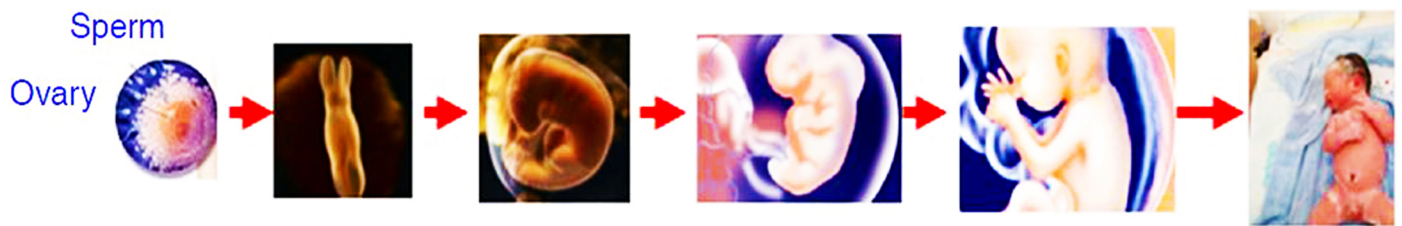
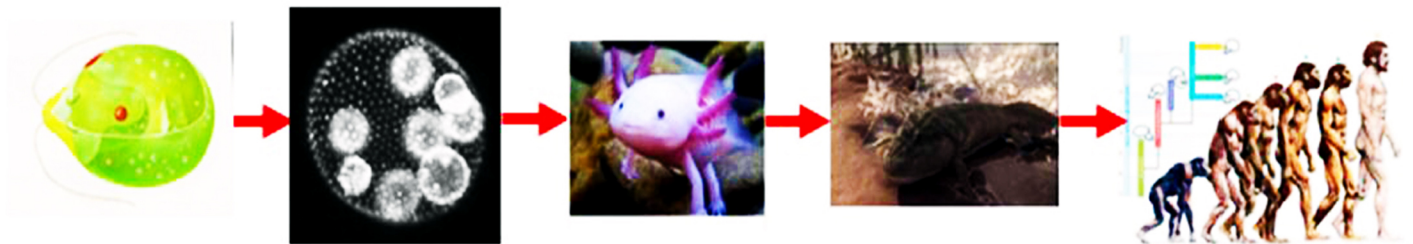


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Radiological diagnosis of hepatocellular carcinoma in non-cirrhotic patients

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ABSTRACT

Hepatocellular carcinoma (HCC) arising in non-cirrhotic livers is relatively rare. Compared with HCC arising in cirrhotic livers they have some quirks. HCC in healthy livers are large tumors at diagnosis, and are detected due to the onset of abdominal symptoms, outside of any scheduled monitoring program. In non-cirrhotic patients, HCC has the same appearance as the classic image of cirrhotic HCC substrate. The presence of capsule, extensive intratumoral necrosis and typical behavior in the dynamic study after administration of intravenous contrast are present in most of the non-cirrhotic livers. In the presence of a suspicious lesion of HCC, we must assess the existence of underlying chronic liver disease. Ultrasound, computed tomography, and conventional magnetic resonance are imaging techniques that have a high specificity for the diagnosis of cirrhosis, but exhibit low sensitivity for diagnosis in the early stages of the disease. In recent years, new imaging methods are being developed to assess emerging liver fibrosis. In particular, in patients without chronic liver disease it is imperative to consider the differential diagnosis with other tumors that may settle in healthy livers with similar radiological characteristics as HCC. Therefore, in the presence of a lesion with pathognomonic radiological characteristics of HCC in the absence of cirrhosis, biopsy is required.

INTRODUCTION

Primary liver cancer is the fifth most common cancer in men and the ninth most common cancer in women, assuming the second leading cause of cancer death worldwide.^[1] Eighty-three percent of new cases occur in developing countries, half of them in China. Its incidence has increased in recent decades, especially in developed countries. In 2015 in Spain, the incidence was 5.172 cases per 100,000 population

and there was an emergence of about 32,000 new cases in the United States.^[1]

Up to 90% of primary liver tumors are hepatocellular carcinoma (HCC). HCC has its origin in hepatocytes, the predominant cells of the liver parenchyma. Around 80-90% arises in a cirrhotic liver. The most commonly associated risk factors are chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV). HBV is the most common cause of HCC



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in underdeveloped countries. In developed countries, most HCC originate in a setting of alcoholic cirrhosis or non-alcoholic steatosis related to obesity. However, there is an incidence of 0.5-1% per year in patients with non-cirrhotic livers.^[2] Usually, such patients are not subject to monitoring prevention programs and so HCC detection is usually late and secondary to symptoms produced by the tumor. Less frequent risk factors are type II diabetes and metabolic syndrome, congenital diseases such as hereditary hemochromatosis, tobacco, parasitic infections or genotoxin intake. The average age at diagnosis of HCC is 63 years old, with an incidence three times higher in men than in women.^[2]

Clinically, it is a silent disease in early stages. When symptoms appear, the most common is abdominal pain (52%).^[3] Less common symptoms are chronic diarrhea, jaundice, fever, or paraneoplastic syndromes such as hypercalcemia or hypoglycemia. It may occur with increased serum levels of alpha-fetoprotein, considered indicative of HCC above 400 ng/dL.^[4] However, this determination has low sensitivity and specificity for diagnosis and for monitoring.

RADIOLOGICAL DIAGNOSIS OF HCC

There are three basic diagnostic tests: computed tomography (CT), magnetic resonance imaging (MRI) and ultrasound (US).

Computed tomography

Proper technique is essential for the accurate assessment of HCC: a baseline study, an arterial phase after administration of intravenous contrast (30-35 s), a portal phase (75-90 s) and a late phase (after 3 min). HCC presents as a single nodular lesion in most cases. Around 20% are multinodular. Without contrast, its density is similar to normal or slightly lower than liver parenchyma. Contrast series shows a typical dynamic behavior. It is a tumor with neoangiogenesis of arterial origin; therefore, it enhances intensely in arterial phase. In portal phase (venous) and late phase, the tumor washes the contrast and becomes hypodense relative to normal parenchyma [Figure 1].

This behavior of early enhancement and late washing (wash in - wash out) is part of the main diagnostic criteria for HCC. Its mosaic appearance is also characteristic with areas of different density within the liver, visible especially in post-contrast phases. The tumor is often encapsulated, identifying one hypodense halo. The capsule enhances more slowly and gradually and uptake usually persists in

later stages. Sometimes, the edges are imprecise, which also determines more aggressive tumors. Growth is usually expansive although there may be transcapsular infiltration into the surrounding parenchyma.

However, a high percentage of patients do not demonstrate pathognomonic HCC criteria, showing atypical features. Thus, in a retrospective study of 243 patients conducted by Lee *et al.*,^[5] the most typical behavior of tumors corresponded to moderately differentiated HCC. A high percentage of cases showed atypical behavior (43.6%). Most of these tumors corresponded histologically to well

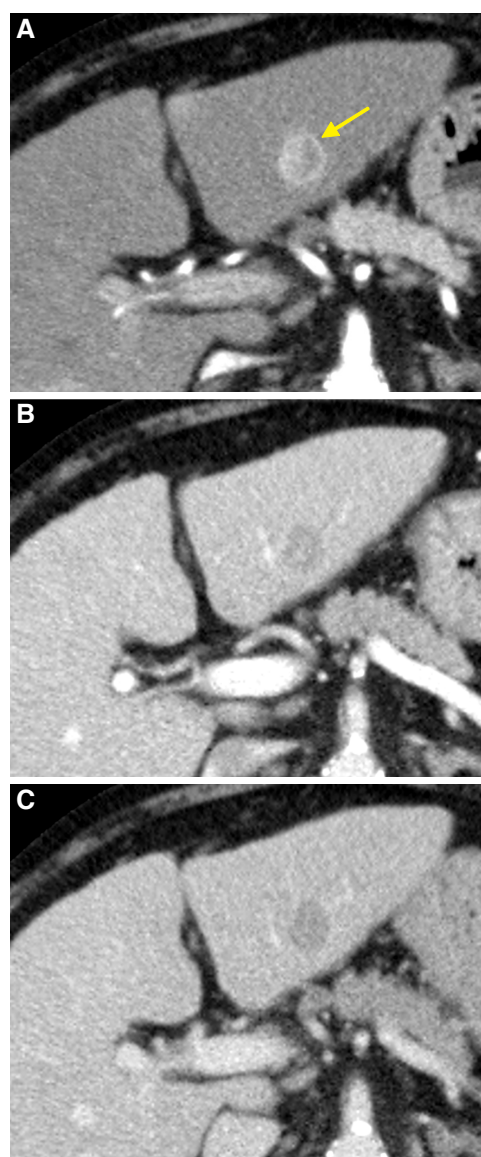


Figure 1: Computed tomography axial planes obtained in arterial phase (A), portal phase (B), and late phase (C). Lesion located in the segment III of left hepatic lobe, heterogeneous enhancement of the lesion is observed in the arterial phase (arrow in A) with washout in the portal and late phases. Mosaic pattern is shown in the arterial and portal phases (yellow arrow)

Table 1: Magnetic resonance for the study of HCC

Studies
FSPGR on phase and opposite phase enhanced on T1
FRFSE enhanced on T2 fat-suppressed
LAVA or dynamic 3D SPGRE
Pre-contrast phase
Post-contrast phase
Arterial phase: 16 s
Portal phase: 60 s
Late portal phase: 180 s
Complementary phases: intermediate or later
Diffusion
B Factor 0 and 600 seg/mm^2

LAVA: liver acquisition with volume acceleration; HCC: hepatocellular carcinoma

differentiated and poorly differentiated tumors. It has also been shown that atypical enhancement and clearing may even be seen in small HCC (< 2 cm).^[6]

Magnetic resonance imaging

MRI is superior to CT in the diagnosis of HCC. The study includes T2 sequences, dual phase-out of phase, dynamic study and diffusion T1 sequences [Table 1].

HCC presents variable signal intensity depending on the degree of fibrosis, necrosis, and fat. It may be hypo, iso, or hyperintense on T1 sequences. On T2 it is generally hyperintense, especially with fat suppression sequences. Gadolinium enhancement shows typical washing as described in CT: enhancement in the arterial phase and typical clearing in portal and late phases [Figure 2]. A mosaic pattern is usually observed.

MRI is also able to distinguish the fat component of the lesion, which is difficult to detect from CT or US. The capsule of the lesion is hypointense on T1 and may present discrete hyperintensity on T2 with tumor infiltration or edema. In MRI, specific contrasts can be used, especially useful in patients who have not obtained a clear diagnosis by basic imaging. One of the most utilized is gadoxetate disodium. This contrast is taken up by hepatocytes, at approximate rates of 50%, and is then excreted into bile canaliculi, and results in an additional hepatocellular phase of imaging. In this phase, contrast is retained not only by normal liver parenchyma but also by regenerative nodules, dysplastic nodules, and nodular focal hyperplasia.^[7] Well differentiated carcinomas may show hyperintensity on hepatobiliary phase; however, most HCC are hypointense.^[8]

CT and conventional MRI have limitations in detecting small HCC. Hepatobiliary phase provides a more accurate diagnosis in small tumors (< 2 cm), which

appear with reduced signal with respect to the surrounding liver, because these tumors do not express the hepatocyte sinusoidal transporter required for uptake.^[9]

Ultrasonography

US is a non-invasive test and more accessible. It is possible to determine the size and morphology of the lesion, its location, and possible vascular involvement. It also provides guidance for percutaneous biopsy. Its echogenicity is variable and non-specific and may be hypo- or hyperechoic. The largest lesions are more heterogeneous and often have hypo- or anechoic necrotic areas. With Doppler color, central or peritumoral

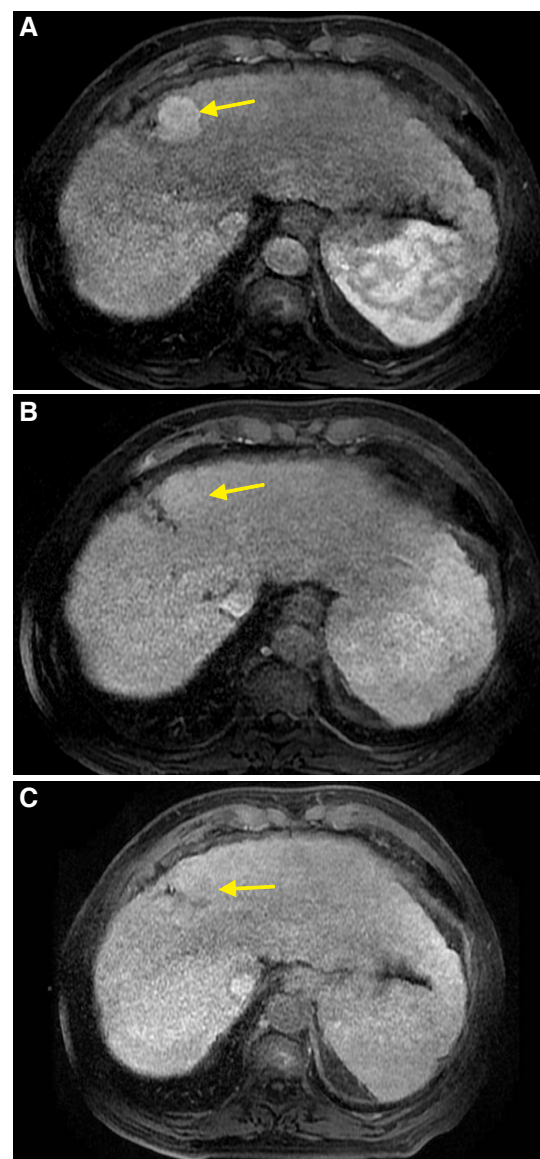


Figure 2: Magnetic resonance imaging. Liver acquisition with volume acceleration dynamic sequences obtained in axial planes at 6 min (A), 9 min (B), and 11 min (C). Enhancement of the lesion (arrow) in early stage (A) and washing (arrow) in the later stages (B and C) is observed

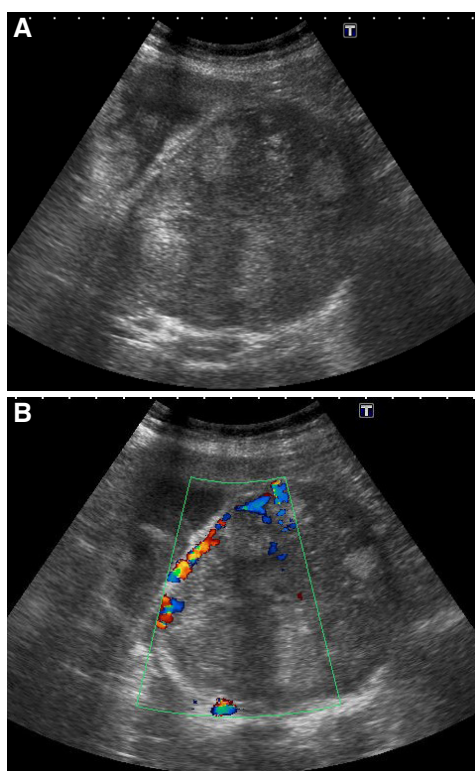


Figure 3: (A) Abdominal ultrasound B-mode showing large heterogeneous mass with hyper- and hypoechoic areas is observed in right hepatic lobe. Peritumoral vascular flow is demonstrated by Doppler (B)

vascular flow can be demonstrated [Figure 3].

According to clinical practice guidelines of the European Association for the Study of the Liver (EASL),^[10] a monitoring program must be carried out in patients at high risk for HCC, which mainly includes patients with liver cirrhosis. Abdominal ultrasound is the diagnostic method used and surveillance is conducted every six months. The main limitation of ultrasound is the detection of small tumors (< 2 cm). They can go undetected in livers with a heterogeneous diffuse nodular pattern base. However, in expert hands, sensitivity is up to 89% and specificity is up to 90%.

Contrast-enhanced ultrasound

Contrast enhanced ultrasound (CEUS) monitors time changes more directly and allows the dynamic study of the lesion. Contrast consists of sulphur hexachloride microbubbles of 2.5 µm of diameter. Since it is not nephrotoxic and presents few secondary effects, it is useful in patients with nephropathies and in those with known adverse reactions to other contrast agents. CEUS is valuable as a diagnostic tool, as a guide for biopsy and as a measure of treatment response.

Similarly to CT and MRI, CEUS shows a typical

vascular pattern in HCC, more frequent in those that are moderately differentiated^[11] [Figure 4]. Contrast agent flows exclusively through the intravascular space, without passing to the interstitial liquid, thus explaining some differences with the typical features found in CT or MRI. However, other reports have not found significant differences. Wilson *et al.*^[12] reported no differences in the dynamic behavior among CEUS, MRI and CT. Giorgio *et al.*^[13] did not find any difference between CEUS and CT. Nevertheless, Liu *et al.*^[14] reported different results for small lesions detected by CEUS and CT. In their report, a good correlation was found between both imaging techniques among lesions greater than 2 cm, but there was a low correlation among lesions measuring 1-2 cm. Possible explanations for this discrepancy are the different distribution of contrast agents, the various thickness of the slices of CT, and the effect of the direct time changes measured with CEUS. A cirrhotic background may also cause atypical patterns due to the progressive arterialization of the small lesions. These results suggest that more research is needed to determine the usefulness of CEUS in the diagnosis of HCC.

On the other hand, some papers found that the presence of wash-in/wash-out in CEUS of liver lesions is highly suggestive of cholangiocarcinoma (CC), thus inducing false positive results of HCC. This was observed by Liu *et al.*^[15] in 92.3% of HCC and in 85.7% of CC found in 819 patients. However, CC lesions had an earlier washout than HCC lesions (media of 27.5 vs. 70.1 s). Up to 68.5% of CC had a ring enhancement, while it was present in just 2.0% of HCC. They concluded that an enhancement and washout time longer than 43 s plus a non-ring enhancement had a 64.1% sensitivity and a 97.4% specificity for HCC lesions equal or smaller than 5 cm.

Ohno *et al.*^[16] observed a linear correlation between blood flow of the lesion and blood flow of the rest of the parenchyma with CEUS in 7 patients, using perflubutane as contrast agent. This activity proves the presence of intratumoral angiogenesis, thus enabling CEUS for measuring response to antiangiogenic therapies, even though the sample size was small in this report.

Nevertheless, the role of CEUS in diagnosis and staging of HCC is limited and it is not considered a first line diagnostic tool in EASL or American Association for the Study of Liver Diseases (AASLD) guidelines.

CEUS is useful for guiding biopsies. Spàrchez *et al.*^[17]

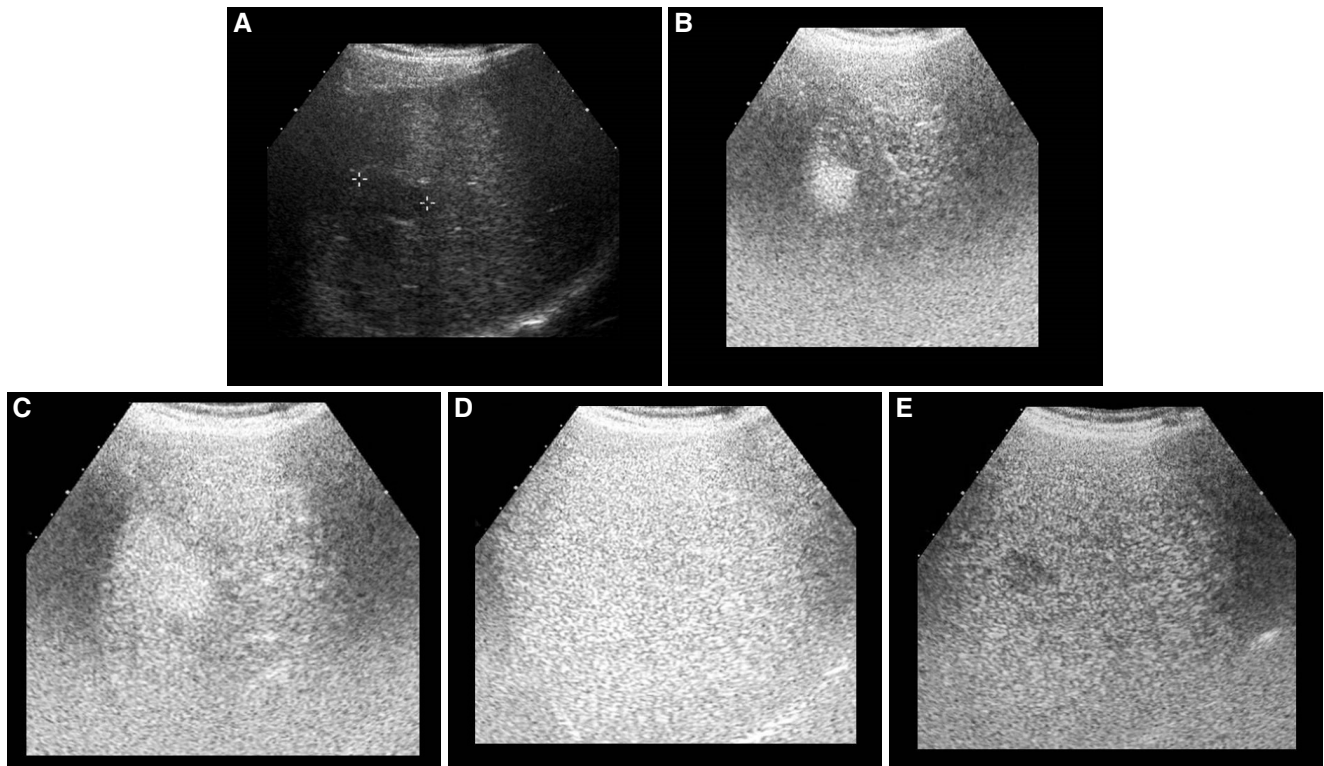


Figure 4: Abdominal ultrasound (A) and with contrast at 23 s (B), 30 s (C), 1 min (D), and 5 min (E). Hypoechoic lesion in right hepatic lobe corresponding to hepatocellular carcinoma with a typical vascular pattern: early uptake in arterial phases (B, C), isoechogenic respect to surrounded liver parenchyma in portal phase (D) and wash-out contrast in late phase (E)

prospectively compared conventional US and CEUS in 171 cirrhotic and non-cirrhotic patients. Biopsy was possible with CEUS in 97.6% of the cases, obtaining one sole sample in 43.0% of them, compared with 23.4% using US. In general, sensitivity was greater with CEUS (96.5% vs. 81.5%), also in cirrhotic patients (95.2% vs. 75.0%), in lesions greater than 6 cm (97.8% vs. 82.0%) and in poorly visualized lesions (100.0% vs. 66.6%). When histology was inconclusive with US a new biopsy was performed with CEUS, obtaining a final diagnosis in every case.

CEUS may be useful also to monitor tumor response to treatment. With antiangiogenic therapy, changes in tumoral vascularization precede changes in tumoral size. A complete response may be considered when there is no enhancement at any time. Irregular enhancement and/or eccentric or peripheral nodules suggest the presence of residual tumor.^[18] Using a quantitative analysis an individualized treatment could be done, but more research is needed to establish this indication for CEUS.

CEUS performed 60 min after radiofrequency ablation or alcoholization of HCC may monitor the efficacy of the treatment.^[18] Gao *et al.*^[19] measured the different peak enhancement of contrast between tumor and surrounding parenchyma and encountered significantly

lower rates in patients with tumor recurrence compared with those without recurrence. On the other hand, the expression levels of basic fibroblast growth factor in the recurrence group were higher than those in the non-recurrence group. Xia *et al.*^[20] and other reports have shown a greater sensitivity of CEUS compared to CT when detecting residual tumor after chemoembolization (58.1% vs. 39.5%).

CEUS has not shown better sensitivity than CT or MRI when looking for late recurrence. Thus, these two techniques are the gold standard for the long term follow-up of patients with HCC.

MANAGEMENT IN DIAGNOSIS OF HCC

The objective is early detection. In early stages, radical treatment and improved prognosis are possible. The pathological diagnosis of the tumor involves biopsy of the lesion. It is an invasive technique including risks such as bleeding or tumor seeding.

In 2001, diagnostic criteria for the management of nodular lesions in the cirrhotic liver were established. These criteria favor an early non-invasive diagnosis, preventing biopsy in some cases. In the latest update of the clinical practice guidelines of the EASL (2012),^[10] the criteria are as follows: (1) nodules > 2 cm can be

diagnosed as HCC directly with one imaging test with typical findings, if there is early enhancement and late washing; (2) nodules from 1 to 2 cm require two different techniques for diagnosis with typical findings; and (3) nodules < 1 cm should be followed by US every 4 months during the first year and then every 6 months.

In nodules between 1 and 2 cm, the AASLD in its latest update (2010)^[21] establish the criteria for a single positive test. However, the EASL does not recommend following this approach in the absence of prospective studies to support it. Both guidelines recommend the use of CT or MRI and limit the use of CEUS. As described in previous sections, intravascular contrast distribution means that in some cases the behavior of the lesion is not typical or obtains false positives in CC. In case of uncertain diagnosis, biopsy of the lesion is required.

RADIOLOGICAL ASSESSMENT OF CIRRHOTIC LIVER COMPARED TO HEALTHY LIVER

In the assessment by imaging techniques of patients without known cirrhosis three questions must be considered: first, differentiating between healthy and cirrhotic liver; second, determining if there are morphological differences or behavior in HCC that occur in cirrhotic liver versus healthy liver; and third, analyzing the management and differential diagnosis according to these characteristics with other tumors that may be seen in healthy liver, with similar radiological characteristics as HCC.

Chronic liver disease, regardless of its etiology, leads to progressive development of liver fibrosis and then to the final and irreversible stage of cirrhosis. The gross morphological changes that occur in cirrhotic livers are easily detectable with any current imaging techniques. In recent years, new imaging methods, from liver elastography of transition to modern diffusion techniques and MRI elastography, have been developed to assess liver fibrosis with the intention of making a diagnosis at an early stage that allows an active treatment for incipient liver fibrosis. In this article we review the spectrum of chronic liver disease findings in different imaging techniques.

US is usually the first technique used and can detect liver cirrhosis and its complications. In the first phase of cirrhosis liver can be enlarged, whereas in advanced stages the liver is usually small with atrophy of the right lobe (predominantly anterior segment) and the medial segment of the left lobe, and relative enlargement of lateral segments of the left, caudate or both lobes. The morphological patterns of chronic liver disease overlap

between the different causes of cirrhosis. However, hypertrophy of the lateral segments, accompanied by atrophy of the right and the left medial lobe segments, occurs frequently in patients with cirrhosis induced by virus. On the other hand, caudate lobe hypertrophy is usually associated with alcoholic cirrhosis.^[3] Several studies have evaluated the ratio between the width of the caudate lobe and the right lobe (C/RL) as an indicator of cirrhosis. Awaya *et al.*^[22] considered a value of C/RL > 0.65 indicative of cirrhosis. The specificity is high (> 90%), but with low sensitivity (43-80%), indicating that the quotient C/RL is a useful measure if abnormal.^[23-25]

Heterogeneous echostructure and multinodular appearance are frequent observations in chronic liver disease. However, its assessment mainly in the initial stages has much variability.^[26] The presence of irregular and nodular surface contour of the liver is considered to be a sign of cirrhosis. This alteration is secondary to the presence of fibrosis and regeneration nodes. This sign is easily visible in the presence of ascites, which allows a better evaluation of liver surface through the liquid (88% sensitivity, 82-95% specificity).^[27] In absence of ascites it is advisable to judge the previous liver surface by high frequency probes (7.5 MHz), increasing the sensitivity in detecting this pattern. Its existence is associated with macronodular cirrhosis.

Fibrosis of liver parenchyma can alter the morphology of the hepatic veins, with alteration in distensibility, causing luminal narrowing because the walls of the hepatic veins are thin. In advanced cases, alteration of venous flow is observed using Doppler-US, with loss of the triphase morphology of the wave flow in the hepatic veins (this condition is called "portalization"). Depending on the degree of fibrosis, intrahepatic arterial branches may be elongated with tortuous appearance with a "corkscrew" morphology, due to the distortion of the underlying liver parenchyma architecture. The wave of the hepatic artery also shows an altered dynamic, with increase of speed secondary to the lower flow of the portal vein.

Another important sign in patients with cirrhosis is detection of portal hypertension. Increased resistance of portal venous blood flow causes increased portal, mesenteric and splenic vein caliber. Thus, the existence of a diameter greater than 13 mm has a sensitivity of 42% and a specificity of 90% for the diagnosis of portal hypertension.^[28] The increase of less than 20% in the diameter of the portal vein with deep inspiration is another sign of portal hypertension, with a sensitivity of 80% and a specificity of 100%.^[29] However, the difficulty in assessing this measurement

and the inter-observer variability make this a poor criterion. In cases of severe portal hypertension there may be reversal of flow in the main vein or intrahepatic branches (centrifugal flow), and even thrombosis of the portal vein and portal cavernoma. Other signs of portal hypertension most commonly found in these patients are the presence of ascites, splenomegaly and porto-systemic collaterals (near the gastroesophageal junction, paraumbilical, retroperitoneal, gastro or spleno-renal and hemorrhoidal). However, conventional US does not usually detect abnormalities in liver morphology in patients with mild cirrhosis. The absence of such changes does not exclude this pathology.^[30]

In the last decade new techniques which quantify the degree of fibrosis have been developed, based on elastography (transient elastography and quantitative elastography) that improve the sensitivity for detection of liver fibrosis. Transient elastography (TE) or FibroScan® is based on the emission of low-frequency elastic waves (50 Hz) and amplitude through the skin to the target organ. There is an inverse relationship between the speed of wave propagation and tissue elasticity (measured in kilopascals, kPa). Thus, there is a higher propagation velocity, with lower tissue elasticity in higher degree of fibrosis. TE has been validated in multiple studies to detect cirrhosis, with a sensitivity of 84-100% and a specificity of 91-96%.^[31]

However, TE has low diagnostic efficiency in obese patients, when there is a narrow intercostal space and the presence of ascites, due to poor acoustic window and depth. Quantitative elastography, based on the strength of acoustic radiation impulse (ARFI), is integrated in a conventional US equipment that generates, through the US transducer, an acoustic pulse on the area of interest to evaluate tissue consistency. The transducer produces an US wave drive that causes a longitudinal displacement and determines the appearance of a wave pulse to the longitudinal tangential cut. The speed of the shear wave in the region of interest is directly proportional to the tissue stiffness and is measured in meters/second. The results are very similar to those achieved with FibroScan®. Both techniques show good reliability to identify patients with significant fibrosis (F2) and severe fibrosis (F3), and are excellent for the diagnosis of liver cirrhosis (F4).^[32,33]

The ARFI system has several advantages compared with TE. With the addition of structural and morphological data to a conventional US, it is a more accurate method of choosing the liver parenchyma fragment to analyze. Also, it avoids structures which distort the results, such as the filling of blood vessels,

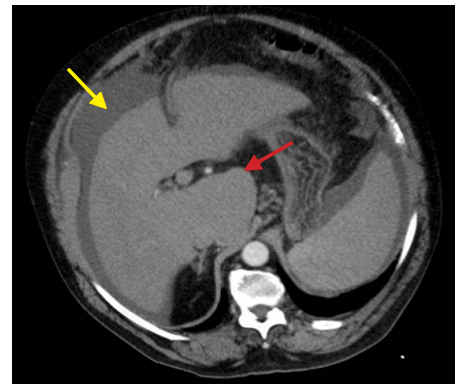


Figure 5: Computed tomography of axial plane in portal phase. Cirrhotic liver: lobed contours (yellow arrow) and moderate hypertrophy of the caudate lobe (red arrow)

gallbladder and ribs or liver capsule. These findings cannot be controlled with the FibroScan®, since it does not have an associated image. Also, with the ARFI elastography adequate results can be obtained in obese patients with a body mass index ≥ 40 kg/m² and even in patients with ascites.^[34]

The CT is a somewhat sensitive technique for the diagnosis of cirrhosis in its early stage. The contrast used should be preferably of a high iodine concentration (350-370 mg/mL) and administered at a high injection rate (4-5 mL/s). CT findings are similar to those observed by US: contour nodularity, right lobe atrophy, hypertrophy of the left lobe and caudate and increased C/RL index [Figure 5].

In early stages of cirrhosis, hepatic hilum widening is identified in 98% of the patients in the absence of other typical morphological findings of cirrhosis. However, this finding is also observed in 11% of patients with healthy liver.^[34] These patients may also show an increase in size and prominence of the interlobular fissure, with increased extrahepatic fat between the medial segment and left lateral liver secondary to atrophy of the medial hepatic segment. Structural changes in the initial phase cannot be readily assessed.

In advanced stages, heterogeneous attenuation with a diffuse distribution can be seen as well as isodense lesions in the surrounding parenchyma, corresponding to regenerative nodules. Some of them may have an increased basal density due to the presence of iron. In the dynamic study it is possible to detect vascular abnormalities as pseudolesions in the subcapsular location and wedge morphology. They have early focal enhancement, being isodense with the rest of the liver parenchyma in the portal phase. They correspond to small arteriportal shunts that are false positives of HCC, both in CT and MRI. In advanced

stages of cirrhosis, it is possible to see peripheral hypodense areas, with retraction of the liver contour and delayed enhancement, corresponding to focal confluent fibrosis. Signs of portal hypertension are similar to those seen with US: portal vein dilation, varicose veins and splenomegaly.

MRI shows greater tissue contrast than CT and US, resulting in increased information on the changes in the structure of the liver parenchyma. In patients with advanced cirrhosis, MRI may show a heterogeneous liver parenchyma with regenerative nodules and fibrous septa or bridges. The regenerative nodules are isointense or hyperintense on T1 sequences and isointense or hypointense on T2 sequences. The fibrous septa are crosslinks of low signal intensity on T1 sequences and high intensity on T2.

