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Editorial board member of *World Journal of Gastroenterology*, José Manuel Ramia, MD, PhD, Associate Professor, Department of Surgery, Hospital Universitario de Guadalajara, Guadalajara 19002, Spain

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Ze-Mao Gong, Director
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Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
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REVIEW

Review article: Update on current and emergent data on hepatopulmonary syndrome

Stergios Soulaidopoulos, Evangelos Cholongitas, George Giannakoulas, Maria Vlachou, Ioannis Goulis

Stergios Soulaidopoulos, Ioannis Goulis, Fourth Department of Internal Medicine, Hippokration General Hospital, Medical School of Aristotle University of Thessaloniki, Thessaloniki 54642, Greece

Evangelos Cholongitas, First Department of Internal Medicine, Laiko General Hospital, Medical School of National and Kapodistrian University of Athens, Athens 11527, Greece

George Giannakoulas, Maria Vlachou, Department of Cardiology, AHEPA University Hospital, Medical School of Aristotle University of Thessaloniki, Thessaloniki 54621, Greece

ORCID number: Stergios Soulaidopoulos (0000-0003-4150-5286); Evangelos Cholongitas (0000-0002-3645-582X); George Giannakoulas (0000-0002-2358-709X); Maria Vlachou (0000-0002-8004-2184); Ioannis Goulis (0000-0002-2765-4157).

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Correspondence to: Evangelos Cholongitas, MD, PhD, Associate Professor, First Department of Internal Medicine, Medical School of National & Kapodistrian University of Athens, Laiko General Hospital, Agiou Thoma 17, Athens 11527, Greece. cholongitas@yahoo.gr
Telephone: +30-6936-378903
Fax: +30-2310-992940

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Abstract

Hepatopulmonary syndrome (HPS) is a frequent pulmonary complication of end-stage liver disease, characterized by impaired arterial oxygenation induced by intrapulmonary vascular dilatation. Its prevalence ranges from 4% to 47% in patients with cirrhosis due to the different diagnostic criteria applied among different studies. Nitric oxide overproduction and angiogenesis seem to be the hallmarks of a complicated pathogenetic mechanism, leading to intrapulmonary shunting and ventilation-perfusion mismatch. A classification of HPS according to the severity of hypoxemia has been suggested. Contrast-enhanced echocardiography represents the gold standard method for the detection of intrapulmonary vascular dilatations which is required, in combination with an elevated alveolar arterial gradient to set the diagnosis. The only effective treatment which can modify the syndrome's natural history is liver transplantation. Although it is usually asymptomatic, HPS imparts a high risk of pretransplantation mortality, independently of the severity of liver disease, while there is variable data concerning survival rates after liver transplantation. The potential of myocardial involvement in the setting of HPS has also gained increasing interest in recent research. The aim of this review is to critically approach the existing literature of HPS and emphasize

unclear points that remain to be unraveled by future research.

Key words: Hepatopulmonary syndrome; Liver cirrhosis; Liver transplantation; Portal hypertension; Contrast echocardiography

Core tip: Hepatopulmonary syndrome (HPS) constitutes a relatively frequent complication of end-stage liver disease, characterized by impairment of arterial oxygenation. The only effective treatment is liver transplantation, improving hypoxemia. While there are controversial data regarding HPS prognosis before and after liver transplantation, the question remains whether HPS constitutes an independent factor of morbidity, providing HPS patients priority for liver transplantation. Furthermore, possible associations with myocardial function, which could support the utility of echocardiographical parameters as markers of HPS, remain yet to be established.

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INTRODUCTION

Liver cirrhosis is often accompanied by complications from the pulmonary system. These include hepatic hydrothorax, portopulmonary hypertension and hepatopulmonary syndrome (HPS). Hepatic hydrothorax affects approximately 6%-10% of patients with end-stage liver disease and is the result of ascetic fluid passage to the pleural space through diaphragmatic defects[1]. Portopulmonary hypertension is characterized by pulmonary vasoconstriction and increased vascular resistance, developing in 2%-8.5% of patients with portal hypertension, combined with poor prognosis[2].

HPS constitutes a pulmonary disorder of chronic liver disease, characterized by poor arterial oxygenation and intrapulmonary vascular dilatations[3]. Although Fluckiger was the first to describe the syndrome in 1884, treating a woman with liver cirrhosis and cyanosis without any other obvious reason for pulmonary disease, the term "Hepatopulmonary Syndrome" was suggested in 1977 by Kennedy and Knudson[4]. Former autopsy studies had previously demonstrated the potential role of pulmonary vascular dilatations in the development of the syndrome[5,6].

The revised diagnostic criteria for HPS comprise the triad of chronic liver disease, pulmonary vascular dilatation and gas exchange abnormalities in the absence of other causes of impaired pulmonary function[7]. Except for chronic liver disease, HPS can coexist with acute or chronic hepatitis, portal hypertension without liver disease, alpha 1 antitrypsin deficiency, Wilson's disease and Abernathy malformation[8,9]. Defining gas exchange abnormalities, an increased alveolar-arterial oxygen gradient (> 15 mmHg or > 20 mmHg for age > 65 years) was suggested as a more sensitive marker of impaired pulmonary function in cirrhotic patients[3]. The presence of intrapulmonary dilatations can be assessed by several methods, but contrast-enhanced echocardiography with agitated saline is considered the gold standard technique[7].

The aim of this review is to provide a critical overview on prevalence, pathogenesis, diagnosis, clinical manifestations, treatment options and current data regarding prognosis before and after liver transplantation in patients with HPS. Upcoming data suggest remarkable associations between the presence of HPS and specific serum markers, clinical signs and echocardiographic parameters which are worthy of discussion.

SEARCH STRATEGY

A literature search was conducted using the online databases Medline, Embase and Scopus until January 2017 for original research papers and review articles concerning pathogenesis, clinical manifestations, diagnosis and management of HPS. Studies evaluating myocardial function in the setting of HPS were also included. The combination of the following terms was used to identify relevant publications: "liver cirrhosis" OR "prevalence" OR "diagnosis" OR "vasodilatation" OR "clinical features" OR "orthodeoxia" OR "platypnea" OR "treatment" OR "liver transplantation" OR "cardiac involvement" OR "myocardial function" AND "hepatopulmonary syndrome". The collected literature was examined for cited articles relevant to the subject to ensure that no important research data were missed. Articles that had been published as full journal articles in English were included. The above terms were used in ClinicalTrials.gov to search for recently completed or ongoing trials on HPS. Not accessible abstracts, conference proceedings or articles not translated in English were excluded.

PREVALENCE AND SEVERITY

Previous studies have used different criteria in terms of diagnostic methodology for HPS. More specifically, different thresholds for alveolar-arterial gradient and partial pressure of oxygen (PaO_2) have been used in order to define gas-exchange abnormalities, leading to a wide range of HPS prevalence rates[10]. Furthermore, different diagnostic methods have been performed to evaluate intrapulmonary dilatations. Based on

Table 1 Hepatopulmonary syndrome-diagnostic criteria

Presence of liver disease and/or portal hypertension AND
Partial pressure of oxygen < 80 mmHg or alveolar–arterial oxygen gradient [P(A-a)O_2 gradient] ⩾ 15 mmHg (or > 20 mmHg for patients > 65-years-old) while breathing ambient air AND
Documented intrapulmonary vascular dilatation by contrast-enhanced echocardiography or lung perfusion scanning with radioactive albumin

Table 2 Hepatopulmonary syndrome-severity classification

Mild	Alveolar-arterial oxygen gradient ⩾ 15 mmHg, partial pressure of oxygen ⩾ 80 mmHg
Moderate	Alveolar-arterial oxygen gradient ⩾ 15 mmHg, partial pressure of oxygen ⩾ 60 mmHg to < 80 mmHg
Severe	Alveolar-arterial oxygen gradient ⩾ 15 mmHg, partial pressure of oxygen ⩾ 50 mmHg to < 60 mmHg
Very severe	Alveolar–arterial oxygen gradient ⩾ 15 mmHg, partial pressure of oxygen < 50 mmHg

reports from several liver transplantation centers, the prevalence of HPS ranges from 4% to 47% in patients with liver cirrhosis[11-14]. The introduction of specific diagnostic criteria (Table 1), including the definition of impaired oxygenation, by the European Respiratory Society Task Force in 2004, provides the opportunity to obtain comparable results from recent studies[3]. The establishment of alveolar-arterial gradient as a more sensitive marker of pulmonary function as well as the screening of asymptomatic patients has led to higher rates of HPS diagnosis. Nevertheless, further well-designed, prospective, multicenter studies are needed for more accurate estimation of the syndrome's prevalence. Interestingly, intrapulmonary vascular dilatations can be detected in 13%-80% of liver transplantation candidates regardless of the development of arterial oxygenation abnormalities[15].

The evaluation of PaO_2 in the arterial blood is crucial for classification of the syndrome. According to arterial blood gas analysis, four severity stages of HPS can be distinguished while the patient is breathing ambient air (Table 2): mild (PaO_2 ⩾ 80 mmHg), moderate (PaO_2 ⩾ 60 and < 80 mmHg), severe (PaO_2 ⩾ 50 to < 60 mmHg), and very severe (PaO_2 < 50 mmHg)[2]. The existing data suggest that the majority of HPS patients are mild or moderate stage, while severe and very severe cases seem to be less common[16,17]. No associations have been demonstrated between the presence or severity of HPS and the severity of liver disease[18]. However, there is restricted data concerning HPS severity assessment, highlighting the need for well-designed HPS protocols in future studies.

PATHOGENESIS AND PATHOPHYSIOLOGY

Intrapulmonary capillary vasodilatations constitute the main anatomic disturbance of HPS leading to impaired arterial oxygenation through ventilation-perfusion mismatch[3,19]. The diameter of the dilated vessels may vary from 15-100 μm and in some cases to 500 μm when HPS is present, whereas normally it ranges between 8 μm and 15 μm[20,21]. Dilatation of pre-capillary and capillary vessels in combination with reduced or absent tone of pulmonary vasculature result in increased pulmonary blood flow, which is also boosted by hyperdynamic circulation in liver disease. In this way, there is an overperfusion of the alveolar capillary bed combined with a decrease in transit time of red blood cells, while ventilation remains unchanged. As a result, an excessive amount of blood passes through the pulmonary circulation without completing gas exchange, leading to increased alveolar arterial gradient and arterial hypoxemia[22], particularly during muscular activity[23].

Oxygen molecules have to cross a longer distance in less time to reach red blood cells in the center of the pulmonary capillaries due to vascular dilatation[24], while an increase in pulmonary capillary wall thickness has also been observed[21,25]. This alteration in oxygen diffusion contributes in the impaired oxygenation of HPS and could be correlated to the abnormal values of carbon monoxide diffusing capacity observed in these patients[14].

Intrapulmonary arteriovenous shunting constitutes another mechanism causing arterial hypoxia in HPS[6]. Mixed blood passes through pleural and pulmonary arteriovenous communications directly into the central circulation, without coming in touch with the alveoli. A few portopulmonary vascular communications can also be observed. The presence of more pronounced vascular dilatations and arteriovenous communications in lower lung zones, as it was suggested by thoracic computed tomography scans, may interpret the mechanism of orthodeoxia, *i.e.* reduction of PaO_2 from supine to upright patient position[26]. Gravitational pulmonary blood flow redistribution leads to overperfusion of these lower lung zones and increased intrapulmonary shunting, perhaps due to a more altered, maladjusted vascular tone[27].

It seems that the severity of arterial hypoxemia is related to the extent of ventilation-perfusion mismatch, intrapulmonary shunting and diffusion impairment[28]. Administration of 100% oxygen [⩾ 300 mmHg (40.0 kPa)] may improve hypoxia in some cases of HPS, as it provides enough pressure to partly overcome the diffusing limitation arising from the dilated pulmonary

vessels[29,30]. However, there is no effect in partial pressure of oxygen when hypoxia is the result of excessive arteriovenous blood shunting.

Pulmonary vasodilatation

Intrapulmonary vascular dilatations seem to be the result of an imbalance between several vasodilators and vasoconstrictors. Much of our knowledge arises from studies on rat experimental models, in which a common bile duct ligation has been performed in order to develop secondary biliary cirrhosis. The increased production of nitric oxide (NO) and carbon monoxide (CO), two pulmonary vasodilators, constitutes the key process for the development of pulmonary vasodilatation[31]. In common bile duct ligation animal models, the proliferation of cholangiocytes is followed by production and secretion of endothelin-1 (ET-1) after the stimulation by transforming growth factor beta-1 (TGFβ-1)[32,33]. The binding of endothelin-1 to its pulmonary receptor ET-1B triggers the activation of endothelial and inducible nitric oxide synthase (eNOS and iNOS) resulting in elevated NO production and NO-induced pulmonary vasodilatation[34,35]. The selective up-regulation of pulmonary ET-1B receptor in response to ET-1 biliary production in experimental portal hypertension has also been suggested[36]. In addition, levels of eNOS and iNOS protein are increased in HPS cirrhotic rats[37,38], while elevated levels of exhaled NO in HPS patients seem to return to normal after liver transplantation[39,40]. Furthermore, NO inhibition by methylene blue administration transiently improves oxygenation, whereas NG-nitro-L-arginine methylester, *via* iNOS inhibition, did not prove to affect hypoxemia of HPS[41-43]. Interestingly, a recent biopsy study comparing explanted livers from 76 patients with cirrhosis found that focal parenchyma extinction as well as vascular lesions, such as intrahepatic portal vein thrombosis, thickening or obstruction of centrilobular veins and sinusoidal proliferation, were more prevalent in those patients with HPS compared to those without, suggesting an association between liver ischemia and the production of proangiogentic and vasodilatation factors[44].

In patients with liver dysfunction, activation and massive accumulation of intravascular macrophages is observed as a result of intestinal bacterial translocation and endotoxemia[45-47]. These macrophages in the pulmonary vasculature produce proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), contributing in the NO-mediated vasodilatation through iNOS activation. Furthermore, ET-1 seems to promote the accumulation of pulmonary monocytes[48]. In support of this theory, TNF-α inhibition by pentoxifylline administration has been shown to improve HPS in rat experimental models[49,50]. Norfloxacin also improved HPS through a reduction in intestinal bacterial load and bacterial translocation[51].

CO produced from the degradation of heme by heme oxygenase, may act as a vasodilator in HPS patients. The latter have elevated levels of arterial carboxyhemoglobin reflecting CO production[52]. Both bacterial accumulation and NO synthesis stimulate heme oxygenase expression[47,53]. Finally, administration of protoporphyrin IX, an inhibitor of heme oxygenase, seems to improve HPS hypoxemia[54].

Angiogenesis

Beside NO-mediated vasodilatation, angiogenesis is considered another crucial mechanism interpreting HPS pathogenesis. Intestinal bacterial translocation and the consequent endotoxemia due to liver dysfunction lead to the recruitment of monocytes and activated macrophages to the lung. These inflammatory cells together with circulating TNF-α stimulate the activation of vascular endothelial growth factor (VEGF) signaling pathways, which are related to angiogenesis[55,56]. The accumulation of CD68+ macrophages in the lungs of common bile duct ligation rats, expressing iNOS and VEGF, has been correlated to the presence of HPS[57]. Remarkably, increased endothelial tube formation and pulmonary artery smooth muscle cell proliferation in HPS plasma was observed. The depletion of CD68+ macrophages improved both histological and hemodynamic features of HPS, while iNOS inhibition disclosed exaggerated vasoconstrictor responses.

TNF-α neutralization in cirrhotic rats has been shown to decrease intrapulmonary shunt as well as alveolar-arterial O_2 gradient[49,58]. The role of specific chemokines, such as the circulating chemokine ligand 1(CX3CL1), in the activation of VEGF is also under investigation[59,60]. Anti-VEGF therapy with sorafenib administration, a kinase inhibitor, was found to improve HPS hypoxia and restrict VEGF-mediated angiogenesis and intrapulmonary shunting in rats with biliary cirrhosis[33,61,62]. Besides, it was recently demonstrated that HPS is independently associated with the presence of hepatocellular carcinoma, an entity also characterized by extensive angiogenesis and VEGF production[62]. Although it can be postulated that VEGF constitutes a regulator of angiogenesis with a possible role in the development of HPS, further studies with measurements of VEGF are needed to unravel the exact pathogenetic pathways. Figure 1 schematically summarizes the main events in HPS pathogenesis.

CLINICAL FEATURES

Progressive dyspnea is the most frequent symptom among HPS patients[63]. In a large cohort of patients listed for liver transplantation, it was found that dyspnea was significantly more frequent in patients with HPS than in those without HPS[64]. However, dyspnea is not specific for HPS, as it is common between patients with liver disease due to complications such as anemia, ascites, hydrothorax and muscular cachexia. Furthermore, HPS can also be asymptomatic, especially

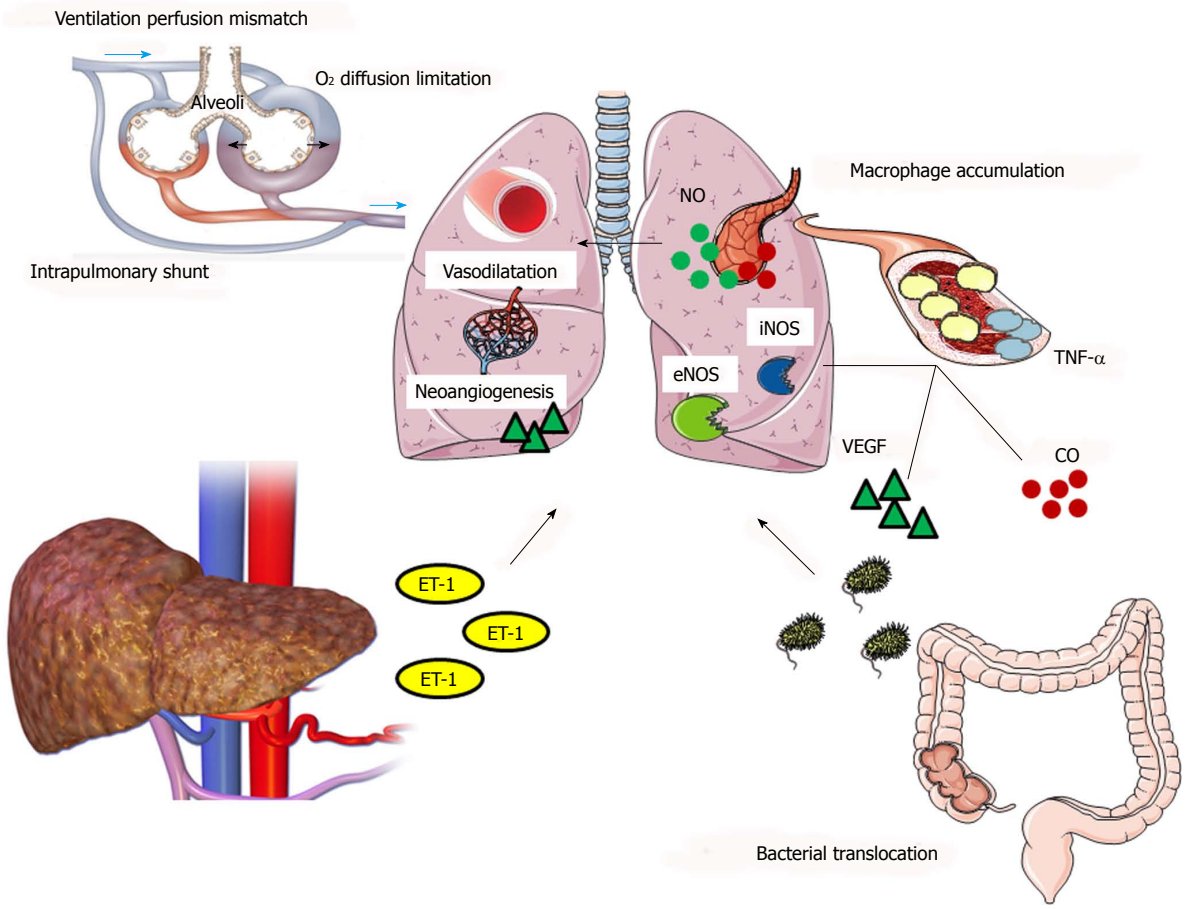

Figure 1 Schematic overview of the main pathways of the pathogenesis of hepatopulmonary syndrome. Liver cirrhosis and portal hypertension lead to endothelin-1 (ET-1) secretion. The binding of ET-1 to its receptor, activates pulmonary endothelial nitric oxide synthase (eNOS), leading to excessive production of nitric oxide (NO), a natural vasodilator. Bacterial translocation and the subsequent pulmonary macrophage accumulation result in the production of inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), which contribute in NO-mediated vasodilatation through inducible nitric oxide synthase (iNOS)-enhanced expression. Carbon monoxide constitutes another pulmonary vasodilator produced by macrophage-induced heme oxygenase-1 (HO-1) increased expression. Pulmonary macrophage accumulation and TNF-α-increased circulation trigger vascular endothelial growth factor (VEGF) pathways, concluding in VEGF-mediated pulmonary angiogenesis. Mixed venous blood passes rapidly, due to hyperdynamic circulation observed in liver cirrhosis, through the dilated capillaries without completing gas exchange. An oxygen (O_2) diffusion limitation occurs, as O_2 molecules need to cross a longer distance to reach the center of dilated vasculature. As a result, there is an impairment of arterial oxygenation due to ventilation perfusion mismatch, also boosted by direct right-to-left shunt through arteriovenous communications.

in those with mild hypoxia and alveolar arterial gradient disturbance, with dyspnea observed more frequently in HPS patients with PaO_2 lower than 70 mmHg[10].

Another form of dyspnea, platypnea, is considered to be pathognomonic for HPS[65]. Platypnea is the condition of worsening dyspnea when patient moves from a supine to an upright position. It is the result of the decrease in PaO_2 in the arterial blood of $\geqslant$ 5% (or $\geqslant$ 4 mmHg) from supine to upright position due to increased perfusion of the basis of the lungs and elevated intrapulmonary shunting, a phenomenon called orthodeoxia[27]. Orthopnea, the worsening of dyspnea in lying position, has also been observed more frequently in patients with HPS[64].

Cyanosis, fatigue, spider naevi and digital clubbing are other clinical findings of HPS[63]. Spider angiomas have been suggested as cutaneous markers of HPS, possibly sharing the same pathogenetic mechanism with HPS, *i.e.* imbalance between vasoconstrictor and vasodilator substances[66]. In addition, digital clubbing has a positive predictive value of 75% in HPS diagnosis[67]. In the same study, dyspnea showed a negative predictive value of 75% in HPS diagnosis, whereas no correlation was found between HPS and splenomegaly, ascites, edema, jaundice, oliguria, and collateral veins. Oxygen desaturation during sleep was also correlated to the presence of HPS in another study[68].

Although none of the aforementioned clinical signs are considered to be specific for HPS and the majority of patients may not present any characteristic symptoms, HPS patients seem to have a worse quality of life and higher New York Heart Association functional class compared to patients without HPS[64]. Therefore, once again there is need for further larger studies to investigate thoroughly the exact clinical features that

may be related to HPS.

DIAGNOSIS

According to the European Respiratory Society Task Force in 2004, HPS diagnosis consists of the following criteria: (1) the presence of liver disease and/or portal hypertension; (2) elevated room air alveolar arterial oxygen gradient (≥ 15 mmHg or ≥ 20 mmHg in patients > 64-years-old; and (3) evidence of intrapulmonary vascular dilatations[3]. Diagnosis should be based on arterial blood gas analysis and alveolar arterial gradient calculation rather than a simple assessment of arterial hypoxemia. Several techniques have been developed for the evaluation of intrapulmonary vasodilatation, but contrast-enhanced echocardiography with agitated saline is considered the gold standard. Modern imaging techniques are also useful for the verification of pulmonary vascular dilatation and right-to-left communications as well as for the exclusion of other pulmonary complications associated with liver disease or lung disease that may coexist with HPS. Furthermore, pulmonary function tests are also valuable to detect abnormalities that may be indicative of HPS or helpful to unmask other underlying lung or cardiac diseases.

The fact that most HPS patients are asymptomatic or manifest nonspecific symptoms in combination with the application of different diagnostic criteria has led to an underestimation of the syndrome in the past. As there is lack of a reliable and simple screening method for diagnosis of HPS, liver transplantation centers should adopt strict diagnostic protocols using unified criteria in order to detect all HPS cases and export comparable results.

Intrapulmonary vascular dilatations

Contrast-enhanced transthoracic echocardiography with agitated saline is considered the cornerstone in the detection of pulmonary vascular dilatations[69]. Normal saline is shaken to produce microbubbles > 10 μm in diameter and is administered to a peripheral vein in the arm while a four-chamber transthoracic echocardiography is performed. Microbubbles are normally trapped in the pulmonary circulation and absorbed by the alveoli as they cannot pass through normal capillaries. However, in the presence of a dilated vascular bed and/or arteriovenous shunting, microbubbles elude pulmonary capture and reach the left cardiac chambers. Left atrial opacification with microbubbles between the fourth and sixth cardiac cycle after the repletion of the right atrial is indicative of intrapulmonary vasodilatation. Notably, the appearance of microbubbles in the left cardiac chambers within less than three cardiac cycles insinuates intracardial shunting and cannot be diagnostic for intrapulmonary vasodilatation[70].

Contrast-enhanced echocardiography constitutes a practical tool for HPS diagnosis. It is a minimally invasive, low-cost technique providing high sensitivity for the qualitative evaluation of intrapulmonary vascular dilatations and shunting. A positive test is not enough for HPS diagnosis, as the two other parameters of the HPS diagnostic triad must be fulfilled. Interestingly, a quantitative classification of intrapulmonary shunting based on the maximum number of microbubbles bypassing to the left ventricle in one still frame has been suggested[71,72]. According to this classification, severity of intrapulmonary shunting can be graded as stage 1 (< 30 microbubbles), 2 (30-100 microbubbles) or 3 (> 100 microbubbles) (Table 3). A possible correlation between this shunt grading and the proposed classification of HPS based on arterial PaO_2 remains to be verified in future studies.

Contrast transesophageal echocardiography is superior to transthoracic echocardiography concerning the sensitivity of the technique in the diagnosis of intrapulmonary vasodilatation[73]. However, it is not preferred for the assessment of HPS in cirrhotic patients due to the risk regarding possible trauma to esophageal varices.

Macroaggregated albumin lung perfusion is performed by injecting technetium-99m-labeled macroaggregated albumin followed by a lung and brain perfusion scanning. Brain uptake of the radionuclide higher or equal to 6% implies intrapulmonary or intracardiac shunting, as the large molecules of radiolabeled albumin are normally trapped in the pulmonary capillary bed[74]. Estimating the pathological retention of the radionuclide in the brain, this technique allows an indirect quantitative assessment of the intrapulmonary shunting. However, it is not as sensitive as contrast echocardiography, especially in early stages of HPS[75], while it cannot distinguish intrapulmonary from intracardial shunting.

Chest radiographs are only useful to exclude concomitant pulmonary disease as they rarely show evidence of dilated vasculature[3]. High resolution computed tomography may also identify large, dilated pulmonary vessels[76].

Pulmonary angiography provides a double contribution in HPS, diagnostic and therapeutic. Two types of HPS can be distinguished on the basis of angiographic findings[77]. Type 1 is characterized by minimally dilated vessels, and type 2 delineated by well-defined arteriovenous communications and resistance to 100% oxygen administration. The invasive character of pulmonary angiography makes it a less convenient method for the diagnosis of HPS.

Arterial oxygenation

Arterial blood gas analysis is required to detect all patients with HPS[78]. The calculation of the alveolar-arterial gradient is proposed as a better diagnostic parameter than the evaluation of the PaO_2 alone to identify those patients with impaired oxygenation. The sensitivity of this marker is attributed to the fact that the

Table 3 Intrapulmonary shunt quantitative classification

Contrast-enhanced echocardiography based on the number of microbubbles passing in the left ventricle	
No shunt	No detection of microbubbles
Stage 1	< 30 microbubbles
Stage 2	30-100 microbubbles
Stage 3	> 100 microbubbles
Macroaggregated albumin lung perfusion	
No shunt	< 6% brain uptake of radiolabeled albumin
Intrapulmonary shunt	⩾ 6% brain uptake of radiolabeled albumin

partial pressure of carbon dioxide ($PaCO_2$) is included to its calculation, so that lower values of $PaCO_2$ lead to an increased alveolar-arterial gradient, reflecting an elevated respiratory effort to maintain normal blood oxygenation, even before PaO_2 is affected. According to the European Respiratory Task Force, alveolar-arterial gradient greater than or equal to 15 mmHg (or ⩾ 20 mmHg in patients > 64-years-old) is indicative of impaired oxygenation, calculated at sea level while the patient is breathing ambient air at rest[3].

The potential role of pulse oximetry as a screening test for the presence of HPS has also been investigated. Lower values of oxygen saturation were measured in HPS patients compared to cirrhotic patients without HPS (96.8% *vs* 98.4%, $P = 0.02$)[79], while pulse oximetry values below 96% presented a sensitivity and specificity of 100% and 88% respectively for detecting patients with $PaO_2 < 60$ mmHg[80]. On the other hand, the utility of pulse oximetry in HPS diagnosis was not confirmed in children with cirrhosis[81]. The difference in oxygen saturation between supine and standing position was suggested as a method to detect HPS[82]. However, the use of low values of oxygen saturation (< 92%) as well as a decrease of ⩾ 4% after change from supine to upright position was unreliable as screening test for diagnosis of HPS. Notably, the majority of HPS patients present oxygen desaturation during sleep, proportional to the syndrome's severity[68]. Finally, the variation in oxygen saturation between supine and standing position was reported as a marker of possible intrapulmonary vascular dilatations[83].

A reduced diffusing capacity for carbon monoxide (DLCO) is the single most common defect among pulmonary function tests that has been correlated to the presence of HPS[14]. However, there is controversial data concerning DLCO as a diagnostic tool for HPS screening[84,85]. In contrast to ventilation-perfusion imbalance, which seems to resolve after liver transplantation, a restricted number of observational studies have suggested a persistence of low DLCO values after liver transplantation due to permanent liver-induced structural vascular changes in the pulmonary vasculature[86,87].

As pulse oximetry fails to detect mild and moderate HPS and the value of other screening markers remains undefined, alveolar-arterial gradient represents, as yet, the most remarkable method for HPS screening

TREATMENT

Liver transplantation constitutes the only established successful treatment that modifies the natural history of HPS, improving arterial hypoxemia within 6-12 mo[88]. The identification of HPS through established diagnostic protocols among liver transplantation candidates in combination with the model for end-stage liver disease (MELD) exception policy to facilitate liver transplantation may achieve an 88% 5-year posttransplantation survival for HPS patients[89]. Besides, oxygen therapy is recommended for those cases with severe hypoxemia[3]. Restricted data report improvement in liver function and oxygenation after 1 year of oxygen supplement[90].

Many pharmaceutical interventions have been studied both in humans and animal models, targeting the syndrome's pathogenetic pathways, without reaching encouraging outcomes. NO-mediated pulmonary vasodilatation and angiogenesis induced by proinflammatory cytokines, which represent the hallmarks of HPS pathogenesis, have constituted the main targets of medical intervention, in an effort to reverse the syndrome's evolutionary process and confirm the assumptions concerning the pathogenetic mechanisms.

Administration of octreotide, a somatostatin analogue inhibiting angiogenesis, failed to improve hypoxemia in patients with HPS[91]. Contrariwise, sorafenib improves experimental HPS by reducing VEGF-mediated angiogenesis and down-regulating eNOS activation through tyrosine kinase receptor inhibition[33,61]. Treatment with antibiotics, such as norfloxacin, in order to reduce endotoxemia and NO production triggered by bacterial translocation, did not improve gas exchange, in contrast to promising results in experimental models[92,93]. Single case reports suggest improvement of HPS after administration of cyclooxygenase inhibitors, such as indomethacin, and immunosuppressants, such as mycophenolate mofetil, but there are no randomized studies to investigate these findings[94-96].

Methylene blue is an oxidizing agent that restricts NO-mediated vasodilatation through blockage of soluble guanylate cyclase stimulation by NO[97]. Intravenous administration of methylene blue reduced intrapulmonary shunting and improved oxygenation

in experimental models and in a restricted number of patients with HPS[98,99].

There are conflicting results regarding the effect of pentoxifylline on HPS. Pentoxifylline is a TNF-α inhibitor that improves HPS in experimental models by reducing TNF-induced NO production through down-regulation of iNOS[50,58]. A dosage of 400 mg of pentoxifylline, three times per day for 3 mo significantly improved oxygenation and decreased TNF-α levels in 9 patients with symptomatic HPS[100]. Nevertheless, another pilot study enrolling 9 patients with advanced HPS reported no significant therapeutic response after pentoxifylline administration, while the drug was poorly tolerated due to gastrointestinal adverse events[101].

N(G)-nitro-L-arginine methylester, a nebulized inhibitor of NO synthesis, did not improve oxygenation in HPS patients, even if a reduction in exhaled NO was recorded[43,102]. Almitrine bismesylate, a potential vasoconstrictor, does not affect impaired oxygenation in HPS[103]. Finally, a few studies have demonstrated that garlic supplementation improves arterial oxygenation and symptoms in HPS[104]. A total reversal of HPS was observed in 14 of 21 patients after 9 mo of garlic treatment, compared to 1 of 20 HPS patients under placebo treatment[105].

Transjugular intrahepatic portosystemic shunting (commonly known as TIPS) was performed as a therapeutic maneuver in a limited number of patients with severe HPS, leading to variable results[106,107]. Although TIPS could be considered as a bridge towards transplantation, there is concern that persistent right-to-left shunting *via* TIPS prevents the reversal of intrapulmonary structural alterations[108]. Embolotherapy has also been performed to treat persistent hypoxemia of HPS, either before or after liver transplantation, in the presence of large arteriovenous communications[109,110].

Clearly, their poor outcomes as well as the small number of enrolled patients make the aforementioned studies insufficient to suggest effective therapeutic options for the management of HPS. In addition, these data underline the complexity of pathogenetic interactions in HPS and outline potential areas of interest and future research.

PROGNOSIS

Despite the relative high prevalence of HPS among cirrhotic patients, there is an inadequate number of prospective studies evaluating the syndrome's impact on overall morbidity and mortality. Once again, the use of varying thresholds, concerning arterial oxygenation, for the diagnosis of the syndrome, has led to ambiguous results about HPS prognosis. The main question remains whether the presence of HPS should be considered as an independent factor for morbidity, giving HPS patients priority to liver transplantation, and whether any correlations between the severity of HPS and the posttransplantation survival rates exist.

A retrospective analysis reported 41% mortality over an approximate 2.5-year period in 22 patients with HPS[77]. Comparing survival rates between cirrhotic patients with HPS and matched for the severity of liver disease by MELD and Child-Pugh score classification and age patients without HPS, who did not undergo liver transplantation, patients with HPS had a worse 5-year survival (23% *vs* 63%, $P = 0.0003$)[111]. Patients with PaO_2 less than 50 mmHg had significantly worse survival rates. Similar results were confirmed by a prospective study that reported lower median survival among HPS subjects compared to nonHPS cirrhotic patients (10.6 mo *vs* 40.8 mo, $P < 0.05$), while the mortality remained higher even after adjusting for age and liver disease severity[112]. Furthermore, HPS was associated with worse quality of life, assessed by the New York Heart Association classification, and higher risk of death compared to nonHPS matched for age, sex and MELD score cirrhotic subjects [hazard ratio = 2.41, 95% confidence interval (95%CI): 1.31-4.41, $P = 0.005$][64].On the other hand, no significant difference in overall survival between HPS and nonHPS transplantation candidates was demonstrated in a prospective study including 316 cirrhotic patients[16]. Notably, even in those studies that reported high HPS-related mortality, the causes of death were mainly attributed to liver dysfunction rather than pulmonary complications.

Liver transplantation is the only therapeutic intervention that reverses HPS between the first 6 to 12 mo, even for cases with severe preoperative hypoxemia[111]. The general policy is prioritizing patients with HPS and hypoxemia for liver transplantation, regardless of the severity of liver disease[113]. Beside poor prognosis of HPS, the progressive aggravation of hypoxemia, estimated at 5.2 mmHg per year, probably boosts the decision for a prompt management[111]. However, there is concern that through this organ allocation policy, HPS patients may be offered a pretransplantation survival advantage over nonHPS cirrhotic transplantation candidates, prompting the need for reassessment of the MELD exception criteria[114].

There is controversial data concerning posttransplantation mortality in HPS transplanted patients. A prospective study suggests higher 6-mo postoperative mortality rates in HPS patients compared to transplanted patients without HPS (33% *vs* 9.25%, $P = 0.0012$)[115]. A PaO_2 of 50 mmHg or less and a macroaggregated albumin shunt fraction > 20% are demonstrated as the most important predictors of mortality following transplantation, suggesting preoperative HPS staging to assess the risk of postoperative mortality[116]. Conversely, no difference in posttransplantation survival between patients with and without HPS was demonstrated in a large prospective study that enrolled 316 patients[16]. One-year posttransplantation survival may reach 93% in HPS patients[117], while the presence of HPS does not seem to affect duration of intensive care unit stay, duration of total hospital stay, rate of pulmonary complications or 3-mo survival after liver

transplantation[118]. Finally, there is a growing number of reports suggesting no differences in short- and long-term posttransplantation morbidity between patients with and without HPS, and no association between the severity of baseline hypoxia and survival after transplantation[17,119].

The discrepancies between the aforementioned studies can be attributed to different methodological approaches and HPS assessment protocols. The possibility of transplantation denial to patients with HPS and significant hypoxemia should always be considered as a confusing factor that may influence the comparison between different research outcomes[120].

HPS AND MYOCARDIAL FUNCTION

Liver cirrhosis is characterized by hyperdynamic circulation as a consequence of systematic vasodilatation[121] in order to preserve normal blood flow. Diastolic dysfunction and impaired cardiac contractile response to stress define cirrhotic cardiomyopathy, another cardiovascular complication strongly associated with chronic liver disease[122]. The possible association between specific markers of cardiac dysfunction and HPS remains an issue of debate.

Right ventricular diastolic dysfunction assessed by Doppler echocardiography was found to be more remarkable in the presence of HPS, in a study enrolling 46 cirrhotic patients, 10 of whom had HPS[123]. Significantly higher right ventricle and right atrial diameters as well as right ventricle wall thickness values were recorded in the HPS group. Moreover, patients with compared to those without HPS had higher estimated mean pulmonary artery pressure (48.9 ± 4.8 mmHg *vs* 40.6 ± 5.3 mmHg, $P < 0.05$) and higher pulmonary vascular resistance (3.97 ± 1.31 Wood's unit *vs* 3.25 ± 0.96 Wood's unit, $P < 0.05$).

Intrapulmonary shunting in the context of liver disease may aggravate hemodynamic imbalance, followed by further increase in cardiac output[124]. Reflecting hyperdynamic circulatory state, left atrial enlargement was associated with the presence of intrapulmonary vasodilatation, both in human and experimental studies[125]. Remarkably, left atrial volume equal or greater than 50 mL was suggested as a strong echocardiographic predictor of HPS in patients with liver cirrhosis (area under the receiver operating characteristic curve: 0.903, sensitivity 86.3%, specificity 81.2%)[126]. Left ventricular enlargement was also proposed as an independent, indirect echocardiographic marker of HPS[127]. In addition, higher systolic myocardial velocity of the mitral valve measured by tissue Doppler imaging technique was independently associated with HPS (odds ratio: 1.428, 95%CI: 1.049-1.943, $P = 0.026$), a finding implying left ventricular systolic dysfunction[127].

In contrast to the previous reports, Voiosu *et al*[128] found no correlations between HPS and echocardiographic markers of systolic or diastolic myocardial dysfunction in 74 patients with liver cirrhosis. Cirrhotic cardiomyopathy did not differentiate between patients with and without HPS, suggesting an independent pathogenetic nature of these complications. The methods and results of previous studies evaluating cardiac involvement in HPS are presented in Table 4.

While hyperdynamic circulation as a response to systemic vasodilatation in liver cirrhosis is well documented, the subsequent myocardial structural changes are not yet fully understood. Increased cardiac output seems to be the main pathogenetic event triggering systemic multifactorial, cellular, neuronal and humoral signaling mechanisms that induce cardiac contractile dysfunction, electrophysiological abnormalities and chronotropic incompetence in the setting of liver cirrhosis[129]. The most prevalent feature of this entity known as cirrhotic cardiomyopathy is silent diastolic dysfunction with impaired ventricular relaxation and ventricular filling, which may become overt after rapid increase in venous return after liver transplantation.

Currently, literature data cannot support an intimate association between cirrhotic cardiomyopathy and HPS[130]. A complicated interaction between different pathogenetic mechanisms is thought to involve myocardial function in the presence of intrapulmonary shunting. The available studies are not only restricted in number but also heterogenous concerning the assessed features of myocardial dysfunction and the evaluated parameters.

The hypothesis is that NO overproduction, which leads to intrapulmonary vasodilatation, is responsible for an intense hyperdynamic circulating state resulting in higher cardiac output and long-term left ventricle myocardial dysfunction. The potential structural myocardial alterations of the right ventricle in the presence of intrapulmonary vasodilatation as well as the effect of HPS hypoxemia on increased myocardial demands also remain to be clarified. Of great importance is to unravel the exact mechanisms affecting cardiac function that differentiate in patients with HPS. In order to extract more accurate results, the assessment tools of myocardial function should be independent of expanded plasma volume and bias correlated to the presence of ascites, diuretic treatment and sodium intake[131].

