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2. VEMDLV prescribing information, Gilead Korea, May 2017.
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Reviews

1 Immunologic strategies and outcomes in ABO-incompatible living donor liver transplantation
Jongwook Oh and Jong Man Kim

7 The fibrogenic process and the unleashing of acute-on-chronic liver failure
Guillermo Nahúm López-Sánchez, Mayra Dóminguez-Pérez, Misael Uribe, and Natalia Nuño-Lámbarri

16 Extrahepatic manifestations of hepatitis E virus: An overview
Fotios S. Fousekis, Ioannis V. Mitselos, and Dimitrios K. Christodoulou

Original Articles

24 A survey on transarterial chemoembolization refractoriness and a real-world treatment pattern for hepatocellular carcinoma in Korea
Jae Seung Lee, Beom Kyung Kim, Seung Up Kim, Jun Yong Park, Sang Hoon Ahn, Jin Sil Seong, Kwang-Hyub Han, and Do Young Kim

33 Serum Wisteria floribunda agglutinin-positive human Mac-2 binding protein level predicts recurrence of hepatitis B virus-related hepatocellular carcinoma after curative resection
Hye Soo Kim, Seung Up Kim, Beom Kyung Kim, Jun Yong Park, Do Young Kim, Sang Hoon Ahn, Kwang-Hyub Han, Young Nyun Park, Dai Hoon Han, Kyung Sik Kim, Jin Sub Choi, Gi Hong Choi, and Hyon-Suk Kim

45 Vimentin as a potential therapeutic target in sorafenib resistant HepG2, a HCC model cell line
Ankita Makol, Harpreet Kaur, Sakshi Sharma, Shruthi Kanthaje, Ramanpreet Kaur, and Anuradha Chakraborti

54 Sensitivity of ultrasound in detecting hepatocellular carcinoma in obese patients compared to explant pathology as the gold standard
Jamak Modaresi Esfeh, Kaveh Hajifathalian, and Kianoush Ansari-Gilani

60 Influence of some methylated hepatocarcinogenesis-related genes on the response to antiviral therapy and development of fibrosis in chronic hepatitis C patients
Waleed Seif Eldin Mohamed Mostafa, Mohammed Hassan Saiem Al-Dahr, Dalia Abdel Hamid Omran, Zeinab Fathy Abdullah, Suzan Hamdy Elmasry, and Mohamed Nabil Ibrahim
Letters to the Editor

70  The management of polycystic liver disease by tolvaptan
Tsuneo Takenaka, Soichiro Miura, and Masaki Kitajima

74  Hyperammonemic encephalopathy: An unusual presentation of fibrolamellar hepatocellular carcinoma
Nimish Thakral and Douglas A. Simonetto

78  Clinical characteristics of portal hypertension complicated by gastroesophageal varices in patients with myeloproliferative neoplasms
Jaejun Lee, Pil Soo Sung, Ki-Seong Eom, Hyun Yang, Soon Kyu Lee, Aung Hlaing Bwa, Angelo Lozada, Jeong Won Jang, Si Hyun Bae, Jong Young Choi, and Seung Kew Yoon
Immunologic strategies and outcomes in ABO-incompatible living donor liver transplantation

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Antibody mediated rejection (AMR) after adult ABO-incompatible living donor liver transplantation (ABO-I LDLT) induced hepatic necrosis or diffuse intrahepatic biliary complications, which were related with poor graft and patient survival. Various desensitization protocols have been used to overcome these problems. Since using rituximab, the outcomes of ABO-I LDLT show a similar survival rate to those of ABO-compatible living donor liver transplantation. However, diffuse bile duct complications still occur after ABO-I LDLT. We have reviewed the past and current immune strategies for desensitization and to provide outcomes and ABO incompatibility-related complications in ABO-I LDLT. (Clin Mol Hepatol 2020;26:1-6)

Keywords: Immunosuppression; Rejection; Graft survival; Complications

INTRODUCTION

In the past, adult ABO incompatible living donor liver transplantation (ABO-I LDLT) had poor graft survival and low patient survival due to hyperacute rejection and a high risk of vascular biliary complication, and it was considered a contraindication. Susceptibility to rejection, including severe hepatic necrosis and diffuse intravascular coagulation disorder within the graft, appears to be due to the blood group antigen expressed in the vascular endothelium and bile ducts after transplantation. Various desensitization strategies have been introduced to overcome the barrier of ABO incompatibility. However, desensitization protocols differ at each center, and the necessity of local infusion, splenectomy, intravenous immunoglobulin (IVIG), and plasmapheresis is controversial. After the rituximab era, the outcome of ABO-I LDLT has been reported in many studies to be comparable to ABO compatible living donor liver transplantation (ABO-C LDLT). Many centers are now trying to simplify protocols. We review the past and current immune strategies for desensitization and to provide outcomes and ABO incompatibility-related complications in ABO-I LDLT.

HISTORY

Thomas Starzl introduced liver transplantation (LT) for the ABO blood group in 1969. In addition, Thomas Starzl proposed the “liver is privileged organ” concept since the liver in contrast to
heart or kidney transplantations, resists acute rejection in animal experimental studies. Therefore, Starzl’s group only reported 11 ABO-I pediatric LT cases in 1979 because of the difficulty of finding compatible small grafts. However, they had shown no evidence of acute rejection in those patients.

In the 1980s and in the early 1990s, ABO incompatible liver transplantation (ABO-I LT) had extremely poor surgical outcomes; severe rejection, hepatic artery thrombosis and intrahepatic bile duct injury were common. Demetris et al. reported a pathological feature, ‘single organ disseminated intravascular coagulation (DIC)’ in a failed ABO-I liver graft. In a control matched study that included 15 ABO-I LT, Sanchez-Urdazpal et al. confirmed increased incidence of cholangitis, bile leak, cellular rejection, and hepatic artery thrombosis in the ABO-I group. To overcome ABO-incompatibility complications, high dose immunosuppression, splenectomy and plasmapheresis were implemented, but these had little effect on the poor outcomes and increased the occurrence of complications, including infection and sepsis.

DESENSITIZATION MANAGEMENT

Plasmapheresis or total plasma exchange

Anti-ABO antibodies are thought to cause antibody mediated rejection (AMR) in ABO-I LT. It has been reported that hepatic necrosis and intrahepatic biliary complications in ABO-I LDLT are closely related to high perioperative anti-A or anti-B antibody titers. Plasmapheresis has been reported as rapidly reducing anti-blood type isoagglutinin titers for ABO-I LDLT. Therefore, plasmapheresis has been applied prior to LT in order to reduce anti-blood antibodies to levels considered safe enough to improve the outcomes of ABO-I LDLT. However, it has also been reported that even if antibody titers are reduced by plasmapheresis prior to ABO-I LDLT, isoagglutinin titers can rise again within 3–7 days after operation. Although plasmapheresis is able to remove antibodies from the peripheral blood prior to ABO-I LT, plasmapheresis cannot suppress the production of new antibodies from the preexisting plasma cells. For this reason, repetitive plasmapheresis has been considered an efficient therapeutic method in patients with a rise in isoagglutinin titers after ABO-I LDLT. It has been previously reported that the target of pre-transplant antibody ABO-titer values following plasmapheresis were less than 1:8, 1:16, 1:32 or 1:64 to prevent posttransplant AMR. The target titer differs markedly by center and a standard target titer has not yet been established.

Splenectomy

Splenectomy has been an important part of the protocol for ABO-I LDLT at many centers because the spleen is the body’s major antibody producing organ, and contains large amounts of B cells and plasma cells. It also fulfils particular functions in blood filtration, phagocytosis, erythrocyte destruction, antigen uptake and potential hemopoiesis. Splenectomy in ABO-I LT carries risks for severe post-transplant infection and portal vein thrombosis, pancreatic fistula, and sepsis. In addition, splenectomy is time-consuming and can cause massive bleeding as a result of splenomegaly in patients with severe liver cirrhosis. However, several studies have reported that splenectomy had not decreased the incidence of AMR. Raut et al. had found no statistically significant difference in anti-ABO immunoglobulin M (IgM) and immunoglobulin G (IgG) antibody titers between splenectomy and non-splenectomy groups. Therefore, the omission of splenectomy in ABO-I LDLT has recently emerged with the prophylactic use of rituximab.

IVIG

The mechanism of action of IVIG is complex and not completely understood. It has been proposed to include the blocking of Fc receptors to non-nuclear cells, direct antibody neutralization, suppression of CD19 presentation to activated B cells, suppression of complements, and suppression of all porous T cells. Several previous studies have reported on the effectiveness of additional IVIG for preventing AMR. Kim et al. had reported that the combination of rituximab, plasmapheresis, and IVIG had excellent results. However, a Japanese study had reported that AMR incidence does not significantly differ between desensitization regimens with or without IVIG.

Local graft infusion therapy

Local infusion therapy was reported in 1998, and involved methylprednisolone, prostaglandin E1, and gabexate mesilate administered via a catheter through the portal vein. This regimen increased survival from 22% to 60%. The theoretical basis of these local infusion agents is that they inhibit different key reactions in single-organ disseminated intravascular coagulation triggered by preformed antibodies against the donor antigen. Prosta-
Rituximab

Rituximab is a monoclonal chimeric human-murine anti-CD20 antibody that depletes B cells by complement-dependent cellular cytotoxicity. It depletes CD20-positive B cells from circulation and lymphoid tissues including the spleen. Thus, rituximab acts as a form of chemical splenectomy. Several previous studies have shown that rituximab’s effect on B cells in peripheral blood lasts for several months, removing cells within 48–72 hours. Moreover, most data show that a single dose of rituximab is sufficient for suppressing B cells in the peripheral blood. The number of B cells in peripheral blood decreased in three days after a single dose of rituximab (375 mg/m²), and the number of cells in the peripheral blood were completely eliminated after more than three weeks. Regular and multiple rituximab doses increased the incidence of fungi and cytomegalovirus infections. Therefore, repeated administration of rituximab may be unnecessary and may increase the risk of serious infection due to long-term hypoglycemia. Currently, most centers administer a single dose of rituximab (300 or 375 mg/m²), two weeks before surgery.

When the outcomes of ABO-I LDLT are divided into before and after the rituximab era, a Japanese nationwide survey revealed that the 3-year survival rates increased from 30% to 80% after the introduction of rituximab. In a study that included 381 adult patients in the Japanese registry of ABO-I LDLT, only the absence of rituximab prophylaxis was a significant risk factor for AMR. Thus, rituximab prophylaxis significantly decreased the incidence of AMR. In that study, the incidence of AMR decreased from 23.5% to 6.2% after the introduction of rituximab. Since the rituximab era, many centers are now trying to simplify the protocol, and avoid splenectomy, local graft infusion, IVIG, and plasmapheresis.

Desensitization trials without plasmapheresis

The role of preoperative ABO antibody titer in rejection of ABO-I LDLT has not yet been established. Several studies have shown no significant correlation between ABO antibody titer and AMR, indicating that high preoperative antibody values have no significant effect on AMR frequency. Another study suggested that sufficient desensitization could be achieved using rituximab alone. In addition, another study included a simplified protocol using rituximab and IVIG without plasmapheresis for ABO-I LDLT. The study reported that the protocol was safe and effective in achieving sufficient desensitization and showed comparable outcomes in patients with titers no higher than 1:64. The incidence of diffuse intrahepatic biliary complications (DIHC) was 3–5%. Considering intraoperative blood loss was much during ABO-I LDLT and very low incidence of DIHC, desensitization protocol without preoperative plasmapheresis required more search for validation.

OUTCOMES

Hyperacute rejection has not been reported in most studies since the use of rituximab. Kim et al. reported 100% patient and graft survivals and no AMR in 22 ABO-I LDLT patients with titers adjusted below 1:32 by total plasma exchange. They reported five biliary complication cases. Song et al. also reported that patient survival, graft survival, and biopsy proven acute rejection were not significantly different between ABO-I LDLT and ABO-C LDLT. They showed that diffuse intrahepatic bile duct complications were observed in 12 cases in the ABO-I LDLT group. A recent study showed that 47 ABO-I LDLT patients who were compared to a 1:2 matched 94 ABO-C LDLT group did not show significant differences in survival and acute rejection, as well as biliary complications. However, three DIHC cases occurred in the ABO-I LDLT patients and progressed to graft failure.

It is not known whether rituximab prophylaxis for desensitization affects hepatocellular carcinoma (HCC) recurrence in ABO-I
LDLT. Kim et al.\textsuperscript{33} reported that ABO incompatibility was not associated with HCC recurrence. The 1-, 2-, and 3-year disease-free survival rates in ABO-I LDLT and ABO-C LDLT groups were 90.3%, 79.7%, and 73.3% and 86.7%, 79.0%, and 75.3%, respectively ($P=0.96$).\textsuperscript{33} The overall patient survival rates for the same period in the ABO-I LDLT and ABO-C LDLT groups were 90.6%, 85.0%, and 81.9% and 88.0%, 83.5%, and 82.5%, respectively ($P=0.77$).\textsuperscript{33} They had shown thatAFP, tumor size, encapsulation and microcirculation invasion were associated with HCC recurrence except in ABO-incompatibility. Propensity score match study had shown comparable recurrence-free survival rates and overall patient-survival outcomes between ABO-I LDLT and ABO-C LDLT groups.\textsuperscript{34}

**COMPLICATIONS**

Egawa et al. reported two types of graft failure in ABO-I LDLT.\textsuperscript{6} The first, ‘liver necrosis’ occurred acutely 1–2 weeks after transplantation, leading to massive graft necrosis within a month. The second, ‘intrahepatic bile duct injury’ presented more slowly 2–3 months after transplantation, with development of extensive irregularities of the intrahepatic bile duct, resulting in graft failure. These reactions were not observed in children <1 year of age, whose ability to produce antibodies against blood group antigens had not yet been established. The 5-year survival rate of recipients younger than 1 year (infants) and 16 years or older (adults) was reported as 76% and 22%, respectively. Because of poor survival outcomes and high incidence of complications, ABO-I LDLT became unpopular and was reserved for emergency transplant surgery only.\textsuperscript{35,36}

Diffuse intrahepatic bile duct complications were significantly higher in ABO-I LDLT than in ABO-C LDLT. Because the targets of isoagglutinin are the bile duct’s epithelium and vascular endothelium of the graft, microvascular thrombotic occlusion of graft bile duct can occur, which causes ischemic cholangiopathy. Although the fulminant hepatic necrosis caused by severe AMR in ABO-I LDLT has been overcome since the introduction of rituximab, the risk of attenuated AMR still remains.\textsuperscript{11} Attenuated AMR can cause DIHC. Unlike fulminant necrosis, DIHC is not always fatal. However, DIHC eventually leads to refractory cholangitis, which leads to sepsis and graft failure. In most cases, DIHC cannot be treated by conventional biliary interventions. The only proven effective treatment is re-transplantation. In addition, DIHC degrades the quality of patient life due to frequent recurrent episodes of cholangitis and the need for intervention and readmission.\textsuperscript{11}

**CONCLUSION**

In conclusion, ABO-I LDLT is a very effective and safe method for extending a raw pool of liver donors. Survival outcomes are now comparable with rituximab prophylaxis and plasmapheresis. However, there is still concern about the high incidence of biliary complication especially DIHC, an intractable form of biliary stenosis that can occur regardless of the isoagglutinin titer. Therefore, we need to closely follow the patient course over several months after ABO-I LDLT even in patients with very low isoagglutinin titers after ABO-I LDLT. In the future, we need to identify certain risks and precautions through studies involving immunology and adaptive mechanisms in ABO-I LDLT.

**Authors’ contributions**

All authors wrote and approved the final version.

**Conflicts of Interest**

The authors of this manuscript have no conflicts of interest to disclose.

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The fibrogenic process and the unleashing of acute-on-chronic liver failure

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Acute-on-chronic liver failure (ACLF) is a life-threatening condition characterized by a rapid deterioration of previously well-compensated chronic liver diseases. One of the main obstacles in ACLF is the lack of knowledge of the pathogenesis and specific broad-spectrum treatments. An excessive systemic inflammatory response has been proposed to explain the pathogenesis of ACLF; this hypothesis involves stellate cells, which are implicated in many liver homeostatic functions that include vitamin A storage, regulation of sinusoidal blood flow, local inflammation, maintenance of the hepatocyte phenotype and extracellular matrix remodeling. However, when there is damage to the liver, these cells are the main target of the inflammatory stimulus, as a result, the secretion of the extracellular matrix is altered. Activated hepatic stellate cells raise the survival of neutrophils by the stimulation of granulocytes colonies and macrophages, which exacerbates liver inflammation and promotes damage to hepatocytes. Elevation of pathogen-associated molecular patterns is related to liver damage by different pathophysiological mechanisms of decompensation, showing ballooning degeneration and cell death with a predominance of cholestatic infection. Moreover, patients with ACLF present a marked elevation of C-reactive protein together with an elevation of the leukocyte count. Chronic liver disease is a complex pathological state with a heterogeneous pathophysiology in which genetic factors of the host and external triggers interact and culminate in hepatic insufficiency. The better understanding of such interactions should lead to a better comprehension of the disease and to the discovery of new treatment targets that will make acute decompensations preventable and even decrease mortality. (Clin Mol Hepatol 2020;26:7-15)

Keywords: Acute-on-chronic liver failure; Hepatitis B, Chronic; Liver cirrhosis; Immunologic factors

INTRODUCTION

Cirrhosis is a progressive chronic liver disease characterized by diffuse fibrosis, severe interruption of intrahepatic venous flow, portal hypertension and hepatic insufficiency. Epidemiological studies indicate the existence of an increase in the prevalence of liver cirrhosis worldwide.1 The natural evolution of cirrhosis is divided into two stages; a compensated cirrhosis, which is defined as the period between the onset of cirrhosis and the appearance of the first major complication of the disease and the decompensated cirrhosis, which defines the period following the development of ascites, gastrointestinal hemorrhage due to rupture of...
esophageal varices and hepatic encephalopathy.

Recent medical studies and the introduction of new and effective treatments of some etiological forms of cirrhosis, especially antivirals, have brought about important changes in our conception of the disease. First, cirrhosis is no longer considered an irreversible progressive disease. In fact, decompensated cirrhosis can again be compensated cirrhosis or even return to pre-cirrhotic stages if the cause of cirrhosis is eliminated. Second, the initial list of organismic or systemic insufficiencies (liver, kidneys and brain) has been expanded and now includes the immune system, the intestine, the heart, the lungs, the adrenal glands, the muscles and the thyroid gland. Third, new mechanisms have been recognized that are implicated in the pathogenesis of cirrhotic complications, such as dysbacteriosis of the microbiota and systemic inflammation. Finally, it is increasingly clear that patients rarely die as a result of irreversible and terminal destruction of the liver. On the contrary, the cause of death in most patients is an acute deterioration of their clinical situation, favored by a triggering event, a syndrome called acute-on-chronic liver failure (ACLF). In critically ill patients, early diagnosis of ACLF could be important for therapeutic decisions.

**FIBROGENIC PROCESS**

Liver fibrosis is a healing process after hepatic injury with a dynamic and potentially reversible process, involving complex molecular and cellular mechanisms that lead to chronic activation of tissue remodeling and repair, however, if the damage persists, this process could advance to cirrhosis even to liver cancer.

Activated hepatic stellate cells are the main cell type responsible for liver fibrogenesis because they express, synthesize and secrete a broad spectrum of extracellular matrix proteins (collagen, glycoproteins and proteoglycans), during chronic liver injury. Additionally, these cells produce pro-fibrogenic and proinflammatory cytokines, chemokines and growth factors, which are vital in the onset of fibrogenesis.

Moreover, activated hepatic stellate cells increase neutrophils survival by the production of granulocyte-macrophage-colony-stimulating factor and interleukin (IL) 15 that exacerbate liver inflammation and increases reactive oxygen species, which are generated by neutrophils that promote hepatocytes damage. Apart from the possible effects on hepatocyte function, the increase in fibrosis significantly modifies the mechanics of blood flow in the liver leading to the formation of collateral vessels and arterial vasodilation progress, which eventually generates esophageal varices and ascites. A high fructose and cholesterol diet activates early pro-fibrotic pathways before the development of proinflammatory signaling or insulin resistance. In contrast, activation of natural killer cells inhibits hepatic fibrosis through the generation of interferon-γ that induces apoptosis and cell-cycle arrest of activated hepatic stellate cells.

Human bone marrow-derived mesenchymal stem cells inhibit the proliferation of activated hepatic stellate cells by the induction of apoptosis through two proteins, antiapoptotic Bcl-2, and pro-apoptotic Bax, which are key proteins to the mitochondria-mediated intrinsic apoptosis. Furthermore, the generation of reactive oxygen species is intimately related to the hepatic fibrogenic process, where nicotinamide adenine dinucleotide phosphate (NADPH) functions as one of the main sources. It is considered that p47phox, an active functional component of the NADPH complex, plays a crucial role in its activity. The mesenchymal stem cells derived from the human bone marrow can restrict the activation of this oxidase by phosphorylation of p47phox, thus inhibiting its signaling pathway, which promotes peroxisome proliferatoractivated receptor γ and inhibits the expressions of collagen α1 and α-smooth muscle actin.

Cirrhosis represents the final stage of the fibrogenic process, with a transition from an asymptomatic compensated stage, which is between cirrhosis appearance and the initial major complication to symptomatic decompensated stage. Patients with compensated cirrhosis are characterized by having minor symptoms or not having any at all, however, if the etiological factor persists, hepatic damage and portal pressure could worsen. The second phase is decompensated cirrhosis, where variceal hemorrhage, jaundice, ascites and hepatic encephalopathy may occur, all of which are associated with poor short-term survival. Once liver cirrhosis is present, there are several processes get underway like monocytes and lymphocytes interaction between the highly specialized sinusoidal endothelial cells, activation of the inflamasome driven by signaling by toll-like receptor (TLR) or interleukin-1 receptor (IL-1R), which leads to expression of inflamasome components such as NOD-like receptors, pro-caspase-1, pro-interleukin-1β, and pro-IL18, as well as nitric oxide elevation.

**DIFFERENCE OF ACUTE-ON-CHRONIC LIVER FAILURE ACORDING TO GEOGRAPHICAL AREA**

In cirrhosis, there is a term called acute decompensation, which
is characterized by severe hepatic disease development, being the primary cause of hospitalization with a high risk of death in the short-term. Commonly these patients are usually diagnosed with ACLF.\[14\]

There are different definitions of ACLF. The first one was described in 2002 by Jalan and Williams to explain how compensated cirrhotic patients show a severe deterioration in hepatic function during 2 to 4 weeks, associated with a precipitating event which leads to a severe clinical worsening with hepatic encephalopathy, jaundice, bacterial infection, ascites, gastrointestinal hemorrhage and/or hepatorenal syndrome.\[3\] There are disparities between the different committees that study the liver, according to the Asian Pacific Association for the Study of the Liver, ACLF is an acute hepatic damage that is manifested as coagulopathy and jaundice which gets complicated over a four-week period by ascites and/or encephalopathy in a patient with a chronic liver disease or cirrhosis and is associated with high mortality; though, bacterial infections are not considered hepatic insults. Patients with cirrhosis and known prior decompensation (jaundice, encephalopathy or ascites) who develop acute deterioration in clinical status —related or unrelated to precipitating events— have acute decompensation, but not ACLF.\[15,16\]

However, the North American Consortium for the Study of End-Stage Liver Disease, states that patients with decompensated cirrhosis and bacterial infections that develop two organ failures: hepatic encephalopathy grades III-IV, septic shock, the need for mechanical ventilation or renal replacement therapy, present ACLF.\[3,17\]

The European Association for the Study of the Liver (EASL), Chronic Liver Failure Consortium decided to apply a more pragmatic approach to define ACLF. According to the results of the EASL-chronic liver failure (CLIF) Acute-on-Chronic Liver Failure in Cirrhosis (CANONIC) study, ACLF is defined as a clinical syndrome characterized by acute decompensated cirrhosis that leads to multiple organ failure and a high mortality rate in the short term (mortality rate within 28 days $\geq 15\%$). In that study, the multiple organ failure was evaluated according to the sequential organ failure assessment score, which is widely used in critically ill patients but with certain modifications to accommodate the clinical setting of patients with cirrhosis which was called CLIF-sequential organ failure assessment score, with a later simplified version

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This table was made by several articles as follows:
Definition: (APASL) Sarin et al.\[16\], (AASLD) Bajaj et al.\[17\]; Duration between insult and ACLF: Bajaj\[15\]; Duration in which there is higher mortality: Bajaj\[17\]; Diagnostic criteria: (APASL) Sarin et al.\[16\], (AASLD) Arroyo et al.\[18\]; What qualifies as precipitants: Bajaj.\[19\]; Predisposition: (APASL) Sarin et al.\[16\], (AASLD) Arroyo et al.\[18\].

APASL, the Asian Pacific Association for the Study of the Liver; AASLD, American Association for the Study of Liver Diseases; EASL, the European Association for the Study of the Liver; CLD, chronic liver disease; ACLF, acute-on-chronic liver failure; TBil, total Bilirubin; INR, International Normalized Ratio; PTA, platelets.
TRIGGERING FACTORS AND PATHOGENIC MECHANISMS IN ACLF

Commonly, patients with acute decompensation or ACLF show an elevation in cell death markers, which increase with clinical severity compared with healthy patients or with stable cirrhosis. Especially from the pathophysiological point of view, since there is a high correlation with the activation of macrophages, the markers of inflammation and the oxidative stress; indicating that cell death is an important feature in ACLF.\textsuperscript{23} Furthermore, there is a difference between the mechanism and the severity according to the etiology from which they are derived.\textsuperscript{23}

The process of decompensation itself can induce organ tolerance possibly because a previous hepatic lesion could produce cellular senescence\textsuperscript{25} which makes the hepatocytes more resistant to apoptosis.\textsuperscript{25} Patients who previously had not suffered from a decompensating event showed to have higher cell death markers and high mortality through mechanisms that have not been described yet.\textsuperscript{14,23}

ACLF triggers can be categorized into two groups: those that directly impact the liver and those that affect it secondarily to a non-hepatic lesion. In the first group, viral hepatitis, drugs and other hepatotoxic substances such as alcohol stand out. In the second group, variceal hemorrhage and bacterial infections show up as the main factors.

