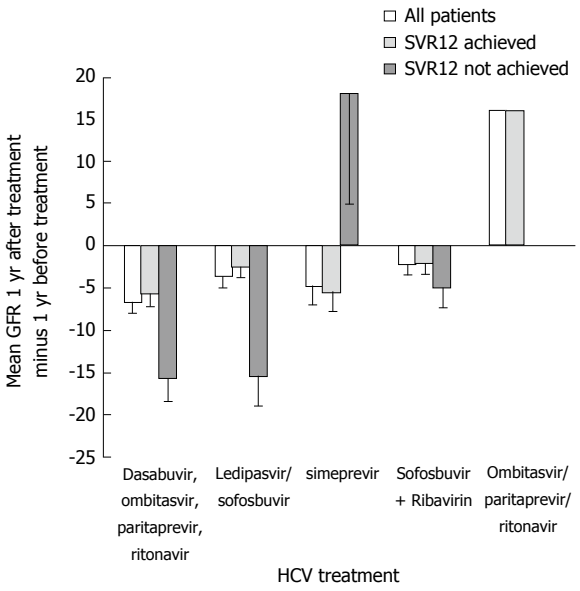

Figure 3 Rate of change in glomerular filtration rate one-year after hepatitis C virus treatment compared to one-year before treatment in relation to achievement of sustained virological response at 12 wk separated by hepatitis C virus treatment type. GFR: Glomerular filtration rate; SVR12: Sustained virological response at 12 wk following therapy; HCV: Hepatitis C virus.

atheromatous disease at the kidney level[11]. There is also clinical and laboratory evidence that suggests that HCV infection may be associated with insulin resistance and susceptibility to diabetes, which may lead to endothelial dysfunction and oxidative stress[11,17,18].

There was no significant difference in renal function decline between those who were treated for HCV and achieved SVR12 and those who were not treated for HCV. These results are similar to previous studies. A meta-analysis looking at the effect of antiviral therapy on HCV-associated CKD showed that HCV RNA clearance with interferon based therapy was not associated with a decrease in serum creatinine in the group that achieved SVR12 compared to the group that did not[19]. However, those who achieved SVR12 did have a decrease in protein excretion[19]. There was inadequate data on proteinuria, given the retrospective design and given proteinuria is infrequently ordered by physicians at our center, thus we were unable to determine the impact of SVR12 on proteinuria.

The fact that there were no significant differences in renal function decline between patients who were not treated with DAAs compared to those who were treated and achieved SVR12, suggests that viral eradication may not be associated improvement in the progression of renal disease. In patients with MPGN and type Ⅱ cryoglobulinemia, there may be virological clearance with DAA therapy, but there may be persistence of cryoglobulinemia, which may lead to persistent renal decline. Circulating cryoglobulins are detected in a large number of patients with HCV, however, only a minority of patients will experience clinical manifestations, thus some cases of cryoglobulinemia may remain undetected[20]. A recent study by Emery *et al*[21], showed

that despite high SVR rates after DAA treatment in patients with HCV associated mixed cryoglobulinemia only 29.4% of symptomatic patients had complete cryoprecipitate clearance despite achievement of SVR12. Work by Gragnani *et al*[22] showed a 100% SVR12 rate, however reported that only 34% of patients had full complete response, defined as disappearance of all the baseline symptoms, with follow-up to 24 wk. However, a recent case series suggests that in patients with HCV and mixed cryoglobulinemia syndrome treated with DAAs that there is an improvement in renal function, even in patients not concomitantly treated with immunosuppression[23].

Another explanation as to why achievement of SVR12 may not improve renal disease progression is that patients may have intrinsic renal disease prior to treatment, such as MPGN, and these patients will have CKD progression despite achieving SVR12; this has been previously described in the literature in case reports[24]. However, other reports have suggested that DAA therapy can result in successful treatment of HCV-associated MPGN with improvement in creatinine and proteinuria[25]. Furthermore, the patient population studied was unique - it is comprised of Veterans who are predominantly male, older in age, have a higher prevalence of CKD compared to the general population, and often have significant co-morbidities associated with CKD, such as diabetes mellitus, hypertension, vascular disease, and cancer[26]. Given the high prevalence of CKD and associated co-morbidities in this veteran population, CKD progression may have occurred despite SVR12 given the other presence of co-morbidities that drive CKD progression.

An alternative explanation could also be that although HCV clearance may have renal sparing effects, there many be a component of direct nephrotoxicity due to DAA therapy. In patients treated with Viekira or ledipasvir and sofosbuvir, there was a greater decline in eGFR in those who did not achieve SVR12 compared to those who achieved SVR12. However, the sample sizes for each treatment group are too small to make any definitive conclusions. Previous treatment with interferon-based therapy was associated with acute kidney injury, however kidney injury has not been attributed to any DAA therapy[27]. Sofosbuvir's circulating metabolite GS-331007 is renally cleared, thus there is a concern of Sofosbuvir use in patients with eGFR < 30 mL/min, but further work is needed to investigate the cases of kidney injury in patients following Sofosbuvir treatment[28].

There is a greater decline in renal function in those who were treated with DAAs and did not achieve SVR12 compared to those who were never treated. However, the group that did not achieve SVR12 following treatment had a greater proportion of cirrhotic patients when compared to the control group who did not undergo treatment. Given that the group who did not achieve SVR12 had a greater proportion of patients with cirrhosis, this group may have been more ill and thus had a higher propensity to undergo complications, such as hepatorenal syndrome, which may contribute to worsening renal function.

Our study has a number of limitations. First, this is a single center study restricted to Veteran health care users; therefore, the results may not be generalizable to non-Veteran populations, given the higher prevalence of baseline CKD and only a few women. Its retrospective nature may result in bias due to confounding variables, including unmeasured patient characteristics. The Modification of Diet in Renal Disease (MDRD) equation used to estimate GFR might be less accurate among patients in hepatitis C and cirrhosis because of abnormalities in protein metabolism as well as muscle wasting. In patients with cirrhosis, serum creatinine is a poor measure of GFR, however it is often used as a surrogate marker[29,30]. Finally, our follow-up time was short due to the recent introduction of DAAs. For our treatment cohort, there was not enough eGFR data two years following treatment, thus we were only able to evaluate eGFR changes one year following treatment. It is possible that the strength and degree of the associations described in the study might differ if the follow up period was extended.

Our study may have implications for clinical practice. Clinicians may be prompted to discuss the need for ESRD surveillance in their patients with HCV prior to treatment with DAAs. The current KDIGO guidelines suggest the patients with HCV be tested annually for proteinuria and eGFR, however the guideline is rated weak given it is based on expert judgment[31]. Given the lack of strong guidelines, it is likely that patients with HCV are not being screened for ESRD.

In summary, we found that there was a lesser decline in renal function in patients who achieved SVR12 compared to those who did not, however there were no significant differences in renal function decline between patients who were not treated compared to those who were treated and achieved SVR12. Additional research is needed to confirm these results in multi-institutional studies with longer duration of follow-up. Further work is required to develop screening guidelines for kidney disease in patients with HCV.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C virus (HCV) is a significant public health issue in the United States and worldwide. The consequences of HCV infection extend beyond the liver, including renal complications. Patients with HCV are at risk for renal function decline and developing end-stage renal disease (ESRD). Chronic kidney disease (CKD) is an important public health problem as it increases the likelihood of adverse outcomes and is associated with high healthcare costs.

Research motivation

Given HCV infection places patients at risk for renal function decline and developing ESRD, it is valuable to understand how the clearance of HCV infection with interferon free, direct acting antiviral (DAA) therapy affects chronic kidney progression. Given the recent introduction of DAA therapy, the impact of HCV clearance on kidney disease has not been fully established.

Research objectives

The authors' principal aim was to determine if the achievement of sustained virological response at 12 wk (SVR12) with interferon-free, DAAs impacts the progression of CKD.

Research methods

The authors retrospectively analyzed medical records of adult patients who initiated hepatitis C treatment with interferon-free DAAs from 2014 to 2016 at the VA Greater Los Angeles Healthcare System. The control group consisted of adult patients who did not undergo hepatitis C treatment, prior to the introduction of DAAs, from 2011 to 2013. Baseline demographic and clinical data were collected. The rate of change in estimated glomerular filtration rate (eGFR) one-year after HCV treatment compared to one-year before treatment was compared between patients who achieved SVR12 to those who did not. The change in eGFR was recorded over two years in patients who did not undergo treatment and compared to those who underwent DAA treatment.

Research results

The findings of the analysis suggest that patients who achieved SVR12 with interferon-free DAAs had a reduced progression of renal disease that was statistically significant compared to patients who did not achieve SVR12. However, there were no significant differences in renal function decline between patients who were not treated with DAAs compared to those who were treated and achieved SVR12. The control group was not statistically different from the cohort of HCV treated patients, except that the there were significantly more patients with cirrhosis and obesity in the cohort who underwent HCV treatment compared to the control group. The control group, however, had significantly more patients with hypertension and congestive heart failure compared to the cohort who underwent HCV treatment.

Research conclusions

There is a lesser decline in renal function in patients who achieved SVR12 compared to those who did not, however there were no significant differences in renal function decline between patients who were not treated compared to those who achieved SVR12. There are several possible explanations for the lack of improvement of CKD progression with viral eradication, such as immune factors related to cyroglobulins, intrinsic renal disease prior to therapy, and that the control group had significantly more patients with cirrhosis compared to the treatment group.

Research perspectives

Additional research is needed to confirm these results in multi-institutional studies with longer duration of follow-up.

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CASE REPORT

De-novo hepatocellular carcinoma after pediatric living donor liver transplantation

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Abstract

De-novo malignancies carry an incidence ranging between 3%-26% after transplant and account for the second highest cause of post-transplant mortality behind cardiovascular disease. While the majority of *de-novo* malignancies after transplant usually consist

of skin cancers, there has been an increasing rate of solid tumor cancers over the last 15 years. Although, recurrence of hepatocellular carcinoma (HCC) is well understood among patients transplanted for HCC, there are increasing reports of *de-novo* HCC in those transplanted for a non-HCC indication. The proposed pathophysiology for these cases has been mainly connected to the presence of advanced graft fibrosis or cirrhosis and always associated with the presence of hepatitis B or C virus. We report the first known case of *de-novo* HCC in a recipient, 14 years after a pediatric living related donor liver transplantation for end-stage liver disease due to biliary atresia without the presence of hepatitis B or C virus before and after transplant. We present this case report to increase the awareness of this phenomenon and address on the utility for screening and surveillance of hepatocellular carcinoma among these individuals. One recommendation is to use similar guidelines for screening, diagnosis, and treatment for HCC as those used for primary HCC in the pre-transplant patient, focusing on those recipients who have advanced fibrosis in the allograft, regardless of etiology.

Key words: Liver transplantation; *De-novo* hepatocellular carcinoma; Living donor liver transplantation; Biliary atresia

Core tip: *De-novo* hepatocellular carcinoma (HCC) is a rare event compared to other *de-novo* malignancies, although the number of reported cases are increasing. The pathophysiology has been related with advanced graft fibrosis, cirrhosis and hepatitis viral serology. We report the first case of *De-novo* HCC 14 years after living related donor liver transplantation for end-stage liver disease due to biliary atresia without positive hepatitis B or C viral serology. Current screening and treatment guidelines have not been well established. This increasing phenomenon challenges us to define the utility of screening and surveillance for hepatocellular carcinoma in these individuals.

Torres-Landa S, Munoz-Abraham AS, Fortune BE, Gurung A, Pollak J, Emre SH, Rodriguez-Davalos MI, Schilsky ML. *De-novo* hepatocellular carcinoma after living donor liver transplantation in a patient with biliary atresia. *World J Hepatol* 2017; 9(36): 1361-1366 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i36/1361.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i36.1361

INTRODUCTION

Hepatocellular carcinoma (HCC), the most common primary malignancy of the liver, is one of the most lethal and prevalent cancers worldwide. However, the use of liver transplantation (LT) is a well proven treatment approach for patients with low stage tumor. The pathogenesis of HCC typically involves chronic liver injury with regeneration, fibrosis and cirrhosis leading to dysplasia within regenerating nodules with an end result of malignancy[1]. HCC development in a liver allograft occurs most often in the setting of prior HCC where it is defined as recurrence[2]. *De-novo* tumor formation that arises in the transplanted graft without evidence of tumor in the previously explanted liver is uncommon and is mainly seen in patients with advanced graft fibrosis or cirrhosis and associated with the presence of hepatitis B or C viral infection[3]. The literature reveals only 15 documented cases of *de-novo* HCC after LT[4-15]. We report the first case of *de-novo* HCC occurring 14 years after a pediatric patient received a living related donor LT for end stage liver disease secondary to biliary atresia.