In this direction, novel promising echocardiographic techniques offering a more accurate assessment of cardiac structure as well as sensitive biomarkers of cardiac dysfunction need further evaluation in future research in order to elucidate possible interactions between pulmonary vasodilatation, hypoxemia and myocardial dysfunction in the context of chronic liver disease. Last but not least, the effect of possible HPS-related myocardial dysfunction on pre- and posttransplantation total survival is yet to be investigated.

CONCLUSION

HPS is a relatively common complication of chronic liver disease, with many of its aspects remaining still

Table 4 Hepatopulmonary syndrome and cardiac involvement

Study	Cirrhotic patients	Parameters assessed	Assessment tools	Associations
Karabulut *et al*[123]	36 without HPS	RV diastolic dysfunction	M-mode ECHO	RV diastolic dysfunction-HPS
	10 with HPS	PVR	TDI	HPS was associated with higher RV wall thickness (0.61 ± 0.13 cm *vs* 0.51 ± 0.10 cm) RVEDD (3.81 ± 0.38 cm *vs* 3.11 ± 0.94 cm)
		Systolic PAP		RA (3.96 ± 0.53 cm *vs* 3.58 ± 0.47 cm), systolic PAP (48.9 ± 4.8 mmHg *vs* 40.6 ± 5.3 mmHg)
				PVR (3.97 ± 1.31 Wood's unit *vs* 3.25 ± 0.96 Wood's unit)
Zamirian *et al*[124]	53 without IPS	LA dimension	M-mode ECHO	IPS was associated with higher LA dimension (4.58 ± 0.54 cm *vs* 3.87 ± 0.63 cm)
	39 with IPS	Cardiac output		Cardiac output (5.62 ± 0.83 L/min *vs* 4.75 ± 0.76 L/min)
Zamirian *et al*[126]	108 without HPS	LA volume	M-mode ECHO	Greater LA volume in HPS (55.1 ± 7.5 mL *vs* 37.1 ± 9.3 mL)
	41 with HPS			LA volume ⩾ 50 mL, AUC: 0.903, sensitivity: 86.3%, specificity: 81.2%
Pouriki *et al*[127]	67 without HPS	Markers of LV and RV diastolic and/or systolic cardiac function	M-mode ECHO	HPS was associated with higher LVEDD (OR = 1.230, 95%CI: 1.036-1.482; *P* = 0.019)
	12 with HPS		TDI	S wave at left lateral wall of MV (TDI) (OR = 1.428, 95%CI: 1.049-1.943; *P* = 0.026)
				S wave lateral ⩾ 13.5 cm/s, AUC: 0.736, sensitivity: 83.3%, specificity: 65.7%
				LVEDD ⩾ 50.5 mm, AUC: 0.724, sensitivity: 75%, specificity: 68.7%
Voiosu *et al*[128]	57 without HPS	Association between HPS and cirrhotic cardiomyopathy	M-mode ECHO	Higher RV wall width in HPS (3.8 ± 1.2 mm *vs* 3.4 ± 0.6 mm)
	17 with HPS		TDI	No association between HPS and cirrhotic cardiomyopathy
				No echocardiographic measurement predictive of HPS

AUC: Area under the curve; ECHO: Echocardiography; HPS: Hepatopulmonary syndrome; IPS: Intrapulmonary shunt; LA: Left atrial; LV: Left ventricle; LVEDD: Left ventricle end diastolic diameter; MV: Mitral valve; OR: Odds ratio; PVR: Pulmonary vascular resistance; PAP: Pulmonary artery pressure; RV: Right ventricle; RVEDD: Right ventricle end diastolic diameter; TDI: Tissue Doppler imaging.

largely unknown. HPS screening with the establishment of standardized protocols among patients with liver disease is crucial in the direction of achieving higher survival rates. Prospective studies evaluating long-term outcomes before and after liver transplantation in large patient cohorts will demonstrate the specific characteristics of HPS requiring management in priority. The precise events that trigger HPS pathogenesis as well as secondary clinical and subclinical vital organ interactions will constitute the field of future research.

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ORIGINAL ARTICLE

Basic Study

Cell culture-adaptive mutations in hepatitis C virus promote viral production by enhancing viral replication and release

Qi Wang, Yue Li, Shun-Ai Liu, Wen Xie, Jun Cheng

Qi Wang, Wen Xie, Jun Cheng, Center of Liver Diseases, Beijing Ditan Hospital, Capital Medical University, Beijing 100015, China

Yue Li, Department of Pathology, Beijing Ditan Hospital, Capital Medical University, Beijing 100015, China

Shun-Ai Liu, Jun Cheng, Institute of Infectious Diseases, Beijing Ditan Hospital, Capital Medical University, Beijing 100015, China

Qi Wang, Shun-Ai Liu, Jun Cheng, Beijing Key Laboratory of Emerging Infectious Diseases, Beijing 100015, China

ORCID number: Qi Wang (0000-0002-0269-1568); Yue Li (0000-0002-7412-7632); Shun-Ai Liu (0000-0002-0004-3217); Wen Xie (0000-0002-7314-8175); Jun Cheng (0000-0001-5579-8353).

Author contributions: Wang Q and Li Y contributed equally to this work; Wang Q and Li Y performed the experiments and analyzed the data; Wang Q and Cheng J designed the research; Wang Q and Li Y wrote the manuscript; Xie W revised the manuscript; Liu SA provided vital reagents and analytical tools.

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Correspondence to: Jun Cheng, MD, PhD, Professor, Center of Liver Diseases, Beijing Ditan Hospital, Capital Medical University, No. 8, Jingshun East Street, Chaoyang District, Beijing 100015, China. chengj0817@ccmu.edu.cn
Telephone: +86-10-84322006
Fax: +86-10-84397196

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Abstract

AIM

To explore hepatitis C virus (HCV) adaptive mutations or combinations thereof responsible for enhanced viral production and investigate the underlying mechanisms.

METHODS

A series of plasmids with adaptive mutations were constructed. After the plasmids were transfected into Huh7.5 cells, we determined the infectious HCV particle titers by NS5A immunofluorescence assays, and detected HCV RNA replication by real-time PCR and protein expression by Western blot. Then we carried out immunoblotting of supernatants and cell

lysates with anti-NS3 to analyze the virus release level. In addition, co-localization of lipid droplets (LDs) with NS5A was measured using confocal laser scanning microscopy. The ratio between the p56 and p58 phosphoforms of NS5A was analyzed further.

RESULTS

The plasmids named JFH1-mE2, JFH1-mp7, JFH1-mNS4B, JFH1-mNS5A, JFH1-mE2/NS5A, JFH1-mp7/NS5A, JFH1-mNS4B/NS5A, JFH1-mE2/p7/NS5A, and mJFH1 were constructed successfully. This study generated infectious HCV particles with a robust titer of 1.61×10^6 focus-forming units (FFUs)/mL. All of the six adaptive mutations increased the HCV particle production at varying levels. The NS5A (C2274R, I2340T, and V2440L) and p7 (H781Y) were critical adaptive mutations. The effect of NS5A (C2274R, I2340T, and V2440L), p7 (H781Y), and NS4B (N1931S) on infectious HCV titers was investigated by measuring the HCV RNA replication, protein expression, and virion release. However, the six adaptive mutations were not required for the LD localization of NS5A proteins or the phosphorylation of NS5A.

CONCLUSION

In this study, we generated infectious HCV particles with a robust titer of 1.61×10^6 FFUs/mL, and found that the viral replication and release levels could be enhanced by some of the adaptive mutations.

Key words: Hepatitis C virus; JFH1; Adaptive mutation; RNA replication; Virion release; Lipid droplet localization

Core tip: In this study, we explored hepatitis C virus (HCV) adaptive mutations or combinations thereof responsible for enhanced viral production and investigated the underlying mechanisms. We generated infectious HCV particles with a robust titer of 1.61×10^6 focus-forming units (FFUs)/mL, and confirmed that the adaptive mutations could enhance viral replication and release. The results were established at the levels of infectious particle titers, HCV RNA, protein expression, virus release, lipid droplet, and NS5A co-localization, and further the ratio between p56 and p58 phosphoforms of NS5A.

Wang Q, Li Y, Liu SA, Xie W, Cheng J. Cell culture-adaptive mutations in hepatitis C virus promote viral production by enhancing viral replication and release. *World J Gastroenterol* 2018; 24(12): 1299-1311 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i12/1299.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i12.1299

INTRODUCTION

Hepatitis C virus (HCV) is a member of the flaviviridae family. HCV infection is a major public health challenge, with an estimated number of 130 to 170 million individuals infected worldwide[1,2]. HCV causes acute and chronic hepatitis, and also leads to permanent liver damage and hepatocellular carcinoma in a significant number of patients, *via* oxidative stress, insulin resistance, fibrosis, liver cirrhosis, and HCV-induced steatosis[3]. Interferon-α-based therapy, in combination with ribavirin, has limited efficacy in approximately 50% of patients and is associated with severe side effects[4]. Direct-acting antivirals (DAAs) targeting NS3/4A, NS5A, and NS5B proteins can lead to higher sustained virological responses than interferon-based regimens, have shorter treatment duration, are orally administered, and have fewer side effects[5].

HCV is an enveloped RNA virus whose replication occurs in the cytoplasm. It consists of a single-stranded 9.6-kb RNA genome of positive polarity with a 5′ internal ribosome entry site (IRES). IRES-driven HCV RNA produces a polyprotein of approximately 3000 amino acids localized to the rough endoplasmic reticulum (ER), where it is cleaved into at least four structural proteins (C, E1, E2, and p7) and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) that play a key role in viral replication, assembly, and pathogenesis[6].

Elucidation of the viral structure and virus-host interaction is an important goal of anti-HCV drug discovery and vaccine development[7]. HCV replicon system has contributed to the study of HCV in the human hepatoma cell line Huh-7[8,9]. The infectious HCV JFH1 cell culture system represents a major advance in anti-HCV drug discovery research[7,10-12]. This model generates infectious viral particles in cell culture (HCVcc) and facilitates the study of HCV life cycle[7,11]. However, HCV JFH1 variant genome (genotype 2a) results in relatively low viral titers[7,13,14].

Several studies suggested that cell culture-adaptive mutations in HCV genomic RNA might potentially increase the production of infectious HCV particles[13,15-18]. Recently, an adaptive HCV JFH1 reporter isolate designated as JFH1-ΔV3-EGFP was identified[19], which produced higher titers (10^6 focus-forming units [FFUs]/mL) of HCV-EGFP reporter virus. Whole genome sequencing analysis showed that JFH1-ΔV3-EGFP included six mutations located in the E2, p7, NS4B, and NS5A regions as follows: D657G in E2; H781Y in p7; N1931S in NS4B; and C2274R, I2340T, and V2440L in NS5A. V2440L and H781Y improved the infectious HCV titers[20,21], while data pertaining to the other mutations are not available. In this study, we explored these mutations or combinations thereof responsible for enhanced viral production and investigated the underlying mechanisms.

MATERIALS AND METHODS

Cell culture

The human hepatoma cell line Huh7.5 was generously provided by Dr. Charles M. Rice[22] (Rockefeller

Table 1 Sequence of primers used for adaptive mutation plasmid construction

Primer	Sequence (5′-3′)
1340-F	CTGGCGTACGTGATGCG
m2310-R	TGTCCCTGTCCTCCAAGCCGCAGCGAT
m2310-F	ATCGCTGCGGCTTGGAGGACAGGGACA
3500-R	GCCCCGTCATACTCACCAC
m2681-R	TGATGTACCAAGCTGCCACGAAGAAG
m2681-F	CTTCTTCGTGGCAGCTTGGTACATCA
5249-F	AATGAGGTCACCCTCACACA
m6132-R	ACGTGGCTTCCTCTGGAAGCAAAGGCA
m6132-F	TGCCTTTGCTTCCAGAGGAAGCCACGT
7791-R	GATGTTGTACAGTACACCTTG
5249-F	AATGAGGTCACCCTCACACA
m7160-R	AGCATGCGCTCCGATGGTATTGAG
m7160-F	CTCAATACCATCGGAGCGCATGCT
m7359-R	TTCTGATGTGGTGCTCTCGCTCAG
m7359-F	CTGAGCGAGAGCACCACATCAGAA
m7658-R	GCACAGGGTGGTATCGTCCTCCT
m7658-F	AGGAGGACGATACCACCCTGTGC
7966-R	CTTGGATCTTGCAGAAT

Table 2 Primer combinations used in adaptive mutation plasmid construction

Fragment	Template	Primers	
		Sense	Anti-sense
mE2-1	JFH1	1340-F	m2310-R
mE2-2		m2310-F	3500-R
mp7-1		1340-F	m2681-R
mp7-2		m2681-F	3500-R
mNS4B-1		5249-F	m6132-R
mNS4B-2		m6132-F	7791-R
mNS5A-1		5249-F	m7160-R
mNS5A-2		m7160-F	m7359-R
mNS5A-3		m7359-F	m7658-R
mNS5A-4		m7658-F	7966-R
mNS4B/NS5A-1	JFH1-mNS5A	5249-F	m6132-R
mNS4B/NS5A-2		m6132-F	7791-R
mE2/p7 -1	JFH1-mp7/NS5A	1350-F	m2310-R
mE2/p7 -2		m2310-F	3500-R

University) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, non-essential amino acids, and 10% fetal bovine serum (Invitrogen) at 37 ℃ in 5% CO_2. All the experiments described in this study were performed using these cells.

Antibodies

The monoclonal antibody to NS5A protein (Abcam), the goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma), and goat anti-mouse IgG conjugated with Alexa Fluor 594 (Invitrogen) were all obtained commercially.

Plasmid construction

Plasmid constructs were based on the consensus sequence of HCV pJFH1, which was kindly provided by Dr. Wakita[10]. JFH1-ΔV3-EGFP and JFH1-AM120 plasmids were kindly provided by Dr. C.H. Hagedorn and Shuang-Hu Liu[19]. The mutations located in HCV genomic RNA are shown in Figure 1. A series of primers for construction of adaptive variants of wild-type HCV JFH1 listed in Table 1 were designed using the pJFH1 sequence and mutations. The pJFH1 plasmid was used as a template for subsequent PCR with Phushion High-Fidelity PCR Master Mix with GC buffer (New England Biolabs) according to the manufacturer's instructions. The preliminary PCR products (mE2-1, mE2-2, mp7-1, mp7-2, mNS4B-1, mNS4B-2, mNS5A-1, mNS5A-2, mNS5A-3, and mNS5A-4) were analyzed by 1% agarose gel electrophoresis, and used for overlap PCR following the combination showed in Tables 2 and 3 to obtain adaptive mutation fragments. The above fragments (mE2, mp7, mNS4B, mNS5A, mE2/NS5A, mp7/NS5A, mNS4B/NS5A, and mE2/p7/NS5A) were sub-cloned into pJFH1 using the appropriate unique restriction enzyme sites such as *Bsi*w I, *Kpn* I, *Nsi* I, *Rsr* II, or *Bsr*G I, to produce JFH1-mE2, JFH1-mp7, JFH1-mNS4B, JFH1-mNS5A, JFH1-mE2/ NS5A, JFH1-mp7/NS5A, JFH1-mNS4B/NS5A, JFH1-mE2/p7/ NS5A, and also mJFH1, which contained all the six mutations. All new clones were sequenced using an ABI 3700-XL (Shanghai Sangon Biotech).

Transfection with HCV RNA

To generate the full-length genomic RNA, pJFH-1 and all plasmids were linearized with *Xba* I. The linearized plasmid DNA was purified and then used as a template for T7 *in vitro* transcription (MEGAscript; Ambion). The RNA genomes were detected by formaldehyde agarose gel electrophoresis as described previously[23], and transfected into cells by electroporation[13].

Immunofluorescence assay

Cells seeded on glass coverslips were infected with HCV. After 48 h, the slips were washed with PBS. Then, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 1% BSA and 1% normal goat serum. The NS5A in cells was detected with a monoclonal antibody and a secondary goat anti-mouse Alexa Fluor 594 antibody (Invitrogen) and visualized by fluorescence microscopy.

Virus titration

The titer of infectious HCV was determined by immunofluorescence assay[7]. Virus titers from supernatants and cell lysates as well were determined using FFUs.

Cell lysates were prepared as described previously[24]. Briefly, cell pellets harvested after trypsinization were washed with PBS, re-suspended in completed culture medium, and lysed in four freeze/thaw cycles at -80 ℃ and 37 ℃. The cell lysates were centrifuged at 4000 rpm for 5 min prior to inoculation into naïve Huh7.5 cells.

Virus titration analysis was conducted by serially diluting the cell supernatants or cell lysates 10-fold in DMEM. The supernatants were used to infect 1×10^4

Table 3 Primers and templates for overlap PCR

Fragment	Template	Primer	
		Upstream	Downstream
mE2	mE2-1 + mE2-2	1340-F	3500-R
mp7	mp7-1 + mp7-2	1340-F	3500-R
mNS4B	mNS4B-1 + mNS4B-2	5249 F	7791-R
mNS5A-3/4	mNS5A-3 + mNS5A-4	m7359-F	7966-R
mNS5A-2/3/4	mNS5A-2 + mNS5A-3/4	m7160-F	7966-R
mNS5A	mNS5A-1 + mNS5A-2/3/4	5249-F	7966-R
mNS4B/NS5A	mNS4B/NS5A-1 + mNS4B/NS5A-2	5249 F	7791-R
mE2/p7	mE2/p7 -1 + mE2/p7-2	1340-F	3500-R

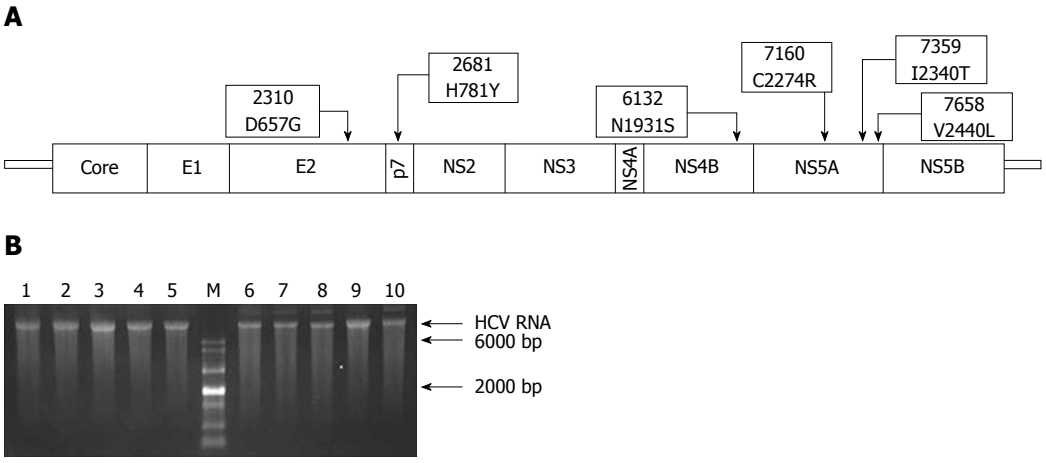


Figure 1 Schematic representation of adaptive mutations used in this study (A) and the electrophoresis results of each mutant virus RNA (B). A: Both nucleotide substitutions (2310, 2681, 6132, 7160, 7359, and 7658) and amino acid substitutions (D657G, H781Y, N1931S, C2274R, I2340T, and V2440L) are shown; B: HCV RNA (500 ng) was analyzed using formaldehyde agarose gel electrophoresis. Lane 1: JFH1; Lane 2: JFH1-mE2; Lane 3: JFH1-mP7; Lane 4: JFH1-mNS4B; Lane 5: JFH1-mNS5A; Lane 6: JFH1-mE2/NS5A; Lane 7: JFH1-mp7/NS5A; Lane 8: JFH1-mNS4B/NS5A; Lane 9: mJFH1; Lane 10: JFH1-mE2/p7/NS5A; M: RNA marker. HCV: hepatitis C virus.

naïve Huh7.5 cells in 96-well plates. The cells were incubated with virus for 2 h at 37 ℃, washed, and incubated with complete DMEM. The level of HCV infection was analyzed 3 and 9 d post-infection by immune- fluorescence staining for NS5A.

Western blot analysis

The Huh7.5 cells infected with HCV RNA were lysed in 50 mmol/L Tris-HCl (pH 7.5) containing 150 mmol/L sodium chloride, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, and proteinase inhibitors (Complete Mini, Roche). Samples were separated by 10% SDS-PAGE and then transferred onto nitrocellulose membranes. The HCV NS5A (p56/p58) was analyzed as described previously[13].

Quantification of HCV RNA by qPCR

Total RNA was extracted with TRIzol (Invitrogen). HCV RNA was measured by qPCR analysis as described previously[25]. β-actin was used as the internal control. The relative quantity of HCV RNA in control and HCV samples was calculated by the comparative Ct (cycling threshold) method using LightCycler480.

Confocal laser scanning microscopy

Cells transfected with HCV RNA with adaptive mutations were seeded onto 24-well plates with cover slips. The cells were treated as previously described[13]. After 48 h, the cells were washed with PBS, fixed with 4% paraformaldehyde, and then permeabilized with 0.2% Triton X-100. Fixed cells were blocked with 1% bovine serum albumin and 1% normal goat serum in PBS. Next, HCV NS5A was analyzed in cells using a NS5A monoclonal antibody and a secondary goat anti-mouse IgG conjugated with Alexa 488 (Invitrogen, dilution of 1:1000). LipidTOX Deep Red (Invitrogen) was used to detect neutral lipids present in lipid droplets (LDs). The slides were counterstained using DAPI (Invitrogen), and examined using an Zeiss LSM 510 Meta confocal laser scanning microscope.

RESULTS

Effect of individual mutations or combinations of adaptive mutations on the production of infectious HCV

A previous study demonstrated that JFH-ΔV3-EGFP variant produces a higher titer of reporter virus[19]. The

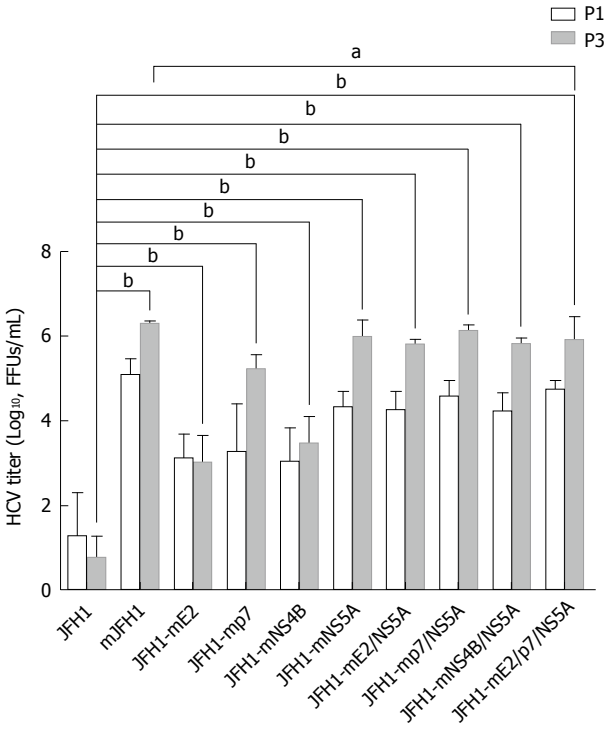


Figure 2 Generation of high titer cell culture-adaptive JFH1 virus. Hepatitis C virus RNA was electroporated into Huh7.5 cells to produce the recombinants of adapted virus in cell culture. The transfected cells were passaged every three days. The infectivity titers of the culture supernatants at day 3 (P1) and day 9 (P3) were measured. Viral titers are expressed as focus-forming units per milliliter (FFUs/mL). The data are presented as mean ± SD (n = 3). HCV: Hepatitis C virus. $^{a}P < 0.05$; $^{b}P < 0.01$.

six adaptive mutations in this variant were located in the E2 (D657G), p7 (H781Y), NS4B (N1931S), and NS5A (C2274R, I2340T, and V2440L) (Figure 1A), respectively. To determine the individual or synergistic combination of these mutations responsible for the increased viral production, recombinant JFH1 genomes containing only one of the selected mutations or four different combinations as shown in Figure 1A were constructed.

Next, ten *in vitro*-transcribed mutant JFH1 RNAs (Figure 1B) were electroporated into Huh7.5 cells to produce recombinants of adapted virus. The transfected cells were sub-cultured every three days. The infectivity titers of supernatants at day 3 (P1) and day 9 (P3) were measured (Figure 2). Viral titers are expressed as FFUs/mL and were assayed in duplicate, which was repeated three times. The data are presented as mean ± SD (n = 3). The HCV titers of wild type JFH1 (JFH1) were extremely low, with a typical titer of 10^2 FFUs/mL. The adaptive mutations in E2, p7, NS4B, and NS5A individually increased the production of infectious HCV titers 2- to 4-fold compared with the levels of JFH1. The NS5A mutations exhibited the greatest effect on the production of infectious HCV particles, with a titer of 1.30×10^6 FFUs/mL at P3. The p7 mutation followed closely, generating a titer of 2.10×10^5 FFUs/mL. Briefly, except for E2, the HCV titers of other variants at P3 were partially higher than those at P1.

To further determine any synergistic effect of the six adaptive mutations on HCV production, we focused on the recombinant viruses with adaptive mutations in different combinations. As shown in Figure 2, JFH1-mE2/NS5A, JFH1-mp7/NS5A, JFH1-mNS4B/NS5A, and JFH1-mE2/p7/NS5A remarkably enhanced the production of infectious HCV, and the mJFH1 produced infectious HCV particles with a robust titer of 1.61×10^6 FFUs/mL 9 d post-transfection.

These results suggest that all the six adaptive mutations increase the HCV particle production. NS5A (C2274R, I2340T, and V2440L) and p7 (H781Y) are the critical adaptive mutations.

HCV RNA replication and protein expression are up-regulated by adaptive mutations

HCV RNA genome replication and structural or non-structural protein expression are early steps in the HCV life cycle. To further confirm our speculation that the robust HCV titers and enhanced virion release were both related to up-regulated RNA replication and protein expression, we determined the relative HCV RNA, NS5A immunofluorescence, and NS3 protein levels in the RNA-transfected Huh7.5 cells on day 3 (P1) and day 9 (P3).

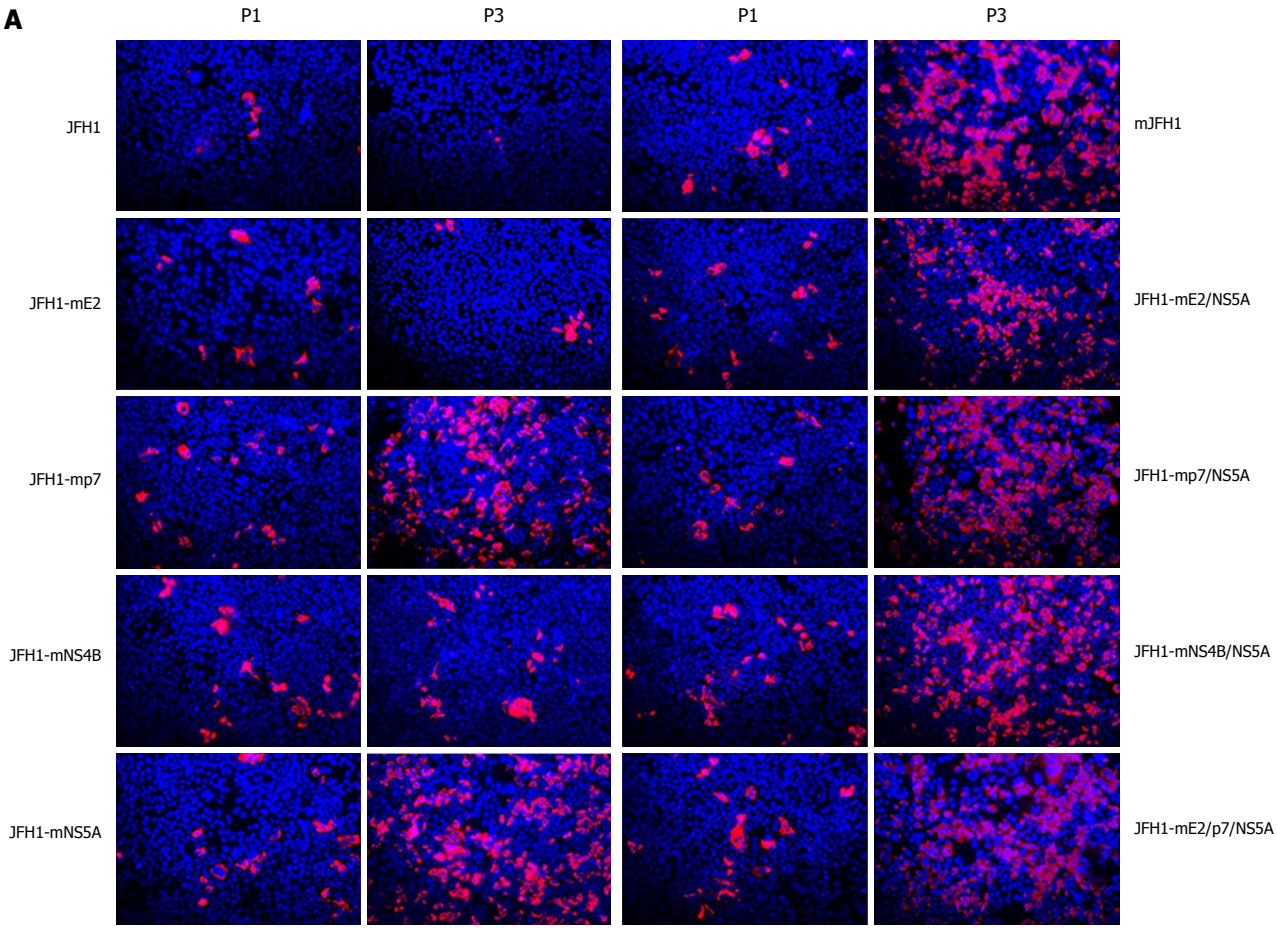
As shown in Figure 3, the expression of NS5A (Figure 3A) and NS3 (Figure 3B) in mutants was up-regulated at different levels during serial passages. The trend was extraordinary obvious in NS5A or p7 mutants. Anti-NS3 Western blot analysis, which was the most widely used for quantitative experiment, yielded consistent results.

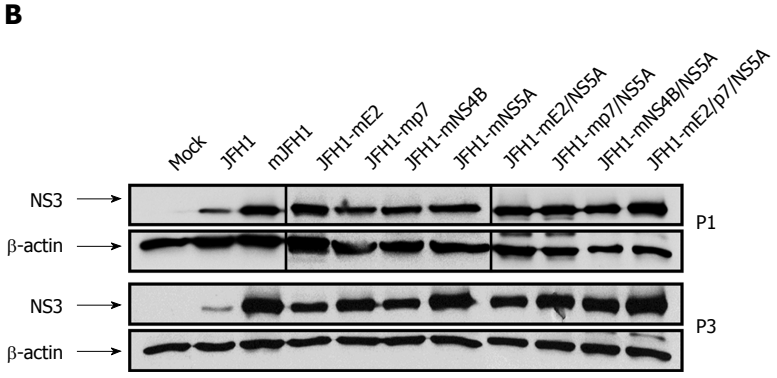
As shown in Figure 3C, the RNA levels of all the mutants were increased compared with JFH1, and mJFH1 was increased 18.7-fold. Interestingly, the results indicated that JFH1-mNS4B expression increased 6.1-fold, and the combination of mutants showed a 7.4-16.8-fold increase.

Taken together, we confirmed that the effect of NS5A (C2274R, I2340T and V2440L), p7 (H781Y), and NS4B (N1931S) on infectious HCV titers was robust, and started with HCV RNA replication and protein expression, followed by virion release.

Adapted variants enhance the efficiency of virus release

Virion release is the last step of the HCV life cycle. To further explore the mechanism underlying the enhanced virus production, the role of adaptive mutations was examined. Ten HCV RNAs (JFH1, JFH1-mE2, JFH1-mP7, JFH1-mNS4B, JFH1-mNS5A, JFH1-mE2/NS5A, JFH1-mp7/NS5A, JFH1-mNS4B/NS5A, mJFH1, and JFH1-mE2/p7/NS5A) were electroporated into Huh7.5 cells. After 3 d, we collected the supernatants and cell lysates, and measured the HCV titers using NS5A immunofluorescence assays. Furthermore, to confirm the infectivity of virions, we carried out immunoblotting of supernatants and cell lysates with anti-NS3, which was extraordinarily consistent with the infectious HCV titers (Figure 4A). As shown in Figure 4B, we also calculated the proportion of extracellular (supernatant)


A
P1
P3
P1
P3
JFH1
mJFH1
JFH1-mE2
JFH1-mE2/NS5A
JFH1-mp7
JFH1-mp7/NS5A
JFH1-mNS4B
JFH1-mNS4B/NS5A
JFH1-mNS5A
JFH1-mE2/p7/NS5A


B
Mock
JFH1
mJFH1
JFH1-mE2
JFH1-mp7
JFH1-mNS4B
JFH1-mNS5A
JFH1-mE2/NS5A
JFH1-mp7/NS5A
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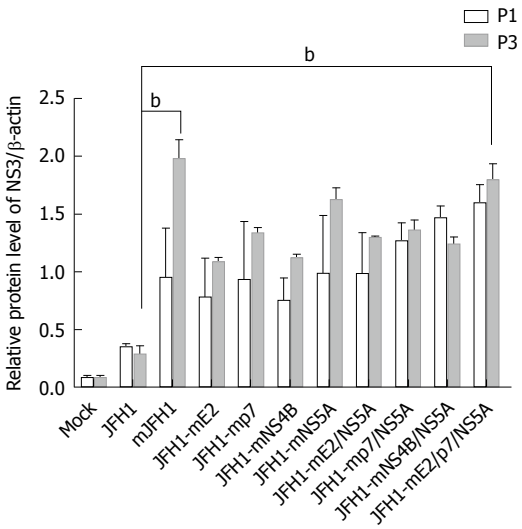
JFH1-mE2/p7/NS5A
NS3
β-actin
NS3
β-actin
P1
P3


P1
P3
b
b
Relative protein level of NS3/β-actin
2.5
2.0
1.5
1.0
0.5
0.0
Mock
JFH1
mJFH1
JFH1-mE2
JFH1-mp7
JFH1-mNS4B
JFH1-mNS5A
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JFH1-mE2/p7/NS5A

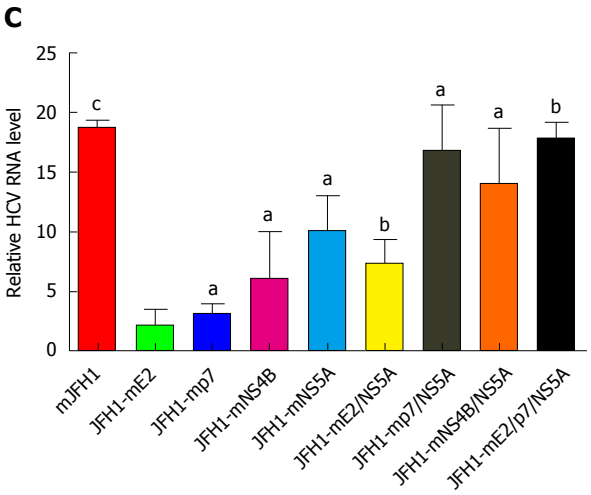

Figure 3 Effects of the adaptive mutations on the hepatitis C virus RNA replication. A: Hepatitis C virus (HCV) RNA was electroporated into Huh7.5 cells to produce the recombinants of adapted virus. The transfected cells were passaged every 3 d. Cells were fixed 48 h after passage and infected cells were identified by fluorescence immunostaining and microscopy. Nuclear DNA was stained with DAPI (blue); B: HCV RNA was electroporated into Huh7.5 cells to produce the recombinants of adapted virus in cell culture. The transfected cells were passaged every 3 d. Cells were lysed at 72 h after passage. The HCV NS3 protein levels were analysis by Western blot. $^{b}P < 0.01$; C: HCV RNA levels in cells 3 d after transfection. Intracellular HCV RNA levels were analyzed by quantitative RT-PCR. The mean ± SD for three independent experiments are presented (qPCR assays, $n = 3$). $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

or intracellular (cell lysate) HCV titers using total titers as 100%. Typically, the supernatant and intracellular HCV titers of JFH1 were 76.31% and 23.69%, respectively, and those of mJFH1 were 94.00% and 6.00%, respectively. Taken together, these findings provide evidence suggesting that the ten variant viruses enhanced the virion release, and the high viral production was linked to the effective virion release. NS5A (C2274R, I2340T, and V2440L) and p7 (H781Y) showed the highest levels compared with the others.

Adaptive mutations are not essential for intracellular LD localization of the NS5A protein

LDs have been reported to play an important role in the HCV virion assembly process[26]. To determine if the six adaptive mutations increased the assembly of HCV at this step, LDs and NS5A were stained in JFH1 and mJFH1 transfected cells and the co-localization of LDs with NS5A was measured. As shown in Figure 5, the LDs were totally covered with NS5A in all cases. However, no significant difference was observed between JFH1 and mJFH1 groups using Image J software and Pearson's correlation coefficient analysis. These results indicated that the six adaptive mutations were not required for LD localization of the NS5A proteins.

Adapted mutations do not affect hyper-phosphorylation of NS5A

Previous studies showed that a ratio between the p56 and p58 phosphoforms of NS5A is required for optimal HCV RNA replication[13,27-29]. JFH1-AM120 is a robust adaptive mutant selected by Liu *et al*[13]. which displays significant switch of p56/p58. In this study, JFH1, JFH1-AM120, and mJFH1 RNA were transfected into Huh7.5 cells, and the total protein was used for Western blot analysis after 3 d (Figure 6). However, we observed no difference in p56 and p58 between the two groups. These results demonstrated that the phosphorylation level of NS5A was not affected by adaptive mutations.

DISCUSSION

Previous studies suggested that *in vitro* adaptive mutations enhance the production of infectious virus[13-17,20,21,30-34]. A high mutation rate in HCV RNA genome is a challenge for successful HCV treatment and vaccine research, although a method to obtain a robust clonal culture of HCV has been unavailable[13]. Liu *et al*[19] demonstrated that a JFH1-ΔV3-EGFP variant produced higher titer of reporter virus. The six adaptive mutations in this variant were located in the E2 (D657G), p7 (H781Y), NS4B (N1931S), and NS5A (C2274R, I2340T, and V2440L) (Figure 1A). However, the mutations responsible for enhanced viral production were not clear. The six mutations in this study were simultaneously located in JFH1-ΔV3-EGFP, which was a reporter EGFP gene chimera virus[19]. The mJFH1 refers to JFH1-ΔV3-EGFP that yielded a robust titer up to 1.61×10^6 FFUs/mL in this study, suggesting that the six mutations are effective adaptive mutations.