Cytoskeleton of hepatocytes and biliary tract cells is formed by cytokeratin 18 (CK18) filaments; therefore after apoptosis or necrosis CK18 is released to the bloodstream establishing as a cell death indicator that can be quantified by enzyme-linked immunosorbent assays, called M65. In apoptotic cells, the CK18 filaments are cleaved by caspases into a small polypeptide M30 that can be detected in serum by specific antibodies, which predict disease severity and survival of patients with ACLF. The rise of M30 and M65 were associated with other liver diseases severity such as cholestasis and chronic HBV infection. This small increase in polypeptides is related to the severity and progression of cirrhosis rather than the etiology of the disease.\textsuperscript{20} Elevation of CK18 impairs liver function as shown in liver biopsies, due to a marked increase in apoptosis observed by terminal deoxynucleotidyl transferase dUTP nick end labeling staining. There is also an activation of neutrophil recruitment signals by IL-8 and anti-inflammatory agents that limit the immunological effect of cell death (IL-10, IL-1RA, scD163).\textsuperscript{21,27} The reactivation of HBV has been the major cause in which the ACLF is presented in most studies of the Asia-Pacific region,\textsuperscript{28} presenting necrosis as the main characteristic.\textsuperscript{29}

Alcohol consumption as an acute precipitant in ACLF is associated with worse outcomes, such as advanced hepatic encephalopathy, elevation of serum creatinine and the presence of infection, in addition to high short-term mortality, when compared with other etiologies.\textsuperscript{30} Also, patients with ACLF show a marked worsening in the circulatory disturbances present in liver cirrhosis. The mechanisms and pathophysiology have not been clearly determined, however, in a study with patients that present severe acute alcoholic hepatitis, blocking the tumor necrosis factor α (TNF-α) using infliximab produced a marked decrease in both portal pressure and the degree of hyperdynamic circulation.\textsuperscript{31} On the other hand, the elevation of pathogen-associated molecular patterns has been related to liver damage by different pathophysiological mechanisms of decompensation, showing ballooning degeneration and cell death with a predominance of cholestatic infection.\textsuperscript{32,33}

When the type of organ failure correlates with mortality, cerebral and circulatory/pulmonary insufficiency is strongly associated in >95% of the cases. However, biliary nephropathy is an important toxic injury caused by elevated levels of bile acids and bilirubin, which is significantly common in patients with ACLF.\textsuperscript{7} In addition, the number of organic failures is also correlated with
mortality, increasing abruptly when there are more than two or-
organic failures to 80% and increase up to 94% when there are
more than 3 and 4 organic failures. Among the available fore-
cast scores, CLIF-C ACLF and Acute Physiology and Chronic
Health Evaluation (APACHE) II present the best performance.

The physiopathological mechanisms that characterize the de-
velopment of ACLF are not completely known, but they generally oc-
cur in the context of an intense systemic inflammatory response.
In the CANONIC study, it was observed that patients with ACLF
presented a marked elevation of C-reactive protein together with
a rise of the leukocyte count. These findings led to the hypothe-
sis that excessive systemic inflammatory response is a basis to ex-
plain the pathogenesis of ACLF.

**ACLF INFLAMMATORY PROCESS**

The majority of the altered cytokines in ACLF are related to che-
motaxis and leukocyte migration, mainly macrophages and mono-
cytes. This disturbance is not just associated with bacterial infec-
tions, given that cytokine levels were different in patients with
bacterial infections out of ACLF when compared to patients with
the illness.

A study conducted by Dr. Barroso shows that patients with
ACLF had elevated levels of C-reactive protein and leukocytosis,
because of an inflammatory event such as bacterial infections and
severe alcoholic hepatitis in 57% of the patients, or to mecha-
nisms that were not identified in the remaining 43%, which sug-
gests an altered immune response that leads to an inadequate in-
flammatory response.

Patients with alcoholism have a neutrophil dysfunction, which
increases the reactive oxygen species and alters phagocytosis to-
gether with a deficient bacterial death. Despite being in a proin-
flammatory state, they are more susceptible to infections, having
a worse prognosis. In some cases, the infections are a conse-
quence of ACLF instead than a cause.

On the other hand, patients with ACLF show higher levels in se-
rum histone 4 compared to controls and it is not only significantly
related to liver damage but also to the failure of other organs, dis-
ease severity and overtime mortality. Extracellular histones have
diverse effects such as coagulation by platelet aggregation, white
blood cell count and elevation of C-reactive protein in plasma, en-
dothelial damage induction and production of cytokines (IL-1β, IL-6,
IL-8, IL-10, IL-18 and TNF-α), which indicates an acute inflamma-
tory response that in turn predisposes host to bacterial translocation
and infection, worsening the inflammatory response. Also, sur-
vi val decrease in ACLF patients is related to the reduced hu-
man leukocyte antigen-D expression on monocytes, which in-
creases the severity and risk of infection. However, interferon-γ,
transforming growth factor-β1, IL-1β and IL-12 levels are normal.

Th9 cells are lymphocytes that produce IL-9 and IL-10, which
migrate to the liver in response to acute damage and regulate the
duration and the intensity of inflammation. In a study conducted
by Dr. Zhijun Su’s group, it was found that the percentage of Th9
cells was lower in patients with ACLF than in healthy patients.
However, patients who died showed a significant increase in IL-9
and IL-10, unlike patients with ACLF who survived and healthy
controls, suggesting that other immune cells could secrete IL-9
and IL-10.

Moreover, patients with ACLF have increased numbers of immu-
noregulatory monocytes and macrophages that express MER re-
ceptor tyrosine kinase and suppress the innate immune response
to microbes. The number of these cells correlates with disease se-
verity and the inflammatory response. MER receptor tyrosine ki-
nase expression positively correlates with IL-6, IL-10 and TNF-α,
which might suggest that these cytokines could be related to the
induction of this in ACLF, therefore it could be said that it may
serve as a prognostic marker for ACLF.

Neutrophil gelatinase-associated lipocalin is a LCN2 gene pro-
ductive protein against bacterial infections given that it binds to
the bacteria siderophores. The neutrophil gelatinase-associated li-
pocalin is another ACLF biomarker in plasma or urine, which can
be used as a prognostic marker in patients with cirrhosis acute
decompensation. As well it has been shown that hepatic LCN2
gene levels are increased in patients with ACLF and the expres-
sion of this gene is correlated with liver function parameters (bili-
rubin, albumin, international normalized ratio and the model for
e nd-stage liver disease score).

**GENETICS OF ACLF**

It has been pointed out that the pathogenesis of the ACLF may
depend on the insult, the immune response, secondary infec-
tions or organ failure; however, the possibility of developing ACLF
could be partially genetically predetermined.

In a genetic association study of candidate genes in twins, it
has been suggested that host genetic factors are critical in deter-
mining the outcome of HBV infection. The HBV infection de-
pends on the interaction between the virus, the hepatocytes and


Guillermo Nahum López-Sánchez, et al.
Acute-on-chronic liver failure insights
Table 2. Genetics of ACLF

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relationship with ACLF</th>
<th>Gene information</th>
<th>Studies</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1846T, C1913A/G</td>
<td>Severity of liver disease and risk of ACLF</td>
<td>Mutation on HBV gene; encodes capsid protein; pre-capsid protein</td>
<td>438 patients with liver diseases were retrospectively reviewed. A1846T was significantly associated with the mortality of ACLF patients within six months after the disease onset, while C1913A led to a significant decrease of core protein expression.</td>
<td>Zang et al. 44</td>
</tr>
<tr>
<td>rs3129859</td>
<td>Prognostic marker for the emergence, severity and survival of ACLF</td>
<td>A/C/G single-nucleotide variation on human chromosome 6</td>
<td>399 HBV-related ACLFs (cases) and 401 asymptomatic HBV carriers (AsCs, as controls). Clinical traits analysis in patients with ACLF showed that the risky rs3129859*C allele was associated with prolonged prothrombin time, faster progression to ascites development and higher 28-day mortality.</td>
<td>Tan et al. 41</td>
</tr>
<tr>
<td>rs2910164 of miR-146a</td>
<td>Deficient immune response and high incidence of infection due to lower serum levels of TNF-α</td>
<td>C/G single-nucleotide variation on human chromosome 5</td>
<td>Case-control study including 717 cases of HBV and 251 cases of ACLF-HBV and 466 cases of chronic hepatitis B. Results showed that the GG homozygote was a protective genotype in terms of susceptibility to ACLF-HBV, compared with CC+GC genotypes.</td>
<td>Jiang et al. 37</td>
</tr>
<tr>
<td>TLR3 C1234T</td>
<td>Inactive response and low recognition response to viral pathogens</td>
<td>Toll-like receptor 3 polymorphism on human chromosome 4</td>
<td>Case-control study including 452 chronic hepatitis B patients and 462 healthy controls. Data showed that subjects carrying 1234CT genotype and TT genotype had 1.42-fold and 2.31-fold increased risk of chronic HBV infection compared to those with CC genotype.</td>
<td>Rong et al. 46</td>
</tr>
<tr>
<td>TLR3 L412F</td>
<td>Lower rejection rate of liver transplantation</td>
<td>Toll-like receptor 3 polymorphism on human chromosome 4</td>
<td>Single-center study of 100 adult patients who received a first whole only liver graft from deceased donors. Homozygous mutant TT genotype for TLR3 L412F was associated with a lower rate of acute rejection compared with the homozygous wild-type genotype.</td>
<td>Citores et al. 48</td>
</tr>
</tbody>
</table>

Some of the gene polymorphisms that explain individual biological differences and how they affect humans to develop acute-on-chronic liver failure (ACLF). HBV, hepatitis B virus; A/C/G, adenine/cytosine/guanine; AsCs, surface antigen carriers; C/G, cytosine/guanine; GG, guanine/guanine; CC, cytosine/cytosine; GC, guanine/cytosine; TNF-α, tumor necrosis factor α; TT, thymine/thymine.

Figure 1. Pathogenic mechanisms in acute-on-chronic liver failure (ACLF). Cirrhosis is a progressive liver disease characterized by diffuse fibrosis, which evolution is divided in compensated and decompensated cirrhosis, where its development shows variceal hemorrhage, jaundice, ascites and hepatic encephalopathy. As the disease develops, reactive oxygen species increase as well as inflammation. A second insult is a trigger for ACLF to occur, leading the patient to multi-organ failure or even death if he does not receive a liver transplant. Upwards arrows indicated ‘an increase’. ROS, reactive oxygen species.
the host immune response; even the same strains of HBV can lead to different levels of pathogenicity and severity, indicating an individual susceptibility to ACLF.⁴¹

Although mortality in patients with ACLF-HBV is 30–70%, most patients with chronic hepatitis B do not suffer from ACLF throughout their lives. This may be due to genetic variations in DNA sequences such as single-nucleotide polymorphism (SNP), which explains the individual biological differences and how they affect humans to develop a disease.⁴¹

Moreover, a few studies showed that mutations of the basal core promoter, or in the core promoter of HBV genome may have different clinical implications.⁴²,⁴³ In 2018 Zang et al. reported that the detection of mutations in the HBV genome in the basal core promoter/core promoter region (A1846T and C1913A) is positively associated with the severity of liver disease and the risk of ACLF.⁴⁴ On the other hand, a genome-wide association study reported that 10 SNPs were associated with HBV-related to ACLF. The analysis showed that human leukocyte D antigen is related as the main locus for susceptibility to ACLF related to HBV. In addition, the responses of allelic CD4+ T cells related to human leukocyte D antigen may be important for the initiation and progression of ACLF related to HBV, which was replicated in four populations. Finally, it is proposed that one of the variants (rs3129859) could be used as a prognostic marker for the appearance, severity and survival of the ACLF related to HBV in patients with chronic HBV infection.⁴¹

SNPs can also modify the immune response and the incidence of infections. The innate immune response serves as the first line of defense against pathogens and the malfunctioning of this lead to an increase in the incidence of bacterial and fungal infections.⁴² The SNP rs2910164 of the mir-146a gene is involved in the regulation of innate immunity and is associated with the susceptibility to ACLF, therefore, a lower genetic risk of developing ACLF. Homozygous subjects for the SNP of the mir-146a gene had a higher survival rate and lower serum levels of TNF-α, which decrease the induction of hepatocytes apoptosis and improve the severity of the disease.⁴³

Genetic polymorphisms of TLRs have been associated with an increased predisposition to infection in several populations; these receptors play a key role in the innate response and the recognition response to viral pathogens.⁴⁵ A case-control study (in a Chinese population) analyzed the correlation between TLR3 C1234T polymorphism and chronic hepatitis B related with ACLF. This receptor plays a key role in the inactive response and the recognition response to viral pathogens. This polymorphism was associated with increased risk of ACLF in chronic hepatitis B patients and may act as a potential marker for the prognosis.⁴⁶ TLR3 agonism may improve antibacterial responses and reduce infectious complications without having a detrimental effect on tissue repair processes.⁴⁷ Furthermore, being homozygous for the polymorphism TLR3 L412 was associated with a lower rejection rate for liver transplantation.⁴⁸ As well, increased expression of TLR4 in mononuclear cells from ACLF patients rises T cells activation, which leads to liver damage, indicating that TLR4 may play a role in the aggravation of ACLF disease.⁴⁹ Previously, the genetic polymorphisms of the TLR4 receptor have been particularly associated with D299G, however, at the polymorphisms of this receptor may not be associated in the predisposition to develop bacterial infections and therefore in the prognosis of cirrhotic patients with ascites (Table 2).⁴¹,⁴⁴,⁴⁶,⁴⁸,⁵⁰

**CONCLUSION**

Chronic liver diseases and cirrhosis are intricate pathological stages with a heterogeneous pathophysiology in which genetic factors of the host and external triggers interact and culminate in a liver failure that leads to multiple clinical complications with a high rate of morbidity and mortality (Fig. 1).

During the last decade several studies have been carried out on the progression of liver damage through the interaction between hepatic parenchymal cell dysfunction and the immune system in the pathological process. These harmful processes can generally lead to the development of fibrosis, however, there are some cases where it does not occur in such a way. This point encourages us to continue basic and clinical researches to elucidate the multiple pathways involved in liver damage and ACLF, leading to the discovery of new treatments that will make acute decompensation preventable and even reduce mortality.

**Author’s contribution**

All authors have contributed to the realization and improvement of the article, also agreed on the content of the manuscript. Guillermo Nahum Lopez-Sanchez, Mayra Dominguez-Perez and Natalia Nuno-Lambarr listed and wrote the article. Misael Uribe revised, contributed with diverse ideas and corrected the final version of the manuscript. The final version have been read and approved by all authors.
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Conflicts of Interest
The authors have no conflicts to disclose.

REFERENCES


Extrahepatic manifestations of hepatitis E virus: An overview

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Hepatitis E virus (HEV) is a significant health problem with approximately 20 million individuals infected annually. HEV infection has been associated with a wide spectrum of extrahepatic manifestations, including neurological, hematological and renal disorders. Guillain-Barré syndrome and neuralgic amyotrophy are the most frequent neurological manifestations. In addition, HEV infection has been observed with other neurological diseases, such as encephalitis, myelitis and Bell’s palsy. Hematologic manifestations include anemia due to glucose-6-phosphate dehydrogenase deficiency, autoimmune hemolytic anemia and severe thrombocytopenia. Membranoproliferative glomerulonephritis and relapse IgA nephropathy with or without coexisting cryoglobulinemia appear to be the most common renal injuries related with HEV infection. Also, HEV infection has been associated with acute pancreatitis and other immune-mediated manifestations, such as arthritis and myocarditis. However, the pathophysiological mechanisms of HEV-related extrahepatic manifestations are still largely unclear. (Clin Mol Hepatol 2020;26:16-23)

Keywords: Hepatitis E; Kidney; Neurologic manifestations; Hematologic diseases

INTRODUCTION

Hepatitis E virus (HEV) is a single-stranded RNA virus and was first discovered in 1983,1 but the viral genome was cloned in 1990.2 It is estimated that HEV causes 20.1 million infections annually, leading to 3.4 million symptomatic cases with acute hepatitis and 70,000 deaths related to acute liver failure.3

HEV has eight genotypes. Genotypes 1 and 2 only infect humans, are detected mainly in Asia and Mexico, respectively and they spread through fecal-oral route. Genotypes 3 and 4 are detected mainly in Europe and North America, circulate in animal species such as pigs, wild boars and deer and occasionally infect humans via consumption of contaminated meat or direct contact.4 Also, genotype 3 has been detected in shellfishes in Scotland and in southern Italy.5,6 Genotypes 5 and 6 have been only reported in wild boar and genotypes 7 and 8 have been identified in camels.7

The diagnostic tests for HEV infection include detection of antibodies against HEV (IgM anti-HEV and IgG anti-HEV) and detection of HEV RNA. IgM anti-HEV is positive during the first month after HEV infection, while IgG anti-HEV represents current or past infection.8 Detection of HEV RNA in blood or stool characterizes chronic or acute HEV infection. Chronic hepatitis E is defined as...
HEV RNA being detectable for at least 3 months. In immunosuppressed patients with chronic HEV infection, antibodies are often undetectable.\textsuperscript{9} HEV infection is usually self-limiting and causes acute mild illness. However, HEV infection during pregnancy, especially in the third trimester may lead to acute liver failure.\textsuperscript{10} Chronic HEV infection is rare and may develop in immunocompromised patients, such as organ transplant recipients.\textsuperscript{11}

In addition, HEV has been associated with a range of extrahepatic manifestations, including a spectrum of neurological symptoms and diseases, hematological disorders, renal diseases, acute pancreatitis, myocarditis, arthritis and autoimmune thyroiditis (Table 1).\textsuperscript{12} However, the pathophysiologic mechanism of extrahepatic manifestations remains unclear. It seems that viral infections trigger a variety of host-defense mechanisms, which may not be restricted to the primary location of infection and can cause cross-reactions between viral epitopes and self-antigens, leading to multisystemic manifestations. Another possible explanation is that HEV replicates not only in liver, but also in other tissues. HEV has been detected in neuronal cells,\textsuperscript{13} human placenta,\textsuperscript{14} breast milk,\textsuperscript{15} and urine.\textsuperscript{16}

### Neurological Manifestations

Several neurological manifestations have been associated with HEV infection and include Guillain-Barré syndrome (acute inflammatory demyelinating polyradiculoneuropathy), neuralgic amyotrophy, encephalitis, myelitis, myositis, peripheral neuropathy, Bell’s palsy and mononeuritis multiplex.\textsuperscript{17} In a prospective multicenter study from United Kingdom, France and Netherlands it was found that 2.4% (11/464) of patients with non-traumatic neurologic injury had evidence of HEV infection.\textsuperscript{18} Also, a study from France demonstrated the neurologic disorders in patients infected with HEV and found that 16.5% of HEV-infected patients reported neurologic symptoms and neurological manifestations were more frequent in immunocompetent patients compared to immunosuppressed patients (22.6% vs. 3.2%, \textit{P}<0.001).\textsuperscript{19} However, a study from China compared the prevalence of acute hepatitis E between 1,117 patients diagnosed with neurological illness and 1,475 healthy controls and found that there was no difference (0.54% vs. 0.68%).\textsuperscript{20} A possible explanation is the geographical distribution of HEV. The study from China was conducted in an area endemic for HEV genotype 4, while the European studies reported cases associated with HEV genotype 3. Therefore, HEV genotype 4 seems not to contribute to neurological disorders.\textsuperscript{21}

#### Guillain-Barré Syndrome

Guillain-Barré syndrome (GBS) is an acute onset immune-mediated disorder of peripheral nervous system and is characterized by acute inflammatory demyelinating polyradiculoneuropathy, causing rapidly progressing symmetric motor paralysis.\textsuperscript{22} HEV infection has been associated with development of GBS. Many studies have reported the high prevalence rate of HEV infection among GBS patients and several case reports have been documented showing the coexistence of acute hepatitis E with GBS. In Netherlands, 201 patients with GBS were compared with 201 healthy controls with a similar distribution in age, sex, and year of sampling and it

### Table 1. Extrahepatic manifestations associated with hepatitis E virus infection

<table>
<thead>
<tr>
<th>System</th>
<th>Manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurological system</td>
<td>Guillain-Barré syndrome, Neuralgic amyotrophy, Encephalitis, Myelitis, Myositis, Peripheral neuropathy, Bell’s palsy, Mononeuritis multiplex, Seizure, Pseudotumor cerebri, Oculomotor palsy, Polyradiculoneuropathy</td>
</tr>
<tr>
<td>Hematological system</td>
<td>Thrombocytopenia, Monoclonal gammopathy of uncertain significance (MGUS), Hemolytic anemia, Aplastic anemia, Hemophagocytic syndrome, CD30 (+) cutaneous T cell lymphoproliferative disorder, Thrombotic thrombocytopenic purpura</td>
</tr>
<tr>
<td>Kidney</td>
<td>Relapse of IgA nephropathy, Cryoglobulinemia, Membranoproliferative glomerulonephritis</td>
</tr>
<tr>
<td>Heart</td>
<td>Myocarditis</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Acute pancreatitis</td>
</tr>
<tr>
<td>Thyroid</td>
<td>Autoimmune thyroiditis, Subacute thyroiditis</td>
</tr>
<tr>
<td>Skeletal system</td>
<td>Poliarthritis, Henoch-Schölein purpura</td>
</tr>
<tr>
<td>Vasculitis</td>
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</tbody>
</table>
was found that the prevalence of acute hepatitis E was higher in patients with GBS compared with healthy controls (5% vs. 0.5%). Additionally, in a similar study in Japan, 4.8% (3/63) of patients with GBS had acute HEV infection preceding the onset illness, while no patients from healthy control group (0/61) suffered from acute hepatitis E. Furthermore, a retrospective cohort study in Belgium found that the prevalence of HEV infection in patients with GBS was 8% (6/73). In all studies, there were no differences regarding course and outcomes of GBS between HEV-related GBS and HEV-unrelated GBS. Also, cases of acute HEV infection have been found in pediatric patients with GBS.

Neuralgic amyotrophy

Neuralgic amyotrophy (NA), also known as Parsonage-Turner syndrome, is an acute and painful unique or multiple mononeuropathy in the upper extremity and is characterized by rapid multifocal motor weakness, amyotrophy and sensory loss. It seems that HEV infection can trigger the development of NA and several studies have been conducted. A cohort study with 64 patients from United Kingdom and Netherlands found that 10% of patients had NA and HEV was not related to age, sex, severity, disease course or outcome. Also, it seems that patients with NA and HEV have a distinct phenotype. A multicenter European study compared 61 HEV-NA patients with 61 NA patients and found that, HEV-NA appears more often predominately bilateral asymmetrical involvement (80.0% vs. 8.6%, P<0.001) and more extensive damage to the brachial plexus. Involvement outside the brachial plexus is more common in HEV-NA (58.5% vs. 10.5%, P<0.01).

Other neurological manifestations

Other neurological cases associated with HEV infection include vestibular neuritis, Bell’s palsy, acute ataxic neuropathy, transverse myelitis, acute encephalitic Parkinsonism, oculomotor palsy, myositis, pseudotumor cerebri, bilateral pyramidal syndrome, polyradiculoneuropathy, and mononeuritis multiplex. In addition, central nervous system infections, such as encephalitis and meningitis, with HEV have been reported and HEV RNA has been demonstrated in serum and cerebrospinal fluid at the time of acute illness. Also, many patients with CNS infection were immunosuppressed as a result of solid organ transplantation.

Pathogenic mechanism

The pathogenic mechanism between HEV and neurological disorders has been not clarified, but it seems that HEV is also neurotropic. Shedding of HEV RNA into the cerebrospinal fluid and intrathecal production of IgM anti-HEV has been detected in a patient with NA and acute HEV infection. Additionally, a study demonstrated that human neuronal-derived cell lines such as neuroepithelioma, desmoplastic cerebellar medulloblastoma, glioblastoma multiforme, glioblastoma astrocytoma and oligodendrocytic cells can support HEV RNA replication. It is recommended that clinicians consider the possibility of HEV infection in patients with neurological disorders and concurrent liver enzyme alteration, especially those with peripheral nerve involvement.

RENAL MANIFESTATIONS

Renal disorders have been reported during HEV infection, including membranoproliferative glomerulonephritis and cryoglobulinemia. A retrospective study assessed kidney function and histology in 51 cases of solid-organ transplant patients during genotype 3 HEV infection and they observed statistically but not clinically significant decrease in estimated glomerular filtration rate (-5 mL/min, P=0.04) during HEV infection. In renal biopsies, glomerular diseases were identified. They included relapse of IgA nephropathy and membranoproliferative glomerulonephritis. The majority of these patients had cryoglobulinemia. After HEV clearance, cryoglobulinemia resolved and proteinuria and renal function improved. Additional cases of HEV-related membranoproliferative glomerulonephritis and membranous nephropathy have been reported. In one case, HEV infection triggered monoclonal gammopathy of renal significance.