CASE REPORT

A 29-year-old male with a history of biliary atresia with failed Kasai procedure complicated with progressive cirrhosis and portal hypertension that received a left lateral segment living donor liver transplant (LDLT) from his biological father at 15 years of age. His immunosuppressive regimen included tacrolimus, and sirolimus. Eleven years after his LDLT, he developed advanced liver fibrosis and portal hypertension that manifested as refractory ascites. He received a splenectomy and a central spleno-renal shunt that eventually failed. He then underwent a side-to-side porto-caval shunt (PCS) at age 27 years. After 2 years with controlled disease, he presented with recurrent ascites and overt hepatic encephalopathy (HE) related to his progressive graft failure. His clinical course was also complicated by severe protein losing enteropathy due to his worsening portal hypertension (sprue was excluded by small bowel biopsy). Other causes of hypoalbuminemia were ruled out (*e.g.,* kidney injury secondary sirolimus, as evidenced by 24-h urine collection with minimal protein and normal creatinine). Liver biopsy at this time showed stage 3-4 fibrosis. In addition, there was a paucity of interlobular bile ducts with degenerative changes in the remaining ducts, features compatible with chronic allograft rejection. A few months after, an abdominal ultrasound of the graft revealed a hepatic mass measuring 2.9 cm × 2.2 cm located in segment 2/3 and no evidence of intrahepatic duct dilation. Dynamic CT imaging showed a 3 cm lesion in the left lateral segment that was slightly hypodense and indeterminate in nature (Figure 1). A dynamic magnetic resonance imaging (MRI) using liver mass protocol was performed due to the indeterminate nature of the lesion on CT and demonstrated increased vascularity of the lesion, raising suspicion for HCC. An ultrasound-guided percutaneous biopsy of the mass revealed a well-differentiated HCC (Figure 2). Chest CT scan and bone scan demonstrated no evidence of extra hepatic disease. Alpha-fetoprotein (AFP) level was slightly elevated at 17 ng/mL (normal < 6 ng/mL).

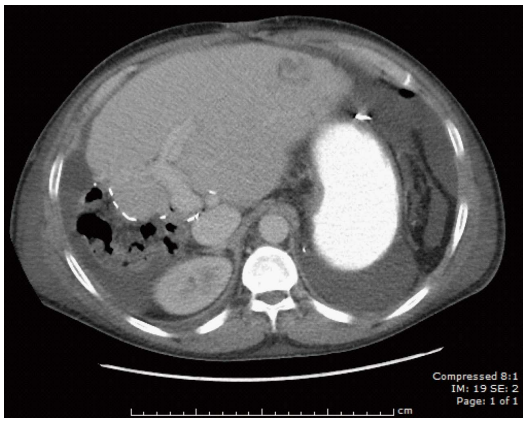

Figure 1 Dynamic computerized tomography scan imaging showing indeterminate 3 cm lesion in the left lateral segment.

Anti-HBcAb (anti-hepatitis B core total antibodies) at this time and a year before showed negative results. The lesion was subsequently treated by percutaneous microwave ablation (PMWA).

Follow up MRI was performed 1.5 mo after the ablation and showed no residual tumor (Figure 3). The patient was subsequently listed for repeat liver transplantation. However, while on the wait-list he developed a second post-transplant malignancy, an EBV negative Burkitt's type lymphoma. He received chemotherapy for the lymphoma but succumbed to complications due to the treatment that was in part limited by his advanced liver disease.

DISCUSSION

Liver transplantation provides the highest survival rates among patients with decompensated cirrhosis and complications of portal hypertension but recipients have a 2-4 fold increased risk of developing *de-novo* malignancies when compared to matched healthy controls[16]. *De-novo* malignancies represent 30% of post-transplant deaths and one of the most common causes of death in patients that survive beyond a year after transplantation[3,17]. Although there is an increased risk of developing malignancies, *De-novo* HCC is uncommon[3,17].

When HCC after transplantation is identified, it is crucial to know if it is a recurrent malignancy as this carries a poor prognosis. Travesani *et al*[17] have proposed an algorithm where they suggest suspicion primary features of a recurrent case including lymph node invasion, macro and microvascular invasion, tumor size > 5 cm, high grade tumor, bi-lobar involvement and high alpha fetoprotein levels. Secondary features include early occurrence, < 2 years, and extra hepatic localization. Without the presence of these characteristics, the suspicion turns towards a *de-novo* HCC. Other common clinical factors that suggest a *de-novo* case, even in patients transplanted for or with a previous HCC, are older donor age, alcoholic liver disease, viral hepatitis,

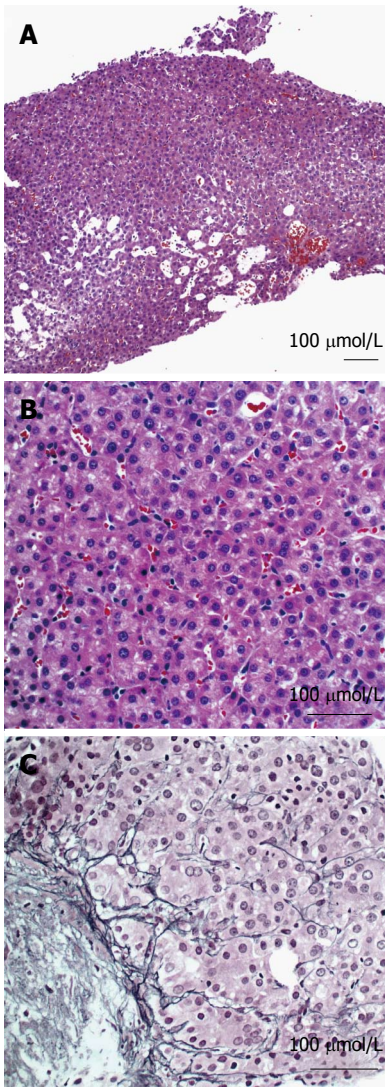

Figure 2 Ultrasound-guided liver biopsy. A: Biopsy of the mass shows a solid growth pattern of hepatocytes (H+E, 100 ×); B: Neoplastic hepatocytes contain hyperchromatic, pleomorphic and enlarged nuclei (H+E, 200 ×); C: Reticulin stain demonstrates thickened trabeculae and decreased staining in the lesion (200 ×).

recurrent liver disease and exposure to environmental carcinogens. Although the clinical features cannot guarantee the distinction with certainty, molecular techniques may permit differentiation of donor from recipient origin[17]. In addition, allografts have a certain degree of hepatocyte chimerism (graft and recipient cells) that also correlates with the degree of hepatic injury and is strongly associated with hepatitis[18].

The pathogenesis of HCC appears to be related to chronic hepatic inflammation that eventually leads to fibrosis and cirrhosis. The inflammatory microenvironment in the liver leads to a proliferative state that can promote dysplasia and eventually malignancy regardless of the underlying liver disease[1]. Graft rejection, which is an immunological surge against detected antigens found within the graft, can generate a chronic inflammatory state[19] and create an environment that promotes oncogenesis and dysplasia. Other well-established risk

Table 1 Modified from Saab *et al*[14]

Patient	Ref.	OLT indication	Age	Gender	Immunosuppression	Interval (yr)	Type of donor	PVS	Approach after *de-novo* HCC
1	Saxena *et al*[4]	HCV and ALD	63	M	CYA, AZA and Pred	7	DD	Yes	Retransplant
2	Levitsky *et al*[5]	HCV and ALD	48	M	CYA, AZA and Pred	5	N/A	Yes	NR
3	Croitoru *et al*[6]	HCV and NAFLD	61	M	CYA and Pred	6	DD	Yes	Retransplant
4	Flemming *et al*[7]	HBV	NR	M	NR	9	DD	Yes	Hepatic Resection
5	Flemming *et al*[7]	HBV	NR	M	NR	8	DD	Yes	Retransplant and Hepatic Resection
6	Torbenson *et al*[8]	HBV	51	M	NR	8.5	DD	Yes	Retransplant
7	Kita *et al*[9]	HBV	43	M	NR	14	NA	Yes	Retransplant
8	Yu *et al*[10]	HBV	36	M	TAC, MMF andPred	2	LD	Yes	RFA
9	Sotiropoulos *et al*[11]	Budd-Chiari Syndrome	61	F	NR	22	NA	Yes	TACE
10	Sotiropoulos *et al*[11]	ALD	65	M	NR	5	NA	NR	RFA
11	Vernadakis *et al*[12]	ALD	59	M	CYA, MMF and Pred	3	DD	No	Hepatic Resection
12	Tamè *et al*[13]	HCV	54	M	TAC and Pred	6	DD	Yes	TACE
13	Saab *et al*[14]	HCV	47	F	TAC and MMF	19	-	Yes	TACE, RFA, Sorafenib, Retransplant
14	Tamè *et al*[13]	SSC	44	M	TAC and Pred	6	DD	Yes	Sorafenib
15	Navarro Burgos *et al*[15]	HCV and HBV	45	M	NR	0.75	DD	Yes	TACE
16	The present case	Biliary atresia	29	M	TAC and Sirolimus	14	LD	No	PMWA

OLT: Orthotropic liver transplantation; DD: Deceased donor; LD: Living donor; PVS: Positive viral serology; HCV: Hepatitis C virus; SSC: Secondary sclerosing cholangitis; ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease; HBV: Hepatitis B virus; NR: Not reported; CYA: Cyclophosphamide; AZA: Azathioprine; Pred: Prednisone; TAC: Tacrolimus; MMF: Mycophenolate mofetil; RFA: Radiofrequency ablation; TACE: Transarterial chemoembolization; PMWA: Percutaneous microwave ablation; M: Male; F: Female; NA: Not available.

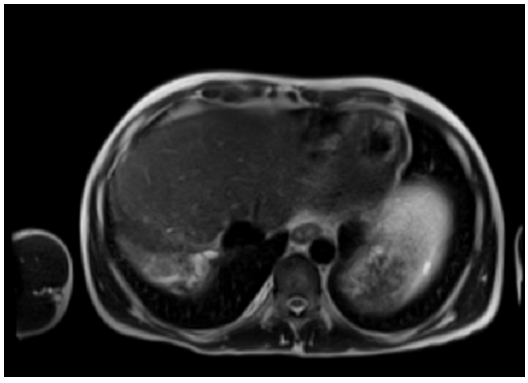

Figure 3 Follow-up magnetic resonance imaging showing the ablation cavity in segment III measuring 2.8 cm × 2.5 cm without evidence of residual tumor.

factors for promoting carcinogenesis include the use of immunosuppressive therapy as it reduces immune surveillance, increased age and gender specific cancer risks, development of insulin resistance and exposure to viral infections (HBV and HCV)[3,16,17].

From the 16 cases of *de-novo* HCC occurrence reported so far in the literature, 14 had positive viral serology (HVB or HCV) (Table 1). In these cases the viral infection likely drove tumorogenesis. However, this is a novel case describing a *de-novo* HCC after a LDLT in a pediatric patient. Our case represents the development of a hepatic tumor in the setting of advanced hepatic fibrosis, likely from chronic allograft rejection, without any underlying viral disease or other chronic infection. Interestingly both biliary atresia itself and Kasai procedure have been associated with the development of HCC[20-22]. However, given the 14 year gap from transplant to development of HCC, this probably did not contribute to the development of HCC in this particular patient.

Although current treatment guidelines for *De-novo* HCC after LT have not been well established, it was suggested that these cases be approached according to the current guidelines for primary and recurrent HCC[17]. The strategies that have been used in the reported cases to date are: re-transplantation (n = 6)[4,6-9,14], trans-arterial chemoembolization (TACE) (n = 4)[11,13-15], hepatic resection (n = 3)[7,12], radiofrequecy ablation (RFA) (n = 3)[10,11,14], medical therapy with Sorafenib (n = 2)[13,14] and PMWA in our case. Two of the reported cases used more than one procedure[7,14].