HCV is a single, positive-strand RNA virus. We focused on key life cycle events in the virus such as replication, expression, assembly, and release. Infectious virion release is the last step and the final objective of the JFH1 system. In our study, we detected variant virus titers initially. Consistent with previous reports[7,14], JFH1 only exhibited a decreased titer of 10^2 FFUs/mL. The other mutants showed increased titers with several orders of magnitude compared with JFH1. Synergistic enhancement of HCV titer was demonstrated obviously. Jiang *et al*[35] suggested that adaptive mutations enhance specific protein-protein interactions among viral proteins and promote the assembly of infectious HCV particles. We speculated that the six mutations involved

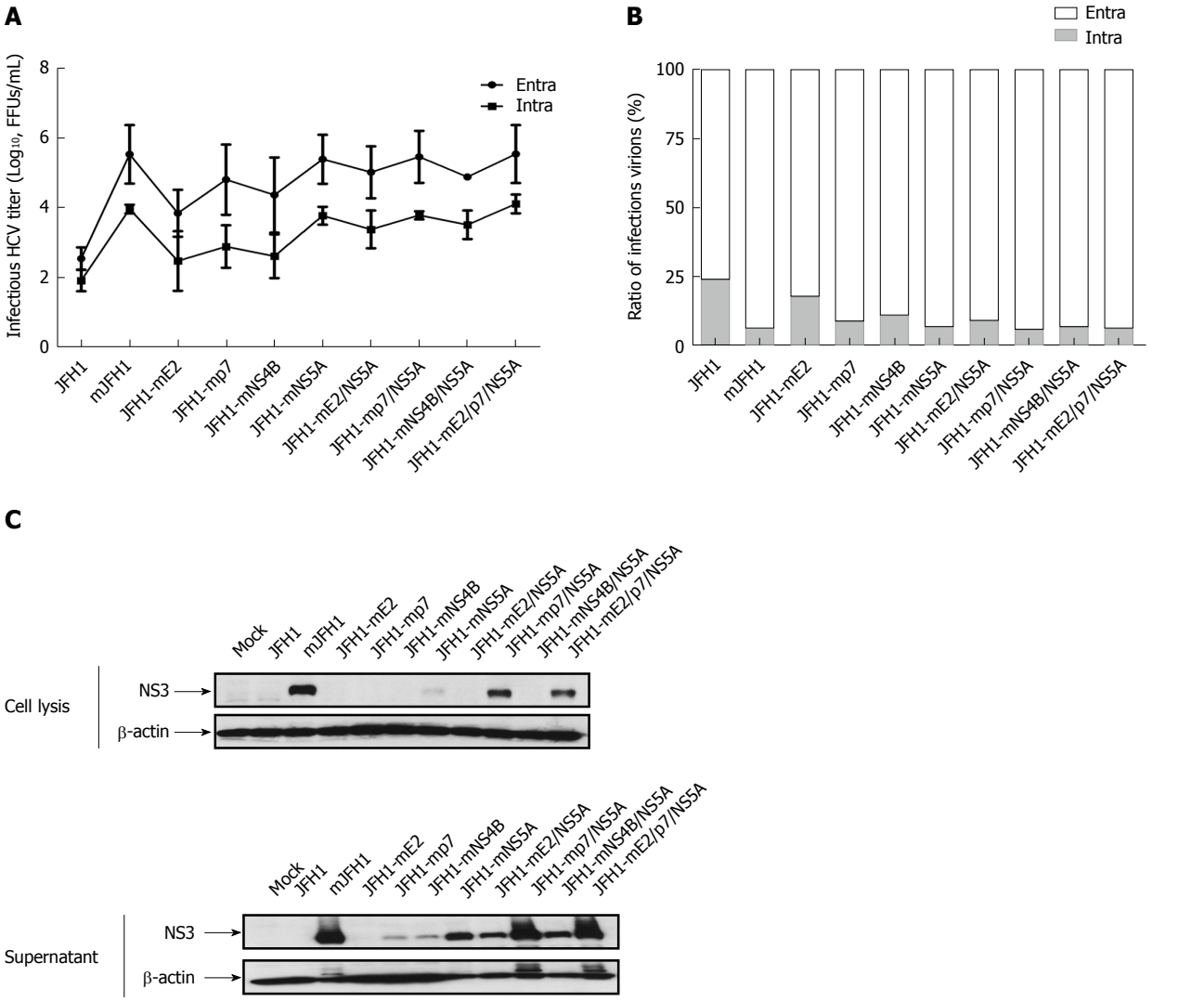

Figure 4 Effect of the adaptive mutations on the virion release. A: Hepatitis C virus (HCV) RNA was electroporated into Huh7.5 cells to produce the recombinants of adapted virus. At 72 h after transfection, the infectivity titers of the culture supernatants and cell lysates were measured. Viral titers are expressed as FFUs/mL. The data are presented as mean ± SD (n = 3); B: HCV RNA was electroporated into Huh7.5 cells to produce the recombinants of adapted virus. At 72 h after transfection, the infectivity titers of the culture media and cell lysates were measured. The extracellular and intracellular viral titers were measured. The relative ratios of infectious virions are shown. The results were from three independent experiments; C: The naive Huh7.5 cells were infected with the culture media and cell lysates. At 72 h after infection, cells were lysed with RIPA buffer, and analyzed by Western blot.

refer to unknown life cycle phases and mechanism as well. Therefore, we analyzed the effect of viral mutations on the distribution of virions in the supernatant and the cell lysate, co-localization of LDs and NS5A, HCV RNA level, NS3 expression, and p56/p58. We found that the adaptive mutations were associated with diverse effects on the life cycle events. The virion release and RNA genome replication were specifically associated with NS5A and p7 mutations.

The transmembrane domains of chimeric E1 and/or E2 HCV glycoproteins were modified to allow transport to and assembly at the cell surface[36]. E2 consisted of three critical domains: a receptor-binding domain (RBD; residues 384-661), the membrane proximal stem-like region of E2 (residues 675-699), and a hydrophobic heptad repeat linking the two domains[37]. Within the RBD, the E2 bound the cellular receptor CD81, leading to receptor-mediated endocytosis of virions[38,39]. Serial studies showed that the mutations in E2 play a role in the HCV life cycle *via* different mechanisms. Tao *et al*[30] demonstrated that the E2 (I414T) mutation had no significant effect on HCV RNA replication and viral entry. However, it enhanced the production of infectious viral particles and decreased the receptor-mediated viral entry. E2 (G451R) altered the relationship between particle density and infectivity, disrupted the co-receptor dependence, and increased virion sensitivity to receptor mimics[40]. The T563I mutation in the E2 protein increased virion viability at 37 ℃. Unfortunately, D657G in E2 improved the HCV titer *via* an unknown mechanism, without any effect on HCV RNA replication or virion release.

As a small membrane polypeptide, the HCV p7 channel plays multiple roles in virus life cycle and

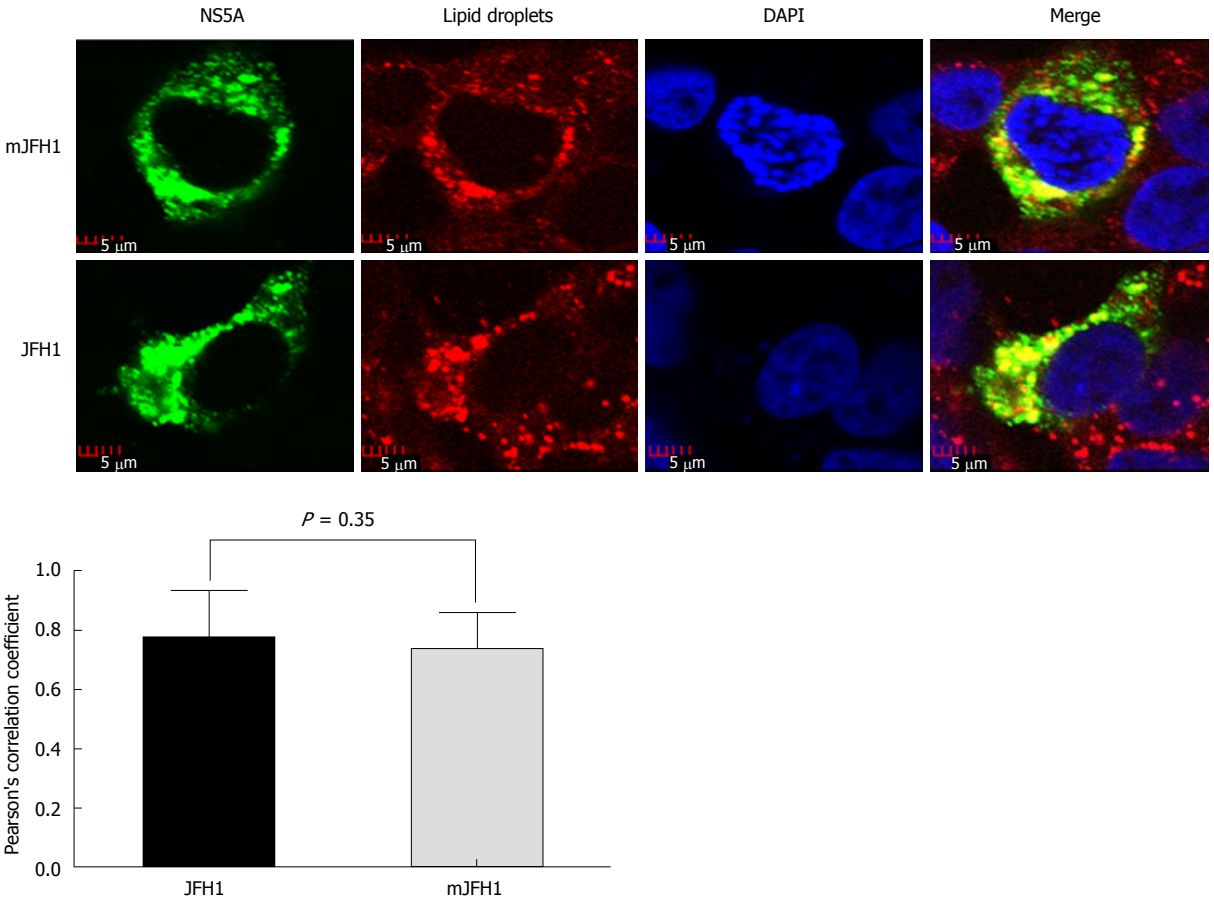

Figure 5 Colocalization analysis of lipid droplets and hepatitis C virus NS5A. JFH1 and mJFH1 RNA was electroporated into Huh7.5 cells to produce the recombinants of adapted virus. At 48 h after transfection, the cells were fixed. Lipid droplets were stained with LipidTOXRed (Red). The HCV NS5A was stained with anti-NS5A antibody (Green). The nucleus was stained with DAPI (Blue). Each triplicate sample of 25 cells was analyzed using Image J software. The degree of co-localization was quantified and compared using Pearson's correlation coefficients.

mediates several biological functions in HCV infection[41]. The p7 consists of six equivalent hydrophobic pockets between the peripheral and pore-forming helices[42]. Generally, p7 is not essential for HCV RNA replication, but required for virion assembly and release[43]. The adaptive mutation N765D in p7 influenced early stages of the HCV life cycle, and increased the infectious HCV titer[15]. Y781H enhances the level of HCV core in the supernatant three- to five-fold, and moderately increases virion assembly and release[21]. In our study, we found similar results, and Y781H enhanced HCV RNA replication 3.1-fold, suggesting its role as a critical initiating agent and a novel mechanism during the HCV life cycle.

HCV NS4B plays an important role in RNA genome replication and virion assembly[44]. NS4B triggers the formation of a viral replication complex[45] similar to the "sponge-like inclusions" observed in the liver of HCV-infected chimpanzee[46]. NS4B (K1846T) increased HCV RNA replication nearly 30-fold[47]. N1931S is located between helices 1 and 2 of the NS4B C-terminus, and was first determined by Li *et al*[48] during HCV RNA replication and virion assembly. Our data suggested that the N1931S increased HCV titer to 10^3 FFUs/mL, which was 10^3-fold compared with JFH1. It significantly enhanced HCV genome replication, and slightly improved virion release. N1931S is a novel mutation in the JFH1 system, and comprehensive studies investigating its role in HCV infection are needed.

HCV NS5A is a phosphoprotein existing in two different forms: a basic phosphorylated NS5A, p56, and a hyperphosphorylatedNS5A, p58. It appears to play an important role in viral replication, since most of the adaptive mutations determined so far are located within the region of NS5A[47]. The three domains in NS5A include: domain I (aa 28-213) coordinating a single zinc atom, and domains II (aa 250-342) and III (aa 356-447), which are less well characterized but are important in RNA replication and/or virion assembly[28]. A previous report suggested that V2440L was located at the NS5A-B cleavage site and decreased the cleavage kinetics[20]. Thus, the mutation C2274R is located in domain II, and the other mutations (I2340T and V2440L) occur in domain III. We analyzed the HCV RNA replication and protein expression. The results showed that the three mutations enhanced HCV RNA replication,

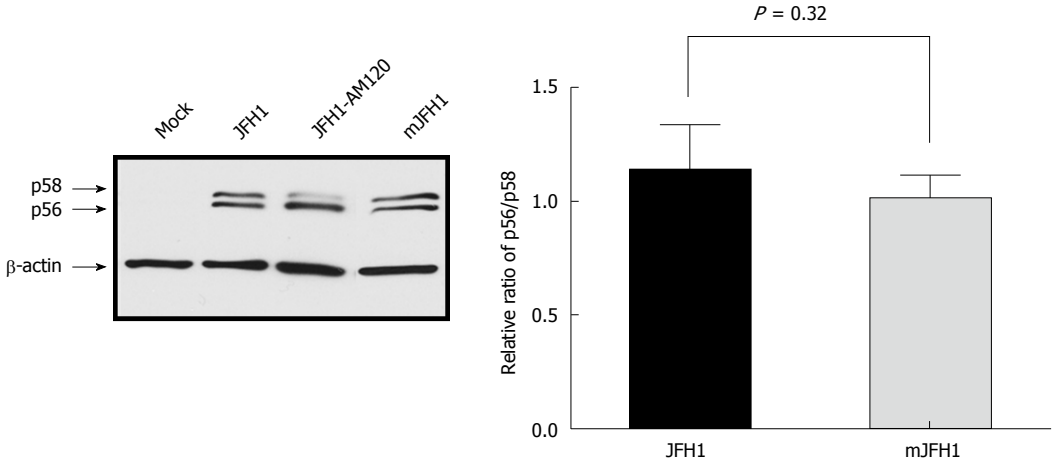

Figure 6 Phosphorylation of NS5A during JFH1 and mJFH1 replication. Huh7.5 cells were transfected with JFH1 or mJFH1 RNA. After three days of culture, cells were lysed for western blot using anti-NS5A and anti-β-actin antibodies. The quantity of p56 and p58 was determined using Image J software and the ratios of p56/p58 are shown. Data are presented as mean ± SD (n = 3). JFH1-AM120 was used as the positive control.

which is consistent with the structure.

A previous study demonstrated that HCV p7 promotes a late step of assembly and release of infectious virions[49] and NS5A plays a major role in regulating the release of infectious virus particles in cell culture[32]. In this study, there were three mutants (C2274R, I2340T, and V2440L) located in NS5A and one located (H781Y) in p7. Our results showed that these mutants obviously promoted the HCV viral particles release (Figure 4). HCV core is located on the cytosolic side of the ER membrane, assembly probably initiates in the cytosol before further maturation, and release occurs by transfer of nascent particles across the ER membrane to enable access to the secretory pathways in hepatocytes[50]. The amino acid changes induced by mutants in NS5A and P7 may be involved in these steps. The specific mechanism needs to be further studied in future.

Previous studies suggested that the up-regulation of p56/p58 ratio might be a critical factor for HCV titer[13] and increased HCV replication since specific mutations reduced NS5A hyper-phosphorylation activating RNA replication[27]. NS5A-p58 levels increased following overexpression of CKI-alpha, CKI-delta, and CKI-epsilon, whereas RNA interference of CKI-alpha alone reduced NS5A hyper-phosphorylation[51]. Here, we detected the status of p56/p58. However, there was no switch between the JFH1 and mJFH1 groups. The two viral proteins including the core and NS5A were localized to LDs, which play an important role in the intracellular assembly of HCV[24,26]. The recruitment of NS5A to LDs was a prerequisite for virion assembly in Huh7.5 cells[26]. Our analysis of the co-localization of NS5A and LDs showed no significant difference between mJFH1 and JFH1, suggesting that these adaptive mutations did not alter virion formation.

The life cycle of HCV is extremely complex, and several details remain unknown. Regulation of host gene expression[52,53], altered association between viral proteins and/or host-cell proteins, and changes in virus *per se*[54] represent obvious mechanisms. In our study, we confirmed that the adaptive mutations led to a robust infectious titer *via* enhanced viral replication and release. It is recommended that DAA regimens can be used for treatment of patients with hepatitis C rather than pegylated interferon/rabivirin[5]. Meanwhile, our study was limited by the reaction of DAAs to above adaptive mutations. Further studies investigating the underlying mechanisms of viral morphogenesis are needed.

In conclusion, we generated infectious HCV particles with a robust titer of 1.61 × 10^6 FFUs/mL in this study. All of the six adaptive mutations increased the HCV particle production at varying levels. The NS5A (C2274R, I2340T, and V2440L) and p7 (H781Y) were critical adaptive mutations. This study confirmed that the JFH1 is still a promising system to study the HCV life cycle. To use adaptive mutations is an effective means to establish a new system with higher infectious HCV virions titer. And the research on molecular mechanism of interaction between viral proteins and/or host-cell proteins should be carried out in depth.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C virus (HCV) causes acute and chronic hepatitis, and leads to permanent liver damage and hepatocellular carcinoma. The infectious HCV JFH1 cell culture system represents a major advance in anti-HCV drug discovery research and facilitates the study of HCV life cycle. However, HCV JFH1 (genotype 2a) merely generates relatively low viral titers. JFH1-ΔV3-EGFP, which includes six mutations located in the E2, p7, NS4B, and NS5A regions, could produce higher titers of HCV-EGFP reporter virus. However, there were no data about which mutations or combinations thereof are responsible for enhanced viral production and the underlying mechanisms.

Research motivation

This JFH1 model generated infectious viral particles in cell culture and facilitated the study of the HCV life cycle, but the low infectious virion titer limits

its application range. Some previous studies have confirmed that adaptive mutations could enhance the virion titer, but the mechanism has not yet been fully elucidated. In this study, we focused on the positive effect of six adaptive mutations located in the E2, p7, NS4B, and NS5A regions, and found that the mechanism was different among them during the procession. These results gave us some new insights into the infectious HCV cell culture system and adaptive mutations.

Research objectives

The main objective of this study was to establish an infectious HCV cell culture system with a robust titer, and to discuss the underlying mechanisms of the adaptive mutations found in previous studies. The results of this study have supplied the researchers with a useful tool. We hope it will be used for the study of viral structure, virus-host interaction, anti-HCV drug discovery, and vaccine development.

Research methods

We investigated JFH1-mE2, JFH1-mp7, JFH1-mNS4B, JFH1-mNS5A, JFH1-mE2/NS5A, JFH1-mp7/NS5A, JFH1-mNS4B/NS5A, JFH1-mE2/p7/ NS5A, and mJFH1, carrying all the six mutations. We analyzed the infectious HCV titer, HCV RNA and NS3 protein levels, viral release capacity, assembly and hyper-phosphorylation of NS5A to determine the role of these mutations in the HCV life cycle. These methods were the routine ways adopted widely in virological and molecular biological research.

Research results

The main findings in this study were as follows: (1) we generated infectious HCV particles with a robust titer of 1.61×10^6 FFUs/mL; (2) The six adaptive mutations increased the HCV particle production at varying levels. The NS5A (C2274R, I2340T, and V2440L) and p7 (H781Y) are critical adaptive mutations. The effect of NS5A (C2274R, I2340T, and V2440L), p7 (H781Y), and NS4B (N1931S) on infectious HCV titers was investigated by measuring the HCV RNA replication, protein expression, and virion release; and (3) the six adaptive mutations were all not required for the lipid droplet localization of NS5A proteins or the phosphorylation of NS5A. To our knowledge, this is a new robust titer related to adaptive mutations from JFH1. The problems that remain to be solved in the future include: (1) how could the adaptive mutations be translated to clinical conditions? (2) are these mutation patterns observed *in vivo*? and (3) would these results be relevant to the resistance to direct-acting antivirals (DAAs)?

Research conclusions

First, this study generated infectious HCV particles with a robust titer of 1.61×10^6 FFUs/mL. Second, all of the six adaptive mutations increased the HCV particle production at varying levels. Third, the NS5A (C2274R, I2340T, and V2440L) and p7 (H781Y) were critical adaptive mutations, but they were not required for the LD localization of NS5A proteins or the phosphorylation of NS5A. Based on the new findings of this study, we proposed that more important adaptive mutations would be addressed in the future, and unknown mechanism of the HCV life cycle would be explained.

Research perspectives

This study re-confirmed that the JFH1 was still a promising system to study the HCV life cycle. To use adaptive mutations was an effective way to establish a new system with higher infectious HCV virion titer. In addition, we also re-confirmed that the molecular mechanism of interaction between viral proteins and/or host-cell proteins is more complex and important.

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ORIGINAL ARTICLE

Case Control Study

Serum interleukin-34 level can be an indicator of liver fibrosis in patients with chronic hepatitis B virus infection

Yin-Qiu Wang, Wen-Jun Cao, Yu-Feng Gao, Jun Ye, Gui-Zhou Zou

Yin-Qiu Wang, Wen-Jun Cao, Yu-Feng Gao, Jun Ye, Gui-Zhou Zou, Department of Infectious Disease, The Second Affiliated Hospital of Anhui Medical University, Hefei 230601, Anhui Province, China

ORCID number: Yin-Qiu Wang (0000-0001-6069-1809); Wen-Jun Cao (0000-0003-1204-592X); Yu-Feng Gao (0000-0003-1822-8161); Jun Ye (0000-0003-4808-5608); Gui-Zhou Zou (0000-0002-4690-5580).

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Correspondence to: Yu-Feng Gao, MAMS, Professor, Department of Infectious Disease, Second Affiliated Hospital of Anhui Medical University, 678 Furong Road, Economic and Technological Development District, Hefei 230601, Anhui Province, China. aygyf@126.com
Telephone: +86-551-63869420
Fax: +86-551-63869400

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Abstract

AIM

To investigate whether serum interleukin (IL)-34 levels are correlated with hepatic inflammation and fibrosis in patients with chronic hepatitis B virus (HBV) infection.

METHODS

In this study, serum IL-34 levels were assessed by enzyme-linked immunosorbent assay in 19 healthy controls and 175 patients with chronic HBV infection undergoing biopsy. The frequently used serological markers of liver fibrosis were based on laboratory indexes measured at the Clinical Laboratory of the Second Affiliated Hospital of Anhui Medical University. Liver stiffness was detected by transient elastography with FibroTouch. The relationships of non-invasive makers of liver fibrosis and IL-34 levels with inflammation and fibrosis were analyzed. The diagnostic value of IL-34 and other liver fibrosis makers were

evaluated using areas under the receiver operating characteristic curves, sensitivity and specificity.

RESULTS

Serum IL-34 levels were associated with inflammatory activity in the liver, and IL-34 levels differed among phases of chronic HBV infection (P = 0.001). By comparing serum IL-34 levels among patients with various stages of liver fibrosis determined by liver biopsy, we found that IL-34 levels ⩾ 15.83 pg/mL had a high sensitivity of 86.6% and a specificity of 78.7% for identifying severe fibrosis (S3-S4). Furthermore, we showed that IL-34 is superior to the fibrosis-4 score, one of the serum makers of liver fibrosis, in identifying severe liver fibrosis and early cirrhosis in patients with HBV-related liver fibrosis in China.

CONCLUSION

Our results indicate that IL-34, a cytokine involved in the induction of activation of profibrogenic macrophages, can be an indicator of liver inflammation and fibrosis in patients with chronic HBV infection.

Key words: Interleukin 34; Hepatitis B virus; Liver fibrosis; Diagnosis

Core tip: Interleukin (IL)-34 is a cytokine involved in the induction of activation of profibrogenic macrophages, which is associated with the severity of liver fibrosis and inflammation. Numerous studies have shown that it has the potential to be a serological indicator of liver fibrosis and inflammation. We investigated the serum IL-34 levels in patients with chronic hepatitis B virus infection, and found the significance of serum levels of IL-34 as a serum target of liver fibrosis associated with chronic hepatitis B virus infection.

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INTRODUCTION

Liver fibrosis is a process accompanied by wound-healing responses caused by chronic injury and inflammation in the hepatic parenchyma, and it often results in serious complications, including portal hypertension and liver failure. It can lead to cirrhosis, which is identified as the final stage of liver fibrosis[1] and can even evolve into hepatocellular carcinoma. Liver fibrosis is often caused by viral infection, toxins and excess alcohol consumption, among others. Chronic hepatitis B virus (HBV) infection is the most common cause of liver fibrosis in China[2].

Chronic HBV infection is characterized by progressive hepatic fibrosis and inflammation. In addition to the key role of hepatic stellate cells, the progression of liver fibrosis depends on the recruitment and accumulation of inflammatory monocytes, which can locally differentiate into macrophages, to the liver[3]. These macrophages activate hepatic stellate cells and promote and perpetuate fibrosis[4,5]. It has already been confirmed that interleukin (IL)-34 is a kind of macrophage differentiation factor that signals *via* the M-CSF receptor (c-fms or CD115)[6,7] and that its serum levels are elevated in hepatitis C virus (HCV)-infected patients and nonalcoholic fatty liver disease patients with advanced liver fibrosis[3,8,9]. Although IL-34 has been identified as a profibrotic factor associated with chronic HCV infection-mediated fibrosis, data on the serum level and role of IL-34 in chronic HBV-infected patients are lacking.

The indication for antiviral therapy depends on HBV DNA levels, aminotransferase levels and/or the grade of inflammation and fibrosis determined by liver biopsy[10]. However, the extent of disease progression is often insufficiently reflected by aminotransferase levels; additionally, liver biopsy has substantial limitations because of the invasive nature of the process[11]. Up to 40% of patients are ineligible for liver biopsy[12]. Therefore, studies are investigating noninvasive methods for detecting fibrosis[13]. These methods rely on biomarkers that are easily determined using one or more serum indexes, such as aspartate transaminase (AST) to platelet ratio index (APRI), fibrosis-4 (FIB-4) score, and fibrosis index (FI)[14]. Although these methods demonstrate adequate diagnostic performance, they still have some limitations. Liver stiffness, measured *via* transient elastography using FibroTouch, can be reliably used to detect fibrosis in most patients; however, this method cannot be used in patients with ascites or obesity, and its performance varies with operator experience[15].

In this study, we assessed the serum level of IL-34 in 175 chronic HBV-infected patients undergoing biopsy. We also analyzed the correlation between IL-34 and other serum indexes that reflect the extent of liver injury and inflammation and evaluated the possibility of using IL-34 level as a marker of liver fibrosis in patients with chronic HBV infection by comparing it with other assessment methods for liver fibrosis.

MATERIALS AND METHODS

Selection of patients

In total, 175 treatment-naive chronic hepatitis B (CHB) patients who had undergone percutaneous liver biopsies at the Department of Infectious Diseases of the Second Affiliated Hospital of Anhui Medical University from January 2014 to March 2016 were

Table 1 Levels of interleukin-34 in patients with different stages of liver fibrosis

Stage	*n*	Median	95%CI
S0 patients and healthy subjects	34	10.05	9.28-11.27
S1-S2 patients	93	11.53	10.38-13.92
S3-S4 patients	67	19.84	17.34-20.63

CI: Confidence interval.

enrolled in this retrospective study. The inclusion criteria were age ⩾ 16 years, history of HBV infection of more than 6 mo and positivity for hepatitis B surface antigen. The exclusion criteria were concomitant infection with the HCV or human immunodeficiency virus, history of antiviral therapy, compensated or decompensated liver cirrhosis, presence of alcoholic liver disease, nonalcoholic fatty liver disease, autoimmune liver diseases, chronic liver diseases due to other causes and renal insufficiency, inadequate biopsy samples, and incomplete clinical data. Nineteen healthy subjects who gave blood on a voluntary basis served as controls, and written informed consent was obtained. This retrospective study was approved by the Ethics Committee of Anhui Medical University. The study was performed in accordance with the 1975 Declaration of Helsinki.

Cytokine quantification

Blood samples were collected at the time of patient presentation at our department, and serum was separated from blood samples by centrifugation. Serum IL-34 levels were quantified by enzyme-linked immunosorbent assay (R and D Systems, United States).

Liver biopsies and fibrosis staging

Percutaneous liver biopsies were obtained using ultrasound-guided biopsy needles. The specimens were then fixed, paraffin-embedded and stained with hematoxylin and eosin. All liver tissues samples were evaluated by board-certified pathologists who were unaware of the patients' clinical history. Liver fibrosis stages (S0-S4) were determined using the Scheuer's classification system. The lack of fibrosis was characterized as S0, mild fibrosis as S1, moderate fibrosis as S2, severe fibrosis as S3-S4 and cirrhosis as S4.

Other laboratory and virological parameters

Other laboratory parameters including AST, alanine transaminase (ALT), gamma-glutamyl transferase, alkaline phosphatase and bilirubin levels, platelet count and virological test results were routinely evaluated prior to liver biopsy at the Clinical Laboratory of the Second Affiliated Hospital of Anhui Medical University.

Transient elastography

Prior to liver biopsy, liver stiffness was determined using the FibroTouch instrument (Wuxi Hayes Kell Medical Technology Co. Ltd., China) operated by experienced technicians. Ten successful acquisitions were performed for each patient. The median value of the 10 measurements was used for analyses. Liver stiffness was expressed in kilopascals (kPa).

Statistical analysis

All statistical analyses were performed using MedCalc 15.8, GraphPad Prism 5.0 and SPSS 17.0. Differences between groups were tested using the Mann-Whitney *U*-test or Wilcoxon-Mann-Whitney test (for continuous variables and nonparametric analyses for independent samples, respectively). Correlation coefficients (*r*) were calculated with nonparametric Spearman's correlation analyses. Receiver operating characteristic (ROC) curves were generated for the assessment of scores predictive of stages of fibrosis. Area under the curve (AUC), sensitivity and specificity were calculated for each factor. The value with the best sensitivity and specificity in AUC analysis (Youden's index) was chosen as the best cut-off. AUCs were compared using the approach described by Hanley and McNeil. $P < 0.05$ (two-sided) was considered significant.

RESULTS

Serum levels of IL-34 among groups of patients with various fibrosis stages

By investigating the serum levels of IL-34 in 19 healthy controls and 175 patients, we found that IL-34 levels were significantly different among the no fibrosis group (S0 patients and healthy subjects), mild to moderate fibrosis group (S1-S2), and advanced fibrosis group (S3-S4) ($P = 0.000$, Kruskal-Wallis test, two-tailed). The median expression level of IL-34 in S0 patients and healthy subjects was 10.05 pg/mL. The mean expression level of IL-34 was 11.53 pg/mL in S1-S2 patients, and the median increased to 19.84 pg/mL in S3-S4 patients (Table 1). We also found a highly statistically significant difference ($P = 0.000$, Kruskal-Wallis test, two-tailed) among HBV patients with different inflammation grades (Figure 1A).

IL-34 levels in different phases of CHB infection

Based on HBV DNA levels, hepatitis B envelope antigen (HBeAg) status and serum aminotransferase levels, patients were classified into four groups according to the European Association for the Study of the Liver guidelines: immune-tolerant patients ($n = 26$), HBeAg-positive hepatitis patients ($n = 24$), HBeAg-negative

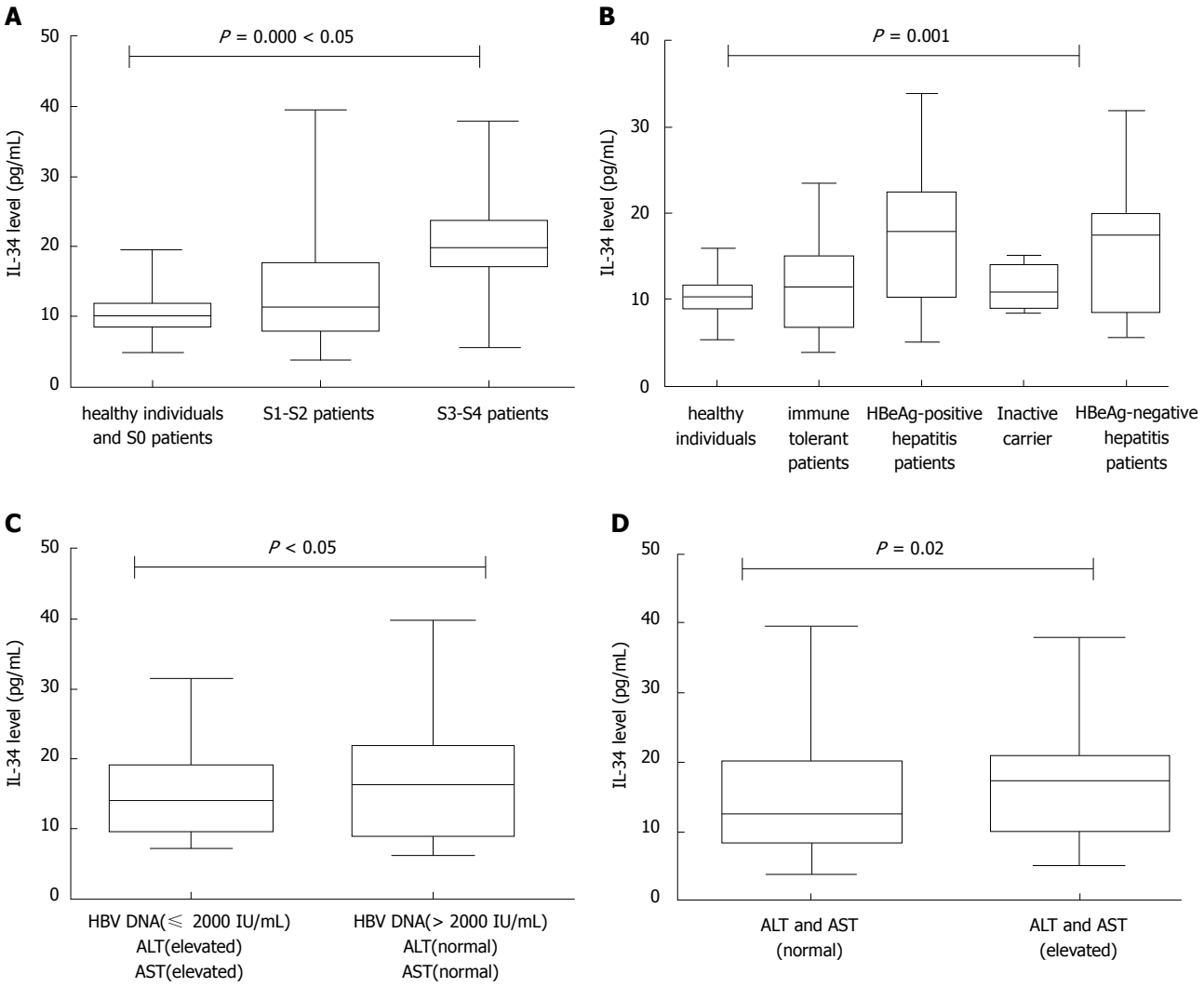

Figure 1 Box-and-whisker plots. A: IL-34 levels in groups of patients with various stages of fibrosis; B: IL-34 levels in groups of different phases of chronic hepatitis B infection; C: IL-34 levels in two groups of HBeAg-negative patients: low viral load (HBV DNA level ≤ 2000 IU/mL) and elevated aminotransferase level; high viral load (HBV DNA level > 2000 IU/mL) and normal aminotransferase level; D: IL-34 levels in group of patients with normal aminotransferase or elevated aminotransferase level. IL: Interleukin; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus.

hepatitis patients (n = 40) and inactive carriers (n = 6)[10]. Furthermore, patients with HBeAg-negative status were stratified into two additional groups: patients with low-replicative hepatitis, characterized by low viral load (HBV DNA level ≤ 2000 IU/mL) and elevated aminotransferase levels (n = 13); and patients with high viral load (HBV DNA level > 2000 IU/mL) and normal aminotransferase levels (n = 26)[10].

Serum IL-34 levels were determined in patients and healthy individuals. Serum IL-34 concentrations ranged from 3.90 pg/mL to 39.56 pg/mL in HBV-infected patients and from 5.39 pg/mL to 15.78 pg/mL in healthy individuals. There were highly significant differences in serum IL-34 levels observed between these groups according to the Kruskal-Wallis test (P = 0.001) (Figure 1B). Patients with HBV infection had the highest IL-34 levels, followed by patients with HBeAg-negative or HBeAg-positive hepatitis. In contrast, inactive HBV carriers and immune-tolerant patients had the lowest IL-34 concentrations. Additionally, there were no differences in serum IL-34 levels among inactive HBV carriers, immune-tolerant patients and healthy individuals.

Correlation between IL-34 levels and other laboratory indexes

In patients with liver fibrosis (chronic HBV infection), there was a significant positive correlation between the serum levels of IL-34 and levels of ALT (r = 0.159, P = 0.036), AST (r = 0.257, P = 0.001), total bilirubin (r = 0.199, P = 0.008), indirect bilirubin (r = 0.225, P = 0.003), gamma-glutamyl transferase (r = 0.178, P = 0.018), alkaline phosphatase (r = 0.214, P = 0.004), and platelet count (r = -0.323, P = 0.000) (Figure 2). IL-34 levels were significantly higher in patients with elevated aminotransferase levels than in patients with

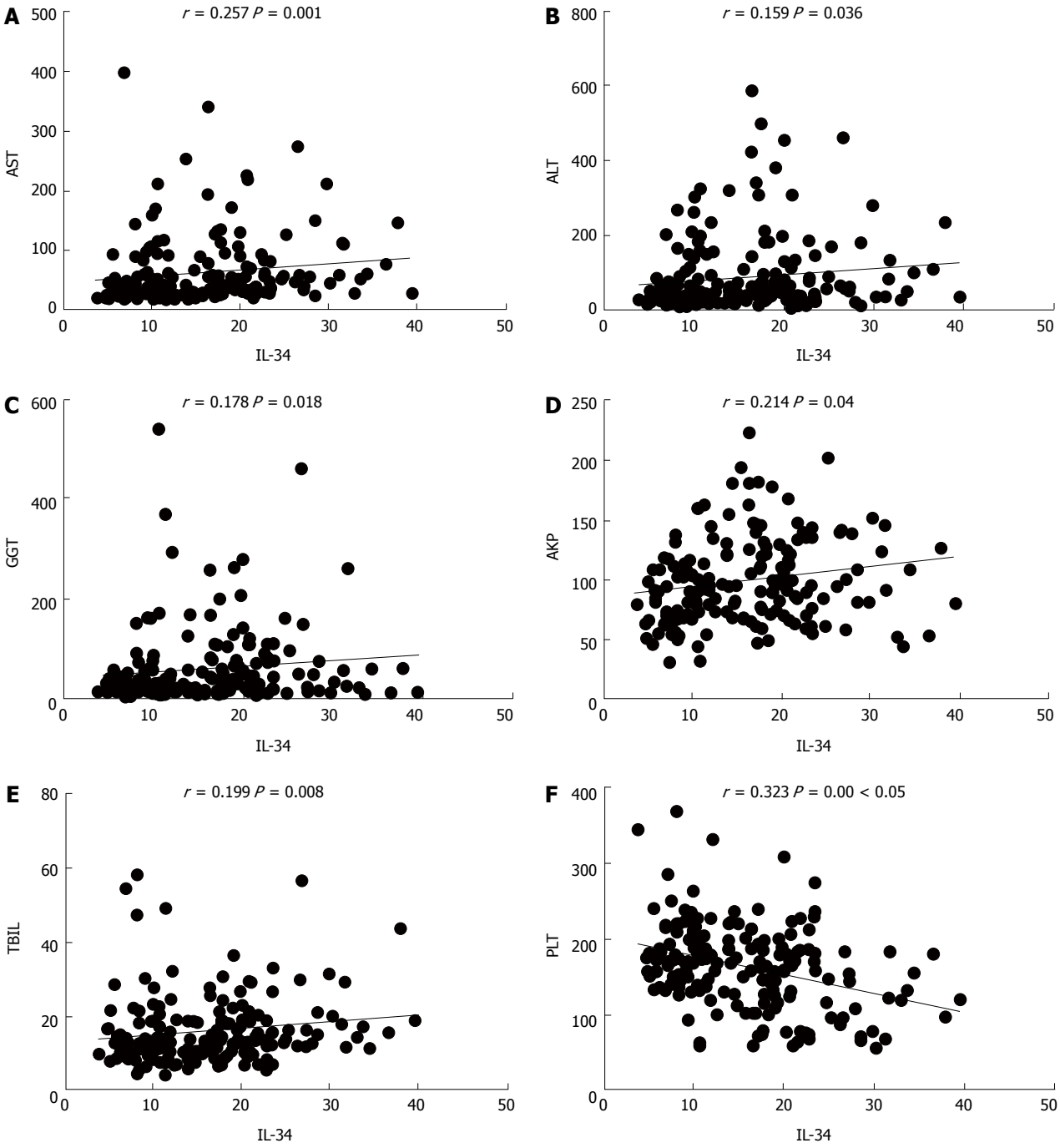

Figure 2 Correlation between IL-34 levels and other laboratory indexes. A: AST; B: ALT; C: GGT; D: AKP; E: TBIL; F: PLT. AKP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase; PLT: Platelet; TBIL: Total bilirubin.

normal aminotransferase levels (P = 0.02) (Figure 1C).

Diagnostic value of IL-34 in predicting severe liver fibrosis

We aimed to determine whether severe liver fibrosis, defined as fibrosis at stages greater than or equal to S3 (S3-S4), in chronic HBV patients is critical for guiding the prognosis and treatment of patients with hepatitis B. Encouraged by our results showing that IL-34 may be a marker of fibrosis stage, we sought to determine whether IL-34 is a marker of severe liver fibrosis (S3-S4) and early cirrhosis (S4). ROC curve analysis resulted in AUCs of 0.829 and 0.836 for severe fibrosis (S3-S4) (Figure 3A) and early cirrhosis (S4) (Figure 3B), respectively. IL-34 levels predicted severe fibrosis (S3-S4) with a sensitivity of 86.6% and a specificity of 78.7%. When IL-34 level > 15.83 pg/mL was used as a cut-off to diagnose severe fibrosis. The sensitivity and specificity of IL-34 on predicting early cirrhosis (S4) are 100% and 64.9%, and the cut-off value is 15.83 pg/mL.