Areas of confluent focal fibrosis, which appear as hypointense lesions on T1 and hyperintense on T2, can also be identified. Contrast media based on gadolinium are accumulated in the extracellular compartment and are deposited on the fibrous tissue in the liver. Thus, most contrast agents based on gadolinium improve signal of liver fibrosis in T1, particularly in the venous phase and equilibrium phase. It is also possible, as with US, to perform an elastography by MRI, quantifying liver stiffness by analyzing the propagation of mechanical waves through the tissue. It allows assessment of all the liver surface, unlike US elastography, which only evaluates the outermost regions. It has high sensitivity (92%) and specificity (95%) for the detection of liver fibrosis.^[35] However, it is a technique of limited availability today, with long turnaround times and cannot be done to livers with iron overload due to noise signal artifacts.

Diffusion technique evaluates the diffusion of the protons of water molecules within tissues. It is routinely used for liver testing. Calculating the apparent diffusion coefficient (ADC) can facilitate the assessment of liver fibrosis. It has been shown that ADC values decrease as liver fibrosis increase. Bakan *et al.*^[36] detected no significant differences in ADC values between stages F0 and F1 and between F1 and F2. Another study, however, showed significant differences in ADC values between the stages F0 and F4.^[37] Together, these findings suggest that diffusion technique is not reliable for distinguishing the early stages of liver fibrosis.

Vascular changes that occur as a result of cirrhosis can be detected after the administration of a paramagnetic contrast agent and can be useful to quantify the state of parenchymal microcirculation. Liver fibrosis decreases portal venous flow, increases arterial blood flow and forms intrahepatic shunts. As is

the case of diffusion and MRI elastography, perfusion measures the liver fibrosis with indirect markers. Hagiwara *et al.*^[38] showed an increase in absolute blood flow, blood fraction, volume of distribution and the mean transit time, and a decreased portal venous fraction in patients with advanced liver fibrosis compared to patients with early-stage fibrosis. However, several factors may affect the correlation between perfusion parameters and fibrosis (cardiac output, fasting, liver congestion, liver inflammation, liver damage, and portal venous flow).

The study of liver fibrosis by molecular MRI is still in its development phase and is emerging as a valuable tool for the non-invasive detection of early-stage liver fibrosis. Compared to normal liver, the amount of type I collagen in fibrotic livers increases significantly (from 36% to 53%).^[39] Therefore, type I collagen can be used as a molecular target for detection of liver fibrosis by molecular MRI. Research on the development of specific radiopharmaceuticals which can target only the extracellular matrix collagen for the diagnosis of early-stage fibrotic livers is underway.

From the above it is concluded that US, CT, and conventional MRI have a high specificity for the diagnosis of cirrhosis, but have a low sensitivity in the early stages of the disease. In pre-cirrhotic patients, the liver parenchyma usually appears normal on MRI or only a mild non-specific heterogeneity of the parenchyma is identified. Using discrete elastography can improve the sensitivity in detecting early cirrhosis. Göbel *et al.*^[40] showed a 10% increase in sensitivity for detection of liver cirrhosis with TE compared to the use of routine screening. They also showed that the combination of TE with conventional US further improves diagnostic accuracy. However, at present, with current imaging techniques, the absence of fibrosis or cirrhosis in patients with lesions suspicious of HCC cannot be confirmed.

Liver biopsy is considered the gold standard for evaluating fibrosis.^[41] However, it is an invasive procedure which can be associated with pain and with a 0.5% risk of complications.^[42] Moreover, this technique has limitations: first, biopsy analyzes a small part of the parenchyma, leading to sampling errors if it has been done in an area with less fibrotic component; second, there is a 20% intra- and inter-observer variability in the histological assessment;^[43] and third, it should be noted that the biopsy does not predict disease progression and therefore additional biopsies would be needed after starting treatment for follow-up.

In the absence of morphological signs of cirrhosis in patients with suspicious lesions of HCC, histological

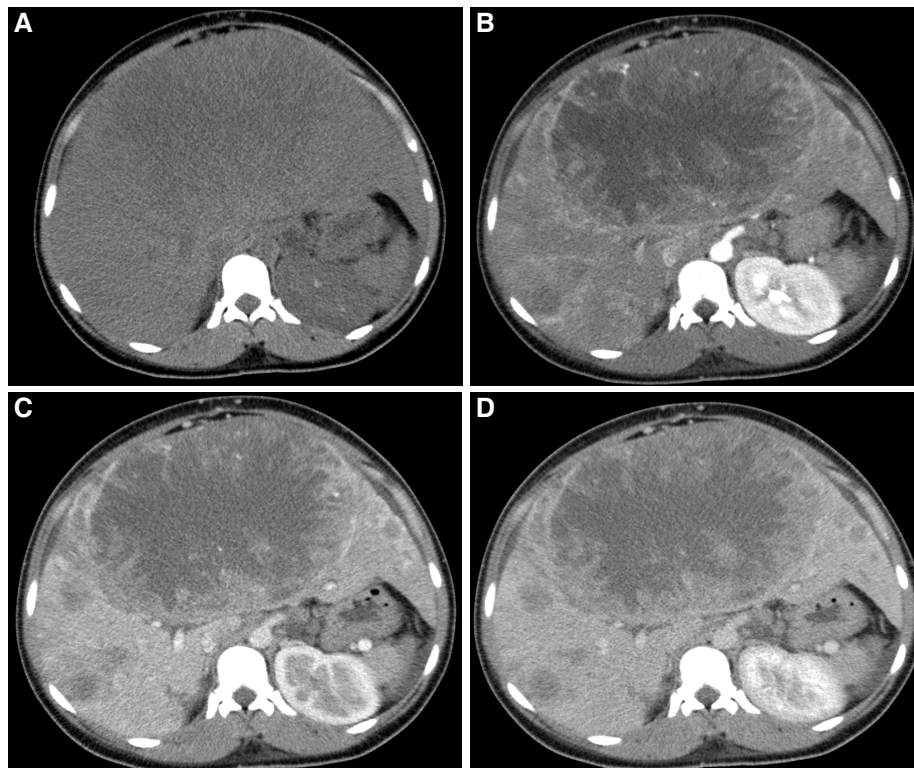


Figure 6: Computed tomography obtained in axial planes (A) and arterial phase (B), portal phase (C), and late phase (D). Voluminous mass, encapsulated, with extensive necrosis and presence of multiple satellite lesions is identified in non-cirrhotic liver. The mass shows peripheral enhancement, predominantly in the arterial phase (B) and no contrast washout are observed in later phases (C, D). The findings are compatible with hepatocellular carcinoma with atypical behaviour

assessment of hepatic parenchyma is a controversial choice. Di Martino *et al.*^[44] demonstrated that non-invasive diagnostic criteria of HCC are present in 90% of cases and that the HCC in non-cirrhotic patients shows a similar pattern of enhancement as HCC in cirrhotic patients. Based on these results it would be reasonable to apply non-invasive diagnostic criteria for HCC in non-cirrhotic patients if they have high levels of α -fetoprotein.

DIFFERENCES OF PRESENTATION OF HCC IN CIRRHOTIC VS. NON-CIRRHOTIC LIVERS

Ninety percent of HCC arise mainly in a liver with established cirrhosis resulting from chronic HCV or HBV infection or alcohol related liver disease.^[45]

Radiologists are used to see the imaging of HCC that arises in cirrhotic livers. In these cases, the tumor is often multifocal or diffuse and small in relation to the screening area visualized in these patients. HCC in non-cirrhotic liver is an uncommon finding for radiologists, presenting with different clinical and treatment options as well as prognosis.^[4,46]

The setting of HCC in non-cirrhotic liver is twice more common in men than in women, but there is

a lower prevalence of male presentation regarding HCC in cirrhotic liver. The average patient age at diagnosis is 65 years old.^[3] There is little literature on the radiological characteristics of this tumor in non-cirrhotic liver. Winston *et al.*^[47] described the characteristics of MRI in 25 patients with HCC in non-cirrhotic liver, compared with 11 patients with HCC in cirrhotic liver. In the group of non-cirrhotic patients, HCC usually presents as large masses (with an average size of 12.4 cm), predominantly solitary or dominant with small satellite lesions (82% of patients) [Figure 6]. In patients with cirrhosis, tumors are generally smaller. Their larger size and extent at time of diagnosis in non-cirrhotic livers could be explained by the non-inclusion of these patients in prevention programs. In healthy livers, there is a predisposition for HCC to occur in the right hepatic lobe.^[48]

The usually well-differentiated HCC is an encapsulated tumor with circumscribed margins, while poorly differentiated HCC is an aggressive tumor that is not encapsulated and has an ill-defined outline [Figure 7]. These findings are more prevalent in HCC in cirrhotic liver whereas the HCC in non-cirrhotic liver is predominantly moderate or well differentiated.^[49] This lesion may contain calcifications, necrosis, haemorrhage, and microscopic and macroscopic fat [Figure 8]. Sometimes,

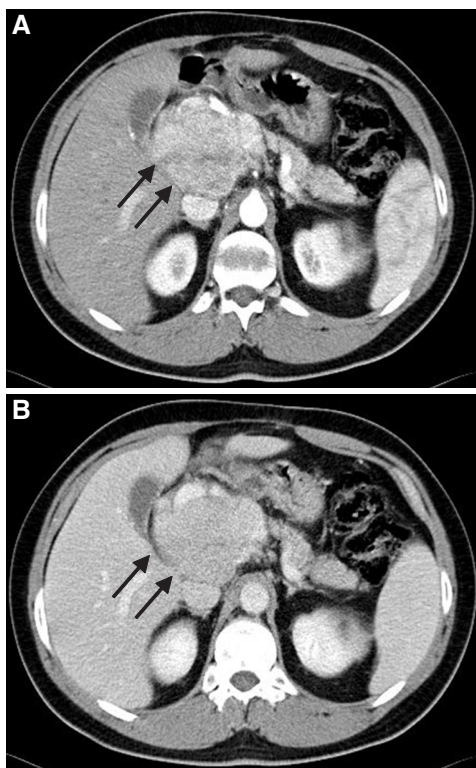


Figure 7: Computed tomography of axial planes in the arterial phase (A) and portal phase (B). Mass in the caudate lobe (arrows), non-capsulated, is identified in non-cirrhotic liver. Lesion presents heterogeneous enhancement in arterial phase (A) and late wash-out (B)

there may be focal dilatation of intrahepatic bile duct [Figure 9]; this finding is secondary to the mass effect produced by these tumors, as already mentioned, and they may reach a large size.

A greater tendency of extrahepatic spread, by direct invasion of adjacent structures or by distant spread as metastasis (20.5% vs. 6.5%, respectively),^[4] has been documented for HCC in non-cirrhotic livers compared to cirrhotic livers. This difference can be explained by a delayed diagnosis.

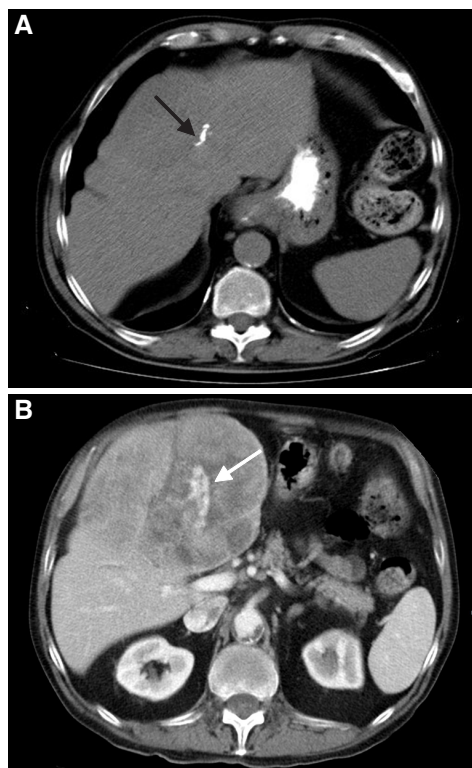


Figure 8: Computed tomography of axial planes in empty (A) and portal phase (B). Liver mass in non-cirrhotic liver with calcifications (black arrow) and important vascular component (white arrow)

In non-cirrhotic patients, HCC has a similar radiologic behavior as in cirrhotic patients. On US, HCC usually appears as a hypoechoic or more often hyperechoic non-specific lesion. In larger size lesions, a heterogeneous echostructure should be observed, due to combining solid and necrotic areas.

In CT studies without contrast the tumor tends to be hypodense relative to the surrounding liver parenchyma. Calcifications can be identified as well as areas of necrosis and hemorrhage. Following administration of intravenous contrast, the tumor typically shows

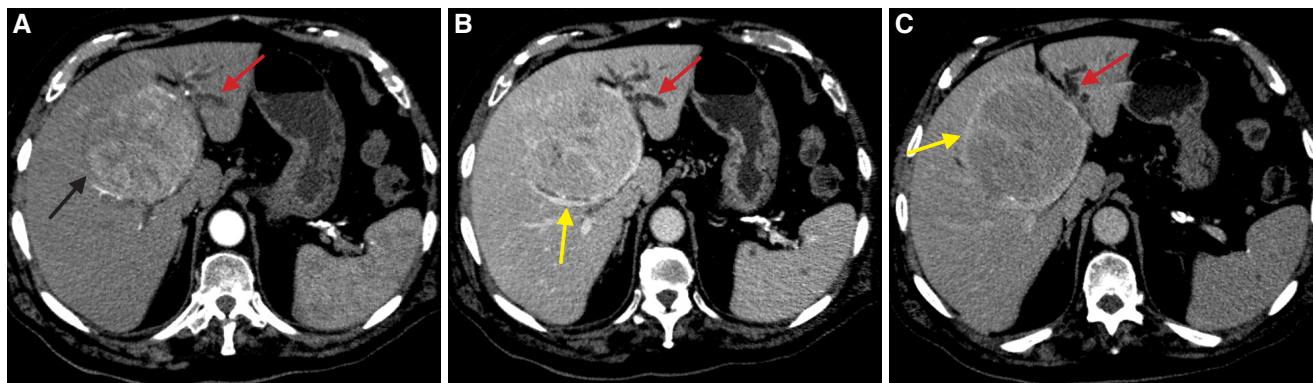


Figure 9: Computed tomography of axial planes obtained in arterial phase (A), portal phase (B), and late phase (C). Non-cirrhotic liver shows mass in right hepatic lobe (black arrow) with typical behavior of hepatocellular carcinoma. Heterogeneous enhancement in the arterial phase (A), and portal phase (B) with wash-out in delayed phase (C). The mass shows enhanced capsule in late phase (yellow arrow) and produces secondary dilatation of the bile duct (red arrow)

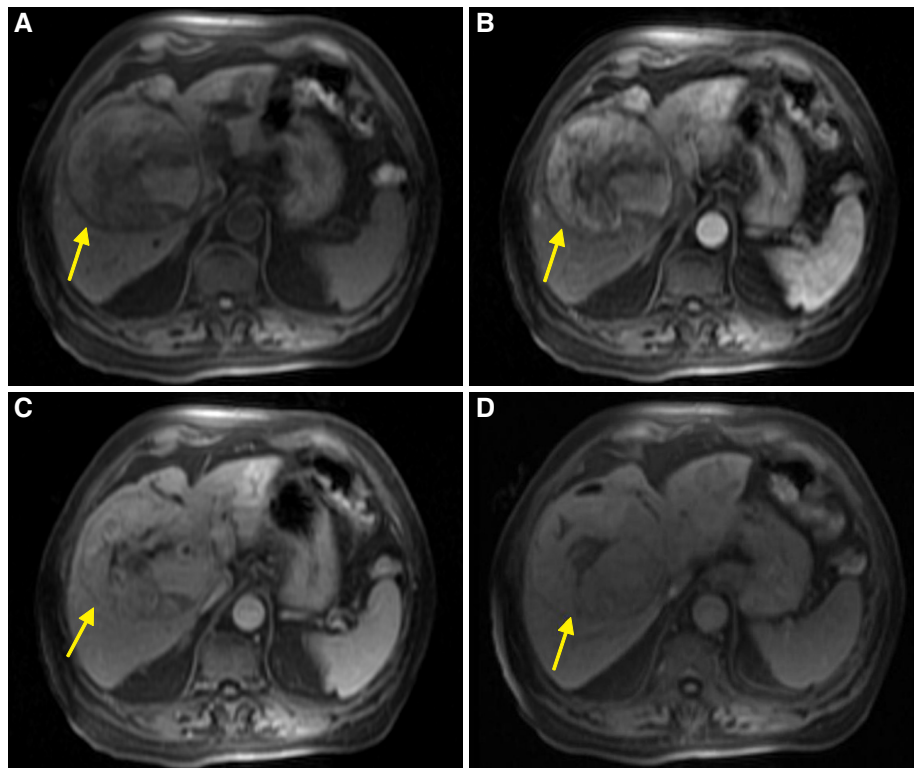


Figure 10: Magnetic resonance imaging of liver acquisition with volume acceleration dynamic sequences in axial planes: empty (A), arterial phase (B), portal phase (C), and delayed phase (D). Non-cirrhotic liver shows mass in the right hepatic lobe (yellow arrows) with necrotic component that presents heterogeneous enhancement in the arterial phase (B), and wash-out in portal phase (C) and delayed phase (D). These findings are compatible with hepatocellular carcinoma with typical behaviour

enhancement during the arterial phase (wash-in), becoming isodense in the early portal phase, and wash-out in the late portal phase and equilibrium with respect to the adjacent liver parenchyma, similar to the HCC in the cirrhotic liver [Figure 9]. Capsular enhancement, when present, is most apparent during the equilibrium phase.

The appearance of HCC on MRI in healthy liver also has the same radiological features as that in cirrhotic liver. On T1 sequences it will be most commonly hypointense relative to the surrounding liver parenchyma, although it may contain hyperintense areas due to the presence of hemorrhage and fat within the lesion. Microscopic fat can be seen in about 10-17% of non-cirrhotic HCC, similar to HCC in cirrhotic livers. It is a finding most often seen in well-differentiated tumors and, therefore, a sign of good prognosis. On T2 sequences, the HCC will be usually isointense or hyperintense. However, well or poorly differentiated tumors can be isointense or hypointense. In dynamic sequences after gadolinium administration, they will show a typical pattern identical to the enhancement on CT [Figure 10].

Usually, there will be an internal enhancement mosaic, also described in previous sections, which become

clearer mainly in the post-contrast study. It may be surrounded by a capsule with a similar behavior: hypointense on T1 and hyperintense on post-contrast study. In 80% of cases there may be a pseudocapsule formed by prominent peritumoral vessels or fibrosis, where iodinated contrast and gadolinium may be retained, producing a circumferential enhancement in the late portal phase or equilibrium phase.

In a retrospective review of 209 patients with diagnosis of HCC in our center over a period of 4 years (January 2010 - December 2014), 23 patients were selected with healthy liver by histological criteria (liver biopsy or surgical resection piece) and/or a combination of clinical, analytical criteria, imaging and hepatic hemodynamics. The average age at diagnosis in these patients was 70 years old, with no significant differences in distribution by sex, as opposed to the higher incidence in males described by other authors.^[3] Most diagnostic testing was initiated by the presence of abdominal pain or abnormal liver profiles, as in other studies.^[50] Twenty-one patients were diagnosed with HCC by biopsy and/or surgery.

Congruent with previous studies, the presentation of HCC was as a single large lesion (65%) or a dominant mass with satellite lesions (35%), with a



Figure 11: Magnetic resonance imaging in dynamic sequences: axial in arterial phase (A), 10 min (B), and coronal plane at 20 min (C). Image A, B, and C show a non-cirrhotic liver with focal lesion (yellow arrows) in segment VI. Lesion is hypovascular in all phases and present atypical behavior for hepatocellular carcinoma

largest mean diameter of 10.7 cm. The right lobe was the most common location (57%). The presence of capsule (60%), well-circumscribed margin (70%), intratumoral necrosis (87%) and a typical behavior (60%) in the dynamic study after administration of intravenous contrast were present in the radiological characteristics in most HCC. Five patients (22%) had distant metastases and 3 (13%) patients had portal vein thrombosis.

DIFFERENTIAL DIAGNOSIS WITH OTHER ENTITIES IN THE CONTEXT OF NON-CIRRHOTIC LIVER

The role of biopsy in the diagnosis of HCC is controversial. Tumor spread after biopsy is unusual, but recent meta-analysis has reported an overall prevalence of 2.7% and an annual rate of 0.9% after performing biopsy.^[51] The AASLD and EASL advocate different guidelines for the diagnosis of HCC using specific imaging criteria.^[52] Biopsy is limited to lesions > 1 cm with indeterminate characteristics in two image techniques. There is no guideline regarding the management of HCC in non-cirrhotic patients compared to that in cirrhotic patients.^[44] However, a lesion with imaging characteristics of HCC in these patients without increased serum levels of alpha-fetoprotein, in a non-endemic area of HCC, makes it necessary to rule out other tumors. Therefore, in these cases performing a biopsy may be recommended.

There are several hypervascular lesions similar to HCC. So, faced with a hypervascular lesion detected with any imaging technique, it is necessary to make a differential diagnosis between several entities such as focal nodular hyperplasia (FNH), hepatocellular adenoma (HA) or other malignancies such as intrahepatic cholangiocarcinoma (ICC), primary neuroendocrine tumors of the liver and hypervascular liver metastases. Moreover, atypical HCC may

present as a hypovascular lesion [Figure 11] or with other characteristics.

FNH [Figure 12] is formed by benign-appearing hyperplastic hepatocytes in normal liver stroma. The typical US appearance is a nodule isoechoic with the normal liver parenchyma. A central scar, containing dense connective tissue and thick arteries, is present in 77% of the cases. This scar appears usually as a hypoechoic area with a central artery that presents low resistance flow in Doppler study. In CT without contrast it is usually seen as a well-defined isodense or slightly hypodense mass compared to liver parenchyma. The scar is hypodense. Following intravenous contrast administration, in the arterial phase there is a homogeneous and intense uptake, with the central scar remaining hypodense. Later, progressive washout makes it isodense in portal and late phases. The central scar, on the contrary, shows a progressive uptake being hypodense or isodense in portal phase and hyperdense in late phase.

MRI may be useful in the characterization of the lesion in order to identify the central scar in a higher number of cases. In both sequences, T1 and T2, FNH may be difficult to distinguish from normal liver parenchyma remaining as an isointense or slightly hypointense mass on T1 and hyperintense on T2. The behavior in the dynamic contrast is similar to CT. Due to the hepatocellular origin of the lesion, when contrast with hepatobiliary elimination is used, the uptake of the lesion remains isointense or slightly hyperintense relative to normal parenchyma, due to increased secretion and excretion of contrast material of the lesion with respect to the remaining liver parenchyma. The key to the differential diagnosis with HCC is the presence of a similar enhancement of liver parenchyma in portal and delayed phases after contrast administration and the retention of hepatoespecific contrast.

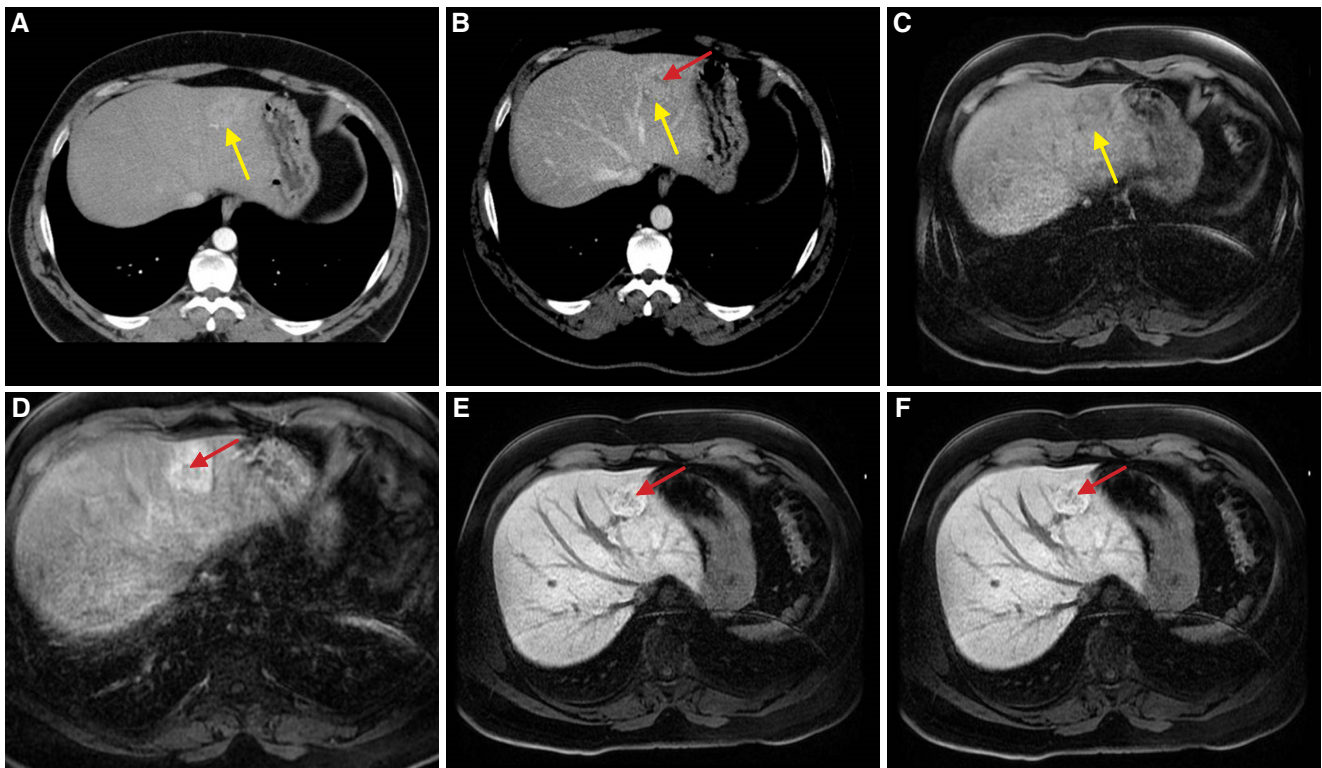


Figure 12: Computed tomography: axial planes obtained in arterial phase (A) and portal phase (B). Focal lesion in left hepatic lobe (yellow arrow) shows enhancement in the arterial phase (A) and is isodense in the portal phase (B) with central scar (red arrow). Magnetic resonance imaging: liver acquisition with volume acceleration dynamic sequences in axial planes: noncontrast phase (C), arterial phase (D), hepatocyte phase (E) and portal phase (F). Focal lesion is hypointense in noncontrast phase (C) with enhancement in arterial (D) and hepatocyte phases (E), with central scar. Lesion is isointense in delayed phase (F). Lesion shows typical radiological findings of focal nodular hyperplasia

Table 2: Magnetic resonance imaging differentiation between the three subtypes of hepatocellular adenoma

Type	T1	T1FF	T2	T1 + C
Inflammatory adenoma	Moderately hyperintense or isointense	No signal drop	Hyperintense, greater peripheral intensity	Enhancement in arterial phase and persists in portal phase and late phase
Mutated HNF1A adenoma	Hyperintense or isointense	Hypointense	Isointense	Enhancement in arterial phase that does not persist in portal phase and late phase
Mutated beta-catenin adenoma	Non specific pattern	Non specific pattern	Non specific pattern	Similar to hepatocellular carcinoma: enhancement in arterial phase and washing in portal phase and late phase

HA is a rare benign tumor. It is currently classified into 4 subgroups depending on their genotype: inflammatory adenoma, mutated HNF1A adenoma, mutated beta-catenin hepatocellular adenoma and unrated. They show different clinical behavior so their management is different.^[53]

HA are hypervascular and heterogeneous lesions caused by foci of bleeding and may contain fat. Using Doppler color, intra-lesional flow can be identified, unlike FNH or HCC, and it does not produce a pulsatile continuous curve. In CT they are well-defined lesions, hypodense to isodense or slightly hyperdense with respect to the parenchyma. They may have a heterogeneous density and/or areas of hemorrhage. In contrast CT they are hypervascular and show

significant enhancement in the arterial phase. In portal and late phases they differ by subtype: inflammatory adenoma shows a persistent enhancement, mutated HNF1A adenoma is isodense regarding the parenchyma, mutated beta-catenin adenoma appears hypervascular in the arterial phase and washes the contrast like HCC [Figure 13].

MRI is the technique of choice for the differentiation of the three subtypes, with the features shown in Table 2. Inflammatory adenoma is the most common subtype. Histologically it is composed of inflammatory infiltrate and dilation of sinusoids. It is the subtype with the higher risk of bleeding. Mutated beta-catenin adenoma is the least common subtype but that which presents the greater risk of malignant transformation

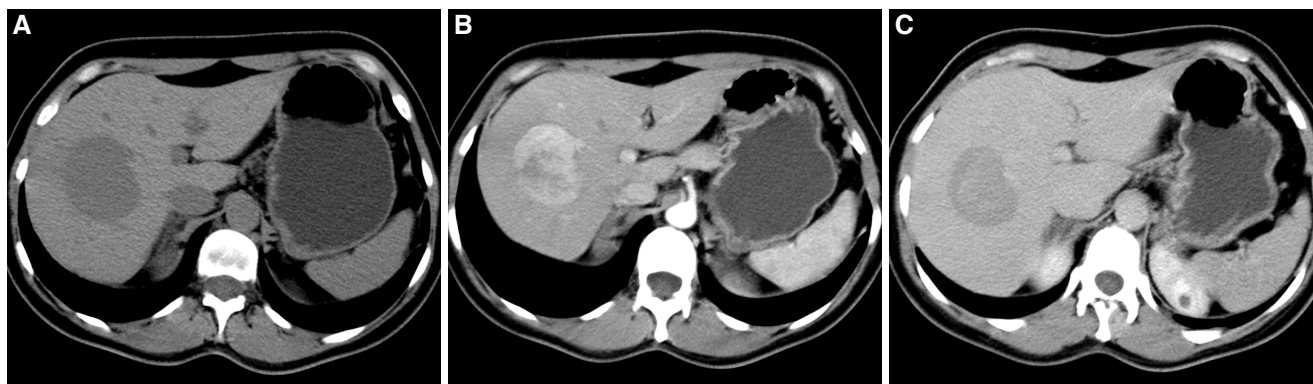


Figure 13: Computed tomography of axial planes obtained in empty (A), arterial phase (B), and portal phase (C). It shows healthy liver with hypodense mass in empty (A) with intense enhancement in the arterial phase (B), and washed-out in portal phase (C). The first radiological and pathological diagnosis was hepatic adenoma. A second biopsy confirmed the diagnosis of hepatocellular carcinoma

(5-10%). It is more common in men with deposition diseases or who consume anabolic steroids.

HCC should be distinguished from ICC with mass growth pattern. Although they are malignant tumors, prognosis and treatment are very different in both entities. The typical enhancement pattern of ICC is a gradual contrast uptake without washing (80% of ICC) or stable contrast uptake without washing (20% of ICC). In arterial phase, it appears as a hypodense mass with incomplete peripheral enhancement. The central part shows a prolonged enhancement in the

late phase, due to the slowness of washing related to the fibrous tissue in the tumor [Figure 14]. The pattern of progressive or stable enhancement in portal and late phases can also sometimes be observed in HCC. Therefore, with this type of pattern we always perform a biopsy for histological diagnosis.

Liver can also be a frequent site of metastatic neuroendocrine tumors from another location [Figure 15]; it is unlikely to be of primary liver origin. The primary hepatic carcinoid tumor appears as a liver mass, usually solid (60%), partially solid with cystic areas

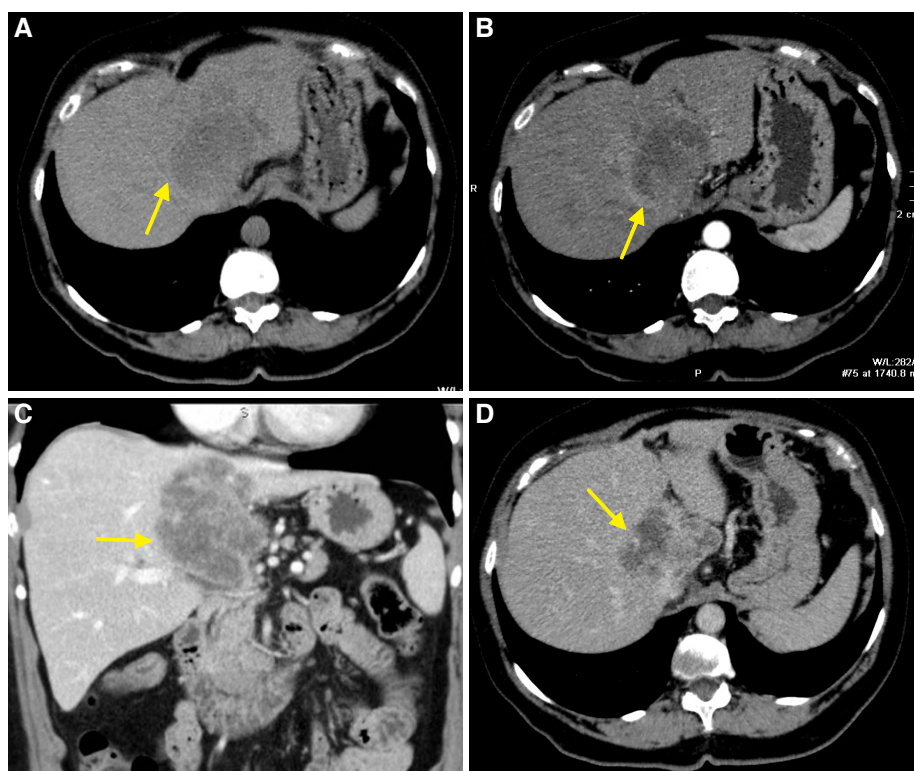


Figure 14: Computed tomography of axial planes obtained in noncontrast phase (A), arterial phase (B), portal phase in coronal plane (C) and axial plane in delayed phase (D). Hepatic mass (yellow arrow) hypodense in noncontrast phase (A), with heterogeneous peripheral enhancement in the arterial phase (B), and portal phase (C), and central uptake in delayed phase (D). This lesion corresponded to cholangiocarcinoma

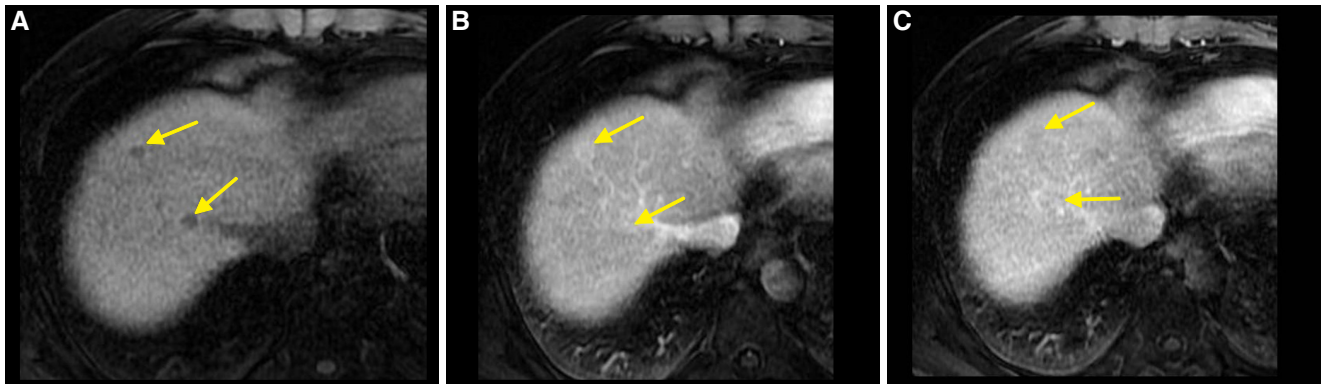


Figure 15: Magnetic resonance imaging in axial planes with T1 sequences in noncontrast phase (A), and arterial contrast phase (B), and portal phase (C). Multiple lesions (yellow arrows) scattered in right hepatic lobe, hypointense in noncontrast phase (A) and uptake in early arterial phase (B), held in portal phase (C) corresponding to liver metastases of pancreatic neuroendocrine tumor

(25%), or predominantly cystic (15%). It shows peripheral enhancement in the arterial phase. In MRI it is hypointense on T1 and hyperintense on T2 and with an enhancement after administration of gadolinium similar to that obtained in CT.