The association between cryoglobulinemia and HEV infection has not been fully investigated. In a study with solid organ recipients, who suffered from HEV infection, the prevalence of cryoglobulinemia was increased during chronic phase of infection (52.9%) compared to acute phase of infection (36.4%) and HEV-negative solid organ recipients (23.6%) (P<0.01). Also, HEV infection was identified as an independent predictive factor for cryoglobulinemia (odds ratio, 2.3). Another retrospective study from Germany compared the prevalence of IgG anti-HEV between patients with cryoglobulinemia and healthy controls. They found that the anti-HEV seroprevalence rate was significantly higher in
patients with essential cryoglobulinemia than in non-essential cryoglobulinemia patients \( (P=0.043) \), suggesting that previous HEV contact might play a role in some cases of cryoglobulinemia that are currently classified as essential.\(^{32}\)

**HEMATOLOGIC MANIFESTATIONS**

**Anemia**

Different patterns of anemia have been reported during HEV infection, including hemolytic anemia due to glucose-6-phosphate dehydrogenase (G-6-PD) deficiency, autoimmune hemolytic anemia (AIHA) and aplastic anemia. Hemolytic anemia may be a complication of acute viral hepatitis and the frequency rate of hemolysis has been reported in up to 23% of patients. The prevalence of hemolytic anemia may rise up to 70% in patients who have G-6-PD deficiency.\(^{51}\) Patients with G-6-PD deficiency have low levels of glutathione in red blood cells, leading to accumulation of oxidants during viral hepatitis and resulting in hemolysis. Several cases of hemolysis in patients with G-6-PD deficiency and acute HEV infection have been reported.\(^{52-54}\) In some cases of hemolysis in patients with acute HEV infection and G-6-PD deficiency, there was development of renal failure, as a result of possible obstruction of renal tubules due to hemoglobin and bilirubin.\(^{55,56}\)

Autoimmune hemolytic anemia has been described in association with a variety of hepatotropic viruses, such as cytomegalovirus, hepatitis A virus (HAV) and hepatitis B virus (HBV).\(^{57}\) AIHA is diagnosed based on clinical presentation, spherocytosis, laboratory findings and positive direct antiglobulin test (DAT). However, DAT was negative up to 15% of AIHA cases. In four published cases of AIHA-related with hepatitis E, the treatment was supportive and their outcomes were favorable.\(^{58-61}\)

Hepatitis-associated aplastic anemia is an uncommon but distinct variant of aplastic in which pancytopenia appears 2 or 3 months after an acute attack of viral hepatitis. Several viruses, such as parvovirus B19, cytomegalovirus, Epstein-Barr virus, HAV and HBV, have been associated with aplastic anemia.\(^{62}\) Three cases of HEV-related aplastic anemia have been reported. In one case there was no response to treatment with cyclosporine and in the second case, the patient expired due to sepsis and in the third case, the patient was treated with thymoglobulin, cyclosporine, corticosteroids, filgastrim and transfusions.\(^{63-65}\)

**Thrombocytopenia**

Thrombocytopenia is a well-recognized complication of infections, including those from hepatotropic viruses. A variety of possible mechanisms of thrombocytopenia have been reported and includes hypersplenism, reduced hepatic production of thrombopoietin, bone marrow suppression by hepatotropic virus or treatment and development of anti-platelet autoantibodies and platelet-associated immune complexes.\(^{56}\) Several cases of HEV-associated thrombocytopenia have been documented. In most cases, thrombocytopenia was self-limited, while in other cases, the patients needed to receive platelet transfusion, intravenous globulin and/ or corticosteroid. It is worth mentioning that, anti-platelet antibodies were detected in some cases of HEV-related thrombocytopenia.\(^{44,61,67,68}\)

**Other hematological diseases**

HEV infection has been related with other less common hematological disorders. Few cases of HEV-related hemophagocytic syndrome have been documented.\(^{69-71}\) Also, HEV infection has been detected in patients with CD30 (+) cutaneous T cell lymphoproliferative disorder\(^{72}\) and monoclonal gammopathy of undetermined significance (MGUS). However, the relation between MGUS and HEV remains uncertain.\(^{73}\) Additionally in one case, thrombotic thrombocytopenic purpura relapse induced by acute hepatitis E transmitted by cryosupernatant plasma. HEV infection treated with ribavirin and thrombotic thrombocytopenic purpura remitted with remission of HEV infection.\(^{74}\)

**ACUTE PANCREATITIS**

A wide variety of infectious agents has been associated with acute pancreatitis and these include viruses, bacteria and parasites. The association between acute pancreatitis and viral hepatitis is well known and HAV, HBV, and hepatitis C virus have been implicated most often.\(^{75,76}\) A proposed pathogenetic mechanism is the development of edema of the ampulla of Vater, causing obstruction of pancreatic fluid flow.\(^{77}\) Several cases of HEV-induced acute pancreatitis have been reported.\(^{78,79}\) In a single-center study from France, 2.1% (16/790) of patients with acute pancreatitis had serological evidence of recent HEV infection with no other discernible cause of pancreatitis.\(^{80}\) The typical profile of a patient is a young male from an endemic area or having recently travelled.
to that area, who develops mild to moderate acute pancreatitis. However, life-threatening complications, such as, acute necrotizing pancreatitis, pseudocyst bleeding and multiorgan failure, have been reported.

OTHER MANIFESTATIONS

Development of many other diseases has been reported during HEV infection, but further studies are needed to establish the association. In previous literatures, three cases of HEV-associated myocarditis have been reported. Furthermore, HEV infection has been correlated with thyroid diseases. These include autoimmune thyroiditis, subacute thyroiditis and Grave’s thyrotoxicosis. In addition, a case of Henoch-Schönlein purpura triggered by acute HEV infection and another case of HEV-induced myasthenia Gravis have been described. Lastly, HEV infection may cause acute polyarthritis.

CONCLUSION

Several extrahepatic manifestations and diseases have been documented during acute and chronic HEV infection. Neurologic diseases are demonstrated to be the most common extrahepatic manifestations of HEV infection, followed by hematological disorders and kidney injury. However, the pathophysiology of these manifestations and the causal relation with HEV infection remain ambiguous. Therefore, further studies are needed to estimate the epidemiological characteristics of HEV-related extrahepatic manifestations and to elucidate their underlying pathogenetic mechanisms.

Author’s contribution

FSF: Data selection, writing, study design, IVM: writing, DKC: Supervision, study design, writing

Conflicts of Interest

The authors have no conflicts to disclose.

REFERENCES

A survey on transarterial chemoembolization refractoriness and a real-world treatment pattern for hepatocellular carcinoma in Korea

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Background/Aims: Transarterial chemoembolization (TACE) is a standard treatment for intermediate-stage hepatocellular carcinoma (HCC), but there is much controversy about TACE refractoriness. The aim of this study was to identify trends in the actual clinical application of TACE and recognition of TACE refractoriness by Korean experts.

Methods: In total, 17 questionnaires on TACE refractoriness were administered to 161 clinicians via an online survey. Multiple answers were allowed for some questions.

Results: Most clinicians agreed that there is a need for standardization of TACE application through specific scoring systems (n=124, 77.0%). TACE refractoriness was predominantly expected by participants when recurrences were detected within 1 month (n=70, 43.5%), there were 4 to 6 tumors (n=77, 47.8%), the maximal tumor size was 3–5 cm (n=49, 30.4%), and when there was insufficient tumor necrosis despite TACE being repeated more than three times (n=78, 48.4%). Overall, sorafenib therapy (n=137) and radiotherapy (n=114) were preferred when repeated TACE was considered ineffective.

Conclusions: Treatment of HCC is often based on the clinical judgment of clinicians because of the heterogeneity among individuals. Experts need to continue discussions on the standardization and sub-classification of HCC treatment guidelines in Korea. (Clin Mol Hepatol 2020;26:24-32)

Keywords: Liver neoplasms; Carcinoma, Hepatocellular; Embolization, Therapeutic; Surveys and questionnaires

Study Highlights
This survey focused to investigate the transarterial chemoembolization (TACE) treatment strategy of Korean clinicians for liver cancer. There were various opinions and differences of domestic medical staff about TACE refractoriness according to hospital size and location, but most of them showed convergence pattern. These results would help to establish the definition of TACE refractoriness and assist in the liver cancer treatment strategies in Korea.

Abbreviations:
ART score, the assessment for retreatment with transarterial chemoembolization score; BCLC, Barcelona-clinic liver cancer; HAP score, the Hepatoma Arterial-embolization Prognostic score; HCC, hepatocellular carcinoma; TACE, transarterial chemoembolization

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INTRODUCTION

Transarterial chemoembolization (TACE) is being widely used as a non-surgical therapy for the simultaneous treatment of chemotherapy and selective ischemia for hepatocellular carcinoma (HCC). On the basis of a higher level of evidence, TACE has been proven to improve the survival rate for patients with intermediate-stage HCC, defined as Barcelona-clinic liver cancer (BCLC) stage B HCC, for which patients are not candidates for curative strategies such as surgical resection, liver transplantation, and local ablative therapies.1-4

Depending on the nature of the tumor, including size, number, growth pattern, and anatomical location, the treatment response is not always easy to obtain from a single session, showing differences in outcomes between reports.5-6 Thus, repeated TACEs are often performed to achieve a sufficient outcome. However, the method for predicting “TACE refractoriness” that does not show a therapeutic response even after repeated TACE treatments is still unclear. Enhanced criteria of TACE refractoriness were proposed by Japanese experts, which considered insufficient treatment response, increase in tumor number, new structural invasion or metastasis, and change in tumor markers.7 In the recent Korean guideline,8-10 sorafenib treatment was recommended if the stage progressed despite more than three repeated TACE treatments within 6 months.

However, in practice, there are too many heterogeneous situations to select a consistent treatment strategy; therefore, the clinical decision of the primary physician often plays a larger role than the formal guidelines in determining the appropriate time to try or switch to other therapies including radiotherapy, radiofrequency ablation, and sorafenib.

Therefore, we conducted an online survey and summarized the results below to identify the trends in the actual clinical application of TACE and the recognition of TACE refractoriness among the clinicians treating HCC in Korea.

MATERIALS AND METHODS

From November 9, 2017 to December 16, 2017, a total of 161 liver cancer clinicians in Korea were enrolled, including 121 gastroenterologists, 15 surgeons, five radiation oncologists, one hematologist, and 19 medical doctors of other fields (Table 1). All participants were working in tertiary medical centers where treatment procedures were performed via an intra-arterial route. Each individual accessed the online survey and selected his/her desired answers among the 17 multiple-choice questions. For 11 of the 17 questions, the participants were allowed to select multiple answers. All participants had worked in their fields for more than 10 years.

To compare practice patterns according to the size of the hospital, participants were divided into two groups: the five highest-volume centers and the lower volume centers. Clinical practice patterns according to the location of the hospitals were also compared by dividing participants into two groups: who were located in metropolitan areas (Seoul and Gyeonggi provinces) that had chance to easily access to the five high-volume centers, and who were located in other provinces.

The General Rules for the Clinical and Pathological Study of Primary liver cancer, developed by the Liver Cancer Study Group of Japan, were used to classify portal vein tumor thrombosis.10 According to these rules, the definition of Vp3 was the presence of a tumor thrombus in the first branches of the portal vein, and the definition of Vp4 was the presence a tumor thrombus in the main trunk of the portal vein and/or the contra-lateral portal vein branch to the primarily involved lobe.

Statistical analyses were conducted using the IBM SPSS Statistics software, version 23.0 (IBM Corp., Armonk, NY, USA), and a two-sided P-value of <0.05 was considered significant. The Chi-square test and Fisher’s exact test, as appropriate, were applied to compare qualitative variables.

RESULTS

Baseline information of the participants are summarized in Table 1.

Table 1. Baseline information of the participants (n=161)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>128 (79.5)</td>
</tr>
<tr>
<td>Clinicians working in the high-volume centers</td>
<td>42 (26.1)</td>
</tr>
<tr>
<td>Clinicians working in Seoul and Gyeonggi province</td>
<td>120 (74.5)</td>
</tr>
<tr>
<td>Specialty</td>
<td></td>
</tr>
<tr>
<td>Gastroenterology and hepatology</td>
<td>121 (75.2)</td>
</tr>
<tr>
<td>Surgery</td>
<td>15 (9.3)</td>
</tr>
<tr>
<td>Radiation oncology</td>
<td>5 (3.1)</td>
</tr>
<tr>
<td>Hemato-oncology</td>
<td>1 (0.6)</td>
</tr>
<tr>
<td>Others</td>
<td>19 (11.8)</td>
</tr>
</tbody>
</table>

Variables are presented as n (%).
Of 128 (79.5%) male and 33 (20.5%) female participants, 42 (26.1%) clinicians were working in the five high-volume centers, and 120 (74.5%) clinicians were working in the medical centers located in Seoul and Gyeonggi provinces.

Questions about the overall perception of TACE

The survey asked participants the following three yes/no questions.

Q1. Standardization of TACE application in Korea is necessary through specific scoring systems such as the Hepatoma Arterial-embolization Prognostic score (HAP score) and assessment for retreatment with TACE score (ART score).
Q2. The effect of TACE would be different depending on individual and tumor characteristics.
Q3. Sub-classification of the intermediate stage is necessary where TACE is recommended as a standard therapy.

Most of the clinicians agreed that there are needs for the standardization of TACE application through specific scoring systems (n=124, 77.0%) and the sub-classification of the intermediate stage HCC where TACE is recommended as standard therapy (n=148, 91.9%). Moreover, most of the respondents (n=157, 97.5%) expected that the effect of TACE would be different depending on individual and tumor characteristics. There was no difference in the answers to the questions depending on the size and location of the hospital, except that the clinicians working far outside of Seoul more strongly raised the need for scoring systems (Fig. 1, Table 2).

Factors affecting TACE treatment response

The survey asked participants the following three questions that allowed multiple answers (Fig. 2, Table 3).

Q4. The patient characteristics affecting the response to TACE. Many clinicians responded that many variables would affect TACE response, especially the tumor size (n=145, 90.1%), number of tumors (n=111, 68.9%), and tumor shape, such as a nodular or infiltrating type (n=116, 72.0%) (Fig. 2A). Clinicians working in the high-volume centers showed a significant tendency to respond

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**Table 2. Responses to three questions (n=161)**

<table>
<thead>
<tr>
<th>Question</th>
<th>Answer</th>
<th>High-volume centers (n=42)</th>
<th>Centers in metropolitan (n=120)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
<td>P-value</td>
</tr>
<tr>
<td>Q1</td>
<td>Yes</td>
<td>124 (77.0)</td>
<td>32 (76.2)</td>
</tr>
<tr>
<td>Q1</td>
<td>No</td>
<td>37 (23.0)</td>
<td>10 (23.8)</td>
</tr>
<tr>
<td>Q2</td>
<td>Yes</td>
<td>157 (97.5)</td>
<td>40 (95.2)</td>
</tr>
<tr>
<td>Q2</td>
<td>Not certain</td>
<td>4 (2.5)</td>
<td>2 (4.8)</td>
</tr>
<tr>
<td>Q3</td>
<td>Yes</td>
<td>148 (91.9)</td>
<td>39 (82.9)</td>
</tr>
<tr>
<td>Q3</td>
<td>No</td>
<td>2 (1.2)</td>
<td>1 (2.4)</td>
</tr>
<tr>
<td>Q3</td>
<td>Not certain</td>
<td>11 (6.8)</td>
<td>2 (4.8)</td>
</tr>
</tbody>
</table>

Values are presented as n (%) unless otherwise indicated. 'Q1' is 'standardization of transarterial chemoembolization (TACE) application in Korea is necessary through the specific scoring systems such as the HAP score and ART score'. 'Q2' is 'the effect of TACE would be different depending on individual and tumor characteristics'. 'Q3' is 'sub-classification of the intermediate stage is necessary where TACE is recommended as a standard therapy'. P-value was calculated using chi-square test and Fisher’s exact test.
that the tumor marker (50.0% vs. 27.7%, P=0.013) and tumor shape (88.1% vs. 66.4%, P=0.009) were important (Table 3).

Q5. Preferred treatment, based on each clinician’s experience, for the patients thought to be poor responders to TACE treatment. Sorafenib therapy (n=113, 70.2%) and radiotherapy (n=109, 67.7%) were predominantly considered when TACE was not expected to be effective and there were also many responses to consider TACE anyway (n=66, 41.0%) or hepatic arterial infusion chemotherapy (HAIC) (n=58, 36.0%) (Fig. 2B). Sorafenib was preferred by clinicians working far outside of Seoul (65.8% vs. 82.9, P=0.039) (Table 3).

Q6. The features that cause repeated TACE to be ineffective when used in patients with tumors localized in the liver. When performing TACE in a patient with a localized HCC in the liver, they suggested that TACE was unlikely to be effective in cases with a higher number of larger sized of tumors (n=92, 57.1%), recurrent tumors, new lesions within a few months after the previous TACE treatment (n=89, 55.3% and n=79, 49.1%, respectively), and insufficient lipiodol uptake after TACE (n=77, 47.8%) (Fig. 2C). The response to tumor marker elevation (n=46, 28.6%) was significantly higher in high-volume centers (40.5% vs 24.4%) (Table 3).

**Expectations for TACE refractoriness**

There were four questions regarding the situations where TACE refractoriness was expected (Fig. 3, Supplementary Table 1).

Q7. How long do you think it would take to detect new lesions or recurrences after TACE in TACE refractoriness?

Q8. If new lesions appear after TACE, how many tumors do you think are maladaptive for repeated TACE?

Q9. If local recurrences occur after TACE, how large is the maximal size of a tumor considered for TACE refractoriness?

Q10. How many times should there be insufficient necrosis or recurrences after repeated TACE for consideration as TACE refractoriness?

Participants replied as follows; TACE refractoriness was predominantly expected when new lesions or recurrence were detected at 1 month (n=70, 43.5%), 3 months (n=43, 26.7%), and 2 months (n=29, 18.0%) after the procedure, respectively. Clinicians working in high-volume centers (66.7% vs. 35.3% for 1 month, P=0.002) or centers near Seoul (50.0% vs. 24.4% for 1 month, P=0.045) preferred to determine TACE refractoriness at an earlier time point (Fig. 3A, Supplementary Table 1). Regarding the number of lesions, 4 to 6 lesions (n=77, 47.8%), below 3 lesions (n=28, 17.4%), and 7 to 10 lesions (n=21, 13.0%) were frequently selected (Fig. 3B). Regarding the size of the largest lesion, 3–5 cm (n=49, 30.4%) and 5–7 cm (n=40, 24.8%) were mostly chosen (Fig. 3C). Regarding the number of insufficient TACE or repeated new lesions, 3 times (n=78, 48.4%) and 2 times (n=62, 38.5%) were mostly selected (Fig. 3D).
Preferred treatment strategies after TACE in specific situations

Participants replied to the questions that allowed multiple answers about the preferred treatment strategies after TACE in the following seven situations; Q11, suspicious TACE failure or refractoriness; Q12, multiple local recurrences with Child-Pugh class A; Q13, Vp3/4 portal vein thrombosis; Q14, main portal vein invasion; Q15, extrahepatic metastasis with Child-Pugh class A; Q16, extrahepatic metastasis with Child-Pugh class B; Q17, insufficient necrosis after at least two treatments of TACE with Child-Pugh class A (Fig. 4, Supplementary Table 2).

Overall, sorafenib (n=137, 85.1%), radiotherapy (n=114, 70.8%) and HAIC (n=62, 38.5%) were preferred when repeated TACE was considered ineffective. Preferred treatment methods in the specific conditions after performing TACE were reported as follows: Repeat TACE (n=111, 68.9%) and sorafenib (n=97, 60.2%) for multiple intrahepatic recurrences with Child-Pugh class A. For Vp3/4 thrombosis and main portal vein invasion, radiotherapy (n=127, 78.9% and n=138, 85.7%, respectively) and sorafenib (n=93, 57.8% and n=106, 65.8%, respectively) were preferred. Sorafenib (n=153, 95.0%) was preferred for extrahepatic recurrence with Child-Pugh class A. In the case of extrahepatic recurrence with Child-Pugh class B, hospice care (n=74, 46.0%) was preferred with other concurrent therapies (sorafenib [n=65, 40.4%], radiotherapy [n=61, 37.9%] and other systemic chemotherapy [n=51, 31.7%], respectively). When the necrosis of tumors with Child-Pugh class A were insufficient after performing more than 2 treatments of TACE, radiotherapy (n=113, 70.2%), sorafenib (n=94, 58.4%) and repeat TACE (n=75, 46.6%) were preferred (Fig. 4). For local control of HCC with advanced stage and Child-Pugh class A liver function, radiotherapy was preferred over repeated trials of TACE treatment in the high-volume centers (Q13, 0.008; Q14, 0.009; Q17, 0.011) or centers in the metropolitan areas (Q14, P=0.032; Q17, P=0.022) (Supplementary Table 2).

Table 3. Responses to three questions allowing multiple answers

<table>
<thead>
<tr>
<th>Question</th>
<th>Answer</th>
<th>No.</th>
<th>High-volume centers (n=42)</th>
<th>Centers in metropolitan (n=120)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Q4</td>
<td>Number of tumor</td>
<td>111 (68.9)</td>
<td>29 (69.0)</td>
<td>82 (68.9)</td>
</tr>
<tr>
<td></td>
<td>Size of tumor</td>
<td>145 (90.1)</td>
<td>39 (92.9)</td>
<td>106 (89.1)</td>
</tr>
<tr>
<td></td>
<td>Tumor marker</td>
<td>54 (33.5)</td>
<td>21 (50.0)</td>
<td>33 (27.7)</td>
</tr>
<tr>
<td></td>
<td>Residual liver function</td>
<td>91 (56.5)</td>
<td>24 (57.1)</td>
<td>67 (56.3)</td>
</tr>
<tr>
<td></td>
<td>Tumor shape (nodular or infiltrating)</td>
<td>116 (72.0)</td>
<td>37 (88.1)</td>
<td>79 (66.4)</td>
</tr>
<tr>
<td>Q5</td>
<td>Sorafenib</td>
<td>113 (70.2)</td>
<td>32 (76.2)</td>
<td>81 (68.1)</td>
</tr>
<tr>
<td></td>
<td>HAIC</td>
<td>58 (36.0)</td>
<td>13 (31.0)</td>
<td>45 (37.8)</td>
</tr>
<tr>
<td></td>
<td>Still perform TACE</td>
<td>66 (41.0)</td>
<td>13 (31.0)</td>
<td>53 (44.5)</td>
</tr>
<tr>
<td></td>
<td>Beads TACE</td>
<td>22 (13.7)</td>
<td>7 (16.7)</td>
<td>15 (12.6)</td>
</tr>
<tr>
<td></td>
<td>Other systemic chemotherapy</td>
<td>16 (9.9)</td>
<td>3 (7.1)</td>
<td>13 (10.9)</td>
</tr>
<tr>
<td></td>
<td>Radiotherapy</td>
<td>109 (67.7)</td>
<td>33 (78.6)</td>
<td>76 (63.9)</td>
</tr>
<tr>
<td>Q6</td>
<td>Insufficient necrotic area</td>
<td>77 (47.8)</td>
<td>16 (38.1)</td>
<td>61 (51.3)</td>
</tr>
<tr>
<td></td>
<td>New lesions within a few months</td>
<td>79 (49.1)</td>
<td>23 (54.8)</td>
<td>56 (47.1)</td>
</tr>
<tr>
<td></td>
<td>Local recurrences within a few months</td>
<td>89 (55.3)</td>
<td>22 (52.4)</td>
<td>67 (56.3)</td>
</tr>
<tr>
<td></td>
<td>Tumor size or number</td>
<td>92 (57.1)</td>
<td>24 (57.1)</td>
<td>68 (57.1)</td>
</tr>
<tr>
<td></td>
<td>Tumor marker elevation</td>
<td>46 (28.6)</td>
<td>17 (40.5)</td>
<td>29 (24.4)</td>
</tr>
<tr>
<td></td>
<td>Short interval between repeated TACE</td>
<td>36 (22.4)</td>
<td>10 (23.8)</td>
<td>26 (21.8)</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>15 (9.3)</td>
<td>6 (14.3)</td>
<td>9 (7.6)</td>
</tr>
</tbody>
</table>

Values are presented as n (%) unless otherwise indicated. ‘Q4’ is ‘the patient characteristics affecting the response to transarterial chemoembolization (TACE)’. ‘Q5’ is ‘preferred treatment based on each clinicians’ experience for the cases thought to be poor responders to TACE’. ‘Q6’ is ‘the features that make repeated TACE ineffective when performed with tumors localized in the liver.’ P-value was calculated using chi-square test and Fisher’s exact test.

HAIC, hepatic arterial infusion chemotherapy.
DISCUSSION

The effects of TACE on HCC and the changes in survival patterns may vary depending on the patient’s residual liver function and the characteristics of the tumor itself, so it is often necessary to repeatedly perform TACE because a single trial does not obtain sufficient results in many cases. It is still controversial whether treatment should be changed during repeated TACE or whether the effect obtained at some time point will help predict a patient’s survival. Although the recent HCC guideline by the Korean Liver Cancer Association suggested that progression of the disease after multiple trials of TACE treatment could be regarded as TACE refractoriness, there has been no actual definition of TACE refractoriness that can help further establish a treatment plan.

Therefore, the survey was conducted to see how medical staff in Korea were actually applying TACE in their work fields. In this survey, tumor number, size, and shape (nodular or infiltrative type) were considered as factors to affect the response to TACE treatment. A short period (about 1–3 months) until the appearance of the new lesions or recurrences, the maximum size of 3–7 cm of local recurrence, and the insufficient necrosis or recurrences after 2–3 times of repeated TACE treatments were considered to be suitable for TACE refractoriness. For multiple local recurrence, participants responded that repeated TACE treatment could be performed. However, for patients with advanced stage or suspected TACE refractoriness, clinicians selected sorafenib and localized radiotherapy more frequently. Radiotherapy was more preferred to be performed by the clinicians in the large medical centers or centers with good accessibility to the larger centers, probably because the high-volume centers had sufficient experience and appropriate facilities to perform radiation therapy.

TACE is useful because it can minimize impairment of hepatic function while improving treatment response and survival rate. However, at a certain point in time, repeated TACE treatments could show refractory patterns that diminish its usefulness and result in necrosis of multifocal lesions, which could lead to aggravation of liver function and worse patient survival. Therefore, it is important to determine a precise definition of TACE refractoriness to maximize the effectiveness of TACE itself and other therapies.