Comparison of IL-34 and several commonly used scores for diagnosing severe liver fibrosis and early cirrhosis

Different fibrosis scores (FIB-4, APRI, Forns and fibrosis-cirrhosis index) have been used to diagnose liver fibrosis or cirrhosis. We compared the performance

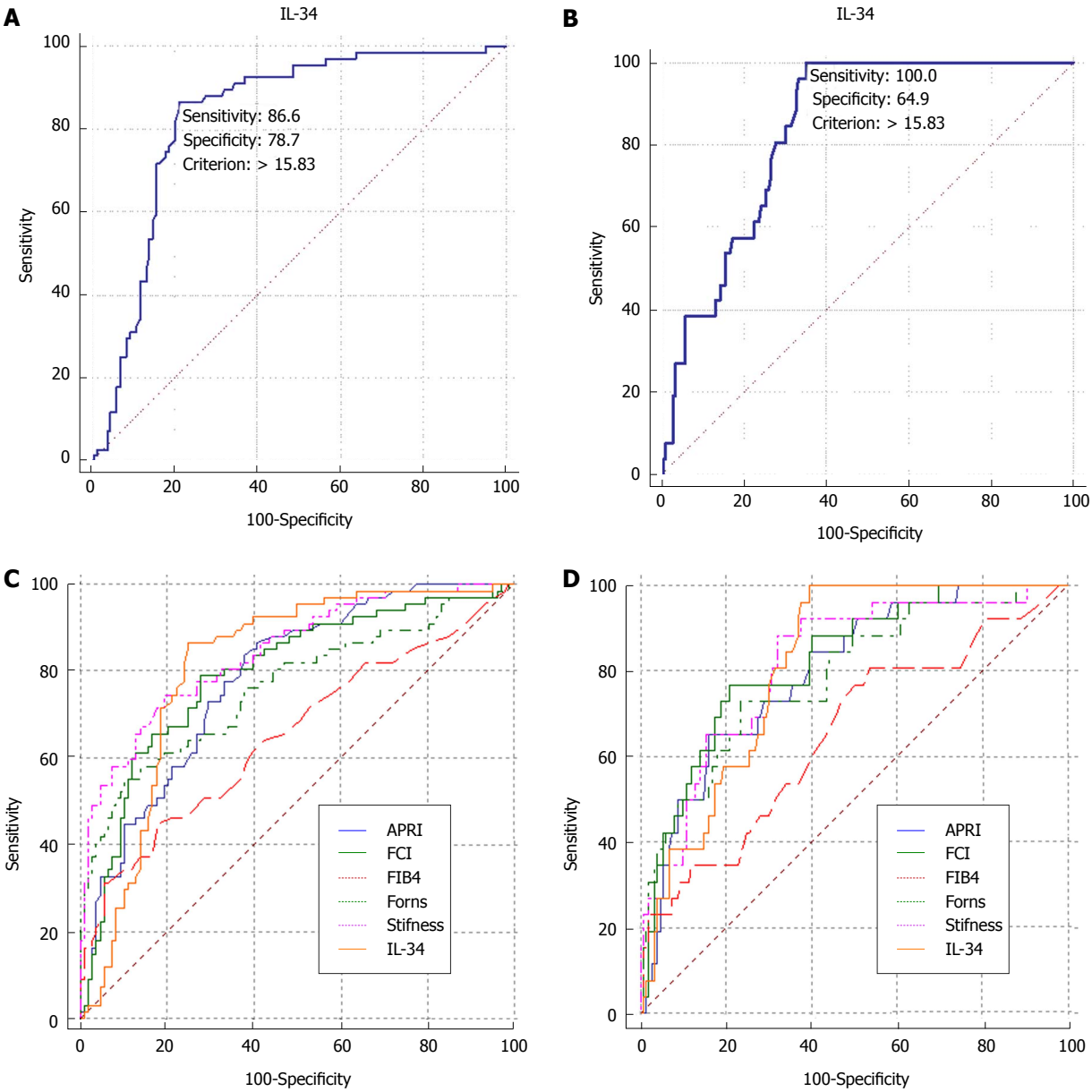

Figure 3 ROC curves, sensitivity and specificity. A: ROC curve analysis for severe fibrosis (S3-S4); B: ROC curve analysis for early cirrhosis (S4); C: AUC comparison of IL-34 level, liver stiffness and other scores for the diagnosis of severe fibrosis (S3-S4); D: AUC comparison of IL-34 level, liver stiffness and other scores for the diagnosis of early cirrhosis (S4). APRI: Aspartate aminotransferase to platelet ratio index; AUC: Area under the curve; FCI: Fibrosis-cirrhosis index; FIB-4: Fibrosis-4; ROC: Receiver operating characteristic.

of IL-34 to the performance of these serum fibrosis scores for the detection of severe liver fibrosis. We conducted a comparative ROC analysis for these scores for individually diagnosing severe liver fibrosis. There were significant differences in AUCs between IL-34 and the FIB-4 score (P = 0.005) in predicting severe fibrosis, indicating that IL-34 was superior to the FIB-4 score. IL-34 was also better than the FIB-4 score in diagnosing early cirrhosis (P = 0.0092). However, for both severe fibrosis and early cirrhosis, the diagnostic accuracy of IL-34 was similar to that of liver stiffness and other scores (Figure 3C and D, Table 2).

DISCUSSION

The correct staging of liver fibrosis is important for guiding the clinical treatment of chronic hepatitis. Liver biopsy, the gold standard for staging liver fibrosis, is invasive and has many limitations[13]. Other recognized noninvasive methods for determining the stage of liver fibrosis also have many disadvantages[16]. Therefore, an increasing number of scholars are investigating noninvasive methods for staging liver fibrosis. In this study, we found that serum IL-34 levels are elevated in HBV-infected patients with severe liver fibrosis (S3-S4) and that IL-34 may be potential marker for differentiating early-stage fibrosis (S0-S2) from late-stage fibrosis (S3-S4) in patients with HBV-related liver fibrosis in China.

This study also clearly demonstrates the diagnostic value of IL-34 as a noninvasive biomarker in the assessment of HBV-related liver fibrosis in patients in

Table 2 Area under the curves for different fibrosis scores for the various stages of fibrosis

	AUC (95%CI)		
	S0 *vs* S1-S4	S3-S4 *vs* S0-S2	S4 *vs* S0-S3
IL-34	0.753 (0.659-0.848)	0.809 (0.743-0.875)	0.815 (0.747-0.883)
APRI	0.714 (0.580-0.847)	0.783 (0.715-0.850)	0.797 (0.710-0.884)
FIB-4	0.577 (0.427-0.727)	0.651 (0.564-0.738)	0.651 (0.529-0.773)
Forns	0.529 (0.405-0.653)	0.762 (0.685-0.839)	0.788 (0.689-0.886)
FCI	0.580 (0.422-0.738)	0.793 (0.723-0.863)	0.822 (0.739-0.906)
Liver stiffness	0.684 (0.565-0.803)	0.844 (0.784-0.903)	0.815 (0.728-0.902)

APRI: Aspartate aminotransferase to platelet ratio index; AUC: Area under the curves; CI: Confidence interval; FCI: Fibrosis-cirrhosis index; FIB-4: Fibrosis-4; IL: Interleukin.

China. We were able to demonstrate IL-34 as predictor of severe liver fibrosis (S3-S4) and early cirrhosis (S4) in HBV-infected patients in China. The AUC of IL-34 was 0.829 for the detection of severe liver fibrosis (S3-S4) and 0.836 for the detection of early cirrhosis (S4). Especially for early cirrhosis (S4) patients, the sensitivity can be up to 100%. This means that it may be possible to avoid missed diagnosis of early cirrhosis. After all, the effective treatments are available to reverse the progress of disease[17]. And, regardless of the situation of ALT and HBeAg, as long as there is an objective basis for cirrhosis, active antiviral therapy is recommended[10].

Compared with other serological models, IL-34 was comparable to the FIB-4 score for the detection of severe liver fibrosis (S3-S4) and early cirrhosis (S4). Even for diagnosing S0 liver fibrosis, IL-34 was comparable to the FIB-4 score (data not shown). Most scholars consider transient elastography to be a promising noninvasive method for the detection of fibrosis in chronic HBV patients[18]. However, this technique is usually only available in specialized centers. Another limitation of transient elastography is that it has a failure rate of approximately 20%, especially in the case of obese individuals[14]. Although the AUC of IL-34 was not significantly different from that of liver stiffness or other fibrosis scores except for FIB-4, IL-34 may be used as a biomarker, as it is sufficient by itself and can be detected in simple-to-obtain samples compared with other established complex fibrosis scores. Perhaps we can also try to combine it with other indicators to improve the effectiveness of disease diagnosis?

Because of the different phases of chronic HBV infection, ranging from stable disease with minimal injury in inactive carriers to rapid cirrhosis development in patients with highly active HBV infection[10], investigations on the mechanisms of liver inflammation and fibrosis together with the establishment of reliable markers for different HBV phases are very meaningful. We showed that serum levels of IL-34, reflective of profibrogenic macrophage activation[3], differ with the phases of HBV infection and are correlated with hepatic inflammation and liver fibrosis.

One of features of hepatotoxic immune responses with increased inflammation and fibrosis in chronic viral hepatitis is the induction of profibrogenic macrophages[3,4]. In accordance with the important role of liver macrophages in HBV-mediated liver damage, we observed high IL-34 levels in patients with HBeAg-positive or HBeAg-negative hepatitis. Patients with HBeAg-positive or HBeAg-negative hepatitis have a high risk of disease progression and development of cirrhosis and hepatocellular carcinoma due to increased hepatic inflammation and fibrogenesis[10,19-21]. In contrast, IL-34 levels in inactive HBV carriers with HBV DNA levels $\leqslant$ 2000 U/mL and normal transaminase levels did not differ from those in healthy subjects, indicating that the low levels of activation of the innate immune system reflect good prognosis[10,19].

Although the IL-34 levels of immune-tolerant patients were markedly different from those of HBeAg-positive or HBeAg-negative hepatitis patients, immune-tolerant patients had comparable IL-34 levels to healthy subjects. This might indicate that if the human immune system fails to respond to HBV, the damage to the liver by the virus is minimal[22]. Liver biopsies in immune-tolerant patients generally show no signs of significant inflammation or fibrosis[23,24]. Given that serum IL-34 concentrations were strongly correlated with aminotransferase levels and could differentiate patients with extensive hepatic inflammation from subjects with reduced inflammatory activity, IL-34 may be used as a potential biomarker for hepatic inflammation.

In summary, IL-34 may aid in the staging of liver fibrosis and diagnosing different phases of HBV infection in China. These processes are critical for guiding the treatment of chronic HBV infection. IL-34 is known to regulate the profibrogenic functions of macrophages by binding to its receptor[3,6,7]. IL-34 and its receptor are highly expressed in hepatocytes in patients with liver fibrosis, mainly in hepatocytes located around fibrotic and inflammatory lesions[3,25]. We hypothesized that by preventing IL-34 from binding with its receptor, the progression of liver fibrosis can be delayed, and inflammation and necrosis of the liver can be prevented. Thus, apart from its above-mentioned function in diagnosis, IL-34 may also be investigated as a therapeutic target for reversing fibrosis.

ARTICLE HIGHLIGHTS

Research background

It is generally believed that the persistence of inflammation plays an important role in the progression of liver fibrosis. Previous studies have shown that interleukin (IL)-34 is an inflammatory cytokine involved in the induction of activation of profibrogenic macrophages, which is associated with the severity of liver fibrosis and inflammation in patients with chronic hepatitis C virus infection and nonalcoholic fatty liver disease.

Research motivation

In order to be helpful to demonstrate the mechanism of liver fibrosis from the perspective of inflammation and provide a new direction for the search of potential new serological diagnostic fibrosis indicators, we investigated the relationship between IL-34 and liver fibrosis in patients with chronic hepatitis B virus (HBV) infection.

Research objectives

This study aimed to investigate whether serum IL-34 levels are correlated with hepatic inflammation and fibrosis in patients with chronic HBV infection.

Research methods

In this study, serum IL-34 levels of 19 healthy controls and 175 patients with chronic HBV infection undergoing biopsy were analyzed.

Research results

We found that the serum IL-34 levels were different among phases of chronic HBV infection and stages of inflammation and fibrosis. We also thought that the serum IL-34 level has potential to diagnose liver fibrosis through comparative analysis of the diagnostic value of IL-34 and other diagnostic methods, except for pathological diagnosis.

Research conclusions

Serum IL-34 level has the potential to be a new indicator of liver inflammation and fibrosis in patients with chronic HBV infection.

Research perspectives

The diagnostic accuracy of serum IL-34 level is not ideal at present. Thus, we can try combining IL-34 with any of other scores and/or with any clinical variable in order to obtain a new "score" with enhanced diagnostic accuracy. Another approach is to increase the sample size for testing.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Model combining pre-transplant tumor biomarkers and tumor size shows more utility in predicting hepatocellular carcinoma recurrence and survival than the BALAD models

Nicha Wongjarupong, Gabriela M Negron-Ocasio, Roongruedee Chaiteerakij, Benyam D Addissie, Essa A Mohamed, Kristin C Mara, William S Harmsen, J Paul Theobald, Brian E Peters, Joseph G Balsanek, Melissa M Ward, Nasra H Giama, Sudhakar K Venkatesh, Denise M Harnois, Michael R Charlton, Hiroyuki Yamada, Alicia Algeciras-Schimnich, Melissa R Snyder, Terry M Therneau, Lewis R Roberts

Nicha Wongjarupong, Gabriela M Negron-Ocasio, Benyam D Addissie, Essa A Mohamed, Nasra H Giama, Michael R Charlton, Lewis R Roberts, Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN 55905, United States

Gabriela M Negron-Ocasio, University of Puerto Rico Medical Sciences Campus, San Juan 00921, Puerto Rico

Nicha Wongjarupong, Roongruedee Chaiteerakij, Department of Medicine, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok 10400, Thailand

Kristin C Mara, William S Harmsen, Terry M Therneau, Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester, MN 55905, United States

J Paul Theobald, Brian E Peters, Joseph G Balsanek, Melissa M Ward, Alicia Algeciras-Schimnich, Melissa R Snyder, Division of Laboratory Medicine, Department of Laboratory Medicine and Pathology, Mayo Clinic College of Medicine, Rochester, MN 55905, United States

Sudhakar K Venkatesh, Department of Radiology, Mayo Clinic College of Medicine, Rochester, MN 55905, United States

Denise M Harnois, Department of Transplantation, Mayo Clinic Florida, Jacksonville, FL 32224, United States

Hiroyuki Yamada, Wako Life Sciences, Incorporated, Mountain View, CA 94043, United States

ORCID number: Nicha Wongjarupong (0000-0001-8676-413X); Gabriela M Negron-Ocasio (0000-0002-8484-8601); Roongruedee Chaiteerakij (0000-0002-7191-3881); Benyam D Addissie (0000-0001-8772-7074); Essa A Mohamed (0000-0002-0307-6241); Kristin C Mara (0000-0001-9824-6049); William S Harmsen (0000-0001-7134-5362); J Paul Theobald (0000-0002-5730-8925); Brian E Peters (0000-0003-2485-0766); Joseph G Balsanek (0000-0003-2929-2871); Mellisa M Ward (0000-0001-9784-9518); Nasra H Giama (0000-0003-2764-866X); Sudhakar K Venkatesh (0000-0002-7514-1030); Denise M Harnois (0000-0001-7307-2900); Michael R Charlton (0000-0002-8766-7494); Hiroyuki Yamada (0000-0002-1827-3758); Alicia Algeciras-Schimnich (0000-0002-4455-6217); Melissa R Snyder (0000-0002-5442-4699); Terry M Therneau (0000-0003-1490-6711); Lewis R Roberts (0000-0001-7885-8574).

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Correspondence to: Lewis R Roberts, MB ChB, PhD, Professor of Medicine, Consultant, Division of Gastroenterology and Hepatology, Mayo Clinic Rochester, 200 First Street SW Rochester, MN 55905, United States. roberts.lewis@mayo.edu
Telephone: +1-507-2664720
Fax: +1-507-2840762

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Abstract

AIM

To assess the performance of BALAD, BALAD-2 and their component biomarkers in predicting outcome of hepatocellular carcinoma (HCC) patients after liver transplant.

METHODS

BALAD score and BALAD-2 class are derived from bilirubin, albumin, alpha-fetoprotein (AFP), Lens culinaris agglutinin-reactive AFP (AFP-L3), and des-gamma-carboxyprothrombin (DCP). Pre-transplant AFP, AFP-L3 and DCP were measured in 113 patients transplanted for HCC from 2000 to 2008. Hazard ratios (HR) for recurrence and death were calculated. Univariate and multivariate regression analyses were conducted. C-statistics were used to compare biomarker-based to predictive models.

RESULTS

During a median follow-up of 12.2 years, 38 patients recurred and 87 died. The HRs for recurrence in patients with elevated AFP, AFP-L3, and DCP defined by BALAD cut-off values were 2.42 (1.18-5.00), 1.86 (0.98-3.52), and 2.83 (1.42-5.61), respectively. For BALAD, the HRs for recurrence and death per unit increased score were 1.48 (1.15-1.91) and 1.59 (1.28-1.97). For BALAD-2, the HRs for recurrence and death per unit increased class were 1.45 (1.06-1.98) and 1.38 (1.09-1.76). For recurrence prediction, the combination of three biomarkers had the highest c-statistic of 0.66 vs. 0.64, 0.61, 0.53, and 0.53 for BALAD, BALAD-2, Milan, and UCSF, respectively. Similarly, for death prediction, the combination of three biomarkers had the highest c-statistic of 0.66 *vs* 0.65, 0.61, 0.52, and 0.50 for BALAD, BALAD-2, Milan, and UCSF. A new model combining biomarkers with tumor size at the time of transplant (S-LAD) demonstrated the highest predictive capability with c-statistics of 0.71 and 0.69 for recurrence and death.

CONCLUSION

BALAD and BALAD-2 are valid in transplant HCC patients, but less predictive than the three biomarkers in combination or the three biomarkers in combination with maximal tumor diameter (S-LAD).

Key words: Alpha-fetoprotein; AFP-L3; Des-gamma-carboxyprothrombin; BALAD; BALAD-2; Hepatocellular carcinoma; Liver transplant; Recurrence; Outcome

Core tip: BALAD score and BALAD-2 class incorporating alpha-fetoprotein (AFP), AFP-L3, and des-gamma-carboxyprothrombin are used to predict survival of patients with hepatocellular carcinoma. However, there were limited numbers of patients who received liver transplant in previous cohorts in which performance of the BALAD was studied. Our study showed that pre-transplant BALAD score and BALAD-2 class are useful for predicting outcome of hepatocellular carcinoma patients receiving liver transplant. However, a more predictive model uses the combination of all three biomarkers using the cut-offs from the BALAD score along with maximum tumor size at the time of transplant.

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INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) in the United States has increased 3-fold in the last 30 years[1]. Currently, liver cancer has also become the second leading cause of cancer-related deaths worldwide[2]. Liver transplant is one of the few curative treatments that can achieve a 5-year survival rate of 70% for some HCC patients. However, to be eligible for a liver transplant, patients with HCC have to meet a rigorous set of criteria. Despite these selection criteria, recurrence of cancer is seen in up to 20% of HCC patients that undergo liver transplantation[3].

This high proportion of recurrences calls into question the liver transplant guidelines used for patients with cancer. For patients with HCC, the Milan and UCSF criteria have been used as standards to determine the eligibility for liver transplant[4,5]. Although adherence to the Milan criteria has been associated with relatively lower recurrence rates after transplantation, it is still considered suboptimal because it relies primarily on tumor morphologic characteristics[6]. Other liver transplant guidelines have been proposed, but similar to the Milan and UCSF criteria, they fail to incorporate the biological behavior of the tumor[6,7].

To achieve more objective models for selection of HCC patients for liver transplant, several serum tumor biomarkers have been evaluated to assess the biological aggressiveness of HCC. Multiple studies suggest that high pre-transplant alpha fetoprotein (AFP), a widely known HCC biomarker, is associated with poor post-transplant outcomes[8] and the AFP model, combining alpha-fetoprotein (AFP) with the tumor number and tumor size, has been proposed and validated to predict HCC recurrence[9]. The BALAD score, a model that incorporates the use of 5 serum biomarkers, has been successful in predicting the survival and recurrence of patients with HCC[10]. In addition to assessing the remnant liver function via the Bilirubin and Albumin levels, the BALAD score incorporates 3 additional serum tumor biomarkers, namely AFP, Lens culinaris agglutinin-reactive AFP (AFP-L3), and des-gamma-carboxyprothrombin (DCP). However, previous studies, including a validation study, have only included a limited number of liver transplant patients[11-14].

The aim of this study was to assess the performance of the discontinuous BALAD and continuous BALAD-2 scores in patients who underwent liver transplant for HCC. In addition, we aimed to assess the utility of each component of the BALAD in predicting outcomes and to develop a more effective model for liver transplant patients.

MATERIALS AND METHODS

Study population and data abstraction

There were 299 patients with HCC who underwent liver transplant between January 2000 and December 2008. Of the 299 patients, 113 had available results of all five biomarkers within two days before the liver transplant. The HCC diagnosis criteria included (1) explanted liver pathology; or (2) a new liver mass with largest diameter of > 1 cm, arterial enhancement and portal venous washout on computed tomography or magnetic resonance imaging. Patients with warfarin use and congenital biliary disorder which could alter the bilirubin level, such as Gilbert disease, were excluded. The transplant selection criteria for the HCC patients during the study period were primarily based on the Milan criteria. Staging within the extended UCSF criteria was accepted in 17 patients based on provider selection and organ availability at the time of transplant. Most patients with intermediate stage disease beyond Milan criteria received locoregional treatment with transarterial chemoembolization prior to liver transplantation. For surveillance for post-transplant HCC recurrence, patients underwent CT scan of the abdomen and chest along with serum AFP at 4, 8, 12, 18, and 24 mo post-transplant.

Patient age, sex, race, etiology of liver disease, date of HCC diagnosis, date of liver transplant, baseline tumor characteristics at the time of diagnosis, and at the time of imaging closest to the transplant (diameter of the largest tumor, tumor number, macrovascular invasion), biomarker results, recurrence date, death date and last follow-up date were abstracted. The Child-Turcotte-Pugh (CTP) class and MELD score were calculated at the time closest to liver transplant in every patient regardless of cirrhosis status. Tumor size and tumor number were also determined from the most recent imaging study prior to the transplant. The Milan and UCSF criteria were also determined from the imaging prior to and closest to the transplant date. HCC recurrence was defined by the presence of new malignant masses seen on imaging, either intrahepatic or extrahepatic metastases, as assessed by the radiologist. The tumor response to treatment was assessed according to the modified Response Evaluation Criteria in Solid Tumors (mRECIST), version 1.0. The survival of patients who were lost to follow-up was obtained using the Accurint system.

BALAD score and BALAD-2 class were calculated based on five biomarkers including total bilirubin, albumin, AFP, AFP-L3, and DCP measured within the two days prior to transplant (Tables 1 and 2). The GALAD and GALAD-z scores were also calculated based on gender, age, and biomarkers within the same period (Table 3).

Measurement of biomarkers

Serum samples were collected and stored at -80 ℃. AFP, AFP-L3, and DCP were measured simultaneously using a liquid-phase binding assay on the μTASWako i30 instrument (Wako Life Sciences Inc., Mountain View, CA, United States). Details of the sample processing and biomarker results were previously published[15].

Statistical analysis

Baseline characteristics were reported as mean ± standard deviation (SD) or median and interquartile range for continuous variables, and percentage for categorical variables. Hazard ratios (HRs) for time to recurrence and death were calculated for each variable and each BALAD score and BALAD-2 class grouping. HRs were presented as HR (95%CI, *P* value). C statistics were used to compare different scores. All analyses were performed using SAS 9 (SAS Institute, Cary, NC, United States). $P < 0.05$ was considered as

Table 1 BALAD score calculation

	0 point	1 point	2 points	3 points
Bilirubin (mg/dL)	< 1.0	1.0-2.0	> 2.0	
Albumin (g/dL)	> 3.5	2.8-3.5	< 2.8	
Summation of these 2 points, then classified as A (0-1), B (2-3), C (4)				
Albumin-Bilirubin	A	B	C	-
No. of elevated markers[1]	0	1	2	3
Summation of these 2 points for BALAD score (0-5)				

[1]Defined by AFP > 400 ng/mL, AFP-L3 > 15%, and DCP > 100 ng/mL.

Table 2 BALAD-2 class calculation

Linear predictor = 0.02 × (AFP - 2.57) + 0.012 × [(AFP-L3) - 14.19] + 0.19 × [ln(DCP) - 1.93] + 0.17 × [(bilirubin)$^{1/2}$ - 4.50] - 0.09 × (albumin - 35.11)
AFP capped at 50000 units. AFP and DCP modeled as /1000 units.
Units: Bilirubin (μmol/L), albumin (g/L), AFP and DCP (ng/mL), AFP-L3 (%).
class 1 (≤ -1.74), class 2 (> -1.74 to -0.91), class 3 (> -0.91 to 0.24), class 4 (> 0.24)

Table 3 GALAD-z and GALAD score calculation

GALAD-z = -10.08 + 0.09 × (Age) + 1.67 × (sex) + 2.34 × log(AFP) + 0.04 × (AFP-L3) + 1.33 × log(DCP)
GALAD score = exp (GALAD-z)/[1 + exp(GALAD-z)]

Sex = 1 for male and 0 for female.

statistically significant.

RESULTS

Demographic characteristics

Of the 113 included patients, the majority were male (*n* = 86, 76%), with viral hepatitis C as the most common liver disease etiology (*n* = 66, 58%) as shown in Table 4. There were 104 (92%) patients with cirrhosis of whom 13 (12%), 76 (67%), and 24 (21%) patients had CTP class A, B, and C cirrhosis, respectively. There were 1 (1%), 39 (35%), 7 (6%), 40 (35%), and 26 (23%) patients with BCLC stage 0, A, B, C, and D HCC, respectively. There were no patients with portal or nodal invasion. BCLC stages C and D were assigned because of poor ECOG performance status and/or CTP class C cirrhosis. The median (range) of total bilirubin and albumin at the time of transplant were 2.3 (0.2-29.5) mg/dL and 3.2 (2.1-5.2) g/dL. For the tumor biomarkers, the median (range) of AFP, AFP-L3, and DCP were 25.3 (0.8-27800) ng/dL, 12 (1-86.5)%, and 1.2 (0.2-1480) ng/mL, respectively. The median waiting time for the included patients was 2.8 (range 0-20) mo.

Of the 113 included patients, 87 (77%) and 96 (85%) were within Milan and UCSF criteria at the time of diagnosis; and 88 (78%) and 105 (93%) were within Milan and UCSF criteria at the time of transplant, respectively. The AFP level was not included in the transplant selection criteria during the study period. Of the 113 patients, 111 patients received TACE, 1 received RFA and 1 received both TACE and RFA prior to liver transplant. Thirty-nine patients (35%) had available imaging for evaluating the locoregional therapy response. Sixty-nine patients had baseline imaging at the time of HCC diagnosis but did not have follow-up imaging after locoregional therapy as most of these patients underwent transplantation shortly after TACE. Another 5 patients had radiology reports in the medical record but did not have the images available for review as the imaging was performed outside Mayo Clinic. Of the 39 patients with imaging available for assessing the treatment response, 29 (74%) were responders (13 complete response and 16 partial response) and 10 (26%) were non-responders (8 stable disease and 2 progressive disease) according to the mRECIST criteria.

According to the explant pathology reports, there were 19, 53, 16, and 2 patients with well-, moderately-, poorly-, and undifferentiated tumors, respectively. There were 23 patients with no report of tumor differentiation. The correlations of the number of elevated tumor biomarkers according to the BALAD score cut-off with the BALAD score are shown in Supplementary Figure 1. There was no correlation between number of elevated tumor biomarkers (P = 0.34), or BALAD score (P = 0.28) with tumor differentiation.

Factors associated with HCC recurrence and death after liver transplant

During a median follow-up of 12.2 years, 38 patients had recurrence and 87 died. The median survival was 10.2 years. The 3-year and 5-year survivals were 74.3% (95%CI: 66.7%-82.8%) and 66.3% (95%CI:

Table 4 Baseline characteristics of 113 hepatocellular carcinoma patients who underwent liver transplant with available biomarker results *n* (%)

Variables	Value
Age, yr, mean ± SD	58.2 ± 8.3
Male sex	86 (76)
Race	
White	91 (80)
Asian	11 (10)
Others	7 (6)
Unknown	4 (4)
Etiology	
Hepatitis virus C	66 (58)
Hepatitis virus B	11 (10)
Alcohol	14 (12)
Non-alcoholic fatty liver disease or cryptogenic	14 (12)
Others	8 (7)
Cirrhosis	104 (92)
CTP class	
A	13 (12)
B	76 (67)
C	24 (21)
MELD score, median (range)	14.2 (6.4–38.6)
ECOG status	
0	57 (50)
1	34 (30)
2	19 (17)
3	3 (3)
Diameter of the largest tumor at the time of transplant by imaging, cm, mean ± SD	2.7 ± 1.6
Tumor number at the time of transplant	
1	73 (64.6)
2	26 (23.0)
3	7 (6.2)
⩾ 4	7 (6.2)
BCLC staging	
Stage 0	1 (1)
Stage A	39 (35)
Stage B	7 (6)
Stage C	40 (35)
Stage D	26 (23)
Within Milan criteria at diagnosis	87 (77)
Within UCSF criteria at diagnosis	96 (85)
Within Milan criteria at transplant	88 (78)
Within UCSF criteria at transplant	105 (93)
AFP model score > 2	26 (23)
Total bilirubin, mg/dL, median (range)	2.3 (0.2-29.5)
Albumin, g/dL, median (range)	3.2 (2.1-5.2)
AFP, ng/mL, median (range)	25.3 (0.8-27800)
AFP > 400 ng/mL	18 (16)
AFP-L3, %, median (range)	12 (1-86.5)
AFP-L3 > 15%	45 (40)
DCP, ng/mL, median (range)	1.2 (0.2-1480)
DCP > 1.2 ng/mL	56 (50)

AFP: Alpha-fetoprotein; AFP-L3: Lens culinaris agglutinin-reactive alpha-fetoprotein; CTP: Child-Turcotte-Pugh; DCP: Des-gamma-carboxyprothrombin.

58.1%-75.6%).

By Cox proportional hazard ratio, the diameter of the largest tumor at the time of transplant was associated with both transplant outcomes with HRs per centimeter of 1.27 (1.04-1.56, $P = 0.02$) for recurrence and 1.21 (1.03-1.41, $P = 0.02$) for death. A neutrophil-lymphocyte ratio of more than 4 also correlated with outcomes, with HRs of 2.24 (1.17-4.26, $P = 0.04$) for recurrence, and 1.66 (1.004-2.73, $P = 0.048$) for death. We did not find any significant increases in risk of recurrence or death for either tumor number or hypothyroidism (Table 5).

Levels of all three tumor biomarkers that exceeded the BALAD score cut-off were associated with increased recurrence and death outcomes in the transplant cohort, whereas albumin and bilirubin, the other components of the BALAD score, were not associated with either outcome. The HRs for recurrence of elevated AFP, AFP-L3, and DCP according to the BALAD score cut-off were 2.42 (1.18-5.00, $P = 0.02$), 1.86 (0.98-3.52, $P = 0.056$), and 2.83 (1.42-5.61, $P = 0.003$), respectively. Similarly, the HRs for death were 3.27 (1.84-5.80, $P < 0.001$), 1.88 (1.14-3.09, $P = 0.01$), and 2.40 (1.43-4.04, $P < 0.001$), respectively. The cumulative incidence of recurrence curve and Kaplan-Meyer survival curve by number of elevated biomarkers are shown in Figure 1A and B, respectively.

BALAD score and BALAD-2 class and risk of HCC recurrence and death

When classified by the BALAD score, there were 14, 31, 33, 23, 9, and 3 patients with BALAD scores of 0 to 5, respectively. By BALAD-2 class there were 29, 30, 34, and 20 patients in BALAD-2 classes 1 to 4, respectively.

For BALAD scores of 1, 2, 3, 4, and 5 *vs* 0, the HRs for recurrence were 0.70 (0.20-2.47), 1.18 (0.37-3.75), 1.99 (0.62-6.36), 2.97 (0.84-10.58), and 5.02 (0.92-27.54); and HRs for death were 1.14 (0.40-3.23), 2.01 (0.75-5.38), 2.73 (0.99-7.51), 4.68 (1.52-14.36), and 17.40 (3.81-79.47), respectively (Figure 2A and B). The HRs per each unit increase in BALAD score for recurrence and death were 1.48 (1.15-1.91) and 1.59 (1.28-1.97). For BALAD-2 classes 2, 3, and 4 *vs* 1, the HRs for recurrence were 0.41 (0.12-1.32), 1.53 (0.66-3.54), and 2.17 (0.90-5.25); and HRs for death were 1.07 (0.50-2.28), 1.76 (0.87-3.54), and 2.45 (1.16-5.17) (Figure 3A and B). The HRs per each unit increase in BALAD-2 class for recurrence and death were 1.45 (1.06-1.98) and 1.38 (1.09-1.76), respectively. A multivariate model of diameter of the largest tumor with BALAD and BALAD-2 was created (Tables 6 and 7). The risk of recurrence was 1.53 (1.17-2.01) per increase of 1 in the BALAD score and 1.42 (1.05-2.03) per increase of one BALAD-2 class. The risk of death was 1.57 (1.27-1.96) per increase of 1 in the BALAD score and 1.37 (1.07-1.76) per increase of 1 BALAD-2 class.

In addition, the HRs for early recurrence were also calculated. Early recurrence was defined as recurrence occurring within 36 mo after transplant. Of the 38 patients with any recurrence, 31 had early recurrence. The BALAD score had better performance for early than overall recurrence with a HR of 1.66 (1.24-2.22) per each unit increase of BALAD score, whereas the BALAD-2 class had similar performance for both recurrence outcomes with a HR of 1.46 (1.04-2.07) per

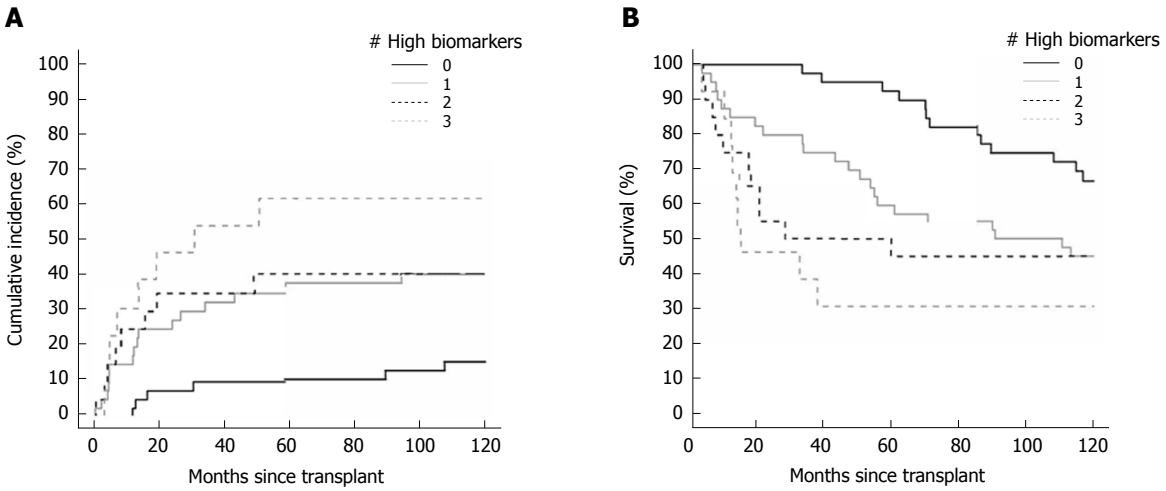

Figure 1 Cumulative incidence of recurrence curve (A) and Kaplan-Meyer survival curve (B) by number of elevated tumor biomarkers.

Table 5 Univariate models for recurrence and death outcome

Variable	Hazard ratio for recurrence		Hazard ratio for death	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
MELD score (per point)	1.03 (0.98-1.09)	0.26	1.05 (1.003-1.09)	0.04[a]
Diameter of the largest tumor at time of transplant (per cm)	1.27 (1.04-1.56)	0.02[a]	1.21 (1.03-1.41)	0.02
Tumor number at time of transplant	1.001 (0.73-1.37)	1.00	0.93 (0.72-1.20)	0.57
Neutrophil lymphocyte ratio > 4	2.24 (1.17-4.26)	0.02[a]	1.66 (1.004-2.73)	0.048[a]
Hypothyroidism	1.26 (0.55-2.85)	0.59	1.54 (0.82-2.90)	0.18
BALAD components				
Albumin (per g/dL)	0.75 (0.41-1.38)	0.36	0.69 (0.43-1.13)	0.14
Bilirubin (per mg/dL)	1.03 (0.98-1.09)	0.21	1.04 (0.995-1.08)	0.08
AFP: > 400 ng/mL	2.42 (1.18-5.00)	0.02[a]	3.27 (1.84-5.80)	< 0.001[b]
AFP-L3 > 15%	1.86 (0.98-3.52)	0.056	1.88 (1.14-3.09)	0.01[a]
DCP > 1.2 ng/mL	2.83 (1.42-5.61)	0.003[b]	2.40 (1.43-4.04)	< 0001[b]
BALAD Score				
0	Reference		Reference	
1	0.70 (0.20-2.47)	0.58	1.14 (0.40-3.23)	0.81
2	1.18 (0.37-3.75)	0.78	2.01 (0.75-5.38)	0.17
3	1.99 (0.62-6.36)	0.24	2.73 (0.99-7.51)	0.052
4	2.97 (0.84-10.58)	0.09	4.68 (1.52-14.36)	0.007[b]
5	5.02 (0.92-27.54)	0.06	17.40 (3.81-79.47)	< 0.001[b]
BALAD Score (per increase of 1)	1.48 (1.15-1.91)	0.002[b]	1.59 (1.28-1.97)	< 0.001[b]
BALAD-2 Score				
1	Reference		Reference	
2	0.41 (0.12-1.32)	0.13	1.07 (0.50-2.28)	0.86
3	1.53 (0.66-3.54)	0.32	1.76 (0.87-3.54)	0.11
4	2.17 (0.90-5.25)	0.09	2.45 (1.16-5.17)	0.02[a]
BALAD-2 Score (per increase of 1)	1.45 (1.06-1.98)	0.02[a]	1.38 (1.09-1.76)	0.008[b]
Within Milan criteria at diagnosis	1.69 (0.84-3.41)	0.14	2.17 (1.25-3.78)	0.006[b]
Within UCSF criteria at diagnosis	1.85 (0.85-4.05)	0.12	3.19 (1.75-5.84)	< 0.001[b]
Within Milan criteria at transplant	1.24 (0.59-2.62)	0.57	1.06 (0.57-1.95)	0.86
Within UCSF criteria at transplant	0.33 (0.05-2.43)	0.28	0.68 (0.21-2.17)	0.51
z-GALAD	1.12 (1.03-1.21)	0.006[b]	1.12 (1.06-1.19)	< 0.001[b]
GALAD score	3.01 (1.14-7.91)	0.03[a]	3.22 (1.48-7.00)	0.003[b]
AFP model cutoff > 2 (explant)	2.82 (1.47-5.41)	0.002[b]	2.83 (1.67-4.82)	< 0.001[b]
AFP model (per increase of 1, explant)	1.42 (1.20-1.68)	< 0.001[b]	1.34 (1.16-1.54)	< 0.001[b]

[a]$P < 0.05$, [b]$P < 0.01$, statistical difference. AFP: Alpha-fetoprotein; AFP-L3: Lens culinaris agglutinin-reactive alpha-fetoprotein; DCP: Des-gamma-carboxyprothrombin.

increase of 1 class (Supplementary Table 1).

Multivariate model of elevated tumor biomarkers combination with tumor size

Based on the results of the univariate analysis, we combined the elevated tumor biomarkers including AFP, AFP-L3, and DCP with diameter of the largest tumor per centimeter increase in diameter (Table 8). In this multivariate model, diameter of the largest tumor and elevated DCP remained significantly associated with recurrence and death, whereas elevated AFP was only associated with death but not with recurrence.

Table 6 Multivariate model for recurrence outcome with BALAD and BALAD-2

Variable	Hazard ratio with BALAD		Hazard ratio with BALAD-2	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Diameter of the largest tumor at time of transplant (per cm)	1.33 (1.07-1.66)	0.02[b]	1.30 (1.05-1.59)	0.014[a]
Neutrophil-lymphocyte ratio	1.55 (0.78-3.14)	0.21	1.76 (0.90-3.49)	0.10
BALAD (per increase of 1)	1.53 (1.17-2.01)	0.002[b]	-	-
BALAD-2 (per increase of 1)	-	-	1.45 (1.05-2.03)	0.02[a]

[a]$P < 0.05$, [b]$P < 0.01$, statistical difference.

Table 7 Multivariate model for death outcome with BALAD and BALAD-2

Variable	Hazard ratio with BALAD		Hazard ratio with BALAD-2	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Diameter of the largest tumor at time of transplant (per cm)	1.24 (1.04-1.48)	0.016[a]	1.20 (1.02-1.42)	0.03[a]
Neutrophil-lymphocyte ratio	1.13 (0.67-1.92)	0.64	1.31 (0.78-2.19)	0.31
BALAD (per increase of 1)	1.57 (1.27-1.96)	< 0.0001	-	-
BALAD-2 (per increase of 1)	-	-	1.37 (1.07-1.76)	0.013[a]

[a]$P < 0.05$, [b]$P < 0.01$, statistical difference.

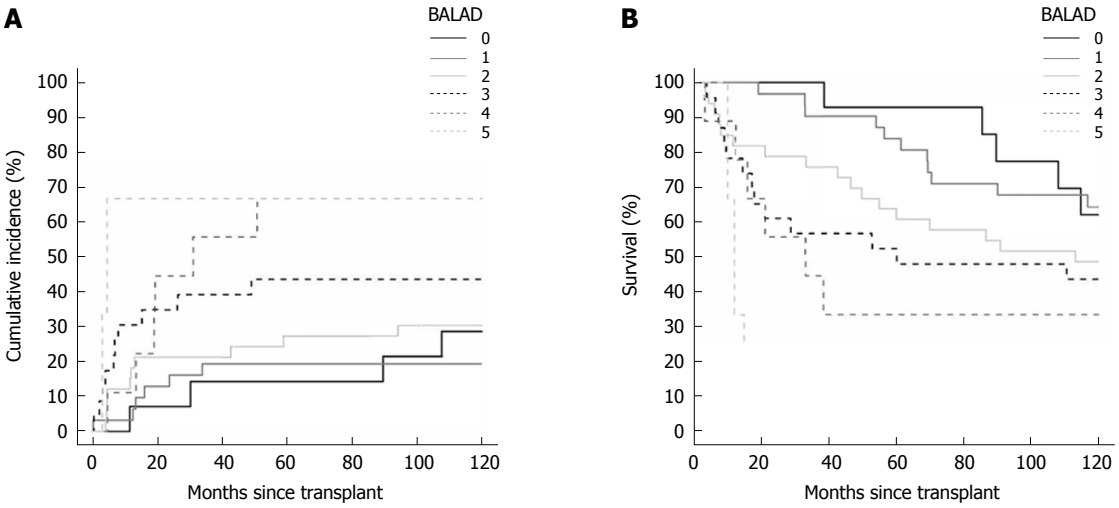

Figure 2 Cumulative incidence of recurrence curve (A) and Kaplan-Meyer survival curve (B) by BALAD score.