Proper age and gender appropriate cancer screening and surveillance is universally practiced among transplant recipients to diagnosis early stage malignancy. Since approximately one-fifth of all post-transplant deaths are related to *de-novo* neoplasms (including *de-novo* HCC)[3] and the incidence of HCC recurrence can be as high as 18.3% after transplant[2], many transplant programs have in place post-LT screening and surveillance for HCC in patients transplanted for HCC along with other

appropriate cancer screening and surveillance[3]. However, these protocols for post-LT screening and surveillance are not uniform amongst centers as there is a general lack of evidence base for deciding on a specific protocol. This remains to be established.

In summary, we describe the first case of *de-novo* HCC after living donor liver transplantation in a patient with a prior history of biliary atresia who developed graft dysfunction and complications of portal hypertension. This case and the increase in reports of *de-novo* development of HCC in liver grafts of patients without HCC prior to LT challenges us to define the incidence of development of HCC in post-LT patients with chronic injury and graft fibrosis to determine when there is utility in recommending screening and surveillance of these individuals for HCC, and design appropriate protocols to carry this out.

ARTICLE HIGHLIGHTS

Case characteristics

A 29-year-old male with a history of biliary atresia with failed Kasai procedure complicated with progressive cirrhosis and portal hypertension that received a left lateral segment living donor liver transplant (LDLT) from his biological father at 15 years of age.

Clinical diagnosis

Biliary atresia, complicated with progressive cirrhosis and portal hypertension that received a left lateral segment living donor liver transplant LDLT.

Differential diagnosis

A case of *de-novo* hepatocellular carcinoma (HCC) (confirmed by ultrasound-guided percutaneous biopsy of the mass) 14 years after a pediatric living related donor liver transplantation for end-stage liver disease without positive hepatitis B or C viral serology.

Imaging diagnosis

Follow up magnetic resonance imaging was performed 1.5 mo after the ablation and showed no residual tumor.

Treatment

The lesion was subsequently treated by percutaneous microwave ablation. The patient was subsequently listed for repeat liver transplantation. Current screening and treatment guidelines have not been well established.

Experiences and lessons

This increasing phenomenon challenges us to define the utility of screening and surveillance for HCC in these individuals.

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CASE REPORT

Autoimmune hepatitis in the setting of human immunodeficiency virus infection: A case series

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

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Abstract

Liver injury in the setting of human immunodeficiency virus (HIV) infection is more commonly attributed to viral hepatitis or highly active antiretroviral treatment (HAART) toxicity. The severity of liver injury is an important cause of morbidity and mortality. The emergence of autoimmune diseases, particularly autoimmune hepatitis (AIH) in the setting of HIV infection, is rare. Previous reports indicate that elevated liver enzymes are a common denominator amongst these patients. We present two patients with HIV infection, on HAART, with virological suppression. Both patients presented with elevated liver enzymes, and following liver biopsies, were diagnosed with AIH. The clinical course of these patients underscore the therapeutic value of corticosteroids, and in some cases, addition of immunosuppression for AIH treatment.

Key words: Liver biopsy; Human immunodeficiency virus; Immunosuppression; Autoimmunity; Autoimmune hepatitis

Core tip: Liver damage is rarely caused by autoimmune disease in the setting of human immunodeficiency virus (HIV) infection. We describe a case series of two patients with a history of HIV, who presented with characteristic elevation in liver enzymes. Both

patients were hepatitis C negative. Liver biopsies followed by histopathology confirmed the diagnosis of autoimmune hepatitis. Case 1 was treated by corticosteroids and azathioprine, while case 2 was treated by corticosteroids only. Both patients reported significant clinical improvement. These cases suggest that liver biopsy should be performed in HIV patients with unknown liver disease. Additionally, they underscore the need for further clinical studies to explore the role of corticosteroids and immunosuppression in the management of autoimmune hepatitis in HIV patients.

Ofori E, Ramai D, Ona MA, Reddy M. Autoimmune hepatitis in the setting of human immunodeficiency virus infection: A case series. *World J Hepatol* 2017; 9(36): 1367-1371 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i36/1367.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i36.1367

INTRODUCTION

Autoimmune hepatitis (AIH) is a rare chronic liver disease which was first reported in the 1950s by the Swedish physician Jan Waldstrom[1]. Patients infected with human immunodeficiency virus (HIV) tend to have impaired immune systems, weakening host defenses against opportunistic pathogens, and autoimmunity[2]. Given that complications of liver disease in the setting of HIV are more likely due to coinfections with hepatitis B (HBV) or hepatitis C (HCV) viruses, antiretroviral drug toxicity, opportunistic infections, or neoplastic disorders, it is very rare to encounter cases of AIH. While the global occurrence of AIH is largely unknown, in Europe and North America it has been estimated at 1.9/100000 incidence and 16.9/100000 prevalence[3]. A review of the literature shows that only 18 cases (excluding our two patients) have been reported[4-11]. Herein, we present two cases of AIH in the setting of HIV infection.

CASE REPORT

Case 1

A 40-year-old male who emigrated from Guyana, diagnosed with HIV since 2009 and started on efavirenz, emtricitabine, and tenofovir disoproxil fumarate (Atripla), with viral suppression and immunological recovery (CD4 cell count 832/mm^3), presented for a follow-up. He was a non-smoker with prior history of alcohol consumption and liver cirrhosis. Laboratory workup showed elevated liver enzymes of alanine aminotransferase (ALT) 302 U/L, and aspartate aminotransferase (AST) 149 U/L. These values decreased over the next 3 mo and increased again, reaching their highest at 14 mo: ALT 465 U/L, and AST 302 U/L. Alkaline phosphatase (ALP) was elevated at 233 U/L, total bilirubin 1.3 mg/dL, direct bilirubin 0.6 mg/dL, and alpha-fetoprotein (AFP) 14 ng/mL.

To elucidate the etiology of elevated transaminases, further laboratory tests were performed. He was immune to HBV virus, nonreactive for HCV antibody and undetected by quantitative PCR assay. White cell count, hemoglobin, hematocrit, platelets, prothrombin time, INR, and albumin were all within normal limits. Iron and copper metabolism in addition to ceruloplasmin and alpha-1-antitrypsin levels were also normal. Autoimmune assay for antinuclear antibodies (ANA) was negative, and smooth muscle antibody (ASMA) immunoglobulin G (IgG) was positive at 60 Units (normal 0-19 Units), suggestive of autoimmune hepatitis. Hemochromatosis gene mutations (H63D and C282Y) screening were negative. IgG level was 2740 mg/dL.

Abdominal ultrasound showed a normal sized liver with slight heterogeneity, suggestive of diffuse liver disease. Abdominal magnetic resonance imaging (MRI) showed obstruction of the right hepatic tip by a blooming artifact of uncertain etiology. A transthoracic percussion guided liver biopsy was performed without complications. Histopathology showed fibrous portal expansion and bridging fibrosis. Portal and periportal inflammatory activity along with piecemeal necrosis was identified (Figure 1). Taken in clinical context, the diagnosis of AIH was confirmed. The patient was started on corticosteroids, and was later prescribed Azathioprine.

The patient was lost to follow-up, but presented 18 mo later and showed a notable improvement in liver enzymes: ALT 112 U/L, and AST 81 U/L. He reported no new symptoms related to liver disease. However, the patient was non-compliant with his medications and repeated laboratory results showed rising liver transaminases again (Figure 2A). The patient was advised to restart his medications.

Case 2

A 44-year-old Hispanic female diagnosed with HIV since 1997 and started on Atripla since 2010 with viral suppression and immunological recovery (CD4 count 823, and viral load undetectable), was admitted for epigastric pain and vomiting. A non-smoker, laboratory workup showed elevated liver chemistries: ALT 155 U/L, AST 136 U/L, ALP 100 U/L, total bilirubin 1.9 mg/dL, AFP 16 ng/mL. She was immune to Hepatitis A (HAV) and HBV, and non-reactive for HCV. ANA (1:640), and ASMA (1:180), were positive suggestive of AIH. Abdominal MRI showed perihepatic fluid and cirrhosis of the liver. Esophagastroduodenoscopy (EGD) revealed a gastric ulcer, which was positive for *Helicobacter pylori* (*H. pylori*) gastritis. Colonoscopy revealed a tubular adenoma. The patient was stabilized and discharged after 6 d.

A liver biopsy was performed without complications. Histopathology showed confluent necrosis infiltrated by dense lympho plasmacytic infiltrates partially replaced by fibrous tissue, as well as bridging fibrous septa that enclosed regenerative nodules, consistent with AIH. The patient was started on prednisone. At the 6th week of steroid therapy, the patient reported notable

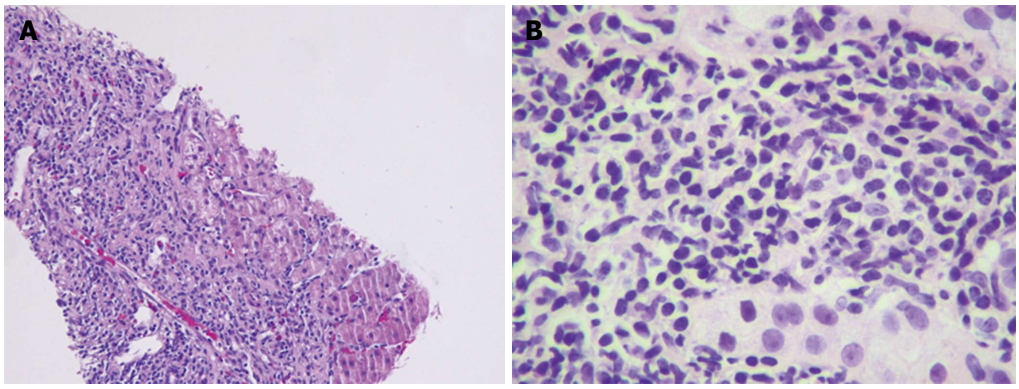

Figure 1 Microscopic examination. A: Microscopic examination reveals mild portal/periportal chronic inflammation; B: Microscopic examination reveals moderate chronic inflammation in a background of cirrhosis. Original magnification, × 20 (A), and × 40 (B).

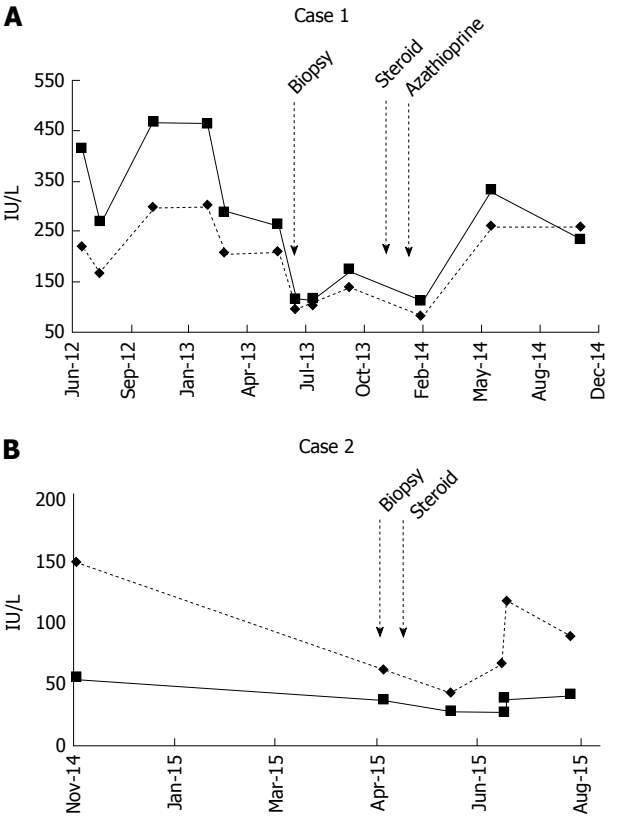

Figure 2 Graphical representation of alanine transferase (straight line) and aspartate transaminase (dashed line) over time (A and B). Time of biopsy and treatment also denoted.

improvement in symptoms, and resolving liver enzyme levels (Figure 2B).

DISCUSSION

HIV is associated with the development of autoimmune disorders such as immune thrombocytopenic purpura, inflammatory myositis, sarcoidosis, Guillain Barre Syndrome, myasthenia gravis, Graves' disease, systemic lupus erythematosus, rheumatoid arthritis, Hashimoto thyroiditis, autoimmune hemolytic anemia, and very rarely, autoimmune hepatitis[12]. Due to its rarity, AIH in the setting of HIV is not often suspected by clinicians, but should be considered when all other etiologies are ruled out.