Many efforts have been made to subclassify the intermediate stage or provide prediction models through other scoring systems. Sub-classification by applying the Milan criteria and up to 7 criteria had been proposed, while classifying HCC patients in the intermediate stage into B1, B2, B3, B4, and Quasi-C groups according to basal liver function and the presence of marginal portal thrombus. The HAP score began as a predictive model for the effectiveness of the first TACE by scoring serum albumin, serum total bilirubin, serum alpha-fetoprotein, and maximum tumor size. Moreover, the modified HAP-II scoring system, which additionally evaluated the presence of multiple tumors, showed efficacy in 280 patients with HCC in Korea. The ART score system, including the Child-Pugh score, serum aspartate aminotransferase, and response evaluation after the first TACE, has not shown efficacy in other studies of HCC patients with chronic hepatitis C in Japan, Italy, and France and chronic hepatitis B in Taiwan, and it was also ineffective in evaluating the efficacy of repeated drug-eluting bead TACE for HCC patients in a Spanish multicenter study. However, combined application of the ART score after classification with the HAP score showed a significant efficacy in predicting TACE refractoriness in a recent multicenter study, and an algorithm for performing repeat TACE in early- and intermediate-stage HCC was suggested, and repeated TACE could be performed for patients with an ART score less than 2.5 after the first TACE.

Some recent studies have shown that obtaining a complete re-
response at an earlier time strongly predicted a favorable survival outcome in patients with HCC. Moreover, BCLC stage, pre-treatment alpha-fetoprotein level, and tumor number were also independent risk factors for overall survival along these studies. This indicates not only the on-treatment response but also baseline tumor characteristics and laboratory data could be used as official predictors for TACE refractoriness in the guideline. Assessing TACE refractoriness as quickly as possible could provide clinicians with the opportunity to make treatment strategies easier (such as localized radiotherapy, TACE, and concurrent therapies) and to increase the effectiveness of these next treatment strategies.

According to the results of this survey, the treatment of HCC favored by participants were not significantly different from the treatments recommended in the BCLC stage or proposed by the various scoring systems or sub-classifications as mentioned above. Of course, the management of HCC is difficult to simply standardize and the clinical judgment of the primary care physician considering individual tumor characteristics, economic situation, and the familial environment is still important, but is difficult without sufficient clinical experience. Therefore, standardization of treatments that many physicians can use is still necessary. There is also a need for experts to continue to discuss the subdivision of guidelines for the treatment of HCC in Korea.

Authors’ contribution
JS Lee and DY Kim: Data acquisition, analysis and interpretation, drafting of manuscript, and statistical analysis.
DY Kim: Study concept and design, data analysis and interpretation, drafting and critical revision of manuscript, and study supervision.
BK Kim, SU Kim, JY Park, SH Ahn, JS Seong, and KH Han: Critical revision of the manuscript.
All authors approved the final version of manuscript.

Figure 4. Voting results for questions about the treatment strategies after transarterial chemoembolization (TACE) for the participants considered in the following situations. CTx, chemotherapy; HAIC, hepatic arterial infusion chemotherapy; HCC, hepatocellular carcinoma; CP, Child-Pugh classification.
Supplementary materials are available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

REFERENCES


Serum *Wisteria floribunda* agglutinin-positive human Mac-2 binding protein level predicts recurrence of hepatitis B virus-related hepatocellular carcinoma after curative resection

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**Background/Aims:** To investigate whether serum *Wisteria floribunda* agglutinin-positive human Mac-2-binding protein (WFA⁺-M2BP) can predict the recurrence of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) after curative resection.

**Methods:** Patients with chronic hepatitis B (CHB) who underwent curative resection for HCC between 2004 and 2015 were eligible for the study. Recurrence was sub-classified as early (<2 years) or late (≥2 years).

**Results:** A total of 170 patients with CHB were selected. During the follow-up period (median, 22.6 months), 64 (37.6%) patients developed recurrence. In multivariate analyses, WFA⁺-M2BP level was an independent predictor of overall (hazard ratio [HR]=1.490), early (HR=1.667), and late recurrence (HR=1.416), together with male sex, des-gamma carboxyprothrombin level, maximal tumor size, portal vein invasion, and satellite nodules (all *P*<0.05). However, WFA⁺-M2BP level was not predictive of grade B-C posthepatectomy liver failure. The cutoff value that maximized the sum of sensitivity (30.2%) and specificity (90.6%) was 2.14 (area under receiver operating characteristic curve=0.632, *P*=0.010). Patients with a WFA⁺-M2BP level >2.14 experienced recurrence more frequently than those with a WFA⁺-M2BP level ≤2.14 (*P*=0.011 by log-rank test), and had poorer postoperative outcomes than those with a WFA⁺-M2BP level ≤2.14 in terms of overall recurrence (56.0 vs. 34.5%, *P*=0.047) and early recurrence (52.0 vs. 20.7%, *P*= 0.001).

**Conclusions:** WFA⁺-M2BP level is an independent predictive factor of HBV-related HCC recurrence after curative resection. Further studies should investigate incorporation of WFA⁺-M2BP level into tailored postoperative surveillance strategies for patients with CHB. *(Clin Mol Hepatol 2020;26:33-44)*

**Keywords:** Hepatitis B; Liver fibrosis; Biomarkers; Hepatocellular carcinoma; Prognosis

**Abbreviations:**

AFP, alpha-fetoprotein; AUC, area under receiver operating characteristic curve; BCLC, Barcelona Clinic Liver Cancer; CHB, chronic hepatitis B; CI, confidence interval; COI, cutoff index; DCP, des-gamma carboxyprothrombin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HR, hazard ratio; ICG R15, indocyanine green retention rate at 15 minutes; INR, international normalized ratio; IQR, interquartile range; PHLF, posthepatectomy liver failure; ROC, receiver operating characteristic; WFA⁺-M2BP, *Wisteria floribunda* agglutinin-positive human Mac-2 binding protein

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INTRODUCTION

If hepatocellular carcinoma (HCC) is detected in an early stage during surveillance, hepatic resection is strongly recommended for non-cirrhotic patients and is considered the mainstay of treatment in cirrhotic patients with well-preserved liver function. Although recent advances in the selection of candidates for resection, optimization of surgical techniques, and perioperative management have improved the postoperative outcomes of patients with HCC, recurrence remains the main cause of an unsatisfactory long-term prognosis after HCC resection. Thus, identification of risk factors for recurrence after curative resection is of paramount importance.

Postoperative recurrence can be divided into early (<2 years) and late (≥2 years) recurrence, which have different underlying mechanisms. Advanced liver fibrosis or cirrhosis is a risk factor for late recurrence, as it contributes to multicentric recurrence in the remnant liver. In addition, advanced liver fibrosis or cirrhosis is closely correlated with short-term poor postoperative outcomes such as postoperative mortality or complications (e.g., liver failure and hepatic decompensation). Thus, preoperative assessment of the fibrotic burden can improve long-term outcomes by enabling selection of patients who will benefit from curative resection. Alternatively, other treatment modalities, such as liver transplantation, can be recommended to patients at greater risk for postoperative recurrence after curative resection.

Efforts have been made to expand the role of noninvasive surrogates for liver biopsy into the surgical setting, beyond simple assessment of the fibrotic burden. Indeed, preoperative liver stiffness values determined by transient elastography are reportedly predictive of the risk of the development of hepatic insufficiency, recurrence, and survival after curative HCC resection. Recently, a glycan-based immunoassay has been introduced; the assay targets Wisteria floribunda agglutinin-positive human Mac-2 binding protein (WFA-M2BP) as a noninvasive biomarker of liver fibrosis and predictor of HCC. Although a recent Japanese study found that WFA-M2BP was predictive of recurrence and survival in patients with early-stage HCC who underwent curative resection, the clinical role of WFA-M2BP in surgical settings is still unclear.

Therefore, we conducted this single-center, retrospective cohort study to investigate the longitudinal prognostic performance of WFA-M2BP in predicting HCC recurrence in patients with hepatitis B virus (HBV)-related HCC who underwent curative resection.

PATIENTS AND METHODS

Patients

Between 2004 and 2015, 492 patients who underwent curative resection for HBV-related HCC were considered eligible. Chronic hepatitis B (CHB) was defined as persistence of serum HBV surface antigen for >6 months. The exclusion criteria were no or an insufficient stored serum sample for WFA-M2BP assays, serum sample collected after resection, insufficient clinical data for statistical analysis, positive resection margin for HCC, co-infection with hepatitis C virus or HIV, and alcohol ingestion of >40 g/day for >5 years.

This study was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board of Severance Hospital. Given its retrospective nature, written informed consent for clinical data was not required.

WFA-M2BP assay

Serum WFA-M2BP was quantified preoperatively through a lectin-Ab sandwich immunoassay using a fully automated immunoanalyzer (HISCL-2000i; Sysmex Co, Hyogo, Japan). For WFA-M2BP assays, the remaining serum samples after HBV DNA quantification during the study period which have been stored at −70°C, were used. Measured levels of analyte conjugated with Wisteria floribunda agglutinin were indexed using the following equation:

\[
\text{Cutoff index (COI)} = \frac{[\text{WFA}^+\text{-M2BP}]_{\text{sample}} - [\text{WFA}^+\text{-M2BP}]_{\text{HC}}}{[\text{WFA}^+\text{-M2BP}]_{\text{NC}2} - [\text{WFA}^+\text{-M2BP}]_{\text{HC}}}.
\]
Postoperative day 5 (compared to the values of the previous day). Postoperative complications were categorized according to the modified Clavien-Dindo classification (Supplementary Table 1). A major complication was defined as grade 3 or above. Recurrence was diagnosed based on the combined findings of the clinical examinations and sub-classified as early (<2 years) or late (≥2 years) recurrence.

**Preoperative work-up, surgery, and follow-up**

Diagnosis of HCC was based on the guidelines proposed by the Korea Liver Cancer Study Group. Preoperative HCC staging was assessed using the Barcelona Clinic Liver Cancer (BCLC) staging system. During follow-up, antiviral therapy was administered in accordance with the guidelines of the Korean Association for the Study of the Liver.

As described in our previous study, all resections were performed by four experienced surgeons (DH Han, GH Choi, KS Kim, and JS Choi). The type and extent of resections were determined according to tumor size, location, and liver reserve function estimated by the Child-Pugh score and indocyanine green retention rate at 15 minutes (ICG R15). Liver resection was defined as minor when two or fewer liver segments were resected, whereas major hepatectomy was defined as the resection of three or more liver segments. Intraoperative ultrasonography was performed routinely to determine tumor location and extent and to exclude the presence of additional lesions in the residual liver. Curative resection was defined as a negative pathological surgical margin and the absence of residual tumor. After discharge, patients were followed up every 3 months with tumor markers (alpha-fetoprotein [AFP] and des-gamma carboxy prothrombin [DCP]) and imaging studies, including abdominal computed tomography or magnetic resonance imaging.

**Defining outcomes**

Posthepatectomy liver failure (PHLF) was defined and graded by the recommendations of the International Study Group of Liver Surgery (ISGLS). PHLF is defined as an increased international normalized ratio (INR) and hyperbilirubinemia on or after postoperative day 5. Grade A PHLF does not require deviations from regular clinical management, whereas grades B and C PHLF require noninvasive (grade B) or invasive treatment (grade C). If the INR or serum bilirubin concentration is increased preoperatively, PHLF is defined as an increasing serum bilirubin concentration on or after postoperative day 5 (compared to the values of the previous day). Postoperative complications were categorized according to the modified Clavien-Dindo classification (Supplementary Table 1). A major complication was defined as grade 3 or above. Recurrence was diagnosed based on the combined findings of the clinical examinations and sub-classified as early (<2 years) or late (≥2 years) recurrence.

**Histological assessment of tumor and non-tumor liver tissue**

As described in our previous study, a histological evaluation of the extracted liver specimens was performed by one experienced pathologist (YN Park) who was blinded to the patients’ clinical information. Gross tumor classification, tumor size and number, vascular invasion, satellite nodule, and Edmondson-Steiner grade were determined. Liver fibrosis was evaluated semiquantitatively in non-cancerous tissues according to the Batts system. Fibrosis was staged on a 0–4 scale: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and a few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.

**Statistical analysis**

Data are expressed as medians (interquartile range [IQR]) or n (%), as appropriate. Differences among continuous and categorical variables were examined using Student’s t-tests (or Mann-Whitney tests) and chi-square tests (or Fisher’s exact tests), respectively.

For longitudinal analysis, patients were followed up until the date of HCC recurrence or the last visit. Cumulative HCC recurrence rates were estimated using the Kaplan-Meier method and compared using log-rank tests. To identify independent risk factors for recurrence, univariate and multivariate Cox regression analyses were performed. In analyses of predictors of early recurrence (<2 years), the analytic population included patients without recurrence during the study period, patients with recurrence after 2 years who were considered to not have had recurrence until the time of recurrence and censored at the same time, and patients with recurrence within 2 years. In analyses of predictors of late recurrence (≥2 years), the analytic population included patients without recurrence, only if their follow-up period was more than 2 years, and patients with recurrence irrespective of follow-up time. To identify independent risk factors for developing grade B-C PHLF, univariate and multivariate binary regression analyses were performed. Hazard ratios (HRs) and corresponding 95%...
confidence intervals (CIs) were calculated. The diagnostic performance of WFA+M2BP was determined using receiver operating characteristic (ROC) curves and the area under ROC curve (AUC). In addition, the cutoff values for predicting recurrence that maximized the sum of sensitivity (Se) and specificity (Sp) (Cutoff_ww+M2BP), the cutoff values that showed positive predictive value (PPV) >80% (Cutoff_ww-risk), and the cutoff values that showed negative predictive value (NVP) >80% (Cutoff_ww-risk) were also calculated. All statistical analyses were performed using SPSS software (v20.0; IBM Corp., Armonk, NY, USA). A P-value <0.05 in two-tailed tests was considered to indicate statistical significance.

RESULTS

Baseline characteristics

After excluding 322 patients according to the exclusion criteria, 170 patients with HBV-related HCC were selected for statistical analyses (Supplementary Fig. 1). The baseline characteristics of the study population before curative resection are summarized in Table 1. The median age of the entire cohort (132 males and 38 females) was 55 years. Most patients had well-preserved liver function of Child-Pugh class A (n=168, 98.8%). The median AFP, DCP, ICG R15, and WFA+M2BP levels were 13.0 ng/mL, 44 mAU/mL, 8.4%, and 1.02 COI, respectively. HCC in BCLC stage 0 and A were identified in 41 (24.1%) and 122 (71.8%) patients, respectively. Of the patients, 126 (74.1%) were receiving antiviral therapy at the time of resection.

Perioperative outcomes and pathological information

Perioperative outcomes and pathological information are summarized in Table 2. A total of 51 (30.0%) patients underwent major hepatectomy. Most patients received open surgery (n=113, 66.5%). The median amount of blood loss was 250 mL and blood

<table>
<thead>
<tr>
<th>Table 1. Baseline characteristics of the study population (n=170)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Variable</strong></td>
</tr>
<tr>
<td>Demographical variables</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>Male sex</td>
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<tr>
<td>Body mass index (kg/m²)</td>
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<tr>
<td>Child-Pugh class A/B</td>
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<tr>
<td>Laboratory variables</td>
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<tr>
<td>Aspartate aminotransferase (IU/L)</td>
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<tr>
<td>Alanine aminotransferase (IU/L)</td>
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<tr>
<td>Platelet count (10⁹/L)</td>
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<td>HBV-DNA levels (log₁₀ IU/mL)</td>
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<tr>
<td>Alpha-fetoprotein (ng/mL)</td>
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<tr>
<td>Des-gamma carboxyprothrombin (mAU/mL)</td>
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<tr>
<td>ICG R15 (%)</td>
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<tr>
<td>WFA+M2BP (COI)</td>
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<tr>
<td>Tumor stage</td>
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<tr>
<td>BCLC stage 0/A/B</td>
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<tr>
<td>On-going antiviral therapy</td>
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</table>

Variables are expressed as median (interquartile range) or n (%).

<table>
<thead>
<tr>
<th>Table 2. Perioperative outcomes and pathological characteristics</th>
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<tbody>
<tr>
<td><strong>Variable</strong></td>
</tr>
<tr>
<td>Operative variables</td>
</tr>
<tr>
<td>Type of hepatectomy (major/minor)</td>
</tr>
<tr>
<td>Surgical approach (open/laparoscopic/robot)</td>
</tr>
<tr>
<td>Blood loss (mL)</td>
</tr>
<tr>
<td>Transfusion</td>
</tr>
<tr>
<td>Postoperative variables</td>
</tr>
<tr>
<td>PHLF grading (0–A/B–C)</td>
</tr>
<tr>
<td>Complications (I/II/IIIa-b/IV-V)</td>
</tr>
<tr>
<td>Tumor pathology</td>
</tr>
<tr>
<td>Maximal tumor size (cm)</td>
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<tr>
<td>Single/multiple tumors</td>
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<tr>
<td>Portal vein invasion</td>
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<tr>
<td>Microscopic vascular invasion</td>
</tr>
<tr>
<td>Satellite nodule</td>
</tr>
<tr>
<td>Edmondson-Steiner grade III-V</td>
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<tr>
<td>Non-tumor pathology</td>
</tr>
<tr>
<td>FO-3/F4 fibrosis</td>
</tr>
<tr>
<td>A1-2/A3 activity grade</td>
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</tbody>
</table>

Variables are expressed as median (interquartile range) or n (%). PHLF, posthepatectomy liver failure.
transfusion was required in 12 (7.1%) patients. Grade B-C PHLF developed in eight (4.8%) patients. Seven (4.1%) patients experienced major postoperative complications; the most common was pleural effusion (n=4). In addition, bile leakage, ascites, and an umbilical incisional hernia developed in one patient each.

The median maximal tumor size was 2.4 cm, and 18 (10.6%) patients had multiple tumors. Portal vein invasion, microscopic vascular invasion, and satellite nodules were identified in 15 (8.9%), 78 (46.2%), and 12 (7.1%) patients, respectively. Regarding non-tumor pathology, 99 (58.2%) patients had F4 fibrosis. The WFA\(^+\)-M2BP level was significantly higher in patients with F4 than those with F0–3 fibrosis (median 1.18 [IQR 0.79–1.86] vs. 0.91 [0.67–1.31]; P=0.045).

**Comparison between patients with and without HCC recurrence**

During the follow-up period (median 22.6 [IQR 13.3–61.0] months), HCC recurred in 64 (37.6%) patients. The cumulative incidence of HCC recurrence at 1, 2, 3, 4, and 5 years was 15.8%, 25.3%, 30.0%, 32.9%, and 34.1%, respectively (Supplementary Fig. 2). The most common site of recurrence was the liver (n=58, 90.6%).

A comparison of baseline characteristics between patients who developed recurrence and those who did not is shown in Table 3. Patients who developed recurrence had a higher HBV-DNA level (median 2.92 vs. 2.16 log_10 IU/mL), higher WFA\(^+\)-M2BP level (median 1.22 vs. 0.91, COI), higher blood loss (median 330 vs. 250 mL), greater maximal tumor size (median 2.6 vs. 2.2 cm), and higher proportions of portal vein invasion (17.5 vs. 3.8%), microscopic vascular invasion (58.7 vs. 38.7%), and satellite nodules (12.9 vs. 3.8%) than patients who did not experience recurrence (all P<0.05).

**Independent predictors of overall, early, and late recurrence**

Among the 64 patients with recurrence, 43 (67.2%) experienced early recurrence and 21 (32.8%) experienced late recurrence. We evaluated independent predictors of overall, early, and late recurrence (Table 4). In univariate analyses, male sex, AFP level, DCP level, WFA\(^+\)-M2BP level, maximal tumor size, multiple tumors, portal vein invasion, microscopic vascular invasion, and the presence of satellite nodules were significantly predictive of overall, early, and late recurrence (all P<0.05), with the exception of borderline significance between multiple tumors and early recurrence (P=0.051). In multivariate analyses, WFA\(^+\)-M2BP was an independent predictor of overall, early, and late recurrence (HR=1.490, 1.667, and 1.416, respectively; all P<0.05), together with other factors such as male sex, DCP, maximal tumor size, portal vein invasion, and satellite nodules.

**Independent predictor of grade B-C PHLF**

Univariate analyses identified aspartate aminotransferase level and major hepatectomy as significantly predictive of grade B-C PHLF (all P<0.05). In subsequent multivariate analyses, major hepatectomy was the only independent predictor of grade B-C PHLF (HR=4.254, 95% CI 1.824–9.919; P=0.001) (Supplementary Table 2).

**Optimal cutoff values of WFA\(^+\)-M2BP to predict recurrence**

Considering the median follow-up period (22.6 months), cutoff values for predicting 2-year recurrence in the whole study population and several subgroups were calculated based on ROC curve analyses (Table 5). The WFA\(^+\)-M2BP cutoff value that maximized the sum of sensitivity (30.2%) and specificity (90.6%) was 2.14 COI (AUC=0.632, P=0.010). Patients with a WFA\(^+\)-M2BP level >2.14 COI had a significantly higher cumulative recurrence rate than those with a WFA\(^+\)-M2BP level ≤2.14 COI (P=0.011 by log-rank test) (Fig. 1). The cumulative incidences of HCC recurrence at 1, 2, 3, 4, and 5 years in patients with WFA\(^+\)-M2BP levels >2.14 COI were 32.0%, 52.0%, 56.0%, 56.0%, and 56.0%, respectively, whereas those in patients with WFA\(^+\)-M2BP levels ≤2.14 COI were 13.1%, 20.7%, 25.5%, 29.0%, and 30.0%, respectively. Using cutoff values for low and high risks of recurrence of 0.57 COI (negative predictive value 89.4) and 3.88 (PPV 89.9%), respectively (Table 5), only 1 (6.2%) of 16 patients with a low risk of recurrence experienced recurrence, whereas 5 (83.3%) of 6 patients with a high risk of recurrence experienced recurrence.

In patients with histological cirrhosis (n=99, 58.2%), the cutoff value of WFA\(^+\)-M2BP that maximized the sum of sensitivity (88.9%) and specificity (50.0%) was 0.94 COI (AUC=0.720, P=0.038) in patients who had undergone major hepatectomy (n=51, 30.0%).
### Table 3. Comparison between patients who did and did not experience hepatocellular carcinoma (HCC) recurrence

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients who developed HCC recurrence (n=64, 37.6%)</th>
<th>Patients who did not develop HCC recurrence (n=106, 62.4%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographical variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>55 (51–59)</td>
<td>55 (51–62)</td>
<td>0.273</td>
</tr>
<tr>
<td>Male sex</td>
<td>55 (85.9)</td>
<td>77 (72.6)</td>
<td>0.057</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.3 (21.6–25.9)</td>
<td>23.6 (21.9–25.0)</td>
<td>0.766</td>
</tr>
<tr>
<td>Child-Pugh class A/B</td>
<td>63 (98.4)/1 (1.6)</td>
<td>105 (99.1)/1 (0.9)</td>
<td>0.717</td>
</tr>
<tr>
<td><strong>Laboratory variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartate aminotransferase (IU/L)</td>
<td>28 (24–39)</td>
<td>27 (22–34)</td>
<td>0.814</td>
</tr>
<tr>
<td>Alanine aminotransferase (IU/L)</td>
<td>30 (20–43)</td>
<td>27 (18–43)</td>
<td>0.739</td>
</tr>
<tr>
<td>Platelet count (10⁹/L)</td>
<td>164 (126–204)</td>
<td>153 (236–196)</td>
<td>0.626</td>
</tr>
<tr>
<td>HBV-DNA levels (log₁₀ IU/mL)</td>
<td>2.92 (1.00–5.01)</td>
<td>2.16 (0.50–3.47)</td>
<td>0.016</td>
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<tr>
<td>Alpha-fetoprotein (ng/mL)</td>
<td>27.4 (5.0–221.3)</td>
<td>8.1 (2.9–82.9)</td>
<td>0.275</td>
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<tr>
<td>Des-gamma carboxyprothrombin (mAU/mL)</td>
<td>55 (27–239)</td>
<td>40 (26–134)</td>
<td>0.341</td>
</tr>
<tr>
<td>ICG R15 (%)</td>
<td>9.4 (6.3–12.2)</td>
<td>8.0 (6.3–11.7)</td>
<td>0.924</td>
</tr>
<tr>
<td>WFA⁺-M2BP (COI)</td>
<td>1.22 (0.79–1.79)</td>
<td>0.91 (0.68–1.41)</td>
<td>0.017</td>
</tr>
<tr>
<td><strong>Tumor stage</strong></td>
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<td></td>
</tr>
<tr>
<td>BCLC stage 0-A/B</td>
<td>59 (92.2)/5 (7.8)</td>
<td>104 (98.1)/2 (1.9)</td>
<td>0.060</td>
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<tr>
<td><strong>On-going antiviral therapy</strong></td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>48 (75.0)</td>
<td>78 (73.6)</td>
<td>0.838</td>
</tr>
<tr>
<td><strong>Operative variables</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Type of hepatectomy (major/minor)</td>
<td>49 (76.6)/15 (23.4)</td>
<td>36 (34.0)/70 (66.0)</td>
<td>0.147</td>
</tr>
<tr>
<td>Surgical approach (open/laparoscopic+robot)</td>
<td>42 (65.6)/22 (34.3)</td>
<td>71 (67.0)/35 (33.0)</td>
<td>0.868</td>
</tr>
<tr>
<td>Transfusion</td>
<td>5 (7.8)</td>
<td>7 (6.6)</td>
<td>0.766</td>
</tr>
<tr>
<td>Blood loss (mL)</td>
<td>330 (150–750)</td>
<td>250 (100–450)</td>
<td>0.018</td>
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<tr>
<td><strong>Postoperative variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHLF grading (0-A/B-C)</td>
<td>59 (96.7)/2 (3.3)</td>
<td>99 (94.3)/6 (5.7)</td>
<td>0.480</td>
</tr>
<tr>
<td>Complications (I-II/IIia-b)</td>
<td>62 (96.9)/2 (3.1)</td>
<td>101 (95.3)/5 (4.7)</td>
<td>0.613</td>
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<tr>
<td><strong>Tumor pathology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal tumor size (cm)</td>
<td>2.6 (2.0–3.8)</td>
<td>2.2 (1.8–3.0)</td>
<td>0.038</td>
</tr>
<tr>
<td>Multiple tumors</td>
<td>10 (15.6)</td>
<td>8 (7.5)</td>
<td>0.097</td>
</tr>
<tr>
<td>Portal vein invasion</td>
<td>11 (17.5)</td>
<td>4 (3.8)</td>
<td>0.003</td>
</tr>
<tr>
<td>Microscopic vascular invasion</td>
<td>37 (58.7)</td>
<td>41 (38.7)</td>
<td>0.016</td>
</tr>
<tr>
<td>Satellite nodule</td>
<td>8 (12.9)</td>
<td>4 (3.8)</td>
<td>0.033</td>
</tr>
<tr>
<td>Edmondson-Steiner grade III-V</td>
<td>34 (56.7)</td>
<td>57 (54.3)</td>
<td>0.767</td>
</tr>
<tr>
<td><strong>Non-tumor pathology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F4 fibrosis</td>
<td>40 (62.5)</td>
<td>59 (55.7)</td>
<td>0.381</td>
</tr>
<tr>
<td>A3 activity grade</td>
<td>40 (62.5)</td>
<td>5 (4.7)</td>
<td>0.993</td>
</tr>
</tbody>
</table>

Variables are expressed as median (interquartile range) or n (%) unless otherwise indicated.