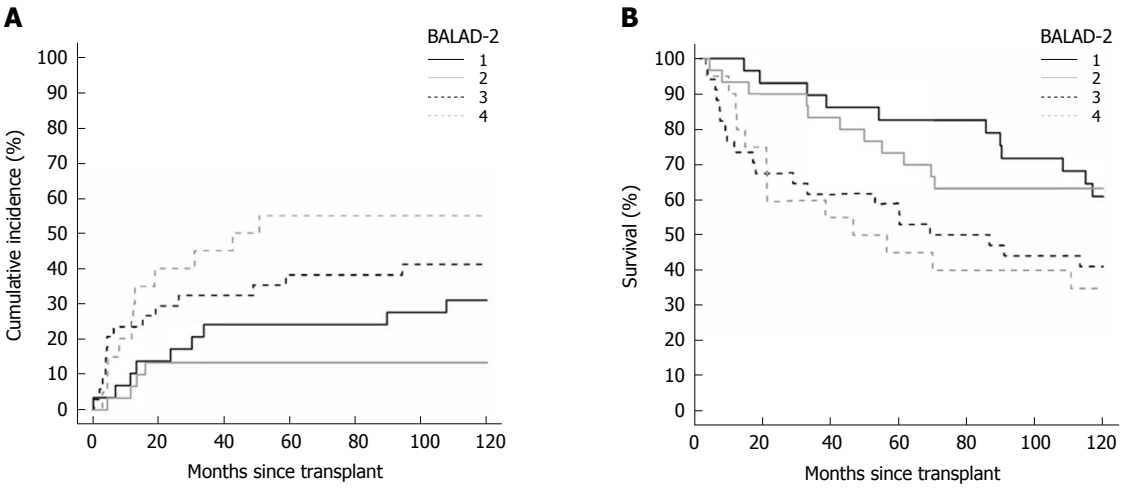

Figure 3 Cumulative incidence of recurrence curve (A) and Kaplan-Meyer survival curve (B) by BALAD-2 class.

Table 8 Multivariate model of biomarkers and tumor size at time of transplant (S-LAD)

Variable	Hazard ratio for recurrence		Hazard ratio for death	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Diameter of the largest tumor at time of transplant (per cm)	1.30 (1.05-1.61)	0.02[a]	1.29 (1.08-1.55)	0.006[b]
AFP: > 400 ng/mL	1.63 (0.70-3.83)	0.26	2.40 (1.19-4.83)	0.02[a]
AFP-L3 > 15%	0.995 (0.46-2.18)	0.99	1.01 (0.54-1.88)	0.98
DCP > 1.2 ng/mL	2.69 (1.28-5.64)	0.009[b]	2.33 (1.31-4.13)	0.004[b]
c-statistic (95%CI)	0.71 (0.62-0.81)		0.69 (0.61-0.77)	

[a]$P < 0.05$, [b]$P < 0.01$, statistical difference. AFP: Alpha-fetoprotein; AFP-L3: Lens culinaris agglutinin-reactive alpha-fetoprotein; DCP: Des-gamma-carboxyprothrombin.

Table 9 Comparison of models to predict outcome of liver transplant patients

Variable	c-statistic (95%CI)	
	For recurrence	For death
Number of elevated biomarkers	0.66 (0.57-0.75)	0.66 (0.59-0.73)
BALAD Score (per increase of 1)	0.64 (0.55-0.73)	0.65 (0.58-0.73)
BALAD-2 Score (per increase of 1)	0.61 (0.52-0.70)	0.61 (0.54-0.68)
Within Milan criteria at diagnosis	0.56 (0.49-0.62)	0.58 (0.54-0.63)
Within UCSF criteria at diagnosis	0.55 (0.49-0.60)	0.59 (0.55-0.63)
Within Milan criteria at transplant	0.53 (0.46-0.59)	0.52 (0.47-0.57)
Within UCSF criteria at transplant	0.53 (0.48-0.58)	0.50 (0.47-0.54)
z-GALAD	0.63 (0.53-0.72)	0.64 (0.56-0.72)
GALAD score	0.63 (0.53-0.72)	0.64 (0.56-0.72)
AFP model (explant model)	0.59 (0.51-0.67)	0.58 (0.51-0.65)

AFP-L3 did not relate to either recurrence or death in this multivariate model. The c-statistics for the combined models were 0.71 (0.62-0.81) and 0.69 (0.61-0.77) for recurrence and death, respectively.

Comparisons to the currently used models

The c-statistic was used to compare models which predict outcome of liver transplant patients. A combination of elevated tumor biomarkers based on the BALAD score cut-offs demonstrated the highest c-statistic for prediction of both recurrence and death, with values of 0.66 (0.57-0.75) and 0.66 (0.59-0.73), respectively. For the outcome of recurrence, BALAD and BALAD-2 (per increase of 1 score/class) showed c-statistics of 0.64 (0.55-0.73) and 0.61 (0.52-0.70), respectively. For the outcome of death, BALAD and BALAD-2 showed c-statistics of 0.65 (0.58-0.73) and 0.61 (0.54-0.68). The c-statistics for the Milan and UCSF criteria at the time of diagnosis and prior to transplant, the GALAD, and AFP explant models are shown in Table 9.

DISCUSSION

The pre-transplant BALAD score and BALAD-2 class had a moderate capability to predict both recurrence and death in liver transplant HCC patients. The most predictive model was the combination of three tumor biomarkers using the cut-offs for the BALAD score. In addition, our study showed that large tumor size, high neutrophil-lymphocyte ratio, and elevated individual tumor biomarkers were associated with recurrence and mortality of patients with HCC who underwent transplant.

Tumor size was found to be significantly related to the outcomes in our cohort with HRs per centimeter of 1.27 for recurrence and 1.21 for death. This supports the use of the Milan and UCSF criteria which are based on tumor size, tumor number and vascular invasion[4,5]. The correlation of increased tumor size and elevated tumor biomarkers with outcomes has been shown in previous cohorts[16,17]. Accordingly, the biomarkers can potentially be used as more convenient predictors of patient outcome.

BALAD and BALAD-2 score contain two major components; the bilirubin-albumin score representing liver functional reserve and the three biomarkers representing tumor biology that independently reflect different characteristics of HCC progression[10]. In our study, by using the cut-off of the tumor markers according to the BALAD score, the three tumor biomarkers individually were predictive for recurrence and mortality. This is concordant with many previous studies of HCC patients receiving transplants[8,18]. High biomarker levels can reflect a poor prognosis, as a high DCP level is related to tumor vascular invasion and portal vein thrombosis[19], whereas a high AFP-L3 level has also been found to be related to vascular invasion and infiltrative growth[20].

The differences between the previous cohorts in which the predictive capability of the BALAD score was shown and our current study is the treatment received and the time of biomarker measurement. The nationwide study of HCC in the Japanese population found that the BALAD score was effective, regardless of the treatment[13]. However, this was concluded with a limited proportion of patients in the cohort receiving liver transplant as a treatment. In contrast to the previous studies of the BALAD score, we found that the c-statistic of the combination of the three biomarkers was the highest among all the tested models, including BALAD and BALAD-2. This finding could be explained by the almost immediate restoration of normal functioning of the liver after liver transplant, and thus consequently the less significant roles of bilirubin and albumin as predictors of outcomes after transplant[21].

By combining the three tumor biomarkers with tumor size, we created a model that is more predictive of both recurrence and survival (S-LAD model). A previous study from our group combined each of the biomarkers with the Milan criteria and found a significant improvement in the ability of the Milan criteria to predict recurrence[15]. In addition to this previous study, as HCC is considered a highly heterogeneous disease[22], the combination of the three biomarkers could further improve the predictive model. The GALAD score is another model that uses the combination of biomarkers with sex and age and which was originally developed for predicting risk of HCC in patients with cirrhosis[23]. Interestingly, the GALAD score also showed good performance in predicting both outcomes in our study. However, age and sex were not found to have any correlation with liver transplant outcomes in our study.

It is important to note that the proportion of recurrences after liver transplant in this study is higher than in previous studies in tertiary care centers[3]. Thirty-eight of the 113 patients (33.6%) with available serum had recurrence. However, when considering all HCC patients who underwent liver transplant during the same period, 43 of 299 patients (14.4%) had recurrence. Per report from the Mayo Clinic Transplant Biorepository, serum samples from patients with non-recurrent HCC were more frequently requested, which led to an unequal availability of the samples from patients with and without HCC recurrence. To control for the effect of the difference in sample availability on this study, we compared the characteristics and survival outcomes of non-recurrent patients without samples to those of patients with samples, finding no substantial differences in their baseline characteristics (Supplementary Table 2).

A major strength of this study is that we were able to assess the performance of BALAD, BALAD-2, and their component tumor biomarkers, and included the largest number of transplant HCC patients evaluated thus far. However, there are several limitations to our study. For most of the patients we did not have biomarker results at the time of diagnosis, as was used in the model development and most of the validation cohorts. Thus, the BALAD score and BALAD-2 class at the time of diagnosis were not available for our study. In addition, with the relatively small number of patients, further validation with a larger cohort is needed.

In conclusion, the combination of the three biomarkers used in the BALAD score along with maximal tumor diameter (S-LAD) was the most predictive model for recurrence and death outcomes for HCC patients receiving liver transplants. However, validation of this new S-LAD model is warranted. Unlike the performance for other HCC treatment modalities, the BALAD score and BALAD-2 class are less predictive for recurrence and death in HCC patients with liver transplant, presumably because liver function is restored after liver transplantation.

ARTICLE HIGHLIGHTS

Research background

Liver transplant is one of the curative treatments for hepatocellular carcinoma (HCC). However, with the limited availability of donor organs, it is essential to select patients who will derive the most benefit from transplant. The alpha-fetoprotein (AFP) model has been widely used for this purpose. In the development cohort of the BALAD model by Toyoda *et al*, liver transplant patients were excluded. In the validation cohort in four countries by Chan *et al*, there were only 21 transplant patients included, and in the Japan Nationwide study from Toyoda *et al*, an unknown number of transplant patients were classified in the other treatment group. There is therefore very limited data on the utility of the BALAD model in patients with liver transplant.

Research motivation

The BALAD model has been shown to be a promising predictor of outcome in hepatocellular carcinoma patients receiving most treatment modalities, but there is very limited data on its performance in hepatocellular carcinoma patients receiving liver transplants. The BALAD model incorporates three tumor biomarkers which represent the underlying biology of hepatocellular carcinomas, as well as the serum bilirubin and albumin, which reflect the extent of the underlying liver dysfunction in patients with chronic liver disease. Individually, the AFP, AFP-L3, and des-gamma-carboxyprothrombin (DCP) have been shown to predict the recurrence and survival of hepatocellular carcinoma patients receiving liver transplants. However, presumably due to replacement of the diseased liver during transplantation, it has been shown that the serum bilirubin and albumin are not predictive of patient outcomes post liver transplant.

Research objectives

We aimed to assess the performance of the discontinuous BALAD and continuous BALAD-2 scores in patients who underwent liver transplant for HCC. Further, we assessed the performance of each component of the BALAD in predicting outcomes and propose a more effective model for liver transplant patients.

Research methods

We included patients with hepatocellular carcinoma receiving liver transplants between 2000 and 2008 for whom blood samples were available to allow testing and calculation of the BALAD scores. Patient characteristics, the components of the BALAD model, BALAD score, and BALAD-2 class were analyzed to calculate hazard ratios for recurrence and death. Currently used predictive models including the Milan and UCSF criteria, GALAD score, and AFP model were compared with the BALAD models using c-statistics. A new multivariate model incorporating the three tumor markers and largest tumor diameter was created from these statistically significant variables. The long follow-up period allows assessment of the long term outcomes of the liver transplant patients.

Research results

113 patients were included in the study. The diameter of the largest tumor at the time of transplant, neutrophil-lymphocyte ratio of more than 4, elevated AFP, AFP-L3, and DCP by BALAD score cut-off were associated with both recurrence and death. The HRs per each unit increase in BALAD score for recurrence and death were 1.48 (1.15-1.91) and 1.59 (1.28-1.97). The HRs per each unit increase in BALAD class for recurrence and death were 1.45 (1.06-1.98) and 1.38 (1.09-1.76), respectively. By c-statistics, a model based on the combination of AFP, AFP-L3, and DCP using the BALAD score cut-off had a higher predictive performance than any of the prior models (0.66 for both recurrence and death). Further, a multivariate model incorporating the three biomarkers and the largest diameter of the tumor, designated the S-LAD model, showed a higher c-statistic than all other models (0.71 for recurrence and 0.69 for death). The main limitation of this study is the need for validation of the S-LAD model.

Research conclusions

BALAD and BALAD-2 are valid in transplant HCC patients, but less predictive than the three biomarkers in combination or the three biomarkers in combination

with largest tumor diameter (S-LAD).

Research perspectives

Due to the limited number of patients included, further cohort studies to assess the performance of the BALAD and S-LAD models in hepatocellular carcinoma patients receiving liver transplant are warranted.

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ORIGINAL ARTICLE

Retrospective Study

Intraoperative frozen section diagnosis of bile duct margin for extrahepatic cholangiocarcinoma

Takayuki Shiraki, Hajime Kuroda, Atsuko Takada, Yoshimasa Nakazato, Keiichi Kubota, Yasuo Imai

Takayuki Shiraki, Keiichi Kubota, Department of Gastroenterological Surgery, Dokkyo Medical University, Tochigi 321-0293, Japan

Hajime Kuroda, Atsuko Takada, Yoshimasa Nakazato, Yasuo Imai, Department of Diagnostic Pathology, Dokkyo Medical University, Tochigi 321-0293, Japan

ORCID number: Takayuki Shiraki (0000-0003-2935-6708); Hajime Kuroda (0000-0001-6546-5317); Atsuko Takada (0000-0001-7042-435X); Yoshimasa Nakazato (0000-0002-0325-9451); Keiichi Kubota (0000-0003-4484-7708); Yasuo Imai (0000-0002-1422-7789).

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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 by written consent.

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Correspondence to: Yasuo Imai, MD, PhD, Department of Diagnostic Pathology, Dokkyo Medical University, 880 Kitakobayashi, Mibu, Shimotsuga, Tochigi 321-0293, Japan. ya-imai@dokkyomed.ac.jp
Telephone: +81-282-872130
Fax: +81-282-861681

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Abstract

AIM

To evaluate the usefulness of frozen section diagnosis (FSD) of bile duct margins during surgery for extrahepatic cholangiocarcinoma (CCA).

METHODS

We retrospectively analyzed 74 consecutive patients who underwent surgery for extrahepatic CCA from 2012 to 2017, during which FSD of bile duct margins was performed. They consisted of 40 distant and 34 perihilar CCAs (45 and 55 bile duct margins, respectively). The diagnosis was classified into three categories: negative, borderline (biliary intraepithelial neoplasia-1 and 2, and indefinite for neoplasia), or positive. FSD in the epithelial layer, subepithelial layer, and total layer was compared with corresponding permanent section diagnosis (PSD) postoperatively.

Then, association between FSD and local recurrence was analyzed with special reference to borderline.

RESULTS

Analysis of 100 duct margins revealed that concordance rate between FSD and PSD was 68.0% in the total layer, 69.0% in the epithelial layer, and 98.0% in the subepithelial layer. The extent of remaining biliary epithelium was comparable between FSD and PSD, and more than half of the margins lost > 50% of the entire epithelium, suggesting low quality of the samples. In FSD, the rate of negative margins decreased and that of borderline and positive margins increased according to the extent of the remaining epithelium. Diagnostic discordance between FSD and PSD was observed in 31 epithelial layers and two subepithelial layers. Alteration from borderline to negative was the most frequent (20 of the 31 epithelial layers). Patients with positive margin in the total and epithelial layers by FSD demonstrated a significantly worse local recurrence-free survival (RFS) compared with patients with borderline and negative margins, which revealed comparable local RFS. Patients with borderline and negative margins in the epithelial layer by PSD also revealed comparable local RFS. These results suggested that epithelial borderline might be regarded substantially as negative. When classifying the status of the epithelial layer either as negative or positive, concordance rates between FSD and PSD in the total, epithelial, and subepithelial layers were 95.0%, 93.0%, and 98.0%, respectively.

CONCLUSION

During intraoperative assessment of bile duct margin, borderline in the epithelial layer can be substantially regarded as negative, under which condition FSD is comparable to PSD.

Key words: Cholangiocarcinoma; Bile duct cancer; Frozen section diagnosis; Permanent section diagnosis; Bile duct margin; Biliary intraepithelial neoplasia; Dysplasia; Indefinite for neoplasia; Borderline lesion; Local recurrence

Core tip: Usefulness of intraoperative frozen section diagnosis (FSD) of bile duct margin for extrahepatic cholangiocarcinoma was investigated. The diagnosis was classified into negative, borderline (biliary intraepithelial neoplasia-1 and 2, and indefinite for neoplasia), or positive, and FSD was compared with permanent section diagnosis postoperatively. In contrast to previous studies, positive FSD in the epithelial layer was significantly associated with local recurrence. Furthermore, borderline FSD in the epithelial layer could be substantially regarded as negative, which could aid surgeons to determine the resection range of the bile duct. Finally, we demonstrated that FSD was reliable enough for pathological diagnosis.

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INTRODUCTION

Bile duct cancer (cholangiocarcinoma: CCA) is a rare malignancy (incidence < 6 cases per 100000 people) in most countries[1], and approximately 8000 people in the United States are diagnosed with CCA annually[2]. It develops in any part of the bile duct system and it is classified into three types based on location: intrahepatic CCA, perihilar CCA (pCCA), and distal CCA (dCCA). The latter two types are grouped as extrahepatic CCA (eCCA). Taken together, CCAs represent the second most frequent liver cancer and up to 3% of all gastrointestinal cancers[1,3]. CCA is generally asymptomatic in the early stages, and a late diagnosis and anatomical complexity of the cancer location result in poor prognosis: Five-year survival rate of eCCA with American Joint Committee on Cancer tumor node metastasis (TNM) stage Ⅰ is 30%, stages Ⅱ and Ⅲ 24%, and stage Ⅳ 2%[2].

Most TNM stage 0, Ⅰ, and Ⅱ CCAs and some stage Ⅲ CCAs are potentially resectable, and complete surgical resection is the only treatment with the potential for cure. The status of the final ductal margin is strongly associated with prognosis of patients with resectable CCA[2,4]. Intraoperative frozen section diagnosis (FSD) of the bile duct margins has traditionally been used to guide the extent of operative resection, but the usefulness of FSD has been controversial until now[5-9]. Because of the rarity and locoregional anatomical complexity of CCA, few centers have substantial clinical experience of managing this disease, and few pathologists have expertise in characterizing resected specimens accurately. In addition, the greatest difficulty of FSD is the low quality of samples because of tissue degeneration and/or destruction during freezing and sectioning. Therefore, production of formalin-fixed and paraffin-embedded samples that reuses frozen samples, and comparison between FSD and permanent section diagnosis (PSD) are mandatory. As a result, alteration of diagnosis often occurs. The primary purpose of this study was to examine reliability of intraoperative FSD to evaluate the margin status. The secondary purpose was to clarify clinical relevance of borderline lesions that could not be definitely determined whether malignant or benign. Borderline in the present study included

Table 1 Clinicopathological characteristics of extrahepatic cholangiocarcinoma

	pCCA (n = 34)	dCCA (n = 40)
Age (yr), median (range)	71.5 (44-82)	72.5 (39-85)
Gender		
Male	23	6
Female	11	34
Preoperative biliary drainage		
Yes	33	38
No	1	2
Procedure		
PD	1	38
HH	27	1
PD + HH	5	0
Bile duct resection	0	1
Others	1	0
Total number of duct margins for frozen section	55	45
Number of duct margins for frozen section		
1	15	35
2	17	5
3	2	0
pT		
pT1/pT2	27	20
pT3/pT4	6	19
Unknown	1	1

dCCA: Distal cholangiocarcinoma; HH: Hemihepatectomy; pCCA: Perihilar cholangiocarcinoma; PD: Pancreaticoduodenectomy.

such lesions as low-grade and intermediate-grade dysplasia (biliary intraepithelial neoplasia (BilIN)-1 and BilIN-2)[10] and lesions indefinite for neoplasia that could not be determined as reactive or neoplastic. For these purposes, we analyzed postoperative local recurrence of eCCA according to the margin status of FSD.

MATERIALS AND METHODS

Patients

We analyzed 74 consecutive patients who underwent hemihepatectomy and/or pancreaticoduodenectomy for eCCA at the Department of Gastroenterological Surgery, Dokkyo Medical University from December 2012 to February 2017. There were 40 cases of dCCA (45 bile duct margins) and 34 of pCCA (55 bile duct margins). The histopathological diagnosis was reviewed by two experienced pathologists (HK and YI), and diagnostic inconsistency between the two pathologists was resolved by discussion. The clinicopathological information was retrospectively retrieved on the electronic medical chart system of the Dokkyo Medical University Hospital (Table 1).

Histopathological analysis

FSD of the resected bile duct margin was performed during the operation. The margin tissue was mounted in WHITE TISSUE-COAT (U.I. Kasei, Amagasaki, Hyogo, Japan), frozen in liquid nitrogen, and thin sections were cut from the frozen blocks using a cryostat. The sections were stained by hematoxylin and eosin and subjected to microscopic diagnosis. At least two, three or more as needed, pieces of frozen sections were examined for each margin. When FSD was positive for malignancy (positive) in the first submitted specimen, additional resection of the margin was performed to the maximal extent possible. Results of the last submitted specimens were analyzed in the present study. After FSD, the tissues were thawed, fixed in formalin, and embedded in paraffin. Thin sections were cut from paraffin-embedded blocks, stained, and observed with microscopy. FSD and PSD were compared with each other.

The surgical margins were diagnosed as either negative for malignancy (negative), borderline, or positive (Figure 1). Borderline included BilIN-1 and 2, and indefinite for neoplasia. We separately assessed the epithelial and subepithelial layers, and made a diagnosis based on both results. The epithelial layer tends to detach from the basement membrane during sample preparation for FSD. In relation to the entire circumference, we defined E1 as 0%-24% remaining epithelium, E2 as 25%-49% remaining epithelium, and E3 as 50%-100% remaining epithelium.

The concordance rate between FSD and PSD was investigated at the margin and patient levels, but survival analysis was performed solely at the patient level; for example, a patient with two negative margins and one positive margin was assigned to the positive group.

The presence or absence of postoperative local recurrence was detected by imaging studies including ultrasonography and computed tomography. The criteria for the local recurrence were defined as mass lesions within the resection field with or without clinical manifestation and/or elevated tumor markers. The

Table 2 Histopathological results of the biliary duct margins

	Negative (%)	Borderline (%)	Positive (%)	Total (%)	*P* value
Margin level					
Total layer					
FSD	41 (41.0)	39 (39.0)	20 (20.0)	100 (100)	0.039
PSD	56 (56.0)	23 (23.0)	21 (21.0)	100 (100)	
Epithelial layer					
FSD	44 (44.0)	41 (41.0)	15 (15.0)	100 (100)	0.078
PSD	59 (59.0)	27 (27.0)	14 (14.0)	100 (100)	
Subepithelial layer					
FSD	87 (87.0)	1 (1.0)	12 (11.0)	100 (100)	0.560
PSD	86 (86.0)	0 (0.0)	14 (14.0)	100 (100)	
Patient level					
Total layer					
FSD	26 (35.1)	31 (41.9)	17 (23.0)	74 (100)	0.134
PSD	36 (48.7)	20 (27.0)	18 (24.3)	74 (100)	

FSD: Frozen section diagnosis; PSD: Permanent section diagnosis.

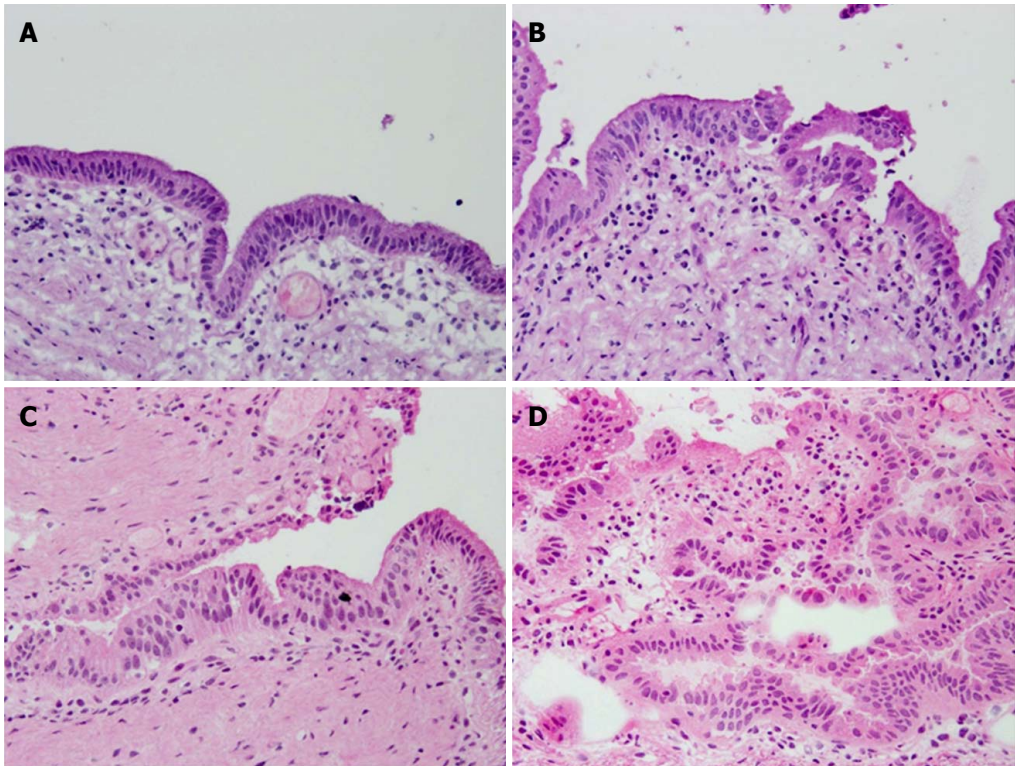

Figure 1 Representative histopathology of BilIN-1, 2, and 3 by frozen section diagnosis. A: Normal mucosa; B: Borderline (BilIN-1); (C) Borderline (BilIN-2); and D: Positive (BilIN-3) (hematoxylin and eosin, 20 ×). BilIN: Biliary intraepithelial neoplasia.

diagnosis of local recurrence was made by the surgeons in charge of each patient.

Statistical analysis

Comparison of categorical data sets between FSD and PSD was performed by the χ^2 test. Local recurrence-free survival (RFS) curves were depicted using the Kaplan-Meier method and analyzed by the log-rank test. $P < 0.05$ was considered significant. Statistical analysis was performed using IBM SPSS Statistics 24 (IBM, Armonk, NY, United States).

RESULTS

Concordance rate between FSD and PSD at the margin level

FSD revealed 41 (41.0%) negative, 20 (20.0%) positive, and 39 (39.0%) borderline out of 100 bile duct margins, while PSD revealed 56 (56.0%) negative, 21 (21.0%) positive, and 23 (23.0%) borderline margins (Table 2). The number of positive margins was similar between FSD and PSD, but the number of negative margins increased and that of borderline decreased

Table 3 Concordance rate between frozen section diagnosis and permanent section diagnosis

	Concordance rate (%)	Sensitivity (%)	Specificity (%)	Positive-predictive value (%)	Negative-predictive value (%)
Original diagnostic results					
Total layer	68.0	85.7	66.1	90.0	90.2
Epithelial layer	69.0	78.6	64.6	73.3	86.4
Subepithelial layer	98.0	85.7	100.0	100.0	98.9
Revised diagnostic results					
Total layer	95.0	85.7	97.5	90.0	97.5
Epithelial layer	93.0	70.0	95.6	73.3	96.5
Subepithelial layer	98.0	85.7	100.0	100.0	98.9

Table 4 Extent of the remaining epithelium and diagnostic results of the bile duct margin

	Extent of the remaining epithelium	Negative (%)	Borderline (%)	Positive (%)	Total (%)
FSD					
	E1 (0%-24%)	22 (66.6)	9 (27.3)	2 (6.1)	33 (100)
	E2 (25%-49%)	8 (38.1)	11 (52.4)	2 (9.5)	21 (100)
	E3 (50%-100%)	14 (30.4)	21 (45.7)	11 (23.9)	46 (100)
PSD					
	E1 (0%-24%)	22 (68.7)	7 (21.9)	3 (9.4)	32 (100)
	E2 (25%-49%)	9 (42.9)	9 (42.9)	3 (14.3)	21 (100)
	E3 (50%-100%)	28 (59.6)	11 (23.4)	8 (17.0)	47 (100)

FSD: Frozen section diagnosis; PSD: Permanent section diagnosis.

significantly in PSD (P = 0.039) (Table 2). The concordance rate between FSD and PSD is summarized in Table 3 as original diagnostic results.

We separately analyzed the status of the surgical margin in the epithelial and subepithelial layers. FSD in the epithelial layer revealed 44 (44.0%) negative, 15 (15.0%) positive, and 41 (41.0%) borderline margins, while PSD revealed 59 (59.0%) negative, 14 (14.0%) positive, and 27 (27.0%) borderline margins. The number of positive margins was similar between FSD and PSD, but the number of negative margins increased and that of borderline decreased in PSD with marginal significance (P = 0.078) (Table 2) (Figure 2A and B).

The extent of the remaining biliary epithelium lining the resected margin might represent the quality of samples especially in evaluating the epithelial layer. A total of 33 samples were E1, 21 were E2, and 46 were E3 in FSD, while a total of 32 samples were E1, 21 were E2, and 47 were E3 in PSD (Table 4). The rate of the remaining epithelium was almost identical between FSD and PSD. More than half of the total margins lacked > 50% of the entire biliary epithelium in FSD and PSD. The rate of negative margins decreased and the rate of borderline and positive margins increased in FSD according to the rate of the remaining epithelium. This suggested proportional sensitivity to the remaining rate and intrinsic difficulty in the assessment of the epithelial layer. The concordance rate between FSD and PSD in the evaluation of the epithelial layer is summarized in Table 3 as original diagnostic results.

In the subepithelial layer, FSD revealed 87 (87.0%) negative, 12 (12.0%) positive, and 1 (1.0%) borderline margins, while PSD revealed 86 (86.0%) negative and 14 (14.0%) positive margins. There was a nearly complete consistency between FSD and PSD (P = 0.560) (Table 2). The concordance rate between FSD and PSD in the evaluation of the subepithelial layer is summarized in Table 3 as original diagnostic results.

Analysis of diagnostic discordance between FSD and PSD

Diagnostic discordance between FSD and PSD was observed in 31 epithelial layers and two subepithelial layers (Table 5). The discordance rate in the epithelial layer was considerably high, while that in the subepithelial layer was very low. The discordance rate in the epithelial layer was somewhat higher in pCCA than dCCA, but there was no significant difference in the discordance rate between pCCA and dCCA in the epithelial layer and subepithelial layer (P = 0.128 and 1.000, respectively). Alteration from borderline to negative in the epithelial layer was the most frequent (20 margins). Less frequently, alterations from negative to borderline (4 margins) and positive to borderline (4 margins) were observed in the epithelial layer (Table 6). Regrettably, alteration from negative to positive was also noted in two margins (Figure 2C and D).

Concordance rate between FSD and PSD at the patient level

FSD revealed 26 (35.1%) negative, 17 (23.0%) positive, and 31 (41.9%) borderline margins in 74 patients with eCCA, while PSD revealed 36 (48.7%) negative, 18 (24.3%) positive, and 20 (27.0%) borderline margins. The number of positive margins was similar between FSD and PSD, but the number

Table 5 Diagnostic discordance between frozen section diagnosis and permanent section diagnosis

	Diagnostic discordance		Total (%)
	Yes (%)	No (%)	
Epithelial layer	31 (31.0)	69 (69.0)	100 (100)
pCCA	21 (38.2)	34 (61.8)	55 (100)
dCCA	10 (22.2)	35 (77.8)	45 (100)
Subepithelial layer	2 (2.0)	98 (98.0)	100 (100)
pCCA	1 (1.8)	54 (98.2)	55 (100)
dCCA	1 (2.2)	44 (97.8)	45 (100)
Total layer	28 (28.0)	72 (72.0)	100 (100)
pCCA	18 (32.7)	37 (67.3)	55 (100)
dCCA	10 (22.2)	35 (77.8)	45 (100)

pCCA: Perihilar cholangiocarcinoma; dCCA: Distal cholangiocarcinoma.

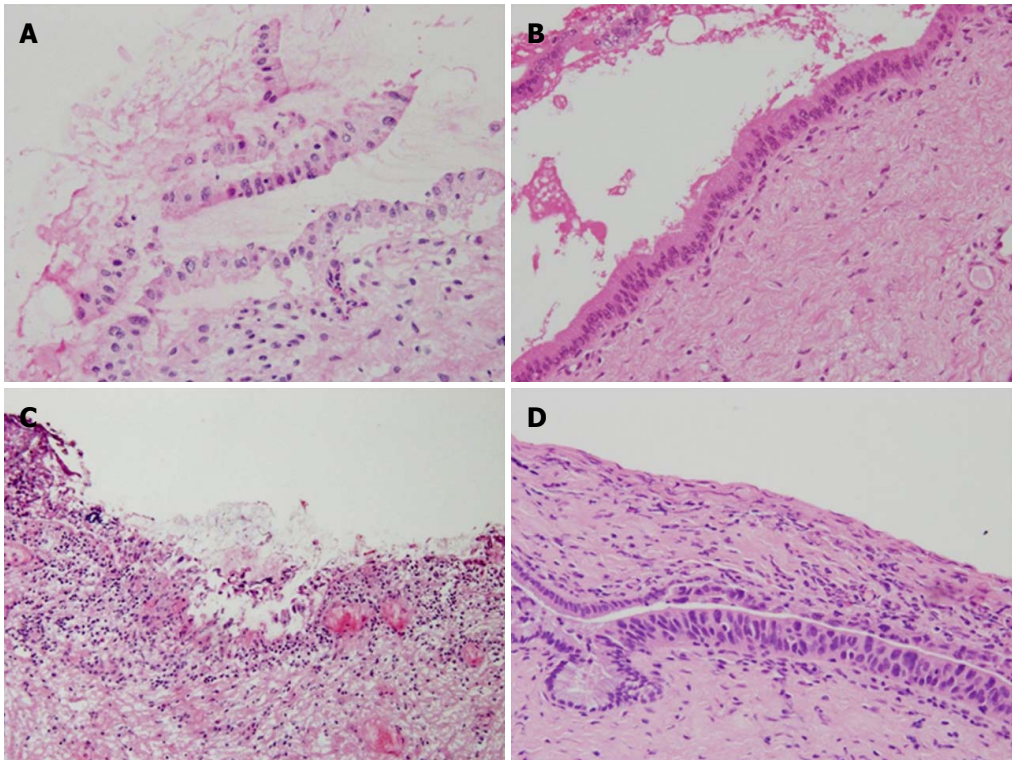

Figure 2 Discordance between frozen section diagnosis and permanent section diagnosis. Bile duct margin of Case 172 (dCCA) prepared for FSD (A) and PSD (B), and that of Case 157 (dCCA) prepared for FSD (C) and PSD (D) (hematoxylin and eosin, 20 ×). These two sets of figures represent the same region of the bile duct margin, respectively. Epithelium was detached from subepithelium, denatured, twisted, and FSD was borderline (BilIN-2) (A), while PSD was negative (B). Epithelium was severely denatured owing to artifacts and FSD was negative (C), while BilIN-3/carcinoma *in situ* appeared in different sections prepared for PSD (D). FSD: Frozen section diagnosis; PSD: Permanent section diagnosis; dCCA: Distal cholangiocarcinoma; BilIN: Biliary intraepithelial neoplasia.

of negative margins increased and that of borderline decreased slightly in PSD (P = 0.134) (Table 2).

Local RFS analysis

The overall follow-up period of the 74 patients from surgery to disease-related death or censoring were 4 to 2343 days (Median, 623 d).

We first performed local RFS analysis based on FSD of the bile duct margin in the total layer. Local RFS rates for 1, 3, and 5 years are listed in Table 7. Patients with positive margins demonstrated a significantly worse survival compared with those with negative or borderline margins (both P < 0.01). In contrast, patients with negative and borderline margins showed comparable prognoses (P = 0.906) (Figure 3A).

We then focused on the status of the epithelial layer, since we thought that diagnosis as borderline was the greatest issue for surgeons in deciding whether to perform additional resection. Patients with borderline and positive margins in the subepithelial layer were excluded from this analysis in order to investigate the pure effect of the status of the epithelial layer. Local RFS rates for 1, 3, and 5 years are listed in Table 7. Patients with positive margins demonstrated a significantly worse survival compared with those with negative or borderline margins (both P < 0.01). In contrast, patients with

Table 6 Details of diagnostic discordance between frozen section diagnosis and permanent section diagnosis

	Epithelial layer (pCCA:dCCA)	Subepithelial layer (pCCA:dCCA)
From negative to borderline	4 (2:2)	0 (0:0)
From negative to positive	1 (0:1)	1 (1:0)
From borderline to negative	20 (13:7)	0 (0:0)
From borderline to positive	2 (2:0)	1 (0:1)
From positive to borderline	4 (4:0)	0 (0:0)
Total	31 (21:10)	2 (1:1)

pCCA: Perihilar cholangiocarcinoma; dCCA: Distal cholangiocarcinoma.

Table 7 Local recurrence-free survival rates of patients according to the status of the bile duct margin evaluated by frozen section diagnosis

	Duration (yr)	Negative	Borderline	Positive
Total layer				
Number of cases		26	31	17
	1	0.916	0.918	0.518
	3	0.769	0.725	0.194
	5	0.769	0.725	0.194
[1]Epithelial layer				
Number of cases		26	30	6
	1	0.916	0.915	0.333
	3	0.769	0.722	0.000
	5	0.769	0.722	0.000

[1]Patients with borderline or positive subepithelial layer were excluded.

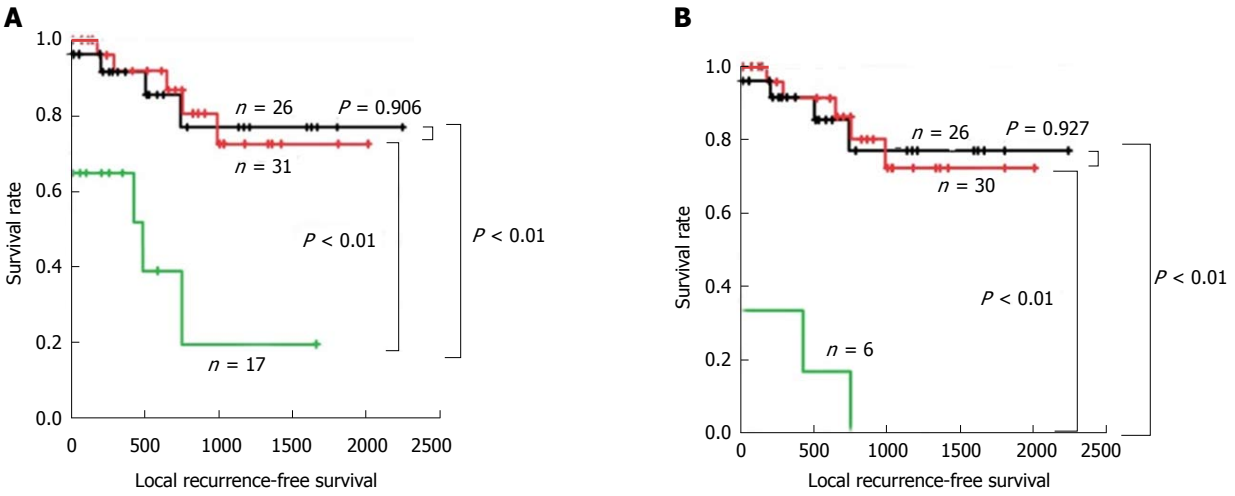

Figure 3 Local recurrence-free survival analysis according to the frozen section diagnosis status. The Kaplan-Meier curves of patients with eCCA according to the status of the bile duct margin evaluated by FSD in total layer (A) and epithelial layer (B). Patients with borderline or positive subepithelial layer were excluded from the analysis in the epithelial layer. Black line: negative; Red line: borderline; Green line: positive; eCCA: Extrahepatic cholangiocarcinoma; FSD: Frozen section diagnosis.

negative and borderline margins showed comparable prognoses (P = 0.927) (Figure 3B).

Local RFS analysis according to the epithelial and total status assessed by PSD demonstrated similar results (Figure 4 and Table 8). Patients with positive margins in the total and epithelial layers demonstrated significantly worse survival compared with those with negative or borderline margins (all P < 0.01). In contrast, patients with negative and borderline margins in the total and epithelial layers showed similar prognoses (both P = 0.896).