There are two clinically relevant types of AIH; namely, type 1 and type 2. Type 1 AIH is referred to as the classic type, typically diagnosed in adulthood, whereas type 2 is diagnosed during childhood[13,14]. Though both types are similar, type 2 AIH can be more severe and difficult to manage. Symptoms associated with AIH include fatigue, pruritus, jaundice, nausea, vomiting, abdominal pain, weight loss, light colored stools, dark colored urine, joint pain, rashes, and loss of menstruation in women[4-11,15,16]. Without adequate therapy, the disease can progress in the form of liver fibrosis. As a result, patients can develop cirrhosis, liver failure, ascites, gastrointestinal bleeding, hepatic encephalopathy, and even hepatocellular carcinoma.

The diagnosis of AIH is established based on the following criteria by the American and European practice guidelines: Hyper-gammaglobulinemia, positive serologic tests including antinuclear and anti-smooth muscle antibodies, and a characteristic hepatic histological appearance, namely interface hepatitis, plasmacytic infiltrate, and regenerative liver-cell rosettes[17]. Other liver diseases such as alpha-1-antitrypsin deficiency, Wilson disease, hemochromatosis, viral hepatitis, drug-induced liver injury, and alcoholic/nonalcoholic liver disease should be ruled out.

The diagnosis of AIH in HIV infected patients pose a diagnostic conundrum because HIV infection is usually considered as being protective against autoimmunity. However, several mechanisms have been proposed by which HIV may subvert and influence host immune regulation. Firstly, it is thought that viral infection triggers a pro-inflammatory milieu, which overrides host regulatory networks. This may lead to the generation of self-perpetuating autoimmune reactions[18]. Genetic susceptibility has also been proposed as an alternative mechanism. AIH is a polygenetic disorder with strong evidence of inheritability. During the maturation of T-cells, the thymus deletes T-cells that react too strongly to self-antigens[19]. Thymic mutations can indeed affect this process and lead to AIH. Furthermore, despite thymic selection, individuals who express HLA haplotypes DR3,

DR4, and hepatocyte enzyme CYP2D6, are more likely to develop AIH[20-23].

Another suggested mechanism is the role of immune reconstitution inflammatory syndrome (IRIS), also known as immune restoration disease. While the pathogenesis of IRIS is speculative, it is thought to occur in patients with significant increase in CD4 cells after initiation of anti-retroviral (ARV) therapy, specifically those who concurrently had low CD4 cells prior to treatment[24,25]. It has been noted that the increase in CD4 count may not be responsible for the inflammatory response, but instead may be due to preexisting perturbations in T-regulatory cells (Tregs), and proinflammatory and regulatory responses such as cytokine imbalances that may significantly contribute to the onset of the syndrome after the initiation of ARV therapy[26].

As far as we know, there are no guidelines for the treatment of AIH in HIV patients. A review of published cases showed that corticosteroids and immunosuppression were reasonably used by other clinicians[4-11]. Case 1 involved the use of corticosteroids and azathioprine, while case 2 used corticosteroids only. While both cases showed resolution of symptoms, it also suggested that additional immunological suppression with azathioprine may not be required for treating AIH.

In conclusion, AIH is a rare and chronic liver disease which seldom presents in HIV-infected patients. A characteristic elevation in liver enzymes is commonly reported in these cases. However, they are often attributed to HAART or other possible liver diseases, particularly viral hepatitis. For this reason, liver biopsies should be performed in HIV patients with an unknown liver disease etiology. Furthermore, patients with AIH in the setting of HIV infection should be treated with corticosteroids. Further research is needed to study the efficacy of corticosteroids with or without the use of immunosuppression.

ARTICLE HIGHLIGHTS

Case characteristics

Case 1: A 40-year-old male diagnosed with human immunodeficiency virus (HIV) since 2009 and started on Atripla, with viral suppression and immunological recovery, presented for a follow-up. Case 2: A 44-year-old Hispanic female diagnosed with HIV since 1997 and started on Atripla since 2010, with viral suppression and immunological recovery, was admitted for epigastric pain and vomiting.

Clinical diagnosis

Case 1: Abdominal ultrasound showed a normal sized liver with slight heterogeneity, suggestive of diffuse liver disease. Case 2: An abdominal magnetic resonance (MRI) imaging was suggestive of cirrhosis of the liver.

Differential diagnosis

Liver cirrhosis, hepatitis, hepatocellular carcinoma.

Laboratory diagnosis

Case 1: Laboratory workup showed elevated liver chemistries: Alanine aminotransferase (ALT) 302 U/L, and aspartate aminotransferase (AST) 149 U/L, alkaline phosphatase 233 U/L, total bilirubin 1.3 mg/dL, direct bilirubin 0.6 mg/dL, and alpha-fetoprotein 14 ng/mL. Case 2: Laboratory workup showed elevated liver chemistries: ALT 155 U/L, AST 136 U/L, alkaline phosphatase 100 U/L, total bilirubin 1.9 mg/dL, and alpha-fetoprotein 16 ng/mL.

Imaging diagnosis

Abdominal MRI imaging was suggestive of liver cirrhosis of uncertain etiology.

Pathological diagnosis

Case 1: A transthoracic percussion guided liver biopsy showed fibrous portal expansion, bridging fibrosis, and portal and periportal inflammatory activity with piecemeal necrosis, consistent with autoimmune hepatitis (AIH). Case 2: Liver biopsy showed confluent necrosis infiltrated by dense lymphoplasmacytic infiltrates partially replaced by fibrous tissue, as well as bridging fibrous septa that enclosed regenerative nodules, consistent with AIH.

Treatment

Case 1 was treated with corticosteroids and azathioprine, while case 2 was treated with corticosteroids only.

Related reports

Review of the literature shows that only 18 cases (excluding our two patients) have been reported.

Term explanation

The occurrence of autoimmune hepatitis in the setting of HIV-infected patients is an extremely rare clinical entity. The global prevalence of AIH is largely unknown. Currently, there are no standardized treatment for AIH.

Experiences and lessons

This report suggest that liver biopsies should be performed in HIV patients with an unknown liver disease etiology. HIV patients diagnosed with AIH should be treated with corticosteroids. Further research is needed to study the clinical efficacy of corticosteroids with or without the use of immunosuppression.

Peer-review

The manuscript describes an interesting and rare association between autoimmune hepatitis and HIV infection in 2 adult patients. The report is concisely and clearly described and it's of potential interest for readers because information on this topic is still scanty. This is very informative and adds new knowledge about autoimmune mechanisms of liver injury in HIV infection.

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CASE REPORT

Sequential tumor-directed and lobar radioembolization before major hepatectomy for hepatocellular carcinoma

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Author contributions: Degrez T, Bouazza F, Delatte P, Gomez Galdon M, Hendlisz A and Flamen P performed research; Vouche M and Donckier V performed research and wrote the paper.

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Abstract

Preoperative radioembolization may improve the resectability of liver tumor by inducing tumor shrinkage, atrophy of the embolized liver and compensatory hypertrophy of non-embolized liver. We describe the case of a cirrhotic Child-Pugh A patient with a segment Ⅳ hepatocellular carcinoma requiring a left hepatectomy. Preoperative angiography demonstrated 2 separated left hepatic arteries, for segment Ⅳ and segments Ⅱ-Ⅲ. This anatomic variant allowed sequential radioembolizations, delivering high-dose ^{90}Yttrium (160 Gy) to the tumor, followed 28 d later by lower dose (120 Gy) to segments Ⅱ-Ⅲ. After 3 mo, significant tumor

response and atrophy of the future resected liver were obtained, allowing uneventful left hepatectomy. This case illustrates that, when anatomic disposition permits it, sequential radioembolizations, delivering different ^{90}Yttrium doses to the tumor and the future resected liver, could represent a new strategy to prepare major hepatectomy in cirrhotic patients, allowing optimal tumoricidal effect while reducing the toxicity of the global procedure.

Key words: Hepatocellular carcinoma; Cirrhosis; Resectability; Radioembolization; Sequential; Efficacy; Safety

Core tip: Preoperative radioembolization may improve resectability of hepatocellular carcinoma in cirrhotic patient, inducing tumor downsizing, atrophy of radio-embolized sector and regeneration of non-embolized liver. We describe a patient with a segment Ⅳ hepatocellular carcinoma where the presence of two separated left hepatic arteries permitted to deliver sequentially high-dose ^{90}Yttrium to the tumor and lower dose to future resected liver, allowing uneventful left hepatectomy 3 mo later. This observation suggests that, when different arterial accesses exist to tumor and future resected non-tumor liver, sequential radioembolization with different radiation doses could represent a new preoperative strategy, optimizing the tumoricidal effect while minimizing the risk of radiation-induced liver damage.

Vouche M, Degrez T, Bouazza F, Delatte P, Gomez Galdon M, Hendlisz A, Flamen P, Donckier V. Sequential tumor-directed and lobar radioembolization before major hepatectomy for hepatocellular carcinoma. *World J Hepatol* 2017; 9(36): 1372-1377 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i36/1372.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i36.1372

INTRODUCTION

Partial hepatectomy (PH) and tumor destruction with radiofrequency (RF) are the first therapeutic options in patients with hepatocellular carcinoma (HCC) and compensated cirrhosis who are not candidates for liver transplantation (LT)[1,2]. However, the feasibility and efficacy of these treatments are dramatically limited by underlying liver disease and high tumor recurrence rates. At the present time, no neoadjuvant treatment has been validated for improving the safety and efficacy of PH and RF in this setting. In particular, locoregional treatment with transarterial chemoembolization (TACE) has failed to demonstrate significant long-term benefits when used before PH or RF for HCC[3-5]. Furthermore, when a major resection of 3 or more segments is indicated in cirrhotic livers, preoperative homolateral portal vein embolization (PVE) is recommended to induce an atrophy of the future resected liver and a compensatory hypertrophy of the future liver remnant (FLR)[6,7]. This strategy, however, leaves the tumor untreated while waiting for liver regeneration, exposing the patient to the risk of tumor progression before the surgery[8].

Selective internal radiotherapy (SIRT), relying on the transarterial embolization of ^{90}yttrium-loaded microspheres (^{90}Y), has become a new tool for treatment of liver tumors. In HCC, SIRT has been demonstrated to improve survival in patients who are not candidates for curative-intent therapies and to allow tumor control while waiting for LT[9-13]. Furthermore, SIRT can be used preoperatively and the feasibility and safety of post-SIRT surgery has been now assessed[14-17]. The tumoricidal effect of SIRT, leading to tumor downsizing, may significantly modify the extent of surgery or allow the resection of initially unresectable tumors. Moreover, regional intra-arterial hepatic embolization with ^{90}Y could also induce the atrophy of the embolized segments and a compensatory hypertrophy of the non-embolized liver[18,19]. This specificity allows for the design of new therapeutic strategies, integrating neoadjuvant SIRT into current surgical approaches to liver tumors, particularly for HCC in cirrhotic patients.

We describe here the case of a patient with centrally-located HCC, treated with sequential intra-tumor and left lobar ^{90}Y embolization before a left hepatectomy. This case illustrates the new possibilities offered by the use of SIRT as a preoperative therapy before major liver resection for HCC in cirrhotic patients.