In addition to the metastatic neuroendocrine tumors, other tumors with hypervascular appearance such as thyroid tumors, renal tumors or melanomas, may present as an initial liver finding. Such lesions are generally multiple and small, unlike the usual presentation of HCC. The uptake curve of hypervascular metastases is typical: very intense and early enhancement in the arterial phase and also very early wash-out in the portal and equilibrium phases. This dynamic behavior is similar to that presented in HCC and therefore, if a primary tumor is not known and there is a small number of lesions, biopsy is essential for the differential diagnosis.

In the absence of typical signs of benign lesion as FNH or inflammatory adenoma and with a suspicion of malignancy, a reliable diagnosis cannot be made and a histologic confirmation is required due to the similarity of the radiologic features of these lesions with typical HCC.

In conclusion, HCC in patients with healthy livers have no significant differences in dynamics and morphological characteristics. However, they are usually diagnosed in more advanced phases and are larger, probably because they are not subjected to screening programs. Due to the similar properties of other benign or malignant lesions, the diagnosis must be made by biopsy unlike in cirrhotic patients, where a lesion with early and late enhancement washing (wash-in and wash-out) is pathognomonic of HCC and a biopsy is not needed. On the other hand, a cirrhotic substrate cannot be ruled out by imaging techniques. Therefore, in the absence of other clinical

and laboratory data suggesting a history of cirrhosis, biopsy should be performed in all lesions with pathognomonic characteristics of HCC.

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Conflicts of interest

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Patient consent

There is no patient involved.

Ethics approval

This review paper is waived for ethics approval.

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Sorafenib from palliative to neoadjuvant chemotherapy in hepatocellular carcinoma with major vascular invasion: experience of two cases

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ABSTRACT

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Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the incidence is higher in cirrhosis. Treatment options depend on tumor stage, status of liver function, and the general condition of the patient. Major vascular invasion is a contraindication for liver transplantation. Sorafenib has been found to be useful in association with transarterial chemoembolization as an effective chemotherapeutic agent to prolong survival in inoperable HCCs. Here we describe our experience where sorafenib was used as palliation but later turned out to be a neoadjuvant. Both cases had major portal vein thrombosis and received sorafenib as palliative therapy. After a mean use of 6 months, both patients had marked tumor response and proceeded to have liver transplantations. Both cases are tumor-free at a median follow up of 13 months.

INTRODUCTION

Hepatocellular carcinomas (HCCs) are the fifth most common cancers in the world. The incidence of HCC is more in the eastern population compared to the west. Incidence is also higher in the cirrhotic livers as compared to the non cirrhotics. Management depends on the tumor stage, status of the liver and general physical status of the patient. Majority of HCC patients at the time of primary consultation

have advanced and incurable. Hence there are many palliative options available to prolong the survival in such group of patients. In patients with early cancers curative treatment options are possible. Curative options include liver resection, liver transplantation, radiofrequency ablation (RFA). Palliative therapeutic options include transarterial chemoembolization (TACE), transarterial radioembolisation, sorafenib, external beam radiotherapy (EBRT), combination chemotherapy regimens. With recent advances in



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liver transplantation various neoadjuvant modalities have evolved over years to make inoperable patients into operable with equivalent survival rates. TACE, RFA and EBRT have been employed as neoadjuvant modalities to reduce the tumor burden. There are resolution chest tomographies (RCTs) going on to assess the effect of neoadjuvant role of TACE with or without sorafenib. Our case reports give a different perspective to these ongoing studies. One case was sorafenib without hepatic artery occlusion and the other one with hepatic artery occlusion.

CASE REPORT

Case 1

A 54-year-old gentleman, a business man from Islamabad, was diagnosed with hepatitis C virus (HCV) infection in 2003 when he was worked up for generalized weakness. For which he received 26 injections of peg-interferon over 3 months and achieved sustained viral response (SVR). He remained relatively asymptomatic till 2015. In September 2015, he developed right upper quadrant pain associated with significant loss of weight. In October 2015, he was diagnosed with HCC in the right lobe with portal vein tumor thrombosis (PVTT) and encasement of right hepatic vein and middle hepatic vein. The alpha fetal protein (AFP) levels rapidly increased to > 50,000 by November 2015. In view of the advanced nature of the disease, he was started on sorafenib 400 mg twice daily in Pakistan. He was reevaluated in our institute and found out to be not a candidate for liver transplantation. Since the cirrhosis was of Child A status, and imaging showed adequate remnant (there was right portal vein thrombosis causing adequate hypertrophy of the left lobe), he was subjected to exploratory laparotomy with the intention of palliative tumor resection on November 24, 2015. But at laparotomy, there was a large mass arising from the right liver with adherence to the colon. There were no signs of any distal metastasis. So the surgery was concluded after doing right hepatic artery ligation. His post procedure period was uneventful and was discharged on November 28, 2015. Tab sorafenib 400 mg bid was continued post operatively. In the second week of April 2016, he developed cutaneous manifestation of drug intolerance, hence discontinued. During this period, the AFP level in January 2016 had decreased to 1,303 and the patient had shown improvement in his general condition. A positron emission tomography-computed tomography (PET-CT) was repeated in April 2016 which showed features of tumor necrosis and bland PVT without any evidence of distant metastasis. His AFP had dramatically decreased to 3 IU/mL [Figure 1]. As he

did not have any radiological signs of viable disease the plan for palliative radiotherapy was cancelled. After assessment for living-donor liver transplantation (LDLT) and after discussion of the case in the liver transplant meeting, it was decided to do LDLT.

On admission, investigations revealed Hb 12.10, TLC 5,860/cu mm, platelet count 198,000/cu mm, prothrombin time/international normalized ratio (PT/INR) 9.40/0.90, urea 25 mg/dL, creatinine 0.70 mg/dL, serum bilirubin 0.60 mg/dL, albumin 3.60 mg/dL. Anti HCV was reactive and HBsAg & HIV were non-reactive. Serum AFP was 3.52 IU/mL. Urine protein/creatinine ratio was 0.24. PET-CT liver showed cirrhotic liver with a small right lobe and multiple SOL's in the residual right lobe and tumor thrombus in right portal veins and main portal veins/left portal veins junction as described, mild ascites. Magnetic resonance imaging upper abdomen showed liver cirrhosis, multiple masses in both lobes of liver (right > left) with tumor thrombus in right, left and main portal vein near portal bifurcation suggestive of HCC, bland thrombus in remaining portal vein, no significant abdominal lymphadenopathy or ascites is seen. High RCT showed no scan evidence of pulmonary metastasis. 2D Echo showed pulmonary artery systolic pressure 22, CVP 5, EF 60% and dobutamine stress echocardiography was negative. Considering the nature of disease and explaining the risk/prognosis to relatives, he was planned for liver transplantation. After optimization and PAC clearance, patient was taken up for surgery on April 21, 2016.

He received a modified right lobe graft with graft recipient weight ratio of > 1 on April 21, 2016. Post operatively he was shifted to the intensive care unit and was extubated on post operative day (POD) 1 according to the protocol. Immunosuppressant were started on POD 1 according to the protocol. Patient was started on liquid diet on POD 2 and gradually increased to normal diet. His lab reports showed a steady improvement with a peak bilirubin of 2.8 and a peak INR of 2.9 on POD 1. His both drains were removed on

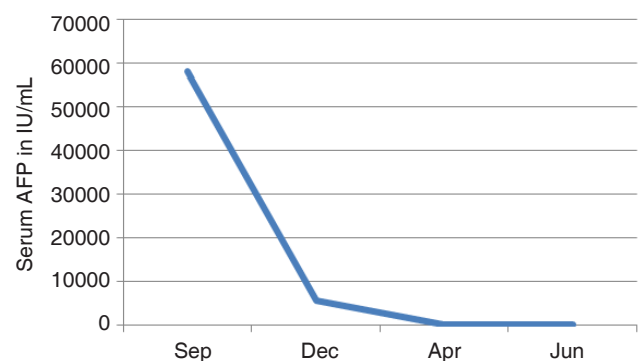


Figure 1: Alpha fetal protein (AFP) trend of case 1

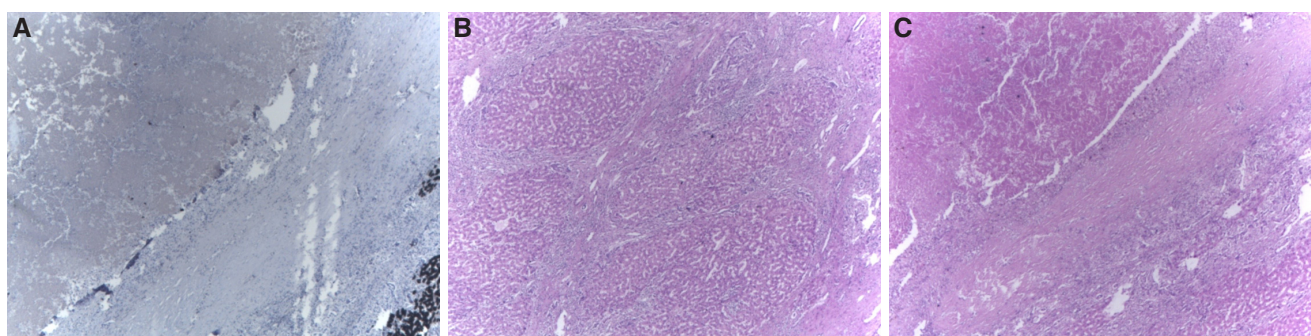


Figure 2: The final istopathology results of case 1. (A) AFP stain showing necrotic tumor, $\times 4$; (B) back ground cirrhosis (HE, $\times 4$); (C) necrotic tumor (HE, $\times 10$)

POD 6. He tolerated immunosuppression well. He was discharged in a stable condition on POD 13.

The final histopathology of the explant specimen did not show any tumor at all. There was complete tumor response to hepatic artery ligation and sorafenib therapy [Figure 2].

At 14 months post transplantation, he has been switched over to everolimus based immunosuppression. Also he is on adjuvant sorafenib treatment. At 13 months post transplantation his serum AFP is normal and PET-CT is normal. Graft functions are normal.

Case 2

A 48-year-old gentleman from Sindh Pakistan was a case of HCV related chronic liver disease. He was diagnosed in 2012 with HCV. He received interferon therapy and achieved SVR. In June 2013 he was diagnosed with HCC and PVT along with elevated AFP. He was given sorafenib treatment. Subsequent follow up revealed normalization of AFP, clearance of PVT and decrease in the tumor size. Sorafenib therapy was discontinued after 4 months owing to intolerance. He was on regular follow up with 3 monthly AFP and CT scan. The AFP was normal and the tumor was more or less constant size of 4.5 cm with no evidence of new lesions elsewhere. In view of the PVT in previous scans, transplantation was deferred by various transplantation centers. However, in June 2015 he developed severe encephalopathy followed by recurrent episodes of minor encephalopathies. In view of hepatic decompensation, he underwent liver transplantation in October 2015. Post transplantation explant biopsy revealed low grade HCC in Milan with no capsular or vascular invasion. He had uneventful post-operative course. At 14 months post transplantation, patient survival and graft survival are good with no tumor recurrence.

DISCUSSION

HCCs are the commonest primary neoplasms of the

liver. They are the fifth most common cancers with 4th commonest malignancy. There are multiple etiologies for HCCs. In general, cirrhotic livers have higher incidence of HCC as compared to non cirrhotics. The duration of cirrhosis is directly proportional to the cumulative incidence of malignancy. HCC has peculiar tumor biology. Curative treatment options for HCC are RFA, resection and liver transplantation.^[1] Of these three, primary liver transplantation has better survival in patients with cirrhosis and HCC.^[2] The indications for liver transplantation in CLD with HCC has been gradually expanding since the publication of Milan criteria. It started from Milan criteria and has reached to any size any number without vascular invasion criteria.^[3] Even in cases of vascular invasion there are case series to prove the efficacy of neoadjuvant radiotherapy (brachy/EBRT) with reasonable recurrence free survival rate.^[4] In case 1 where the intention was purely palliative but later on patient ended up with successful liver transplantation. Initial look up of the case was suggestive of hopeless situation. Hence we abandoned the resection attempt after ligation of the hepatic artery. There was no decompensation in the post-operative period. He received sorafenib as palliative chemotherapy protocol. Decision making for liver transplantation was crucial in this case. However, we went by basic tumor assessment methods like serum AFP, PET avidity and contrast enhancement of tumor and thrombus. Since all three parameters were negative he was taken up for transplantation. There are trials which showed improved survival in HCC patients who had received TACE+ sorafenib instead of either one alone. However, there is no case report so far in the literature where a patient with massive portal vein tumor thrombus has had complete tumor response after hepatic artery blockage and sorafenib therapy. We do not know whether the response was purely to Hepatic artery ligation or it is cumulative response to sorafenib also.^[5]

The case 2 we described received sorafenib with palliative intent. But follow-up evaluation with CT

scan and serum AFP revealed good tumor response in the form of clearing of portal vein thrombosis and reduction of AFP.

These two cases give us additional hope that PVT is not the end of the story in HCC patients. Though today the standard of care for HCC with PVTT is EBRT followed by reassessment and transplantation once tumor thrombus clears.^[6] We believe that sorafenib plays definite role as a neoadjuvant therapy.

In conclusion, high AFP and major vascular invasion should not be considered as end points in treatment of HCC patients. Neoadjuvant modalities are to be employed followed by reevaluation for transplantation. Since final conclusion needs high experience with more number of cases individual discretion is advised before offering transplantation in these patients.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent is not needed as there is no disclosure

of any personal information of patients.

Ethics approval

This is not needed as it is not an experimental study.

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Further understanding of mechanisms involved in liver cancer chemoresistance

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ABSTRACT

An important limitation for the success of chemotherapy in the treatment of primary liver cancer (hepatocellular carcinoma, hepatoblastoma and cholangiocarcinoma) is the marked efficacy of mechanisms of chemoresistance (MOC). These have been previously classified into five groups depending on whether they result in: a reduced drug uptake or enhanced drug export (MOC-1); poor intracellular activation of prodrugs or higher inactivation of active drugs (MOC-2); changes in the molecular targets that impairs the action of the drug by increasing the activity of the metabolic route to be inhibited or stimulating alternative routes (MOC-3); ability of tumor cells to repair drug-induced modifications in the target molecule, usually DNA (MOC-4); and the activation or inhibition of intracellular signaling pathways that lead to a change in the balance between pro- and anti-apoptotic factors favoring tumor cell survival (MOC-5). Nevertheless, novel information appeared over the last few years has recommended to consider two additional groups, MOC-6 and MOC-7, based on changes in tumor microenvironment, mainly hypoxia and acidity, and epithelial-mesenchymal transition, respectively. These contribute to the defensive armamentaria developed or enhanced in liver cancer cells to resist the pharmacological attack, which accounts for a negligible beneficial effect of commonly used antitumor drugs and only a modest response to novel targeted therapies based on tyrosine kinase inhibitors, such as sorafenib. Therefore, further advances are urgently needed to better understand the molecular and cellular bases of the chemoresistant barrier and help scientists in this field to develop new tools able to overcome cancer cell defenses.

INTRODUCTION

An important limitation for the success of chemotherapy in the treatment of primary liver cancer [hepatocellular carcinoma (HCC), hepatoblastoma (HPB) or cholangiocarcinoma (CCA)] is the marked efficacy of mechanisms of chemoresistance (MOC) that have

previously been classified into five groups^[1] depending on whether they result in: reduced drug uptake or enhanced drug export (MOC-1); poor intracellular activation of pro-drugs or higher inactivation of active drugs (MOC-2); changes in the molecular targets that impairs the action of the drug by increasing the activity of the target route to be inhibited, or the appearance



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or stimulation of alternative routes (MOC-3); ability of tumor cells to repair drug-induced modifications in the target molecule, usually DNA (MOC-4); and the activation or inhibition of intracellular signaling pathways that lead to a change in the balance between pro- and anti-apoptotic factors favoring tumor cell survival (MOC-5). Nevertheless, novel information on the role of several adaptive mechanisms involved in liver cancer chemoresistance has emerged in the last few years. These regard the existence of cancer stem cells with particularly poor sensitivity to anticancer drugs, the interference with the inflammatory processes and cytokine expression, cellular autophagy status, changes in tumor microenvironment and phenotypic transition of cancer. This situation recommends considering at least two additional MOC that we propose to be classified into MOC-6 and MOC7.

CHEMORESISTANCE DUE TO CHANGES IN TUMOR MICROENVIRONMENT (MOC-6)

Two peculiar features characterizing tumor microenvironment, i.e. hypoxia and acidity, play an important role in tumor progression, metastasis and response to chemotherapy. Several *in vitro* studies have demonstrated that hypoxia induces enhanced resistance to antitumor drugs, such as cisplatin, doxorubicin, etoposide, melphalan, 5-fluorouracil, gemcitabine, and docetaxel.^[2] The family of hypoxia-inducible transcription factors (HIFs) represents the main mediator of the hypoxic response and is widely upregulated in human cancers. HIF-1 and to a lesser extent HIF-2, the oxygen-regulated HIF isoforms, have been associated with chemotherapy failure. Thus, HIF-1 inhibition reverses multidrug resistance in colon cancer cells.^[3] Moreover, silencing HIF-1 in tumor cells results in increased sensitivity to anticancer drugs.^[4] Several mechanisms and pathways that may underlay HIF-1-mediated chemotherapy resistance in tumor cells under hypoxia have been described. These include: (1) HIF-1-mediated regulation of drug efflux through the activation of transport proteins such as the multidrug resistance 1 (MDR1) gene (*ABCB1*), the multidrug-resistance-associated protein 1 (MRP1, gene symbol *ABCC1*) and the lung resistance protein (LRP) or major vault protein (MVP, gene symbol *MVP*);^[5] (2) HIF-1-mediated inhibition of drug-induced DNA damage.^[6] This effect is partially mediated via transcriptional down-regulation of topoisomerase II in human tumor cells;^[6] (3) HIF-1 functions as a robust suppressor of apoptosis and functional interference with HIF-1 results in enhanced cell death upon treatment with chemotherapeutic agents in tumors of different origins. The molecular nature of this phenomenon was mostly accounted for

by anti-apoptotic target genes of HIF-1, which include *Bcl-xL*, *Bcl-2*, *Mcl-1*, *NF-kB* and *BIRC5*;^[7] (4) HIF-1-dependent decrease of the DNA-damage response-activated senescence, which is partly accountable for the anti-tumor effect of different chemotherapeutic agents;^[7] (5) HIF-1-dependent induction of autophagy which confers a survival advantage to tumor cells and protects them from drug-induced death signals.^[7] HIF-1 target genes such as *BNIP3* (Bcl-2/adenovirus E1B 19-kDa interacting protein 3) and *BNIP3L* (Bcl-2/adenovirus E1B 19-kDa interacting protein 3 like), members of the so-called BH3-only subfamily of Bcl-2 family proteins that antagonize the activity of the pro-survival proteins Bcl-2 and Bcl-xL, have suggested to be involved in the hypoxia-induced autophagy.^[8] A role of HIF-1 independent mechanisms in hypoxia-induced drug resistance in cancer cells has also been reported. These mechanisms are still poorly understood, but pathways involving phosphoinositol-3-kinase (PI3K), nuclear factor kappa-B (NF-kB), cyclooxygenase-2 (COX-2), activator protein-1 (AP-1), c-Jun, Pim-1, and STAT-3 have been reviewed and have been suggested to participate in MOC-6.^[2]

Regarding the role of acidic environment in MOC-6, it should be taken into account that, as a result of the active acid production through glycolysis, which occurs in tumor cells even in the presence of oxygen, there is the need of extruding a large amount of H⁺ to survive. The mechanisms activated in tumor cells to efficiently eliminate protons include up-regulation of ion pumps, such as vacuolar H⁺-ATPase (V-ATPase), and transporters, such as Na⁺/H⁺ exchanger (NHE), together with an increased turnover of acidic vesicles. The low extracellular pH (pHo) may severely affect drug uptake. For instance, acidic pHo reduces the uptake of chemotherapeutic drugs that behave as weak bases, such as anthracyclines and Vinca alkaloids, and, hence, reduces their cytotoxicity by preventing these compounds from reaching their intracellular targets.^[9] Thus, the possibility that basic drugs could be protonated and neutralized in a higher proportion by the acidic pHo of tumor environment has to be considered.^[10] It has been demonstrated that compounds able to disrupt tumor pH homeostasis may reverse multidrug resistance phenotype and indirectly inhibit the growth of the tumors. Thus, treatment with sodium bicarbonate induced alkalization of pHo and tumor growth inhibition in animal models.^[9] Moreover, lysosomotropic agents that induce modification of the pHo vs. intracellular pH (pHi) gradient and alkalization of intracellular acidic vesicles may reverse anthracycline resistance in chemoresistant cells.^[11] In addition, H⁺-pump inhibitors induce drug-resistance reversion in chemoresistant

human melanoma cells and increased sensitivity to cytotoxic drugs in chemoresistant cell lines.^[12]

Several strategies are currently being developed to overcome tumor chemoresistance associated to microenvironment acidity including inhibition of deprotonation mechanisms using drugs such as inhibitors of proton transporters NHE-1, carbonic anhydrases, monocarboxylate transporters and proton pumps (PPI).^[13,14] A multicentre historically controlled trial has been performed to evaluate the activity of a pre-treatment administration of the PPI esomeprazole as chemosensitizer during neoadjuvant chemotherapy based on methotrexate, cisplatin and adriamycin in patients with osteosarcoma.^[15] The analysis of the resected tumors after neoadjuvant therapy revealed that pretreatment with the PPI increases the effectiveness of the polychemotherapy at the tumor level. This was particularly evident in the histological chondroblastic subtype which normally shows poor histological response. This study provides evidences that PPI may be beneficially added to standard regimens in combination to conventional chemotherapy. Other strategies involves the use of induced tumor acidity as an attractant for antitumor drugs such as cyclooxygenase inhibitors and photoactivatable cytotoxic agents such as acridine orange and imidazoacridinones, with tropism for acid environments, where they are activated.^[13,14]

CHEMORESISTANCE DUE TO PHENOTYPE TRANSITION OF TUMOR CELLS (MOC-7)

The epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose cell-cell interactions and polarity, and acquire a phenotype with mesenchymal characteristics, i.e. enhanced migratory behavior, invasive ability, and resistance to apoptosis activation. Under physiological circumstances during intrauterine life, EMT occurs transiently during embryogenesis and organ development, and after birth in association with wound healing, tissue regeneration and organ fibrogenesis in the context of normal morphogenesis. EMT also takes place in some types of cancer, including HCC and CCA, in cells that have previously undergone genetic changes affecting oncogenes and/or tumor suppressor genes, which favors carcinogenesis.^[16] Carcinoma cells that have acquired a mesenchymal phenotype lose E-cadherin expression and express mesenchymal markers, such as N-cadherin, alpha-smooth muscle actin (α -SMA), fibroblast-specific protein 1 (FSP1), vimentin, and desmin. Commonly, carcinoma cells that have lost epithelial phenotype appear in the external layer of primary tumors and they are considered to be the

cells that eventually enter into further steps of the invasion-metastasis process.

In liver cancer, a relationship between enhanced chemoresistance and EMT has also been recently described.^[17] Poor differentiated liver cancer cell lines, such as HLE, HLF and SK-Hep1, expressing high levels of mesenchymal markers were more invasive and resistant to cisplatin, doxorubicin and sorafenib than other well-differentiated liver cancer cells, such as Hep3B, HepG2 and Huh7. It has been suggested that the development of a more invasive capability and chemoresistance in tumor cells could be attributed to EMT. Clinical observations support the concept that poorer differentiated HCC are more refractory to chemotherapy based on inhibitors of receptors with tyrosine kinase activity (TKI).^[18] Moreover, patients with undifferentiated tumors have a worse prognosis.^[17]

Although signals triggering EMT in carcinoma cells are not well known, different signaling pathways have been involved in this process.

(1) Transforming growth factor-beta (TGF- β) signaling pathway. TGF- β has been suggested to play an important role in promoting EMT in liver tumor cells.^[19] In a study carried out with gemcitabine-resistant MzChA-1 cells from human biliary tract cancer, a relationship between an increase in TGF- β expression, EMT and enhanced invasive activity was found.^[20] SMAD proteins are intracellular proteins belonging to the TGF- β pathway. It has been demonstrated that down-regulation of microRNA-145 (miR-145) in human HPB and HCC cells, such as HepG2 and Huh7, respectively, increases resistance to doxorubicin through enhancement of SMAD3 expression.^[21] A relationship between overexpression of SMAD2 and SMAD4 and enhanced EMT resulting in mesenchymal phenotype and reduced sensitivity to sorafenib and doxorubicin has been found both *in vitro* and in HCC patients.^[22] A down-regulation of miR-125b, a microRNA whose expression is strongly suppressed in HCC, has been suggested to be involved in the acquisition of chemoresistance in this type of tumor cells.

(2) Epidermal growth factor receptor (EGFR) signaling pathways. EMT status in HCC cells is also considered to be a determinant of sensitivity to EGFR inhibitors.^[23] Amphiregulin, a ligand of the EGFR, which is not expressed in healthy liver, is up-regulated during chronic liver injury, the background on which most liver tumors develop. Overexpression of amphiregulin in SK-Hep1 cells enhanced their proliferation rate, anchorage-independent growth, drug resistance, and *in vivo* tumorigenic potential.^[24] Another signal able

to induce EMT via modulation of EGFR pathways in HCC cells is galectin-1 (Gal-1).^[25] Dysregulation of Gal-1 expression in HCC cells leads to an over-activation of FAK/PI3K/AKT and H-Ras/Raf/ERK pathways resulting in enhanced phosphorylation of AKT, mTOR and p70 kinases and up-regulation of the $\alpha\beta 3$ integrin expression. A consequence of the dysregulation of these pathways is EMT induction and higher resistance to sorafenib. Moreover, high levels of Gal-1 in tumors are associated with impaired sorafenib response and reduced overall survival of patients with HCC.^[25]

(3) Cell-adhesion proteins involved in intracellular signaling networks. An example is CD44, a stem cell marker that besides being the cell-surface receptor of the hyaluronic acid has been suggested to play functions as a co-receptor for several tyrosine kinase receptors.^[26] In a recent study carried out with human liver tumor cell lines in culture and implanted in nude mice, it has been demonstrated that cells showing a mesenchymal-like phenotype and high expression of CD44 were refractory to sorafenib-induced cell death.^[19] In contrast, epithelial-like cells were more sensitive to sorafenib-induced apoptosis. The authors of this study have proposed that the appearance of a mesenchymal phenotype in tumor cells could be used as a marker to predict the lack of response of HCC to sorafenib.

CONCLUSION

In sum, in addition to the classical MOC-1 to MOC-5, two additional mechanisms of chemoresistance must be included in the defensive armamentarium developed or enhanced in liver cancer cells to overcome the pharmacological attack, MOC-6 and MOC-7, based on changes in tumor microenvironment and EMT, respectively. This accounts for a negligible response to the commonly used antitumor drugs and only a modest response to novel targeted therapies based on TKIs, such as sorafenib. Further advances are urgently needed to better understand the bases of the chemoresistance barrier, which in the future may enlarge the list of MOCs by including for instance autophagy mechanisms.^[27] This knowledge is required to develop new tools able to demolish or inactivate cancer cell defenses against chemotherapy.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

There is no patient involved.

Ethics approval

This review paper is waived for ethics approval.

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Antioxidant activity and free radical-scavenging of cape gooseberry (*Physalis peruviana* L.) in hepatocellular carcinoma rats model

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ABSTRACT

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Aim: Oxidative damage of cellular components by free radicals and other reactive oxygen molecules is believed to be associated with the development of degenerative diseases. The aim of the present study was to evaluate the antioxidant capacity and free radical scavenging activity of cape gooseberry juice (CGJ) in diethylnitrosamine-(DENA) and CCl₄ (3 mL/kg b.w.)-induced hepatocellular carcinoma (HCC) rats model. **Methods:** The rats were divided into 4 groups (6 rats each group). Group 1 (control): the rats of this group did not receive any treatments; group 2 (CGJ): rats were daily administered cape gooseberry juice at a dose of 1 mL/kg b.w.; group 3 (HCC): the rats treated with a single intraperitoneal injection of fresh DENA (200 mg/kg body weight) and received a subcutaneous injection of CCl₄ (3 mL/kg/week); group 4: (HCC + CGJ): rats were treated with DENA (200 mg/kg b.w.) and CCl₄ (3 mL/kg b.w. per week) in addition to daily administered cape gooseberry juice at a dose of 1 mL/kg b.w. **Results:** Treatment



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with DENA plus CCl₄ induced a significant increase in tumor marker level, alpha-fetoprotein level, and liver function enzymes activity as well as elevated levels of malondialdehyde. This suggests oxidative stress accompanied with a significant decrease in antioxidant biomarkers including glutathione, total antioxidant capacity, superoxide dismutase and catalase in the examined tissues. However, the administration of GGJ could reduce these changes to control levels. **Conclusion:** CGJ is a promising candidate as a free radical scavenger and antioxidant processor in an HCC rats model. This beneficial effect was achieved by antagonizing free radicals generation and the enhancement of the antioxidant defense mechanisms, resulting in marked improvement of hepatic biomarkers.

INTRODUCTION

Oxygen radical generation and lipid peroxidation have been implicated in the pathogenesis of various diseases and the toxic action of a wide range of compounds.^[1] Involvement of free radicals, generation of oxygen radicals and enhancement of lipid peroxidation have been shown to play an important role in hepatocellular carcinoma (HCC).^[2,3] Amelioration of the deleterious effects of oxidative stress associated with HCC using synthetic compounds causes undesirable side effects. Therefore, natural agents could be the most prudent strategy and the most effective agents for protecting humans from various diseases.^[4,5] Furthermore, there is the growing popularity of natural functional food ingredients that are believed to be safer, healthier and less subject to hazards than their artificial counterparts.^[6,7]

One of the most important natural diets with anti-oxidant properties is berries, among the most widely consumed fruits in the human diet. Berry fruits, wild or cultivated, are proven as a traditional and rich source of bioactive compounds, possessing important biological substances such as flavonoids minerals, vitamins, and phenolic acids.^[8,9] One key berry fruit is, cape gooseberry (*Physalis peruviana*), a herbaceous plant which belongs to the Solanaceae family. Its fruit is also known as golden berry, ground cherry and in Egypt, harankash. The fruit of the cape gooseberry is highly nutritious, containing high levels of macronutrients and essential minerals such as magnesium, calcium, potassium, sodium, and phosphorus, as well as micronutrients such as iron and zinc,^[10,11] the fruit also contains vitamins A, B and C, in addition to β -carotene, α -carotene and β -cryptoxanthin. In addition, the fruit contains polyunsaturated fatty acids (e.g. linoleic acid and oleic acid). These bioactive compounds have nutritional value, medicinal properties, and an antioxidant property that can prevent peroxidative damage of liver cells.^[12-14] Cape gooseberry extracts show antioxidant activity,^[15,16] anti-inflammatory activity,^[17,18] and anti-hepatotoxic^[18] and anti-proliferative effects on hepatoma cells.^[19] This fruit

also has excellent potential as a food-based strategy for anti-diabetic and anti-hypertensive products.^[20]

Therefore, the objective of this study was to investigate the antioxidant properties of cape gooseberry juice as a potential source of natural functional substances against lipid peroxidation and scavenging capacities towards free radicals in different tissues of experimental HCC rats model.

METHODS

Chemicals

Diethylnitrosamine (DENA) and carbon tetra chloride (CCl₄) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DENA was freshly dissolved in sterile 0.9% saline and given to rats at a single dose of 200 mg/kg b.w.^[21] CCl₄ was given to rats at a dose of 3 mL/kg b.w. per week.^[22,23]

Animals

Healthy male albino rats (*Rattus rattus*), 8 weeks old (150-170 g) were purchased from Institute of Ophthalmic Disease Research, Cairo, Egypt. Rats were housed in cages at regulated temperature (22-25 °C). They were kept under good ventilation under a photoperiod of 12-h light/12-h darkness schedule with lights-on from 06:00 to 18:00. They all received a standard laboratory diet (60% ground corn meal, 10% bran, 15% ground beans, 10% corn oil, 3% casein, 1% mineral mixture and 1% vitamins mixture), purchased from Meladco Feed Company (Aubor City, Cairo, Egypt) and supplied with water *ad libitum* throughout the experimental period. Animals received humane care and the present study complies with the animal care guidelines. The local committee approved the design of the experiments, and the protocol conforms to the guidelines of the National Institutes of Health (NIH).