HBV, hepatitis B virus; ICG R15, indocyanine green retention rate at 15 minutes; WFA⁺-M2BP, *Wisteria floribunda* agglutinin-positive human Mac-2 binding protein; BCLC, Barcelona Clinic Liver Cancer; PHLF, posthepatectomy liver failure.
Table 4. Independent predictors of overall, early (<2 years), and late (≥2 years) recurrence

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall recurrence</th>
<th>Early recurrence</th>
<th>Late recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uni</td>
<td>Multi</td>
<td>Uni</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Demographical variables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.517</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Male sex</td>
<td>0.042</td>
<td>2.480</td>
<td>1.181–5.205</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>0.984</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Child-Pugh class B (vs. A)</td>
<td>0.405</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Laboratory variables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>0.806</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>0.943</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Platelet count (10⁹/L)</td>
<td>0.647</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HBV-DNA levels (log₁₀ IU/mL)</td>
<td>0.959</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AFP (ng/mL)</td>
<td>&lt;0.001</td>
<td>1.000</td>
<td>1.000–1.000</td>
</tr>
<tr>
<td>DCP (mAU/mL)</td>
<td>0.003</td>
<td>1.000</td>
<td>0.999–1.000</td>
</tr>
<tr>
<td>ICG R15 (%)</td>
<td>0.805</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WFA–M2BP (COI)</td>
<td>&lt;0.001</td>
<td>1.490</td>
<td>1.110–2.001</td>
</tr>
<tr>
<td>Tumor variables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal tumor size (cm)</td>
<td>&lt;0.001</td>
<td>1.333</td>
<td>1.045–1.700</td>
</tr>
<tr>
<td>Multiple tumors (vs. single)</td>
<td>0.043</td>
<td>1.605</td>
<td>0.756–3.405</td>
</tr>
<tr>
<td>Portal vein invasion</td>
<td>0.001</td>
<td>3.101</td>
<td>1.373–6.599</td>
</tr>
<tr>
<td>Microscopic vascular invasion</td>
<td>0.003</td>
<td>1.320</td>
<td>0.714–2.443</td>
</tr>
<tr>
<td>Satellite nodule</td>
<td>0.002</td>
<td>2.395</td>
<td>1.012–5.664</td>
</tr>
<tr>
<td>ES III-V (vs. I-II)</td>
<td>0.267</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Histological variables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F4 fibrosis (vs. F0-3)</td>
<td>0.433</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A3 activity grade (vs. A0-2)</td>
<td>0.451</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>On-going antiviral therapy</td>
<td>0.475</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

HR, hazard ratio; CI, confidence interval; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HBV, hepatitis B virus; AFP, alpha-fetoprotein; DCP, des-gamma carboxyprothrombin; ICG R15, indocyanine green retention rate at 15 minutes; WFA–M2BP, Wisteria floribunda agglutinin-positive human Mac-2 binding protein; ES, Edmondson-Steiner.
Postoperative outcomes according to WFA\(^+\)-M2BP
cutoff value

Postoperative outcomes according to preoperative WFA\(^+\)-M2BP
cutoff value are shown in Table 6. Patients with WFA\(^+\)-M2BP levels
>2.14 (n=25, 14.7%) had a significantly higher risk of overall re-
currence (56.0% vs. 34.5%) and early recurrence (52.0% vs.
20.7%) than patients with WFA\(^+\)-M2BP levels ≤2.14 (all \(P<0.05\)).
Although the risk of late recurrence was higher in patients with
WFA\(^+\)-M2BP levels >2.14 compared to those with WFA\(^+\)-M2BP
levels ≤2.14, the association was of marginal significance
(\(P=0.072\)). The risks of grade B-C PHLF and major complications
were statistically similar (all \(P>0.05\)) (Table 6).

DISCUSSION

Serum levels of WFA\(^+\)-M2BP may be useful as a marker of the
degree of liver fibrosis. WFA\(^+\)-M2BP is secreted by hepatic stellate
cells and enhances synthesis of Mac-2 by Kupffer cells, which ren-
ders hepatic stellate cells fibrogenic.\(^{20}\) WFA\(^+\)-M2BP is a “dough-
nut-shaped” glycoprotein, changes in the N-glycan structure of
which mirror the extent of liver fibrosis.\(^{21}\) Automated rapid assays
for these changes have been developed for noninvasive assess-
ment of fibrotic burden. Indeed, the diagnostic accuracy of WFA\(^+\)-
M2BP for assessing the degree of liver fibrosis has been demon-
strated in patients with CHB, chronic hepatitis C, non-alcoholic
fatty liver disease, and biliary atresia.\(^{21-26}\) In addition, WFA\(^+\)-M2BP
levels are predictive of the risk of development of HBV- and HCV-
related HCC.\(^{11,23}\)

Our study has several strengths. First, because the significant

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**Table 5. WFA\(^+\)-M2BP cutoff values for 2-year recurrence and corresponding diagnostic indices**

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Cutoff values</th>
<th>AUC</th>
<th>95% CI</th>
<th>(P)-value</th>
<th>Se (%)</th>
<th>Sp (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entire population</td>
<td>0.632</td>
<td>0.534–0.730</td>
<td>0.010</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutoff(_{\text{low-risk}})</td>
<td>2.14</td>
<td></td>
<td></td>
<td></td>
<td>30.2</td>
<td>90.6</td>
<td>65.9</td>
<td>68.3</td>
</tr>
<tr>
<td>Cutoff(_{\text{high-risk}})</td>
<td>0.57</td>
<td></td>
<td></td>
<td></td>
<td>97.7</td>
<td>11.8</td>
<td>40.1</td>
<td>89.4</td>
</tr>
<tr>
<td>Cutoff(_{\text{high-risk}})</td>
<td>3.88</td>
<td></td>
<td></td>
<td></td>
<td>11.6</td>
<td>99.2</td>
<td>89.9</td>
<td>65.0</td>
</tr>
<tr>
<td>Histological cirrhosis</td>
<td>0.720</td>
<td>0.608–0.833</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutoff(_{\text{low-risk}})</td>
<td>0.94</td>
<td></td>
<td></td>
<td></td>
<td>88.9</td>
<td>50.0</td>
<td>54.7</td>
<td>86.9</td>
</tr>
<tr>
<td>Cutoff(_{\text{high-risk}})</td>
<td>0.94</td>
<td></td>
<td></td>
<td></td>
<td>88.9</td>
<td>50.0</td>
<td>54.7</td>
<td>86.9</td>
</tr>
<tr>
<td>Cutoff(_{\text{high-risk}})</td>
<td>3.88</td>
<td></td>
<td></td>
<td></td>
<td>11.1</td>
<td>98.6</td>
<td>84.4</td>
<td>62.1</td>
</tr>
<tr>
<td>Major hepatectomy</td>
<td>0.706</td>
<td>0.513–0.898</td>
<td>0.038</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutoff(_{\text{low-risk}})</td>
<td>2.31</td>
<td></td>
<td></td>
<td></td>
<td>45.5</td>
<td>97.5</td>
<td>88.3</td>
<td>81.1</td>
</tr>
<tr>
<td>Cutoff(_{\text{low-risk}})</td>
<td>0.69</td>
<td></td>
<td></td>
<td></td>
<td>90.9</td>
<td>37.5</td>
<td>37.7</td>
<td>90.8</td>
</tr>
<tr>
<td>Cutoff(_{\text{low-risk}})</td>
<td>2.31</td>
<td></td>
<td></td>
<td></td>
<td>45.5</td>
<td>97.5</td>
<td>88.3</td>
<td>81.1</td>
</tr>
</tbody>
</table>

WFA\(^+\)-M2BP, Wisteria floribunda agglutinin-positive human Mac-2 binding protein; AUC, area under receiver operating characteristic curve; CI, confidence interval; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.

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**Figure 1.** Cumulative incidence of hepatocellular carcinoma (HCC) recurrence according to WFA\(^+\)-M2BP level. The cumulative incidence increased significantly in patients with WFA\(^+\)-M2BP levels >2.14 than in those with WFA\(^+\)-M2BP levels ≤2.14 (log-rank test, \(P=0.011\)). WFA\(^+\)-M2BP, Wisteria floribunda agglutinin-positive human Mac-2-binding protein.
correlation between fibrotic burden assessed using WFA⁺-M2BP and the risk of HCC development was confirmed in previous studies, the ability of WFA⁺-M2BP to predict the risk of HCC recurrence due to remnant fibrotic liver after curative resection should be investigated. Although serum levels of WFA⁺-M2BP measured at the time of curative resection are reportedly an independent predictor of HCC recurrence and survival in patients undergoing curative resection of HCC, most of the patients in the study had HCV-related HCC (68.8%). Because the natural course of disease and the antiviral treatment strategy for HCV and HBV differ, it is important to validate the role of WFA⁺-M2BP in patients with HBV-related HCC undergoing resection. The recent reports that WFA⁺-M2BP levels are lower in patients with CHB than in those with CHC also support the need for validation of WFA⁺-M2BP in patients with HBV-related HCC. To the best of our knowledge, this is the first report to focus on the prognostic value of WFA⁺-M2BP for HBV-related HCC recurrence and survival in patients undergoing curative resection.

Second, we comprehensively investigated the role of WFA⁺-M2BP in predicting overall, early, and late recurrence as well as PHLF; the results suggested that WFA⁺-M2BP is predictive of all types of recurrence. Although early recurrence is generally associated with tumor factors, not those of the background liver, WFA⁺-M2BP levels were independently associated with the risk of early recurrence in our study, together with several tumor factors such as AFP, DCP, maximal tumor size, portal vein invasion, and the presence of satellite nodules. However, the ability of WFA⁺-M2BP levels to predict early recurrence warrants further investigation.

Third, fibrosis stage (F0–3 vs. F4) was not predictive of recurrence in this study. This may be because the overall sample size was not sufficient to evaluate the prognostic accuracy of histological stage in terms of recurrence. In addition, more than half of the patients had histological cirrhosis (58.2%). These factors might cause spectrum bias and potentially false-negative results. Also, it can be postulated that the linear expression of WFA⁺-M2BP with using the best cutoff values to predict recurrence might be better in predicting recurrence than histological information with step-wise crude expression. Furthermore, previous studies have reported other surrogates for liver fibrosis (such as transient elastography) to be predictive of HCC recurrence after curative resection in patients with CHB, not histologic fibrosis stage. However, because the predictive accuracy of histological stage has been reported, our results warrant further investigation.

Fourth, we determined the optimal cutoff level of WFA⁺-M2BP for predicting overall recurrence. This value (2.14) stratified subgroups with different risks of overall recurrence. Interestingly, our cutoff was lower than that of a previous study involving patients with HCV-related HCC undergoing curative resection (vs. 3.0). This is likely because over half of the study population had F4 stage fibrosis, and because HBV-related cirrhosis tends to be micronodular, leading to a lesser fibrotic burden than HCV-related cirrhosis. A lower cutoff WFA⁺-M2BP level in patients with HBV has been reported by others. In addition, a lower fibrotic burden in each fibrosis stage among patients with HBV-related chronic liver disease has been reported by previous studies of other surrogates for liver fibrosis (e.g., transient elastography). We also proposed clinically relevant cutoff values to enable identification of low- and high-risk patients. Only 6.2% of low-risk patients experienced recurrence, compared to >80% of high-risk patients. These cutoff values might facilitate development of improved follow-up and management strategies.

This study also had several weaknesses. First, due to the insufficient sample size, a binary risk stratification of the study population using the calculated cutoff values was adopted. However, because a certain proportion of patients experienced recurrence of HCC despite a WFA⁺-M2BP level lower than the cutoff value, fu-
ture large-scale studies should involve multi-stage detailed risk stratification. Second, the effect of WFA+M2BP levels at the time of resection on overall survival could not be investigated because of the relatively short follow-up period (median, 22.6 months) and low mortality rate (n=17, 10.0%). Further studies with long-term follow-up are required to resolve this issue. Third, because this was a retrospective study and more than half of the participants were excluded, which might have caused selection bias, the results should be interpreted with caution. In addition, the distribution of each fibrosis stage was skewed (F1 in 1 [0.6%] patient, F2 in 25 [14.7%] patients, F3 in 45 [26.5%] patients, and F4 in 99 [58.2%] patients), which might resulted in the statistically non-significant prognostic value of F4 vs. F0–3. Probably due to the similar reason, fibrosis grade as an ordinal variable and different binary stratification such as F3–4 vs. F0–2 were significant neither. Finally, noninvasive surrogates for liver fibrosis, including WFA+M2BP level, can predict the risk of developing PHLF after curative resection. However, we could not demonstrate that WFA+M2BP was predictive of grade B-C PHLF in patients with HBV-related HCC undergoing curative resection. Major hepatectomy was the only independent predictor of grade B-C PHLF in this study.

In conclusion, our findings indicate a significant association between WFA+M2BP level and the risk of HBV-related HCC recurrence after curative resection. However, it should be validated in further studies whether current strategies to select optimal candidates for resection and to evaluate the risk of postoperative recurrence can be optimized according to preoperative WFA+M2BP values.

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Conflicts of Interest
None to declare for all authors.

SUPPLEMENTARY MATERIALS
Supplementary materials are available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

REFERENCES


Vimentin as a potential therapeutic target in sorafenib resistant HepG2, a HCC model cell line

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Departments of Experimental Medicine and Biotechnology and 2Hepatology, Postgraduate Institute of Medical Education & Research, Chandigarh, India

Background/Aims: Hepatocellular carcinoma (HCC) is the most common liver cancer with high mortality rate in patients suffering from liver diseases. The drug of choice used in advanced-stage of HCC is sorafenib. However, adaptive resistance has been observed in HCC patients undergoing long-term sorafenib treatment, lowering its effectiveness. Hence, it is important to overcome drug resistance to improve overall management of HCC. Here, we have identified a candidate biomarker for sorafenib resistance in a HCC model cell line, HepG2.

Methods: Initially, comparative proteomic profiling of parental HepG2 [HepG2 (P)] and sorafenib-resistant HepG2 [HepG2 (R)] cells was performed via MALDI (matrix-assisted laser desorption/ionization) which revealed the deregulation of vimentin in HepG2 (R) cells. Gene and protein level expression of vimentin was also observed through quantitative real-time polymerase chain reaction (qRT PCR) and fluorescence-activated cell sorting (FACS), respectively. Furthermore, withaferin A was used to study regulation of vimentin expression and its significance in sorafenib resistance.

Results: Both gene and protein level of vimentin expression was found to be downregulated in HepG2 (R) in comparison to HepG2 (P). Interestingly, the study demonstrated that withaferin A further lowered the expression of vimentin in HepG2 (R) cells in a dose-dependent manner. Also, inhibition of vimentin lowered ABCG2 expression and decreased cell viability in parental as well as sorafenib resistant HepG2 cells.

Conclusions: Hence, our study for the first time highlighted the probable therapeutic potential of vimentin in sorafenib resistant HepG2, a HCC model cell line. (Clin Mol Hepatol 2020;26:45-53)

Keywords: Carcinoma, Hepatocellular; Sorafenib resistance; Vimentin; Withaferin A

Study Highlights
The present study highlights the significance of vimentin as potential therapeutic target as its inhibition with withaferin A results to overcome sorafenib resistance as well as reduces cancer cells viability.

Abbreviations:
conc., concentrations; DMSO, dimethyl sulfoxide; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GRP78, glucose regulated protein 78; HCC, hepatocellular carcinoma; HepG2 (P), HepG2 parental; HepG2 (R), HepG2 sorafenib resistant; IC, inhibitory concentration; MALDI, matrix-assisted laser desorption/ionization; MEM, minimum essential media; PBS, phosphate-buffered saline; PMF, peptide mass fingerprinting; qRT PCR, quantitative real-time polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TOF, time of flight

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths globally. HCC, the primary liver cancer, is regarded as the third most common malignancy worldwide. The major risk factors of HCC include hepatitis C and B viral infections, fungal metabolite aflatoxin A1 exposure, alcohol intake, obesity, and non-alcoholic fatty liver diseases. HCC is generally asymptomatic in nature and hence, is mainly diagnosed in the advanced stage. The lack of primary screening leads to an increased mortality to incidence ratio. Furthermore, sorafenib, an orally active multikinase inhibitor, is the first-line of therapy showing a significant survival benefit in patients with advanced HCC. Sorafenib has a direct effect on the components of tyrosine kinase signalling pathways which are generally deregulated in HCC. Sorafenib remarkably prolonged the survival rates in advanced HCC patients as illustrated by randomized controlled clinical trials. However, the management of HCC is still debatable. The reason behind this, is the development of adaptive drug resistance against sorafenib therapy. Global analysis of the expressed cellular proteins may aid to identify the differentially expressed proteins between parental cells and drug resistant cancer cells. Besides, malignant cell culture models are also suitable for application of proteomic techniques in order to recognize particular protein that might be associated with a characterized phenotype of the malignant cells.

Recent studies have demonstrated that various alterations in the cytoskeletal proteins may be one of the important mechanisms involved in the development of drug resistance in cancer cells that may in turn be associated with altered drug efflux pumps. Among them, vimentin is the cytoskeletal protein which belongs to the family of intermediate filament and is one of the markers of epithelial-mesenchymal transition (EMT). The vimentin expression in cancers is associated with increased tumor invasion and proliferation. Deregulation of vimentin expression and its relation to tumor metastasis has been discussed in various cancers such as gastrointestinal tumors, prostate carcinoma, and breast cancer. A relation between vimentin expression and drug resistance has been demonstrated in ovarian cancer as the downregulation of vimentin is associated with acquired drug resistance to cisplatin. Sorafenib-resistant cancer cells may undergo EMT, however, various studies have shown that sorafenib downregulate this process in HCC cells. During the exposure of mouse primary hepatocytes to sorafenib, EMT gets diminished due to decrease in transforming growth factor β signalling. A study by van Malenstein et al. in 2013, also have shown that long term exposure of sorafenib to HepG2 cells leads to development of resistance due to activation of EMT. But no such study has yet been revealed EMT cytoskeleton proteins as a potential target for treatment along with sorafenib. Further, the exact role of vimentin is not well established in sorafenib-resistant HCC.

In this study, we have unveiled the expression pattern of vimentin in parental as well as sorafenib-resistant HepG2 cells and further, highlighted the significance of vimentin as a plausible therapeutic target in sorafenib resistant HCC model cell line, HepG2.

MATERIALS AND METHODS

Cell lines and cell culture

This study was performed on HepG2 cell line that procured from cell repository of National Centre for Cell Sciences, Pune, India. HepG2 cells were then cultured in minimum essential media (MEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 0.15% (v/v) sodium bicarbonate, 1 mM sodium pyruvate and 2 mM L-glutamine. HepG2 cells were maintained at 37°C in 5% CO₂ and 95% humidity in a CO₂ incubator (Thermo Heraeus HERAcell® 240; Thermo Scientific, Waltham, MA, USA). Antibiotics streptomycin (100 μg/mL) and penicillin (100 U/mL) were supplemented in the media for maintenance of the cultures.

Chemicals

The media and antibiotics for cell culture were procured from HiMedia (Chandigarh, India), Sigma-Aldrich (St. Louis, MO, USA), and ThermoFisher Scientific. Sorafenib was procured from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Withaferin A was procured from Cayman Chemicals Co-USA (Cayman Chemical, Ann Arbor, MI, USA). Both sorafenib and withaferin A were dissolved in dimethyl sulfoxide (DMSO) to prepare 1 mM stock and 20 mM stocks respectively for further use in cell lines.

Samples preparation for matrix-assisted laser desorption/ionization (MALDI)

The total protein isolated from both HepG2 parental [HepG2 (P)] and HepG2 sorafenib-resistant [HepG2 (R)] cells was estimated following bicinchoninic acid method (Sigma-Aldrich). The proteins were separated by sodium dodecyl sulfate polyacrylamide gel
electrophoresis (SDS-PAGE) according to the method of Laemmeli (1970). After washing the gel with water, the spot of interest was excised and 200 μL of distaining solution was added to the gel, vortexed and incubated for 5 minutes and then the supernatant was discarded in order to destain the gel. Gel particles were then treated with 10 mM dithiotreitol/50 mM NH₄HCO₃ (freshly prepared), followed by incubation for 45 minutes at 56°C and then immediate cooling of tubes at room temperature. The gel pieces were treated with light sensitive 55 mM idoacetamide prepared in freshly prepared 50 mM NH₄HCO₃ for 30 minutes at room temperature and then washed with wash buffer. Freshly prepared trypsin enzyme solution (30 ng) was added to the gel, incubated at 37°C overnight. Following overnight incubation, the supernatant was collected and 5 μL of extraction buffer containing 60% ACN+ 0.1% TFA in 25 mM NH₄HCO₃ was added to the gel, vortxced and incubated for 10 minutes. Supernatant was collected into the same tube in which supernatant collected from trypsin digestion was added. The above process was then repeated first with extraction buffer containing 70% ACN+ 0.1 TFA in 25 mM NH₄HCO₃ and then with 90% ACN+ 0.1% TFA in 25 mM NH₄HCO₃. The extracted peptides were stored at –20°C for MALDI analysis.

MALDI-time of flight (TOF) mass spectroscopy

MALDI spectra were acquired using an Ultraflex TOF/TOF mass spectrometer (BrukerDaltonic, Hamburg, Germany). The resulting peaks were subjected to MASCOT search (www.matrixscience.com) and parameters were set as carbamidomethyl modification of cysteine, one missed cleavage was allowed for trypsin (Sigma-Aldrich). Once protein was identified it was further fragmented and subjected to MS-MS analysis. Protein MASCOT score, sequence coverage, and number of peptides matched were used to confirm the protein using SwissProt database (www.uniprot.org).

RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction (qRT PCR) analysis

Total RNA was isolated from both HepG2 (P) as well as HepG2 (R) cells using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Finally, RNA concentration was quantified and processed for cDNA synthesis. cDNA synthesis was carried out from the intact RNA by using Verso cDNA Synthesis Kit (Thermofischer). qRT PCR was performed on Lightcycler® 96 (Roche, Bremen, Germany) using SYBR Green I master (Roche) detection method to check relative expression of target genes using specific primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), vimentin and ABCG2 genes. The relative mRNA levels were calculated using the formula $2^{-\Delta\Delta Ct}$, where $\Delta Ct = (Ct$ target gene - Ct internal control). Each set of primers were designed to target specifically exon-exon junction in cDNA and was commercially synthesized by Sigma-Aldrich, USA. The gene expression was normalized using GAPDH acting as internal control. The primer sequences are:

- **ABCG2** (F)-GTGGCCTTGGCCTTGTGATGAT (R)-GATGGCAAGGGAA-CAGAAAA
- **GAPDH** (F)-CCATCTTCCAGGAGCAGA (R)-GGTCAT-GAGTCTTCCACGAT
- **Vimentin** (F)-CCGGTGCAATCGTGATCTCTGGG (R)-ATTCAAGTCT-CAGCGGGCTC

Protein expression: flow cytometry

HepG2 (P) and HepG2 (R) cells were cultured in a 12-well plate. After 24 hours of treatment, cells were trypsinized and washed twice with phosphate-buffered saline (PBS) in dark and cell pellet was resuspended in PBS. The cells were then fixed using 4% paraformaldehyde solution and again washed using PBS. After washing, the cells were permeabilized using permeabilizing buffer (1% BSA + 0.01% Triton X 100 + 0.01% Sodium azide in 1X PBS) and washed with PBS. Then blocking buffer (5% BSA in 1X PBS) was used to blocking the cells. Antibody solution was added after blocking to cells and incubated for 1 hour at room temperature. The expression of vimentin was then analyzed using flow cytometry.