CASE REPORT

A 70-year-old man with a past history of alcohol consumption presented with a liver tumor. Contrast-enhanced magnetic resonance imaging (MRI) demonstrated a 40 mm mass in segment IV with vascular characteristics of HCC (arterial wash-in and portal wash-out) and features of cirrhosis (Figure 1A and B). Blood tests, including liver function and alpha-fetoprotein, were normal and the patient was classified as Child-Pugh A, with a MELD score of 7. Complete work-up did not demonstrate extra-hepatic metastasis. Accordingly, the tumor corresponded to Okuda stage 1 and BCLC stage A. Due to the patient's age, the comorbidities, and the patient's preferences, LT was not recommended during multidisciplinary meeting. Therefore, a left hepatectomy (resection of segments II-III-IV) was proposed and, due to the presence of cirrhosis, preoperative treatment to modulate FLR volume and function was indicated. Analysis of liver volumes on angio-CT scan showed a total liver volume (TLV) of 2339 mL, a tumor volume of 36 mL, a left liver volume (segments II, III, IV) of 812 mL, and an FLR volume (segments I, V, VI, VII, VIII) of 1527 mL, corresponding to a FLR/TLV of 65% and an FLR/body weight ratio of 0.68. On the basis of our previous experience[20] and in relation to the proximity of the tumor to the portal bifurcation that might

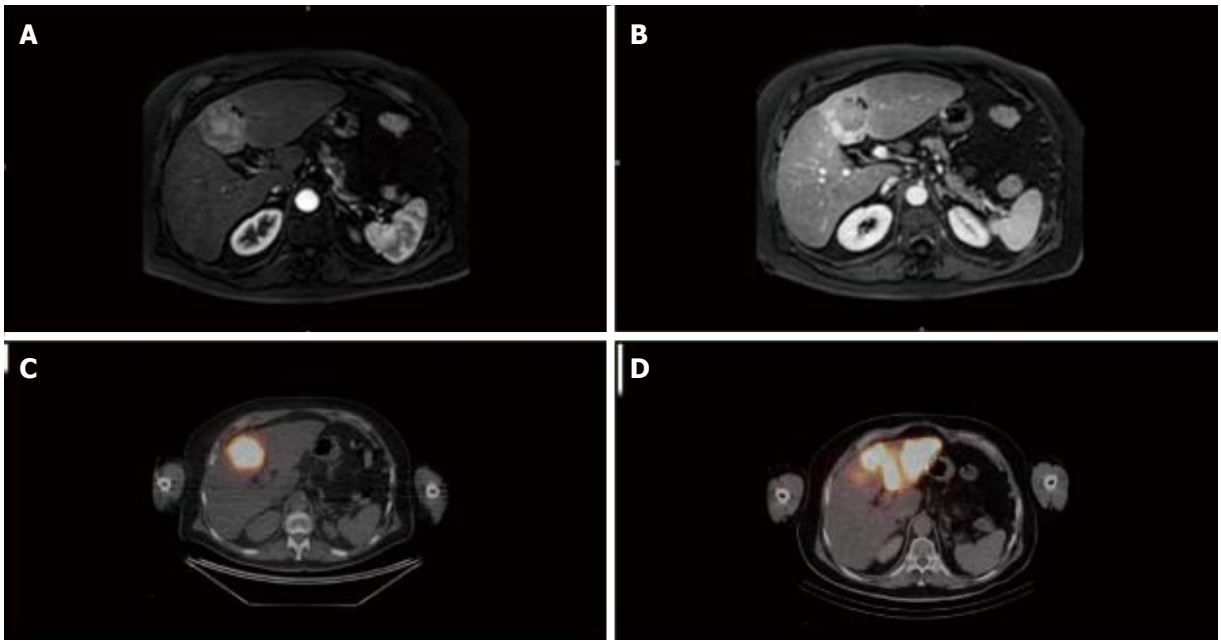

Figure 1 Preoperative imaging. A and B: Baseline contrast-enhanced magnetic resonance imaging (MRI). Contrast-enhanced MRI demonstrated a 40 mm mass in segment IV of the liver with arterial wash-in (A) and wash-out on the portal venous phase (B) and features of cirrhosis (irregular surface, relative hypertrophy of segment I); C and D: Selective intra-tumor deposition of 90Y microspheres after first SIRT session (C) and deposition of 90Y microspheres to segments II and III after the second SIRT session (D).

preclude the chance for resection in case of progression, SIRT was preferred to PVE as preoperative treatment.

Simulation of SIRT with ^{99}Tc macroaggregated albumin showed no extra-hepatic deposition and excellent tumor targeting. In addition, the angiography demonstrated a variant hepatic arterial anatomy characterized by a left hepatic artery arising from the right gastric artery, a segment IV artery arising from the gastroduodenal artery and a right hepatic artery arising normally from the celiac trunk. Therefore, 2-step SIRT using different ^{90}Y doses was decided upon in order to maximize the dose of ^{90}Y selectively delivered to the tumor and to minimize the potential toxicity related to intense radioembolization of a large liver volume. First, ^{90}Y hyperselective radioembolization of the segment IV artery to the tumor was performed, allowing the delivery 161 Gy to segment Ⅳ (Figure 1C). No side effects related to this procedure were observed. Twenty-eight days later, the left hepatic artery was catheterized and ^{90}Y microspheres injected, allowing for the delivery of 120 Gy to segments II and III (Figure 1D). No side effects were observed following this procedure. At day 110 after the second SIRT, contrast-enhanced MRI showed a significant tumor response (size reduction of the tumor diameter from 40 to 34 mm and complete necrosis on arterial phase) (Figure 1D). On the same examination, segments Ⅱ, Ⅲ, and Ⅳ measured 545 mL, corresponding to a 34% reduction, and FLR measured 1643 mL, corresponding to a minimal increase of 2%. At day 115 after the second SIRT, a left hepatectomy, partially extended to segment Ⅴ, was performed. Operative exploration confirmed the cirrhosis while the entire left lobe appeared as atrophic and fibrotic (Figure 2A). The surgery proceeded uneventfully. Intraoperative blood losses were 800 mL and no blood transfusions were required. Postoperative course was unremarkable clinically and biologically (minimal values of PT, peak INR, and total bilirubin respectively of 56%, 1.3, and 1.5 mg/dL on day 3 after surgery) and the patient was discharged on day 14. On macroscopic examination of the operative specimen, small foci of cancer cells < 5 mm were observed within a tumor necrotic/fibrotic zone of 55 mm in diameter (Figure 2b). Pathological examination demonstrated a margin-free resection and a major tumor response as indicated by approximately less than 10% of residual cancer cells (Figure 2C and D).

DISCUSSION

PH remains the treatment of choice in patients with large HCC and compensated cirrhosis without significant portal hypertension and who are not candidates for LT[1]. When a major resection is required, preoperative PVE to adapt the FLR is currently considered as the standard procedure. The present case illustrates that neoadjuvant SIRT before surgery may represent now an alternative to this classical sequence. The rationale for considering the use of SIRT before PH for HCC in cirrhotic patients relies on several factors. The first is that SIRT is an effective local treatment for HCC[13]. Thus, if liver surgery would ultimately be found to be infeasible, the patient would still receive an efficient anti-tumor therapy. Secondly, when ^{90}Y microspheres are administered both selectively in the tumor and

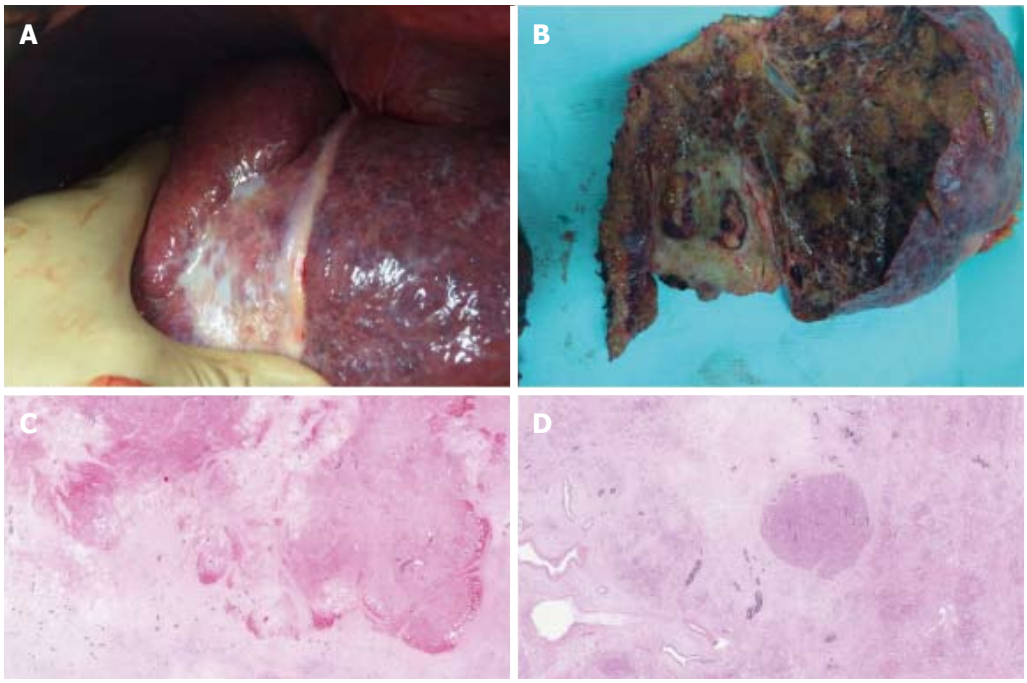

Figure 2 Intra- and postoperative images. A: Intraoperative view showing the cirrhosis and the post-selective internal radiotherapy (SIRT) relative atrophy of the left liver; B: Resected specimen showing small residual cancer cells foci with the necrotic and fibrotic zone targeted by segment IV high-dose SIRT; C: Pathological view showing massive necrosis and fibrosis together with the presence of microspheres; D: Pathological view showing a residual hepatocellular carcinoma focus, surrounded by necrosis and fibrosis together with the presence of microspheres.

regionally in the future resected liver segments (radiation lobectomy), SIRT has the unique capacity to induce an effective tumoricidal effect together with the atrophy of the future resected liver and a compensatory hypertrophy of the FLR. As compared with preoperative PVE, this may reduce the risk of tumor progression while waiting for functional and volumetric adaptation of the FLR. Finally, and as described for TACE[21], response to SIRT may potentially serve as a predictive factor both for the safety and the efficacy of the surgery. The feasibility of major liver resection after ^{90}Y radiation lobectomy has been assessed. However, particularly in cirrhotic livers, such large liver volume irradiation exposes the patient to the risk of radiation-induced liver disease (RILD)[22]. In the present case, the hepatic arterial anatomy allowed to perform a 2-step SIRT, delivering first high ^{90}Y dose to the segment Ⅳ tumor, followed by an ablative but safe irradiation dose to left lobe (segments Ⅱ and Ⅲ). As a dose-tumor response correlation was demonstrated over 170 Gy[23] and FLR volume modulation was found for doses approximating 120 Gy[18], such sequential procedures may potentially optimize the neoadjuvant effect of the treatment while reducing the toxicity and the risk of RILD. At 3 mo after SIRT, we observed volumetric effects within the embolized regions, as indicated by significant tumor shrinking and left lobe atrophy. In contrast, virtually no increase of the non-embolized FLR was detected, potentially related to the relatively short time period between SIRT and surgery[18]. Despite the absence of significant volumetric regeneration of the right liver, no sign of liver insufficiency has been observed after the left hepatectomy, potentially in relation with favorable initial FLR/TLV ratio. Finally, this case indicates that, despite the so-called ablative ^{90}Y dose given to the tumor, a complete pathological response was not obtained, highlighting the need to still resect these irradiated tumors whenever possible.

In conclusion, when distinct arteries to the tumor and to the future resected liver can be selectively catheterized, sequential ^{90}Y embolization with modulated doses to the tumor and to the future resected liver could represent a new strategy for improving the safety and the efficacy of neoadjuvant radioembolization before major liver resection in cirrhotic patients. The potential oncological benefit of this therapeutic combination remains to be evaluated.

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ARTICLE HIGHLIGHTS

Case characteristics

A seventy years old patient presented with a segment Ⅳ liver tumor.

Clinical diagnosis

Due to the presence of alcohol-related cirrhosis, a diagnosis of hepatocellular

carcinoma was suspected.

Differential diagnosis
Differential diagnosis included other solid liver tumors, primary or secondary.

Laboratory diagnosis
Laboratory data, including alpha-fetoprotein were not contributive.

Imaging diagnosis
Contrast-enhanced magnetic resonance imaging demonstrated a 40 mm mass in segment Ⅳ of the liver with vascular characteristics of hepatocellular carcinoma, such as arterial phase wash-in and portal phase wash-out and features of cirrhosis. Angiography demonstrated two separated left hepatic arteries, for segment Ⅳ and for segments Ⅱ and Ⅲ, allowing selective access to the tumor and to the future resected liver.

Pathological diagnosis
On operative specimen, pathology confirmed the diagnosis of hepatocellular carcinoma and a major response to preoperative radioembolization as indicated by less than 10% residual cancer cells.

Treatment
Left hepatectomy was preceded by sequential radioembolizations, delivering high-dose radiation to the tumor and then, lower dose to the future resected liver. This 2-steps approach aimed to maximize tumoricidal effect while limiting the risks for radiation-induced liver disease and liver insufficiency.

Related reports
In such cases of hepatocellular carcinoma requiring a major hepatectomy in patients with compensated cirrhosis, resectability is dramatically limited by the risk of postoperative liver insufficiency.