Hepatocellular rats model

Experimental hepatocellular carcinoma rats were subjected by a single intraperitoneal injection of freshly prepared DENA (200 mg/kg body weight), then 2 weeks later received a subcutaneous injection of CCl₄ once every week (3 mL/kg b.w.) for 10 weeks to

Table 1: Effect of CGJ administration on the tumor marker AFP and liver function enzymes activity in control and different treated rat groups (means \pm SE)

Parameters groups	Serum				Liver		
	AFP (ng/mL)	ALT (U/mL)	AST (U/mL)	ALP (IU/L)	ALT (U/g)	AST (U/g)	ALP (U/g)
Control	0.99 \pm 0.10	34.66 \pm 1.02	40.16 \pm 0.62	93.63 \pm 0.38	60.77 \pm 0.67	18.77 \pm 0.34	33.03 \pm 0.67
CGJ	1.00 \pm 0.94	33.16 \pm 1.06	41.00 \pm 0.60	91.56 \pm 0.50	59.99 \pm 2.49	18.90 \pm 0.33	32.14 \pm 1.12
HCC	2.57 \pm 0.28 ^{a,b}	50.50 \pm 1.76 ^{a,b}	60.50 \pm 0.99 ^{a,b}	153.11 \pm 1.68 ^{a,b}	42.41 \pm 0.51 ^{a,b}	15.16 \pm 0.26 ^{a,b}	54.99 \pm 1.12 ^{a,b}
HCC + CGJ	1.25 \pm 0.66 ^{a,b,c}	37.33 \pm 1.05 ^c	45.63 \pm 0.63 ^{a,b,c}	105.65 \pm 0.38 ^{a,b,c}	52.16 \pm 0.61 ^{a,b,c}	16.98 \pm 0.31 ^{a,b,c}	43.50 \pm 1.10 ^{a,b,c}

^a $P \leq 0.05$ vs. control, ^b $P \leq 0.05$ vs. CGJ, ^c $P \leq 0.05$ vs. HCC. HCC: hepatocellular carcinoma; CGJ: cape gooseberry juice; AFP: alpha-fetoprotein; ALT: alanine transaminase; AST: aspartate transaminase; ALP: alkaline phosphatase

promote the carcinogenic effect of DENA.^[23]

Preparation of cape gooseberry juice

Cape gooseberry (*Physalis peruviana*) was purchased from local markets at Mansoura, Egypt. Fruits were washed, cut into small pieces and freshly prepared juice [500 g cape gooseberry juice (CGJ) up to 500 mL distilled water, where each 1 mL juice contains 1 g cape gooseberry]. The cape gooseberry juice (1 mL/kg b.w.) was shaken well just before oral administration by gavage.^[17]

Experimental design

After 2 weeks of acclimatization, the rats were classified into 4 groups (6 rats/group) and treated for 12 weeks as follows: group 1 (control) rats did not receive any treatments; group 2 (CGJ): rats were orally administered with cape gooseberry juice (1 mL/kg b.w.); group 3 (HCC) rats were treated with a single intraperitoneal injection with DENA freshly dissolved in sterile 0.9% saline (200 mg/kg b.w.) and 2 weeks later given a subcutaneous injection of CCl₄ (3 mL/kg b.w. per week) for 10 weeks to promote the carcinogenic effect of DENA; group 4 (HCC + CGJ) rats were treated with DENA (200 mg/kg b.w.) and CCl₄ (3 mL/kg b.w. per week) plus CGJ (1 mL/kg b.w.).

Blood collection and tissue preparation

At the end of the experimental period (12 weeks), blood samples were collected from overnight rats, centrifuged at 860 *g* for 20 min at 4 °C and the separated sera were frozen at -20 °C for future biochemical analysis. Then the rats were sacrificed by cervical dislocation and the tissues (liver, kidney, brain and testes) removed and decapsulated. These tissues were weighed and homogenized in saline solution. The homogenates were centrifuged at 860 *g* for 20 min at 4 °C and the supernatants were frozen at -20 °C for further analysis.

Biochemical analysis

Alpha-fetoprotein (AFP) level was estimated by immunoenzymatic colorimetric method according

to Acosta.^[24] Aspartate transaminase (AST) activity, alanine transaminase (ALT) activity and alkaline phosphatase (ALP) were measured using colorimetric kits purchased by ABC Diagnostic Kit, Cairo, Egypt.^[25,26] Malondialdehyde (MDA) content was determined by the methods of Ohkawa *et al.*^[27] Reduced glutathione (GSH) was analyzed based on the method of Prins and Losse.^[28] Superoxide dismutase (SOD) and catalase (CAT) activities were assayed as described by Niskikimi *et al.*^[29] and Bock *et al.*^[30] respectively. Total antioxidant capacity (TAC) was determined using commercial Biodiagnostic kits (Dokki, Giza, Egypt) according to the methods of Koracevic *et al.*^[31]

Statistical analysis

Data were subjected to statistical significance tests using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test.^[32] The statistical analysis was carried out using SPSS 12.00 software. The results were expressed as mean \pm SE and the differences were considered significant at $P \leq 0.05$.

RESULTS

The results of the present study [Table 1] recorded that the HCC rats treated with DENA and CCl₄ resulted in a significant increase in serum AFP level compared to the control level, indicating the development of HCC in rats. This elevation in AFP was accompanied by the elevation of serum and liver ALT, AST and ALP activity. The results in rats treated with CGJ alone were comparable to results in the control rats group in most of the estimated parameters. However, the administration of CGJ to the HCC rats was associated with a significant improvement in all the tested parameters where the treatment succeeded in reducing the elevation level of AFP, ALT, AST and ALP in both serum and liver [Table 1].

Moreover, the administration of CGJ to HCC rats succeeded in restoring oxidative stress through decreases in MDA level and induced a significant improvement in the antioxidant biomarkers by the observed increase in GSH, TAC, SOD and CAT in all

Table 2: Effect of CGJ administration on oxidative stress and antioxidant biomarkers in liver of control and different treated rat groups (means \pm SE)

Parameters groups	MDA (nmol/g)	GSH (mg/g)	TAC (mmol/L)	SOD (U/g)	CAT (μ mol/s/g)
Control	512.11 \pm 0.64	19.46 \pm 0.54	95.86 \pm 0.03	892.99 \pm 1.18	190.73 \pm 1.19
CGJ	510.11 \pm 0.59	19.99 \pm 0.64	97.89 \pm 0.13	894.90 \pm 1.21	194.73 \pm 1.89
HCC	701.80 \pm 2.91 ^{a,b}	10.06 \pm 0.46 ^{a,b}	28.11 \pm 0.23 ^{a,b}	416.99 \pm 1.18 ^{a,b}	132.55 \pm 1.38 ^{a,b}
HCC + CGJ	517.11 \pm 0.59 ^c	17.48 \pm 0.44 ^{a,b,c}	75.47 \pm 0.31 ^{a,b,c}	841.71 \pm 1.77 ^{a,b,c}	180.68 \pm 1.35 ^{a,b,c}

^a $P \leq 0.05$ vs. control, ^b $P \leq 0.05$ vs. CGJ, ^c $P \leq 0.05$ vs. HCC. HCC: hepatocellular carcinoma; CGJ: cape gooseberry juice; MDA: malondialdehyde; GSH: glutathione; TAC: total antioxidant capacity; SOD: superoxide dismutase; CAT: catalase

Table 3: Effect of CGJ administration on oxidative stress and antioxidant biomarkers in the kidney of control and different treated rat groups (means \pm SE)

Parameters groups	MDA (nmol/g)	GSH (mg/g)	TAC (mmol/L)	SOD (U/g)	CAT (μ mol/s/g)
Control	90.73 \pm 1.12	42.56 \pm 0.54	72.86 \pm 0.23	69.99 \pm 0.34	95.56 \pm 1.54
CGJ	93.43 \pm 1.16	42.99 \pm 0.64	77.89 \pm 0.13	70.79 \pm 0.64	96.99 \pm 0.64
HCC	190.93 \pm 1.79 ^{a,b}	20.06 \pm 0.46 ^{a,b}	20.21 \pm 0.53 ^{a,b}	13.49 \pm 0.66 ^{a,b}	30.16 \pm 1.46 ^{a,b}
HCC + CGJ	105.77 \pm 1.19 ^{a,b,c}	29.48 \pm 0.44 ^{a,b,c}	37.47 \pm 0.31 ^{a,b,c}	37.48 \pm 0.94 ^{a,b,c}	70.48 \pm 1.44 ^{a,b,c}

^a $P \leq 0.05$ vs. control, ^b $P \leq 0.05$ vs. CGJ, ^c $P \leq 0.05$ vs. HCC. HCC: hepatocellular carcinoma; CGJ: cape gooseberry juice; MDA: malondialdehyde; GSH: glutathione; TAC: total antioxidant capacity; SOD: superoxide dismutase; CAT: catalase

Table 4: Effect of CGJ administration on oxidative stress and antioxidant biomarkers in the brain of control and different treated rat groups (means \pm SE)

Parameters groups	MDA (nmol/g)	GSH (mg/g)	TAC (mmol/L)	SOD (U/g)	CAT (μ mol/s/g)
Control	75.31 \pm 0.64	35.46 \pm 0.50	75.06 \pm 0.13	63.80 \pm 0.34	62.43 \pm 0.29
CGJ	76.44 \pm 0.99	35.99 \pm 0.64	77.00 \pm 0.12	63.97 \pm 0.64	62.43 \pm 0.29
HCC	117.80 \pm 1.71 ^{a,b}	13.76 \pm 0.16 ^{a,b}	18.25 \pm 0.12 ^{a,b}	18.25 \pm 0.12 ^{a,b}	25.58 \pm 1.70 ^{a,b}
HCC + CGJ	95.71 \pm 0.99 ^{a,b,c}	23.48 \pm 0.24 ^{a,b,c}	44.38 \pm 0.94 ^{a,b,c}	44.38 \pm 0.94 ^{a,b,c}	48.50 \pm 1.05 ^{a,b,c}

^a $P \leq 0.05$ vs. control, ^b $P \leq 0.05$ vs. CGJ, ^c $P \leq 0.05$ vs. HCC. HCC: hepatocellular carcinoma; CGJ: cape gooseberry juice; MDA: malondialdehyde; GSH: glutathione; TAC: total antioxidant capacity; SOD: superoxide dismutase; CAT: catalase

Table 5: Effect of CGJ administration on oxidative stress and antioxidant biomarkers in the testes of control and different treated rat groups (means \pm SE)

Parameters groups	MDA (nmol/g)	GSH (mg/g)	TAC (mmol/L)	SOD (U/g)	CAT (μ mol/s/g)
Control	14.24 \pm 0.02	25.02 \pm 0.24	76.88 \pm 1.23	50.04 \pm 0.24	48.94 \pm 0.12
CGJ	14.43 \pm 0.06	25.28 \pm 0.24	77.98 \pm 1.13	50.22 \pm 0.14	48.97 \pm 0.24
HCC	54.96 \pm 0.17 ^{a,b}	9.06 \pm 0.16 ^{a,b}	35.55 \pm 0.33 ^{a,b}	10.41 \pm 0.22 ^{a,b}	16.03 \pm 1.12 ^{a,b}
HCC + CGJ	31.95 \pm 0.19 ^{a,b,c}	19.48 \pm 0.04 ^{a,b,c}	57.77 \pm 0.33 ^{a,b,c}	31.52 \pm 0.23 ^{a,b,c}	30.48 \pm 0.44 ^{a,b,c}

^a $P \leq 0.05$ vs. control, ^b $P \leq 0.05$ vs. CGJ, ^c $P \leq 0.05$ vs. HCC. HCC: hepatocellular carcinoma; CGJ: cape gooseberry juice; MDA: malondialdehyde; GSH: glutathione; TAC: total antioxidant capacity; SOD: superoxide dismutase; CAT: catalase

the examined tissues; liver, kidney, brain and testis, indicating the antioxidant activity of CGJ [Tables 2-5].

DISCUSSION

Recently, there has been growing interest in dietary bioactive compounds obtained from natural sources which have a therapeutic effect against various diseases including chemoprotective properties against cancer.^[5,33] HCC is a common disease, being the third leading cause of death worldwide.^[34] The current study suggests that treatment with DENA and CCl₄ is a good model for the induction of HCC in rats.^[35] The data also showed increased AFP in HCC rats. Increase of this protein may be due to hepatotoxic agents or hepatocarcinogens that are frequently associated with HCC. Increased glycoprotein AFP levels is considered

a good marker for various malignancies including testicular, bile duct, pancreatic, stomach, colon and hepatic cancer.^[36,37] Moreover, the observed elevation of serum AST, ALT and ALP and the decrease in ALT and AST in the liver in HCC rats supports earlier findings.^[38] These findings may be due to damage to hepatocytes caused by exposure to DENA resulting in hepatic dysfunction and subsequent leakage of these enzymes from the neoplastic cell into circulation.^[39] Or, the findings may be due to the release of enzymes from normal tissue by tumors or possibly the effect of tumors on remote tissue, leading to leakage of enzyme and release into the blood.^[40] In a related concern it has been suggested that there is an increase in the levels of these transaminases activity in serum of HCC patients.

In concurrence with the above findings, elevated serum

aminotransferase activity is more specific for liver injury due to damage to the liver cell membrane.^[40] As well, alkaline phosphatase is used as a specific tumor marker for making diagnoses in the early detection of cancer.^[41] This enzyme is involved in the transport of metabolites across cell membranes, in protein synthesis, secretory activities and glycogen metabolism. It is a membrane-bound enzyme, and its alteration is likely to affect the membrane permeability that produces derangement in the transport of metabolites.^[42]

The observed increases of serum and liver ALP in HCC rat groups may be due to altered gene expression.^[43] In the current study, the HCC rats group suffered from severe oxidative stress in various organs, achieved by elevation of MDA level and depletion of antioxidant enzymes. This may be due to the conversion of cellular poly-unsaturated fatty acids to the toxic product MDA which has a cytotoxicity and inhibitory action on cellular protective enzymes.^[44] HCC caused by carcinogenic DENA generally reflects instability of liver metabolism associated with free radicals species (ROS) generation, which leads to oxidative stress and alterations in antioxidant defense mechanisms.^[35,45] Increased level of MDA has been reported during DENA-induced hepatocarcinogenesis. This dynamic action may further lead to uncompromised production of free radicals overwhelming the cellular antioxidant defense.^[46,47] Moreover, HCC causes depletion of SOD and CAT activity as well as TAC and GSH contents in all observed organs. Such studies support the current findings, as the current study showed a significant decrease in the activities of antioxidant enzyme in the liver of animals treated with carcinogen.^[35] Antioxidants are substances that either directly or indirectly protect cells against adverse effects of xenobiotics, drugs, carcinogens and toxic radical reactions.^[48] The observed decrease in SOD activity in liver, kidney, brain and testes suggests the inactivation of antioxidant enzymes; this is possibly due to increased superoxide radical production or to an inhibition by H_2O_2 as a result of corresponding decrease in the activity of catalase which selectively degrades H_2O_2 .^[49] The decreased GSH, SOD and CAT observed in the HCC group of rats may be due to accumulation of lipid peroxidation that was seen to increase during carcinogenesis.

The accompanying reduction in the activity of SOD, CAT and depletion of GSH content suggests induction of oxidative stress in the organs studied. SOD is considered the first line of defense against deleterious effects of oxygen free radicals in the cells by catalyzing of superoxide radicals (O_2^-) to H_2O_2 and molecular oxygen. CAT is also responsible for the detoxification

of H_2O_2 , which is an effective inhibitor of SOD.^[50,51] The reduction in the activity of CAT may reflect the inability of tissues to eliminate H_2O_2 . CAT protects SOD against inactivation by H_2O_2 , while SOD protects CAT against inhibition by (O_2^-). Thus, the balance of this enzyme system may be essential to eliminating ROS generated in the tissues. In this area, GSH represents an important defense mechanism in protecting cells against ROS.^[52] On the other hand, the enormous impacts of CGJ supplementation in alleviating oxidative stress in all organs in the HCC rats may be attributed to either a direct effect of many antioxidant compounds of CGJ as free radical scavengers, or to enhancement of cellular antioxidant defense functions. This occurs through modulating the alteration in GSH content and antioxidant enzymes activity. An amelioration of AFP after supplementation of CGJ to HCC rats may be due to the antioxidant activity of CGJ. Additionally, the observed increase in alterations to liver enzymes, including ALT, AST and ALP, in HCC-received CHJ rats may be due to the improvement of the functional status of hepatocytes with preservation of cellular architecture leakage of intracellular enzymes through membrane-stabilizing activity.^[18,53]

Previous studies have suggested that CGJ is a significant source of natural antioxidative compounds.^[54] These components may have a wide variety of chemical structures that could react with radicals by donating protons (free radical quenching), radical addition, redox reaction (electron transfer) and radical combination.^[12] Natural antioxidants could prevent the deleterious effects of toxic agents by scavenging free radicals and other ROS or by modulation of the inflammatory response. The supplementation of CGJ to the HCC rats model resulted in amelioration of oxidative stress. The improvement of the antioxidants defense mechanism is considered a favorable indicator for anti-lipid peroxidative properties and antioxidant activity through high levels of antioxidant compounds such as polyphenols and similar flavonoids.^[55,56]

The observed decreases in MDA in HCC rats that received CGJ may be due to free radicals scavenging, a potential mechanism by which CHJ can act as an anti-inflammatory and antioxidant to protect the liver and other organs. Therefore, dietary consumption of cape gooseberry may be a highly effective potential antioxidant and protective agent against oxidative stress in liver toxicity.^[57,58]

In view of the present results, it was observed that CGJ supplementation showed a significant antioxidant status as manifested by elevation of GSH, TAC, SOD and CAT in serum and various organs. Many plant

secondary metabolites act as potent antioxidants and it has been demonstrated that free radical scavenger/antioxidants such as SOD, CAT, TAC and reduced glutathione (GSH) prevent the tissue damage induced by different toxicants.^[59] The first line of defense against superoxide anion (O_2^-), H_2O_2 and (OH), the major ROS which induce cell degeneration by increasing LPO of cell membrane lipids, is the family of enzymes SOD and CAT that convert O_2^- to H_2O_2 . The toxic end products of peroxidation induce damage to the structural and functional integrity of cell membranes, break DNA strands, and denature cellular proteins. The natural cellular antioxidant enzyme SOD is an important enzyme as because it is found virtually in all aerobic organisms. O_2^- is the only known substrate for SOD which is considered to be a stress protein, which is synthesized in response to oxidative stress.^[60]

In conclusion, there is a significant relationship between HCC and free radical-mediated oxidative stress demonstrated by increased levels of MDA as well as decreased levels of anti-oxidant parameters in the examined organs of rats. The obtained data also strongly suggested the antioxidant activity of cape gooseberry supplementation, as evidenced by the greatly positive effect on reduced oxidative stress as well as improvement in the cellular anti-oxidant defense system antioxidant status. The underlying mechanisms for this protective effect may be through various nutritional constituents due, at least in part, to their synergistic anti-oxidant capacity as well as scavenging free radicals. Thus, blocking the oxidative stress pathway may be of therapeutic value in treatment of liver injury. These results suggest that CGJ-enriched diets should be added to diet regimens to develop a new therapeutic strategy for treatment of diseases associated with free radicals generation. The fractionation and bioavailability of the main constituents of cape gooseberry, which are responsible for the anti-oxidant activity, will be an important area of study in the future.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

There is no patient involved.

Ethics approval

The local committee approved this study and the protocol conforms to the guidelines of the National Institutes of Health.

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Comparison of p53 and prohibitin expression in the spectrum of hepatitis, cirrhosis, and hepatocellular carcinoma

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ABSTRACT

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Aim: To investigate the correlation of p53 and prohibitin (PHB) expression in the spectrum of hepatitis, cirrhosis, and human hepatocellular carcinoma (HCC). **Methods:** Hepatic biopsies from patients with HCC ($n=60$), cirrhosis (CIR, $n=30$), hepatitis C virus (HCV, $n=30$), and normal livers (NL, $n=20$) were examined for immunohistochemical expression of RelA/p65, tumor necrosis factor receptor-1 (TNFR1), TNF-related apoptosis-inducing ligand (TRAIL), p53, and PHB. The samples were also analysed by nuclear factor kappa B (NFkB) Southwestern histochemistry and a transferase-mediated dUTP-biotin nick-end labelling assay. **Results:** Expression of NFkB and RelA/p65 was detected increasingly from NL to CIR, but had a diminished labelling in the HCC cases ($P < 0.05$). Expression levels of TNFR1 and TRAIL followed the same pattern ($P < 0.05$). Apoptosis was increased in HCC, but was progressively reduced from CIR to NL ($P < 0.05$). p53 and PHB nuclear expressions were amplified in cases of HCC, but diminished in NL, HCV, and CIR ($P < 0.05$). **Conclusion:** These results suggest that in addition to well-understood sequences of proinflammatory events such as TNF-induced NFkB activation and NFkB/TRAIL pathway-mediated apoptosis, development of HCC is also influenced by regulation of p53 and PHB tumour suppressor function. Additional studies are necessary to explain the contradictory mechanisms of the tumour microenvironment observed in the sequence of HCV, CIR, and HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC), the most frequent primary hepatic cancer, is the third highest cause of cancer-related death worldwide.^[1] Accumulation of genetic and epigenetic alterations results in the development of HCC.^[2] Therefore, the molecular

pathways involved in hepatic cancer have been the focus of numerous studies.

Activation of the transcriptional factor nuclear factor kappa B (NFkB), an important modulator of inflammatory and cell survival responses,^[3] has been associated with hepatic carcinogenesis.^[4] NFkB may be activated in



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hepatocytes as a consequence of chronic inflammation, which occurs in viral hepatitis.^[5] However, little is known about the interaction of NFκB activation and tumour microenvironment in the sequence of hepatitis C virus (HCV), cirrhosis (CIR), and HCC.

The inflammatory cytokine tumor necrosis factor-α (TNFα) participates in the control of cellular proliferation and differentiation and cell death.^[6] Although TNFα was initially identified as inducing cell death in some tumours, an association between activation of the TNFα/NFκB pathway by inflammation and hepatocarcinogenesis has also been reported.^[7] Binding of TNFα to TNF receptor-1 (TNFR1) results in NFκB activation and may induce hepatocyte survival and proliferation.^[8] Additionally, activated NFκB can be considered as a protector of TNFα-induced apoptosis.^[9]

TNF-related apoptosis-inducing ligand (TRAIL) has been demonstrated as a proapoptotic mediator of various tumour cells.^[10] Similarly, TRAIL caused cytotoxic effects in transformed hepatocytes of HCC, perhaps related to inhibition of NFκB survival signalling.^[11] In contrast, participation of TRAIL in hepatocyte's apoptosis during chronic hepatic diseases remains controversial.

The *p53* gene is a classical tumor suppressor gene. *p53* mutations occur in diverse human cancers, including HCC. *p53* tumor suppressor function involves cell cycle control, transcriptional regulation, and apoptosis.^[12] In spite of the *p53* mutation being rare in HCC not induced by aflatoxin, the high incidence of HCV-related HCC justifies novel studies even in this context. NFκB linked to HCV non-structural 5A (NS5A) protein inhibits the *p53* tumor suppressor role and leads to cell survival and hepatocarcinogenesis.^[12]

Human prohibitin (PHB), a pleiotropic protein, was identified first as a potential tumour suppressor^[13] and has been implicated in cellular differentiation, antiproliferation, and morphogenesis.^[14] Although a liver-specific deletion of PHB has been observed in a wide spectrum of liver injury types, fibrosis, and hepatocarcinogenesis in mice,^[15] PHB has also been reported to be overexpressed in cases of human HCV.^[16] On the other hand it has been suggested that PHB may have a pivotal role in cellular proliferation and malignant transformation.^[17] Thus, the role of PHB in development of human hepatic cancer remains controversial.

Our study aimed to compare the importance of the *p53* and PHB pathway through the NFκB signalling in the spectrum of HCV, CIR, and HCC, considering the

potential differences in the pathogenesis of human hepatic cancer.

METHODS

Tissue specimens

Primary liver carcinoma samples were obtained from the Department of Pathology at the University Hospital of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil. Twenty samples of normal liver from autopsies with causes other than liver disease (NL), 30 cases of HCV, 30 cases of CIR associated with HCV and 60 cases of HCC related to HCV-CIR were selected for this study [Table 1]. Patients with evidence of any other cause of liver disease were excluded. The study was approved by the local Ethics Committee (number 1611/2011).

Immunohistochemistry

Liver preparations were submitted for immunohistochemical analysis. Sections were incubated with monoclonal primary antibodies specific for TNFR1, TRAIL, RelA/p65, and *p53* (Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution 1:100) and PHB (Thermo Fisher Scientific Waltham, MA, USA, dilution 1:100). Following this, a secondary antibody (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc.) was applied. Next, the slides were incubated with avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit) and developed with Vector NovaRED kit (Vector Laboratories Inc.) for 5 min. The slides were counterstained with Harris haematoxylin and mounted with Permount (Biomedex, Foster City, CA, USA). Percentages of nuclear RelA/p65 and *p53*, cytoplasmic TNFR1 and TRAIL, and nuclear/cytoplasmic PHB-positive cells were obtained blindly for each case at least 10 representative high-power fields (40×) by two of the authors (LDP and LNR). For statistical purposes, the samples were scored as follows: -, no stained cells; +, weak or moderate staining in less than 25% positive cells; ++, moderate to strong staining in 25-50% positive cells; and +++, strong staining in more than 50% positive cells.^[18]

Southwestern histochemistry analysis

Non-radioactive *in situ* detection of NFκB in paraffin-embedded liver tissue preparations was performed using the Southwestern histochemistry method, with digoxigenin labelling and detection kits (Roche Applied Science, Indianapolis, USA). Briefly, synthetic sense DNAs (Imprint Genetics Corporation, Hialeah, USA), which contain sequences of NFκB, were used as probes. After annealing with the complementary sequence, the DNA probe was labelled with digoxigenin.

Table 1: Clinicopathologic variables of NL, CIR, HCV, and HCC

Clinicopathologic variables	Etiology (% cases)				P value
	NL (n = 20)	HCV (n = 30)	CIR (n = 30)	HCC (n = 60)	
Age (years)					0.5530
≤ 50	0	40	20	35	
> 50	100	60	80	65	
Gender					0.4116
Male	60	80	70	80	
Female	40	20	30	20	
Cirrhosis					0.0001
Absence	100	100	0	3	
Presence	0	0	100	97	
Serum AFP (µg/L)					0.0001
≤ 20	100	100	100	40	
> 20	0	0	0	60	

NL: normal liver; CIR: cirrhosis; HCV: hepatitis C virus; HCC: hepatocellular carcinoma

The percentage of NFκB-positive cells was obtained blindly for each case, at least 10 representative high-power fields (40×) by two of the authors (LDP and LNR). For statistical purposes, the samples were scored similarly as was presented in the immunohistochemistry section.^[18]

TUNEL assay

Paraffin-embedded liver tissue sections were deparaffinised and incubated with 20 µg/mL proteinase K (Promega Corporation, Madison, USA). A DeadEnd peroxidase *in situ* apoptosis detection kit (DeadEnd TUNEL; Promega Corporation) was used for transferase-mediated dUTP-biotin nick-end labelling (TUNEL) staining. The percentage of TUNEL-positive cells was obtained blindly, for each case at least 10 representative high-power fields (40×) by two of the authors (LDP and LNR). For statistical purposes, the samples were scored similarly as was presented in the immunohistochemistry section.^[18]

Statistical evaluation

Statistical analysis was performed using GraphPad Prism v4.0 software (GraphPad Software, Inc., San Diego, CA). Association between the expression of TNFR1, TRAIL, NFκB, RelA/p65, p53, PHB, and TUNEL and histologic variables was determined by Fisher's exact test (2 groups) or a chi-square test (3 or more groups). One-way analysis of variance followed by Dunn's post-test was also performed. All tests were two-tailed, and $P < 0.05$ was considered significant.

RESULTS

Study tissue specimens

The most relevant data concerning clinic and pathological variables was the occurrence of cirrhosis

or viral hepatitis in almost all HCC cases, as well as absence of cirrhosis or other inflammatory conditions in the normal liver ($P = 0.0001$). Additionally, the level serum α-fetoprotein was > 20 µg/L in the majority of HCC cases, but ≤ 20 µg/L in the other types ($P = 0.0001$) [Table 1].

Expression and clinicopathological features

Table 2 summarizes the comparative TNFR1, TRAIL, NFκB, nuclear RelA/p65, p53, nuclear PHB and cytoplasmic PHB and TUNEL expression by HCC, CIR, HCV, and NL.

TNFR1 expression was higher in CIR [Figure 1C] in comparison to HCC [Figure 1D] and HCV [Figure 1B] ($P = 0.0294$ and $P = 0.0037$, respectively), as well as in HCV compared to NL [Figure 1A] ($P = 0.0313$) [Figure 1Q]. TRAIL expression was amplified in HCC [Figure 1H] compared to CIR [Figure 1G] and HCV [Figure 1F] ($P = 0.0377$ and $P = 0.0371$, respectively), as well as in HCV in relation to NL [Figure 1E] ($P = 0.0462$) [Figure 1R].

NFκB expression was increased in CIR [Figure 1K] in comparison to HCC [Figure 1L] ($P = 0.0464$). NFκB expression was also higher in CIR compared to HCV [Figure 1J] ($P = 0.0031$), as well as in HCV [Figure 1D] in relation to NL [Figure 1I] ($P = 0.0477$) [Figure 1S]. In a pattern similar to that of NFκB, nuclear RelA/p65 expression was increased in CIR [Figure 1O] compared to HCC [Figure 1P] and HCV [Figure 1N] ($P = 0.0228$ and $P = 0.0426$, respectively), as well as in HCV relative to NL [Figure 1M] ($P = 0.0288$) [Figure 1S].

Since p53 expression was almost solely found in HCC [Figure 2D], this group presented a higher p53 expression relative to NL [Figure 2A], HCV [Figure 2B], and CIR [Figure 2C] ($P = 0.011$, $P = 0.014$ and $P = 0.0013$, respectively). However, no difference in p53 expression was observed between CIR and HCV ($P = 0.9421$), as well as in HCV in relation to NL ($P = 0.9421$) [Figure 2M].

Cytoplasmic PHB expression was augmented in CIR [Figure 2G] in relation to HCC [Figure 2H] ($P = 0.0001$), whereas PHB cytoplasmic expression was similar between CIR and HCV [Figure 2F] ($P = 0.4468$). PHB cytoplasmic expression was increased in HCV, CIR, and HCC in comparison to NL [Figure 2E] ($P = 0.0088$). Because PHB nuclear expression was almost exclusively observed in HCC, this group presented a higher PHB nuclear expression in contrast to CIR, HCV, and NL ($P = 0.0041$, $P = 0.0011$ and $P = 0.0011$, respectively) [Figure 2N].

Table 2: Comparative TNFR1, TRAIL, NFkB, nuclear RelA/p65, p53, nuclear PHB, cytoplasmic PHB and TUNEL expression by NL, CIR, HCV, and HCC

Markers	Expression	NL	HCV	CIR	HCC	<i>P</i> < 0.05
		(<i>n</i> = 20)	(<i>n</i> = 30)	(<i>n</i> = 30)	(<i>n</i> = 60)	
TNFR1	Negative	18	3	3	32	
	+	2	24	9	13	CIR vs. HCC CIR vs. HCV HCV vs. NL
	++	0	3	15	12	
	+++	0	0	3	3	
TRAIL	Negative	18	12	9	14	
	+	2	18	21	18	HCC vs. CIR HCC vs. HCV HCV vs. NL
	++	0	0	0	18	
	+++	0	0	0	10	
RelA/p65	Negative	16	3	0	21	
	+	4	18	9	19	CIR vs. HCC CIR vs. HCV HCV vs. NL
	++	0	9	15	13	
	+++	0	0	6	7	
NFkB	Negative	18	0	3	26	
	+	2	6	18	22	CIR vs. HCC CIR vs. HCV HCV vs. NL
	++	0	15	6	10	
	+++	0	9	3	2	
p53	Negative	17	27	27	20	
	+	3	3	3	26	HCC vs. CIR HCC vs. HCV HCC vs. NL
	++	0	0	0	12	
	+++	0	0	0	2	
Cytoplasmic PHB	Negative	16	3	3	35	
	+	4	5	7	23	CIR vs. HCC CIR vs. HCV HCV vs. NL
	++	0	10	11	2	
	+++	0	12	9	0	
Nuclear PHB	Negative	18	27	24	19	
	+	2	3	6	24	HCC vs. CIR HCC vs. HCV HCC vs. NL
	++	0	0	0	13	
	+++	0	0	0	4	
TUNEL	Negative	17	3	2	4	
	+	3	16	18	15	HCC vs. CIR HCC vs. HCV HCC vs. NL
	++	0	11	10	32	
	+++	0	0	0	9	

TNFR1: tumor necrosis factor receptor-1; TRAIL: TNF-related apoptosis-inducing ligand; NFkB: nuclear factor kappa B; PHB: prohibitin; TUNEL: transferase-mediated dUTP-biotin nick-end labelling; NL: normal liver; CIR: cirrhosis; HCV: hepatitis C virus; HCC: hepatocellular carcinoma

The percentage of apoptosis was increased in HCC [Figure 2L] in contrast to CIR [Figure 2K] and HCV [Figure 2J] ($P = 0.0054$ and $P = 0.0217$, respectively). Moreover, the percentage of apoptosis was increased in HCV in relation to NL [Figure 2I] ($P = 0.0161$) [Figure 2O].

DISCUSSION

The findings of the present study reveal the importance of p53 and PHB participation in human

hepatic cancer development, which is dependent on different pathogenic mechanisms of the tumour microenvironment.