MTT assay

HepG2 (P) and HepG2 (R) cells were seeded in a 96-wells plate (Corning Inc, Corning, NY, USA) with density of 5,000 cells per well in MEM with 10% FBS. After 24 hours of incubation, various concentrations (conc.) of withaferin A and sorafenib were added to MEM with 0.5% FBS, a total volume of 200 μL per well. After 24 hours, sterile 20 μL MTT solution was added to each well and plates were incubated for 4 hours at 37°C, 5% CO₂. Media containing MTT was then removed from the each well and formazan crystals formed were dissolved in DMSO (200 μL) and incubated for 5 to 15 minutes at room temperature in dark. The absorbance was then measured at 570 nm using a microtiter plate reader by infiniteM200PRO microplate reader (Tecan, Männedorf, Switzerland).
Crystal violet assay

This analysis was done to assess the growth inhibition pattern of HepG2 (P) and HepG2 (R) cells under different conditions. HepG2 cells (2,000 per well) were seeded in a 24-well plate in triplicate. After 24 hours of treatment, the cells were washed with PBS (1X) and later fixed using 4% paraformaldehyde in PBS (1X) for 15 minutes (200 μL per 12-well). Then, the cells were stained with 0.1% crystal violet (50 mg crystal violet powder in 5 mL ethanol/45 mL water) for 20 minutes (100 μL per 12-well). Subsequently, after washing with PBS, 500 μL 10% acetic acid was added to each well and incubated for 20 minutes on shaker. Finally, 0.5–1 mL of stain was taken out and absorbance was checked at 590 nm.

Statistical analysis

For the statistical analysis the experimental group differences were calculated by Student’s t-test or one-way ANOVA with Bonferroni’s correction for multiple comparisons. Drug concentrations effect in two cell types were assessed by two-way ANOVA with Sidak’s correction. All data were analyzed by GraphPad Prism (v.4.00; GraphPad Software, San Diego, CA, USA). Data were presented as the mean and standard error of the mean. Significance level was set at 0.05.

RESULTS

Stored HepG2 (P) and HepG2 (R) cells were recultured to perform the present experiments. As earlier (Ankita Makol, Ph.D. thesis, 2017) the HepG2 cell line was treated with minimal concentrations of sorafenib inhibitory concentration (IC)-20 dissolved in DMSO, continuously for long period followed by further fractional dose elevation to mimic the clinical settings. As a result, sorafenib-resistant HepG2 cell line was established. The chemoresistance nature of HepG2 cells was characterized in view of microscopic analysis (Fig. 1A) and dose-response assessment (IC50) in presence of different doses of the sorafenib (Fig. 1B). The IC50 value of resistant cells was 2.25 fold higher than parental cells.

Protein profiling and identification of differentially expressed proteins in HepG2 cells

In order to detect candidate protein marker which may have role in development of resistance against sorafenib, the comparative protein profiling of HepG2 (P) and HepG2 (R) cells was done using SDS-PAGE followed by coomassie staining (Fig. 2A). The six fragments (1, 2, 3, 4, 5, and 6) on the coomassie stained gel (Fig. 2B) were selected for MALDI-TOF-MS analysis. Among them peptide mass fingerprinting (PMF) of each in-gel digested four samples along with the respective theoretical weight, protein coverage, peptide matches which were documented from Swiss-Prot for each samples (Supplementary Table 1). The identified proteins were further confirmed by MS analysis. The analysis resulted in the identification of four proteins which were differentially expressed in HepG2 (P) and HepG2 (R) cells.

According to PMF analysis, sample 1 (excised protein band) was identified to be glucose regulated protein 78 (GRP78) which is highly expressed in HepG2 (R) as compared to HepG2 (P) cells whereas sample 2 was identified to be actin, highly expressed in HepG2 (R) in comparison to HepG2 (P) cells. On the contrary,
sample 3 was identified to be TRAP1 that was highly expressed in HepG2 (P) cells as compared to HepG2 (R) cells. Sample 4 was identified to be vimentin with theoretical mass 50 kDa which was similar to observed molecular weight (53,676 Da), protein coverage 44%, peptide matches 31. Vimentin was further confirmed by MS-MS analysis. Two peptides of molecular weight 1,572 Da and 1,094 Da were analysed which showed the identification with peptides of vimentin which was highly expressed in HepG2 (P) cells in comparison to HepG2 (R) cells (Supplementary Fig. 1).

Expression of vimentin in HepG2 (P) and HepG2 (R) cells

The expression level of vimentin was examined in both HepG2 (P) and HepG2 (R) cells at mRNA levels by qRT-PCR. Vimentin gene expression was resulted to be downregulated in HepG2 (R) cells in comparison to HepG2 (P) (Fig. 3C). These results were further confirmed at protein level through analysis of vimentin by flow cytometry. The protein expression analysis using phycoery-
therin labelled anti-vimentin antibodies (mouse anti-human antibodies) revealed higher expression of vimentin in HepG2 (P) cells as compared to HepG2 (R) cells (Fig. 3A, B).

Vimentin expression in response to sorafenib

In the presence of different concentrations of sorafenib (1.0, 2.5, 5.0, and 7.5 μM), the relative expression levels of vimentin in HepG2 (P) and HepG2 (R) cells has shown deregulated behaviour. Moreover, at sorafenib conc. 5 μM, vimentin expression was significantly decreased in HepG2 (R) cells. Therefore, this concentration was selected for further experiments (Fig. 4).

Vimentin expression in relation to withaferin A and its combination with sorafenib

Withaferin A is a steroidal lactone found in the restorative plant, Withania somnifera (Ashwagandha). It is reported as a potent inhibitor of vimentin, as it is known to cause the aggregation of tetrameric form of vimentin. We found that gene expression levels of vimentin was downregulated in the HepG2 (R) cells when treated with increasing concentrations of withaferin A. In HepG2 (R) cells, the vimentin expression was significantly decreased at withaferin A drug concentration of 0.25 μM (IC-50 value; >6 fold decrease).

Further, we also examined the gene expression of vimentin in HepG2 (R) cells in response to the combination of withaferin A and sorafenib as compared to the untreated ones. It was observed that when HepG2 (R) cells were treated with the combination of sorafenib (5 μM) and withaferin A (0.25 μM; IC-50 value), the gene expression of vimentin showed a significant decrease in comparison to untreated cells (Fig. 5).

Effect of vimentin inhibition on sorafenib resistance: expression analysis of ABCG2

ABCG2 transporters are known to be commonly associated with the development of drug resistance in majority of cancers. Elucidating the targeted effect of withaferin A on the ABCG2 gene expression in HCC cells, we analyzed the effects of its incremented doses as well as in combination with sorafenib.

As expected, the ABCG2 gene expression was higher in HepG2 (R) cells as compared to HepG2 (P) cells (Fig. 6A). Further, the levels of ABCG2 gene were observed to decrease with 0.25 μM (IC-50 value) withaferin A treatment in HepG2 (R) cells and at drug concentrations of 0.5 μM, the levels of ABCG2 were observed to decrease significantly (Fig. 6B).

Effect of vimentin inhibition on cell viability of HepG2 cells

The effect on cell viability via withaferin A treatment and combination of withaferin A with sorafenib was assayed through crystal violet staining. This analysis depicted the dose dependent cytotoxic effect of withaferin A and combination of withaferin A with sorafenib. The cell number significantly decreased with increasing concentration of withaferin A as well as with the combination of sorafenib and withaferin A (Fig. 7A).
The cell viability of HepG2 (P) and HepG2 (R) cells was also confirmed through the MTT assay. On treating the cells with different concentrations of withaferin A as well as in combination with sorafenib (5 μM), the cell viability was found to decrease significantly in both HepG2 (P) and HepG2 (R) cells (Fig. 7B).

As increasing concentration of withaferin A as well as its combination with sorafenib, the only first-line therapy for advanced HCC has a direct effect on cell viability of both HepG2 (P) and HepG2 (R) cells.

Hence, inhibition of vimentin might involve modulating sorafenib resistance as well as proliferation of HCC.

**DISCUSSION**

HCC is the primary cancer of liver and the leading cause of death in patients with liver diseases. The first-line systemic therapy of choice for advanced HCC is sorafenib that is known to extend the median survival time moderately by 2–3 months. However, long-term exposure of sorafenib has been reported to induce adaptive resistance in patients. The main processes which are known to have a role in sorafenib resistance broadly include hypoxic microenvironment, autophagy and EMT etc. EMT is a process of acquiring more metastatic and invasive properties similar to mesenchymal cells and is stated as losing the normal epithelial properties, like cellular polarity and cell-cell contact by the epithelial cells. Vimentin, a cytoskeleton protein of 57 kDa, is a highly conserved and broadly expressed protein of the type III Intermediate Filament protein family. Vimentin expression is restricted to mesenchymal cells. Vimentin has gained much attention in cancer biology as a sanctioned marker of EMT, lately. EMT is a cellular re-programming process in which the epithelial cells lose their cellu-
lar polarity, downregulation of the epithelial markers like E-cadherins and keratin, and acquires a mesenchymal phenotype that results in change of shape and shows increased motility of cells. The process of EMT is related to the development of resistance against certain chemotherapeutic drugs and vimentin expression has been observed to be upregulated in various tumor cell lines and tissues. Thus, we would like to study the role of vimentin in proliferation as well as sorafenib resistance in hepatocellular carcinoma.

In present study, the proteomic profiling of parental [HepG2 (P)] and sorafenib-resistant [HepG2 (R)] cells revealed the downregulation of vimentin in HepG2 (R) cells as compared to HepG2 (P) cells. This result was further confirmed at mRNA level by qRT PCR and at protein level by flow cytometry analysis. A study in 2016 by Yi Huo et al. also reported the similar pattern of downregulation of vimentin in cisplatin resistant ovarian cancer cells. Then, we investigated the effect of different doses of sorafenib on gene expression of vimentin and at 5 μM sorafenib conc. there is significant downregulation of vimentin expression. Further, to evaluate significance of vimentin inhibition in sorafenib resistance, a well known inhibitor of vimentin was used that mediates its aggregation and disassembly. Withaferin A successfully lowered down the levels of vimentin in HepG2 (R) cells.

In HCC sorafenib resistance, ABCG2 is one of the prominent efflux pumps belonging to the family of ABC (ATP binding cassette) transporters, involved in efflux of sorafenib out of resistant cancer cells.25,26 We analyzed the effect of withaferin A on ABCG2 expression in HepG2 (R) cells to understand its targeted action. In our study, withaferin A significantly decreased the levels of ABCG2 gene in HepG2 (R) cells which indicated towards the probable role of vimentin in the establishment of sorafenib resistance in HCC cells.

Studies reported about the cytotoxic effect of Withaferin A in cancer cells.27 To evaluate its cytotoxic nature our both crystal violet as well as MTT assays have demonstrated that using increasing dose of withaferin A as well as in combination with sorafenib both resulted to decrease cell viability of both HepG2 (P) and HepG2 (R) cells significantly.

Thus, the modulation of vimentin may result in the shift in proliferation as well as sorafenib resistance in HCC. Hence, our results demonstrated that vimentin is an eminent marker as well as its significance as a potential therapeutic target to treat sorafenib resistance in HCC.

This study connotes that vimentin is crucial for cell survival as its inhibition resulted in cell cytotoxicity in both HepG2 (P) and HepG2 (R) cells. Nonetheless, vimentin expression in resistant HCC cells was observed to be lowered, the inhibition further has proved detrimental to the resistant cells. Further, deciphering the specific resistance mediators, the effect of withaferin A was checked on ABC transporter gene. For the first time, the present study suggesting that withaferin A leads to a decrease in the expression of ABCG2 gene in sorafenib-resistant HCC [HepG2 (R)] cells. The inhibitor, withaferin A, mediating its anti-cancer action through cell apoptosis significantly decreased the expression of vimentin as well as cell viability in a dose dependent manner. This observed shift in resistant cells towards a parental-cell profile further hints the effect of vimentin in sorafenib resistance as its inhibition with withaferin A as a combination therapy along with only current targeted drug, sorafenib.

However, our study has some limitations. All experiments were performed using only one human HCC cell line (HepG2) and it was not possible to perform in vivo experiments. Thus, future research in this area is required to completely understand the mechanism and validate this study.

Authors’ contribution
AM, HK, SS, SK, RK were participated in the study concept and its design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript. Anuradha Chakraborti participated in study supervision, data interpretation, and critical review of the manuscript for important intellectual content.

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Conflicts of Interest
The authors have no conflicts to disclose.

SUPPLEMENTARY MATERIALS
Supplementary materials are available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

REFERENCES


Sensitivity of ultrasound in detecting hepatocellular carcinoma in obese patients compared to explant pathology as the gold standard

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Background/Aims: The American Association for the Study of Liver Diseases recommends ultrasound (US) screening for hepatocellular carcinoma (HCC) among cirrhotic patients, regardless of body mass index (BMI), every 6 months. We examined US sensitivity for diagnosis of HCC in obese patients.

Methods: Liver transplant patients data with HCC in explant was used (January 2012-December 2017). All patients underwent liver US within 3 months of diagnosis of HCC. Number/size of HCC lesions were extracted from radiologic and pathologic reports. Obesity was defined as BMI ≥30 kg/m².

Results: One hundred sixteen patients were included. 80% were male, with mean BMI of 31 kg/m². The most common underlying liver disease was hepatitis C virus (62%). At the time of diagnosis, median number of HCC lesions was 2 (interquartile range [IQR], 1–3), and median size of the largest lesion was 2.5 cm (IQR, 1.75–3.9). Overall sensitivity of US study for detection of HCC was 33% (95% confidence interval [CI], 29–48%). Sensitivity was 77% (95% CI, 62–93%) in patients with BMI<30 and 21% (95% CI, 11–30%) in patients with BMI≥30 (P<0.001). Size of the largest HCC lesion (P=0.290) and number of lesions (P=0.505) were not different between groups. Computed tomography (CT) scan detected HCC in 98% of the obese patients with negative US.

Conclusions: Sensitivity of US for detection of HCC is significantly lower among obese patients compared to overweight and normal weight patients. These patients may benefit from alternating between US and a different imaging modality.

Keywords: Liver cirrhosis; Neoplasms; Obesity

Study Highlights
The American Association for the Study of Liver Diseases (AASLD) recommends ultrasound (US) screening for hepatocellular carcinoma (HCC) among patients with cirrhosis, regardless of body mass index (BMI), every 6 months. However based on this study sensitivity of US for detection of HCC is significantly lower among obese patients compared to overweight and normal weight patients. These patients may benefit from alternating between US and a different imaging modality, i.e., computed tomography or magnetic resonance imaging.

Abbreviations:
AASLD, American Association for the Study of Liver Diseases; AFI, alfa-fetoprotein; BMI, body mass index; CI, confidence interval; CT, computed tomography; HCC, hepatocellular carcinoma; IQR, interquartile range; MRI, magnetic resonance imaging; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; US, ultrasound

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related death in the world.¹ HCC usually happens in the context of pre-existing chronic liver disease, and its clinical diagnosis is difficult, commonly necessitating using an imaging modality for diagnosis.² As cirrhosis is the single most important risk factor for HCC in North America, national and international clinical guidelines recommend surveillance of all patients with cirrhosis for HCC.²⁻⁵ Imaging the liver or using blood levels of biomarkers have been studied as potential surveillance strategies. Measuring the plasma levels of alfa-fetoprotein (AFP) for screening of HCC has poor accuracy,⁶,⁷ and its use for surveillance, especially as a stand-alone test is discouraged by current practice guidelines.⁸ Although contrast-enhanced liver computed tomography (CT) or magnetic resonance imaging (MRI) have been validated as accurate diagnostic tests for HCC,⁸,⁹ their use for surveillance is costly and accumulating radiation exposure over time in case of CT is problematic. Therefore currently a liver ultrasound (US) every 6 months is recommended as the surveillance strategy of choice for patients with cirrhosis.²⁻⁴

Use of ultrasound for HCC surveillance in obese patients has been reported to be controversial due to potentially decreased accuracy.⁵,¹⁰,¹¹ Prevalence of obesity has been increasing both in United States and globally.¹² Moreover, obesity is a major risk factor for non-alcoholic fatty liver disease (NAFLD)¹³⁻¹⁵ and recent studies show an increase in prevalence of NAFLD and non-alcoholic steatohepatitis (NASH) cirrhosis, as well as an increase in NAFLD as the underlying liver disease in patients with HCC.¹⁶⁻²⁰ Given these trends which lead to increasing frequency of obesity among patients with cirrhosis and HCC,²¹,²² it is important to evaluate the impact of obesity on the accuracy of US for surveillance of HCC.

In this retrospective study, we evaluated the accuracy of US for surveillance of HCC among liver transplant patients, using pathologic examination of the explant as the gold standard for diagnosis of HCC. The effect of patients’ body mass index (BMI) on the accuracy of US for HCC surveillance was assessed and compared to the results of MRI and CT in such population.

MATERIALS AND METHODS

Study population and data collection

The study protocol was approved by the Cleveland Clinic Institutional Review Board. Between January 2012 and December 2017, consecutive liver transplant patients who were diagnosed with HCC based on the pathologic examination of their explant entered the study. In each explant, the number of HCC lesions, their diameter, and the histological differentiation of the tumor were recorded. For each liver transplant, patient’s gender, age, BMI at the time of transplant, underlying liver disease, and date of transplant were recorded. Obesity was defined as BMI ≥30. Electronic medical records of these patients were reviewed and data was extracted on the date of diagnosis of HCC. Also, the method of HCC diagnosis was recorded (i.e., US, CT, MRI, or diagnosed during the examination of the explant).

For patients whose HCC was first diagnosed based on a screening US, data was extracted from the US findings, including date of US, number of HCC lesions, and size of the largest lesion. For patients whose HCC was diagnosed based on a diagnostic test other than US, same data was extracted from a screening US within a 3-month period prior from the date of diagnosis of HCC. Similar data on presence or absence of HCC and characteristics of lesions were collected on CT and MRI findings when applicable.

Sensitivity of US for diagnosis of HCC was calculated and compared to the gold standard which was diagnosis of HCC in explant pathology. US sensitivity in diagnosis of HCC was compared between different BMI subgroups. Additionally, diagnostic utility of CT and MRI imaging was evaluated for diagnosis of HCC.

Statistical analysis

All analysis was done with stata data analysis and statistical software (version 11.2 SE; StataCorp LLC, College Station, TX, USA). Variables are reported as number (percentage), mean (standard deviation) or median (interquartile range, IQR). Categorical variables are compared between groups with chi-square test. Interval variables are compared between groups with Wilcoxon rank-sum and t-tests. Confidence intervals (CIs) for proportions are calculated using normal distribution approximation. P-value less than 5% was defined as clinically significant. All P-values are two-sided.

RESULTS

Patient characteristics

Between January 2012 and December 2017, 169 consecutive
liver transplantations were confirmed to have HCC in pathological examination of the explant. One hundred and sixteen patients had at least one screening US within the 3-month period of the date of diagnosis of HCC and were included in the study. Main risk factor for HCC in all these patients was known diagnosis of cirrhosis. AFP was elevated in 83% of the patients (mean of 48±12). In all cases HCC was identified in the background of cirrhosis. On pathology exam, 66% of the patients had well-differentiated HCC, 4% had poorly-differentiated HCC.

73% of these patients received at least one type of locoregional therapy prior to liver transplant (including radiofrequency ablation/micro wave ablation in 41%, transarterial chemoembolization and bland embolization in 25% and transarterial radioembolization in 7% of the patients). The majority of study population was male and obese (80% and 65%, respectively). Mean BMI was 31 kg/m² (range, 20–43). Overall 62% of the patients had a BMI ≥30 kg/m² and 38% of the patients had BMI <30 kg/m². The most common underlying liver disease was chronic hepatitis C virus infection in 72 patients (65.0%), followed by alcoholic cirrhosis in 16 (13.7%), and NASH in eight patients (6.8%). Less common etiologies for cirrhosis were cryptogenic cirrhosis, chronic hepatitis B virus infection, primary biliary cholangitis, hemochromatosis and alpha 1 antitrypsin deficiency, in order of frequency.

The mean Model for End-Stage Liver Disease Na score at the time of transplant was 18. 13% of the patients were Child-Turcotte-Pugh class A, 34% class B and 53% class C at the time of transplant. On average patients had 2.0 (IQR, 1–3) HCC lesions on pathologic examination with the average size of the largest lesion being 2.5 cm (IQR, 1.75–3.9) (Table 1). In all patients, post transplantation immunosuppression regimen consisted of different combinations of tacrolimus, or cyclosporine, and mycophenolate mofetil and prednisone.

### Diagnosis of HCC and sensitivity of US

Among the 116 patients, 38 (32.7%) had their HCC diagnosed by US. Sixty-five (56.0%) were diagnosed by CT, three (2.6%) with MRI, and 10 (8.6%) were diagnosed after transplant by examination of the explant. The method of HCC diagnosis was significantly different depending on patient’s body habitus and weight. While in the majority of patients (59%) with BMI <30, the sensitivity of US was 77% (CI 62–93), while in the group with BMI ≥30 it was significantly lower (21% 11–30) (Table 2).

### Table 1. Characteristics of patients with HCC

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>116</td>
</tr>
<tr>
<td>Male</td>
<td>93 (80)</td>
</tr>
<tr>
<td>Age, mean (range)</td>
<td>58 (48–71)</td>
</tr>
<tr>
<td>BMI</td>
<td>31±4.4</td>
</tr>
<tr>
<td>BMI ≥30</td>
<td>75 (65)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>42 (7)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>15 (17)</td>
</tr>
<tr>
<td>MELD</td>
<td>18±5</td>
</tr>
<tr>
<td>CTP score (%)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>13</td>
</tr>
<tr>
<td>B</td>
<td>34</td>
</tr>
<tr>
<td>C</td>
<td>53</td>
</tr>
<tr>
<td>Number of HCC lesions*</td>
<td>2 (1–3)</td>
</tr>
<tr>
<td>Size of largest HCC lesion* (cm)</td>
<td>2.5 (1.75–3.9)</td>
</tr>
</tbody>
</table>

Values are presented as means±standard deviation, number (%), or median (interquartile range).

HCC, hepatocellular carcinoma; BMI, body mass index; MELD, Model for End-Stage Liver Disease; CTP, Child-Turcotte-Pugh.

*Based on pathological examination of the explant.

### Table 2. Characteristic of HCC lesions

<table>
<thead>
<tr>
<th>Characteristics of patients and their HCC lesions</th>
<th>BMI &lt;30</th>
<th>BMI ≥30</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>41 (35)</td>
<td>75 (65)</td>
<td>NA</td>
</tr>
<tr>
<td>Mean BMI</td>
<td>26±2.5</td>
<td>33±2.9</td>
<td>0.152</td>
</tr>
<tr>
<td>Mean age</td>
<td>57±9</td>
<td>59±7</td>
<td>0.152</td>
</tr>
<tr>
<td>Male</td>
<td>33 (81)</td>
<td>60 (80)</td>
<td>0.950</td>
</tr>
<tr>
<td>HCC first diagnosed by</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>US</td>
<td>24 (59)</td>
<td>14 (19)</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>15 (37)</td>
<td>50 (67)</td>
<td></td>
</tr>
<tr>
<td>MRI</td>
<td>1 (2)</td>
<td>2 (3)</td>
<td></td>
</tr>
<tr>
<td>Pathology</td>
<td>1 (2)</td>
<td>9 (12)</td>
<td></td>
</tr>
<tr>
<td>Number of HCC lesions at diagnosis*</td>
<td>1 (1–2)</td>
<td>1 (1–2)</td>
<td>0.734</td>
</tr>
<tr>
<td>Size of largest HCC lesion at diagnosis* (cm)</td>
<td>2.2 (1.8–4)</td>
<td>2 (1.8–3)</td>
<td>0.373</td>
</tr>
<tr>
<td>Number of HCC lesions in the explant*</td>
<td>2 (1–3)</td>
<td>1 (1–3)</td>
<td>0.545</td>
</tr>
<tr>
<td>Sensitivity of US (CI) (%)</td>
<td>77 (62–93)</td>
<td>21 (11–30)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation, number (%), or median (interquartile range) unless otherwise indicated.

HCC, hepatocellular carcinoma; BMI, body mass index; NA, not applicable; US, ultrasound; CT, computed tomography; MRI, magnetic resonance imaging.

*As reported at diagnosis with US, CT, MRI or pathological exam of explant.

†As reported on pathological exam of the explant.
the diagnosis was made with US; in the majority of patient (67%) with BMI ≥30, HCC diagnosis was made with CT scan (P-value <0.001) (Table 2).

Overall sensitivity of a single US for detection of HCC was 33% (95% CI, 24–41%) in the study population. When sensitivity of US was calculated for each subgroup of patients based on their BMI, the sensitivity was 59% (95% CI, 43–74%) in non-obese patients, as compared to sensitivity of 19% (95% CI, 10–28%) in obese patients (P-value <0.001). Age, gender, number and size of largest HCC lesion at diagnosis were not significantly different between non-obese and obese patients (Table 2).

Seventeen non-obese patients (41%) and 61 obese patients (81%) had falsely negative US within 6 months of HCC diagnosis detected by another diagnostic method. Sixteen out of 17 non-obese patients (94%) and 52 out of 61 obese patients (85%) with falsely negative US had been evaluated for HCC with either CT or MRI. Alternate imaging with either CT or MRI had a sensitivity of 100% in both groups for diagnosis of HCC.

DISCUSSION

American Association for the Study of Liver Diseases (AASLD) recommends screening for HCC in cirrhotic patients using ultrasound every 6 months.21 The reported sensitivity of unenhanced ultrasound for detection of HCC varies significantly based on different studies (between 34% and 100%).22 However, it has been shown that sensitivity of ultrasound drops significantly in obese patients.23 AASLD recommendations regarding HCC screening has been made regardless of the BMI of the patient. Considering the lower sensitivity of US in detecting HCC in obese patients, current HCC recommendations can potentially cause a delay in diagnosis of HCC in this patient population. This can be one of the reasons for the worse prognosis and larger tumor size in patients with NASH induced HCC.24

This needs extra attention given the high prevalence and growing number of obese population in the United States. Based on the report form Centers for Disease Control and Prevention, the prevalence of obesity was 36.5% (crude estimate) among United States adult population during 2011–2014, which is more than one-third of this population. From 1999–2000 through 2013–2014, a significant increase in obesity was observed in adults.25 Lower sensitivity of ultrasound in obese patients and risk of delay in timely diagnosis of HCC, has a significant effect on prognosis, treatment options (cure vs. palliative measures) as well as on long-term survival post liver transplant. Mazzaferro et al.26 showed a higher post transplantation recurrence rates in patients with single lesion larger than 5 cm or multiple nodules any of which exceeds 3 cm. This is the rationale behind Milan criteria, used by United Network for Organ Sharing.