Experiences and lessons
This case indicates that, when arterial anatomy allows it, sequential radioembolizations with different radiation doses to the tumor and to the future resected liver could represent a new strategy to maximize the tumoricidal effect while preserving the atrophic effect but reducing the risk of radiation-induced liver injury.

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CASE REPORT

Primary biliary cholangitis metachronously complicated with combined hepatocellular carcinoma-cholangiocellular carcinoma and hepatocellular carcinoma

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Author contributions: Ide R and Oshita A made conception and design of this case report; authors other than Ide R and Oshita A, Nishisaka T, Nakahara H, Aimitsu S and Itamoto T contributed to collection and interpretation of data; Ide R and Oshita A wrote the draft manuscript, and other authors performed critical revision of the manuscript; all authors gave final approval of the version to be published; Oshita A has overall responsibility and guarantees the scientific integrity.

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Abstract

Primary biliary cholangitis (PBC) is a progressive cholestatic liver disease characterized by the presence of highly specific antimitochondrial antibodies, portal inflammation and lymphocyte-dominated destruction of the intrahepatic bile ducts, which leads to cirrhosis. While its pathogenesis remains unclear, PBC that shows histological progression to fibrosis carries a high risk of carcinogenesis; the same is true of viral liver diseases. In patients with PBC, the development of hepatocellular carcinoma (HCC) is rare; the development of combined hepatocellular carcinoma and cholangiocellular carcinoma (cHCC-CCC) is extraordinary. Herein, we report a rare case of PBC metachronously complicated by cHCC-

CCC and HCC, which, to the best of our knowledge, has never been reported. We present a case report of a 74-year-old Japanese woman who was diagnosed as PBC in her 40's by using blood tests and was admitted to our department for further management of an asymptomatic liver mass. She had a tumor of 15 mm in size in segment 8 of the liver and underwent a partial resection of the liver. Subsequent pathological findings resulted in the diagnosis of cHCC-CCC, arising from stage 3 PBC. One year after the initial hepatectomy, a second tumor of 10 mm in diameter was found in segment 5 of the liver; a partial resection of the liver was performed. Subsequent pathological findings led to HCC diagnosis. The component of HCC in the initial tumor displayed a trabecular growth pattern while the second HCC showed a pseudoglandular growth pattern, suggesting that metachronous tumors that arise from PBC are multicentric.

Key words: Primary biliary cholangitis; Combined hepatocellular carcinoma and cholangiocellular carcinoma; Hepatocellular carcinoma

Core tip: Primary biliary cholangitis (PBC) is a progressive cholestatic liver disease characterized by the presence of highly specific antimitochondrial antibodies, portal inflammation and lymphocyte-dominated destruction of the intrahepatic bile ducts, which leads to cirrhosis. While its pathogenesis remains unclear, PBC that shows histological progression to fibrosis carries a high risk of carcinogenesis; the same is true of viral liver diseases. In patients with PBC, the development of hepatocellular carcinoma is rare; the development of combined hepatocellular carcinoma and cholangiocellular carcinoma (cHCC-CCC) is extraordinary. Herein, we report a rare case of PBC metachronously complicated by cHCC-CCC and HCC, which, to the best of our knowledge, has never been reported.

Ide R, Oshita A, Nishisaka T, Nakahara H, Aimitsu S, Itamoto T. Primary biliary cholangitis metachronously complicated with combined hepatocellular carcinoma-cholangiocellular carcinoma and hepatocellular carcinoma. *World J Hepatol* 2017; 9(36): 1378-1384 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i36/1378.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i36.1378

INTRODUCTION

Primary biliary cholangitis (PBC)[1] is a progressive cholestatic liver disease characterized by the presence of a highly specific antimitochondrial antibody, portal inflammation, and lymphocyte-dominated destruction of the intralobular bile ducts, which lead to cirrhosis. According to recent and relatively large cohort studies conducted in European countries, the United States and Japan, the development of hepatocellular carcinoma (HCC) is estimated to be 0.7%-3.6%; this frequency increases as histological stages progress[2]. While its pathogenesis remains unclear, PBC cases that display histological progression to fibrosis are at a high risk of carcinogenesis; the same is true of viral liver diseases[3,4]. Although some cases of PBC complicated by HCC have been reported[5-8], to our knowledge, a case of PBC with cholangiocellular carcinoma (CCC) has never been described. In patients with PBC, the development of combined hepatocellular carcinoma and cholangio cellular carcinoma (cHCC-CCC) is extremely rare[9]. Herein, we report a case of PBC metachronously complicated by cHCC-CCC and HCC.

CASE REPORT

A 74-year-old Japanese woman was diagnosed as PBC in her 40's by using blood tests. Imaging studies, including abdominal ultrasonography (US) and computed tomography (CT), and tumor markers consisting of alpha fetoprotein (AFP) and protein induced by vitamin K absence (PIVKA-II) were checked up every 6 mo to 12 mo[4]. She was admitted to our department for further management of an asymptomatic liver mass. The patient denied alcohol consumption. Hepatitis B virus antigen and anti-hepatitis C virus antibody tests were negative. Liver function test results, with daily intake of 600 mg of ursodeoxycholic acid, were stable. Serum levels of AFP, PIVKA-II, carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 19-9 and the L3 fraction of AFP were all within normal limits (Table 1).

Abdominal US, dynamic CT, and magnetic resonance imaging (MRI) showed a liver tumor of 15 mm in size in segment 8 of the liver. Since the tumor was located in the peripheral lesion and was in contact with the middle hepatic vein (MHV), we performed partial resection of the liver in segment 8 including partial resection of MHV. Hematoxylin-eosin (HE) staining revealed two components consisting of the trabecular type of HCC and CCC, resulting in the definitive diagnosis of cHCC-CCC. According to the classification for the severity of PBC[10,11], the hepatic parenchyma, excluding carcinomatous tissue, showed stage 3 PBC (Figure 1). In the immunohistochemistry, the component of HCC was negative for AFP but positive for cytokeratin (CK) 18 and hepatocyte, while that of CCC was positive for CK7 and CK19. The components of both HCC and CCC are positive for the epithelial cell adhesion molecule (EpCAM) (Figure 2).

One year after the initial hepatectomy, tumor marker levels for AFP, PIVKA-II, CEA and CA 19-9 were within normal limits; only AFP-L3 isoform level was elevated (Table 2). Dynamic CT and MRI showed a peripheral tumor of 10 mm in diameter in segment

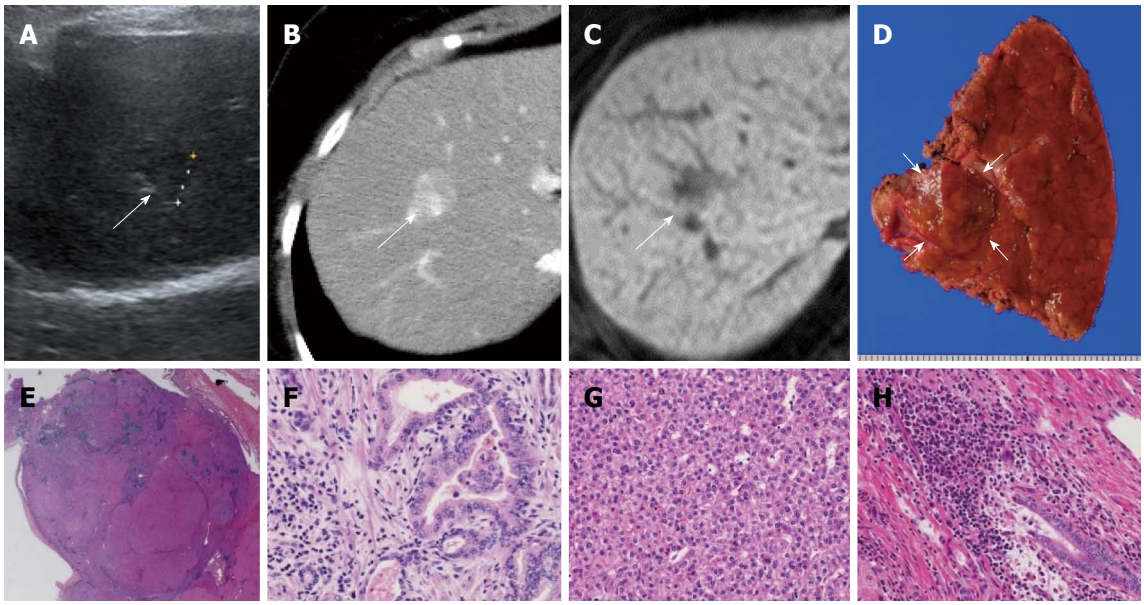

Figure 1 The initial tumor. A: Low-echoic tumor of 15 mm in size in segment 8 in US; B: The enhanced tumor on the early phase in dynamic CT; C: Low-intensity tumor on the hepatocyte phase in MRI; D: Cut surface of the 15-mm solid mass in segment 8; E: HE staining of the resected specimen; F: Adenocarcinoma in the component of CCC; G: HCC with a trabecular pattern; H: Dense fibrous tissue was formed and intrahepatic biliary ducts were showing destruction, while a loose lymphoid aggregate indicated stage 3 of primary biliary cirrhosis. CCC: Cholangiocellular carcinoma; CT: Computed tomography; HCC: Hepatocellular carcinoma; HE: Hematoxylin-eosin; MRI: Magnetic resonance imaging; US: Ultrasonography.

Table 1 Laboratory data on the initial hepatectomy

WBC	5800/μL	ALP	228 U/L	PIVKA-II	18 mAU/mL
RBC	432 × 10^4/μL	γ-GTP	65 U/L	AFP	3 ng/mL
Hb	13.0 g/dL	ChE	280 IU/L	AFP-L3	0.5%
Ht	38%	BUN	14.5 mg/dL	CEA	1.2 ng/mL
Plt	22.6 × 10^4/μL	Cr	0.54 mg/dL	CA 19-9	7 U/mL
PT	77.3%	T-Chol	203 mg/dL	ANA	× 40
PT-INR	1.04	TG	77 mg/dL	AMA	× 640
TP	7.9 g/dL	ICG-R15	8.3%	AMA-M2	158 Index
Alb	4.2 g/dL	Glucose	109 mg/dL	HBs Ag	(-)
TBil	0.5 mg/dL	CRP	0.2 mg/dL	HBs Ab	(-)
AST	19 U/L	IgG	1760 mg/dL	HBc Ab	(-)
ALT	14 U/L	IgM	305 mg/dL	HCV Ab	(-)
LDH	183 U/L				

AFP: Alpha-fetoprotein; AFP-L3: A Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein; Alb: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AMA: Antimitochondrial antibody; AMA-M2: Anti-mitochondrial M2 antibody; ANA: Antinuclear antibodies; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; CA 19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; ChE: Cholinesterase; Cr: Creatinine; CRP: C-reactive protein; γ-GTP: Gamma-glutamyltransferase; Hb: Hemoglobin; HBcAb: Hepatitis B core antibody; HBsAb: Hepatitis B surface antibody; HBsAg: Hepatitis B virus antigen; HCVAb: Hepatitis C virus antibody; Ht: Hematocrit; ICG-R15: 15-min retention rates of indocyanine green test; IgG: Immune globulin G; IgM: Immune globulin M; LDH: Lactate dehydrogenase; PIVKA-II: Prothrombin-induced vitamin K absence II; Plt: Platelet; PT: Prothrombin time; PT-INR: Prothrombin time international normalized ratio; RBC: Red blood cell count; TBil: Total bilirubin; T-Chol: Total cholesterol; TG: Triglyceride; TP: Total protein; WBC: White blood cell count.

5 of the liver. Since it was not possible to detect the tumor with intraoperative US, partial resection of the liver on the basis of the anatomical structure, including the Glissonean sheath and the hepatic vein, was performed. HE staining revealed a pseudoglandular pattern of HCC (Figure 3). In the immunohistochemistry, recurrent HCC was negative for AFP and EpCAM but positive for CK18 and hepatocyte (data not shown). There was no recurrence and/or metastasis 10 mo after re-hepatectomy.

DISCUSSION

While some cases of PBC complicated by HCC have been reported[5-8], only 1 case of PBC with cHCC-CCC has been reported[9]. The present case of PBC was metachronously complicated by both cHCC-CCC and HCC; to the best of our knowledge, such a case has never been reported.