TNF α cytokine is increased in patients with chronic HCV infection.^[19] TNF α and TNF-receptors levels are augmented during chronic HCV infection and result in disease progression.^[20] Similarly, we found a progressive increase of TNFR1 expression in cases of HCV and CIR in comparison to NL. Indeed, TNF α

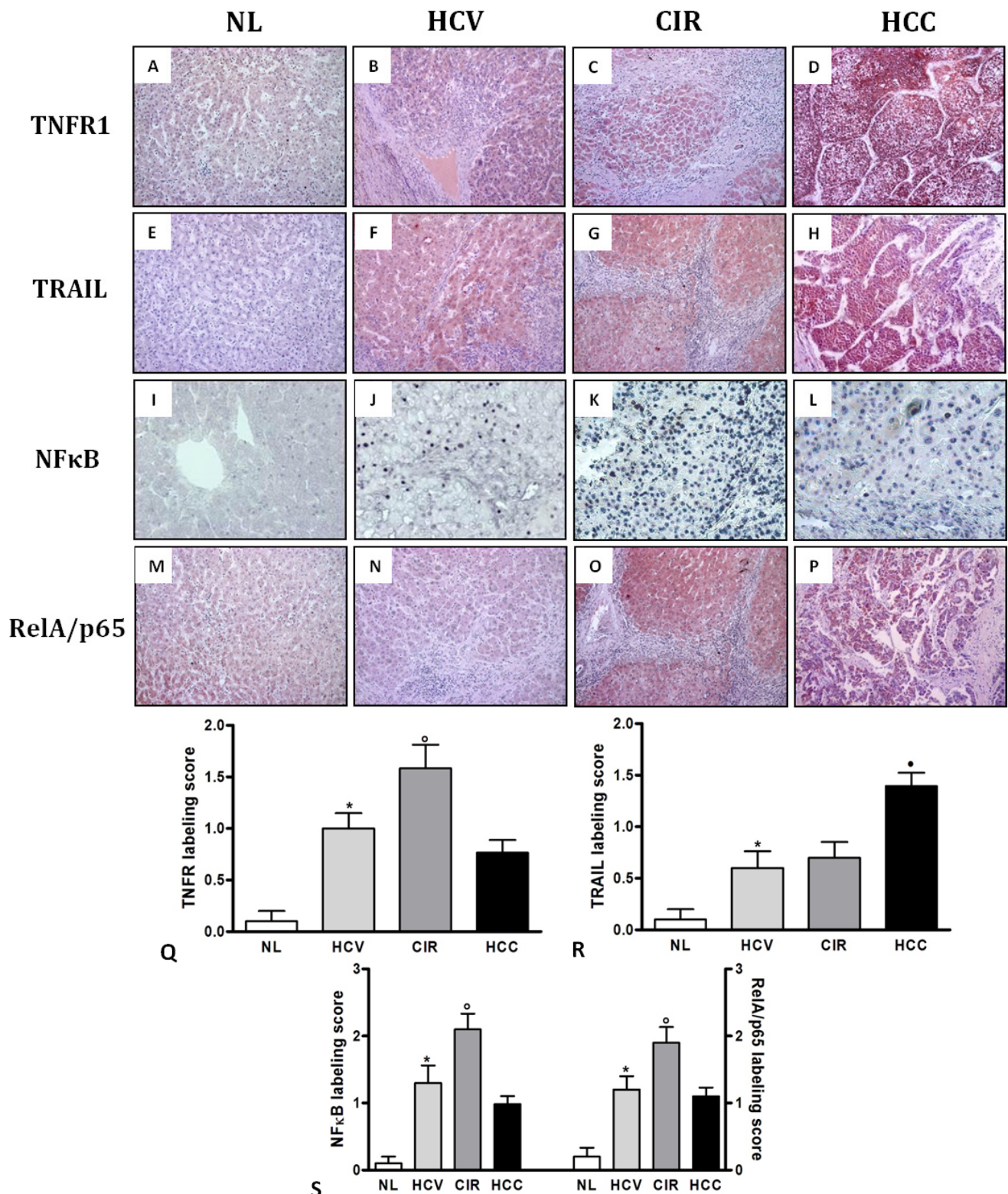


Figure 1: Comparative expression of TNFR1 (immunohistochemistry, $\times 200$), TRAIL (immunohistochemistry, $\times 200$), NFκB (Southwestern histochemistry, $\times 200$) and RelA/p65 (immunohistochemistry, $\times 200$) by NL, HCV, CIR and HCC. A: low expression of TNFR1 in NL; B: increased expression of TNFR1 in HCV; C: high expression of TNFR1 in CIR; D: strong expression of TNFR1 in HCC; E: minimal expression of TRAIL in NL; F: increased expression of TRAIL in HCV; G: augmented expression of TRAIL in CIR; H: high expression of TRAIL in HCC; I: low expression of NFκB in NL; J: high expression of NFκB in HCV; K: marked expression of NFκB in CIR; L: increased expression of NFκB in HCC; M: minor expression of RelA/p65 in NL; N: increased expression of RelA/p65 in HCV; O: evident expression of RelA/p65 in CIR; P: high expression of RelA/p65 in HCC; (Q, R, S) comparison of the TNFR1 (Q), TRAIL (R), NFκB and RelA/p65 (S) labeling scores by NL, HCV, CIR and HCC. ^{*}P < 0.05 vs. NL; ^oP < 0.05 vs. HCV; ^{*}P < 0.05 vs. CIR. TNFR1: tumor necrosis factor receptor-1; TRAIL: TNF-related apoptosis-inducing ligand; NFκB: nuclear factor kappa B; NL: normal liver; CIR: cirrhosis; HCV: hepatitis C virus; HCC: hepatocellular carcinoma

proinflammatory stimuli can be responsible for NF κ B activation, which results in protection of hepatocytes from apoptosis.^[9] Moreover, the HCV core protein potentiates NF κ B activation and chronically activated

NF κ B leads to infected hepatocytes survival and consequent HCV infection persistence.^[21]

In the present study, RelA/p65 nuclear labelling

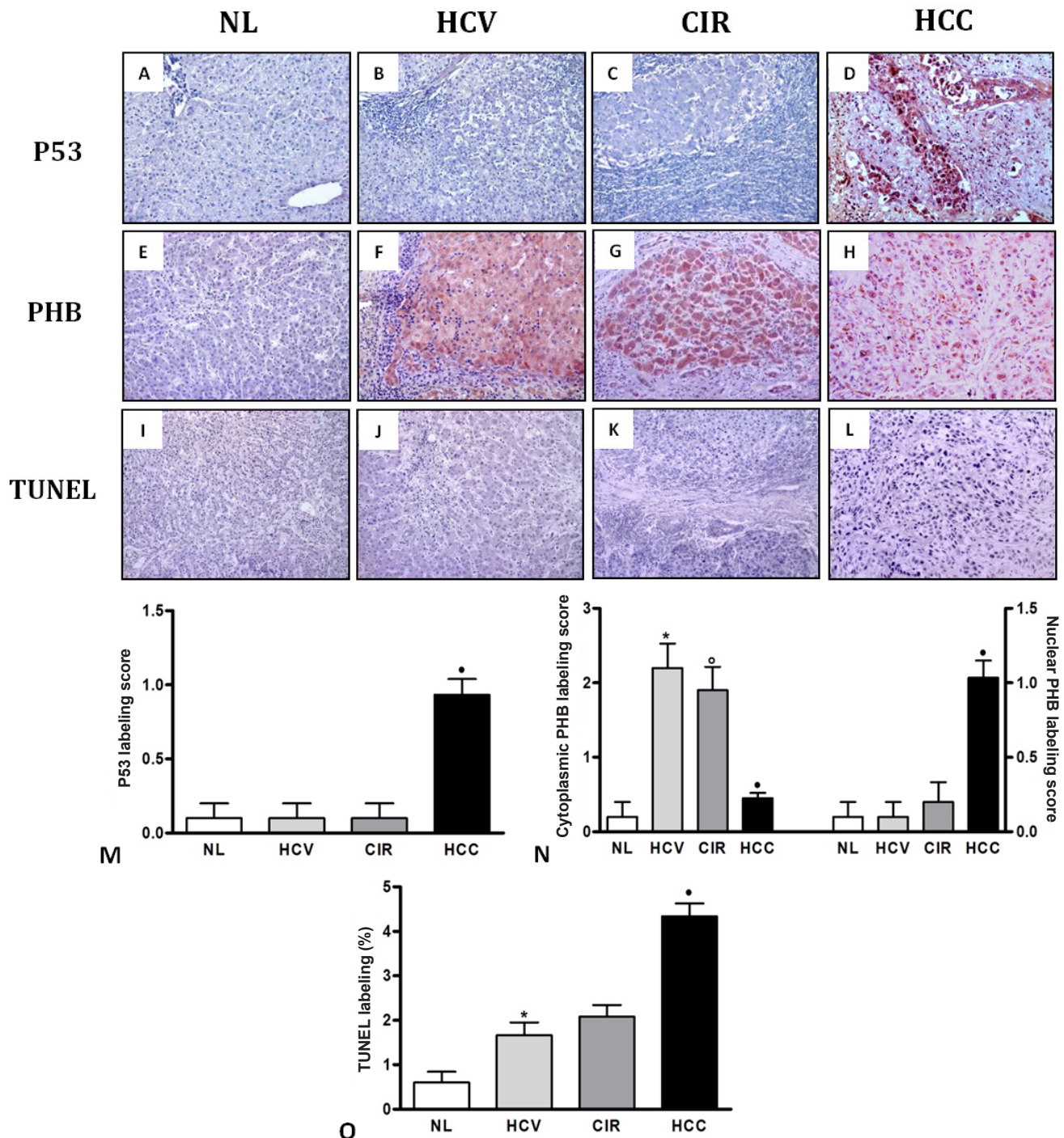


Figure 2: Comparative expression of P53 (immunohistochemistry, $\times 200$), PHB (immunohistochemistry, $\times 400$) and TUNEL (immunoassay, $\times 200$) by NL, HCV, CIR and HCC. A: absence of P53 expression in NL; B: low expression of P53 in HCV; C: minor expression of P53 in CIR; D: high nuclear and cytoplasmic expression of P53 in HCC; E: slight cytoplasmic expression of PHB in NL; F: augmented cytoplasmic expression of PHB in HCV; G: high cytoplasmic expression of PHB in CIR; H: strong nuclear and cytoplasmic expression of PHB in HCC; I: slight expression of apoptosis in NL; J: increased expression of apoptosis in HCV; K: augmented expression of apoptosis in CIR; L: marked expression of apoptosis in HCC; (M, N, O) comparison of the P53 (M), cytoplasmic and nuclear PHB (N) and TUNEL (O) labeling scores by NL, HCV, CIR and HCC. * $P < 0.05$ vs. NL; ^o $P < 0.05$ vs. HCV; $P < 0.05$ vs. CIR. PHB: prohibitin; TUNEL: transferase-mediated dUTP-biotin nick-end labelling; NL: normal liver; CIR: cirrhosis; HCV: hepatitis C virus; HCC: hepatocellular carcinoma

followed the same profile of activated NF κ B expression by Southwestern histochemistry, as previously reported.^[22] Thus, these data were discoursed together as activated NF κ B expression. We observed a crescent augmentation of activated NF κ B expression in HCV and CIR in relation to NL. NF κ B activation may be induced by TNF α secondarily to the increase of proinflammatory status in HCV and CIR. Since TNF α -induced NF κ B activation acts as an important survival factor for hepatocytes,^[9] higher activated NF κ B expression could explain the slight number of apoptotic hepatocytes in HCV and CIR samples in comparison to HCC. In addition, constant activation of NF κ B during chronic liver disease performs as an early molecular change during HCC progression.^[11] Nevertheless, in the later stages of hepatocarcinogenesis, NF κ B inhibition can accelerate development of HCC by cellular proliferation.^[23] Thus, in spite of the reduction of activated NF κ B expression in HCC patients which may be related to the significant increase of apoptosis, other mechanisms could be associated with this phenomenon.

TRAIL expression was increased and accompanied by decreased NF κ B activation and frequent apoptosis in the cases of HCC when compared to HCV and CIR. In addition to inducing apoptosis in normal hepatocytes,^[24] TRAIL can mediate NF κ B inhibition with consequent apoptosis in transformed hepatocytes.^[11] This finding may be related to the attempt of preventing tumorigenesis during chronic inflammatory disease. Moreover, increase of TRAIL expression in HCC with consequent reduction of NF κ B activation and augment of hepatocytes apoptosis may occur by a p53-independent pathway.^[25]

p53 has been widely considered as a tumor suppressor gene associated with induction of apoptosis in different types of neoplasms, including HCC.^[26] Furthermore, p53 is usually less expressed during HCV than in HCC.^[27] Accordingly, we found p53 expression almost solely in HCC, without significant changes during chronic liver inflammation. Increased p53 expression was also followed by augmentation of apoptosis in HCC. Moreover, the RelA/p65 subunit can inhibit p53 activation, as well as p53 also suppresses NF κ B transcriptional effects.^[28] Perhaps during HCV and CIR, the inflammatory stimulus induces an increase of TNF α with consequent NF κ B activation and p53 inhibition, which leaked from hepatocytes from apoptosis. Concerning HCC, which present a less evident inflammatory process, TNF α -induced NF κ B activation is discrete and, in addition to inhibition of NF κ B activation by p53, augments apoptosis. Furthermore, another possible suppressor

tumor gene, *PHB*, has been associated with an increase of p53 activity in the induction of apoptosis in cancer.^[29]

The role of *PHB*, in particular, is not fully understood. There are controversies because both antitumorigenic and protumorigenic functions have been reported for *PHB*, depending on its subcellular localization.^[30] In addition to regulation of various cellular functions, the mitochondrial location of *PHB* is mainly engaged in reducing damage caused by oxidative stress in the chronic phase of various diseases.^[31] In our HCV and CIR samples, we detected a high expression of cytoplasmic PHB, likely due to chronic inflammation associated with these diseases. Moreover, in spite of PHB reducing NF κ B activation mediated by TNF α , which permits occurrence of apoptosis, high levels of TNF α can also inhibit tumour suppressor *PHB* activity during chronic inflammation.^[32] This regulatory mechanism may explain increased NF κ B activation and higher hepatocytes survival in HCV and CIR in comparison with HCC, despite high cytoplasmic PHB expression in HCV and CIR. In addition, the failure of apoptosis of mutated hepatocytes may represent a primary event in the hepatocarcinogenesis associated with chronic liver disease.

Our data show that PHB expression was exclusively identified as nuclear labelling in HCC. It has been reported that nuclear PHB appears to be essential for regulation of cellular processes such as apoptosis, proliferation, and gene transcription.^[30] PHB was also detected in the nucleus associated with retinoblastoma and p53, inducing changes in transcription factors, resulting in cell cycle inhibition and induction of apoptosis.^[33] The tumour suppressor action of *PHB* was associated with nuclear expression in cells of several neoplasms.^[29,34] In agreement, the HCC group presented exclusively a PHB nuclear expression in contrast to CIR and HCV. Because NF κ B may be acting as an antiapoptotic mediator, and once inhibited by the action of PHB in HCC, it may increase the rate of apoptosis in these cases.

In conclusion, our results suggest that, in addition to well-understood sequences of proinflammatory events such as TNF-induced NF κ B activation and NF κ B/TRAIL pathway-mediated apoptosis, development of human hepatic cancer may be influenced by regulation of p53 and PHB tumour suppressor function. Another possibility would be that expression of p53 and PHB may have been altered as a consequence of an already-established hepatocellular carcinoma. Additional studies are necessary to explain the contradictory mechanisms of the tumour

microenvironment observed in the sequence of HCV, CIR, and HCC.

Authors' contributions

Casuistic retrieval and samples analysis: L.M. Della Porta
Statistical analysis and writing: F.S. Ramalho
Samples analysis and discussion: C.A. Oliveira
Histological and immunohistochemical preparations and analysis: D.M. Silva
Southwestern histochemistry and analysis: M.J. Augusto
Paper scientific design and writing: L.N.Z. Ramalho

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Conflict of interests

There are no conflicts of interests.

Patient consent

Each patient was informed of the study and gave their consent.

Ethics approval

The study was approved by the local Ethics Committee (nº1611/2011).

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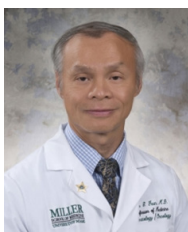
Immunotherapy for hepatocellular carcinoma: the force awakens in HCC?

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ABSTRACT

Systemic therapy for hepatocellular carcinoma (HCC) has been disappointing. The only drug approved by Food and Drug Administration recently has been sorafenib. Sorafenib has modest benefits with a low response rate and an improvement in time to progression of only 2-3 months. Multiple randomized trials, which compare the new agent to sorafenib as either first line or second line therapy, have been negative, showing no improved clinical benefit. Recently, in a large phase III randomized trial, regorafenib has shown superiority to placebo as a second line treatment for HCC. However, this drug has multiple side effects and is not well tolerated by many patients. The clinical benefit is also modest. Clearly, new approaches to treat advanced HCC are still needed. There is data showing that HCC is immunogenic and the immune system can be stimulated to attack these cancer cells. This article will briefly review immunotherapy as a promising treatment for HCC.

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INTRODUCTION

There is a strong rationale to evaluate immunotherapy in this disease. Hepatocellular carcinoma (HCC) is typically an inflammation-associated cancer and can be immunogenic. Both hepatitis B virus (HBV) and hepatitis C virus (HCV) are known to be risk factors for the development of HCC. The HCC from HCV

typically develops in a setting of long standing liver cirrhosis. On the other hand, HCC may develop from HBV even in the absence of liver cirrhosis. Increasingly, HCC appears to be developing from nonalcoholic steatohepatitis (NASH) with obesity being a risk factor. Studies in mice have shown that dietary factors and genetic obesity can promote liver inflammation and tumorigenesis. This appears to be



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mediated by enhancing interleukin (IL)-6 and tumor necrosis factor (TNF) expression. With increasing incidence of obesity in the western countries, NASH will become a greater risk factor for HCC.

Another supporting factor is that spontaneous regression have been reported in HCC.^[1] Spontaneous regression has been reported in other cancers as well and is thought to be mediated by immune response in the host. Although infrequent, these occurrences suggest it may be possible to enhance the immune system against certain malignancies.

In addition, more than 50% HCC patients develop spontaneous cellular or humoral immune response against NY-ESO-1.^[2] Furthermore, tumor-associated antigens (TAA)-specific CD8+ T-cell immune responses have been described [most studied include AFP, glypican-3, NY-ESO-1, SSX-2, MAGE-A, human telomerase-reverse transcriptase (h-TERT)].^[3] More than 50% HCC patients had HCC-infiltrating TAA-specific CD8+ T-cells and these cell numbers correlated with progression-free survival.^[4]

In HCC, the number of T regulatory cells increased both in peripheral blood and inside tumor itself.^[5] Intratumoral T regs correlated with disease progression and poor prognosis.^[6] Activation of T-cell infiltration (CD4+, CD8+, natural killer cells) has been observed after liver ablation.^[7,8] Together the data shows that inflammation is a common feature seen in HCC and this tumor can elicit an immune response.

REASONS FOR HCC IMMUNE TOLERANCE

If HCC is typically an inflammation-associated cancer and can be immunogenic, why is there immune tolerance? There are several factors which may be involved.

On a cellular level, the liver is a site for myeloid-derived suppressor cells (MDSC) which can inhibit effector T-cell function and decrease natural killer (NK) cell cytotoxicity and cytokine production. The frequency of MDSCs correlates with progression-free survival in HCC after radiofrequency ablation.^[9] It has been suggested that MDSCs interact with Kupffer cells to induce programmed death-1 (PD-1) expression and MDSCs may help expand T regs.^[10] Depletion of T regs or MDSCs may prompt spontaneous immune responses against α -fetoprotein (AFP).^[11,12]

A new subset of immune suppressive cells has been described in HCC called regulatory dendritic cells (DCs) which can suppress T-cell activation via IL-10

and indoleamine 2,3-dioxygenase (IDO) production.^[13] Unexpectedly, it was found that these dendritic cells expressed high levels of cytotoxic T-lymphocyte antigen-4 (CTLA-4) and PD-1. CTLA-4 was discovered to be essential for IL-10 and IDO production. This finding represents a target for immunotherapy as well as one possible explanation for immune tolerance.

Human HCC tumor-infiltrating CD4+ CD69+ T regs are higher than conventional CD4+ CD25+ Foxp3+ T regs and correlates with tumor progression.^[14] These T regs do not express CD25 or Foxp3 but express high levels of mouse transforming growth factor beta 1 (mTGF- β 1), PD-1, CTLA-4 and could suppress CD4 T-cell proliferation via mTGF- β 1.^[14] The percentage of these T regs in tumors correlated significantly with tumor progression.

Failure of HCC-associated antigen production presentation by antigen presenting cells is due to decreased expression of HLA class 1 molecules and ineffective antigen processing.^[15,16]

Increase in T regulatory cells, invariant NK T-cells, MDSC and tumor-associated macrophages may play a role and a decrease in CD4+ T helper cells has been reported.^[4,11,17-20]

There is an increase in CD4+, CD25+ T regs within tumor infiltrating lymphocytes (TILs) which is associated with decrease in number and function of CD8+ T-cells.^[5,17]

T-cell (CD4+) exhaustion and apoptosis have been associated with chronic HCV infection.^[21] The CD4+ T-cells of chronic HCV-infected patients displayed increased surface expression of TRAIL and expression of other immune exhaustion molecules. In addition, indoleamine 2,3-dioxygenase activity is increased and IDO is a T-cell proliferation-limiting enzyme. Other molecules associated with T-cell exhaustion and apoptosis signaling in peripheral blood mononucleocytes from chronically infected HCV patients have been described.^[21]

LAG-3 expression has been found to be significantly up-regulated in tumor infiltrating CD8+ T-cells in HCC patients and a severe functional defect was detected in tumor infiltrating HBV-specific CD8+ T-cells at the tumor site.^[22] Because LAG-3 is an inhibitory molecule that helps to downregulate T-cell responses, there was a correlation between LAG-3 expression and HBV-specific CD8+ T-cell dysfunction.

Thus, the immunosuppression that is seen in HCC is

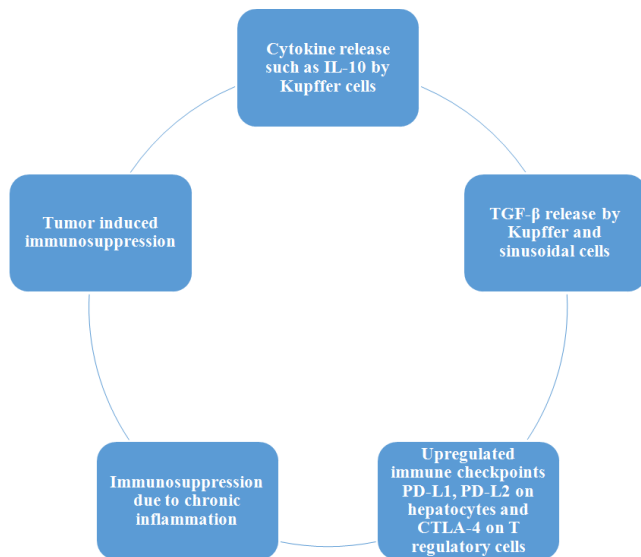


Figure 1: Selected known mechanisms of immune tolerance in HCC. HCC: hepatocellular carcinoma; IL-10: interleukin-10; TGF-β: transforming growth factor beta; PD: programmed death; CTLA: cytotoxic T-lymphocyte antigen

complex and involves a balance of multiple factors [Figure 1]. The factors which are involved in tumor progression include hepatic tolerance to various antigens, chronic inflammation which predisposes to immunosuppression and liver cancer dependent immune tolerance. Poorly counter-balancing this involves antitumor immune response.

RATIONALE FOR IMMUNOTHERAPY FOR HCC

NK cells have been divided into two major groups, one mainly cytotoxic and the other mainly involved in cytokine secretion. In HCV-related hepatocellular carcinoma a prevalent cytotoxic NK phenotype was found in HCC patients with longer time to tumor recurrence and overall survival.^[23] This suggests a role for NK cells in the immune response against HCC and a rationale for immunotherapy using NK enhancing therapy.

Pim kinases are downstream effector molecules of certain oncogenes. Pim-3 expression occurs in certain solid tumors and is highly expressed in HCC tissues and cell lines. In HCC Pim-3 is associated with acceleration of HCC development and has been found to inhibit apoptosis by phosphorylating the proapoptotic BH3-only protein BAD. A dual-function vector with both immunostimulatory and Pim-3-silencing effects inhibited Hepa1-6 cell growth by regulating expression of apoptosis-related proteins and inducing secretion of type I interferons.^[24] NK cells, CD4⁺ T and CD8⁺ T-cells and macrophages were required for effective tumor inhibition and CD4⁺ T-cells were demonstrated to play helper role in NK cell activation. This novel bi-

functional vector represents a novel immunotherapy approach to treat HCC.

PD-1/PD-L1 pathway has been associated with T-cell exhaustion in chronic hepatitis virus infection.^[25] Inhibition of T-cell activation is due to PD-1 ligation, which causes recruitment of SHP-2 phosphatase. This in turn inhibits PI3K activity and downstream activation of AKt, with dampening of T-cell receptor signaling. PD-1/PD-L1 involvement in chronic viral infection-associated T-cell exhaustion was first shown in a murine lymphocytic choriomeningitis model.^[26] PD-1/PD-L1 blockade can produce functional recovery of T-cells, cytokine secretion, cytotoxic capability and decreased viral load.

IMMUNOTHERAPY TRIALS IN HCC

Interferons

Immunotherapy has been used for HCC in the past with limited success. Interferon alpha was combined with systemic chemotherapy and modest activity was observed. In one study, 26 patients with advanced HCC received intravenous cisplatin, doxorubicin, and 5-fluorouracil combined with subcutaneous administered human recombinant α-interferon-2a (PIAF regimen).^[27] The disease control rate was 50% (4 partial responses and 9 stable disease). The 1-year survival rate was 24.3% and median survival time was 6.0 months. A modified PIAF showed superior response rate and survival compared to standard dose PIAF.^[28] A randomized trial of PIAF versus standard dose doxorubicin showed that PIAF had more activity and greater toxicity, particularly myelosuppression.^[29] Since myelosuppression was greater than single agent doxorubicin, this treatment regimen is best for younger patients with good performance status and adequate bone marrow reserve.

Checkpoint inhibitors

Checkpoint inhibitors have demonstrated activity in a number of cancers including HCC. CTLA-4 blockade has been evaluated using Tremelimumab.^[30] In this study the drug was given at 15 mg/kg intravenously every 90 days. Twenty patients were treated and 17 patients were assessable for response. In these 17 patients, the overall response rate was 17.6% and disease control rate was 76.4%. The median time to tumor progression was 6.48 months. Toxicity was mainly hepatic. There were > grade 3 AST and ALT elevation in 45% patients and 25%, respectively. Serum bilirubin elevated occurred in 10%. Skin rash occurred in 5%. An antiviral effect was noted. FoxP3⁺ natural T reg expanded and viral hepatitis C load decreased.

A dose escalation study of the PD-1 inhibitor Nivolumab was initially reported at ASCO in 2015 and subsequently updated at ASCO 2016.^[31] In the initial report, 43 patients were treated in dose-escalation phase. Median of 6 (range 1-42) doses of Nivolumab administered. One dose-limiting toxicity (DLT) with grade 2 hepatic decompensation occurred at 10 mg/kg in uninfected cohort. No maximum tolerated dose was identified. The 3 mg/kg dose was selected for dose expansion in uninfected and HCV-infected cohorts. Thirty patients had discontinued therapy (26 patients due to progressive disease, 2 due to complete response (CR), 2 due to adverse events (1 due to increase in bilirubin, 1 due to treatment-related increase in AST/ALT and hepatitis). Responses usually occurred within 3 months of drug initiation. The overall response rate was 15% (10% CR and 5% partial response, PR). The preliminary overall 1-year survival was 62%. This is superior to the 1-year overall survival of 30% reported in phase III trials after sorafenib failure.

An update at ASCO 2016 showed the following: the dose escalation part involved 48 patients and the expansion part involved 214 patients.^[32] The dose of nivolumab for the dose escalation ranged from 0.1 mg/kg to 10 mg/kg, while in the dose expansion study nivolumab was given at 3 mg/kg every 2 weeks.

A total of 10 patients had HCV, 15 patients had HBV and 23 patients were uninfected. Safety data was presented for 48 patients. Of these patients 79% had treatment-related adverse events (TRAEs) of any grade, including 25 % with grade 3-4. The most common TRAEs were rash, pruritus and elevation of AST, ALT, lipase and amylase. AST and ALT and lipase/amylase occurred more frequently in this group of patients compared to other nivolumab-treated patient populations. Most of these TRAEs were noted to be asymptomatic and reversible. Five of 7 patients responded within 3 months of start of treatment.

Response was ongoing beyond 24 months in 1 patient who stopped treatment with a complete response. The median duration of response was 17 months, range 6-24 months. Stable disease occurred out to 12-18 months. Altogether, 3 patients had a complete response and four had a partial response; the disease control rate was 65%. The expansion phase was continuing with nivolumab at 3 mg/kg. In conclusion, Nivolumab has shown manageable safety and toxicity in HCC patients, including HBV and HCV infected patients. Antitumor activity was observed across all dose levels and cohorts. The response was durable in many patients and the 1-year overall survival rate was encouraging.

An investigator-initiated, first in-human phase II trial of the PD-1 inhibitor pembrolizumab was started at the University of Miami (ClinicalTrials.gov NCT02658019). The dose of pembrolizumab is 200 mg intravenously every 3 weeks. So far, 10 patients have been treated. It is too early yet to assess response. Correlative studies include tumor staining for PD-L-1, and changes in hepatitis B and hepatitis C viral titers. In addition, several representative cytokines associated with T-cell activation and suppression in serum and peripheral blood mononuclear cells will also be analyzed pre- and post-treatment. T-cell proliferation and activation in response to T cell receptor (TCR) and CD28 signals require IL-2, IL-12, IFN- γ stimulation, but can be suppress by immunosuppressive cytokines such as IL-10 and TGF- β . Changes in these cytokines may be important to predict response/toxicity to therapy.

Dendritic cell vaccine

Another approach to immunotherapy is by infusion of autologous dendritic cell vaccine.^[33] Patients with advanced HCC were infused with mature autologous dendritic cells pulsed *ex vivo* with a liver tumor cell lysate and compared to a control group. There were 15 patients in each group. In terms of response for the treated group, 2 patients (13.3%) had partial response, 9 (60%) had stable disease and 4 (26.7%) patients had progressive disease. Serum gamma interferon and CD8+ T-cells both were increased after dendritic cell vaccination. The median survival time for the treated group was 7 months versus 4 months for the untreated group. The side effects of the dendritic cell vaccine were minimal with low-grade fever and mild bone aches.

Viral oncolytics

A viral oncolytic immunotherapy approach has been studied using a vaccinia virus.^[34-36] These therapies have been designed to replicate selectively within HCC cells and produce cell lysis, while inducing tumor-specific immunity. JX-594 (Pexa-Vec) is a vaccinia virus with a disrupted viral thymidine kinase gene with insertion of human granulocytic-macrophage colony-stimulating factor and β -galactosidase transgenes both for immune stimulation and replication assessment. A phase I trial of JX-594 demonstrated feasibility and tolerability and produced some responses.^[35,36] A randomized, dose finding trial was performed with direct infusion of the vaccinia virus into liver tumors (days 1, 15 and 29). Patient survival duration was significantly longer with high dose (median 14.1 months) compared to low dose (6.7 months). Responses were observed in both injected and noninjected tumors within both dose groups.

FUTURE DIRECTION

Immunotherapy, especially checkpoint inhibitors, has recently shown promise in a number of cancers, including HCC. How to improve current immunotherapy for HCC? Understanding the mechanisms of resistance and methods to potentiate response remain a challenge. It has been shown that intrahepatic HCV-specific CD8 T-cells from patients with chronic HCV infection were highly PD-1 positive, very dysfunctional, and unexpectedly refractory to PD1/PD L-1 blockade.^[37] This functional impairment was HCV-specific and directly correlated with the level of PD-1 expression. The highly PD-1 positive intrahepatic CD8 T-cells were more exhausted with increased CTLA-4 and with reduced CD28 and CD 127 than circulating T-cells. Thus, a novel therapeutic approach is to combine CTLA-4 with PD-1 blockade. Indeed, studies have shown that there is a synergistic reversal of intrahepatic HCV-specific CD8 T-cell exhaustion by combining anti-CTLA-4 antibody with PD-1 inhibitor.^[38] This has therapeutic implications as both anti-CTLA-4 antibody and PD-1 inhibitors alone have shown activity in HCC.^[30-32] As demonstrated in the treatment of malignant melanoma, the combination has been tolerable and antitumor response may be greater than single agent used alone. However, toxicities including more autoimmune reactions such as diarrhea, hypophysitis and hepatitis may be more frequent and the dose and tolerability of combination therapy will need to be carefully defined. Drug-induced hepatitis is the main concern and may limit patients with significant liver impairment including those with a history of autoimmune diseases such as autoimmune hepatitis.

Currently, a study is being planned to combine anti-CTLA-4 antibody with anti-PD-1 inhibitor in advanced, unresectable HCC.