In a study done by Chalasani et al.27 in 1999, 84% of the 473 members of AASLD indicated use of some form of surveillance practice, with majority (69%) using ultrasound as the only imaging modality. In our study, ultrasound sensitivity in detecting HCC was assessed in the entire studied population as well as in different BMI sub-groups. Overall sensitivity of a single US for detection of HCC was 33% (95% CI, 24–41%) in the study population. It was shown that, sensitivity of ultrasound drops significantly as BMI increases. US sensitivity in detecting HCC was 59% (95% CI, 43–74%) in non-obese patients with BMI <30, as compared to sensitivity of 19% (95% CI, 10–28%) in obese patients with BMI ≥30 (P-value <0.001).

This study has some limitations. First, this is a retrospective study with all its inherent limitations. Second, liver ultrasound was performed by different radiologists and therefore prone to interobserver variability. The other limitation of this study is possibility of missed sub centimeter HCCs on the explants which can be missed on 5–10 mm sectioning.

Theoretically, using an alternative method of imaging for HCC screening, CT scan or MRI, can potentially decrease the rate of missed cases of HCC and reduce the delay in diagnosis and treatment of this cancer. However, performing CT scan every 6 months (as recommended by AASLD), imposes a significant accumulative radiation dose over years. One option would be applying different methods of imaging, i.e., alternating ultrasound and CT scan or MRI every 6 months.

Based on this study, and due to the concern with US sensitivity in detecting HCC –especially in obese patients– and given the prognostic significance of earlier diagnosis; a more sensitive imaging modality especially in obese population appears to be a necessity.

Authors’ contribution
Jamak: Designed the study, Collected the data, wrote the paper
Kaveh: Performed the analysis
Kianoush: Wrote the paper

Conflicts of Interest
The authors have no conflicts to disclose.
REFERENCES


Influence of some methylated hepatocarcinogenesis-related genes on the response to antiviral therapy and development of fibrosis in chronic hepatitis C patients

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Background/Aims: Epigenetics involved in multiple normal cellular processes. Previous research have revealed the role of hepatitis C virus infection in accelerating methylation process and affecting response to treatment in chronic hepatitis patients. This work aimed to elucidate the role of promoter methylation (PM) in response to antiviral therapy, and its contribution to the development of fibrosis through hepatocarcinogenesis-related genes.

Methods: A total of 159 chronic hepatitis Egyptian patients versus 100 healthy control group were included. The methylation profile of a panel 9 genes (SFRP1, p14, p73, APC, DAPK, RASSF1A, LINE1, O6MGMT, and p16) was detected in patients' plasma using methylation-specific polymerase chain reaction (MSP).

Results: Clinical and laboratory findings were gathered for patients with combined pegylated interferon and ribavirin antiviral therapy. Regarding the patients' response to antiviral therapy, the percentage of non-responders for APC, O6MGMT, RASSF1A, SFRP1, and p16 methylated genes were significantly higher versus responders (P<0.05). Of the 159 included patients, the most frequent methylated genes were SFRP1 (102/159), followed by p16 (100/159), RASSF1A (98/159), then LINE1 (81/159), P73 (81/159), APC (78/159), DAPK (66/159), O6MGMT (66/159), and p14 (54/159). A total of 67/98 (68.4%) cases of RASSF1A methylated gene (P=0.0.024), and 62/100 (62%) cases of P16 methylated gene (P=0.03) were associated with mild-degree fibrosis.

Conclusions: To recapitulate, the PM of SFRP1, APC, RASSF1A, O6MGMT, and p16 genes increases in chronic hepatitis C patients, and can affect patients' response to antiviral therapy. The RASSF1A and P16 genes might have a role in the distinction between mild and marked fibrosis. (Clin Mol Hepatol 2020;26:60-69)

Keywords: Peginterferon alfa-2b; Ribavirin; Fibrosis; Hepatitis C, Chronic
INTRODUCTION

Chronic liver disease may be defined as a disease of the liver that lasts over a period of 6 months. It comprises liver pathologies such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma.\textsuperscript{1} Hepatitis C virus (HCV) infection is one of the causes that associated with chronic liver diseases. Infections with the HCV are pandemic, and the World Health Organization (WHO) estimates a world-wide prevalence of 3%. In Middle Europe, about 1% of the population is infected, mostly with genotype 1 (85% in Austria). In developing countries, chronic hepatitis C (CHC) is the most prominent cause for liver cirrhosis, hepatocellular carcinoma and liver transplantation.\textsuperscript{2}

Ribavirin/pegylated-interferon combination therapy is currently the most effective treatment for hepatitis C infection. Clearance of this HCV can be predicted by a sustained virological response (SVR).\textsuperscript{3} The main predictors of SVR are HCV genotype, stage of fibrosis, baseline HCV RNA levels, the dose and duration of therapy, IL28B polymorphism, body mass index (BMI), age, insulin resistance, gender, the levels of alanine aminotransferase (ALT), gamma glutamyl-transferase (GGT), and co-infection with human immunodeficiency virus (HIV) or other hepatotropic virus.\textsuperscript{4} Many authors have found that different types of cancer, including hepatocellular carcinoma (HCC), show distinct DNA methylation profiles; suggesting the existence of cancer-type specific methylation signatures.\textsuperscript{5} Others have shown that the presence of hepatitis viruses, especially HCV, could play a role in accelerating the methylation process which is involved in HCC development, potentiate the progression of HCV related liver disease and affect its response to treatment.\textsuperscript{6,7}

Molecular pathogenesis of hepatocarcinogenesis still unclear. However, it has been revealed that epigenetic changes, especially global DNA hypomethylation concomitant with locus-specific DNA hypermethylation in gene promoters, plays vital roles in carcinoma progression.\textsuperscript{8,9} DNA methylation markers could be utilized to detect human cancers in blood, plasma, secretion, or exfoliated cytology specimens and predict the risk of cancer development.\textsuperscript{10,11} Thus, cell free DNA circulating in plasma of chronic liver disease patients may represent a promising non-invasive alternative for HCC screening and monitoring. Progression from chronic hepatic inflammation to the fibrotic/cirrhotic stage is supported by numerous core pathways, observed in other fibrotic diseases, as well as tissue- or injury-specific pathways that are only activated in particular conditions.\textsuperscript{12,13}

Therefore, the present work was applied to verify the previous results,\textsuperscript{7,14} and elucidate the role of promoter methylation (PM) in the response to antiviral therapy, and its contribution to the development of fibrosis using some hepatocarcinogenesis-related genes such as SFRP1, p14, p73, APC, DAPK, RASSF1A, LINE1, O6MGMT, and p16.

MATERIALS AND METHODS

Patient specimens

This study was done on 159 Egyptian patients with chronic genotype 4 hepatitis C in addition to 100 healthy control group. These patients were eligible for ribavirin/pegylated interferon combination therapy. Selection of patients was based on clinical and histological examinations. Inclusion criteria were morphologic evidence of chronic hepatitis, normal renal function (normal creatinine level), normal prothrombin time, elevated hepatic function (elevated bilirubin, aspartate aminotransferase and ALT levels), normal cardiac enzymes, HIV-antibody (Ab) negative by ELISA, hepatitis B surface antigen (HBsAg) negative by ELISA and hepatitis B virus (HBV) DNA negative by polymerase chain reaction (PCR), and anti-HCV positive by ELISA. Informed consents were obtained from all the participants enrolled in the study, which was performed in accordance with the declaration of Helsinki, local and national laws.

Laboratory investigations

They were done, and HCV RNA was quantified using quantitative real time PCR\textsuperscript{15} at baseline, after 12, 24, 48, and 72 weeks of anti-viral therapy. Histological examination was done on core needle biopsies to determine the grade of necro-inflammation and the stage of fibrosis according to the Metavir scoring system prior to treatment. For the steatosis assessment tool, it was confirmed histologically, and expressed as % values of fatty changed. Also, it was checked by abdominal ultrasonography, each criterion of none, minimal, mild, and moderate steatosis was demonstrated in Table 1. Clinical and laboratory follow up were done for every patient to report any adverse side effects and treatment response according to interferon treatment guidelines.

DNA extraction

DNA was extracted from patient’s plasma before receiving riba-
virin/pegylated interferon combination therapy, according to the previously published protocol. DNA was extracted through a phenol/chloroform treatment. Briefly, equal volume of buffer equilibrated phenol (pH 7.0–7.5) was added to samples and vortexed. The upper aqueous layer was removed with a "cut down" pipette tip, and an equal volume of phenol/chloroform (1:1) was then added to the aqueous supernatant and vortexed. The upper aqueous layer was removed again in a similar fashion, and an equal volume of chloroform/isoamyl (24:1) was then added and vortexed. Sodium acetate (3 M) (pH 4.7–5.2) was added to the aqueous supernatant, followed with ice-cold ethanol. Samples were then incubated overnight at -8°C. After decantation of the liquid, the DNA pellet was recovered and dissolved in sterile water. The purity and integrity of the DNA was confirmed by carrying out β-actin gene amplification.

**Bisulphate conversion and methylation-specific polymerase chain reaction (MSP)**

After DNA extraction, it was subjected to bisulfite treatment using EZ DNA methylation kit that uses 300 ng of the extracted nucleic acid. This was followed by MSP using the primer sequences and the methylation-specific PCR conditions illustrated in Table 2. DNA methylation of CpG islands for SFRP1, p14, p73, APC, DAPK, RASSF1A, LINE1, O6MGMT, and p16 genes was determined using specific primers for methylated (M) and unmethylated (UM) DNA.

---

**Table 1.** Clinico-pathological features of 159 Egyptian patients with chronic genotype 4 hepatitis C

<table>
<thead>
<tr>
<th>Variable</th>
<th>Responders (n=81)</th>
<th>Non-responders (n=78)</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>37.8±6.8</td>
<td>40.8±9.0</td>
<td>38.1±8.9</td>
<td>0.462</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>75/12</td>
<td>60/12</td>
<td>135/24</td>
<td>0.805</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.51±3.86</td>
<td>70.06±11.79</td>
<td>49.7±8.9</td>
<td>0.03*</td>
</tr>
<tr>
<td>WBC (2.5–30.0x10³/mm³)</td>
<td>6.5±2.4</td>
<td>6.3±1.7</td>
<td>6.4±2.1</td>
<td>0.149</td>
</tr>
<tr>
<td>Hemoglobin (9–17 gm/dL)</td>
<td>14.2±1.5</td>
<td>14.2±1.7</td>
<td>14.2±1.6</td>
<td>0.641</td>
</tr>
<tr>
<td>Platelets (100–600x10³/mm³)</td>
<td>188.1±63.6</td>
<td>209.3±65.7</td>
<td>197.7±64.8</td>
<td>0.683</td>
</tr>
<tr>
<td>Albumin (3.5–5.5 gm/dL)</td>
<td>4.2±0.4</td>
<td>4.1±0.3</td>
<td>4.2±0.5</td>
<td>0.207</td>
</tr>
<tr>
<td>Total bilirubin (0.1–1.2 mg/dL)</td>
<td>0.78±0.21</td>
<td>0.80±0.33</td>
<td>0.79±0.27</td>
<td>0.116</td>
</tr>
<tr>
<td>ALT (0–42 IU/L)</td>
<td>101.1±181.11</td>
<td>121.35±401.73</td>
<td>118.11±231.11</td>
<td>0.617</td>
</tr>
<tr>
<td>AST (0–42 IU/L)</td>
<td>52.30±30.23</td>
<td>62.35±32.73</td>
<td>57.23±31.59</td>
<td>0.155</td>
</tr>
<tr>
<td>ALP (0–290 IU/L)</td>
<td>124.81±72.43</td>
<td>107.81±64.30</td>
<td>116.47±68.45</td>
<td>0.406</td>
</tr>
<tr>
<td>AFP (0–10 ng/mL)</td>
<td>5.3±7.3</td>
<td>7.2±10.4</td>
<td>10.1±1.5</td>
<td>0.346</td>
</tr>
<tr>
<td>HCV viral load (IU/mL)</td>
<td>193.000±108.000</td>
<td>338.000±237.000</td>
<td>244.231±101.000</td>
<td>0.789</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>63 (77.7)</td>
<td>57 (73.1)</td>
<td>120 (75.5)</td>
<td>0.03*</td>
</tr>
<tr>
<td>Marked (F3 &amp; F4)</td>
<td>18 (22.3)</td>
<td>21 (26.9)</td>
<td>39 (24.5)</td>
<td></td>
</tr>
<tr>
<td>Necroinflammatory activity</td>
<td>69 (85.1)</td>
<td>72 (92.3)</td>
<td>141 (88.6)</td>
<td>0.01*</td>
</tr>
<tr>
<td>Steatosis</td>
<td>12 (14.9)</td>
<td>6 (7.7)</td>
<td>18 (11.4)</td>
<td>0.459</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation or number (%).

M, male; F, female; BMI, body mass index; WBC, white blood cells; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; AFP, α-fetoprotein; HCV, hepatitis C virus.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>AT (°C)</th>
<th>MgCl₂ (mM)</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFRP1</td>
<td>MF TTT AGT AAA TCG AAT TCG GC</td>
<td>60</td>
<td>4.5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>MR TAA AAT ACG CGA AAC TCC TAC G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UF TTT TAG TAA ATT GAA TTT GTT TGT GA</td>
<td>60</td>
<td>4.5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>UR TAA AAT ACA CAA AAC TCC TAC AAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p14</td>
<td>MF GTTTAAAGGGCGGCGTACGC</td>
<td>54</td>
<td>4.5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>MR AAAAACTCCTACTCGCAGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UF TTTTGTTGTTAAGGGTGTTAGT</td>
<td>56</td>
<td>4.5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>UR CACAAAAACCCTCACATCAACAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p73</td>
<td>MF GGACGTAGCCGAATCGGGGTC</td>
<td>62</td>
<td>4.5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>MR ACCCGAACATCGACTGCGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UF AGGGGATGTAGGAATGGGTTT</td>
<td>62</td>
<td>4.5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>UR ATCACAACCCAAAAATCAACATCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APC</td>
<td>MF TATTCGGGAGTGGCGGTC</td>
<td>62</td>
<td>4.5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>MR TCAACGAACCTCCCGAGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UF GTTGTGTTGTTGAGTGTTGTTT</td>
<td>59.2</td>
<td>4.5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>UR CCAATCAACAAACTCCCAACAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAPK</td>
<td>MF GATAGTGCGATCGAGTTACGT</td>
<td>59</td>
<td>4.5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>MR CCCCTCAAACGCGGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UF GGAGGATGTGGATGAGTTGAATGTT</td>
<td>59</td>
<td>4.5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>UR CAAATCCAACAAACTCCCAACAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RASSF1A</td>
<td>MF TCGTGTTGTTATGGATTGGATT</td>
<td>54.4</td>
<td>4.5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>MR CGATTAACCCGATCTTCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UF TGGTGTGTTGTTGATGGATTGGT</td>
<td>52</td>
<td>4.5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>UR TACAACTCCTCCAACACAC</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>LINE1</td>
<td>MF GCCCGAGTCGAAGTGGGC</td>
<td>60</td>
<td>4.5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>MR CTCCGGATCAAATATAAATAATATAATCTCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UF ATTTTAGTTATTTTGGAAGTGCTGAGGC</td>
<td>60</td>
<td>4.5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>UR GCAATCTCGACTCTACAACAACTCCCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O6MGMT</td>
<td>MF TTTGAGCTGTTGAGTTGGCTG</td>
<td>56</td>
<td>3.5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>MR GCACCTTCGAAAACGAAAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UF TTTGTTTTGTTAGTTTTGAGTTTTTGT</td>
<td>56</td>
<td>4.5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>UR AACTCCACACTCTCCAACACACACAA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Negative control specimens (without DNA) were included in each PCR set. PCR products were analysed on 4% ethidium bromide-stained agarose gel and visualized under UV.

**Statistical analysis**

Statistical analysis was done using IBM SPSS Statistics 21.0 (International Business Machines Corporation Company, New York, NY, USA). For categorical variables, percentages were calculated, and differences were analysed with chi square tests and Fisher’s exact test when appropriate. Continuous variables were analysed as mean±standard deviation or median and range as appropriate. Differences among continuous variables with normal distribution were analysed by Student’s t-test; comparison between three groups was done using Kruskel-Wallis test (non-parametric analogue for analysis of variance). P-value which is less than (0.05) was considered statistically significant.

**RESULTS**

**Clinico-pathological features of the patients**

The demographic, laboratory, and histopathological data of the 159 patients (81 responders and 78 non-responders) are illustrated in Table 1. No significant difference was observed between the two groups (responders and non-responders) regarding age, sex, haematological parameters, liver profile, HCV viral load. However, a significant difference was found in other variables such as BMI, Fibrosis, necroinflammatory activity, and steatosis (Table 1).

**HCV RNA results**

For HCV RNA levels by RT-PCR technique, there was no significant difference (P=0.789) between responders (193.000±108) and non-responders (338.000±237) for the 159 CHC patients before treatment (Table 1). HCV RNA results at different treatment end points and follow up of our patients were done to detect treatment response as shown in Table 3.

**Methylation frequency of the studied genes in plasma samples**

The methylation frequency of SFRP1, p14, p73, APC, DAPK, RASSF1A, LINE1, 6MGMT, and p16 genes for chronic HCV-4 Egyptian patients and healthy control group is shown in Table 4, Figure 1, 2. Regarding the patients’ response to antiviral therapy, the percentage of non-responders for APC, O6MGMT, RASSF1A, SFRP1, and p16 methylated genes were significantly (P <0.05) higher versus responders (Table 4, Fig. 2), where it was 75.6%, 75.8%, 69.4%, 63.7%, and 65% versus 24.4%, 24.2%, 30.6%, 36.3%, and 35% for APC, O6MGMT, RASSF1A, SFRP1, and p16 methylated genes respectively.

Of the 159 included patients, the most frequent methylated genes were SFRP1 (102/159), followed by p16 (100/159), RASSF1A (98/159), then LINE1 (81/159), P73 (81/159), APC (78/159), DAPK (66/159), O6MGMT (66/159), and p14 (54/159) (Fig. 1).

**Promotor methylation index**

It defined as the ratio between the number of methylated genes and the total number of the studied genes for each sample was calculated for all patients. For methylation index, no significant difference was found between responders and non-responders (2.65±1.31 and 2.71±1.23; P=0.67) respectively. Also, there is no significant difference between mild fibrosis (F1 and F2) and marked fibrosis (F3 and F4) except for RASSF1A (P=0.024) and p16 (P=0.03) methylated genes (Table 5).
DISCUSSION

The foremost predictors of response to interferon-based HCV therapy included both patient and viral factors. Patient factors that were associated with worse response to interferon-based therapy included male gender, older age, high BMI, advanced liver fibrosis, history of failed treatment, black race, non-CC IL28B genotype, and the presence of certain comorbid conditions, such as HIV coinfection, insulin resistance, or diabetes. Viral factors that were associated with worse response included non-genotype-2 infection, high viral load, and unfavourable viral kinetics during treatment.\(^4,20\)

Some authors have revealed that hepatitis viruses infection might play a role in fast-tracking the methylation process which is involved in HCC development, and affect its response to treatment.\(^6,19,21,22\) Progression from chronic hepatic inflammation to the fibrotic/cirrhotic stage is supported by numerous core pathways, observed in other fibrotic diseases, as well as tissue- or injury-specific pathways that are only activated in particular conditions.\(^12,13\)

In an early work done by our group,\(^16\) detection of APC, FHIT, p15, p16 and E-cadherin-PM (range, 67.9–89.2%) had been done in the plasma and tissues of 28 chronic HCV and/or HBV-associated HCC patients, with a high concordance for all studied genes. However, no significant association was found, in this study, between the methylation status of any gene and the presence of hepatitis virus infection. This was partially attributed to the small sample size in this study. Then, we assessed the contribution of methylation status to the development and progression of HCV-associated HCC and CH in Egyptian patients using a specific panel of genes (APC, FHIT, p15, p14, p16, DAPK1, CDH1, RARb, RASSF1A, O6MGMT).\(^19\) We found that HCV infection may contribute to hepatocarcinogenesis through enhancing PM of certain genes. A panel of 4 genes (APC, p73, p14, O6MGMT) successfully classified cases into HCC or CH with high accuracy (89.9%), sensitivity (83.9%) and specificity (94.7%). A more extended confirmatory study, including 516 Egyptian patients with HCV-related liver disease (208 HCC, 108 liver cirrhosis, 100 CHC, and 100 controls), was then performed to detect PM of P14, P15, P73 and Mismatch repair gene (O6MGMT) in patient’s plasma by using EpiTect Methyl qPCR Array technology.\(^23\) The candidate genes selection (SFRP1, p14, p73, APC, DAPK, RASSF1A, LINE1, O6MGMT, and p16) of the present work was analyzed by the Gene Expression Profiling Interactive Analysis database.

### Table 3. HCV RNA of the 159 CHC patients at different treatment end points

<table>
<thead>
<tr>
<th>HCV RNA (+/-) by RT-PCR after weeks</th>
<th>Non-responder (n=78)</th>
<th>Responder (n=81)</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCR-W12</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>69 (46.0)</td>
<td>81 (54.1)</td>
<td>150 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>9 (100.0)</td>
<td>0 (0.0)</td>
<td>9 (100.0)</td>
<td>0.032*</td>
</tr>
<tr>
<td>Total</td>
<td>78 (49.1)</td>
<td>81 (50.9)</td>
<td>159 (100.0)</td>
<td></td>
</tr>
<tr>
<td><strong>PCR-W24</strong></td>
<td></td>
<td></td>
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<td>81 (50.9)</td>
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Values are presented as number (%).

HCV, hepatitis C virus; CHC, chronic hepatitis C; RT-PCR, reverse transcription-polymerase chain reaction; W, weeks.

*P-value <0.05 is considered significant.
In the current study, significant efforts had been done to elucidate the role of PM to the response to antiviral therapy and its contribution to the development of fibrosis using some hepatocarcinogenesis-related genes. Percentage of non-responders for APC, O6MGMT, RASSF1A, SFRP1, and p16 methylated genes were significantly (P <0.05) higher than those in responders. The most frequent methylated genes in the 159 CHC patients was SFRP1 (102/159), followed by p16 (100/159), RASSF1A (98/159), then LINE1 (81/159), P73 (81/159), APC (78/159), DAPK (66/159), O6MGMT (66/159), and p14 (54/159). In a previous study done by Iyer et al., they detected a high frequency of 5 methylated genes (APC, FHIT, p15, p16 and E-cadherin) which ranged from 67.9% to 89.2% in the plasma and tissues of 28 chronic HCV and/or HBV-associated HCC patients. Although, no significant association was found in his study between the methylation status of any gene and the presence of hepatitis virus infection which could be attributed to the small sample size. Also, in a previous study done by our group, we assessed the contribution of methylation status

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<td>Control (n=100)</td>
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<td>(n=78; 49.1%)</td>
<td>(n=81; 50.9%)</td>
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Values are presented as number (%). M, methylated; UM, unmethylated. *P-value <0.05 is considered significant.
to the development and progression of HCV-associated HCC and CH in Egyptian patients using a specific panel of genes (APC, FHIT, p15, p73, p14, p16, DAPK1, CDH1, RARb, RASSF1A, O6MGMT). We found that HCV infection may contribute to hepatocarcinogenesis through enhancing the promoter methylation of certain genes. On the other hand, Huang et al.,\textsuperscript{14} determined whether methylation status in plasma could be employed for monitoring the multistep carcinogenesis, multiplex MSP was applied to assay the methylation status for p16, SFRP1, and LINE1 in plasma specimens of 119 HCC patients, 105 LC patients, 52 patients with benign lesions and 50 healthy people. Therefore, Huang et al.\textsuperscript{14} found that the modification in the expression of p16, SFRP1, and LINE1 genes might be involved in the process of hepatocarcinogenesis.

The present work has shown that the most frequent methylated genes in the 159 CHC patients were SFRP1, p16, RASSF1A, APC, and O6MGMT, where they were 102 (64.2%), 100 (62.9%), 98 (61.6%), 78 (49.1%), and 66 (41.5%), respectively. This finding does not go well with the previous study done by Zekri et al.\textsuperscript{7} where they found that O6MGMT had the highest methylation frequency among HCV infected patients (64.2%) followed by p73 (50.9%), APC (49.1%), RASSF1A/DAP-kinase (41.5%), and p14 (34%). This discrepancies in results might be attributed to a small sample size of his study, where it was done on 53 CHC cases comparing to 159 chronic HCV patients and 100 healthy controls of the current work.

For the PM of the studied genes and degree of fibrosis, 67/98 (68.4%) cases of RASSF1A methylated gene ($P=0.0.024$) and 62/100 (62%) cases of p16 methylated gene ($P=0.03$) were associated with mild fibrosis. This finding was close to the results that found by Zekri et al.\textsuperscript{7} where they stated that only PM of the RASSF1A gene was significantly associated with mild fibrosis in the studied patients ($P=0.0019$). However, his study was done on six genes (p14, p73, APC, DAPK, RASSF1A, and O6MGMT) of 53 chronic HCV patients while our study was applied on nine genes (SFRP1, p16, p73, APC, DAPK, RASSF1A, LINE1, O6MGMT, and p16) of 159 CHC patients. This finding might be explained by the fact that DNA methylation modification is played by the HCV core protein which inhibit the expression of the CDKN2A gene, that encodes for p16INK (inhibitor of cell proliferation) by up-regulat-
ing the methyltransferases DNMT1 and DNMT3b.\textsuperscript{24,25} Moreover, HCV core protein also increases the methylation of RASSF1A promoter, a negative regulator of the Ras pathway, by inducing the histone methyltransferase SMYD3.\textsuperscript{25,26} Therefore, our results provide an evidence for the role of RASSF1A, and p16 genes in the induction of fibrogenesis in chronic HCV patients.