While the etiology of PBC remains unknown, it is well known that the intrahepatic bile ducts are to be destructed slowly and progressively, leading to cirrhosis[12]. PBC

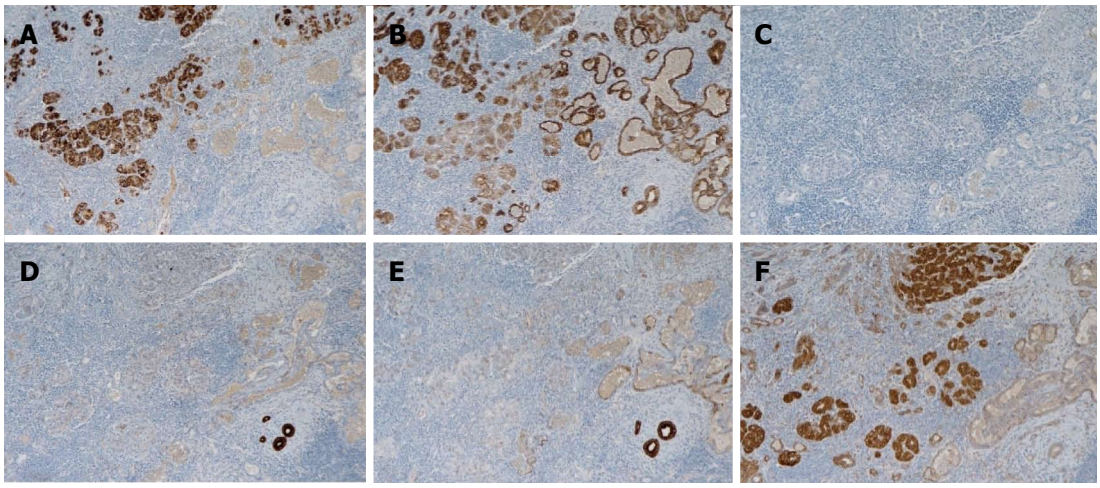

Figure 2 Immunohistochemistry findings for the initial tumor. A: HCC component stained positive for hepatocyte; B: HCC component stained positive for CK18; C: Both HCC and CCC components stained negative for alpha fetoprotein; D: CCC component stained positive for CK7; E: CCC component stained positive for CK19; F: Epithelial cell adhesion molecule stained positive for the HCC component and weakly positive for the CCC component. CCC: Cholangiocellular carcinoma; CK: Cytokeratin; HCC: Hepatocellular carcinoma.

Table 2 Laboratory data on the re-hepatectomy

WBC	3600/μL	AST	29 U/L	PIVKA-II	28 mAU/mL
RBC	397 × 10^4/μL	ALT	18 U/L	AFP	5 ng/mL
Hb	12.0 g/dL	LDH	186 U/L	AFP-L3	11.7%
Ht	35.9%	ALP	300 U/L	CEA	1.0 ng/mL
Plt	22.3 × 10^4/μL	γ-GTP	79 U/L	CA 19-9	29 U/mL
PT	77.3%	ChE	211 IU/L	ICG-R15	7.4%
PT-INR	1.12	BUN	16.1 mg/dL	Glucose	138 mg/dL
TP	7.3 g/dL	Cr	0.6 mg/dL	CRP	0.2 mg/dL
Alb	3.8 g/dL	T-Bil	0.4 mg/dL		

AFP: Alpha-fetoprotein; AFP-L3: A Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein; Alb: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; ChE: Cholinesterase; Cr: Creatinine; CRP: C-reactive protein; γ-GTP: Gamma-glutamyltransferase; Hb: Hemoglobin; Ht: Hematocrit; ICG-R15: 15-min retention rates of indocyanine green test; LDH: Lactate dehydrogenase; PIVKA-II: Prothrombin-induced vitamin K absence II; Plt: Platelet; PT: Prothrombin time; PT-INR: Prothrombin time international normalized ratio; RBC: Red blood cell count; T.Bil: Total bilirubin; TP: Total protein; WBC: White blood cell count.

occurs more often in middle-aged women and is often asymptomatic in its early stage[13,14]. The frequency of HCC development in patients with PBC is estimated to be 0.7%-3.6%. While this frequency increases as the histological stages progress[2,5,6,9,11,15-20], the carcinogenic mechanism of primary liver cancer in PBC remains unclear. Although our patient's PBC progressed to stage 3 of 4, when primary liver cancer was found, she had no liver cirrhosis symptoms.

Few studies have evaluated the imaging characteristics of cHCC-CCC, and no studies have evaluated the ability of preoperative imaging to determine diagnosis. The appearance of HCC and CCC is well known on contrast-enhanced MRI and CT. The histological composition and relative ratio of CCC and HCC components within cHCC-CCC appear to dictate the imaging appearance. Tumors may show features typical of HCC, such as arterial enhancement, washout and pseudocapsule, whereas other regions within the tumor show progressive or delayed enhancement, necrosis and possible ductal dilation more akin to CCC[21]. The cHCC-CCC display enhancement patterns resembling CCC or HCC in comparable proportion on both contrast-enhanced US and CT[22]. Some suggest that the combination of imaging features and tumor markers may be helpful in preoperative diagnosis of cHCC-CCC[23]. In our case, since dynamic CT showed arterial enhancement and washout imaging, we performed initial hepatectomy expected for HCC.

Allen *et al*[24] classified cHCC-CCC into three subtypes: type A, "double cancer" representing cases in which HCC and CCC exist separately; type B, "combined" type, HCC and CCC components existing contiguously, but independently; and type C, "mixed" type, consisting of truly combined HCC and CCC components originating from the same tumor. Based on the morphological findings from HE staining, the present case was classified as mixed type cHCC-CCC.

In recent years, the ability of hepatic precursor cells to differentiate into hepatocytes and bile duct cells,

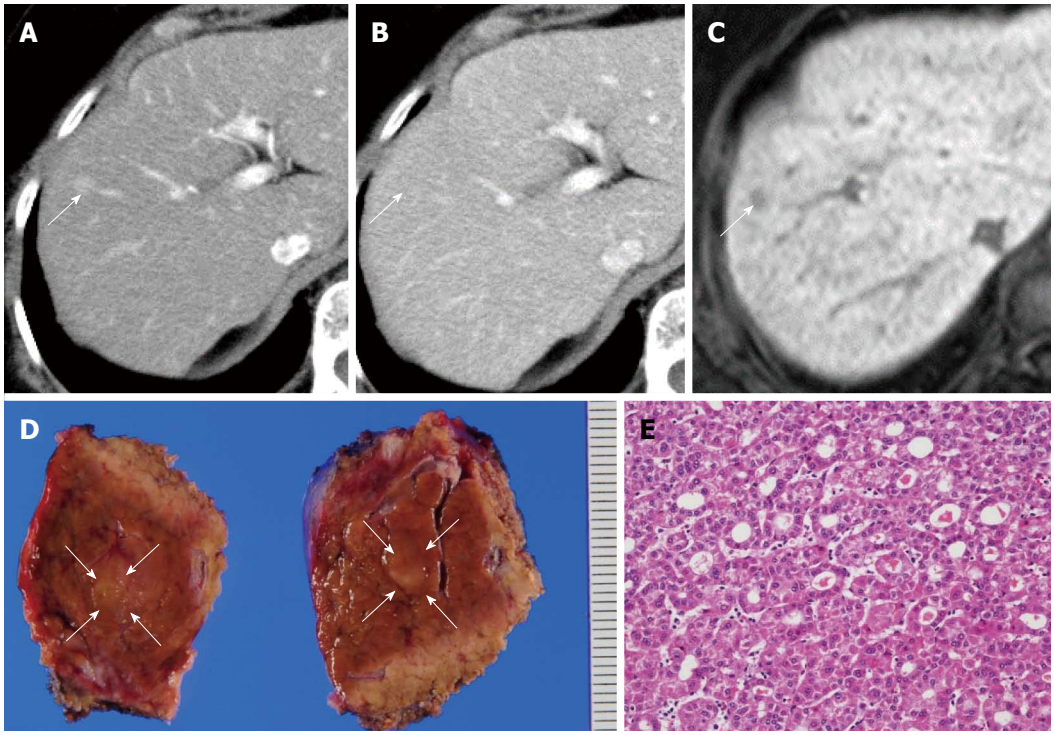

Figure 3 The second tumor. A: The enhanced tumor of 10 mm in diameter in segment 5 on the early phase in dynamic CT; B: The iso-density tumor on the delayed phase; C: Low intensity tumor on the hepatocyte phase in MRI; D: Cut surface of the 10-mm solid mass in segment 5; E: HE staining showing a pseudoglandular pattern of HCC. CT: Computed tomography; HCC: Hepatocellular carcinoma; HE: Hematoxylin-eosin; MRI: Magnetic resonance imaging.

and of hepatic stem cells to proliferate and differentiate have been proposed. As candidate stem cells, cells derived from the Herring duct or small oval cells may be able to differentiate into hepatocytes and bile duct cells[25-27]. Carcinogenesis of the precursor cells has been suggested as a developmental mechanism for cHCC-CCC with tissue components of HCC and CCC. In the present case, as Theise *et al*[28] indicated, the result of EpCAM immunohistochemistry (a stem cell marker), might be consistent with that of mixed type cHCC-CCC.

The pathological results of the initial tumor showed the trabecular pattern in the component of HCC, while that of the second tumor showed the pseudoglandular pattern in HCC. Immunohistochemistry also revealed the different pattern, which led the authors to speculate that the second tumor did not recur from the HCC component of cHCC-CCC, but the multicentric development of PBC-derived metachronous tumors.

In conclusion, we herein report a rare case of PBC metachronously complicated by both cHCC-CCC and HCC. In patients with PBC, it is necessary to check up not only liver function but also carcinogeneses, including HCC, CCC and cHCC-CCC.

ARTICLE HIGHLIGHTS

Case characteristics

A 74-year-old Japanese woman was diagnosed as primary biliary cholangitis (PBC) in her 40's by using blood tests. Imaging studies, including abdominal ultrasonography (US) and computed tomography (CT), and tumor markers consisting of alpha fetoprotein (AFP) and protein induced by vitamin K absence (PIVKA-II) were checked up every 6-12 mo. She was admitted to the authors' department for further management of an asymptomatic liver mass.

Differential diagnosis

Combined hepatocellular carcinoma and cholangiocellular carcinoma (cHCC-CCC), hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CCC) were considered from imaging tests.

Laboratory diagnosis

In the initial surgery, serum levels of AFP, PIVKA-II, carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 19-9, and the L3 fraction of AFP were all within normal limits. One year after the initial hepatectomy, tumor marker levels for AFP, PIVKA-II, CEA, and CA 19-9 were within normal limits; only AFP-L3 isoform level was elevated.

Imaging diagnosis

The authors diagnosed both the first and second tumors as HCC from the imaging findings.

Pathological diagnosis

First, hematoxylin-eosin (HE) staining revealed two components, consisting of the trabecular type of HCC and CCC, resulting in the definitive diagnosis of cHCC-CCC. Second, HE staining revealed a pseudoglandular pattern of HCC.

Treatment

The first one was that the tumor was involved in middle hepatic vein (MHV). If radiofrequency ablation was performed, the cooling effect around the MHV would have occurred, leading to the insufficient ablation. The second one was that the tumor was not detected using US preoperatively. Moreover, the tumor was not detected even with intraoperative contrast-enhanced US. Therefore,

the authors performed partial resection on the basis of the anatomical structure, including the Glissonean sheath and the hepatic vein.

Related reports

This report relates to this reference: Kobayashi M, Furuta K, Kitamura H, Oguchi K, Arai M, Koike S, Nakazawa K. A case of primary biliary cirrhosis that complicated with combined hepatocellular and cholangiocellular carcinoma. *Clin J Gastroenterol* 2011; 4: 236-241.

Term explanation

PBC: Primary biliary cholangitis, is marked by slow progressive destruction of the intrahepatic bile ducts, which leads to cirrhosis.

Experiences and lessons

In patients with PBC, it is necessary to check up not only liver function but also carcinogenesis including HCC, CCC and cHCC-CCC.

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CASE REPORT

Eosinophilic cholangitis treatment with budesonide

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Author contributions: De Roza MA and Lim CH designed the report and analyzed the data; Lim CH reported and monitored outcomes; De Roza MA wrote the report; Both De Roza MA and Lim CH made critical revisions before final approval of the report.