Another approach is to apply different modalities together. There is a rationale to evaluate combinatorial therapy for HCC. Adding checkpoint inhibitor to TACE or liver ablation seems reasonable. TACE and liver ablation can both increase T-cell infiltration including NK cells.^[7,39] Another study showed that CD4/CD8 ratio and number of B cells and natural killer cells were significantly decreased in HCC patients prior to treatment.^[40] When compared to pretreatment levels, the CD4+ and CD4/CD8 ratio decreased but the CD8+ cells increased in the TACE group. In the TACE + RFA group, the CD4/CD8 ratio and the natural killer cells and the CD8+ cells increased. On the other hand, the CD3+, CD8+, CD4/CD8 ratio and natural killer cell increased in the RFA group. More recently,

tremelimumab has been combined with subtotal ablation (TACE, RFA or cryoablation) in patients with either HCC or biliary tract carcinomas.^[41] In this study 14 patients had TACE, 19 had RFA, including 9 with biliary tract cancers and 5 had cryoablation. No dose limiting toxicity was noted during the trial. Seventeen patients were evaluable for response for lesions outside of TACE/RFA-treated lesions. Of these 4 patients or 23.5% had confirmed partial response. This is noteworthy that 10 of 12 patients with quantifiable HCV had marked reduction in viral load. Liver biopsies were done at week 6. These showed increase in CD8+ T-cells only in patients with clinical response. Furthermore, in peripheral blood mononuclear cells, there was a statistically significant change in CD4/T reg and CD8/T reg ratio in clinical responders. The median time to tumor progression for evaluable HCC patients was 5.7 months.^[41]

Chemotherapy has been used for lymphodepleting prior to adoptive T-cell therapy. This lymphodepletion has been shown to enhance immune reconstitution by the transferred cells and increase tumor specific responses. Low dose cyclophosphamide can impair T regulatory cells and can unmask AFP- specific CD4+ T-cell responses in patients with advanced HCC.^[12] Adoptive T-cell therapy itself, which uses a patient's own T lymphocytes genetically altered to enhance anti-tumor activity, expanded *in vivo* and then infused into the patient, has a number of problems. Problems include the need for surgery to obtain tumor-reactive TIL cells and the expansion of the TIL cells from tumors. Alternatively, adoptive transfer of bulk T lymphocytes can be procured from peripheral blood and expanded *in vivo* to generate large number of T lymphocytes before infusing back into the patient. Problems with this approach include tumor cells can have low antigen presentation and most tumor antigens are normally expressed as self-antigens. Thus, the T-cell receptor (TCR) may have low affinity for these self-tumor antigens.

To overcome these obstacles, T-cells have been genetically engineered to stably express transgenes using viral transduction, often with vectors from gamma retroviruses or lentiviruses. Molecularly engineered TCRs have certain advantages including the ability to target all cellular proteins and not just cell surface epitopes. Genetically altering the T-cells to express a tumor antigen specific TCR is one way to target a specific tumor antigen (chimeric antigen receptor-T-cells or car-T-cells). TCR engineered T-cell transfer using human TCRs targeting AFP is currently in clinical trial in several institutions, including our Center. Known toxicities to this approach include

side effects from lymphodepleting chemotherapy, cytokine release syndrome, and possible autoimmune toxicities. Car-T-cell treatment has been successful to treat various hematologic malignancies including lymphoid leukemia and acute myeloid leukemia.

Adding checkpoint inhibitor to sorafenib therapy is also reasonable. Sorafenib reduces hepatic infiltrated T regs by suppressing TGF- β signal.^[42] However, sorafenib has other effects which may inhibit the immune system. For example, sorafenib but not sunitinib, appears to have a detrimental effect on dendritic cell phenotype and can inhibit cytokine secretion, migration ability and T-cell stimulatory capacity, while not affecting the function and phenotype of T-cells.^[43] Sorafenib has been shown to inhibit JAK-STAT signal transduction in human immune cells.^[44] The immune effects of sorafenib were dose dependent. At pharmacologic doses of sorafenib, the drug decreased T effector cell activation by down regulating CD25 surface expression. At low doses, sorafenib produced T effector cell activation, with significant increase in T effector cell proliferation, IL2 secretion and up regulation of CD25 cell surface expression and could reduce T regulatory cell suppression. Thus, the dose of sorafenib used may be critical for the desired immune effects. Other actions of sorafenib on the immune system include: (1) inhibit NK function; (2) increase MDSC; and (3) upregulate PD-L1 expression. In mice bearing orthotopic HCC, sorafenib upregulated tumor-specific T effector cell function, while the proportion of PD-1 expressing CD8+ T-cells and regulatory T-cells were reduced.^[45] In addition, the function of T regulatory cells was inhibited. In another study mouse and human HCC tumor samples expressing low pERK showed intense inflammatory infiltrating cells and significant enrichment of CD8+ that expressed PD-1.^[46] Patients with pERK PD-1 positive tumors had worse prognosis than pERK PD-1 negative tumors. PD-1 immunotherapy could complement sorafenib by targeting tumor cells resistant to sorafenib.

Recently, PD-L1 expression in HCC was shown to be significantly associated with markers of tumor aggressiveness including high AFP serum levels, satellite nodules, macrovascular invasion, microvascular invasion, poor histologic differentiation progenitor subtype (cytokeratin 19 expression).^[47] High PD-L1 expression in immune cells in the tumor microenvironment also correlated with high serum AFP levels, macrovascular invasion, poor differentiation, high PD-1 expression and lymphoepithelioma subtype.

Targeting glypican-3 (GPC3) immunologically is a novel approach. GPC3 is a member of the glypican family of heparin sulfate proteoglycans on the cell

surface. This molecule is overexpressed in 80% of HCC and when present, carries a poor prognosis.^[48,49]

Not only is it a prognostic factor and important for cell proliferation by stimulating Wnt signaling, but it is a tumor specific and becomes an attractive target as a tumor antigen.^[50] Clinical trials have started with both peptide-based vaccine^[51] or anti-GPC3 antibodies.^[52,53]

The phase I trial of peptide-based vaccine showed the treatment was well tolerated and one partial response and several stable disease responses were observed.^[51]

There was a correlation between overall survival and GPC-3 specific CTL response. Two recent phase I studies of antibody therapy against glypican-3 were reported recently.^[52,53] In the first study, a recombinant, humanized monoclonal antibody against GPC3 was performed in advanced HCC.^[52] There were no dose-limiting toxicities (DLT) noted. The most common side effects include transfusion reactions (35%), fatigue and pyrexia and diarrhea. Stable disease was seen in several patients. A phase I study of this antibody in Japanese patients with advanced HCC was performed.^[53] The most common toxicity was lymphocytopenia, natural killer cell decrease, C-reactive protein increase and pyrexia. Infusion reactions were observed in 62% patients. No DLT or maximum tolerated dose (MTD) was noted. There were no partial or complete responses but stable disease was noted in several patients. A phase I trial of combination of anti GC33 antibody with sorafenib in 40 patients was reported.^[54] There were 3 DLTs seen (grade 3 hyponatremia, grade 3 hyponatremia and hypoglycemia and grade 3 ALT increase). One partial response and 6 stable diseases were reported. No MTD was obtained with combining the antibody to GC33 with sorafenib at a dose of 400 mg bid daily. A phase I trial of T-cell redirecting bispecific antibody against glypican-3 is another approach.^[55]

Another clinical trial for HCC starting at University of Miami is the study of a bifunctional fusion protein that will combine PD-L1 antibody with the soluble extracellular domain of TGF- β receptor type II as a TGF- β neutralizing "trap". This compound (developed by Merck) will target two major mechanisms of immunosuppression, PD-1/PD-L1 axis as described previously and TGF- β . TGF- β is known to have growth inhibitory effects on normal epithelial cells and can act as tumor suppressor in early stage cancer development. Later as the tumor advances, TGF- β loses its ability to suppress cancer and various cancers can actually produce this molecule which acts as a stimulatory molecule for cell growth and division. Then, TGF- β can downregulate the effector function of T cytotoxic cells and natural killer cells, while inducing differentiation of CD4+ T-cells to T reg cells. In mice,

the blocking of TGF- β signaling in T-cells or deletion of TGF- β from T-cells resulted in decreased PD-1 expression in tumor-infiltrating CD8+ T-cells.^[56] Further studies in mice model of HCC showed that TGF- β increased the PD-L1 expression in dendritic cells which led to T-cell apoptosis and increased number of CD25+, Foxp3+ T regulatory cells.^[56,57] Thus, there is a strong rationale to target two pathways of immunosuppression in HCC simultaneously.

In summary, immunotherapy represents a novel approach to treat advanced hepatocellular carcinoma. Immune cells have been found in HCC specimens that appear “fatigued” or suppressed. New checkpoint inhibitors and other agents may awaken these immune cells to attack the tumor. Early results from clinical trials suggest it is feasible to do so and encouraging antitumor responses were been observed. The challenge is to further define what patients may benefit from this approach, how to predict or reduce toxicities, particularly liver toxicity, and how best to combine this therapy with other known modalities of treatment.

Authors' contributions

Manuscript writing and editing: L.G. Feun
Manuscript review, participating in the phase II trial of Pembrolizumab in HCC, and performing the laboratory correlates: Y.Y. Li, M. Wangpaichitr, C.J. Wu, N. Savaraj

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Conflicts of interest

There are no conflicts of interest.

Patient consent

There is no patient involved.

Ethics approval

This review paper is waived for ethics approval.

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Attenuation of liver stiffness in sorafenib-treated patients with advanced hepatocellular carcinoma

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ABSTRACT

Aim: Sorafenib is a multi-tyrosine kinase inhibitor and the standard therapy for advanced hepatocellular carcinoma (HCC). This retrospective study aimed to observe the anti-fibrotic effect of sorafenib in patients with advanced HCC. **Methods:** Seventeen patients with advanced HCC were recruited. Shear wave velocity (SWV) using acoustic radiation force impulse elastography and non-invasive serum markers for liver fibrosis, such as the aspartate aminotransferase (AST) to alanine aminotransferase ratio (AAR), the AST to platelet ratio index, the fibrosis-4 index and the Lok index, were recorded at the beginning of sorafenib treatment and 3-6 months after sorafenib treatment in 2014-2015. **Results:** Nine (52.9%) patients achieved disease control status and 8 had progressive disease after a mean duration of 11.1 months with sorafenib treatment. The mean SWV decreased from 2.37 m/s at the beginning to 1.90 m/s after sorafenib treatment ($P < 0.01$). This trend was observed in patients with and without liver cirrhosis (from 2.49 to 2.06 m/s, $P = 0.06$, and from 2.32 to 1.69 m/s, $P < 0.05$, respectively). Among the non-invasive serum markers, no statistically significant differences were observed except for the AAR in the cirrhotic group. **Conclusion:** Sorafenib has potential antifibrotic effects in patients with advanced HCC.

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INTRODUCTION

Liver fibrosis is a wound-healing response and a common consequence of hepatic inflammation/injury caused by a variety of etiologies, such as infection, drugs, metabolic disorders or immune attack.^[1]

Platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) are important for the sustained activation and proliferation of hepatic stellate cells (HSCs), which are activated and transformed into myofibroblasts during liver injury.^[2,3] The treatment of liver fibrosis by curing/controlling underlying liver



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diseases or interfering with receptor/ligand interactions has been reported in clinical trials or observational studies.^[4]

The inhibition of tyrosine kinase receptors for proliferative cytokines, such as PDGF, VEGF and fibroblast growth factors (FGF), could reverse liver fibrosis. The binding of PDGF to PDGF receptor (PDGFR)- β activates Ras and sequentially propagates the stimulatory signal via the phosphorylation of the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) pathway,^[5] which regulates protein synthesis, transcription of profibrogenic genes, proliferation, cell cycle control and apoptosis in HSCs.^[6] The anti-fibrotic effect of imatinib, which occurs via the targeting of PDGF, has been observed in mouse and rat studies.^[7] Sorafenib is a multi-tyrosine kinase inhibitor that targets the receptor tyrosine kinases VEGF receptor (VEGFR) and PDGFR- β and inhibits the activation of Raf/ERK signaling pathways.^[8] Sorafenib is the standard therapy for the treatment of advanced hepatocellular carcinoma (HCC).^[9,10] Recent studies have shown that sorafenib can induce anti-fibrotic effects by reducing HSC proliferation and enhancing apoptosis.^[6,11] Sorafenib also attenuates liver fibrosis and injury through the up-regulation of signal transducer and activator of transcription 3 (STAT3) phosphorylation in hepatocytes or through STAT3 inhibition in HSCs.^[12,13]

Liver biopsy has been considered to be a “gold standard” for the assessment of liver fibrosis.^[14] However, a number of well-known characteristics, such as the associated risk of morbidity, including the risk of bleeding and perforation, inter-observer variability in the interpretation of biopsies, sampling variability in the context of accurate staging, monetary costs and the turnover time for results, limit the clinical application of liver biopsy.^[15] Non-invasive methods that use serum biologic markers or elastography via ultrasound and magnetic resonance imaging-based techniques have emerged recently for the indirect assessment of liver fibrosis. Acoustic radiation force impulse (ARFI) elastography is an ultrasound-based technique for quantifying the mechanical properties of tissue stiffness.^[16,17] ARFI has been utilized in comparison with various stages of liver fibrosis and shows good diagnostic accuracy in predicting hepatic fibrosis.^[18,19]

We conducted an observational case-series study to assess liver fibrosis/stiffness using ARFI elastography among sorafenib-treated patients with advanced HCC to explore anti-fibrotic effects and the correlation with non-invasive methods.

METHODS

Patients

Patients with HCC were treated for recurrence after resection or advanced HCC as stage C or stage III-IV according to the Barcelona Clinical Liver Cancer staging system or the 7th edition of American Joint Committee on Cancer/Union for International Cancer Control) staging system respectively from May 2014 to July 2015.^[20-22] A total of 17 consecutive patients with advanced HCC were recruited retrospectively for this observational study in the clinic of Chang Gung Memorial Hospital (CGMH) fourteen patients had previously undergone surgical resection and tumor recurrence developed in the follow-up period. Sorafenib was administered as salvage treatment. The remaining three patients were unresectable with typical imaging findings. The status of advanced HCC included major portal vein thrombosis ($n = 5$) and distant metastasis ($n = 12$, 5 in the lung, 2 in the bone, 2 in the peritoneum, 1 in the bone and peritoneum, 1 in a lymph node and 1 in the adrenal gland). The treatment of HCC was based on clinical practice guidelines,^[20,21] and all patients were under the care of the liver cancer team of the Linkou branch of CGMH. The daily oral dosage of sorafenib was administered and adjusted with toxicity evaluation and without drug interruption in the observation period. The dosage was deescalated with toxicity intolerance from 800 mg to 400 mg.

This study was approved by the Institutional Review Board (IRB) of CGMH, Linkou branch (IRB No. 103-1747B). All methods of data collection were performed in accordance with the relevant guidelines and regulations of IRB in CGMH.

ARFI elastography measurements

The ARFI elastography examinations were performed with an Acuson S2000 ultrasound (Siemens Medical Solutions, Mountain View, California, USA) with ARFI technology equipment, a curvilinear array transducer operating at 4 MHz (4C1) and the virtual touch tissue quantification system every 3 months. With the liver parenchyma free of visible hepatic tumors, blood vessels and bile ducts, as confirmed by conventional ultrasonic images, 10 valid measurements of shear wave velocity (SWV, m/s) were made by a single experienced examiner (Chen YC) with the patients holding their breath for a few seconds. The results of ARFI elastography were expressed as the median of the 10 SWV measurements in the liver parenchyma. The SWV measurements in this study were validated using the ratio of the interquartile range (IQR) to the median value, which is currently used to assess the

validity of transient elastography. An IQR/median ratio of less than 0.3 is considered to indicate a homogeneous set of measurements.^[23,24]

Non-invasive serologic indexes: AAR, APRI, FIB-4 index, and Lok index

The non-invasive serologic index values at the beginning of sorafenib treatment and 3 months post-treatment were compared. The aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio (AAR), the AST to platelet ratio index (APRI), the fibrosis-4 index (FIB-4), and the Lok index for the non-invasive assessment of liver fibrosis were examined at 3 months interval.^[25-28] The variables of AST, ALT, international normalized ratio and platelets were recorded at the time of ARFI elastography.

Treatment response of HCC

The treatment response of HCC to sorafenib was assessed based on the modified response evaluation criteria in solid tumors every 3-6 months after sorafenib administration and the treatment protocol was continued if the treatment response was disease control, including complete response (CR), partial response (PR), and stable disease (SD).^[29] Four types of response are defined as: (1) CR, which indicates the disappearance of any intratumoral arterial enhancement in all target lesions; (2) PR, which indicates a decrease of at least 30% in the sum of the diameters of viable (enhancement in the arterial phase) target lesions, taking as a reference the baseline sum of the diameters of the target lesions; (3) SD, which includes any cases that do not qualify for either PR or progressive disease (PD); and (4) PD, which indicates an increase of at least 20% in the sum of the diameters of viable (enhancing) target lesions, taking as reference the smallest sum of the diameters of viable (enhancing) target lesions recorded since treatment started. All patients have regular assessment every 2 or 3 months as 1 cycle and sorafenib administration was discontinued if the treatment response of PD was identified by liver CT assessment or if the patient's clinical condition deteriorated. All patients survived in the observation period; 1 lost to follow-up in 1 year; and the others were disease free or shifted to second line treatment. We used Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 for adverse events severity evaluation and there was no grade IV adverse event in this study.

Statistical analysis

Clinical data were evaluated with descriptive statistics. The SWV measurements of the liver parenchyma

were expressed as the median for each patient. For the overall values, the values of SWV measurements were expressed as the mean \pm standard deviation. A paired *t*-test was performed for a paired comparison of variables before and 3-6 months after treatment with sorafenib, including SWV measurements and non-invasive serologic indices. A *P* value < 0.05 was considered to indicate a significant difference. Statistical analysis was performed using the Statistical Package for the Social Sciences (version 19.0, IBM SPSS Statistics, New York, USA).

RESULTS

The baseline clinical characteristics of the 17 patients with advanced HCC are shown in Table 1. The mean age was 59.7 ± 10.2 years, and 14 (82.4%) patients were males. Ten (58.8%) patients had chronic hepatitis B infection, 5 (29.4%) patients had chronic hepatitis C infection and 10 (58.8%) patients had liver cirrhosis. The mean duration of sorafenib treatment was 11.1 months, and the mean survival time was 15.1 months (range: 8.3-19.1 months; 95% confidence interval: 13.3-17.0 months) [Table 1].

The clinical characteristics of the case series are shown in Table 2. Two patients achieved CR, 1 patient achieved PR, 6 patients experienced SD and 8 patients experienced PD after a mean of 2.5 cycles of sorafenib treatment (range 1-4 cycles). The disease control rate, including CR, PR, and SD in the first 2 cycles was 52.9%. A 64-year-old male patient (No. 11) suffered from recurrence of mesentery HCC after the initial surgical resection and achieved a CR after 4 months of sorafenib treatment. A 34-year-old female patient (No. 12) experienced lung metastases 6 weeks after partial hepatectomy. She had a complete

Table 1: Baseline clinical characteristics

Characteristics	Data
Patient No.	17
Age, years	59.7 ± 10.2
Male, %	14 (82.4)
Liver cirrhosis, %	10 (58.8)
HBV/HCV/none	10/5/2
Albumin, g/dL	4.1 ± 0.4
AST, U/L	56.2 ± 37.3
ALT, U/L	44.6 ± 24.5
Bilirubin, mg/dL	0.8 ± 0.3
INR	1.13 ± 0.08
Leukocytes, $10^3/L$	$6,288.0 \pm 2,198.0$
Hemoglobin, mg/dL	13.5 ± 1.7
Platelets, $10^9/L$	171.5 ± 57.2
Duration of sorafenib, months	8.5 ± 3.9
Survival time, months	11.1 ± 4.0

HBV: hepatitis B virus; HCV: hepatitis C virus; AST: aspartate transaminase; ALT: alanine transaminase; INR: international normalized ratio

Table 2: The clinical characteristics of 17 advanced HCC patients with sorafenib treatment

No.	Age, years	Gender	Personal history	HBV	HCV	Cirrhosis	Distant metastasis	ARFI elastography, median, m/s		Dosage (mg)	Staging	Outcome	Treatment cycles	Survival time (months)
								Before	After					
1	59	Male	Nil	+	-	Yes	Lung	3.45	2.75	400	IV	PD	1	14.6
2	67	Male	Nil	-	+	No	Lung	2.38	3.44	400	IV	SD→PD	2	17.6
3	75	Male	Peptic ulcer, chronic lung disease	+	-	Yes	Bone	2.98	1.88	400	IV	PD	2	14.3
4	68	Male	DM, HTN	-	-	No	Bone, peritoneum	2.99	1.23	400	IV	PD	2	10.0
5	50	Male	Nil	+	-	No	Lung	2.86	1.78	600	IV	PD	3	17.2
6	61	Female	HTN	+	-	Yes	Lung	1.00	1.06	400	IV	PD	2	11.2
7	61	Male	DM, HTN	+	-	Yes	PVT	2.68	2.69	600	III	PD	3	9.0
8	52	Male	Nil	-	+	No	PVT	2.42	1.93	600	III	SD	3	13.1
9	68	Female	Nil	-	+	Yes	PVT	1.29	2.03	400	III	SD	4	19.1
10	54	Male	Nil	+	-	Yes	PVT	3.54	3.64	600	III	PD	3	13.1
11	64	Male	Peptic ulcer, HTN	+	-	Yes	Peritoneum	2.65	1.42	600	IV	CR*	3	12.3
12	34	Female	Nil	-	-	No	Lung	1.09	0.90	800	IV	CR*	4	13.6
13	56	Male	Peptic ulcer, HTN	+	-	No	Lymph node	1.29	1.42	600	IV	SD	3	11.6
14	70	Male	DM, HTN	+	-	Yes	Peritoneum	1.35	1.69	600	III	SD	2	9.9
15	46	Male	HTN	+	-	No	Bone	1.37	1.70	600	IV	SD	2	9.5
16	69	Male	DM, HTN, chronic lung disease	-	+	Yes	Adrenal gland	2.37	1.71	600	IV	SD	2	8.3
17	61	Male	Nil	-	+	Yes	PVT	2.68	1.71	600	III	PR	2	8.9

*One case with post-operative mesentery recurrence had another surgery for resection after 4 months of sorafenib treatment. The other case experienced multiple lung metastases after partial hepatectomy and had a complete pathologic response for lung metastases after sorafenib treatment. These pulmonary lesions enlarged initially and regressed thereafter. HBV: hepatitis B virus; HCV: hepatitis C virus; ARFI: acoustic radiation force impulse; DM: diabetes mellitus; HTN: hypertension; PVT: partial response; PD: progressive disease; SD: stable disease; CR: complete response; PR: partial response

pathological response after 9 months of sorafenib treatment. Both patients also had decreased SWV (liver stiffness) during sorafenib treatment, as indicated by ARFI elastography [Table 2]. Of the 9 patients with decreased liver stiffness, all of the reductions of SWV by ARFI elastography were > 10% from baseline, whereas there was no statistical difference in the change in SWV after sorafenib treatment between patients with and without a treatment response (decreased SWV in 5 and 4 patients with and without a treatment response, respectively, $P = 1.000$).

The paired comparison of SWV, the AAR, the APRI, the FIB-4, and the Lok index between the beginning of sorafenib treatment and the end of treatment with sorafenib is shown in Table 3. The mean SWV was 2.37 ± 0.83 m/s at the beginning of sorafenib treatment, which decreased to 1.90 ± 0.64 m/s 3 months after sorafenib treatment ($P < 0.01$). However, there were no statistically significant differences in the non-invasive serum markers of AAR (1.39 vs. 1.15, $P = 0.05$), APRI (1.14 vs. 1.31, $P = 0.52$), FIB-4 (3.50 vs. 3.65, $P = 0.77$), and the Lok index (0.63 vs. 0.41, $P = 0.30$) between the beginning of sorafenib treatment and the end of treatment [Table 3]. The decline of the mean SWV was also significant (2.32 vs. 1.69 m/s, $P < 0.05$), whereas the differences in the AAR, APRI, FIB-4 and the Lok index were not significant in the

7 patients without cirrhosis. Among the 10 patients with cirrhosis, the mean AAR decreased significantly after sorafenib treatment (1.61 vs. 1.19, $P = 0.04$). The observed differences in the mean SWV by ARFI elastography, the APRI, the FIB-4 and the Lok index were not statistically significant.

DISCUSSION

To our knowledge, this investigation is the first study to evaluate the anti-fibrotic effect of sorafenib based on changes in liver parenchymal stiffness using ARFI elastography. The results of the present study showed significantly reduced stiffness of the liver parenchyma based on the SWV after short-term sorafenib treatment (reduction from 2.42 to 1.91 m/s in 3-6 months, $P < 0.01$), and this trend was observed in both cirrhotic and non-cirrhotic patients [Table 3].

In addition to its clinical application in advanced HCC treatment due to its ability to inhibit tumor-cell proliferation and tumor angiogenesis,^[9,10] sorafenib has been demonstrated to have anti-fibrotic effects *in vivo* and *in vitro*.^[6,11,13] These anti-fibrotic effects have been reported to occur through the inhibition of the Raf/ERK signaling pathway, which reduces HSC proliferation and enhances apoptosis.^[6,8,11,13] As observed in the present study, the decline of the

Table 3: Comparison of SWV, AAR, APRI, FIB-4 and the Lok index at the beginning of sorafenib treatment and 3 to 6 months after sorafenib treatment

Group	Overall (n = 17)			Cirrhosis (n = 10)			Non-cirrhosis (n = 7)		
	Beginning	After	P	Beginning	After	P	Beginning	After	P
SWV	2.42 ± 0.78	1.91 ± 0.64	< 0.01	2.49 ± 0.76	2.06 ± 0.73	0.06	2.32 ± 0.87	1.69 ± 0.45	< 0.05
AAR	1.39 ± 0.64	1.15 ± 0.36	0.05	1.61 ± 0.73	1.19 ± 0.39	0.04	1.10 ± 0.34	1.10 ± 0.36	0.96
APRI	1.14 ± 1.05	1.31 ± 0.51	0.52	1.07 ± 1.02	1.48 ± 0.53	0.25	1.24 ± 1.16	1.08 ± 0.39	0.70
FIB-4	3.50 ± 2.45	3.65 ± 1.28	0.77	3.89 ± 2.29	4.38 ± 0.86	0.52	2.94 ± 2.74	2.62 ± 1.05	0.69
Lok	0.63 ± 1.27	0.41 ± 0.77	0.30	1.16 ± 1.31	0.67 ± 0.65	0.49	-0.13 ± 0.78	0.05 ± 0.83	0.38

SWV: shear wave velocity, m/sec; AAR: aspartate/alanine aminotransferase ratio; APRI: aspartate aminotransferase-platelet ratio index; FIB-4: fibrosis-4 index; Lok: Lok index

mean SWV was suggestive of the attenuation of liver parenchymal stiffness after sorafenib treatment.

Although liver biopsy is a well-known method for assessing liver fibrosis, it is not possible to perform repeated liver biopsy for the assessment of liver fibrosis in patients with advanced HCC because of possible complications and ethical issues. The development of non-invasive methods based on serum markers offers an alternative approach for clinical practice. These markers are classified as direct markers that reflect the pathophysiology of liver fibrogenesis and represent components of the extracellular matrix; indirect markers use routine laboratory data and reflect the consequences of liver damage.^[15,25] However, liver biochemistry and platelet counts could change over time in patients with deteriorating advanced HCC, and non-invasive serum markers, such as the AAR, the APRI, the FIB-4 or the Lok index, may be inadequate for assessing liver fibrosis in such patients.

With the advantage of combination with conventional B-mode ultrasound, ARFI technology can be easily used for the evaluation of liver parenchyma free of hepatic tumors, blood vessels and bile ducts, which cannot be achieved by transient elastography (Fibroscan®). Therefore, ARFI elastography may be the better choice among non-invasive methods for evaluating the severity and serial changes of liver fibrosis during sorafenib treatment.

There are some limitations in the present study. First, this study is a case-series study, and the number of patients is small. However, the stiffness of the liver parenchyma was observed to decrease, as indicated by a reduced SWV, after sorafenib treatment. Furthermore, the results of the present study have provided a basis for future prospective large-scale studies that test the anti-fibrotic efficacy of sorafenib in the liver parenchyma. Second, the follow-up duration may be too short to see long-term changes in SWV or the stiffness of the liver parenchyma during sorafenib treatment. Third, no control group was included for

comparison. Nevertheless, this study can be viewed as a pilot study to explore the anti-fibrotic effect of sorafenib in patients with advanced HCC.

In conclusion, sorafenib has potential anti-fibrotic effects and efficacy in patients with advanced HCC. Large-scale, long-term, and randomized control studies are needed to confirm the results of this study.

Authors' contributions

Study design and ARFI evaluation: M.C. Yu, Y.C. Chen
Data collection and manuscript writing: C.F. Hung, D. Liu
Clinical practice for HCC patients as the guideline at this institute: T.H. Wu, C.W. Lee, K.T. Pan, C.T. Wang, H.Y. Chai

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Each patient was informed of the study and gave their consent.

Ethics approval

This study was approved by the Institutional Review Board (IRB) of CGMH, Linkou branch (IRB No. 103-1747B).

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Late recurrence of hepatocellular carcinoma after liver transplantation

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ABSTRACT

Aim: Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths worldwide and liver transplant (LT) prolongs survival. However, 15-20% will experience recurrent HCC, most occurring within 2 years of LT. HCC patients with late recurrences (> 5 years after LT) may have distinctive clinical/biological characteristics. **Methods:** A retrospective review was conducted of 88 patients who underwent LT for HCC during 1993-2015, analyzing demographics, clinical factors, explant pathology, and outcome. **Results:** Median follow-up was 6.4 years. HCC recurred in 15 (17.0%) patients with mean time to recurrence of 3.96 ± 3.99 years. Five patients reoccurred > 5 years post-LT. All late recurrences involved males in their 50s, recurring at 8.5 years on average. Recurrences occurred in chest wall (2), liver (2), lung (2), bone (1) and pelvis (1), with multifocal involvement in 2 patients. Four patients died within 18 months of late recurrence. The fifth patient is alive after ablation of liver recurrence and treatment with sorafenib and everolimus. **Conclusion:** One-third of post-LT patients with recurrent HCC experienced late recurrence. Although the sample size makes it difficult to identify significant risk factors, this study highlights the importance of long-term follow-up and need for biomarkers to identify patients at risk for late recurrences.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer with 782,000 new cases and 745,000 deaths annually worldwide.^[1] The best treatments for HCC include liver resection and liver transplantation (LT). However, most patients present at advanced stage and are not candidates for these potentially curative therapies. LT, although limited by the shortage of donor livers, has superior disease-free survival, with improved 5-year survival of 70%

compared to 10% in untreated HCC.^[2] Despite receiving optimal therapy with transplantation, up to 20% of patients may experience recurrent HCC. Most of these recurrences occur within 2 years following transplantation. Although there are no clear guidelines on how to treat these recurrences, surgical resection is the preferred treatment option. Other locoregional therapies such as radiofrequency ablation (RFA) and transarterial chemoembolization (TACE) may be options, and sorafenib can be considered for more diffuse, unresectable disease.^[3,4]



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Unfortunately there is little evidence of the survival benefits following treatment for recurrent HCC following transplant. In addition, few studies have examined risk factors in recurrent HCC after transplant or prognostic factors for survival after recurrence. Moreover, while tumor recurrence tends to happen within the first two years following transplantation, late recurrence can occur and the pathobiology underlying these cases is not well understood. This study aims to identify and characterize cases of late recurrent HCC after transplantation in Hawaii, a state with a high burden of liver disease and cancer due to a large population of Asians and Pacific Islanders with viral hepatitis.^[5-7]

METHODS

This is a retrospective analysis of 88 patients who underwent LT for HCC from 1993 to 2015. All patients were referred to a group of physicians associated with the medical center with the only LT program in the state of Hawaii. It is also the primary referral center for hepatobiliary surgery for American Samoa, Guam, Saipan, and the Marshall Islands. This clinic and the transplant center were initially affiliated with Hawaii Medical Center-East (formerly St. Francis Medical Center) and after 2012, the Queens Medical Center. This center sees about 60-70% of the HCC cases in Hawaii. This study was approved by the University of Hawaii Institutional Review Board.

HCC was diagnosed histologically by percutaneous biopsy or at surgery. The diagnosis of HCC was made with only imaging if a contrast-enhanced study [dynamic computed tomography (CT) or magnetic resonance imaging (MRI)] showed typical arterial enhancement with “washout” in the venous phase as described by the American Association for the Study of Liver Disease guidelines.^[8] All patients received transplant livers from deceased donors. For immunosuppression after LT, patients all received basiliximab for induction, steroids, tacrolimus, and mycophenolate mofetil. All patients were weaned off of steroids after 6 months and maintenance immunosuppression was continued with tacrolimus or tacrolimus/mycophenolate.

Information on demographics, medical history, laboratory results, tumor characteristics, treatment, and survival was collected via clinical interviews. Demographic data included age, gender, birthplace, and the patient's self-reported ethnicity. Data collected on medical history included diabetes mellitus, hyperlipidemia, smoking, and risk factors for HCC including viral hepatitis, alcohol abuse (defined

as greater than 2 alcoholic beverages daily for at least 10 years), and other chronic liver diseases. Information was based on available medical records and interviews by a single physician.

Laboratory data collected included serum bilirubin, albumin, prothrombin time, creatinine, alanine aminotransferase, aspartate aminotransferase, platelet count and alpha-fetoprotein (AFP). Laboratory data that was used for the study had been obtained within 2 weeks of initial visit or drawn at the time of the visit. Serum bilirubin, prothrombin time with international normalized ratio (INR) and creatinine were used to calculate the Model for End-stage Liver Disease (MELD) score. Dynamic imaging with CT or MRI was performed to determine if Milan criteria were met (single tumor ≤ 5 cm or up to 3 tumors ≤ 3 cm each, no vascular invasion, no extrahepatic spread). Patients who met Milan criteria initially or who could be downstaged with locoregional therapy to meet Milan criteria were considered for liver transplantation.

We also noted the type of locoregional therapy that was performed before LT including RFA and TACE. Pathology reports were also examined to determine the size and number of HCC lesions present, the amount of tumor necrosis, the location of tumors, and presence of vascular invasion.

Data analysis was performed using Microsoft Excel and Statistical Package for the Social Sciences software to identify potential predictors for recurrent HCC. Fisher's and chi-square analysis was performed and *P* values < 0.05 were considered significant.

Five patients were identified as having a “late” recurrence, defined as the diagnosis of HCC occurring more than 5 years after the date of LT. Late recurrence cases were examined in detail for post-LT course, use of immunosuppression, site of recurrence, treatment for recurrence, and response to treatment.