Based on the results of survival analysis, borderline margins in the epithelial layer were regarded substantially as negative. Histopathological diagnosis in the epithelial layer was reclassified into positive or negative, and the concordance rate between FSD and PSD was revised (Table 3). The concordance rates in the total, epithelial, and subepithelial layers were 95.0%, 93.0%,

Table 8 Local recurrence-free survival rates of patients according to the status of the bile duct margin evaluated by permanent section diagnosis

	Duration (yr)	Negative	Borderline	Positive
Total layer				
Number of cases		36	20	18
	1	0.940	0.862	0.667
	3	0.710	0.790	0.356
	5	0.710	ND	ND
[1]Epithelial layer				
Number of cases		36	20	6
	1	0.940	0.862	0.333
	3	0.710	0.790	0.167
	5	0.710	ND	ND

[1]Patients with borderline or positive subepithelial layer were excluded. ND: Not determined.

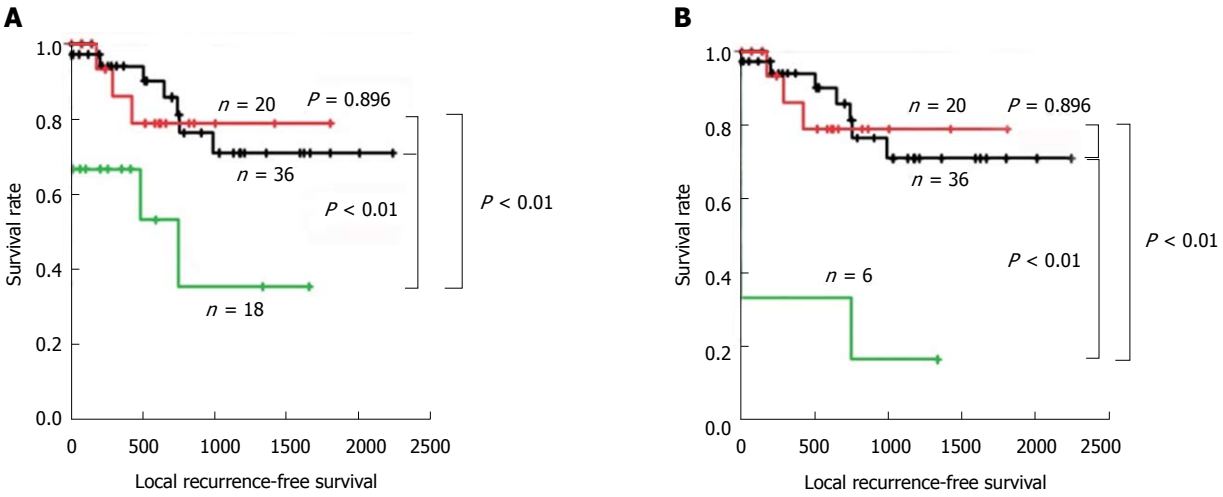

Figure 4 Local recurrence-free survival analysis according to the permanent section diagnosis status. The Kaplan-Meier curves of patients with eCCA according to the status of the bile duct margin evaluated by PSD in total layer (A) and epithelial layer (B). Patients with borderline or positive subepithelial layer were excluded from the analysis in the epithelial layer. Black line: negative; Red line: borderline; Green line: positive; eCCA: Extrahepatic cholangiocarcinoma; PSD: Permanent section diagnosis.

and 98.0%, respectively. These results suggest that FSD is a reliable method to evaluate margin status of the bile duct intraoperatively.

DISCUSSION

The status of the bile duct margin has been assessed by intraoperative FSD for complete resection of eCCA. However, the usefulness of FSD is controversial owing to the frequent discordance between FSD and PSD. Okazaki *et al*[5] reported that concordance rate between FSD and PSD was only 56.5%, and concluded that FSD should not be carried out for patients with a high risk of hepatic failure. Yamaguchi *et al*[7] reported that diagnosis of resected bile duct margin was altered from FSD to PSD in five of 20 patients with gall bladder or bile duct cancer who underwent surgical resection. Endo *et al*[9] reported that discrepancies between FSD and PSD were observed in 10 of 101 patients with pCCA who underwent surgery. In the present study, we experienced diagnostic discordance in 28 of 100 duct margins between FSD and PSD. This high discordance rate was probably due to the grouping method of histopathological results. The margin status was classified into either positive or negative by Yamaguchi *et al*[7] and either positive/suspicious or negative in the study by Endo *et al*[9], in which only invasive carcinoma was diagnosed as positive. In contrast, the margin status in the present study was grouped into three categories of positive, borderline, or negative. Diagnostic discordance in this study was only 2% if only invasive cancer was classified as positive.

Discordance between FSD and PSD was observed in 28 bile duct margins; 31 in the epithelial layer and two in the subepithelial layer. The most frequent alteration was borderline to negative in the epithelial layer. During frozen sample preparation, epithelium easily detaches from basement membrane, and becomes twisted, folded, and overlapped. The nuclei are often swollen because of rapid freezing, which

makes it difficult to discriminate the epithelium from dysplasia and/or carcinoma *in situ*. We assume that the low quality of the frozen section sample may have been the greatest cause of the discordance (Figure 2A and B). The low quality of the frozen section sample was also demonstrated by the low remaining rate of the epithelium for histopathological evaluation. In this study, more than half of the epithelium was lost during sample preparation, and this might have resulted in underdiagnosis in the epithelial layer. In light of the similar extent of the remaining epithelium between FSD and PSD, the epithelial layer might be lost during resection for intraoperative diagnosis by surgeons. We experienced two cases of alteration from negative to positive. These were caused by sampling different sections within the bile duct margin (Figure 2C and D). The cut-surface of the permanent histology was different from that of frozen section histology. We did not overlook cancer cells within frozen section samples. Marked stromal cell infiltration into the tumor is an inherent characteristic of CCA, which may fundamentally underlie inaccurate FSD. Mucosal inflammation caused by the catheter for preoperative biliary drainage may also mislead the frozen diagnosis[5,7]. It may cause regenerative atypia of normal mucosa with thick and multilayered atypical epithelial cells and immature mesenchymal cells, which may be misdiagnosed as malignant epithelium showing sarcomatous changes. In this study, the rate of borderline was significantly higher in the epithelial layer than in the subepithelial layer on FSD, and borderline epithelial margins significantly decreased but borderline subepithelial margins did not change on PSD. This may be partly explained by the fact that almost all patients underwent biliary drainage tube insertion preoperatively (Table 1).

In the present study, patients with negative margins by FSD demonstrated a significantly favorable local RFS compared with patients with positive margins, suggesting that FSD is useful for complete resectability and predicting good prognosis. Positive margins in this study included the presence of cancer cells in the epithelial and/or subepithelial layers, of the bile duct submitted for diagnosis. However, clinical significance of positive surgical margins of the bile duct is controversial. Some authors reported no correlation between positive margins and postoperative local recurrence of pCCA[11]. In contrast, a strong correlation has been reported by other authors[9,12-14]. For example, pCCA patients with positive bile duct margins by paraffin section histology demonstrated significantly worse disease-specific survival compared with those with negative bile duct margins[9]. Bile duct margin was evaluated as positive only when invasive cancer was confirmed histologically[9]. In addition, local recurrence of gall bladder and bile duct cancer was slightly associated with the margin status by paraffin section histology, that is, 4/7 positive patients *versus* 9/37 negative patients (P = 0.081)[7]. In the patients with positive margins, local recurrence occurred only when cancer cells were observed in the subepithelial layer[7]. In the study of middle and distal bile duct cancer, PSD of the hepatic-side duct margin predicted local recurrence with marginal significance, that is, 2 of 6 (33%) positive patients *versus* 4 of 45 (9%) negative patients (P = 0.08)[4]. Localization of cancer cells in the surgical margin was not described in that study[4].

It has been reported that the presence of epithelial dysplasia at the bile duct margin confirmed postoperatively is not associated with survival of patients who undergo R0 resection[9,12,13]. Yamaguchi *et al*[7] also reported that local recurrence occurred in neither of the two patients with carcinoma *in situ* of the bile duct margin by permanent histopathology. In the present study, local recurrence was observed in all six patients with positive margins in the epithelial layer by FSD, suggesting the need for accurate intraoperative diagnosis of BilIN-3/severe dysplasia/*in situ* carcinoma. However, FSD of the bile duct is often difficult even for experienced pathologists. In addition, there is some interobserver variation in the evaluation of the grade of biliary dysplasia. In contrast, diagnosis of invasive carcinoma in the subepithelial layer is easier, especially when there is perineural invasion. Analysis of a greater number of cases is awaited to clarify the significance of BilIN-3/*in situ* carcinoma in the bile duct margin.

One of the main purposes of this study was to determine the relevance of borderline lesions, consisting of BilIN-1 and 2 and indefinite for neoplasia, diagnosed intraoperatively. Approximately 40% of the epithelial layer was diagnosed as borderline, while only 1% of the subepithelial layer was diagnosed as borderline by FSD. We thought that the difference was due to the following reasons: (1) intrinsic borderline lesion, such as BilIN-1 and 2, is defined as a diagnostic category in the epithelial lesion; (2) epithelium is more vulnerable to artifacts than subepithelial stromal tissue is; and (3) impact of preoperative biliary drainage tube insertion. Because our patients had pCCA and dCCA, which are locoregionally different tumors, we investigated only local recurrence rate and not overall survival rate. By survival analysis, patients with borderline margins in the epithelial layer demonstrated a comparable local RFS compared with patients with negative margins. These data suggested that epithelial borderline lesions might be interpreted substantially as negative margins and that additional ductal resection might not be necessary in such institutions as having well-experienced pathologists.

On the other hand, it is quite likely that some borderline margins may ultimately turn out to be positive in a larger series with more diverse pathologist. Hence, if the first margin is borderline and additional margin can be safely obtained, additional ductal resection will be desirable to achieve negative margin as the local recurrence is very high in positives.

In the present study, four of 26 patients with negative frozen margins had local recurrence. PSD was also negative in all these patients. This may have

been because CCA sometimes shows discontinuous longitudinal spread or tumorigenesis from separate foci along the bile duct[14,15].

In conclusion, FSD of the bile duct margin was reliable enough to provide useful information for deciding the extent of resection of eCCA regardless of technical limitations in sample preparation. Positive margins in the epithelial layer was significantly associated with local recurrence, while the borderline margins demonstrated a similar local recurrence rate to that of negative margins. Although it is desirable to achieve negative margin if the first margin is borderline, epithelial borderline lesions could be regarded substantially as negative margins in such institutions as with well-experienced pathologists.

ARTICLE HIGHLIGHTS

Research background

Cholangiocarcinoma (CCA) is a rare malignancy with poor prognosis. Complete surgical resection is the only treatment with the potential for cure, and the status of the final ductal margin is strongly associated with prognosis. Intraoperative frozen section diagnosis (FSD) of the bile duct margins has traditionally been used to guide the extent of operative resection, but its usefulness has been controversial until now.

Research motivation

Because of the rarity and locoregional anatomical complexity of CCA, few centers have substantial clinical experience of managing this disease, and few pathologists have expertise in characterizing resected specimens accurately. In addition, quality of FSD samples is very low. Hence, discordance between FSD and permanent section diagnosis (PSD) that reuses frozen samples often occurs.

Research objectives

The primary purpose of this study was to examine reliability of intraoperative FSD to evaluate the margin status. The secondary purpose was to clarify clinical relevance of borderline lesions that could not be definitely determined whether malignant or benign. Borderline in the present study included such lesions as low-grade and intermediate-grade dysplasia [biliary intraepithelial neoplasia (BilIN)-1 and BilIN-2] and lesions indefinite for neoplasia.

Research methods

We retrospectively analyzed 74 consecutive patients who underwent surgery for extrahepatic CCA (eCCA) from 2012 to 2017, during which FSD of bile duct margins was performed. They consisted of 40 distant CCAs (dCCAs) and 34 perihilar CCAs (pCCAs) (45 and 55 bile duct margins, respectively). The diagnosis was classified into three categories: negative, borderline, or positive. FSD in the epithelial layer, subepithelial layer, and total layer was compared with corresponding PSD postoperatively. Then, association between FSD and local recurrence was analyzed. The concordance rate between FSD and PSD was investigated at the margin and patient levels, but survival analysis was performed solely at the patient level.

Research results

Analysis of 100 duct margins revealed that original concordance rate between FSD and PSD was 68.0% in the total layer, 69.0% in the epithelial layer, and 98.0% in the subepithelial layer. The extent of remaining biliary epithelium was comparable between FSD and PSD, and more than half of the margins lost > 50% of the entire epithelium, suggesting low quality of the samples. In FSD, the rate of negative margins decreased and that of borderline and positive margins increased according to the extent of the remaining epithelium, suggesting proportional sensitivity to the remaining rate and intrinsic difficulty in the assessment of the epithelial layer. Diagnostic discordance between FSD and PSD was observed in 31 epithelial layers and two subepithelial layers. Although the discordance rate in the epithelial layer was somewhat higher in pCCA than dCCA, there was no significant difference between them in the epithelial layer and subepithelial layer. Alteration from borderline to negative was the most frequent (20 of the 31 epithelial layers). Less frequently, alterations from negative to borderline (4 margins) and positive to borderline (4 margins) were observed in the epithelial layer. Although some authors reported no correlation between positive margins and postoperative local recurrence, in the present study patients with positive margin in the total and epithelial layers by FSD demonstrated a significantly worse local recurrence-free survival (RFS) compared with patients with borderline and negative margins. On the other hand, patients with borderline and negative margins in the total and epithelial layers by FSD revealed comparable local RFS. Patients with borderline and negative margins in the epithelial layer by PSD also revealed comparable local RFS. These results suggested that epithelial borderline might be regarded substantially as negative in such institutions as having well-experienced pathologists. However, if the first margin is borderline and additional margin can be safely obtained, additional ductal resection will be desirable to achieve negative margin, because it is quite likely that some borderline margins may ultimately turn out to be positive in a larger series with more diverse pathologist and the local recurrence is very high in positive margins. When classifying the status of the epithelial layer either as negative or positive, concordance rates between FSD and PSD in the total, epithelial, and subepithelial layers were 95.0%, 93.0%, and 98.0%, respectively. These results suggest that FSD is a reliable method to evaluate margin status of the bile duct intraoperatively.

Research conclusions

FSD of the bile duct margin was reliable enough to provide useful information for deciding the extent of resection of eCCA regardless of technical limitations in sample preparation. In contrast to the previous reports, positive margins in the epithelial layer was significantly associated with local recurrence, while the borderline margins demonstrated a similar local recurrence rate to that of negative margins. Although negative margin is desirable, epithelial borderline lesions could be regarded substantially as negative in such institutions as with well-experienced pathologists. These findings would aid surgeons to determine the resection range of the bile duct and better manage the patients with eCCA.

Research perspectives

Intraoperative FSD of the bile duct margins has traditionally been used to guide the extent of operative resection, but the usefulness of FSD has been controversial until now. In the present study, we clearly demonstrated that FSD was reliable enough for pathological diagnosis by comparing FSD and PSD and based on the results of survival analysis. In addition, in contrast to some previous reports, we demonstrated that positive FSD in the epithelial layer was significantly associated with local recurrence and that borderline FSD in the epithelial layer could be substantially regarded as negative. Our results may be partly due to a relatively large number of eCCA cases. This study also highlighted the need for precise and detailed histopathological diagnosis. In this respect, the future challenge is more objective differential diagnosis of BilIN-1, 2, and 3 by FSD. Development of morphometric analysis, special staining procedure, immunohistochemistry, and molecular diagnostics which can be available over a short time of intraoperative FSD are awaited. It will be also necessary to develop the training program of pathologists who can make a correct diagnosis of bile duct margin by intraoperative FSD.

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ORIGINAL ARTICLE

Clinical Trials Study

Correlation between serum vitamin B12 level and peripheral neuropathy in atrophic gastritis

Guo-Tao Yang, Hong-Ying Zhao, Yu Kong, Ning-Ning Sun, Ai-Qin Dong

Guo-Tao Yang, Hong-Ying Zhao, Yu Kong, Ning-Ning Sun, Ai-Qin Dong, Department of Third Neurology, Cangzhou Central Hospital, Cangzhou Clinical Medical School of Hebei Medical University, Cangzhou 061001, Hebei Province, China

Hong-Ying Zhao, Department of Elderly Internal Medicine, Cangzhou Central Hospital, Cangzhou Clinical Medical School of Hebei Medical University, Cangzhou 061001, Hebei Province, China

Yu Kong, Department of Second Digestion, Cangzhou Central Hospital, Cangzhou Clinical Medical School of Hebei Medical University, Cangzhou 061001, Hebei Province, China

Ning-Ning Sun, Department of First Digestion, Cangzhou Central Hospital, Cangzhou Clinical Medical School of Hebei Medical University, Cangzhou 061001, Hebei Province, China

ORCID number: Guo-Tao Yang (0000-0001-8760-1092); Hong-Ying Zhao (0000-0002-2313-8569); Yu Kong (0000-0002-7423-5051); Ning-Ning Sun (0000-0002-1711-6451); Ai-Qin Dong (0000-0002-1362-4520).

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Correspondence to: Dr. Guo-Tao Yang, MD, Department of Third Neurology, Cangzhou Central Hospital, Cangzhou Clinical Medical School of Hebei Medical University, Gaojiao District, Cangzhou 061001, Hebei Province, China. yangguotao3815@cz_hospital.ac.cn
Telephone: +86-317-2179510
Fax: +86-317-2179510

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Abstract

AIM

To explore the correlation between serum vitamin B12 level and peripheral neuropathy in patients with chronic atrophic gastritis (CAG).

METHODS

A total of 593 patients diagnosed with chronic gastritis by gastroscopy and pathological examination from

September 2013 to September 2016 were selected for this study. The age of these patients ranged within 18- to 75-years-old. Blood pressure, height and weight were measured in each patient, and the body mass index value was calculated. Furthermore, gastric acid, serum gastrin, serum vitamin and serum creatinine tests were performed, and peripheral nerve conduction velocity and *Helicobacter pylori* (*H. pylori*) were detected. In addition, the type of gastritis was determined by gastroscopy. The above factors were used as independent variables to analyze chronic gastritis with peripheral neuropathy and vitamin B12 deficiency risk factors, and to analyze the relationship between vitamin B12 levels and peripheral nerve conduction velocity. In addition, in the treatment of CAG on the basis of vitamin B12, patients with peripheral neuropathy were observed.

RESULTS

Age, *H. pylori* infection, CAG, vitamin B9 and vitamin B12 were risk factors for the occurrence of peripheral nerve degeneration. Furthermore, CAG and *H. pylori* infection were risk factors for chronic gastritis associated with vitamin B12 deficiency. Serum vitamin B12 level was positively correlated with sensory nerve conduction velocity in the tibial nerve (R = 0.463). After vitamin B12 supplementation, patients with peripheral neuropathy improved.

CONCLUSION

Serum vitamin B12 levels in patients with chronic gastritis significantly decreased, and the occurrence of peripheral neuropathy had a certain correlation. CAG and *H. pylori* infection are risk factors for vitamin B12 deficiency and peripheral neuropathy. When treating CAG, vitamin B12 supplementation can significantly reduce peripheral nervous system lesions. Therefore, the occurrence of peripheral neuropathy associated with vitamin B12 deficiency may be considered in patients with CAG. Furthermore, the timely supplementation of vitamin B12 during the clinical treatment of CAG can reduce or prevent peripheral nervous system lesions.

Key words: Chronic gastritis; Chronic atrophic gastritis; Vitamin B12; Peripheral neuropathy

Core tip: The general situation and peripheral nerve conduction velocity of 593 patients with chronic gastritis were compared. We found that serum vitamin B12 levels in patients with chronic gastritis significantly decreased, and the occurrence of peripheral neuropathy had a certain correlation. Vitamin B12 supplementation can significantly reduce peripheral nervous system lesions. The occurrence of peripheral neuropathy associated with vitamin B12 deficiency may be considered in patients with chronic atrophic gastritis. Timely supplementation of vitamin B12 during the clinical treatment of chronic atrophic gastritis can reduce or prevent peripheral nervous system lesions.

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INTRODUCTION

Chronic atrophic gastritis (CAG) is a common chronic digestive system disease, and its main clinical manifestations include excessive abdominal pain, bloating and abdominal discomfort. Some patients may develop numbness and other neurological disease symptoms, and its pathological features are gastric mucosal and inherent glandular atrophy[1,2]. In addition, gastric mucosal and inherent glandular atrophy lead to gastric acid. Furthermore, internal factors, such as the insufficient secretion of substances, affect vitamin B12 (VitB12) absorption[3,4], which in turn leads to lack of VitB12 *in vivo*[5-9]. Related studies have shown that VitB12 and folic acid deficiency can affect homocysteine metabolism, which leads to impaired neurons, causing peripheral neuropathy[10-12]. Therefore, numbness and other neurological symptoms that may be related to VitB12 and folic acid deficiency should be considered in patients with CAG.

No clinical evidence published to date has confirmed the relationship between these two. Furthermore, no study has reported the supplementation of VitB12 during the occurrence and prognosis of peripheral neuropathy in patients with CAG. Moreover, the association of patients with CAG and peripheral neuropathy remains unclear. Therefore, in the present study, through the treatment of peripheral neuropathy in patients with chronic gastritis, the clinical characteristics were analyzed and the possible risk factors were screened out to identify viable preventive measures and interventions, thereby playing a guiding role in the clinical treatment of CAG. The details are reported as follows.

MATERIALS AND METHODS

Object of study

Outpatients diagnosed with chronic gastritis by gastroscopy and pathological examination in our hospital from September 2013 to September 2016 were selected for this study. Exclusion criteria: (1) patients < 18-years-old or > 75-years-old; (2) patients who received drugs to treat gastritis within the past 2 wk; (3) patients who received VitB12 supplements and folic acid drugs within the past 2 wk; (4) patients whose other

systems or organs are good, patients with malignant neoplasms, severe cardiovascular, cerebrovascular, liver or kidney disease, patients with primary disease of the hematopoietic system and patients with mental illness; (5) or patients who are pregnant and lactating. Finally, a total of 593 patients were included in the study. Among these patients, 295 were male and 298 were female. The average age of these patients was 46.5 ± 12.8 years, their mean blood pressure was 130.54 ± 19.96 mmHg/96.56 ± 9.70 mmHg, and their average body mass index (BMI) value was 21.16 ± 2.34. This research program and its experimental design were approved by the Ethics Committee of our institute. All patients provided signed informed consent.

Detection methods and groupings

Measurement of nerve conduction velocity: The Dantec Keypoint EMG/evoked potential (Denmark) was used at room temperature (25 ℃). The median nerve, ulnar nerve, tibial nerve and sural nerve sensory and motor nerve conduction velocity of patients were routinely detected. Nerve conduction velocity was lower than the average conduction velocity in healthy young people, and was less than three times the standard deviation, or the same nerve conduction velocity difference of > 10%; that is, peripheral nerve conduction velocity abnormality. These abnormalities were measured again. Hence, there were two results for the abnormal diagnosis for peripheral neuropathy. In our hospital, the motor nerve conduction velocity reference value was as follows: median nerve: 57.8 ± 6.2; ulnar nerve: 55.36 ± 4.65; tibial nerve: 44.96 ± 2.57; and sural nerve: 50.17 ± 3.62. The sensory nerve conduction velocity reference value was as follows: median nerve: 55.18 ± 4.26; ulnar nerve: 50.27 ± 4.53; tibial nerve: 52.43 ± 3.62; and sural nerve: 47.65 ± 6.47. These patients were divided into two groups, according to these results: peripheral neuropathy group, and no peripheral neuropathy group.

Determination of serum creatinine, serum gastrin and vitamin levels: 5 mL of venous blood was collected from all patients after 1 d of fasting. After anticoagulation, the collected samples were centrifuged. Then, after the serum was separated, the sample was frozen and stored in aliquots at -20 ℃ for testing. Serum creatinine and serum gastrin were detected using a Hitachi 7060 automatic biochemical analyzer (Japan), and serum vitamin was detected by immunoenzyme analysis. All related operations were performed by highly experienced personnel, in strict accordance with instrument instructions. The VitB12 normal reference value in our hospital was > 160 ng/L.

Gastric juice analysis: Patients were instructed to fast for 8-12 hr prior to their examination in the morning. The nasogastric tube was placed into the stomach through the nose, and overnight net pumping of fasting gastric juice was performed. Pentagastrin was subcutaneously injected to stimulate gastric acid secretion, and gastric juice suction was continued for 1 hr. Then, the maximum amount of gastric acid secreted by the patient was recorded.

Gastroscopy: Patients were instructed to fast for 6-8 hr prior to the examination. After the antifoaming agent was administered and pharyngeal anesthesia was performed, an Olympus GIF-XQ230 gastroscope (Japan) was used for the examination. For the endoscopy of each patient, two tissue samples were collected from the antrum and curvature of the gastric body, respectively. The specimens were immediately fixed in methanol after collection. After the specimens were conventionally fixed, the tissues were embedded, sliced, dyed and microscopically observed by experienced hospital laboratory personnel to identify the type of chronic gastritis.

***H. pylori* detection:** Each patient underwent the following tests for *H. pylori* detection: (1) rapid urease test; (2) 13C urea breath test; and (3) pathological examination. If the results revealed two or more signs of *H. pylori* positivity, the patient was diagnosed with *H. pylori* infection.

Intervention method

In addition to the conventional treatment of chronic gastritis, each patient was supplemented for vitamin deficiency according to their condition. The supplementation of VitB12 for CAG patients with peripheral neuropathy was based on the primary disease treatment and control of risk factors that lead to VitB12 deficiency.

Specific methods: In the treatment of CAG or the radical treatment of *H. pylori* on the basis of conventional medication, patients were intramuscularly injected with 0.5 mg of VitB12 once a week. Then, the VitB12 level and peripheral nerve conduction velocity (tibial nerve sensory nerve) of each patient were determined *in vivo* after diagnosis; that is, at the start of the medication, before the start of the medication, 1-3 mo after the medication, and 6 mo after the medication, respectively. The data were recorded and compared.

Statistical analysis

SPSS 19.0 was used for statistical analysis. The age and incidence of peripheral neuropathy in each group was used for count data, and analyzed by χ^2-test. Age, blood pressure, serum creatinine, gastric acid, serum gastrin and serum vitamin levels, and nerve conduction velocity measurement data were expressed as mean ± SD. *T*-test was used to compare between groups. The multivariate regression analysis of chronic gastritis with peripheral neuropathy was performed by logistic regression analysis. The correlation analysis between VitB12 and peripheral nerve conduction velocity

Table 1 Comparison of the peripheral nerve conduction velocity of patients with or without peripheral neuropathy

Item	Sensory nerve conduction velocity				Motor nerve conduction velocity			
	With peripheral nerve damage	Without peripheral nerve damage	*t*	*P* value	With peripheral nerve damage	Without peripheral nerve damage	*t*	*P* value
Median nerve	50.10 ± 7.80	52.30 ± 8.90	-2.733	0.006	54.20 ± 8.70	56.20 ± 10.70	-2.129	0.034
Ulnar nerve	49.40 ± 8.10	51.50 ± 9.20	-2.556	0.011	50.30 ± 9.40	51.30 ± 8.60	-1.230	0.219
Tibial nerve	38.30 ± 3.20	44.20 ± 7.60	-9.563	0.000	50.4 ± 8.70	55.60 ± 9.80	-5.931	0.000
Sural nerve	45.40 ± 5.00	50.80 ± 8.30	-7.622	0.000	46.70 ± 7.90	51.10 ± 9.00	-5.479	0.000

was analyzed by Pearson analysis. The multivariate regression analysis of chronic gastritis with VitB12 deficiency was performed using logistic regression analysis. The level of serum VitB12 and folic acid were compared using one-way ANOVA after 1-3 mo and 6 mo. $P < 0.05$ was considered statistically significant.

RESULTS

Groupings and the comparison of peripheral nerve conduction velocity between the two groups

A total of 593 patients with chronic gastritis were included in the present study. Among these patients, 162 had peripheral neuropathy (peripheral neuropathy group) and 431 had no peripheral neuropathy (no peripheral neuropathy group). The peripheral nerve conduction velocity in these two groups was compared. The ulnar-median nerve, tibial nerve and sural nerve sensory and motor nerve conduction velocity, and ulnar nerve sensory nerve conduction velocity were lower in patients with peripheral neuropathy, compared to patients without peripheral neuropathy, and the difference was statistically significant ($P < 0.05$). There was no significant difference in nerve conduction velocity between these two groups ($P > 0.05$; Table 1).

Comparison of the general situation of patients in the peripheral neuropathy and no peripheral neuropathy groups

In comparing the general information of patients in these two groups, it was revealed that age, *H. pylori* infection rate and the prevalence of CAG were higher in patients in the peripheral neuropathy group than in patients in the no peripheral neuropathy group, while BMI, serum vitamin A, vitamin B9 (folic acid) and VitB12 were lower than in patients in the no peripheral neuropathy group, and the differences were statistically significant ($P < 0.05$). Moreover, the difference in sex, blood pressure, serum creatinine, VitB1, VitB6 and VitE between these two groups were not statistically significant ($P > 0.05$; Table 2).

Peripheral neuropathy multivariate logistic regression analysis results

A further factorial analysis was performed on factors that were statistically significant in the univariate analysis. Age, BMI, *H. pylori* infection, endoscopic results (CAG), vitamin A, VitB9 (folic acid) and VitB12 were included in the analysis. The logistic regression analysis results revealed that BMI, gastric acid, serum gastrin and vitamin A had no significant effect on peripheral neuropathy, and the difference was not statistically significant ($P > 0.05$). On the contrary, age (P =0.037), *H. pylori* infection (P = 0.000), CAG (P = 0.000), VitB9 (P = 0.034) and VitB12 (P = 0.000) had a significant effect on peripheral neuropathy. Further analysis revealed that based on odds ratio (OR) values, the following factors effected peripheral neuropathy (arranged in descending order according to occurrence): VitB12, CAG, *H. pylori* infection, VitB9 and age (Table 3).

Correlation analysis of serum VitB12 levels and sensory nerve conduction velocity in the tibial nerve for patients with chronic gastritis

The correlation between serum VitB12 and peripheral nerve conduction velocity in patients with chronic gastritis was analyzed. Results are shown in Figure 1. There was a positive correlation between serum VitB12 levels and peripheral nerve conduction velocity (r = 0.631, P = 0.000).

Comparison of the general situation of patients with or without VitB12 deficiency

In comparing the general situation of chronic gastritis patients with VitB12 deficiency and normal VitB12 levels, it was found that age, *H. pylori* infection rate, the prevalence of CAG and serum gastrin levels were significantly higher in patients with VitB12 deficiency than in patients with normal VitB12 levels ($P < 0.05$), while BMI values and folic acid levels were low in patients with normal VitB12 levels; and, the difference was statistically significant ($P < 0.05$). However, the difference in sex, blood pressure and serum creatinine levels between both groups of patients was not statistically significant ($P > 0.05$; Table 4).

Multivariate logistic regression analysis results for chronic gastritis patients with VitB12 deficiency

Further factorial analysis was performed on factors that were statistically significant for the univariate analysis. Age, BMI value, *H. pylori* infection, endoscopy results (CAG), gastric acid and serum gastrin were included in the analysis. The logistic regression analysis results revealed that BMI values, gastric acid and serum gastrin had no significant effect on VitB12 deficiency,

Table 2 Comparison of the general situation of patients in the peripheral neuropathy and no peripheral neuropathy groups

Item	Peripheral neuropathy group	No peripheral neuropathy group	t/c^2	*P* value
Age in yr	50.50 ± 13.90	45.00 ± 12.40	4.653	0.000
Sex, % male	50.60	49.50	0.068	0.795
Systolic blood pressure in mmHg	130.17 ± 18.98	128.35 ± 20.32	0.989	0.323
Diastolic blood pressure in mmHg	75.34 ± 10.32	77.02 ± 9.45	-1.880	0.061
BMI in kg/m^2	19.26 ± 2.15	21.88 ± 2.27	-12.703	0.000
Gastroscopy results, % prevalence of chronic atrophic gastritis	76.50%	59.20%	15.418	0.000
Helicobacter pylori infection, %	86.40	56.40	46.452	0.000
Gastric acid in mmol	6.80 ± 3.70	17.80 ± 3.50	-33.570	0.000
Serum gastrin in pg/mL	532.42 ± 167.33	208.43 ± 44.12	36.968	0.000
Serum creatinine in μmol/L	78.60 ± 17.20	76.50 ± 12.40	1.643	0.101
VitA in ng/mL	0.267 ± 0.269	0.383 ± 0.336	-3.944	0.000
VitB1 in nmol/L	79.40 ± 20.70	82.60 ± 17.50	-1.884	0.060
VitB6 in mmol/L	30.90 ± 14.80	32.70 ± 15.60	-1.269	0.205
VitB9 in ng/mL	9.06 ± 3.81	10.60 ± 3.27	-2.495	0.013
VitB12 in pg/mL	170.20 ± 111.20	216.40 ± 149.80	-2.731	0.007
VitE in μmol/L	31.60 ± 5.48	33.20 ± 6.37	-1.346	0.181

BMI: Body mass index; Vit: Vitamin.

Table 3 Peripheral neuropathy multivariate logistic regression analysis results

Influencing factor	β	SE	Wald value	OR	95%CI		*P* value
Age	0.140	0.056	4.658	1.150	1.030	1.283	0.034
BMI	-0.139	2.321	3.097	0.871	0.009	82.261	0.089
Helicobacter pylori infection, infected = 1; uninfected = 0	1.541	0.124	7.816	4.670	3.662	5.955	0.000
Gastric acid	1.332	1.469	1.158	3.790	0.213	67.465	0.886
Serum gastrin	1.545	2.497	1.796	4.690	0.035	626.127	0.375
Endoscopy results, atrophic gastritis = 1; nonatrophic gastritis = 0	1.663	0.197	8.562	5.276	3.586	7.762	0.000
VitA	0.039	0.127	1.562	1.041	0.811	1.334	0.645
VitB9	0.871	0.359	4.162	2.390	1.183	4.830	0.037
VitB12	1.883	0.236	9.364	6.571	4.137	10.434	0.000

BMI: Body mass index; CI: Confidence interval; OR: Odds ratio; SE: Standard error; Vit: Vitamin.

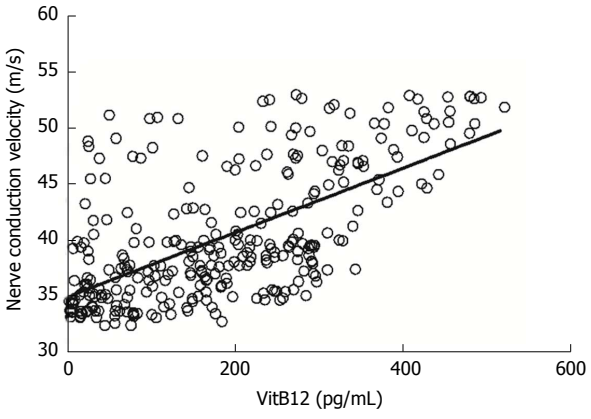

Figure 1 Correlation analysis of serum VitB12 level and sensory nerve conduction velocity in the tibial nerve of patients with chronic gastritis. Vit: Vitamin.

and the difference was not statistically significant ($P > 0.05$); while age ($P = 0.037$), *H. pylori* infection ($P = 0.000$) and CAG ($P = 0.000$) had a significant effect on VitB12 deficiency. Further analysis revealed that based on OR values, the following factors affected VitB12 deficiency (in descending order): CAG, *H. pylori* infection and age (Table 5).

Changes of serum VitB12 levels and nerve conduction velocity in patients after supplementation with VitB12

Chronic gastritis in patients with VitB12 deficiency occurred mainly due to slow atrophic gastritis and *H. pylori* infection. In the present study, atrophic gastritis and radical *H. pylori* infection were treated based on the supplementation of VitB12 in patients. These results revealed that compared with untreated patients, serum VitB12 levels gradually increased ($F = 5.241$, $P < 0.05$); and after 1 mo of treatment, the differences were statistically significant ($T = 4.647$, $P = 0.000$). Furthermore, nerve conduction velocity gradually accelerated ($F = 3.172$, $P < 0.05$; Table 6, Figures 2 and 3).

DISCUSSION

CAG is a common digestive system disease, which commonly causes *H. pylori* infection, bile reflux, vasoactive factors and cytokine changes. It has been generally accepted that CAG occurs under the joint action of

Table 4 Comparison of the general situation of patients with or without vitamin B12 deficiency

Item	VitB12 deficiency, n = 207	Normal VitB12 level, n = 386	t/c^2	P value
Age in yr	51.70 ± 14.70	44.3 ± 11.80	6.666	0.000
Sex, % male	51.60	48.80	0.481	0.488
Systolic blood pressure in mmHg	132.13 ± 19.37	129.35 ± 20.06	1.628	0.104
Diastolic blood pressure in mmHg	75.26 ± 11.44	76.31 ± 9.37	-1.202	0.230
BMI in kg/m^2	18.36 ± 3.22	22.45 ± 2.39	-17.529	0.000
Helicobacter pylori infection, %	87.6	74.8	12.949	0.000
Gastroscopy results, % prevalence of chronic atrophic gastritis	86.50	51.80	70.180	0.000
Serum creatinine in μmol/L	78.60 ± 17.20	76.5 ± 12.40	1.709	0.088
Gastric acid in mmol	7.90 ± 4.20	17.60 ± 3.50	-29.955	0.000
Serum gastrin in pg/mL	432.85 ± 137.62	219.49 ± 47.98	27.516	0.000

BMI: Body mass index; Vit: Vitamin.

Table 5 Multivariate logistic regression analysis of VitB12 deficiency

Influencing factor	β	SE	Wald value	OR	95%CI		P value
Age	0.519	0.149	4.865	1.680	1.255	2.250	0.023
BMI	1.477	1.325	0.004	4.380	0.326	58.795	0.957
Helicobacter pylori infection, positive = 1; negative = 0	1.730	0.279	7.218	5.640	3.264	9.745	0.000
Endoscopy results, atrophic gastritis = 1; nonatrophic gastritis = 0	2.145	0.364	9.645	8.546	4.187	17.442	0.000
Gastric acid	0.948	1.269	1.024	2.580	0.214	31.032	0.762
Serum gastrin	1.479	2.226	2.549	4.390	0.056	344.567	0.267

BMI: Body mass index; CI: Confidence interval; OR: Odds ratio; SE: Standard error; Vit: Vitamin.

Table 6 Changes in serum VitB12 levels and nerve conduction velocity in patients after half a year of VitB12 supplementation

Item	0 mo	1 mo	2 mo	3 mo	6 mo
VitB12 in pg/mL	158.70 ± 104.50	237.20 ± 156.40	481.50 ± 164.60	614.80 ± 186.70	635.20 ± 174.80
Nerve conduction velocity in m/s at 0 mo	40.10 ± 5.50	40.30 ± 4.70	41.60 ± 7.40	42.70 ± 5.90	45.80 ± 5.80

Vit: Vitamin.

various factors, and its development process is caused by the long evolution of multiple genes. Its main clinical manifestations include stomach pain, fullness, ruffian nausea, belching and acid reflux. Some patients may also experience numbness and present other nervous system symptoms. In the course of disease development, gastric mucosal and inherent gland atrophy, decreased gastric acid secretion and other serious effects may disrupt the absorption of nutrients[13-15].

VitB12 is one of the essential vitamins that can improve folic acid utilization, and in turn promote homocysteine metabolism[16-18]. Studies have shown that VitB12 and folic acid deficiency lead to homocysteine metabolism, and is inhibited by the role of axons and myelin in Schwann cells, leading to neuronal damage and peripheral neuropathy[19]. Another study revealed that VitB12 deficiency can lead to neuronal myelination[20-22]. However, at present, the relationship between these two has not been clinically confirmed. Furthermore, the effect of VitB12 on the occurrence and outcome of peripheral neuropathy in patients with CAG remains unclear.

In the present study, by comparing the effects of different factors on peripheral nerve conduction velocity and serum VitB12 levels, it was found that VitB12 deficiency may be a major risk factor for CAG patients with peripheral neuropathy, while CAG and *H. pylori* infection may be risk factors for chronic gastritis patients with VitB12 deficiency. Simultaneously, this study confirmed that treating the primary disease with the supplementation of VitB12 can significantly improve peripheral neuropathy symptoms, suggesting that the timely supplementation of VitB12 can prevent or improve CAG in patients with peripheral neuropathy symptoms.

Analysis of the influencing factors of peripheral neuropathy

In comparing the general situation of patients with or without peripheral neuropathy, it was found that age, *H. pylori* infection rate, CAG, BMI, serum vitamin A,

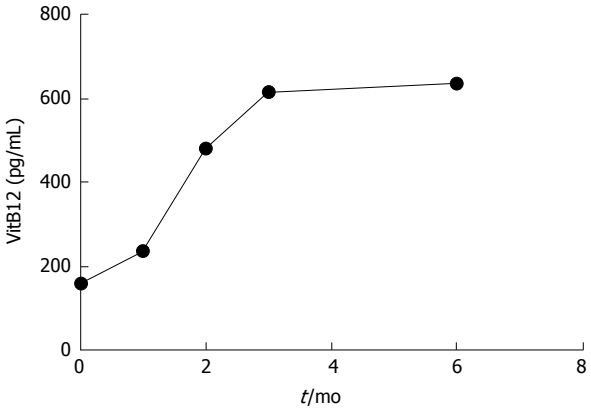

Figure 2 Trend changes in serum VitB12 level. Vit: Vitamin.