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Abstract

Eosinophilic cholangitis is a rare cause of deranged obstructive liver function tests. It has been described as a great mimicker for malignant biliary strictures and bile duct obstruction. There are only case reports available on treatment experience for eosinophilic cholangitis. A large proportion of patients present with biliary strictures for which they have undergone surgery or endoscopic treatment and a small proportion was given systemic corticosteroid. We share our treatment experience using budesonide which has fewer systemic side effects to prednisolone and avoids invasive management.

Key words: Eosinophilic cholangitis; Budesonide; Biliary stricture; Eosinophilia; Obstructive liver function test

Core tip: Eosinophilic cholangitis is a rare cause of obstructive liver function tests and secondary sclerosing cholangitis. Peripheral eosinophilia is the most useful laboratory hint for the diagnosis thus avoiding invasive endoscopic or surgical treatment. It is normally treated with a prolonged duration of corticosteroids, risking the development of corticosteroid adverse effects. We describe our successful experience with budesonide, an alternative treatment option which has a higher first pass effect resulting in fewer systemic side effects.

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INTRODUCTION

Eosinophilic cholangitis is a rare cause of obstructive liver function tests. It has been described as a great mimicker for malignant biliary strictures and bile duct obstruction. There are only case reports available on treatment experience for eosinophilic cholangitis. A large proportion of patients present with biliary strictures for which they

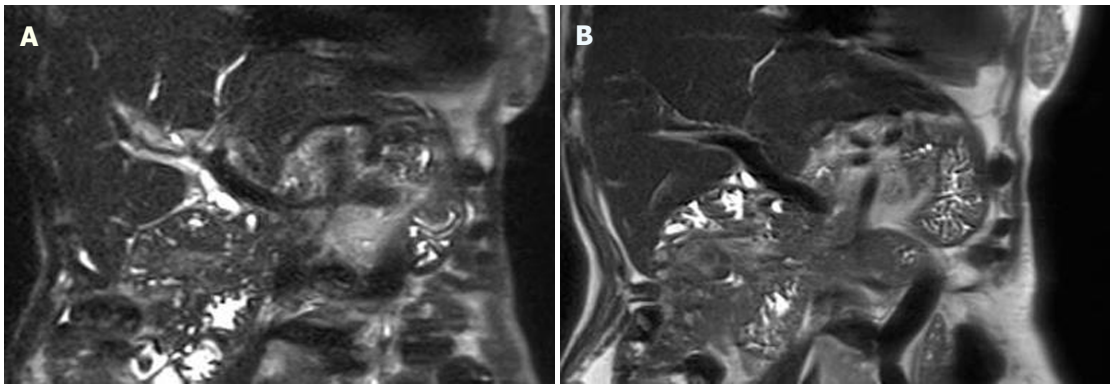

Figure 1 T2 magnetic resonance imaging. A: T2 magnetic resonance imaging segment Ⅷ biliary stricture before treatment; B: T2 magnetic resonance imaging after budesonide showing resolution of segment Ⅷ stricture.

have undergone surgery or endoscopic treatment. A smaller proportion was given corticosteroid treatment and most involved the use of systemic corticosteroids such as prednisolone.

CASE REPORT

Our patient is a 75-year-old Chinese retired lady. She does not smoke, consume alcohol or substances. Past medical history of note is hypertension and septic arthritis with a right first metatarsal osteomyelitis for which she underwent a Ray's amputation and was discharged to a step-down facility for slow stream rehabilitation.

She presented with deranged liver function tests (LFT), done during routine follow up at her rehabilitation centre. She was otherwise asymptomatic with no abdominal pain, fever, nausea, vomiting or diarrhoea. She did not take any supplements or over the counter medications. She was prescribed two weeks of antibiotics (one week of cefazolin followed by one week of oral Augmentin) for osteomyelitis which was treated with ray's amputation. However, her antibiotic course was completed almost 2 mo prior to presentation.

Her baseline LFT (taken during admission for osteomyelitis) was unremarkable except for a mildly raised Alkaline Phosphatase which we attributed to her bone infection. Her baseline LFT was as such: Albumin 38 g/L (normal range 40-51 g/L), bilirubin 11 μmol/L (normal range 7-32 μmol/L), alkaline phosphatase (ALP) 126 U/L (normal range 39-99 U/L), alanine aminotransferase (ALT) 14 U/L (normal range 6-66 U/L), aspartate aminotransferase (AST) 24 U/L (normal range 6-66 U/L).

She was referred to us 2 mo later with a predominantly cholestatic LFT and eosinophilia with markedly raised serum IgE levels. Her test results are as follows: Albumin 34 g/L, (normal range 40-51 g/L), bilirubin 20 μmol/L, (normal range 7-32 μmol/L), ALP 803 U/L, (normal range 39-99 U/L), ALT 234 U/L, (normal range 6-66 U/L), AST 145 U/L, (normal range 6-66 U/L), GGT 667 U/L (normal range 14-94 U/L), total leukocyte count 7.75 × 10^9/L (normal range 4.0-10.0^9/L), eosinophils 23.1% (normal range 0-6%), eosinophil absolute count 1.79 × 10^9/L (normal range 0.04-0.44 × 10^9/L), IgG, serum 12.08 g/L (normal range 5.49-17.11 g/L), IgA, serum 2.54 g/L (normal range 0.47-3.59 g/L), IgE, serum 1064 IU/ml (normal range 18-100 IU/mL).

Anti-MPO, Anti-PR3, Antinuclear Antibody, Anti Liver Antibodies (including M2, LKM-1, LC-1, SLA/LP) and Anti Smooth Muscle Antibody were all negative.

Serologies for hepatitis A, B, C, E and Human Immunodeficiency Virus were negative as well. Her renal function was normal.

She has no history of allergies or atopy and stool samples sent for parasites were negative twice. She had no new symptoms, had a good appetite without weight loss and was well and stable with no other organ involvement.

She underwent an ultrasound of the abdomen which showed a prominent pancreatic duct and biliary sludge in the gallbladder. It was normal otherwise with a negative sonographic Murphy's sign. There were no gallstones, no biliary tree dilation and the common bile duct (CBD) measured 5 mm.

She was further investigated with a magnetic resonance cholangiopancreatography (MRCP) which showed stones in the gallbladder with no evidence of cholecystitis. There was also prominence of the CBD at 9mm without a centrally obstructing stone, stricture or definite mass. The pancreatic duct was prominent with borderline dilated calibre but no obstructing lesion was detected. There were also several prominent/borderline dilated subsegmental ducts in segment VIII, V and II, and underlying strictures with mild periportal oedema (Figure 1).

Our patient went on to do an endoscopic ultrasound (EUS) for further evaluation of her CBD and PD prominence and exclude an ampullary lesion. The EUS showed a mildly thickened CBD wall which was unremarkable endosonographically. The biliary tree was not dilated. No intervention was done as there were no significant endosonographic abnormalities.

Our working diagnosis was Eosinophilic Cholangitis

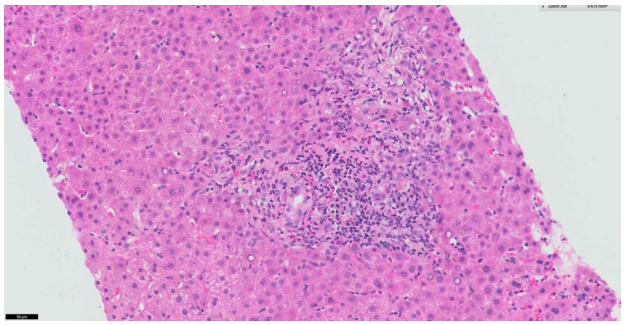

Figure 2 Histology from liver biopsy at 20 × magnification with HE staining: Portal and bile duct inflammation with up to 18 Eos/HPF. There is bile ductular proliferation and portal tract oedema.

in view of the biliary strictures and dilation seen on MRCP with eosinophilia and raised serum IgE.

We have excluded biliary stones and an ampullary tumour. Autoimmune and viral serology were also negative. Drug induced liver injury was unlikely as she had no exposure.

A liver biopsy was performed which confirmed portal and bile duct inflammation with a significant number of eosinophils of up to 18 per HPF (Figure 2). There was mild to moderate portal inflammatory cell infiltrate, predominantly composed of neutrophils and lymphocytes with moderate numbers of eosinophils. There was also bile ductular proliferation and portal tract oedema. No evidence of ductopenia, florid duct lesion, cholestasis, granuloma or neoplasia. Special stains did not show evidence of fibrosis. There was no conspicuous HBsAg, copper-associated protein, PASD positive or significant iron deposits. No increase in IgG4 positive cells were noted on immunohistochemistry.

Our patient was started on oral budesonide 9 mg/d. After one month of oral budesonide, her eosinophilia resolved and her LFT showed marked improvement with almost halved ALP (476 U/L) and ALT (125 U/L) values. Her LFT normalised after 6 mo. The patient declined a repeat liver biopsy but a repeat MRCP was done at 4 mo of treatment and showed overall improvement of the biliary dilation and strictures seen previously. Her oral budesonide was tapered down after 6 mo and subsequently discontinued after 9 mo.

DISCUSSION

Eosinophilic cholangitis (EC) is an uncommon and unknown cause of indeterminate biliary stricture and there is no consensus on a diagnostic criterion available. Matsumoto *et al*[1] proposed the following findings to diagnose EC: (1) Wall thickening or stenosis of the biliary system; (2) histopathological findings of eosinophilic infiltration; and (3) reversibility of biliary abnormalities without treatment or following steroid treatment.

The degree of eosinophilic infiltration has not been established either. In fact, there are case reports of Eosinophilic cholangitis with normal liver biopsies[2]. As a general guideline, Eos/HPF are significant when > 15 in the gastrointestinal tract but this has not been specified in EC[3]. Peripheral eosinophilia and obstructive liver function tests results are helpful laboratory findings to consider the diagnosis of EC. However, peripheral eosinophilia is only present in about two-third of cases[4].

A review of 23 cases of eosinophilic cholangitis showed that eight (34.8%) had complete resolution of symptoms with surgery alone and seven (30.4%) improved with the use of oral corticosteroids. The remaining six cases needed a combination of surgery and oral corticosteroids for resolution[4]. Most treatment experience with steroids for eosinophilic cholangitis was with prednisolone.

Budesonide is a corticosteroid immunosuppressive agent that results in interference with cytokine production and inhibition of T lymphocyte activation. It is a second-generation corticosteroid with an affinity for the glucocorticoid receptor that is approximately 15 times greater than that of prednisolone. When taken orally, it has a 90% first-pass metabolism in the liver, allowing it to reach high intrahepatic concentrations before its elimination, significantly limiting its systemic effects[5]. Budesonide has been compared to prednisolone and found to be more effective with fewer adverse effects than prednisolone for liver specific disease such as autoimmune hepatitis[6]. It is prescribed at a dose of 9 mg once a day and shown to be effective in patients with active Crohn's disease and autoimmune hepatitis[7]. Hence, we chose to use budesonide at a dose of 9 mg once a day for our patient based on known evidence of its efficacy at this dose.

EC is a benign condition and should be managed with a trial of corticosteroids before considering more invasive treatment. A recent retrospective study showed an EC prevalence of 2.2% from a cohort of 135 cases of sclerosing cholangitis and post-hoc diagnosis of EC was ascertained in 30% (3/10) of patients where no cause of indeterminate biliary stricture was identified[8]. Our patient was on oral budesonide treatment for 9 mo with biochemical resolution of her eosinophilia and liver function test. She did not exhibit adverse effects from budesonide therapy on outpatient follow up. This case report is the first, to our knowledge, to treat EC with budesonide.

ARTICLE HIGHLIGHTS

Case characteristics

Deranged liver function test with a cholestatic pattern, eosinophilia, raised IgE, intrahepatic biliary stricture.

Clinical diagnosis

Eosinophilic cholangitis.

Differential diagnosis

Biliary stone, pancreaticobiliary malignancy, drug induced liver injury.

Laboratory diagnosis

Eosinophilic cholangitis.

Imaging diagnosis
Biliary stricture and dilation.

Pathological diagnosis
Eosinophilic cholangitis.

Treatment
Budesonide 9 mg once a day.

Related reports
There are no previous reports of treating eosinophilic cholangitis with Budesonide. But there are reports of successful treatment with prednisolone. Please see reference No. 2.

Experiences and lessons
This is a rare case of eosinophilic cholangitis and the first time in literature, to be successfully treated with budesonide. The patient did not experience any side effects or steroid toxicity. In the future, with further evidence, budesonide might be a reasonable first line treatment for eosinophilic cholangitis as it is safer than prednisolone.

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