RESULTS

Of 1,200 patients in our database of patients treated for HCC, 88 underwent LT for HCC and had the following characteristics were shown in **Table 1**: mean age 56.6 years, 83% male, 54.5% Asian, 10.2% Pacific Islanders, 58% hepatitis B positive, 61.4% hepatitis C positive, 30.7% with diabetes, and 46.6% with normal AFP (< 20). Locoregional therapy was performed in 67 patients (76.1%) with 26 receiving only RFA and 17 received only TACE. Cases with single tumors less than 2.5 cm in easily accessible locations were chosen for RFA. Cases with larger

Table 1: Baseline characteristics of patients in study (n = 88)

Characteristics	Data, n (%)
Age, years, mean ± SD (range)	56.6 ± 6.1 (41-72)
Males	73 (83.0)
Ethnicity	
Asian	48 (54.5)
Pacific Islanders	9 (10.2)
Black	1 (1.1)
Hispanic	2 (2.3)
White	26 (29.5)
Mixed	2 (2.3)
Hepatitis B	51 (58.0)
Hepatitis C	54 (61.4)
Diabetes	27 (30.7)
AFP, mean ± SD	669.7 ± 3,739.6
Patients receiving locoregional therapy	67 (76.1)
RFA only	26 (29.5)
TACE only	17 (19.3)

AFP: alpha-fetoprotein; RFA: radiofrequency ablation; TACE: transarterial chemoembolization

tumors and multiple tumors were treated with TACE. Median duration of follow-up was 6.4 years (mean 6.8 years, range 8 days-17.2 years).

Univariate analysis suggested the presence of microvascular invasion as seen on pathology and size of the largest tumor in the explant to be predictors of recurrence of HCC after transplant. Other factors including age, gender, race, presence of hepatitis B or C, diabetes, AFP level, locoregional treatment, and presence of 4 or more tumors in the explanted liver

did not predict recurrence [Table 2].

Recurrent HCC occurred in 15 cases (17.0%) with mean time to recurrence of 3.96 ± 3.99 years. Seven patients recurred within 2 years and 5 recurred > 5 years post-LT. All late recurrences involved males in their 50s, recurring at a mean 8.5 years (range 5.2-13.4 years). Explanted livers showed 1 with vascular invasion, 2 with > 4 tumors and 2 with single tumors. Recurrences occurred in chest wall (2), liver (2), lung (2), bone (1) and pelvis (1), with 2 patients having recurrent tumors in multiple sites. Four patients died within 18 months of late recurrence. The fifth patient is alive for 3 years after ablation of liver recurrence and treatment with sorafenib and everolimus. Table 3 lists details of the late recurrence cases and Table 4 shows a comparison of early and late recurrence cases. Clinical summaries of the 5 cases of late recurrence are reported below.

Case 1

The first case is a 53-year-old Chinese male with hepatitis B cirrhosis and 2 liver masses (3.4 and 2.1 cm). AFP was 2,397 ng/mL and liver biopsy showed a well-differentiated HCC. He underwent RFA followed by TACE and LT 1 month later. The explanted liver showed multifocal HCC without microvascular invasion including 4.5 cm, 3.0 cm and 1.8 cm masses with 95-100% necrosis, and a 1.8 cm caudate lobe mass with 10% necrosis.

Table 2: Characteristics of patients with recurrence vs. without recurrence, n (%)

Characteristics	Recurrence (n = 15)	No recurrence (n = 73)	P value
Age, years, mean ± SD	58.1 ± 3.1	56.3 ± 6.5	0.30
Males	11 (73.3)	62 (85.0)	0.28
Asians	10 (66.7)	38 (52.1)	0.40
Hepatitis B	8 (53.3)	21 (28.8)	0.08
Hepatitis C	7 (46.7)	47 (64.4)	0.25
Diabetes	6 (40.0)	21 (28.8)	0.54
Imaging: largest tumor size, cm, mean ± SD	3.1 ± 1.2	2.9 ± 1.3	0.60
Explant: largest tumor size, cm, mean ± SD	3.9 ± 2.0	2.4 ± 1.2	0.001
Explant: largest tumor > 3 cm	6/11* (54.5)	19/66* (28.8)	0.16
Imaging: no. of tumors, mean ± SD	1.2 ± 0.6	1.3 ± 0.6	0.60
Explant: no. of tumors, mean ± SD	3.1 ± 3.6	2.0 ± 1.9	0.13
Explant: > 4 tumors	5/13* (38.5)	10/67* (15.0)	0.06
Well differentiated tumor	2/13* (15.4)	15/58* (25.9)	0.72
Met milan criteria	13 (86.7)	57/72* (79.2)	0.73
AFP at diagnosis, ng/mL, mean ± SD	829.9 ± 2,401.1	637.7 ± 3,966.1	0.86
AFP > 500 ng/mL	2/14	7/70	0.64
AFP > 1,000 ng/mL	2/14	5/70	0.33
Lab MELD score, mean ± SD	11 ± 3.9	12 ± 3.8	0.74
Received locoregional therapy	12 (80.0)	55 (75.3)	1.00
Waiting time (from diagnosis to LT), days, mean ± SD	193.4 ± 163.9	315.8 ± 367.3	0.21
Explant liver met Milan criteria	7/13* (53.8)	47/67* (70.1)	0.33
Explant liver with microvascular invasion	5/13* (38.5)	1/59* (1.7)	0.001

*Data not available for all patients. No. of cases for which data was available is indicated. AFP: alpha-fetoprotein; MELD: Model for End-stage Liver Disease; LT: liver transplantation

Table 3: Late recurrence cases (more than 5 years after LT)

Age/ gender	Time to recurrence (years)	Site of recurrence	Preop biopsy	AFP pre- LT (ng/mL)	ESLD	Size of largest tumor on imaging (cm)	Explant vascular invasion	Explant with 4+ tumors	Treatment of recurrence	Status after recurrence
53/M	11.3	Chest wall, liver, lung	Yes	2,387	HBV	3.4	No	Yes	Resect chest wall mass, left liver, everolimus	Deceased, 8 months
57/M	5.9	Chest wall	Yes	34	HCV	2.0	No	No	Resect chest wall, radiation	Deceased, 12 months
59/M	5.2	Pelvic mass, bone	Yes	7	NASH	4.0	Yes	No	Resect pelvic mass, sorafenib	Deceased, 18 months
58/M	6.6	Liver	Yes	46	HCV	2.3	No	No	RFA, everolimus, sorafenib	Living, 44 months
59/M	13.4	Lung	No	10.2	HBV	2.2	No	Yes	None	Deceased, 2 months

AFP: alpha-fetoprotein; LT: liver transplantation; ESLD: end-stage liver disease; HBV: hepatitis B virus; HCV: hepatitis C virus; NASH: non-alcoholic steatohepatitis; RFA: radiofrequency ablation

Table 4: Characteristics of patients with early recurrence vs. late recurrence on initial presentation, *n* (%)

Characteristics	Early recurrence (<i>n</i> = 10)	Late recurrence (<i>n</i> = 5)	<i>P</i> value
Age, years, mean ± SD	58.7 ± 3.4	57 ± 2.5	0.34
Males	6 (60.0)	5 (100.0)	0.23
Asians	7 (70.0)	3 (60.0)	1.00
Hepatitis B	6 (60.0)	2 (40.0)	0.61
Hepatitis C	5 (50.0)	2 (40.0)	1.00
Diabetes	4 (40.0)	2 (40.0)	1.00
Imaging: largest tumor size, cm, mean ± SD	3.3 ± 1.3	2.8 ± 0.9	0.48
Explant: largest tumor size, cm, mean ± SD	4.7 ± 2.0	2.3 ± 1.0	0.05
Explant: largest tumor > 3 cm	5/8* (62.5)	1/4* (25.0)	0.55
Imaging: no. of tumors, mean ± SD	1.2 ± 0.6	1.2 ± 0.5	1.00
Explant: no. of tumors, mean ± SD	3.3 ± 4.3	2.8 ± 2.9	0.82
Explant: > 4 tumors	3/8* (37.5)	2 (40.0)	1.00
Well differentiated tumor	0/8* (0)	2 (40.0)	0.13
Met milan criteria	9 (90.0)	4 (80.0)	1.00
AFP at diagnosis, ng/mL, mean ± SD	1,014.9 ± 2,950.0	496.8 ± 1,056.8	0.72
AFP > 500 ng/mL	1/9*	1	1.00
AFP > 1,000 ng/mL	1/9*	1	1.00
Lab MELD score, mean ± SD	12 ± 4.2	11 ± 3.5	0.71
Received locoregional therapy	9 (90.0)	3 (60.0)	0.24
Waiting time (from diagnosis to LT), days, mean ± SD	222.6 ± 185.2	135.0 ± 102.0	0.35
Explanted liver met Milan criteria	3/8* (37.5)	4 (80.0)	0.27
Explanted liver with microvascular invasion	4/8* (50.0)	1 (20.0)	0.56

*Data not available for all patients. No. of cases for which data was available is indicated. AFP: alpha-fetoprotein; MELD: Model for End-stage Liver Disease; LT: liver transplantation

Immunosuppression consisted of steroids, mycophenolate, and tacrolimus with eventual wean to tacrolimus monotherapy. Hepatitis B was controlled with monthly hepatitis B immune globulin injections and lamivudine, but he was eventually switched to adefovir and then tenofovir. Seven years post-LT, he was found to have a hepatitis B surface Ag escape mutant.

Eleven years post-LT, the patient noted a prominent xiphoid process. CT scan showed an 8.9 cm mass involving the left lobe of the liver, xiphoid and anterior chest wall. AFP was 60,000 ng/mL and bone scan was negative. He underwent *en-bloc* resection of recurrent HCC with partial resection of the diaphragm,

pericardium, pleural and sternum, and left lateral segment of liver. After surgery, he was given sorafenib and immunosuppression was changed to very low dose tacrolimus and everolimus 0.5 mcg twice daily. His AFP reached a nadir of 1,097 ng/mL, but then increased to 60,000 ng/mL. He eventually developed lung metastases and died 8 months after the surgery.

Case 2

This patient was a 57-year-old Caucasian male who presented with decompensated hepatitis C cirrhosis and a 2.3 cm well-differentiated HCC. He underwent RFA via an intercostal approach at the right upper abdomen/chest wall. Seven months after the biopsy,

the patient underwent LT. Explanted liver demonstrated 2 well differentiated HCC in the right lobe (1.5 cm and 1.0 cm) with 60% necrosis and 3 non-necrotic satellite nodules measuring 0.2 to 0.3 cm. No microvascular invasion was noted.

Four years after LT, the patient was found to have 1.1 cm solid nodule in the right chest wall at the 8th rib. Needle biopsy showed necrosis and fibroinflammatory tissue reaction with a focus of metastatic HCC. Complete wide excision of this mass showed no additional HCC. Long-term immunosuppression consisted of low dose tacrolimus.

Six years post-LT, he developed another 2.4 cm soft tissue mass in the right lateral chest wall. This was thought to be a needle tract seeding of tumor related to a previous biopsy and RFA. Wide surgical resection was performed and revealed metastatic HCC with necrosis.

Seven years post-LT, he developed a persistent cough and CT scan showed a 1.8 cm mass in the left lower lung and AFP was 3 ng/mL. He underwent a left thoracotomy and wedge excision of a 1.7 cm moderately differentiated squamous cell lung cancer (node negative). No additional therapy was given for his lung cancer.

He was disease free from both lung cancer and HCC up until 9 years post-LT when he began to complain of right rib pain. AFP was 140 ng/mL. CT scan showed a multilobulated mass in the right chest wall involving the 8th and 9th ribs and adjacent diaphragm, which was separate from the liver. He underwent radiation and refused sorafenib. He eventually expired from this 1 year later.

Case 3

The third case is a 59-year-old Japanese male with non-alcoholic steatohepatitis with variceal bleeding episode. He was found to have a 3 cm liver mass and biopsy showed poorly differentiated HCC. Within 4 months of diagnosis, he received LT. His explanted liver showed a 3.8 cm moderately differentiated HCC with lymphovascular invasion. Immunosuppression consisted of basiliximab, steroids, mycophenolate mofetil and tacrolimus. Maintenance immunosuppression was with low dose tacrolimus.

Five years after transplant, a routine AFP was noted to be 70 ng/mL. His AFP continued to increase but multiple imaging tests were negative. A few months later, repeat CT scan showed a 3 cm mass in the pelvis between the internal and external iliac arteries.

He underwent surgical resection and pathology showed a 5.3 cm HCC.

Post-operatively, his immunosuppression was changed to very low dose tacrolimus and sirolimus. Sorafenib was also added. His AFP continued to increase and he also developed skeletal metastases. He expired 18 months after recurrence of HCC.

Case 4

The next case is a 66-year-old Puerto-Rican male with hepatitis C cirrhosis and a 2.3 cm mass adjacent to the inferior vena cava. AFP was 46 ng/mL. Liver biopsy demonstrated HCC and he underwent TACE followed by LT 4 months later. The explanted liver showed a 2.0 cm moderately differentiated HCC with 20% necrosis and no vascular invasion. Immunosuppression consisted of basiliximab, steroids, mycophenolate, and tacrolimus; he was gradually weaned to tacrolimus monotherapy.

Six years after transplant, AFP was noted to be 216 ng/mL. CT scan showed a nonspecific 1.0 cm hypovascular lesion in the left lobe which increased to 2.2 cm on subsequent imaging. Immunosuppression was changed to very low dose tacrolimus and everolimus 0.5 mg bid. Sorafenib was also added. He underwent RFA and subsequent CT scan showed no new lesions, but AFP increased to 10,385 ng/mL 1 month later. MRI scan showed a suspicious 5.4 cm mass in the left lobe. Stereotactic body radiation (SBRT) was planned and AFP decreased to 8,243 ng/mL. When he arrived for SBRT simulation, the lesion could not be found. AFP decreased to 2.1 ng/mL. CT scan now showed no liver lesion and resolution of the previously seen liver mass. All subsequent AFP tests have been normal. His hepatitis C was successfully treated with sofosbuvir and simeprvir. He is currently on everolimus and sorafenib and has no evidence of liver disease on imaging 44 months after diagnosis of recurrent HCC.

Case 5

The final case is a 59-year-old Korean male with end stage liver disease due to hepatitis B. During the LT evaluation, he was found to have a 2.2 cm hypervascular mass. AFP was 10.2 ng/mL. He underwent LT without any locoregional therapy preoperatively. The explanted liver showed a multifocal HCC with at least 7 lesions. Immunosuppression consisted of tacrolimus and steroids.

Post-LT, he had no episodes of rejection, infection, or liver dysfunction. His hepatitis B was well-controlled with lamivudine and hepatitis B immune

Table 5: Review of literature on the incidence of recurrence of HCC after LT

Authors	Year	Area	No. of LT patients	Recurrence rate	Median follow-up	Mean follow-up
Roayaie <i>et al.</i> ^[4]	2004	USA (NY)	311	18.3%	51.9 months	
Hwang <i>et al.</i> ^[9]	2011	Korea	87	1.3%	75 months	
Lee <i>et al.</i> ^[10]	2014	Korea	69	44.9%	24.5 months	
Schraiber <i>et al.</i> ^[11]	2016	Brazil	206	15.5%	43.6 months	49.8 months
Hanounieh <i>et al.</i> ^[12]	2011	USA (OH)	92	13.0%	19.5 months	
Andreou <i>et al.</i> ^[13]	2016	Germany	364	25.0%	78 months	
Kondili <i>et al.</i> ^[14]	2007	UK	104	11.5%	36 months	47 months
Escartin <i>et al.</i> ^[15]	2007	Spain	184	15.2%		
Varona <i>et al.</i> ^[16]	2015	Spain	109	7.0%	42 months	
Lai <i>et al.</i> ^[19]	2013	Italy, Brussels	422	14.5%	4.9 years	
Agopian <i>et al.</i> ^[20]	2015	USA (CA)	865	13.5%	29.7 months	
Parfitt <i>et al.</i> ^[21]	2007	Spain	75	26.7%		8 years
Chok <i>et al.</i> ^[23]	2011	Hong Kong	139	17.3%	55 months	
Zou <i>et al.</i> ^[26]	2008	China	303	15.8%		
Rodriguez-Peralvarez <i>et al.</i> ^[27]	2013	Spain	219	17.6%	51 months	
Nissen <i>et al.</i> ^[28]	2011	USA (CA)	122	10.7%	32.7 months	
Pfiffer <i>et al.</i> ^[29]	2011	Germany	139	17.3%	37.2 months	
Marelli <i>et al.</i> ^[30]	2008	UK	100	18.0%	29 months	
Sharma <i>et al.</i> ^[40]	2012	USA (MI)	94	18.0%	2.2 years	
Wai <i>et al.</i> ^[41]	2012	Singapore	77	38.0%		953 days
Iacob <i>et al.</i> ^[42]	2013	Romania	38	13.2%		22 months

HCC: hepatocellular carcinoma; LT: liver transplantation

globulin injections. Approximately 13.5 years after LT, he complained of persistent cough and was found to have a large pleural effusion. CT scan showed multiple small pulmonary nodules and hilar/mediastinal lymphadenopathy. CT guided biopsy of a chest wall mass showed metastatic HCC. A video-assisted thoracoscopy and pleurodesis was performed. The patient opted not to have any further treatment and died in hospice about 2 months after diagnosis of recurrent HCC.

DISCUSSION

LT is the best treatment for localized HCC in terms of long-term disease free survival. Despite this, patients do have a chance of recurrent HCC that varies from 1.3% to 44.9% depending on individual series.^[9,13] Multiple studies have determined that microvascular invasion, poor tumor grade, larger tumor diameter, and higher AFP are associated with increased recurrence after transplant.^[14-18] Other factors that have been reported to contribute include age, bilobar involvement, multiple lesions, absence of necrosis, tumor beyond Milan criteria, elevated neutrophil-to-lymphocyte ratio, microsatellitosis, and previous liver resection.^[19-22] Two studies have found that the time between LT and HCC recurrence affects prognosis, with worse outcomes associated with early recurrence within 2 years.^[23,24]

Once a patient develops a recurrence after transplant, prognostic factors associated with decreased survival

include major vascular invasion, poorly-differentiated tumor, unresectable disease, and bone metastases.^[4,25] Our small study is consistent with these larger studies in that larger tumors and microvascular invasion on the explanted liver were associated with increased recurrence. Four patients with late recurrence died within 18 months, suggesting that although their initial course after transplantation appeared to be favorable, recurrence at any time threatens survival.

Recurrent HCC tends to occur early or within 2 years of LT. There have been reported cases of recurrent HCC beyond 5 years; however these cases may become more prevalent as more patients are living longer after LT for HCC. Table 5 demonstrates the current literature on recurrence after LT for HCC, which may suggest a trend toward a higher proportion of recurrences with longer follow-up when all cases are considered.^[26-30] Castroagudin *et al.*^[31] in 165 cases, reported a 10.9% recurrence with 78% of these recurrences occurring within the first 3 years, but they had 3 recipients that had recurrences after 7, 9, and 10 years. In our study, the recurrence rate was 17% with a third of our recurrence cases occurring beyond 5 years.

In terms of the site of recurrence, most of the cases of recurrent HCC after LT have been reported to involve extrahepatic (38.5-53%) or both extrahepatic and intrahepatic sites (31-38.5%). In general, intrahepatic recurrence is more common in cases of early recurrence, while more extrahepatic involvement is

seen in cases of late recurrence.^[23,32] The lungs are the most common site of extrahepatic involvement, followed by bone involvement. In our late recurrence patients, 4 out of 5 had extrahepatic involvement. Our one case of continued survival after late recurrent HCC (currently over 44 months) had just hepatic involvement. This could potentially be a case of *de novo* HCC developing in the transplanted liver, the mechanism of which may differ from the biological mechanisms involved in early HCC recurrence.

Treatment of HCC recurrence after transplant involves surgical resection when possible as it has been shown to be associated with a survival advantage.^[4,33] Unfortunately, in many cases, patients present with disseminated disease and surgery is not feasible. Other options for treatment include TACE, RFA, high-intensity focused ultrasound ablation, stereotactic body radiation therapy, and modulation of immunosuppressants.^[34] Sorafenib, a multikinase inhibitor that improves progression-free and overall survival in patients with advanced HCC, has also shown promising results in treatment of HCC recurrence post-LT with a modest survival benefit and manageable adverse effects.^[35,36] Combination therapy with sorafenib and an mTOR inhibitor such as everolimus has also been used in practice, though longer follow-up studies are needed to assess the benefits versus increased toxicity of such a regimen in recurrent HCC.^[37,38] Our 5 patients with late recurrence were treated with various combinations of resection, RFA, sorafenib, and everolimus. Our single surviving patient had undergone ablation, has been on everolimus/sorafenib, and had resolution of a previously seen intrahepatic lesion.

Once a patient develops recurrence, survival is rather dismal despite efforts to treat these patients. Median survival for patients with recurrence has been reported to be between 8.7 months to 18.3 months from time of recurrence.^[4,18,25,33] Our 5 late recurrence cases ranged greatly in survival time after diagnosis of recurrence (2 months-over 44 months).

Efforts have been made to better identify molecular factors that predict recurrence after liver resection for HCC. Kim *et al.*^[39] in a cohort of 72 patients in Korea performed gene expression studies on archived tissue samples. They identified a 233 gene signature that was significantly associated with late recurrence after liver resection. From this, they also developed and validated a 4 and 20 gene predictors from the full 233 gene predictors, however this was in a population of primarily hepatitis B HCC. Perhaps similar molecular studies are needed, especially in transplant patients

to identify those patients with the potential for late recurrence.

This study is limited by its small sample size and small number of identified cases of late recurrence, which renders it difficult to identify trends and factors that may predispose a patient to develop recurrent HCC. However, our study provides detailed clinical information characterizing five cases of late HCC recurrence after LT, in the hopes that it may benefit other researchers in elucidating the characteristics associated with this fortunately infrequent post-LT complication. A notable observation from this study was that not all of the late recurrences occurred in the liver. This is notable because all the patients underwent LT of primary treatment of HCC. Thus, it can be inferred that the patients experiencing extrahepatic recurrences did so as a consequence of indolent metastases present at the time of transplant. In the 2 cases with intrahepatic recurrence, it is not possible to conclude whether the recurrences were in fact, new tumors arising in the transplanted liver. However, in both cases, patients had difficulty to control viral hepatitis and it is possible that hepatitis and fibrosis predisposed them to recurrent HCC in the liver. Because nearly one-third of our post-LT patients with recurrent HCC experienced recurrence more than 5 years after LT, our study highlights the importance of long-term follow-up with imaging every 6-12 months and the need for biomarkers to identify patients who may be at risk for late recurrences. We encourage future studies to further characterize patients with late recurrence of HCC and perhaps molecular studies could help better identify those patients at greatest risk for recurrence to allow physicians to monitor these patients more vigilantly.

Authors' contributions

Study design: S.A. Kwee, L.L. Wong
Data analysis: J.A. Zhang
Manuscript preparation: J.A. Zhang, L.L. Wong
Critical review of manuscript: S.A. Kwee

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Conflicts of interest

Dr. Wong is on the speaker bureau for Bayer Healthcare. Dr. Kwee and Ms. Zhang have no conflicts of interest to report.

Patient consent

This is a retrospective study and the Institutional

Review Board did not require patient consent for this study.

Ethics approval

This study was approved by the Institutional Review Board at the University of Hawaii.

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Updates on hepatorenal syndrome and strategies bridging to liver transplantation

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acute kidney injury

ABSTRACT

Hepatorenal syndrome is not an uncommon life-threatening complication arising from liver cirrhosis. The diagnostic criteria for this syndrome have been revised throughout the years, with recent revisions aimed at improving earlier diagnosis and treatment. Liver transplantation remains the only definitive treatment for hepatorenal syndrome. Due to the scarcity of liver grafts, many patients die waiting. This review focuses on the different strategies to bridge patients to liver transplantation and to improve the postoperative outcome.

DEFINITION OF HEPATORENAL SYNDROME

Hepatorenal syndrome (HRS) is the deterioration of renal function resulting from cirrhosis.^[1] Portal hypertension leads to splanchnic vasodilatation, accompanied by gradual decrease in systemic vascular resistance.^[2] The fall in systemic arterial pressure, or so-called “arterial under filling”, is compensated by an increase in cardiac output by activating the renin-angiotensin-aldosterone system and sympathetic nervous system, which causes vasoconstriction of renal arteries.^[3] Resulting renal hypoperfusion, together with sodium

and water retention and hypoalbuminemia due to poor synthetic function of the liver, causes decreased glomerular filtration rate (GFR), ascites, and edema.^[1,4]

Diagnostic criteria of HRS have been revised throughout the years. They were initially defined by the International Ascites Club (IAC) in 1996, based on major and minor criteria to characterize the occurrence of renal failure in cirrhotic patients.^[5] Major criteria can be summarized as the presence of liver failure and portal hypertension and acute renal failure, while excluding shock, ongoing sepsis, nephrotoxic drug, hypovolemia, nephrotic syndrome,



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and obstructive uropathy.^[6] Urinary output, sodium, osmolality and red blood cells, and serum sodium were included as minor criteria [Table 1]. These criteria were subsequently revised in 2007 to improve accuracy and applicability.^[7] Minor criteria were excluded. Ongoing bacterial infection without septic shock was no longer an exclusion criterion [Table 1].

Two types of HRS have been described. Type-1 HRS is characterized by acute onset and rapidly progressing kidney failure with a doubling of serum creatinine (corresponding to a 50% reduction of creatinine clearance) in less than 2 weeks. The prognosis is poor, with only 10% of patients surviving longer than 90 days. Type-2 HRS presents as a less severe and more gradual decline in renal function associated with refractory ascites. The differential diagnosis between the two types is based on the rate of progression and extent of renal impairment.^[3,8] In this review, we mainly focus on type-1 HRS as it is more clinically relevant in terms of strategies bridging to liver transplantation. Treatment of type-2 HRS with terlipressin and albumin does not appear to have beneficial effects either in pretransplantation or in posttransplantation outcomes.^[9]

According to the IAC criteria, acute renal failure is defined as an increase in serum creatinine (sCr) of $\geq 50\%$ from baseline to a final value > 1.5 mg/dL (133 μ mol/L). However, the threshold value of 1.5 mg/dL has been challenged. Meanwhile, new definition of acute renal failure, now termed acute kidney injury (AKI), has been developed and validated in patients without cirrhosis. Combining the emerging evidence and consensus of the experts, the IAC revised the criteria of AKI in patients with cirrhosis (type-1 HRS) in 2015.^[10] In the new definition, AKI is defined as a sCr increase of ≥ 0.3 mg/dL (26.5 μ mol/L) within 48 h or of $\geq 50\%$ from baseline within 7 days [Table 1]. Three stages of AKI and responses to treatment were also defined. The implementation of the new criteria is to allow earlier treatment of patients with type-1 HRS, which may lead to a better outcome instead of having to wait until the sCr reaches ≥ 2.5 mg/dL.^[11]

STRATEGIES TO BRIDGE TO LIVER TRANSPLANTATION

Medical treatment aims to stabilize the patients until liver transplantation and to optimize their pre-transplant clinical conditions.^[4] Treatment strategies target the underlying pathophysiological mechanism of HRS, including exerting splanchnic vasoconstriction and renal vasodilatation in combination with volume expansion.^[12,13] Many studies have suggested that

the use of vasopressor plus volume expansion with intravenous albumin improves prognosis of HRS. A significant proportion of patients was successfully bridged to liver transplantation.^[9,13-16] Human serum albumin has been introduced as a plasma expander since 1940 and it has been proved useful in the management of HRS.^[17] The additive effects provided by vasoconstrictors and albumin infusion improve outcome compared to monotherapy with either agent.^[18] A meta-analysis has demonstrated that increments of 100 g in cumulative albumin dose were associated with a significantly increased survival, which provides evidence on the important role of albumin in improving outcome of treating HRS.^[19]

The most commonly used vasopressor is terlipressin. Terlipressin is a prohormone of lysine-vasopressin (three glycyl residues and lysine-vasopressin). The glycyl residues are cleaved from the prohormone by endothelial peptidases, allowing prolonged release of lysine-vasopressin.^[20,21] This mechanism enables divided-dose administration by prolonging the half-life of terlipressin, in contrast to the need for continuous infusion as with vasopressin. Terlipressin acts on the V1 receptors expressed on vascular smooth muscle cells in the splanchnic circulation.^[22] The vasoconstrictive effect corrects the circulatory dysfunction and intrarenal vasoconstriction, which lowers the levels of renin and serum creatinine and improves the urine output. As a result of breaking the vicious cycle, the kidney regains its normal self-regulatory function. Gluud *et al.*^[15] performed a meta-analysis in 2012 involving 6 randomized controlled trials of terlipressin (with or without albumin) vs. placebo, with a total of 309 patients. Use of terlipressin was associated with reduced mortality with a relative risk of 0.76 (95% CI 0.61-0.95). Concurrent use of terlipressin and albumin increased the number of patients with reversal of HRS.

Side-effects of terlipressin include abdominal cramps and diarrhea, cardiac tachyarrhythmia and chest pain, as well as cyanosis and livedo reticularis. Ischemia of bowel or skin and extremities is one of the rare complications.^[23] The adverse effects of terlipressin may be minimized by means of intravenous infusion rather than bolus injections as shown in a recent randomized controlled study in Italy.^[24] Although most commonly used and studied, terlipressin is expensive and unavailable in many countries. Other vasoconstrictive agents are used as well. An association between increase in arterial pressure and therapeutic response has been found.^[25]

Noradrenaline, a catecholamine with predominantly alpha-adrenergic activity, is widely available and inexpensive and has been used for the treatment of

Table 1: Comparison of three versions of the International Ascites Club diagnostic criteria of HRS-1

1996	2007	2015
Major criteria	Criteria	Criteria
Chronic or acute liver disease with advanced hepatic failure and portal hypertension	Presence of cirrhosis with ascites	Presence of cirrhosis with ascites
Low glomerular filtration rate: sCr > 1.5 mg/mL or 24 h sCr clearance < 40 mL/min	sCr > 1.5 mg/dL	Diagnosis of acute kidney injury (increase in sCr \geq 0.3 mg/dL or 1.5 times over baseline)
No sustained improvement in renal function following diuretic withdrawal and expansion of plasma volume with at least 1,500 mL of isotonic saline	No improvement of sCr after at least 2 days of diuretic withdrawal and volume expansion with albumin (1 g/kg of body weight per day up to a maximum of 100 g/day)	No improvement of sCr after at least 2 days of diuretics withdrawal and volume expansion with albumin (1 g/kg of body weight per day up to a maximum of 100 g/day)
Absence of shock, ongoing bacterial infection	Absence of shock	Absence of shock
No treatment with nephrotoxic drugs or gastrointestinal or renal fluid losses	No current or recent treatment with nephrotoxic drugs	No current or recent treatment with nephrotoxic drugs
Proteinuria < 0.5 g/day and no evidence of obstructive nephropathy or parenchymal renal disease on ultrasound	No macroscopic signs of structural kidney injury: normal findings on renal ultrasonography, absence of proteinuria > 500 mg/day and absence of microhematuria	No macroscopic signs of structural kidney injury: normal findings on renal ultrasonography, absence of proteinuria > 500 mg/day and absence of microhematuria
Additional criteria		
Urinary volume < 0.5 L/day		
Urinary sodium < 10 mmol/L		
Urinary osmolality > plasma osmolality		
Urinary red blood cells < 50/HPF		
Serum sodium < 130 mmol/L		

HRS: hepatorenal syndrome; sCr: serum creatinine; HPF: high power field

HRS since 2002.^[26] A meta-analysis in 2014 identified four small randomized trials comparing noradrenaline and terlipressin in the treatment of HRS.^[27] The 4 studies comprising 154 patients showed no differences between terlipressin and noradrenaline in reversal of HRS, mortality at 30 days, and recurrence of HRS. Adverse events, mainly abdominal cramps, were less common with noradrenaline.

Midodrine is another alpha-adrenergic agent commonly used in the United States as an alternative to terlipressin, and is used in combination with octreotide and albumin. Skagen *et al.*^[28] reported a case control study comparing 75 HRS patients who received the triple therapy with a historical cohort of 87 HRS patients who did not. It showed a significantly better transplant-free survival, overall survival and renal function at 1 month.

Besides the use of vasopressors and albumin, transjugular intrahepatic portosystemic shunt (TIPS) and extracorporeal albumin dialysis were also used to treat HRS in some centers. TIPS is a percutaneously created low-resistance channel between portal vein and hepatic vein with the aim of reducing the portal pressure by shunting blood from the portal to the systemic circulation.^[29] Few studies have evaluated the effectiveness of TIPS in treating HRS. In 6 out of 7 patients with type 1 HRS, renal function improved 30 days after TIPS, which was associated with a significant

reduction in activity of the renin-angiotensin and sympathetic nervous systems.^[30] Another small study also demonstrated an improvement in renal function after TIPS in 18 patients with type 2 HRS awaiting orthotopic liver transplant.^[31] A non-randomized comparative study of 41 HRS patients (31 with TIPS performed, and another 10 in which TIPS was contraindicated due to advanced liver failure), type 1 and 2 included, showed that renal functions improved 2 weeks after TIPS and with better survivals.^[32] However, the study was heavily biased towards the intervention arm due to patient selection. Wong *et al.*^[33] further demonstrated in their case series the additional benefit of TIPS on top of Midodrine, octreotide and albumin, in improving renal function and sodium excretion for type 1 HRS patients. Despite these evidence, TIPS is a risky procedure, if not contraindicated, in HRS patients requiring liver transplantation who have advanced liver failure. Procedural-related mortality was also reported.^[32] Thus, the role of TIPS in bridging HRS patients to liver transplantation remains limited to selected patients.

Renal replacement therapy (RRT) has been provided to cirrhotic patients with acute kidney injury, with indications no different from other patients with acute kidney injury. However, the renal failure was caused by HRS in only 13% of these patients.^[34] The 3-month survival was only 15% in these patients without liver transplant, which was the lowest comparing to

parenchymal renal failure, hypovolemia or infection associated renal failure.

Molecular adsorbent recirculating system (MARS) was used in bridging fulminant hepatic failure and acute on chronic hepatic failure patients to orthotopic liver transplantation.^[35] It represents a cell-free liver dialysis or albumin dialysis, and helps to remove albumin-bound substances accumulating in liver failure.^[36] A randomized controlled trial by Mitzner *et al.*^[37] compared type 1 HRS patients treated with volume expansion, dopamine, and haemodynamic filtration vs. the same plus MARS. The result showed a significantly better survival for treatment group at 1 month. Even though there was improvement of 1-month survival, one criticism of the study was that it only had one long-term survivor (more than 1 month) and thus it had little clinical relevance. The improvement in serum creatinine and bilirubin may merely reflect the effect of albumin dialysis, without a significant change in liver and renal function.^[38] Further trials to evaluate this strategy will be needed.