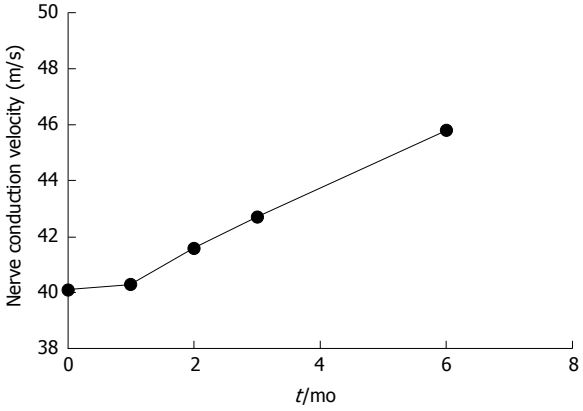

Figure 3 Trend changes in nerve conduction velocity.

vitamin B9 (folic acid) and VitB12 were the possible risk factors for peripheral neuropathy in patients with chronic gastritis. Based on further logistic multivariate regression analysis, it was found that age, *H. pylori* infection, CAG, VitB9 and VitB12 were risk factors for peripheral neurodegeneration. Among these factors, VitB12, *H. pylori* infection and CAG exhibited a higher relative risk. In addition, age is also one of the risk factors for CAG[23-28], which may be due to its long course; that is, pathogenic factors take a long period of time to become a risk factor for peripheral neuropathy.

Correlation between peripheral nerve conduction velocity and serum VitB12 level

In the assessment of peripheral nerve conduction velocity, it was found that this was more obvious in lower limb peripheral neuropathy, and was particularly evident in tibial nerve sensory nerves in the lower limb. Therefore, the correlation between serum VitB12 level and peripheral conduction velocity was analyzed by tibial nerve sensory nerve conduction velocity. The correlation analysis revealed that peripheral nerve conduction velocity was positively correlated with serum VitB12 levels (R = 0.463); that is, as serum VitB12 levels decreased, the degree of peripheral neuropathy gradually increased.

Analysis of influencing factors for VitB12 deficiency

The above studies show that serum VitB12 levels in patients with chronic gastritis were associated with the risk factors for peripheral neuropathy. In order to explore the etiology of VitB12 in patients with chronic gastritis in the present study, the general situations of chronic gastritis patients associated with VitB12 deficiency were compared. Based on further logistic multivariate regression analysis, it was found that *H. pylori* infection and CAG were independent risk factors for chronic gastritis with VitB12 deficiency. Among these factors, *H. pylori* infection can lead to VitB12 deficiency[29-31]. Furthermore, *H. pylori* infection is one of the common causes of CAG[32-40]. The possible cause for VitB12 deficiency is the damage induced by *H. pylori* infection on gastric mucosal cells[41,42], which reduces gastric acid secretion and affects the separation of VitB12 from food[43]. At the same time, the reduction in gastric mucosal secretion of vitamin C and stomach pH value is affected by the increased absorption of vitamin B[44,45].

Effects of VitB12 supplementation on peripheral neuropathy

Peripheral neuropathy can be treated by VitB12 supplementation. A large number of studies have shown that VitB12 can significantly improve nervous system diseases in patients, such as spinal cord subacute combined disease and reversible myelopathy[46-50]. In the present study, the management of VitB12 deficiency may be a risk factor (CAG and *H. pylori* infection). On this basis, by comparing patients with CAG on the basis of conventional treatment without VitB12 supplementation (0 mo), and after 1-3 mo and 6 mo of treatment, the serum VitB12 level and peripheral nerve conduction velocity trend revealed that serum VitB12 level and nerve conduction velocity gradually increased after treatment. As shown in Figures 2 and 3, it can be observed that the increase in peripheral nerve conduction velocity was faster than that of serum VitB12 levels. It can be speculated that the speed of peripheral nerve conduction was accelerated due to elevated serum VitB12 levels. Hence, VitB12 supplementation can improve peripheral neuropathy.

Limitations and outlook

In the present study, the subjects collected for the experiment all came from our hospital, which may give rise to some limitations. However, there were significant differences in VitB12 and nerve conduction velocity between these two groups. Hence, there can still be a certain degree of response to the relationship between these two. In subsequent studies, a multi-center and multi-region joint cooperation should be conducted to expand the sample size and improve the sample representation, in order to provide results with a higher

degree of confidence.

Summary

In summary, in the present study, we analyzed the risk factors of chronic gastritis with peripheral neuropathy. Furthermore, the correlation between serum VitB12 level and peripheral neuropathy was analyzed. The level of serum VitB12 in patients with chronic gastritis was a risk factor for peripheral neuropathy, and serum VitB12 levels and the severity of peripheral neuropathy were positively correlated. In addition, CAG and *H. pylori* infection were the major risk factors for VitB12 deficiency in patients with chronic gastritis.

By comparing the peripheral nerve conduction velocity after VitB12 supplementation, it was found that the treatment of CAG and the control of *H. pylori* infection while supplementing with VitB12 can significantly reduce peripheral neuropathy. This suggests that the timely supplementation of VitB12 may become a treatment or even prevent the occurrence of CAG in patients or the occurrence of peripheral neuropathy. However, it remains to be further studied whether this can be applied to this population.

ARTICLE HIGHLIGHTS

Research background

The main clinical manifestations of chronic atrophic gastritis are excessive abdominal pain, bloating and abdominal discomfort. It is known that the insufficient secretion of substances would affect vitamin B12 (VitB12) absorption. VitB12 and folic acid deficiency can affect homocysteine metabolism, which leads to peripheral neuropathy. Therefore, the occurrence of patients with chronic atrophic gastritis numbness and other nervous system symptoms may be related to VitB12 and folic acid deficiency

Research motivation

At present, there are no studies reporting the effect of VitB12 supplementation on the occurrence and outcome of peripheral neuropathy in patients with chronic atrophic gastritis. The causes of peripheral neuropathy in patients with chronic atrophic gastritis are also not clear. Therefore, it is necessary to explore the risk factors of peripheral neuropathy in patients with chronic atrophic gastritis.

Research objectives

This study aimed to explore the clinical features of peripheral neuropathy in patients with chronic atrophic gastritis and to screen out the possible risk factors in order to find out the feasible prevention and intervention measures for the clinical treatment of chronic atrophic gastritis

Research methods

In total, 593 patients diagnosed with chronic gastritis were involved and their gastric acid, serum gastrin, serum vitamin and serum creatinine tests, peripheral nerve conduction velocity and *Helicobacter pylori* (*H. pylori*) were detected. In addition, the type of gastritis was determined by gastroscopy. All detected results were used to analyze the relationship between VitB12 levels and peripheral nerve conduction velocity.

Research results

H. pylori infection and chronic atrophic gastritis were independent risk factors for chronic gastritis associated with VitB12 deficiency. The separation of VitB12 from food was affected because *H. pylori* infection in gastric mucosal cells damage gastric acid secretion (reducing it). This study also found that the serum VitB12 and nerve conduction velocity gradually increased after VitB12 supplement treatment, suggesting that VitB12 supplementation can improve peripheral neuropathy.

Research conclusions

This study found that serum VitB12 is a risk factor for peripheral neuropathy in patients with chronic gastritis, and serum vitamin B12 is positively correlated with the severity of peripheral neuropathy. Chronic atrophic gastritis and *H. pylori* infection are the main risk factors of VitB12 deficiency in patients with chronic gastritis. In addition, timely VitB12 supplementation may be an effective treatment and even a prevention method of peripheral neuropathy in patients with chronic atrophic gastritis.

Research perspectives

Although this study has demonstrated serum VitB12 level is related to peripheral neuropathy in patients with chronic atrophic gastritis, it is still limited since it's a single center study. Future research should be designed as a multicenter study, and a large sample size is needed to make the findings more credible.

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ORIGINAL ARTICLE

Observational Study

Successful combination of direct antiviral agents in liver-transplanted patients with recurrent hepatitis C virus

Christian Rupp, Theresa Hippchen, Manuel Neuberger, Peter Sauer, Jan Pfeiffenberger, Wolfgang Stremmel, Daniel Nils Gotthardt, Arianeb Mehrabi, Karl-Heinz Weiss

Christian Rupp, Theresa Hippchen, Manuel Neuberger, Peter Sauer, Jan Pfeiffenberger, Wolfgang Stremmel, Daniel Nils Gotthardt, Karl-Heinz Weiss, Department of Internal Medicine IV, University Hospital of Heidelberg, Heidelberg 69120, Germany

Christian Rupp, Peter Sauer, Interdisciplinary Endoscopy Unit, University Hospital of Heidelberg, Heidelberg 69120, Germany

Arianeb Mehrabi, Department of General, Visceral and Transplantation Surgery, University Hospital of Heidelberg, Heidelberg 69120, Germany

ORCID number: Christian Rupp (0000-0003-2808-9230); Theresa Hippchen (0000-0002-0163-7084); Manuel Neuberger (0000-0003-4573-5134); Peter Sauer (0000-0003-2164-4035); Jan Pfeiffenberger (0000-0002-4741-3617); Wolfgang Stremmel (0000-0002-8545-1753); Daniel Nils Gotthardt (0000-0001-9978-9766); Arianeb Mehrabi (0000-0001-6163-1525); Karl-Heinz Weiss (0000-0002-6336-9935).

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Correspondence to: Christian Rupp, MD, Department of Internal Medicine IV, University Hospital of Heidelberg, Im Neuenheimer Feld 410, Heidelberg 69120, Germany. christian_rupp@med.uni-heidelberg.de
Telephone: +49-6221-5636817
Fax: +49-6221-568904

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Abstract

AIM

To analyze the safety and efficiency of direct-acting

antiviral (DAA) regimens in liver-transplanted patients with hepatitis C virus (HCV) reinfection.

METHODS

Between January 2014 and December 2016, 39 patients with HCV reinfection after liver transplantation were treated at our tertiary referral center with sofosbuvir (SOF)-based regimens, including various combinations with interferon (IFN), daclatasvir (DAC), simeprivir (SIM) and/or ledipasvir (LDV). Thirteen patients were treated with SOF + IFN ± RBV. Ten patients were treated with SOF + DAC ± RBV. Fiveteen patients were treated with fixed-dose combination of SOF + LDV ± RBV. One patient was treated with SOF + SIM + RBV. Three patients with relapse were retreated with SOF + LDV + RBV. The treatment duration was 12-24 wk in all cases. The decision about the HCV treatment was made by specialists at our transplant center, according to current available or recommended medications.

RESULTS

The majority of patients were IFN-experienced (29/39, 74.4%) and had a history of hepatocellular carcinoma (26/39, 66.7%) before liver transplantation. Sustained virological response at 12 wk (SVR12) was achieved in 10/13 (76.9%) of patients treated with SOF + IFN ± RBV. All patients with relapse were treated with fixed-dose combination of SOF + LDV + RBV. Patients treated with SOF + DAC + RBV or SOF + LDV + RBV achieved 100% SVR12. SVR rates after combination treatment with inhibitors of the HCV nonstructural protein (NS)5A and NS5B for 24 wk were significantly higher, as compared to all other therapy regimens (P = 0.007). Liver function was stable or even improved in the majority of patients during treatment. All antiviral therapies were safe and well-tolerated, without need of discontinuation of treatment or dose adjustment of immunosuppression. No serious adverse events or any harm to the liver graft became overt. No patient experienced acute cellular rejection during the study period.

CONCLUSION

Our cohort of liver-transplanted patients achieved high rates of SVR12 after a 24-wk course of treatment, especially with combination of NS5A and NS5B inhibitors.

Key words: Hepatitis C virus; Recurrence; Direct acting antivirals; Liver transplantation; Sustained virological response

Core tip: We examined the safety and efficiency of novel direct-acting antiviral agents (DAAs) in liver-transplanted patients with recurrence of hepatitis c virus (HCV) infection in a real-world cohort at our tertiary care center. In conclusion, DAAs are safe and very efficient in HCV patients after liver transplantation, even in case of recurrent cirrhosis or history of relapse after pegylated-interferon therapy. The high sustained virological response rates in our cohort, despite many patients with recurrent cirrhosis, may argue for a 24-wk therapy period in patients with risk factors for therapy failure in a posttransplant setting.

Rupp C, Hippchen T, Neuberger M, Sauer P, Pfeiffenberger J, Stremmel W, Gotthardt DN, Mehrabi A, Weiss KH. Successful combination of direct antiviral agents in liver-transplanted patients with recurrent hepatitis C virus. *World J Gastroenterol* 2018; 24(12): 1353-1360 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i12/1353.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i12.1353

INTRODUCTION

Recurrent infection with hepatitis C virus (HCV) following liver transplant (LT) treatment is the leading cause of liver graft loss and death in liver-transplanted patients infected with HCV[1]. In patients with detectable HCV RNA at the time of transplantation, HCV universally recurs. In such cases, HCV infection shows an accelerated course, with progression to advanced fibrosis within 5 years post LT in the majority of patients. Fundamental steps in understanding and deciphering the HCV replication system in the last 2 decades has opened up the way for development of highly effective new antiviral drugs[2-4].

Before introduction of the direct-acting antiviral (DAA) therapies, treatment options for recurrent HCV in liver-transplanted patients were limited, due to significant drug-drug interactions and severe side effects. The approval of DAAs has revolutionized HCV treatment. Nowadays, well-tolerated, interferon (IFN)-free and highly efficient treatment options are available for HCV-infected patients[5-8]. In most cases, DAA administration before liver transplantation prevents HCV recurrence[9].

Despite the growing number of successfully treated patients, HCV recurrence after orthotopic LT remains one of the most challenging clinical situations[10-12]. Thus, analysis of real-world cohorts of LT recipients may provide valuable insights into the safety and efficacy of DAA treatment in these cohorts[13-17]. Herein, we present the first experience of liver-transplanted patients with HCV recurrence at our tertiary care center.

MATERIALS AND METHODS

Study cohort

The study cohort comprised all liver-transplanted patients treated with a DAA regimen at the Heidelberg University Hospital. In total, 39 patients were included. The baseline characteristics are depicted in Table 1. All patients included in the study were treated with DAAs. All

Table 1 Baseline characteristics of study cohort *n* (%)

Characteristic		Data
Sex	Male	28 (71.8)
	Female	11 (28.2)
Age (yr)		58.6 (range: 45.8-72.3)
Immunosuppression	Cyclosporine	19 (48.7)
	Tacrolimus	18 (46.2)
	Sirolimus	1 (2.6)
	Everolimus	1 (2.6)
	Mycophenolate mofetil	21 (53.8)
Liver histology	F0-2	7 (17.9)
	F3	15 (38.5)
	F4	17 (43.6)
Liver function, CTP	A	17 (43.6)
	B	2 (5.1)
Risk factors	Interferon-experienced	29 (74.4)
	History of HCC	26 (66.7)
HCV genotype	1	24 (61.5)
	2	1 (2.6)
	3	13 (33.3)
	4	1 (2.6)

CTP: Child-turcotte-pugh; HCC: Hepatocellular carcinoma.

patients were at least 6-mo post LT before the antiviral therapy was started. In all patients, corticosteroids had been discontinued successfully, by tapering over a 3-mo to 6-mo period and immunosuppressive therapy reduced to a long-term dosage. Immunosuppression was achieved by cyclosporine in 19 (48.7%) patients, tacrolimus in 18 (46.2%) patients, and sirolimus 1 (2.6%) or everolimus in 1 (2.6%) patient, respectively. Comedication with mycophenolate mofetil was administered in 21 (53.8%) patients. Patients with a history of hepatocellular carcinoma (HCC) before liver transplantation accounted for 26 (66.7%). All patients with HCC before LT met the Milan-criteria. Three patients [3 (7.7%)] have been already retransplanted at least once. The study covered the period from January 2014 to December 2016. The outcomes of all patients in the study were followed until June 2017.

HCV treatment

HCV treatment was administered by the outpatient clinic at our tertiary center. The decision about the HCV treatment was made by specialists at our transplant center, according to current available or recommended medications. Patients were treated according to recommendations of available drugs that carried approval by the United States Food and Drug Administration and the European Medicines Evaluation Agency. As different drugs became approved during the course of this study, the therapy regimens were adapted. In the beginning, 400 mg sofosbuvir (SOF) was combined with pegylated (Peg)-IFN (180 μg once weekly, dosage modifications according manufacturers' recommendations) and ribavirin (RBV). After introduction of 60 mg daclatasvir (DAC), 150 mg simeprivir (SIM) and fixed-dose combination of 400 mg SOF with 90 mg ledipasvir (LDV), IFN-containing regimens were no longer perpetuated.

Statistical analysis

Calculations were carried out using PASW Statistics 22. Frequencies were compared using a χ^2 test or the Fisher's exact test, where appropriate. Continuous data were compared using the nonparametric Wilcoxon rank-sum test.

Ethic approval

Written informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected by the prior approval by the institution's human research committee. The study was approved by the local ethics committee of Heidelberg University as well.

RESULTS

Baseline characteristics

The baseline characteristics of the study cohort are presented in Table 1. The male to female ratio was 3:1. The median age at beginning of antiviral therapy was about 5 years above the median age of first liver transplantation (53.8 years; range: 23.4-68.4 years). Immunosuppression was achieved mainly by cyclosporine or tacrolimus, with only 2 of the patients receiving sirolimus or everolimus, respectively; half of the patients received comedication with mycophenolate mofetil.

Recurrent cirrhosis occurred in 17 (43.6%) patients, with the majority of cases having relatively low severity [Child-Turcotte-Pugh (CTP) score A] and 2 of the cases having mid-severity (CTP score B). Nearly two-thirds of the patients in the total study cohort were treatment experienced, with an IFN-containing regimen. 26 (66.7%) patients had a history of hepatocellular carcinoma (HCC) before liver transplantation. The median time since transplantation was 4.6 years, ranging from 5.5 mo to 22.7 years. The most common HCV genotypes were 1 and 3, respectively, with genotypes 2 and 4 being relatively rare. The median viral load before therapy was 1.43×10^6.

Therapy regimen

Nine patients were treated with SOF + RBV, five of who received the Peg-IFN combination therapy. In general, the treatment duration was 12 wk in cases of stable liver function and up to 24 wk in cases with known risk factors of therapy failure (*e.g.*, recurrent cirrhosis or treatment-experience). One patient received SOF + RBV for 48 wk, as she was awaiting liver transplantation. Eighteen patients were treated with the fixed-dose combination of SOF plus LDV, either with (n = 15) or without (n = 3) RBV for 24 wk.

Table 2 Hepatitis C virus treatment regimens

n	Therapy	SVR24
5	IFN + SOF + RBV	4/5 (80.0%)
8	SOF + RBV	6/8 (75.0%)
9	DAC +SOF + RBV	9/9 (100.0%)
1	DAC + SOF	1/1 (100.0%)
13	LDV + SOF + RBV	13/13 (100.0%)
2	LDV + SOF	2/2 (100.0%)
1	SIM + SOF + RBV	1/1 (100.0%)

DAC: Daclatasvir; IFN: Interferon; LDV: Ledipasvir; RBV: Ribavirin; SIM: Simeprevir; SOF: Sofosbuvir; SVR24: Sustained virological response at 24 wk.

Table 3 Viral load throughout treatment period

Time (wk)	Mean	Min	Max
T (0)	3268770	45600	25200000
T (4)	25812.1	0	771000
T (8)	22.8	0	268
T (12)	4.1	0	101
T (24)	0	0	0

Ten patients received SOF in combination with DAC, either with (n = 6) or without (n = 4) RBV for 24 wk. One patient was treated with a combination of SOF plus SIM and RBV for 24 wk (Table 2). Clinical and laboratory baseline characteristics were not different between the different regimen cohorts.

Safety

All patients completed antiviral treatment. No serious adverse events occurred that required hospitalization or discontinuation of therapy. No adaption of immunosuppression was necessary during the course of treatment. No patient experienced acute cellular rejection of the graft during the study period. Side effects attributable to the antiviral therapy were fatigue (14/39, 35.9%), anemia (11/39, 28.2%) and irritability (6/39, 15.4%). Side effects concerning blood cell count were attributable to concomitant therapy with RBV. In patients without RVB therapy, no anemia or thrombocytopenia occurred. All side effects disappeared after therapy was finished.

Sustained virological response

At the end of the study period, all patients had attained Sustained virological response (SVR) at 24 wk (SVR24). Of the thirty-nine patients, three patients experienced relapse after the first therapy with SOF + RBV, including those with (n = 1) or without (n = 2) the Peg-IFN for 24 wk. Relapse occurred within 4 wk after the end of therapy. All patients with relapse were retreated with fixed-dose combination of SOF + LDV and achieved SVR24.

The viral loads detected during therapy are shown in Table 3. In the majority of patients HCV was undetectable between weeks 4 through 8 of the antiviral therapy. Only 2 patients had detectable viral load after 12 wk of treatment. In both of these cases, no HCV was detectable after 24 wk of treatment and no relapse occurred. There was no association between viral load at the beginning or during the course of therapy and risk for relapse.

Liver function

The model for end-stage liver disease (MELD) score remained stable or improved in 20 (51.3%) patients until the end of therapy. At 12 wk after end of therapy, improved or stable MELD score was found in 21 (53.8%) patients. At 24 wk after end of therapy, the majority of patients (32/39, 82.1%) had at least stable or improved MELD score (Figure 1).

Risk factors for relapse

We assessed several clinical and laboratory risk factors associated with treatment failure. We found no association of sex, age, immunosuppression, HCV genotype, viral load, CTP score or MELD score with treatment failure. In addition, there was no association found for any of these factors with SVR. When comparing different therapy regimens, we were able to demonstrate superior rates of SVR at 12 wk (SVR12) for a combination of inhibitors of the HCV nonstructural protein (NS)5A and NS5B administered for 24 wk, as compared to all other regimens (29/29 *vs* 10/13; P = 0.007).

Overall graft and host survival rates and prevalence of HCC

During the study period, 1 patient underwent re-transplantation and 1 patient died because of progredient liver failure. Both had achieved SVR24 after successful antiviral therapy. During the study period, no HCC was detected in any patient, especially not in those who had had HCC before the LT. No other malignant disease became overt in our cohort during the study period.

DISCUSSION

The availability of new antiviral drugs poses new questions about the optimum timing and duration of treatment to prevent HCV recurrence after liver transplantation[18]. Facing good tolerance and low drug-drug interactions, antiviral treatment seems to be acceptable for both before and after transplantation[19-21]. Yet, antiviral therapy after liver transplantation remains challenging in this difficult-to-treat population[22,23]. On the one side, antiviral therapy should not interfere with immunosuppression; on the other side, stimulation of the immune system might compromise liver graft function. With the introduction of DAAs, a new era for treatment of HCV-infected patients has begun.

A growing amount of studies have confirmed the efficiency and safety of DAAs in LT recipients[24-26].

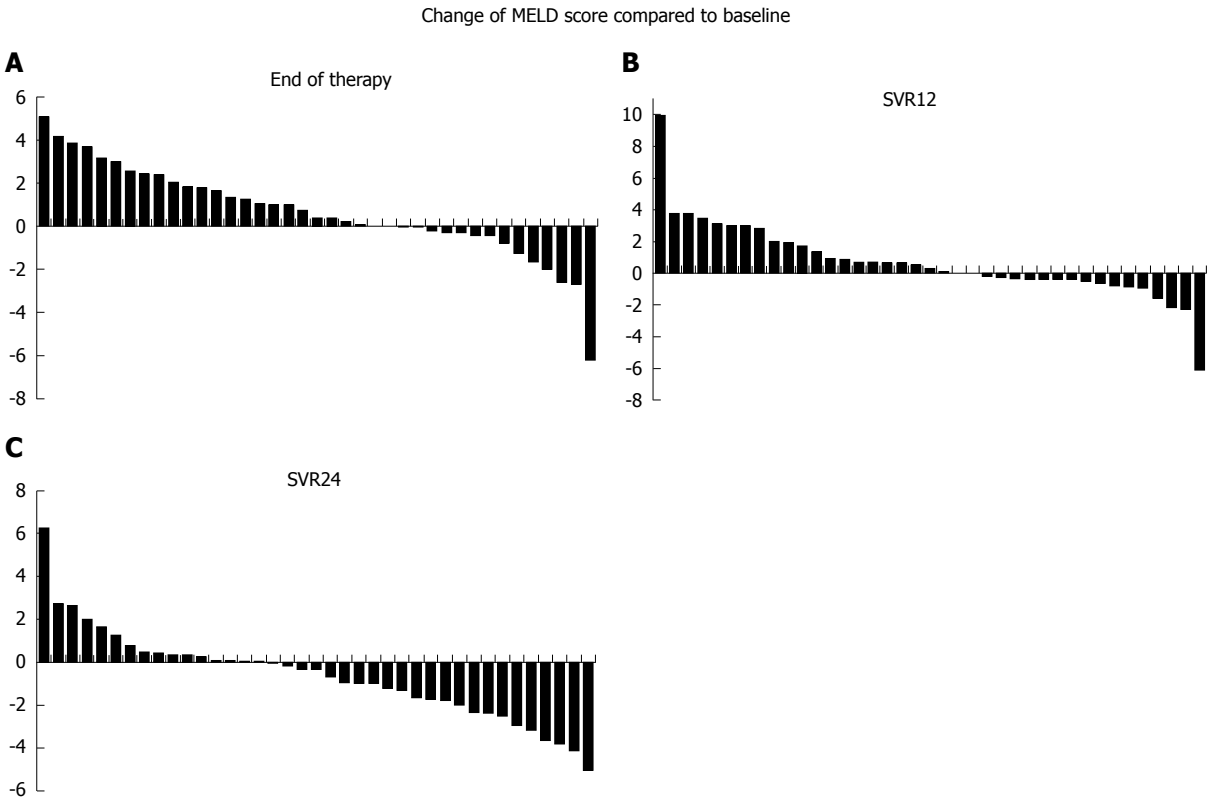

Figure 1 Model for end-stage liver disease score after the end of antiviral treatment. Differences in MELD score compared to baseline at A: the end of therapy; B: 12 wk after the end of treatment; C: and, 24 wk after the end of treatment. Each column indicates one patient. MELD: Model for end-stage liver disease.

Several therapy regimens have been successfully tested so far[14]. We report here about the first experiences with liver-transplanted patients and HCV reinfection at our tertiary care center. To the end of the study period, all patients had reached SVR12. In this study we showed also SVR24 rates, to rule out the possibility of delayed relapse in our patients, like rarely seen in patients treated with interferon and ribavirin. As all three relapses to DAA therapy appeared already within 4 wk after cessation of therapy we believe SVR12 is sufficient to determine successful HCV eradication. We had decided on a 24-wk treatment period for the majority of patients, as most patients had already relapsed or shown nonresponse with past administered IFN-containing HCV therapies. Furthermore, most patients had already developed recurrent cirrhosis, representing another risk factor for therapy failure[27].

HCV therapy was well tolerated in all our patients, and there was no case of therapy termination necessitated for any patient due to side effects or adverse events. In our cohort, most patients received RBV in addition to the DAA[28]. Side effects concerning affectation of the blood count might be attributable to the comedication with RBV. Importantly, we recognized no serious harmful effects on transplant function, as no patient experienced an episode of acute cellular rejection or required re-transplantation during or immediately after the antiviral therapy. Most patients showed improvement of liver function after the end of therapy, which might improve graft survival in the future[29].

One patient underwent re-transplantation at 1 year after successful antiviral therapy, and another patient died due to progredient liver failure after more than 2 years after reaching SVR12. Both patients had recurrent cirrhosis and were transplanted more than 5 years ago. These patients might represent a subgroup of patients that have reached a point of no return, as HCV infection has already caused severe damage to the liver graft, which cannot be reverted even by successful antiviral therapy[29-32].

Liver function remained stable in most patients during the course of therapy and improved within 24 wk after end of therapy in more than 80% of patients. This is in line with other studies of posttransplant patients and emphasizes the importance of antiviral therapy for liver graft protection. Importantly, there was no HCC recurrence despite a high number of patients with HCC prior to transplantation in our cohort[33-35].

We were not able to identify any potential risk factors for therapy failure according to the clinical or laboratory parameters used in our study. In particular, we found no correlation with successful antiviral therapy and viral load, genotype, age, immunosuppression or liver function. Additionally, we found no different outcome between patients treated with RBV or without, which might underline the advantage of an RBV-free regimen[28]. When comparing different therapy regimens, we were able to demonstrate superior SVR12 rates for a combination of NS5A and NS5B inhibitors at 24 wk, as compared to all other regimens. However, this study was not designed nor powered to answer this

question.

In conclusion, DAAs are safe and very efficient in HCV patients after liver transplantation, even in cases of recurrent cirrhosis or history of relapse after Peg-IFN therapy. The high SVR rates in our cohort, despite the many patients with recurrent cirrhosis, may argue for a 24-wk therapy period in patients with risk factors for therapy failure in a posttransplant setting.

ARTICLE HIGHLIGHTS

Research background

Recurrent infection with hepatitis C virus (HCV) following liver transplant (LT) treatment is the leading cause of liver graft loss and death in liver-transplanted patients infected with HCV. Before introduction of the direct-acting antiviral (DAA) therapies, treatment options for recurrent HCV in liver-transplanted patients were limited, due to significant drug-drug interactions and severe side effects. The approval of DAAs has revolutionized HCV treatment.

Research motivation

Despite the growing number of successfully treated patients, HCV recurrence after orthotopic LT remains one of the most challenging clinical situations. Thus, analysis of real-world cohorts of LT recipients may provide valuable insights into the safety and efficacy of DAA treatment in these cohorts.

Research objectives

To analyze the safety and efficiency of DAA regimens in liver-transplanted patients with HCV reinfection in a real-world cohort.

Research methods

The study cohort comprises all liver transplanted patients that were treated with direct acting antiviral regimen at the Heidelberg University Hospital from January 2014 to December 2016. In total 39 patients were included. Clinical and laboratory baseline characteristics were collected at entry into the study. All patients were at least six months liver transplanted before antiviral therapy was started. HCV treatment was administered by the outpatient clinic at our tertiary center. The decision about the HCV treatment was made by specialists at our transplant center, according to current available or recommended medication. Patients were treated according recommendations of available drugs after approval by FDA and EMEA. As different drugs were approved during the course of this study therapy regimen were adapted. In the beginning Sofosbuvir was combined with pegylated interferon (Peg-INF) and ribavirin. After introduction of Daclatasvir, Simeprivir and fixed-dose combination of Sofosbuvir with Ledipasvir interferon containing regimen were no longer perpetuated.

Research results

At the end of the study period, all thirty-nine patients had attained SVR at 24 wk (SVR24). Sustained virological response at 12 wk (SVR12) was achieved in 10/13 (76.9%) of patients treated with SOF + IFN ± RBV. All patients with relapse were treated with fixed-dose combination of SOF + LDV + RBV. Patients treated with SOF + DAC + RBV or SOF + LDV + RBV achieved 100% SVR12. SVR rates after combination treatment with inhibitors of the HCV nonstructural protein (NS)5A and NS5B for 24 wk were significantly higher, as compared to all other therapy regimens ($P = 0.007$). Liver function was stable or even improved in the majority of patients during treatment. All antiviral therapies were safe and well-tolerated, without need of discontinuation of treatment or dose adjustment of immunosuppression. No serious adverse events or any harm to the liver graft became overt. No patient experienced acute cellular rejection during the study period.

Research conclusions

In conclusion, DAAs are safe and very efficient in HCV patients after liver transplantation, even in cases of recurrent cirrhosis or history of relapse after Peg-IFN therapy. The high SVR rates in our cohort, despite the many patients with rrecurrent cirrhosis, may argue for a 24-wk therapy period in patients with risk factors for therapy failure in a posttransplant setting.

Research perspectives

HCV recurrence after orthotopic LT can be safely and efficiently treated with DAAs. Optimal timing and duration of antiviral therapy remains undetermined. Patients at risk for relapse need to be identified before initiation of therapy. Long-term effects of successful antiviral therapy, especially in patients with advanced recurrent cirrhosis, need to be analyzed in future.

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ORIGINAL ARTICLE

Randomized Clinical Trial

Daclatasvir plus asunaprevir in treatment-naïve patients with hepatitis C virus genotype 1b infection

Lai Wei, Fu-Sheng Wang, Ming-Xiang Zhang, Ji-Dong Jia, Alexey A Yakovlev, Wen Xie, Eduard Burnevich, Jun-Qi Niu, Yong Jin Jung, Xiang-Jun Jiang, Min Xu, Xin-Yue Chen, Qing Xie, Jun Li, Jin-Lin Hou, Hong Tang, Xiao-Guang Dou, Yash Gandhi, Wen-Hua Hu, Fiona McPhee, Stephanie Noviello, Michelle Treitel, Ling Mo, Jun Deng

Lai Wei, Peking University People's Hospital and Peking University Hepatology Institute, Beijing 100044, China

Fu-Sheng Wang, 302 Military Hospital of China, Beijing 100039, China

Ming-Xiang Zhang, the Sixth People's Hospital of Shenyang, Shenyang 110006, Liaoning Province, China

Ji-Dong Jia, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China

Alexey A Yakovlev, Saint-Petersburg State Healthcare Institution 'Clinical Infectious Hospital n.a. S.P. Botkin', Saint-Petersburg 191167, Russia

Wen Xie, Beijing Ditan Hospital, Capital Medical University, Beijing 100015, China

Eduard Burnevich, I.M. Sechenov First Moscow State Medical University, Moscow 119991, Russia

Jun-Qi Niu, The First Hospital of Jilin University, Jilin 1300021, Jilin Province, China

Yong Jin Jung, SMG-SNU Boramae Medical Center, Seoul 07061, South Korea

Xiang-Jun Jiang, Qingdao Municipal Hospital, Qingdao 266011, Shandong Province, China

Min Xu, Guangzhou No. 8 People's Hospital, Guangzhou 510060, Guangdong Province, China

Xin-Yue Chen, Beijing Youan Hospital, Capital Medical University, Beijing 100069, China

Qing Xie, Shanghai Ruijin Hospital, Jiaotong University School of Medicine, Shenyang 200025, Liaoning Province, China

Jun Li, TheFirst Affiliated Hospital with Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Jin-Lin Hou, Hepatology Unit, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China

Hong Tang, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Xiao-Guang Dou, China Medical University, Shengjing Hospital, Shenyang 110004, Liaoning Province, China

Yash Gandhi, Wen-Hua Hu, Stephanie Noviello, Michelle Treitel, Bristol-Myers Squibb, Princeton, NJ 08540, United States

Fiona McPhee, Bristol-Myers Squibb, Wallingford, CT 06492, United States

Ling Mo, Jun Deng, Bristol-Myers Squibb, Shanghai 200040, China

ORCID number: Lai Wei (0000-0003-2326-1257); Fu-Sheng Wang (0000-0002-8043-6685); Ming-Xiang Zhang (0000-0001-6519-3497); Ji-Dong Jia (0000-0002-4673-8890); Alexey A Yakovlev (0000-0003-4163-5769); Wen Xie (0000-0002-7314-8175); Eduard Burnevich (0000-0002-7251-4284); Jun-Qi Niu (0000-0002-9857-6520); Yong Jin Jung (0000-0001-8785-2254); Xiang-Jun Jiang (0000-0001-8786-9654); Min Xu (0000-0003-0099-9101); Xin-Yue Chen (0000-0003-0099-2398); Qing Xie (0000-0002-2582-8803); Jun Li (0000-0002-1961-7188); Jin-Lin Hou (0000-0001-8230-8583); Hong Tang (0000-0002-9790-6225); Xiao-Guang Dou (0000-0002-5293-6009); Yash Gandhi (0000-0001-7637-9617); Wen-Hua Hu (0000-0002-8763-609X); Fiona McPhee (0000-0002-9321-4483); Stephanie Noviello (0000-0003-2705-1097);

Michelle Treitel (0000-0001-9488-7113); Ling Mo (0000-0001-8272-4210); Jun Deng (0000-0002-6390-7430).

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Correspondence to: Jun Deng, MD, Virology Clinical Research, Bristol-Myers Squibb, 55F Wheelock Square, 1717 West Nanjing Road, Shanghai 200040, China. daniel.deng@bms.com
Telephone: +86-21-23218405
Fax: +86-21-32303705

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Abstract

AIM

To assess daclatasvir plus asunaprevir (DUAL) in treatment-naïve patients from mainland China, Russia and South Korea with hepatitis C virus (HCV) genotype 1b infection.

METHODS

Patients were randomly assigned (3:1) to receive 24 wk of treatment with DUAL (daclatasvir 60 mg once daily and asunaprevir 100 mg twice daily) beginning on day 1 of the treatment period (immediate treatment arm) or following 12 wk of matching placebo (placebo-deferred treatment arm). The primary endpoint was a comparison of sustained virologic response at posttreatment week 12 (SVR12) compared with the historical SVR rate for peg-interferon plus ribavirin (70%) among patients in the immediate treatment arm. The first 12 wk of the study were blinded. Safety was assessed in DUAL-treated patients compared with placebo patients during the first 12 wk (double-blind phase), and during 24 wk of DUAL in both arms combined.

RESULTS

In total, 207 patients were randomly assigned to immediate (n = 155) or placebo-deferred (n = 52) treatment. Most patients were Asian (86%), female (59%) and aged < 65 years (90%). Among them, 13% had cirrhosis, 32% had *IL28B* non-CC genotypes and 53% had baseline HCV RNA levels of ⩾ 6 million IU/mL. Among patients in the immediate treatment arm, SVR12 was achieved by 92% (95% confidence interval: 87.2-96.0), which was significantly higher than the historical comparator rate (70%). SVR12 was largely unaffected by cirrhosis (89%), age ⩾ 65 years (92%), male sex (90%), baseline HCV RNA ⩾ 6 million (89%) or *IL28B* non-CC genotypes (96%), although SVR12 was higher among patients without (96%) than among those with (53%) baseline NS5A resistance-associated polymorphisms (at L31 or Y93H). During the double-blind phase, aminotransferase elevations were more common among placebo recipients than among patients receiving DUAL. During 24 wk of DUAL therapy (combined arms), the most common adverse events (⩾ 10%) were elevated alanine aminotransferase and upper respiratory tract infection; emergent grade 3-4 laboratory abnormalities were infrequently observed, and all grade 3-4 aminotransferase abnormalities (alanine aminotransferase, n = 9; aspartate transaminase, n = 6) reversed within 8-11 d. Two patients discontinued DUAL treatment; one due to aminotransferase elevations, nausea, and jaundice and the other due to a fatal adverse event unrelated to treatment. There were no treatment-related deaths.

CONCLUSION

DUAL was well-tolerated during this phase 3 study, and SVR12 with DUAL treatment (92%) exceeded the

historical SVR rate for peg-interferon plus ribavirin of 70%.

Key words: Asunaprevir; Daclatasvir; Direct-acting antiviral; Chronic hepatitis C; Liver disease; NS3; NS5A; Genotype 1b

Core tip: This phase 3, placebo-controlled study assessed the efficacy and safety of daclatasvir (NS5A inhibitor) plus asunaprevir (NS3/4A protease inhibitor) in treatment-naïve patients from mainland China, Russia and South Korea with hepatitis C virus (HCV) genotype 1b infection. The rate of sustained virologic response at posttreatment week 12 among patients in the immediate treatment arm was 92%, which was significantly higher than the historical comparator rate (70%). The combination was well tolerated during 24 wk of treatment. These results demonstrate that for countries such as China, where interferon-based combinations are still widely used for the treatment of HCV genotype 1b, daclatasvir/asunaprevir offers a more efficacious and tolerable alternative with a shorter treatment duration.

Wei L, Wang FS, Zhang MX, Jia JD, Yakovlev AA, Xie W, Burnevich E, Niu JQ, Jung YJ, Jiang XJ, Xu M, Chen XY, Xie Q, Li J, Hou JL, Tang H, Dou XG, Gandhi Y, Hu WH, McPhee F, Noviello S, Treitel M, Mo L, Deng J. Daclatasvir plus asunaprevir in treatment-naïve patients with hepatitis C virus genotype 1b infection. *World J Gastroenterol* 2018; 24(12): 1361-1372 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i12/1361.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i12.1361

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a significant health burden across Asia[1], and affects 5-7 million people in China alone[2]. Without effective treatment, patients can develop severe complications, such as hepatocellular carcinoma (HCC)[3,4], for which HCV infection has become one of the most common causes in Asian and Western countries[5,6].

DUAL is an all-oral combination of daclatasvir (pan-genotypic NS5A inhibitor with *in vitro* activity against genotypes 1-6)[7,8] and asunaprevir (NS3 protease inhibitor with *in vitro* activity against genotypes 1 and 4-6)[9]. This regimen has demonstrated efficacy in several phase 3 studies of patients infected with HCV genotype 1b[10-13], the predominant genotype in East Asia[14-16], including those with characteristics known to attenuate response to interferon (IFN)-based treatment[17-19]. DUAL also has a superior safety profile compared with IFN-based combinations[20] and in April 2017 became the first all-oral, nonribavirin-containing combination for chronic HCV infection to gain approval in China[21].

In this study, we evaluated the efficacy and safety of DUAL in treatment-naïve patients from mainland China, South Korea and Russia with HCV genotype 1b infection.