In conclusion, the PM of SFRP1, APC, RASSF1A, O6MGMT, and p16 genes increases in CHC patients. These methylated genes can significantly affect patients’ response to antiviral treatment, whereas RASSF1A and p16 genes are involved in the process of fibrogenesis and possibly will have a role in the distinction between mild and marked fibrosis in those patients.

Ethics approval and consent to participate

This study was performed in compliance with relevant laws and institutional guideline and in accordance with the ethical standards of the Declaration of Helsinki. The Institutional Review Board (IRB) of the NCI approved the protocol. Informed written consent was obtained from all patients and individuals enrolled in the study.

Author’s contribution

WSM conceived and designed the study with inputs from DAO. WSM and MHS were responsible for the supervision and coordination of the project. ZFA and MNI performed most of the in vitro experiments. DAO, ZFA, YMS, and HES collected the clinical specimens. WSM performed statistical analysis of the data. The first draft of the manuscript was written by WSM and DAO then MHS, YMS, HES, MNI contributed to revise and review the manuscript. All authors read and approved the final manuscript before submission.

Conflicts of Interest

The authors have no conflicts to disclose.

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ALL: correlation with phenotype and molecular subgroup. Leukemia 2003;17:1845-1850.


Dear Editor,

Polycystic liver disease is a genetically heterogeneous disorder, involving derangements on at least three different chromosomes.\(^1,2\) Some patients with polycystic liver disease may develop complications as the result of massive hepatomegaly or progress to advanced liver disease. In the symptomatic polycystic liver disease patient, surgical therapy and/or liver transplantation remain the mainstay of therapy.\(^2,3\) Although the benefits of tolvaptan, an inhibitor of vasopressin type 2 (V2) receptor, on the progression of renal dysfunction in autosomal dominant polycystic kidney disease (ADPKD) are well established,\(^4\) the influences on liver cysts have not been well delineated. Indeed, only one report was seen until now.\(^5\) We recently experienced a case suggesting the efficacy of tolvaptan to manage polycystic liver disease. The present case indicated that tolvaptan reduced liver as well as kidney volume in ADPKD, presumably by shrinking cysts.

A 37-year-old woman with family history of ADPKD complained abdominal fullness. Figure 1 showed her clinical course. Physical examination blood pressure of 144/102 mmHg and hepatomegaly, the liver and kidney function were normal (Table 1, prothrombin time-international normalized ratio: 1.02). Abdominal computed tomography (CT) showed polycystic liver and kidneys (Fig. 2). The liver and the total kidney volume were 8,674 and 1,024 mL, respectively (Fig. 3). The CT scan data were transferred to the workstation (Ziostation system 610, Amin Co., Ltd., Tokyo, Japan) to generate 3D image and assess organ volume. Left and right kidney volumes were combined to calculate the total kidney volume. Since she preferred medical management, irbesartan (200 mg/day) was first prescribed after counselling on family planning.\(^6\) A year later, her blood pressure dropped to 115/84 mmHg. Although her liver volume was essentially unchanged to 8,781 mL, kidney volume was increased to 1,194 mL. She started to take tolvaptan (60 mg/day) to limit the growth of renal cysts.\(^4\) Of note, Japanese Ministry of Health, Labour and Welfare approved to apply large doses of tolvaptan (up to 120 mg/day) for the patients with progressive ADPKD. Since then, she has been visiting our office once a month (Fig. 1). No adverse reactions including hepatic events were found at any visits (Table 1). Another year later, her kidney and liver volume were reduced to 1,047 and 7,846 mL (Fig. 3), suggesting that cysts in both kidneys and liver were shrunk. Notably, her abdominal fullness was improved, and abdominal CT revealed near-complete disappearance of a large hepatic cyst (74 mm in diameter) in the S6 segment (Fig. 2). She neither complained abdominal pain or discomfort, nor exhibited abnormal liver function test at any visits, implicating that hepatic cyst rupture was unlikely. She is still taking irbesartan (200 mg/day) and tolvaptan (60 mg/day) without any side effects.

Recent studies show that cholangiocyte autophagy, which is associated with activation of the cyclic adenosine monophosphate (cAMP)-protein kinase A and of cAMP response element-binding

Abbreviations:
ADPKD, autosomal dominant polycystic kidney disease; cAMP, cyclic adenosine monophosphate; CT, computed tomography; FSH, follicle-stimulating hormone; TEMPO, Tolvaptan Efficacy and Safety in Management of Autosomal Dominant Polycystic Kidney Disease and its Outcomes; V2, vasopressin type 2

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protein signaling pathway, contributes to cystogenesis in polycystic liver disease.\(^7\) Further, follicle-stimulating hormone (FSH) receptors are seen in biliary epithelial cells from normal and ADPKD patients.\(^8\) FSH increases c-AMP in cholangiocytes, inducing biliary growth via ERK. In addition, Mancinelli et al. have demonstrated that cholangiocytes express V2 receptors that are upregulated in the liver of ADPKD patients, and that vasopressin causes an increase in the proliferation and cAMP, a second messenger of V2 receptor, in human cholangiocytes from the cystic epithelium.\(^9\) Indeed, tolvaptan inhibits the vasopressin-induced increase in cAMP in cholangiocytes.

Clinically, somatostatin analogues can be used for medical management of polycystic liver disease.\(^10\) Lanreotide (120 mg) reduced liver volume by 3% in 6 months. Somatostatin analogues are now mainly used for endocrine diseases as they exhibit severe adverse effects such as gallstone. An aberrant relation between intracellular calcium and c-AMP is proposed as the mechanism of liver cyst formation.\(^3,7\) As discussed, tolvaptan reduces c-AMP in cholangiocytes.\(^9\) Although 14 out of 135 ADPKD patients experienced hepatic events in Tolvaptan Efficacy and Safety in Management of Autosomal Dominant Polycystic Kidney Disease and its Outcomes (TEMPO) extension Japan trial, they recovered after the interruption of tolvaptan.\(^11,12\)

Collectively, our case is consistent with the notion that tolvaptan suppresses liver cyst growth by reducing cAMP to inhibit autophagy and proliferation of cholangiocytes in polycystic liver dis-

![Timeline of the case](https://example.com/timeline.png)

**Figure 1.** Timeline of the case. We asked the patient to admit when starting tolvaptan to circumvent severe adverse reactions such as dehydration and liver dysfunction, and advised her to take the amount of fluid similar to urine volume. The administration of tolvaptan increased urine volume from 1,250 to 3,700 mL/day. CT, computed tomography.

<table>
<thead>
<tr>
<th>Table 1. Time course of blood biochemical profile</th>
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<td><strong>Before 12</strong></td>
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<td>Alb (g/dL)</td>
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<tr>
<td>TB (mg/dL)</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
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<td>GGT (U/L)</td>
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<tr>
<td>Cr (mg/dL)</td>
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<td>Na (mEq/L)</td>
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</table>

Tolvaptan was started after measurements at time 0.

Alb, albumin; TB, total bilirubin; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma glutamyl transpeptidase; Cr, creatinine; Na, sodium.

http://www.e-cmh.org https://doi.org/10.3350/cmh.2019.0026
Figure 2. Abdominal computed tomography scan was obtained at her first visit (before one year), prior to administration of tolvaptan (0 year) and a year after tolvaptan treatment (after one year), in a patient with polycystic liver disease. The scan shows disappearance of the previously visualised liver cyst in the S6 segment. R, right; L, left.

Figure 3. Time course of the liver and the total kidney volume. Tolvaptan was started at 0 year. Tolvaptan treatment for a year induced approximately 10% reductions in liver (A) and kidney (B) volume.
ease, and suggests that tolvaptan treatment can be performed safely in patients with polycystic liver disease. These provide potential applicability of tolvaptan for polycystic liver disease patients with normal liver function to manage hepatomegaly, and warrant further clinical investigations to examine the effects of tolvaptan as a novel agent militating against liver cyst progression.

Authors’ contribution
TT: Data acquisition and drafting the manuscript.
SM: Data interpretation and drafting the manuscript.
MK: Data interpretation and critical revision of the manuscript.
All authors approved the final version of manuscript.

Acknowledgements
The patient gave us an informed consent for this publication.

Conflicts of Interest
The authors have no conflicts to disclose.

REFERENCES
Dear Editor,

Fibrolamellar hepatocellular carcinoma (FLHCC) is a rare tumor, historically thought to be a form of primary hepatocellular carcinoma (HCC). FLHCC affects primarily younger individuals (5–35 years of age), however there appears to be a double peak of incidence at ages 5–30 and 70–79.1 In contrast to primary HCC, the individuals affected with FLHCC classically lack a history of underlying liver disease or cirrhosis.1 The incidence of this rare entity varies geographically, while it represents less than 1% of primary liver tumors in the United States, in Mexico it represents 5.8% of all primary liver cancers.1

Currently, curative surgical resection remains the primary treatment modality for FLHCC. Unfortunately, there are limited therapeutic options for patients who are afflicted with unresectable or metastatic disease. From a clinical standpoint there has been limited success in the management of advanced unresectable cancers with cytotoxic chemotherapeutic regimens and the outcomes remain poor.1,2

Similar to other abdominal tumors, FLHCC may present with nausea, abdominal fullness, abdominal pain and/or weight loss. A distinguishing feature however, is the increased rate of paraneoplastic manifestations. There have been reports of FLHCC presenting with tumoral hormone production, such as androgen aromatization resulting in gynecomastia, thyroid hormone and β-HCG production.3,4

Hyperammonemia is a rare and dreaded complication of chemotherapy for certain malignancies, including gastrointestinal stromal tumors, neuroendocrine tumors and myeloma. Several cases have been recently reported of hyperammonemia associated with FLHCC, even in the absence of recent chemotherapy. Here we present a case of aggressive FLHCC presenting with hyperammonemic encephalopathy, in the absence of underlying liver dysfunction.

A 32-year-old female with metastatic FLHCC presented with elevated transaminases thought to be secondary to nivolumab-associated hepatitis. She was diagnosed with FLHCC at the age of 26 and initially treated with 2 stages of chemoembolization, followed by surgical removal of her remaining cancer 6 months later. Given recurrent metastatic disease, involving the gallbladder, mediastinal lymph nodes and pleura (Fig. 1), the patient received different regimens of systemic chemotherapy. Sorafenib, Ponatinib, Gemcitabine, Oxiplatin and Folfox were administered sequentially in the listed order, starting 4 years prior to presentation and discontinued about a year before symptoms started. The last chemotherapeutic intervention was nivolumab given every 2 weeks at a standard dose for 3 months and discontinued 2 months prior to presentation. The patient had mild to moderate transaminitis in the setting of Nivolumab use and this was discon-
Continued. Additionally, interval cross-sectional imaging revealed that disease progression. The patient received dexamethasone around this time for transaminitis and persistent nausea.

Approximately 10 days later, patient was hospitalized at a local hospital for acute confusion with an elevated ammonia level of 204 µmol/L. Lactulose was initiated with improvement. Her liver enzymes remained elevated although her bilirubin, alkaline phosphatase, prothrombin time and serum albumin levels remained within normal limits, indicating preserved synthetic liver function. Approximately a month later, her symptoms recurred and she was transferred to our facility. Lactulose was continued and Rifaximin was added (Fig. 2). Viral hepatitis serologies, CMV PCR and HSV were negative. An ultrasound guided liver biopsy was performed and it was negative for drug induced hepatitis or other etiologies, but it revealed mild steatosis, presumably secondary to the glucocorticoid treatment. Repeat contrast-enhanced abdominal imaging was negative for portosystemic shunts or additional findings. Plasma amino acids elevated glutamine with significant decrease.

Figure 1. (A, B) Contrast enhanced chest computed tomography imaging showing the tumor (white arrow) and pleural metastasis (black arrow).

Figure 2. Time-course of ammonia levels during treatment and associated interventions. PO, per os.
in her essential amino acids which was reflective of hyperammonemia and severe catabolic stress. However, arginine and citrulline were not significantly decreased, ruling out primary inborn error of urea metabolism. Furthermore, genetic testing was obtained and it was negative for urea cycle disorders.

Unfortunately, the patient’s mental status continued to deteriorate and it progressed to West Haven grade 4 encephalopathy, requiring intubation for airway protection. Her ammonia level at this point was 300 µmol/L, despite lactulose and rifaximin, and a decision was then made to commence ammonia scavenging therapy with sodium phenylacetate 55 mL/m² along with 120 mL arginine and start D10W continuous infusion to avoid protein catabolism. Repeat embolization of the largest hepatic tumor was performed for possible intratumoral portosystemic shunting, but with no benefit. Subsequently to the patient was transitioned to glycerol phenyl butyrate 2.5 mL TID and 3 g citrulline q.i.d. The ammonia levels decreased, reaching a nadir of 57 µmol/L, but rose again following a single dose of prednisone for a truncal rash. Steroids were withheld as they were thought to be contributing to the elevated ammonia levels by inducing a catabolic state. The ammonia level further decreased and her mental status returned to baseline. Patient was dismissed home on L-citrulline, glycerol phenylbutyrate, lactulose and rifaximin. Unfortunately her course continued to fluctuate with multiple readmissions for recurrent encephalopathy. Given progression of her malignancy and extremely poor quality of life, the patient and the family opted to transition to comfort measures and hospice. The patient deceased several weeks later.

Although most frequently seen in advanced liver disease, portosystemic shunting and urea cycle disorders, the potential causes of hyperammonemic encephalopathy (HAE) are many. As such, a thorough evaluation and avoiding premature closure are important.

There have been reports of HAE occurring as a complication or rather in conjunction with FLHCC. Only nine such cases have been published in the literature to this date. The hypothesized mechanisms of hyperammonemia in FLHCC are similar to other tumors, including chemotherapy-induced tissue necrosis leading to increased nitrogen levels and overloading the urea cycle. Additionally, a recent hypothesis has been proposed by Sulaiman and colleagues suggesting HAE as a paraneoplastic manifestation of FLHCC. They speculate that FLHCC might release an inhibitor of the ornithine transcarbamylase (OTC) enzyme or that there might be increased activity of ornithine decarboxylase. An experimental study in animal models has demonstrated an inverse relationship between OTC activity and rate of growth of hepatomas. An interesting phenomenon observed in this study was a significant decrease in OTC activity with induced starvation of hepatomas. Another study has also demonstrated significant metabolic changes on FLHCC tumor cells, including upregulation of glycolysis and suppression of Kreb’s cycle. These findings might be a step toward elucidating the possible mechanisms involved in FLHCC-related paraneoplastic phenomena. Furthermore, it is likely that the pre-existing hyperammonemia has been exacerbated by the development of a catabolic state induced by chemotherapy and the use of steroids.

Currently, there are no standard guidelines for the treatment of hyperammonemia in the setting of FLHCC. Treatment focuses on management of the acute crisis and prevention of future recurrences. A three step approach is recommended: reduction of nitrogen load by avoiding catabolic states, removing excess ammonia and correcting precipitating causes. Chapuy et al. have proposed an algorithm for the diagnosis and treatment of hyperammonemia in the setting of FLHCC. However, further research is required to validate these recommendations in the management of this condition.

In summary, we report a severe case of FLHCC-related hyperammonemic encephalopathy exacerbated by corticosteroid use, with only partial response to standard therapy, including ammonia scavengers. We hope with this case to increase awareness of this severe complication in patients with FLHCC.

Authors’ contribution

Douglas A. Simonetto was involved in direct patient care.

Both the authors, Douglas A. Simonetto and Nimish Thakral were involved in literature search, manuscript writing and development of hypothesis of the paraneoplastic phenomena.

Conflicts of Interest

The authors have no conflicts to disclose.

REFERENCES


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Dear Editor,

Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) which include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) are characterized by erythrocytosis, leukocytosis, thrombocytosis, bone marrow hypercellularity, splenomegaly, and extramedullary hematopoiesis. A rare complication of Philadelphia-negative MPNs is portal hypertension (PHT). Previous reports have indicated that the incidence of PHT in patients with MPN is approximately 7% to 18%. The first manifestation of the disease may be complications of PHT, such as bleeding gastroesophageal varices (GEVs). Typically, endoscopic variceal ligation and sclerotherapy is used to control GEVs, and a transjugular intrahepatic portosystemic shunt (TIPS) is applied for variceal bleeding or refractory ascites. MPNs are often associated with Janus kinase 2 (JAK2) V617F mutations. JAK2 is a nonreceptor tyrosine kinase expressed by all hematopoietic stem cells; it relays the signal to induce cell proliferation when cells are stimulated by external cytokines or growth factors. The JAK2 V617F mutation may be a risk factor for splanchnic circulation thrombosis in patients with MPN and subclinical MPN, although the detailed mechanism has not been elucidated.

Abbreviations:
CT, computed tomography; ET, essential thrombocytemia; GEV, gastroesophageal varices; HVPG, hepatic venous pressure gradient; JAK2, Janus kinase 2; LDLT, living donor liver transplantation; MPN-U, myeloproliferative neoplasm unclassified; MPN, myeloproliferative neoplasm; PHT, portal hypertension; PMF, primary myelofibrosis; PV, polycythemia vera; TIPS, transjugular intrahepatic portosystemic shunt

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JAK2 mutation frequency in splanchnic circulation thrombosis or extramedullary hematopoiesis is associated with a high PHT incidence in patients with MPN. However, it is not clear whether specific subgroups of patients with MPN are more susceptible to the development of PHT or whether the JAK2 V617F mutation is associated with PHT in patients with MPN.

In this retrospective study with a large cohort of MPNs, we evaluated the clinical characteristics of patients with PHT complicated by GEVs and Philadelphia-negative MPNs. Most previous reports of PHT in MPN are case reports or case series, and detailed clinical characteristics of patients are generally lacking. The mechanisms underlying PHT in MPNs are still unclear. Increased blood flow into the portal system through the enlarged spleen is one of the primary causes of the development of PHT. The intrahepatic obstruction of the portal system due to myeloid metaplasia or sinusoidal change also increases portal pressure. Thrombosis of the portal vein due to blood vessel endothelium damage also induce PHT.

We designed a retrospective single-center study. This study was approved by the Institutional Review Board of Seoul St. Mary’s Hospital (KC19RESI0476). The medical records of all patients diagnosed with MPNs at Seoul St. Mary’s Hospital between January 2009 and December 2018 were reviewed. BCR-ABL1-negative MPNs primarily include PV, ET, PMF, and myeloproliferative neoplasm unclassified (MPN-U). The clinical diagnosis of PV, ET, PMF, and MPN-U was conducted in accordance with the 2016 WHO classifications.

Normal hepatic venous pressure gradient (HVPG) is typically 1 to 5 mmHg, and significant PHT is considered as an increase in HVPG ≥10 mmHg, leading to the development of complications of PHT. Owing to the inability to measure HVPG or to perform endoscopy, PHT was evaluated by the existence of GEVs, as verified by abdominal computed tomography (CT). An esophageal varix was radiologically defined as an enhancing nodular tubular structure protruding into the esophageal lumen. A previous report demonstrated that CT showed a 90% sensitivity in the detection of large (>5 mm in diameter) GEVs. In our study, to increase the specificity of clinically significant GEV detection, the threshold diameter for esophageal varix was set as 5 mm on abdominal CT. Data were collected at the time of the abdominal CT. Quantitative variables are expressed as medians (interquartile range), whereas percentages are reported for qualitative data. Comparisons between groups were performed using the Mann-Whitney test. Values of P<0.05 were considered statistically significant.

Two hundred and twenty eight patients with MPN underwent contrast-enhanced abdominal CT at least once between January 2009 and December 2018 (Table 1, 2). Among these 228 patients, 50 (21.9%) were diagnosed with PV, 72 patients (31.6%) had ET, 84 patients (36.8%) had PMF, and 22 patients (9.7%) did not have a specific diagnosis within the MPN-U category.
not meet the criteria for these three diseases and were classified as MPN-U. A total of 11 patients among 228 patients had GEVs, as confirmed by abdominal CT. A total of 130 out of 228 patients had the JAK2 V617F mutation and all patients with GEV had the JAK2 V617F mutation (57% vs. 100%, P = 0.003). The incidence of the JAK2 V617F mutation in each group was as follows: 68% (34/50), PV; 52% (37/72), ET; 56% (47/84) PMF; 45% (10/22), MPN-U.

Among 11 patients having GEVs, five had PV, two had ET, three had PMF, and the remainder were classified as having MPN-U. The median age was 60, and five subjects were male. The median spleen size was 16.9 cm. No patients had chronic viral hepatitis or other chronic liver diseases such as hemochromatosis or autoimmune hepatitis (Table 1, 2). Three had portal vein thrombosis without abnormalities in coagulation factor assays. All these patients were evaluated for the liver function (international normalized ratio, albumin, total bilirubin, presence of ascites, and presence of hepatic encephalopathy), and were categorized into Child-Pugh class A, indicating that the liver function was well-preserved (Table 1, 2). Three patients had variceal bleeding, which had not been lethal and controlled by endoscopic ligation. Seven patients received hydroxyurea and two patients received ruxolitinib (a JAK1, 2 inhibitor), but both of the medication resulted in no changes in variceal size by CT imaging.

A 53-year-old patient was diagnosed with PMF in 2016 at Seoul St. Mary’s Hospital. Ruxolitinib treatment was started after the diagnosis. On August 4, 2017, he visited the emergency room presenting with hematemesis, and esophageal varix bleeding was documented. The bleeding was successfully stopped by endoscopic ligation. Prominent varix formation (F3) was detected and TIPS was performed on August 10, 2017. In September of 2017, he developed hepatic encephalopathy and visited the hospital again. He and his family decided to undo the shunt and TIPS closure was performed on September 25, 2017. Within 1 month of the procedure, the patient presented with recurrent hematemesis. Esophageal varix bleeding was noted again and successfully ligated by endoscopy. The disease activity of PMF was stable, and the patient underwent successful living donor liver transplantation (LDLT) on November 24, 2017.

About 1 month after LDLT, in December of 2018, endoscopy revealed that varices were nearly resolved (Fig. 1A, B). We reviewed histologic findings of the recipient liver (Fig. 1C). We detected dif-
fuse myeloid metaplasia at sinusoids, verifying the previous hypothesis based on pathophysiology that extramedullary myeloid metaplasia plays an important role in PHT of MPNs. By Masson’s trichrome staining, we found a lack of fibrotic changes in the liver parenchyma, indicating that liver parenchymal injury is not the main pathophysiological factor.

Our analysis shows that about 5% of patients with MPN who underwent abdominal CT exhibited PHT, and three among 11 patients with PHT had variceal bleeding. Interestingly, our present study showed a strong correlation between the JAK2 V617F mutation and PHT. In our study, however, ruxolitinib did not have promising results for the treatment of variceal bleeding. One patient had recurrent variceal bleeding and underwent LDLT, which dramatically decreased portal pressure.

Several previous studies have demonstrated an association between the JAK2 V617F mutation and PHT and/or portal vein thrombosis.1,14 It is possible that the blood flow velocity in portal venous systems is slow, leading to prolonged interactions between blood and endothelial cells. Increased blood cells due to the clonal expansion by JAK2 V617F mutation could provoke thrombosis in the portal venous system. Hydroxyurea and JAK inhibitors are primary treatment options in MPNs. In a previous report on a patient who received ruxolitinib for PMF, PHT improved significantly, allowing discontinuation of all medications for ascites and GEVs.3 However, another report showed no effect of hydroxyurea and ruxolitinib on regression of PHT.2 Our data also showed no effect of these drugs on the size change of GEVs. The discrepancy may stem from the degree of PHT. In our study, we defined PHT as having GEVs more than 5 mm on CT, which may reflect severe PHT by MPNs.

Our study had some limitations, including the retrospective design and the use of abdominal CT to detect PHT, rather than HVPG measurement. However, owing to the inconvenience and invasiveness of the procedure, we could not recommend it for every patient.

In summary, patients with MPN can develop PHT and life-threatening events such as variceal bleeding, particularly when the patient has JAK2 V617F mutation. In one patient, LDLT resolved PHT, suggesting that liver transplantation, rather than JAK inhibitors alone, is a therapeutic candidate when MPNs are well-controlled. Our findings also suggest that gastrointestinal endoscopy or abdominal CT should be performed to evaluate PHT in patients with Philadelphia-negative MPNs.

Authors’ contributions
Jaejun Lee, Pil Soo Sung, and Ki-Seong Eom contributed to conception, design, acquisition, analysis, and interpretation of data. Aung Hlaing Bwa, Angelo Lozada, Hyun Yang and Soon Kyu Lee contributed to acquisition and interpretation of data. Jeong Won Jang, Si Hyun Bae, Yong Young Choi, and Seung Kew Yoon contributed to conception and design. Jaejun Lee and Pil Soo Sung wrote the manuscript.

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Conflicts of Interest
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<td>[1] Identify the committee(s) approving the study protocol and include a statement of compliance with ethical regulations.</td>
<td>□</td>
</tr>
<tr>
<td>[2] An acknowledgement of persons who made a assistance and provided special reagents may be included. Grant and financial support related with the work should be specifically stated.</td>
<td>□</td>
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<td>[3] Please state any conflicts of interest.</td>
<td>□</td>
</tr>
<tr>
<td>[4] All citations in the paper have a complete and accurate reference in the reference list. The number of references in case reports should be 20 or less, and 10 or less in special topics.</td>
<td>□</td>
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</table>

4) Tables and Figures

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
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<tbody>
<tr>
<td>[1] Prepare tables on individual sheets of paper, double spaced and numbered consecutively with Arabic numerals in the order of their appearance in the text.</td>
<td>□</td>
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<tr>
<td>[3] Figure legends should be typed consecutively on a separate sheet of paper.</td>
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<tr>
<td>[4] Figures should be supplied in the JPG or TIFF format at a final resolution of 600 dpi or higher. The number of figures in case reports should be 5 or less.</td>
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