MATERIALS AND METHODS

Study design and treatment

This was a phase 3, double-blind, placebo-controlled study (ClinicalTrials.gov number, NCT02496078) of DUAL, conducted between August 2015 and February 2017 in treatment-naïve patients from mainland China, South Korea and Russia with chronic HCV genotype 1b infection. Patients were randomly assigned (3:1) to receive DUAL (daclatasvir 60 mg tablet once daily and asunaprevir 100 mg soft capsule twice daily) for 24 wk either immediately (immediate treatment arm) or after 12 wk of matching placebo (placebo-deferred treatment arm) *via* an interactive voice-response system, and stratified according to the presence or absence of cirrhosis. Treatment was blinded to patients, investigators and the sponsor until week 12, and was open label thereafter.

The study was conducted according to local laws and regulatory requirements, and in accordance with Good Clinical Practice, as defined by the International Conference on Harmonization and the principles of the Declaration of Helsinki. Written informed consent was gained prior to study initiation.

Patients

The study population comprised male and female patients aged $\geqslant$ 18 years (body mass index: 18-35 kg/m^2) with chronic HCV genotype 1b infection (HCV RNA of $\geqslant$ 10000 IU/mL at screening) and no prior exposure to any IFN formulation, ribavirin or direct-acting antiviral agent for HCV. Patients with compensated cirrhosis were included (enrollment capped at approximately 25%). Cirrhosis status was defined by a hierarchical algorithm based on available biopsy, Fibroscan® or Fibrotest® (BioPredictive, Paris, France) and aspartate transaminase (AST):platelet ratio index (APRI) data. Patients were considered noncirrhotic if they met one of the following criteria: liver biopsy within 36 mo of screening showing absence of cirrhosis; Fibroscan® result of $\leqslant$ 9.6 kPa within 1 year of baseline/day 1; or FibroTest® score of $\leqslant$ 0.48 with APRI of $\leqslant$ 1 (performed during screening). Patients were considered cirrhotic if they met one of the following criteria: liver biopsy showing cirrhosis any time prior to screening; Fibroscan® showing cirrhosis or results of > 14.6 kPa within 1 year of baseline; or FibroTest® score of > 0.75 and an APRI of > 2 (at screening). Both sets of criteria are listed in decreasing hierarchical order.

Key exclusion criteria included: HCV infection other

than genotype 1b; evidence of a medical condition contributing to chronic liver disease other than HCV, or of decompensated liver disease (*e.g.,* history or presence of ascites, bleeding varices, or hepatic encephalopathy); diagnosed or suspected HCC or other malignancies; uncontrolled diabetes or hypertension; moderate to severe depression (well-controlled mild depression was permitted); total bilirubin ⩾ 34 μmol/L (or ⩾ 2 mg/dL) unless the patient had a documented history of Gilbert's disease; alanine aminotransferase (ALT) ⩾ 5 × the upper limit of normal; albumin < 3.5 g/dL; alpha-fetoprotein > 100 ng/mL (patients with alpha-fetoprotein 50-100 ng/mL required a liver ultrasound, and those with findings suspicious of HCC were excluded); hemoglobin < 8.5 g/dL; absolute neutrophil count < 0.5×10^9 cells/L; and, platelet count < 50×10^9 cells/L.

Study assessments

HCV RNA was quantified using the COBAS® *Taq*Man® assay v2.0 (Roche Molecular Diagnostics, Pleasanton, CA, United States) with a lower limit of quantitation (LLOQ) of 25 IU/mL. HCV genotype and subtype were determined using the RealTime HCV Genotype II assay (Abbott Molecular, Des Plaines, IL, United States); if the results were inconclusive, the Versant HCV Genotype 2.0 assay (Siemens, Erlangen, Germany) or population-based sequencing of the NS5A region was employed. *IL28B* rs12979860 single-nucleotide polymorphisms were identified using PCR amplification and sequencing (*Taq*Man assay; Applied Biosystems, Waltham, MA, United States).

Treatment failure comprised: virologic breakthrough, defined as any confirmed > 1 $\log_{10}$ increase in HCV RNA from nadir, or increase in HCV RNA ⩾ LLOQ after confirmed HCV RNA < LLOQ target detected or not detected (TD or TND) during treatment; HCV RNA < LLOQ but still detectable at end of treatment (EOT); or, relapse, defined as HCV RNA ⩾ LLOQ in any posttreatment window following HCV RNA < LLOQ TND at EOT.

Resistance testing was performed using population-based sequencing (threshold ⩾ 20% of a viral population) of the NS5A and NS3 regions on all available plasma samples at baseline, and on the samples of patients experiencing treatment failure with HCV RNA ⩾ 1000 IU/mL.

Safety was monitored based on incidence of adverse events (AEs) and abnormalities in clinical laboratory assessments, vital signs and physical examinations.

Study endpoints

The primary efficacy outcome was the proportion of patients, randomly assigned to the immediate treatment arm, achieving a sustained virologic response (HCV RNA < LLOQ, TD or TND) at posttreatment week 12 (SVR12), and the primary endpoint was comparison of this outcome against a historical SVR rate of 70% associated with peg-IFN plus ribavirin treatment.

SVR12 in the placebo-deferred treatment arm was a secondary endpoint. Safety-related secondary endpoints included the incidence of AEs, serious (S)AEs, discontinuations due to AEs, deaths, and grade 3-4 laboratory abnormalities observed during the 12-wk double-blind phase (DUAL *vs* placebo), and in both arms during 24 wk of treatment with DUAL. Efficacy-related secondary endpoints included SVR12 according to rs12979860 single-nucleotide polymorphisms in the *IL28B* gene; the proportion of patients achieving HCV RNA < LLOQ, TD or TND and TND only, in each treatment arm at on-treatment weeks 1, 2, 4, 6, 8, and 12, both on-treatment weeks 4 and 12, EOT, and post-treatment weeks 4 and 24.

Statistical analysis

The statistical methods used in this study were reviewed by the biometrics group at Bristol-Myers Squibb. The primary objective was to determine whether SVR12 among patients in the immediate treatment arm would be significantly higher than the historical 70% SVR rate associated with peg-IFN plus ribavirin. The lower bound of a two-sided 95% confidence interval (CI) for SVR12 was used to compare to the historical SVR rate; if it exceeded 70%, it was concluded that the primary objective was met and SVR12 for patients in the immediate treatment arm was significantly higher than the SVR rate associated with peg-IFN plus ribavirin. A sample size of approximately 150 patients would have provided a 95%CI with a lower bound exceeding 70% for a corresponding SVR12 rate of approximately 77.3% or higher, while an SVR12 rate of 90% would have provided a lower bound not less than 85%. Missing HCV RNA data at posttreatment week 12 were imputed using the next value carried backwards approach, where the next and closest available HCV RNA measurement after posttreatment week 12 was utilized instead.

RESULTS

Patient disposition

In total, 229 patients were enrolled, of whom 207 were randomly assigned to the immediate (*n* = 155) or placebo-deferred (*n* = 52) treatment arms.

Of 155 patients assigned to the immediate treatment arm, all completed the 12-wk double-blind phase, 148 completed 24 wk of treatment with DUAL, and 151 completed 24 wk of follow-up; seven discontinued treatment with DUAL due to lack of efficacy (*n* = 6) or AEs (*n* = 1), and four discontinued follow-up after posttreatment week 12 due to withdrawal of consent (*n* = 3) or inability to attend the visit due to an accident (*n* = 1).

Of 52 patients randomly assigned to placebo-deferred treatment, 51 completed the 12-wk double-blind phase, 44 completed 24 wk of treatment with DUAL, and 48 completed 24 wk of follow-up; one discontinued placebo due to an SAE (hepatitis E),

Table 1 Baseline demographics and disease characteristics *n* (%)[1]

Characteristic	Immediate treatment, *n* = 155[2]	Placebo-deferred treatment, *n* = 52	Overall, *n* = 207[2]
Age, median (range) years	49 (18-73)	49 (23-69)	49 (18-73)
< 65 yr	142 (92)	45 (87)	187 (90)
⩾ 65 yr	13 (8)	7 (14)	20 (10)
Male	61 (39)	23 (44)	84 (41)
Race			
Asian	132 (85)	45 (87)	177 (86)
White	23 (15)	7 (14)	30 (15)
Country			
Mainland China	119 (77)	42 (81)	161 (78)
Russia	23 (15)	7 (14)	30 (15)
South Korea	13 (8)	3 (6)	16 (8)
HCV RNA, median (range) $\log_{10}$ IU/mL	6.78 (3.1-7.6)	6.86 (5.6-7.6)	6.79 (3.1-7.6)
⩾ 6 million IU/mL	79 (51)	31 (60)	110 (53)
IL28B genotype			
CC	107 (69)	34 (65)	141 (68)
CT	43 (28)	17 (33)	60 (29)
TT	5 (3)	1 (2)	6 (3)
Cirrhosis	19 (12)	7 (14)	26 (13)

[1]Unless otherwise stated; [2]Includes one patient from mainland China who was subsequently reclassified as having HCV genotype 1a infection by phylogenetic analysis of the HCV NS5A sequence. HCV: Hepatitis C virus.

seven discontinued treatment with DUAL due to lack of efficacy (*n* = 6) or AEs (*n* = 1), and two discontinued follow-up after posttreatment week 12 due to withdrawal of consent (*n* = 1) or initiation of alternative HCV therapy (*n* = 1).

Baseline characteristics

The majority of patients were Chinese (77.8%) and female (60.6%); among them, 12.6% had compensated cirrhosis, 31.9% had *IL28B* non-CC genotypes, 53.1% had baseline HCV RNA ⩾ 6 million IU/mL and 9.7% were aged 65 years or older (Table 1). These data include six patients who were found not to meet the study enrollment criteria after treatment initiation; one of these patients, from mainland China, was reclassified as having genotype 1a infection, and five had received prior treatment with ribavirin and/or IFN regimens.

Efficacy endpoints

The study met its primary endpoint, with SVR12 achieved by 142 (91.6%, 95%CI: 87.2-96.0) patients in the immediate treatment arm (including the patient with HCV genotype 1a infection), significantly above the 70% historical comparator (Figure 1). SVR12 was comparable between patients from mainland China (110/119, 92.4%) and Russia (22/23, 95.7%), although lower among the smaller cohort of patients from South Korea (10/13, 76.9%). SVR12 in this arm was also comparable between patients with (17/19, 89.5%) and without (125/136, 91.9%) cirrhosis, with *IL28B* CC (96/107, 89.7%) and non-CC genotypes (46/48, 95.8%), aged < 65 (130/142, 91.5%) and ⩾ 65 (12/13, 92.3%) years, with baseline HCV RNA < 6 million (72/76, 94.7%) and ⩾ 6 million (70/79, 88.6%) IU/mL, and between male (55/61, 90.2%) and female (87/94, 92.6%) patients (Figure 2). HCV RNA declined rapidly from baseline, and by week 4 was undetectable in 140 (90.3%) patients.

SVR12 rates in the placebo-deferred treatment arm, overall and according to selected baseline characteristics, are provided in Figures 3 and 4.

Treatment failure

Thirteen (8.4%) patients in the immediate treatment arm failed to achieve SVR12. Six patients experienced virologic breakthrough [mainland China (*n* = 4), South Korea (*n* = 1), and Russia (*n* = 1)], one patient from mainland China had detectable HCV RNA at EOT, and six patients relapsed [mainland China (*n* = 4) and South Korea (*n* = 2)] (Figure 1).

Treatment failure in the placebo-deferred treatment arm is described in Figure 3.

Resistance analysis

Resistance analyses were conducted at baseline for 154 patients in the immediate treatment arm (excluding the patient with HCV genotype 1a infection) (Tables 2 and 3). Daclatasvir resistance-associated polymorphisms at NS5A amino acid positions L31 or Y93H preexisted in 17 (11.0%) patients, 9 of whom (52.9%) achieved SVR12. By contrast, SVR12 was achieved by 132 of 137 (96.4%) patients without baseline NS5A-L31 or NS5A-Y93H, and was comparably high among patients with (17/19, 89.5%) and without (115/118, 97.5%) cirrhosis who did not have baseline resistance-associated polymorphisms.

The asunaprevir resistance-associated polymorphism NS3-D168E preexisted in one (0.6%) patient who did not achieve SVR12; this patient also had NS5A-Y93H at baseline. Of the 13 patients in the immediate treatment

Table 2 SVR12 in hepatitis C virus genotype 1b-infected patients with and without resistance-associated polymorphisms at baseline (immediate treatment arm) *n* (%)

	All patients - immediate treatment arm							
	With RAPs at baseline				Without RAPs at baseline			
	Mainland China	Russia	South Korea	Overall	Mainland China	Russia	South Korea	Overall
NS5A-L31M/V	1/1 (100)	1/1(100)	0	2/2 (100)	108/117 (92.3)	21/22 (95.5)	10/13 (76.9)	139/152 (91.4)
Y93H	7/13(53.8)	0	0/2 (0)	7/15 (46.7)	102/105 (97.1)	22/23 (95.7)	10/11(90.9)	134/139 (96.4)
L31M/V or Y93H	8/14 (57.1)	1/1 (100)	0/2 (0)	9/17 (52.9)	101/104 (97.1)	21/22 (95.5)	10/11(90.9)	132/137 (96.4)
NS3-D168E	0/1 (0)	0	0	0/1 (0)	109/117 (93.2)	22/23 (95.7)	10/13 (76.9)	141/153 (92.2)

RAP: Resistance-associated polymorphism; SVR12: Sustained virologic response at posttreatment week 12.

Table 3 SVR12 in cirrhotic and non-cirrhotic hepatitis C virus genotype 1b-infected patients with and without resistance-associated polymorphisms at baseline (immediate treatment arm) *n* (%)

	Patients with cirrhosis - immediate treatment arm							
	With RAPs at baseline				Without RAPs at baseline			
	Mainland China	Russia	South Korea	Overall	Mainland China	Russia	South Korea	Overall
Patients with cirrhosis								
NS5A-L31M/V	0	0	0	0	15/16 (93.8)	0	2/3 (66.7)	17/19 (89.5)
Y93H	0	0	0	0	15/16 (93.8)	0	2/3 (66.7)	17/19 (89.5)
L31M/V or Y93H	0	0	0	0	15/16 (93.8)	0	2/3 (66.7)	17/19 (89.5)
NS3-D168E	0	0	0	0	15/16 (93.8)	0	2/3 (66.7)	17/19 (89.5)
Patients without cirrhosis								
NS5A-L31M/V	1/1 (100)	1/1 (100)	0	2/2 (100)	93/101 (92.1)	21/22 (95.5)	8/10 (80.0)	122/133 (91.7)
Y93H	7/13 (53.8)	0	0/2 (0)	7/15 (46.7)	87/89 (97.8)	22/23 (95.7)	8/8 (100)	117/120 (97.5)
L31M/V or Y93H	8/14 (57.1)	1/1 (100)	0/2 (0)	9/17 (52.9)	86/88 (97.7)	21/22 (95.5)	8/8 (100)	115/118 (97.5)
NS3-D168E	0/1 (0)	0	0	0/1 (0)	94/101 (93.1)	22/23 (95.7)	8/10 (80.0)	124/134 (92.5)

RAP: Resistance-associated polymorphism; SVR12: Sustained virologic response at posttreatment week 12.

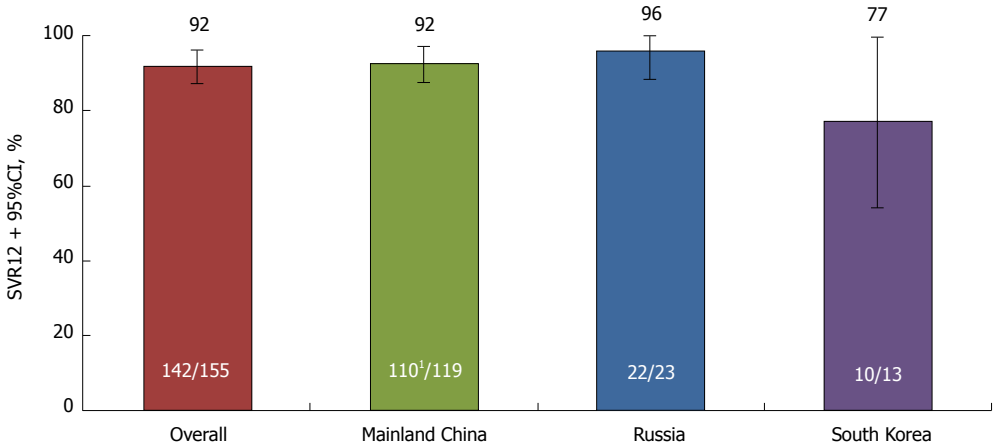


Figure 1 SVR12 in the immediate treatment arm. [1]Includes the patient with genotype 1a infection; [2]On-treatment HCV RNA ⩾ LLOQ after < LLOQ, or increased > 1 $\log_{10}$ over nadir; [3]Posttreatment HCV RNA ⩾ LLOQ after < LLOQ without detectable target at EOT. EOT: End of treatment; HCV: Hepatitis C virus; LLOQ: Lower limit of quantitation; SVR12: Sustained virologic response at post-treatment week 12.

arm who failed to achieve SVR12, 8 (61.5%) had the NS5A-Y93H polymorphism at baseline, including the patient who also had baseline NS3-D168E. At treatment failure, all 13 patients had emergent NS5A-L31 and/or NS5A-Y93H substitutions, while 10 of these patients also had emergent NS3-D168 substitutions (A/E/H/V/Y).

The impact of baseline resistance-associated polymorphisms on SVR12 in the placebo-deferred arm

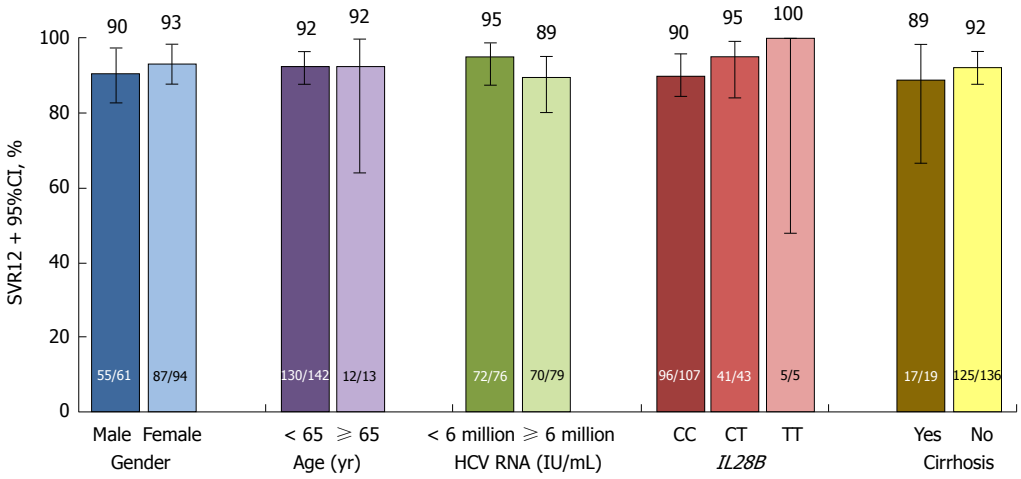
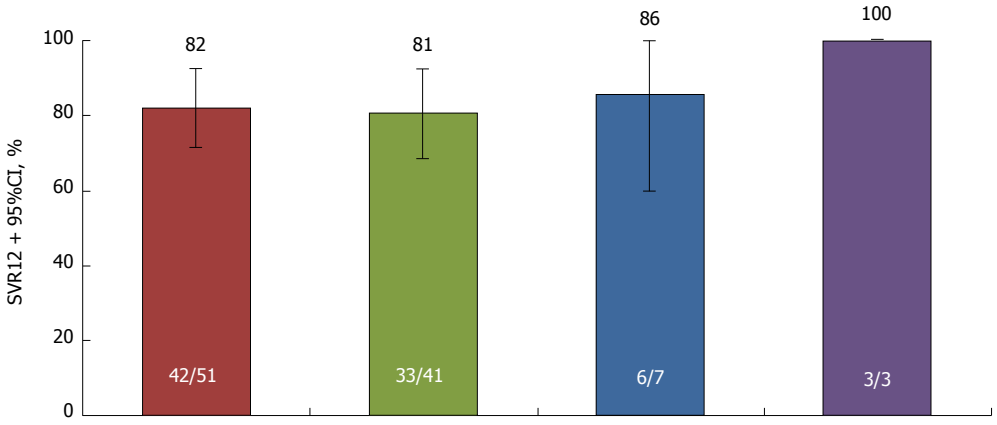


Figure 2 SVR12 according to selected baseline characteristics in the immediate treatment arm. HCV: Hepatitis C virus; SVR12: Sustained virologic response at posttreatment week 12.



	Overall	Mainland China	Russia	South Korea
Virologic breakthrough, [1]*n*	7	7	0	0
Detectable HCV RNA at EOT, *n*	0	0	0	0
Relapse, [2]*n*	1	1	0	0
Other, [3]*n*	1	0	1[4]	0

Figure 3 SVR12 in the placebo-deferred treatment arm. [1]On-treatment HCV RNA ≥ LLOQ after < LLOQ, or increased >1 $\log_{10}$ over nadir; [2]HCV RNA < LLOQ (TND) at EOT followed by HCV RNA ≥ LLOQ at any follow-up visit; [3]Other nonresponders included patients who had HCV RNA < LLOQ (TND) at EOT, but with missing posttreatment week 12 data; [4]Death, not considered related to study therapy (stab wound). EOT: End of treatment; HCV: Hepatitis C virus; LLOQ: Lower limit of quantitation; SVR12, Sustained virologic response at post-treatment week 12.

is shown in Tables 4 and 5.

Safety and tolerability

The safety outcomes observed during the 12-wk double-blind phase are summarized in Table 6. Five (3.2%) patients in the immediate-treatment arm had SAEs considered related [study drug overdose (*n* = 2)] or unrelated to treatment [ventricular extra-systoles (*n* = 1), acute cholecystitis (*n* = 1) and intervertebral disc protrusion (*n* = 1)], and three (5.8%) patients in the placebo-deferred treatment arm had SAEs [ALT elevation (*n* = 1), coronary artery disease (*n* = 1), and hepatitis E virus infection plus liver injury (*n* = 1; leading to study discontinuation)] while receiving placebo. No treatment-related deaths were observed during the study.

The most common AEs (any grade) occurring in > 5% of patients in either arm during the initial 12-weeks of treatment with DUAL (immediate treatment arm) compared with placebo (placebo-deferred arm) were elevated ALT (3.2% *vs* 23.1%), elevated AST (1.3% *vs* 15.4%), hypertension (7.1% *vs* 7.7%), upper respiratory tract infection (6.5% *vs* 5.8%), platelet count decrease (1.9% *vs* 7.7%) and pyrexia (0.6% *vs* 5.8%). The most common grade 3-4 laboratory abnormalities during this period (DUAL *vs* placebo) were related to ALT (0.6% *vs* 9.6%), AST (0.6% *vs* 5.8%), total bilirubin (0.6% *vs* 0%) and hemoglobin (1.9% *vs* 0%).

The safety outcomes observed during 24 wk of

Table 4 SVR12 in hepatitis C virus genotype 1b-infected patients with and without resistance-associated polymorphisms at baseline (placebo-deferred treatment arm) *n* (%)

	All patients - placebo-deferred treatment arm							
	With RAPs at baseline				Without RAPs at baseline			
	Mainland China	Russia	South Korea	Overall	Mainland China	Russia	South Korea	Overall
NS5A-L31M/V	0	0	0	0	33/41 (80.5)	6/6 (100)	3/3 (100)	42/50 (84.0)
Y93H	2/8 (25.0)	0	0	2/8 (25.0)	31/33 (93.9)	6/6 (100)	3/3 (100)	40/42 (95.2)
L31M/V or Y93H	2/8 (25.0)	0	0	2/8 (25.0)	31/33 (93.9)	6/6 (100)	3/3 (100)	40/42 (95.2)
NS3-D168E	0	0	0	0	33/41 (80.5)	6/6 (100)	3/3 (100)	42/50 (84.0)

RAP: Resistance-associated polymorphism; SVR12: Sustained virologic response at posttreatment week 12.

Table 5 SVR12 in cirrhotic and noncirrhotic hepatitis C virus genotype-1b-infected patients with and without resistance-associated polymorphisms at baseline (placebo-deferred treatment arm) *n* (%)

	Patients with cirrhosis - placebo-deferred treatment arm							
	With RAPs at baseline				Without RAPs at baseline			
	Mainland China	Russia	South Korea	Overall	Mainland China	Russia	South Korea	Overall
Patients with cirrhosis								
NS5A-L31M/V	0	0	0	0	3/5 (60.0)	1/1 (100)	1/1 (100)	5/7 (71.4)
Y93H	1/3 (33.3)	0	0	1/3 (33.3)	2/2 (100)	1/1 (100)	1/1 (100)	4/4 (100)
L31M/V or Y93H	1/3 (33.3)	0	0	1/3 (33.3)	2/2 (100)	1/1 (100)	1/1 (100)	4/4 (100)
NS3-D168E	0	0	0	0	3/5 (60.0)	1/1 (100)	1/1 (100)	5/7 (71.4)
Patients without cirrhosis								
NS5A-L31M/V	0	0	0	0	30/36 (83.3)	5/5 (100)	2/2 (100)	37/43 (86.0)
Y93H	1/5 (20.0)	0	0	1/5 (20.0)	29/31 (93.5)	5/5 (100)	2/2 (100)	36/38 (94.7)
L31M/V or Y93H	1/5 (20.0)	0	0	1/5 (20.0)	29/31 (93.5)	5/5 (100)	2/2 (100)	36/38 (94.7)
NS3-D168E	0	0	0	0	30/36 (83.3)	5/5 (100)	2/2 (100)	37/43 (86.0)

RAP: Resistance-associated polymorphism; SVR12: Sustained virologic response at posttreatment week 12.

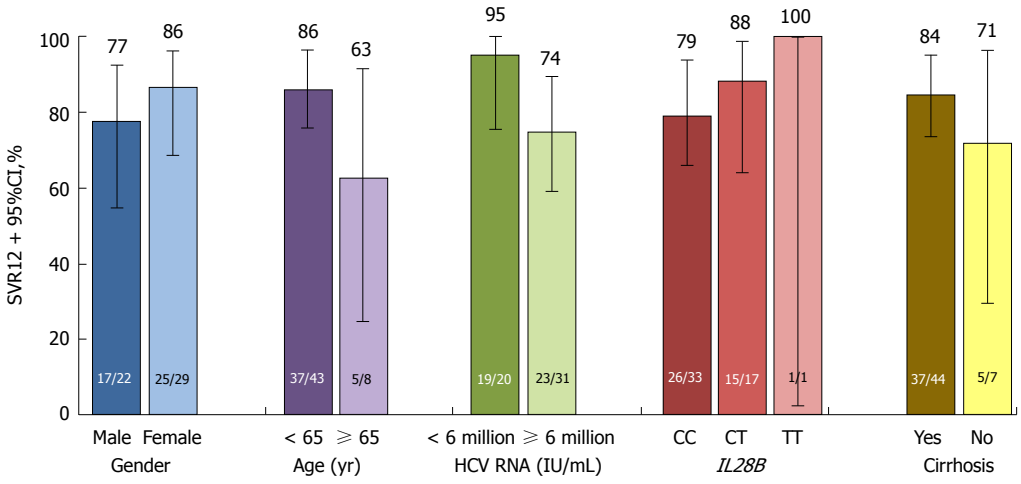


Figure 4 SVR12 according to selected baseline characteristics in the placebo-deferred treatment arm[1]. [1]Reasons for patients not achieving SVR12 included virologic breakthrough (*n* = 7), relapse (*n* = 1) or other (*n* = 1; death, not considered related to study therapy). HCV: Hepatitis C virus; SVR12: Sustained virologic response at post-treatment week 12.

DUAL treatment in either arm are summarized in Table 7. Two (1.3%) patients in the immediate treatment arm had SAEs deemed unrelated to treatment [appendicitis (*n* = 1) and retinal detachment (*n* = 1)] in addition to the five patients with SAEs during the 12-wk double-blind phase. One (2.0%) patient in the placebo-deferred treatment arm (excluding the patient who discontinued during the 12-wk double-blind phase) discontinued due to fatality unrelated to treatment (stab wound). One patient in the immediate treatment arm discontinued after twice meeting the biochemical criteria for Hy's law. On day 118, treatment was interrupted for this

Table 6 Safety during the 12-wk double-blind period *n* (%)

Parameter	Immediate treatment, *n* = 155	Placebo-deferred treatment, *n* = 52
AEs leading to discontinuation	0 (0)	1 (2)[1]
Serious AEs	5 (3)[2]	3 (6)[1,3]
AEs (any grade), ⩾ 5%		
ALT elevation	5 (3)	12 (23)
AST elevation	2 (1)	8 (15)
Hypertension	11 (7)	4 (8)
Upper respiratory tract infection	10 (6)	3 (6)
Platelet count decrease	3 (2)	4 (8)
Pyrexia	1 (1)	3 (6)
On-treatment grade 3-4 laboratory abnormalities		
ALT	1 (1)	5 (10)
AST	1 (1)	3 (6)
Total bilirubin	1 (1)	0 (0)
Hemoglobin	3 (2)	0 (0)

[1]Hepatitis E virus infection and liver injury (*n* = 1); [2]Treatment related: Study drug overdose (*n* = 2); Unrelated to treatment: Ventricular extrasystoles (*n* = 1), acute cholecystitis (*n* = 1) and intervertebral disc protrusion (*n* = 1); [3]ALT elevation (*n* = 1) and coronary artery disease (*n* = 1). AE: Adverse event; ALT: Alanine transaminase; AST: Aspartate transaminase.

Table 7 Safety during 24 wk of daclatasvir plus asunaprevir treatment in either arm *n* (%)

Parameter	Immediate treatment, *n* = 155	Placebo-deferred treatment, *n* = 51[1]	Overall, *n* = 206
AEs leading to discontinuation	1 (1)[2]	1 (2)[3]	2 (1)
Serious AEs	7 (5)[4,5]	1 (2)[3]	8 (4)
Deaths	0 (0)	1 (2)[3]	1 (< 1)
AEs (any grade), ⩾ 5%			
ALT elevation	17 (11)	5 (10)	22 (11)
Upper respiratory tract infection	13(8)	8(16)	21(10)
Hypertension	11 (7)	6 (12)	17 (8)
AST elevation	13 (8)	3 (6)	16 (8)
INR elevation6	11 (7)	2 (4)	13 (6)
Blood bilirubin elevation	12 (8)	0 (0)	12 (6)
Fatigue	5 (3)	6 (12)	11 (5)
On-treatment grade 3-4 laboratory abnormalities			
ALT	7 (5)[2]	2 (4)[7]	9 (4)
AST	5 (3)[2]	1 (2)[7]	6 (3)
Total bilirubin	1 (1)	0 (0)	1 (< 1)
Hemoglobin	3 (2)	0 (0)	3 (1)
Platelets	1 (1)	0 (0)	1 (< 1)
Absolute lymphocyte count	0 (0)	1 (2)	1 (< 1)
Absolute neutrophil count	1 (1)	0 (0)	1 (< 1)
Lipase	3 (2)	0 (0)	3 (1)

[1]Excludes the patient who discontinued during the double-blind phase; [2]Jaundice and nausea, which followed concomitant but reversible treatment-related ALT, AST and total bilirubin elevations (patient met the biochemical criteria for Hy's law; aminotransferases, jaundice and nausea resolved off-treatment and patient achieved SVR12); [3]Fatality (stab wound) unrelated to treatment; [4]Treatment related: Study drug overdose (*n* = 2); [5]Unrelated to treatment: Ventricular extrasystoles (*n* = 1), acute cholecystitis (*n* = 1), intervertebral disc protrusion (*n* = 1), retinal detachment (*n* = 1) and appendicitis (*n* = 1); [6]No grade 3-4 INR laboratory abnormalities were observed; [7]One patient experienced vomiting, decreased appetite and myalgia (all resolved), plus grade 3 ALT and AST abnormalities (both reversible), and interrupted DUAL treatment for 2 d (patient achieved SVR12). AE: Adverse event; ALT: Alanine transaminase; AST: Aspartate transaminase; INR: International normalized ratio.

patient until day 124 due to grade 3 ALT (320 U/L) and AST (237 U/L), grade 2 bilirubin (36.3 μmol/L), and grade 1 alkaline phosphatase (201 U/L). By day 133, the patient's AST level had improved to 195 U/L (grade 3), but levels of ALT (223 U/L) and blood bilirubin (37.6 μmol/L) remained elevated. On day 141, the patient's blood bilirubin and ALT levels had improved to 32.5 μmol/L (grade 2) and 155 U/L (grade 2), respectively; however, he was diagnosed with grade 2 AST (152 U/L) and grade 2 AEs of jaundice and nausea. Given this patient's already elevated levels of ALT, AST and alkaline phosphatase, he met the biochemical criteria for Hy's law for a second time and discontinued treatment the next day. All events resolved by day 152 and the patient achieved SVR12.

The most common AEs (any grade) occurring in > 5% of patients during 24 wk of treatment with DUAL in either treatment arm were elevated ALT (11%), upper respiratory tract infection (10%), hypertension (8%), elevated AST (8%), elevated international normalized

ratio (6%), elevated blood bilirubin (6%) and fatigue (5%). The most common grade 3-4 laboratory abnormalities were related to ALT (4%), AST (3%), hemoglobin (1%) or lipase (1%) (Table 7).

DISCUSSION

In this study, SVR12 was achieved by 91.6% of patients with HCV genotype 1b infection who were randomly assigned to receive immediate treatment with DUAL. With the lower bound of the corresponding 95%CI (87.2%) greater than the prespecified 70% threshold, the primary endpoint was met, confirming that DUAL is more efficacious than peg-IFN plus ribavirin in patients with HCV genotype 1b infection.

SVR12 was comparable between patients from mainland China (92.4%) and Russia (95.7%). By contrast, SVR12 was lower among patients from South Korea (76.9%); however, this was a small cohort and two of the three patients experiencing virologic failure had the NS5A-Y93H polymorphism at baseline, which has been shown to reduce SVR in patients with HCV genotype 1b infection receiving DUAL[18,22,23]. SVR12 was also lower among patients in the placebo-deferred arm following treatment with DUAL (42/51, 82.4%); however, again this was a small cohort and six of the eight patients with virologic failure had the NS5A-Y93H polymorphism at baseline. Nonetheless, consistent with the results of other phase 3 studies, SVR12 was high overall and largely unaffected by characteristics known to attenuate response to IFN, namely cirrhosis, *IL28B* non-CC genotypes, male sex, advanced age, and high baseline HCV RNA[10-13]. Virologic failure in the immediate treatment arm tended to coincide with the presence of baseline NS5A polymorphisms at L31M or Y93H, consistent with previous observations[18]. Although the prevalence of NS5A-L31 or NS5A-Y93H was relatively low in this study (11.0%), the observed SVR12 rates were, consistent with previous reports, higher among patients without these baseline polymorphisms (132/137, 96.4%), including those with cirrhosis (17/19, 89.5%), compared with cirrhotic patients with these baseline polymorphisms (9/17, 52.9%).

During the 12-wk double-blind phase, SAEs and AEs leading to discontinuation were infrequently observed in the immediate (5/155, 3.2% and none) and placebo-deferred (3/52, 5.8% and 1/52, 1.9%) treatment arms. However, although the AE profiles were broadly comparable between the two arms, elevations of ALT and AST were more common among patients receiving placebo (12/52, 23.1% and 8/52, 15.4%) compared with those receiving DUAL (5/155, 3.2% and 2/155, 1.3%). Consistent with this, grade 3-4 ALT and AST laboratory abnormalities during the blinded phase were more common among patients receiving placebo compared with those receiving DUAL. These elevations most likely reflected ongoing inflammation from untreated HCV infection; indeed, ALT and AST levels in most of these patients had begun to decrease by week 2 of open-label treatment with DUAL. One patient in the immediate treatment arm met the criteria for Hy's law during treatment with DUAL; however, following treatment discontinuation, the events resolved and the patient achieved SVR12.

DUAL was well tolerated during 24 wk of treatment in both arms, consistent with findings from other phase 3 studies[10-12,19]. SAEs (8/206, 3.9%) and AEs leading to discontinuation (2/206, 1.0%) were infrequently observed and, except for two cases of study drug overdose, no SAEs were deemed treatment related. Emergent grade 3-4 laboratory abnormalities were similarly uncommon. The most common grade 3-4 laboratory abnormalities were related to ALT (9/206, 4.4%) and AST (6/206, 2.9%), however these reversed rapidly (median reversal times: 11.0 and 8.5 d for ALT and AST abnormalities, respectively) during or after treatment, and their incidences were comparable with those observed in other studies[10,24-26].

A limitation of this study was the absence of a direct IFN-based comparator for the primary efficacy endpoint. However, despite the continuing importance of IFN-based treatment across much of Asia, it was felt that including an IFN-based treatment arm in the study design would have been unethical. Peg-IFN is associated with a high burden of systemic AEs that include "flu-like" symptoms, neutropenia and thrombocytopenia[27], while ribavirin is associated with hemolytic anemia, birth defects, nausea, rash, itching, coughing and hyperuricemia[28,29]. The result is a combination with poor treatment adherence and a high rate of study discontinuations due to AEs[30]. Comparing DUAL, an all-oral combination with superior efficacy and safety profiles, to peg-IFN plus ribavirin, a combination containing an injectable drug with inferior efficacy and safety profiles, would therefore have lacked clinical equipoise. We also acknowledge that some patients were denied access to DUAL for 12 wk during the double-blind phase; however, as liver disease progresses slowly in patients with HCV infection, we do not believe that giving placebo instead of active treatment for 12 wk in compensated, treatment-naïve patients posed any ethical concerns.

In conclusion, the findings of this study showed that the all-oral DUAL combination of daclatasvir plus asunaprevir was highly effective and well tolerated in treatment-naïve patients from mainland China, Russia and South Korea with HCV genotype 1b infection. For patients in China, where IFN-based combinations have been considered the standard of care for HCV infection, DUAL was the first all-oral, nonribavirin-containing combination to gain approval, providing patients with access to a more efficacious and tolerable alternative for the treatment of HCV genotype 1b infection, with an easier route of administration and shorter treatment duration. DUAL is also predicted to be a cost-effective treatment alternative for HCV genotype 1b in China[31]. In addition, in countries such as Japan, where all-

oral regimens are considered the standard of care for the treatment of HCV genotype 1b infection, DUAL is expected to be cost-saving compared with sofosbuvir/ledipasvir, with similar health outcomes[32].

ARTICLE HIGHLIGHTS

Research background

Chronic hepatitis C virus (HCV) infection is a significant health burden across Asia, and affects 5-7 million people in China alone. Without effective treatment, patients can develop severe complications, such as cirrhosis or hepatocellular carcinoma. Previous therapies for the treatment of chronic HCV infection have been based on a combination of peg-interferon and ribavirin, both of which are associated with a high burden of adverse events (AEs) that contribute to poor treatment adherence and high rates of treatment discontinuations.

Research motivation

Daclatasvir plus asunaprevir (DUAL) is an all-oral combination of daclatasvir, an HCV NS5A inhibitor, and asunaprevir, an NS3 protease inhibitor. This regimen has previously demonstrated efficacy in several phase 3 studies of patients infected with HCV genotype 1b, including those characteristics known to attenuate response to interferon-based therapies. In this study, we sought to evaluate the efficacy and safety of DUAL in treatment-naïve patients from mainland China, South Korea and Russia.

Research objectives

The primary efficacy objective of the study was to measure the rate of sustained virologic response at posttreatment week 12 (SVR12) and to determine if this rate was significantly higher than the historical rate of 70% associated with peg-interferon plus ribavirin. Safety was monitored based on incidence of AEs and abnormalities in clinical laboratory assessments, vital signs and physical examinations.

Research methods

This was a phase 3, double-blind, placebo-controlled study of DUAL in treatment-naïve patients from mainland China, South Korea and Russia with chronic HCV genotype 1b infection. Patients were randomly assigned (3:1) to receive DUAL (daclatasvir 60 mg tablet once daily and asunaprevir 100 mg soft capsule twice daily) for 24 wk either immediately (immediate treatment arm) or after 12 wk of matching placebo (placebo-deferred treatment arm).

Research results

An SVR12 rate of 91.6% (95% confidence interval: 87.2-96.0) was observed among patients in the immediate treatment arm, which was significantly higher than the historical comparator rate (70%). SVR12 was largely unaffected by cirrhosis (89%), age $\geqslant$ 65 years (92%), male sex (90%), baseline HCV RNA $\geqslant$ 6 million (89%), or *IL28B* non-CC genotypes (96%), although SVR12 was higher among patients without (96%) than among those with (53%) baseline NS5A resistance-associated polymorphisms (at L31 or Y93H). DUAL was well tolerated during 24 wk of therapy in this study; the most common AEs ($\geqslant$ 10% in the combined arms) were elevated alanine aminotransferase and upper respiratory tract infection. Two patients discontinued DUAL treatment; one due to aminotransferase elevations, nausea and jaundice and the other due to a fatality unrelated to treatment. There were no treatment-related deaths.

Research conclusions

This study demonstrates that the all-oral DUAL combination of daclatasvir plus asunaprevir was highly effective and well tolerated in treatment-naïve patients with HCV genotype 1b infection from mainland China, Russia and South Korea.

Research perspectives

These findings suggest that for patients in many Asian countries, such as China, where interferon-based combinations have been considered the standard of care for HCV infection, DUAL offers a more efficacious and tolerable alternative for the treatment of HCV genotype 1b infection, with an easier route of administration and shorter treatment duration.

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