LIVER TRANSPLANTATION

Liver transplantation is the definitive treatment of HRS. However, due to the scarcity of liver grafts, most patients died while awaiting transplantation.^[2] Acute liver decompensation with type-1 HRS has worse outcome after liver transplantation than that without HRS. Chok *et al.*^[39] reported 104 patients with acute liver decompensation who received living donor liver transplantation. Among them, 33 patients had HRS. These 33 patients had longer stay in the intensive care unit, more hemodialysis, more blood transfusions, worse postoperative renal function at 1 year and poorer overall survival. However, 5-year overall survival was still nearly 80%, which is satisfactory. The authors concluded that living donor liver transplantation should be considered for such patients. Other centers also reported similar outcome.^[40,41] Some patients with a longer duration of type-1 HRS before liver transplantation were reported to have non-reversal of HRS after transplantation. Wong *et al.*^[42] analyzed the 15 patients with non-reversal of HRS among the 62 HRS patients with liver transplantation. They found a 6% increased risk of non-reversal with each additional day of pre-transplant dialysis. This has illustrated that timely liver transplantation can improve the outcome of HRS patients.

PERIOPERATIVE USE OF TERLIPRESSIN AND REVERSAL OF HRS IN LIVER TRANSPLANTATION

There are little data regarding the role of perioperative

use of terlipressin in liver transplantation. Patients with reversal of HRS before liver transplantation were reported to have a similar postoperative outcome to patients without HRS.^[43] However, Rodriguez *et al.*^[9] reported a contradicting result. In their cohort of 46 patients with type-2 HRS who underwent liver transplantation, 15 patients received terlipressin and albumin and had reversal of type-2 HRS. The remaining 31 patients had either relapse or no response or did not receive terlipressin and albumin. The 2 groups had no significant differences with respect to development of postoperative acute kidney injury, frequency of chronic kidney disease at 1 year, and 1-year and 3-year survival.

A randomized controlled trial was conducted to compare the hemodynamic effects of perioperative terlipressin infusion during living donor liver transplantation.^[44] In this trial, intraoperative terlipressin infusion significantly decreased hepatic and renal arterial resistive indices, portal venous blood flow and systemic arterial pressure with lower systemic vascular resistance. The need for intraoperative vasoactive support was reduced. Terlipressin was continued for three postoperative days. Postoperative renal function was better in the terlipressin group.

FUTURE PERSPECTIVES

HRS is a life-threatening complication of liver cirrhosis and carries a poor prognosis. With a better understanding of the pathophysiology and advances in therapeutic strategies, there is hope to reduce its prevalence and improve patient outcome. Vasopressor treatment, such as that with terlipressin together with volume expander (i.e. albumin), has been shown to be an important strategy to stabilize patients and bridge them to liver transplantation, which is the only definitive treatment. It would be interesting to know the impact on prognosis in future after revising the diagnostic criteria and initiating treatment in an earlier phase. Moreover, studies showed contradicting results on whether the short-term survival benefit of terlipressin in patients with HRS, or the reversal of HRS, would translate into a better long-term outcome after liver transplantation. Further well-designed trials are needed to address this question.

Authors' contributions

Design of the review: K.S.H. Chok

Literature review and manuscript writing: C.Y. Cheung

Manuscript revision: K.S.H. Chok

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There are no conflicts of interest.

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There is no patient involved.

Ethics approval

This review paper is waived for ethics approval.

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Primary hepatic lymphoma: is it a disease entity?

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ABSTRACT

It is now widely accepted that lymphoma is a cancer of the lymphatic cells in the immune system and a type of blood cancer that develops when T or B lymphocytes of the white blood cells display uncontrolled proliferation and cellular immortality. Currently, a number of authors have published case reports or case series in which these lesions are defined as primary hepatic lymphoma. This minireview discusses several aspects of the entity of primary hepatic lymphoma, especially the dilemmas in diagnosis.

INTRODUCTION

Malignant lymphoma is a tumor disease originated from hematopoietic cells typically presented as an apparently circumscribed solid tumor of lymphoid cells.^[1,2] It is now widely accepted that lymphoma is a cancer of the lymphatic cells in the immune system which protect the body against diseases and infection. It is a type of blood cancer that develops when T or B lymphocytes of the white blood cells display uncontrolled proliferation and cellular immortality.^[3,4] Lymphoma is composed of cells that look naïve or resemble lymphocytes, histiocytes or plasma cells.^[5,6] Sometimes the origin of the lymphoma can not be simply decided, like in the instance of natural killer-

cell lymphoma or immunodeficiency-associated lymphoproliferative disorders.^[7,8] Lymphomas are usually seen in lymph nodes, spleen, blood, bone marrow, brain, gastrointestinal tract and skin or other normal structures where lymphoreticular cells exist, but very rarely in the liver.^[9-11] Lymphomas with disseminated disease may enter the blood stream and present as leukemia, notably of the lymphocytic type.^[12,13] The classification of lymphoma is based on clinical manifestation, cell morphology, cytochemistry, genetics and immunophenotype to determine the entity with clinical significance.^[13] With the advance in imaging technology, more and more lesions of lymphoma were detected involving the liver at presentation.^[14] Currently, a number of authors have published case reports or case series



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in which these lesions are defined as primary hepatic lymphoma and described as a malignancy that is very rare and frequently misdiagnosed, but has a rather better prognosis by the combination of surgery and chemotherapy as the treatment than primary hepatic carcinoma.^[15-17] This paper discusses several aspects of the entity of primary hepatic lymphoma, especially the dilemmas in diagnosis.

CLASSIFICATION OF LYMPHOMA

In 1965, Ata *et al.*^[18] reported, for the first time, a case of primary reticulum cell sarcoma of the liver. Later in 1971, Torres *et al.*^[19] described primary reticulum cell sarcoma of liver in cancer. Since then, cases and case series have been accumulated reporting the characteristics of primary hepatic lymphoma and stating that this disease is extremely rare with the absence of lymphoproliferative disease outside the liver.^[20,21]

Some authors defined primary hepatic lymphoma as a very rare malignant tumor with the features of liver involvement and without involvement of other organs and tissues including bone marrow, lymph nodes, the spleen and peripheral blood until at least 6 months after diagnosis.^[14,22] However, Caccamo's criteria which many authors have cited for the diagnosis of primary hepatic lymphoma were proposed in 1986 and based on the data of a single patient who was complicated with liver cirrhosis, Kaposi's sarcoma, and involvement of gastric mucosa and abdominal lymph nodes.^[22,23] In many of the case reports, the patient follow-up was not long enough as "at least 6 months after diagnosis" and some diagnoses were determined just after the biopsy even when there were enlarged retroperitoneal lymph nodes and bone marrow infiltration by lymphoma.^[11,14,15]

Is there indeed a disease entity named primary hepatic lymphoma? The definition of the word or special term "primary" is the key point to answer the question. The authors of this paper consulted several dictionaries and encyclopedias including online medical dictionaries, especially the National Institutes of Health's Web site, i.e. MedlinePlus, produced by the National Library of Medicine.^[24] The definition of the word "PRIMARY" can be summarized in two explanations. The first one is general: first in order of time, place, development, or importance. However the second is medical: not derived from any other source or cause, especially the original condition or a group of symptoms in disease processes, such as a primary infection or a primary tumor, arising spontaneously (idiopathic, efforts to find the original tumor have failed), being an initial tumor or

place specifically of cancer.^[24]

Some authors stated that primary hepatic lymphoma should be differentiated from hepatitis, hepatic metastasis, primary hepatic tumors and secondary hepatic lymphoma, however, they attempted to prove that a liver lymphoma was primary even when bone marrow or portal lymph nodes were involved and interpreted that this involvement was metastasis but not infiltration.^[25,26]

Secondary hepatic lymphomas are defined by some authors as liver lymphomas with extra hepatic foci of lymphoma and found at the first onset or afterwards, or systemic lymphoma with secondary hepatic involvement. This description is also conflicting with the above definition of primary lymphoma.^[27]

The WHO classification of lymphoma is the generally accepted interpretation. In this system, lymphomas are classified by the normal cell type that looks most like the tumor and interpreting cytogenetic, molecular or phenotypic features.^[28] The three main groups are the T cell, B cell, and natural killer cell lymphomas. Less common groups are identified and listed in the subtypes. It has been debated that this classification needs validating in a large series of patients before publication.^[2,3] Interestingly in this classification, the term "primary" is used but not specified, like primary effusion lymphoma, primary central nervous system lymphoma, *etc.*, and a number of subtypes are described as "provisional entities" [Table 1].

ESTABLISHMENT OF DIAGNOSIS

Clinical manifestations usually suggest diseases involving the liver but not the lymphatic and immune system.^[29,30] Many cases are diffuse large B cell lymphoma and the patients show B-symptomatology of weight loss, fever, and night sweats, as well as fatigue and lethargy.^[26] Laboratory study on hepatitis virus infection and serum l-lactate dehydrogenase provides reference to treatment rather than diagnosis of lymphoma. Serum levels of α -fetoprotein and other common tumor markers are usually normal.^[31,32] Imaging modalities are very important tools for detecting liver tumors and lymphoma, although the majority of the diagnoses of liver lymphoma are established afterwards which is quite different from the imaging diagnosis of mediastinal or retroperitoneal lymphoma.^[33]

Image modalities

For evaluating primary liver tumors, currently useful image modalities include ultrasound, computed

Table 1: WHO classification subtypes of lymphoma

Subtypes	Group members
Mature B-cell neoplasms	ALK+ large B-cell lymphoma B-cell prolymphocytic leukemia Burkitt lymphoma/leukemia B-cell chronic lymphocytic leukemia/small cell lymphoma Diffuse large B cell lymphoma Epstein-Barr virus-positive diffuse large B-cell lymphoma of the elderly Extranodal marginal zone B cell lymphoma (mucosa-associated lymphoid tissue lymphoma) Follicular lymphoma Hairy cell leukemia Intravascular large B cell lymphoma Lymphoplasmacytic lymphoma Mantle cell lymphoma Nodal marginal zone B cell lymphoma Plasma cell neoplasms Plasmablastic lymphoma Primary cutaneous follicle center lymphoma Primary mediastinal (thymic) large B-cell lymphoma Splenic marginal zone lymphoma
Mature T cell and natural killer (NK) cell neoplasms	Adult T cell leukemia/lymphoma Anaplastic large cell lymphoma Aggressive NK cell leukemia Angioimmunoblastic T cell lymphoma Blastic NK cell lymphoma Enteropathy-associated T-cell lymphoma Extranodal NK/T-cell lymphoma, nasal type Hepatosplenic T-cell lymphoma Mycosis fungoides/Sezary syndrome Peripheral T-cell lymphoma not otherwise specified Primary cutaneous CD30-positive T-cell lymphoproliferative disorders T-cell large granular lymphocytic leukemia T-cell prolymphocytic leukemia
Hodgkin lymphoma	Classical Hodgkin lymphomas Lymphocyte depleted or not depleted Lymphocyte-rich Mixed cellularity Nodular sclerosis Nodular lymphocyte-predominant Hodgkin lymphoma
Immunodeficiency-associated lymphoproliferative disorders	Associated with a primary immune disorder Associated with methotrexate therapy Associated with the human immunodeficiency virus Post-transplant Primary central nervous system lymphoma

tomography (CT), magnetic resonance imaging (MRI), positron emission tomography and computed tomography (PET/CT), and digital subtraction angiography (DSA). The following description of these modalities is mainly based on the results from retrospective studies.^[34]

Ultrasound is the most sensitive of image modalities to find liver lymphoma showing hypoechoic liver lesion with irregular margins. When contrast is used, the tumor is inhomogeneously hyperenhanced in the arterial phase and hypoenhanced in the portal and late phases, similar to the images of hepatocellular carcinoma.^[35]

Lymphomas detected in the liver by plain CT scan usually present as homogeneous shadows of low density, with irregular demarcations.^[16] A very low density area in the center might indicate necrosis. When 3-phase contrast-enhanced scan is used, the lesions will not be enhanced at the arterial and portal phase, and will be slightly enhanced at the delayed phase with a border sharply contrasted with the normal

neighboring tissue.^[24] Some liver lymphomas reported as primary or secondary may shrink or vanish after treatment when demonstrated by CT scan, but no change in density or enhancement of the remaining lesions are found. In addition, diffuse liver infiltration by lymphoma can be detected by CT scan only when there are areas of density change in the swelling liver. But lymphoma infiltration without density change in an enlarged liver cannot be revealed by CT. It is impossible, of course, to definitely exclude lymphoma infiltration within a liver which is normal in size and CT density. CT is now commonly used for lymphoma staging.^[15,36]

Liver lymphomas present heterogeneous signals on MRI image with features of hypointense in T1-weighted sequences but hyperintense in T2-weighted sequences.^[33] Although MRI has the advantage in specifically characterizing liver lesions over all other imaging modalities, it often fails to distinguish primary hepatic lymphoma from other liver masses.^[37] In a report, MRI presented almost the same imaging features for lymphoma and sarcoidosis.^[11]

Table 2: Several pathological subtypes of lymphomas and their possible immunophenotypes

Pathological subtypes	Immunophenotypes
B-cell chronic lymphocytic leukemia/lymphoma	CD5, CD19, CD20
Burkitt's lymphoma	BCL6, CD10, CD19, CD20, CD22
Diffuse large B-cell lymphoma	CD4, CD8, CD10
Follicular lymphoma	CD10, CD19, CD20
Mucosa-associated lymphoid tissue lymphoma	CD20, CD45, CD79 α
Mantle cell lymphoma	CD5, CD19
Mixed-cellularity subtype of Hodgkin lymphoma	CD2, CD4, CD15, CD30
Mycosis fungoides	CD4, CD8
Nodular sclerosis form of Hodgkin lymphoma	CD15, CD20, CD30
Peripheral T-cell lymphoma-not-otherwise-specified	CD3, CD43, CD45, CD45RO
Precursor T-cell leukemia/lymphoma	TdT, CD2, CD7, CD45, CD99

PET/CT is used to improve the detection range, response evaluation, and prognosis prediction of lymphoma. With the help of 18F-fluorodeoxyglucose (FDG), this modality provides high sensitivity in evaluating most liver lesions and is invaluable for finding extrahepatic lesions. However, false positive findings are common in inflammatory or metastatic lesions.^[38,39] However, the problem of specificity can be partially solved by percutaneous needle biopsy.

Celiac trunk angiography of known lesions shows very scarce contrast staining, tiny feeding arteries, obvious shifting of the hepatic artery/its tributaries, and the absence of enlarged tumor blood vessels. When a small amount of lipiodol is injected into the feeding arteries during the DSA procedure, no deposit of lipiodol can be observed in the lesions.^[40]

None of the above modalities produce specific image features for the diagnosis of liver lymphoma. Other than explorative laparotomy, puncture biopsy under the guidance of ultrasound or CT is of paramount importance for establishing the diagnosis of liver lymphoma by acquiring specimens for pathological and immunohistochemical examination.^[5] Moreover, core puncture needle is much more reliable than fine needle to obtain adequate samples for study.^[41]

Pathology and immunohistochemistry

The clinical diagnosis is usually established by a pathologist (commonly a hemato-pathologist) after the examination of the biopsy specimens.^[4] Hematoxylin and eosin stain of liver specimen may show infiltration of large lymphoid cells. Immunohistochemistry may show positive Ki67, positive CD3, CD5, CD30, CD40, etc.^[39] Fluorescence *in situ* hybridization is applied for tumor genetics and flow cytometry is used for quantitative analysis of cells.^[15] The pathological classification of subtypes is essential for treatment decision and outcome prediction [Table 2]. On the other hand, newly developed lesions in the liver of lymphoma patients can not be the same disease, as other kinds of tumor may occur because of immune compromise or liver diseases.^[42]

CONCLUSION

Liver is a poor “soil” for malignant lymphoma as a “seed” to grow. Most reports about primary hepatic lymphoma are published in the form of case studies and no prospective researches have been found till now. It is difficult for the radiologist to define lymphomas in the liver as primary or secondary, even when they really are lymphomas. The differentiation of primary lymphoma from secondary also puzzles the pathologist and all involved multidisciplinary oncological specialists. That another malignant tumor may develop in the liver of patients with lymphomas makes things more complicated.

Most of the reported cases are diffuse large B cell lymphomas but this type is usually aggressive and involves multiple organs. In addition, according to the above definition provided by some authors, the establishment of the diagnosis of primary hepatic lymphoma is a retrospective process that should be decided at least 6 months after the initial diagnosis of lymphoma. So, the diagnosis of primary hepatic lymphoma at first liver biopsy is problematic and conflicting with this definition.

Lymphomas are often detected in the liver by CT incidentally or general screening for lesions in the patients. The term “primary hepatic lymphoma” is imprecise and less informative for referral to a proper treatment and can be confused with the subtype hepatosplenic T cell lymphoma of WHO classification. Current criteria for the diagnosis of primary hepatic lymphoma are outdated. The differentiation must be made between primary and secondary disease at first, and efforts should be directed to find the original tumor. This “provisional entity” is still controversial, and additional researches and discussions on criteria for its diagnosis are warranted to clarify their significance for consensus and refinement.

Authors' contributions

Collection of literature data: F. Xiong

Design, writing and revision of the paper: Y.S. Guan

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Conflicts of interest

There are no conflicts of interest.

Patient consent

There is no patient involved.

Ethical approval

This review paper is waived for ethics approval.

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MELD score and AST-to-platelet ratio index predict long-term survival in patients with a small hepatocellular carcinoma following non-transplant therapies: a pilot study

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ABSTRACT

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Hepatocellular carcinoma, ablation, liver resection, transplant

Aim: Liver transplantation (LT) is the most effective treatment for long-term survival from hepatocellular carcinoma (HCC); however, insufficient donors limit therapy. The authors sought to identify characteristics that predicted long-term survival after non-transplant therapies in patients with small HCC. **Methods:** In a database of 1,050 HCC patients, the authors identified those with single HCC ≤ 3.0 cm, who underwent hepatic resection (HR, $n = 16$), radiofrequency ablation (RFA, $n = 55$), or LT ($n = 23$) with 5-year follow-up. Overall survival (OS) and odds-ratios (OR) for survival after HR/RFA were calculated for MELD score, platelet count, creatinine, albumin, AST/platelet ratio index (APRI), international normalized ratio, and bilirubin. **Results:** LT patients had 3- and 5-year OS of 82.6% and 73.9% compared to HR/RFA patients with 3- and 5-year OS of 40.8% and 33.8%. The strongest predictors of survival after HR/RFA were MELD < 10 [OR 4.43, 95% confidence interval (CI) 1.85-10.58] and APRI ≤ 0.5 (OR 4.25, 95% CI 1.63-11.08). HR/RFA patients with both MELD < 10 and APRI ≤ 0.5 had 3- and 5-year OS of 77.3% and 72.7%. **Conclusion:** Patients with MELD < 10 and APRI ≤ 0.5 who undergo HR/RFA have survival approaching LT. Perhaps patients who meet these criteria can safely undergo non-transplant therapy and donor livers can be allocated to patients with a greater need.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver. Worldwide, there were 782,000 new cases in 2012 and HCC is the

second leading cause of cancer-related mortality with 745,000 deaths.^[1,2] Advanced stage at diagnosis and poor underlying liver function present major challenges to treatment. Potential curative therapies for HCC include hepatic resection (HR) and liver



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transplantation (LT). LT is viewed as the optimal treatment for HCC as it treats both the tumor and the underlying liver disease.^[3] However, the inadequate number of available donors significantly limits use of LT. Prolonged waiting times lead to dropout from the waiting list due to tumor progression exceeding criteria for LT, or death due to liver failure.^[4] While overall survival (OS) and recurrence-free survival are both higher in patients undergoing LT compared to HR, prior studies have found that resection in patients with a single tumor less than 3.0 cm in size may have comparable survival to those undergoing LT.^[5] Similarly, radiofrequency ablation (RFA), while not a curative therapy, is a safe and effective alternative to HR in patients who are not surgical candidates. Direct comparisons of overall survival between HR and RFA are limited by the degree of hepatic dysfunction in the patients who are offered resection versus ablation, but retrospective studies suggest that survival after RFA may not differ significantly from that of HR in certain patient populations.^[6,7]

Prognosis is also affected by the degree of hepatic dysfunction, patient comorbidities, and tumor biology. Increasing evidence suggests that tumor size is a surrogate marker of tumor biology and surgical outcomes. Tumors less than 3.0 cm have been shown to be well-differentiated, contained within the capsule and have better prognosis.^[8] Smaller tumors have a higher likelihood of being successfully treated by non-transplant therapies. Therefore, our goal is to identify characteristics in patients with small HCC (≤ 3.0 cm) that predict comparable long-term survival after HR or RFA versus LT, as these patients may be able to undergo non-transplant therapy and allow allocation of donor livers to those most in need.

METHODS

Patients

This is a retrospective analysis of 94 patients out of a cohort of 1,050 HCC cases referred over a 22-year period (1993-2014) to our group of physicians associated with the only liver transplant program in Hawaii, and the only referral center for liver disease/surgery for American territories of the Pacific Basin (including Samoa, Guam, Saipan, and the Marshall Islands). Patients also included foreign nationals from China, Japan, Korea, and the Philippines, who sought medical care in the USA. About 75% of the overall cohort had some type of viral hepatitis with about 41% with hepatitis C, 38% hepatitis B and 4-5% coinfecting with both. This center sees about 65-70% of the HCC cases in Hawaii. This study was approved by the University of Hawaii Institutional Review Board.

HCC diagnosis

Patients with either a histological or clinical diagnosis of HCC were considered for inclusion. Histological diagnosis of HCC was made either from liver biopsy or examination of the resected liver. Patients without histologic diagnosis, but a history of chronic liver disease, mass > 2 cm in size on dynamic imaging and one of the following (1) arterial uptake with venous washout seen on computed tomography scan or magnetic resonance imaging or (2) alpha-feto protein (AFP) > 200 ng/mL.

Study design

Inclusion criteria were the following: (1) patients with a single tumor ≤ 3.0 cm; (2) treatment with HR, RFA, or LT; and (3) either minimum follow-up of at least 5 years or death prior to the 5-year mark. We excluded 865 patients with multiple tumors or tumors ≥ 3.0 cm. Of the remaining 185 patients, 69 were lost to follow-up prior to the 5-year mark or were enrolled less than 5 years prior to the time of data analysis and 22 received another therapy (chemoembolization, Yttrium-90 or sorafenib) or no therapy. The final study population included 94 patients: 55 patients underwent RFA as their sole therapy, 16 underwent HR and 23 had LT.

Demographic/medical data were collected prospectively via clinical interview and chart analysis, and the data retrospectively analyzed. Patient characteristics chosen for analysis were: age ≤ 50 years, age ≤ 60 years, gender, presence of hepatitis B and/or hepatitis C, alcohol use (defined as 2 or more alcoholic drinks/day for 10 years), obesity [defined as body mass index (BMI) ≥ 30], smoking, diabetes mellitus, AFP (stratified as normal versus abnormal with normal < 20 ng/dL), tumor size ≤ 1.5 cm, presence of cirrhosis, serum bilirubin ≤ 1.2 mg/dL, albumin ≥ 2.5 g/dL, albumin ≥ 3.0 g/dL, international normalized ratio (INR) ≤ 1.2 , INR ≤ 1.7 , presence of ascites, Child-Turcotte-Pugh (CTP) score $\leq A$, CTP score $\leq B$, platelet count ≥ 100 , creatinine ≤ 1.0 mg/dL, AST-to-platelet ratio index (APRI) ≤ 0.5 , APRI ≤ 1 , and MELD score < 10 . Laboratory data used for the study was obtained within 2 weeks of the initial visit. Exception points were added to the MELD scores of patients with HCC whose tumors met Milan criteria, in order to balance their risk of tumor progression and dropout to that of non-HCC patients. Because the number of added exception points fluctuated throughout the study period based on united network for organ sharing guidelines, the raw MELD score rather than the adjusted MELD score was used in the analysis for consistency. APRI was categorized based on initial description by Wai *et al.*^[9] Of patients with an APRI of ≤ 0.5 , 85% would not have significant fibrosis (defined

Table 1: Characteristics of study population by group

Characteristic	HR (n = 16)	RFA (n = 55)	LT (n = 23)	Total (n = 94)	P value
Age (years), mean ± SD	58.6 ± 8.3	65.4 ± 10.8	54.3 ± 6.2	62.0 ± 11.0	< 0.001 ^{*,†}
Male, n (%)	13 (81.3%)	37 (67.3%)	21 (91.3%)	71 (75.5%)	0.067
Cirrhosis, n (%)	12 (75%)	51 (91.1%)	23 (100.0%)	86 (91.5%)	0.020 [‡]
HBV, n (%)	6 (37.5%)	15 (27.3%)	8 (34.8%)	29 (30.9%)	0.661
HCV, n (%)	7 (43.8%)	31 (56.4%)	14 (60.1%)	52 (55.3%)	0.555
Diabetic, n (%)	1 (6.3%)	16 (29.1%)	4 (17.4%)	21 (22.3%)	0.125
BMI, mean ± SD	22.7 ± 3.3	25.4 ± 5.5	29.4 ± 4.9	26.0 ± 6.0	< 0.001 ^{*,†}
Ascites, n (%)	0 (0.0%)	18 (32.7%)	8 (34.8%)	26 (27.7%)	0.025 ^{*,†}
Serum bilirubin (mg/dL), mean ± SD	1.1 ± 0.7	1.5 ± 1.0	1.8 ± 1.1	1.5 ± 1.0	0.097
MELD score, mean ± SD	8 ± 2	11 ± 4	11 ± 4	11 ± 4	0.016 ^{*,†}
Platelet count (x10 ³ /μL), mean ± SD	139 ± 46	119 ± 69	109 ± 76	121 ± 69	0.394
Tumor size (cm), mean ± SD	2.0 ± 0.6	2.3 ± 0.5	2.3 ± 0.5	2.3 ± 0.5	0.114

**P* < 0.05, HR vs. RFA; [†]*P* < 0.05, RFA vs. LT; [‡]*P* < 0.05, HR vs. LT. HR: hepatic resection; RFA: radiofrequency ablation; LT: liver transplantation; HBV: hepatitis B virus; HCV: hepatitis C virus; BMI: body mass index

as an Ishak score of 3 or more), and of patients with an APRI of ≤ 1.00, 98% would not have cirrhosis (defined as an Ishak score of 5 or 6). Thus we chose the cutoff values of APRI ≤ 0.5 and ≤ 1.00 for our study. Outcome measures included: 3- and 5-year survival and recurrence categorized as early (< 2 years) vs. late (≥ 2 years).

Statistical analysis

One-way analysis of variance was used to identify significant differences between the baseline characteristics of the three study groups defined by continuous variables: age, BMI, serum bilirubin, MELD score, platelet count, and tumor size. For groups in which a difference was identified, the Tukey post-hoc analysis was applied to determine which of the three comparisons (HR vs. RFA, HR vs. LT, RFA vs. LT) contained the difference. The chi-squared test was used to identify significant differences between the baseline characteristics of the 3 groups defined by categorical variables: gender, presence of cirrhosis, presence of hepatitis B or C, presence of diabetes, and presence of ascites. For groups in which a difference was identified, the Fisher's exact test was used to determine which of the 3 comparisons contained the difference.

Odds ratios (OR) were calculated for both 3-year and 5-year OS for each of the patient characteristics, in each of the groups HR, RFA, LT. The OS for HR and RFA groups were calculated both separately and as a composite (HR/RFA), and compared against OS for patients undergoing LT. Results were expressed as OR with 95% confidence interval (CI). Only results with a *P* value < 0.05 were considered statistically significant.

RESULTS

Baseline characteristics

The demographics of the patients included in this

study are outlined in Table 1. The patients were 71 men and 23 women with a mean age of 62 ± 11 years and 1 HCC tumor with a mean size of 2.3 ± 0.5 cm. The majority of patients (73.4%) were Asian or Pacific Islander. The RFA group differed significantly from the HR and LT groups with respect to age (mean age of 65.4 vs. 58.6 and 54.3 years respectively). The HR group differed significantly from the RFA and LT groups with respect to presence of ascites and MELD score: 0% with ascites in HR group vs. 32.7% and 34.8%, respectively. The mean MELD score in the HR group was 8, compared to 11 in the RFA and LT groups. Patients in the LT group had a significantly higher BMI than patients in the HR and RFA groups (29.4 vs. 22.7 and 25.4, respectively). Finally, the HR and LT groups differed significantly with respect to cirrhosis: 75% in the HR vs. 100% of LT patients.

Of the 23 patients who underwent LT, 15 underwent locoregional therapy before LT including 2 patients who underwent resection, 8 RFA and 6 transcatheter arterial chemoembolization (TACE) procedures; 2 patients received both RFA and TACE while awaiting transplant. Mean waiting time for LT was 355 days (range 120-720 days). Mean MELD score was similar between the LT and RFA groups. Most patients who underwent LT received MELD exception points in order to qualify for transplant as only 3 patients had MELD above 15.

Overall survival

Overall 3-year and 5-year survival in all patients undergoing LT was significantly higher than patients in the HR, RFA, and composite HR/RFA groups [Table 2]. The 3-year survival was 82.6% in the LT group, 62.5% in the HR group, 34.5% in the RFA group, and 40.8% in the composite HR/RFA group. Similarly, 5-year survival rates were 73.9%, 56.3%, 27.3%, and 33.8% respectively.

Table 2: The 3-year and 5-year OS after LT, HR, and RFA

Survival	LT	HR	RFA	HR/RFA
3-year OS	82.6%	62.5%	34.5%	40.8%
5-year OS	73.9%	56.3%	27.3%	33.8%

LT: liver transplantation; HR: hepatic resection; RFA: radiofrequency ablation; OS: overall survival

Patient characteristics significantly affecting survival

Patient characteristics with statistically significant ORs for both 3-year and 5-year OS were: MELD < 10, creatinine \leq 1 mg/dL, and APRI \leq 0.5 [Table 3]. Characteristics with significant ORs inversely correlating with 3-year and 5-year OS were age > 60 years and presence of diabetes. Serum bilirubin \leq 1.2 mg/dL, serum albumin \geq 3.0 g/dL, and CTP score \leq 6 approached but did not reach significance.

Modified OS

Modified 3-year and 5-year OS was calculated for patients who underwent HR or RFA with the characteristics in Table 3, and compared with 3-year and 5-year survival after LT [Figure 1]. APRI \leq 0.5 was associated with a 3-year OS of 68.0% and 5-year OS of 64.0%, and MELD < 10 was associated with a 3-year OS of 64.9% and 5-year OS of 54.1%. Patients who underwent HR or RFA with both MELD < 10 and APRI \leq 0.5 (22 out of 71 patients) had a modified 3-year OS of 77.3% and 5-year OS of 72.7%. Diabetes mellitus was associated with a 3-year and 5-year OS of 17.6% following HR/RFA.

Recurrence

Of the 71 patients that underwent HR/RFA, 31 patients had documented recurrence, or 43.6%. Twenty-seven of these 31 patients underwent subsequent treatment including 1 patient who underwent repeat resection, 16 patients who underwent RFA, 9 who underwent

TACE, and 6 patients who received chemotherapy (5 patients received more than 1 treatment modality for recurrence). Forty-four patients (including 3 out of the 31 patients with recurrence) did not have any documented subsequent therapy, so their causes of death were unclear. Among the 22 patients who underwent HR/RFA and had both MELD < 10 and APRI \leq 0.5, 13 patients had a documented recurrence (59.1%). In the remaining 49 HR/RFA patients, 18 patients had a documented recurrence (36.7%). Two of the 23 patients who underwent LT had recurrence (8.7%): 1 patient had a local recurrence which was treated with RFA and sorafenib, and 1 patient underwent excision of a metastatic lesion on the chest wall.

The average time to recurrence among all HR/RFA patients was 935 days. Among the 22 patients with MELD < 10 and APRI \leq 0.5, 5 patients had early recurrence (38.4%), and mean time to recurrence was 1,107 days (range 169-3,380 days). For the other 49 HR/RFA patients, 1 patient had recurrence for which time to recurrence was unknown, and 11 patients (64.7%) had early recurrences. The average time to recurrence in this group was 803 days (range 188-2,664 days). There was a trend toward late recurrences in the low MELD/APRI group compared to the other patients (61.5% vs. 35.3%), however this was not statistically significant ($P = 0.27$).

DISCUSSION

Determining the most appropriate initial therapy for early HCC is challenging given the need to balance procedural morbidity and mortality with long-term recurrence rates. LT has been shown in multiple retrospective studies and a meta-analysis to have superior long term, recurrence-free survival compared to HR.^[10-14] However, the scarcity of donor livers is a

Table 3: Patient characteristics significantly affecting 3-year and 5-year OS

Characteristic	n	3-year OS OR (95% CI)	P value	5-year OS OR (95% CI)	P value
MELD < 10	54	4.43 (1.85, 10.58)	0.0008	2.77 (1.19, 6.46)	0.0181
APRI \leq 0.5	31	4.25 (1.63, 11.08)	0.0031	4.09 (1.59, 10.50)	0.0034
Creatinine \leq 1.0 mg/dL	55	6.28 (2.09, 18.86)	0.0010	4.15 (1.39, 12.36)	0.0107
Diabetes	22	0.22 (0.07, 0.66)	0.0070	0.32 (0.11, 0.97)	0.0438
Age > 60 years	41	0.42 (0.18, 0.96)	0.0396	0.32 (0.14, 0.75)	0.0089
Bilirubin \leq 1.2 mg/dL	46	2.28 (0.99, 5.24)	0.0520	2.19 (0.95, 5.06)	0.0677
Albumin \geq 3.0 g/dL	67	3.50 (1.22, 10.07)	0.0201	2.36 (0.82, 6.78)	0.1093
CTP score \leq 6	58	2.23 (0.95, 5.21)	0.0649	2.00 (0.84, 4.74)	0.1155

OS: overall survival; OR: odds ratio; CI: confidence interval; MELD: Model for End Stage Liver Disease; APRI: AST-to-platelet ratio index; CTP: Child-Turcotte-Pugh

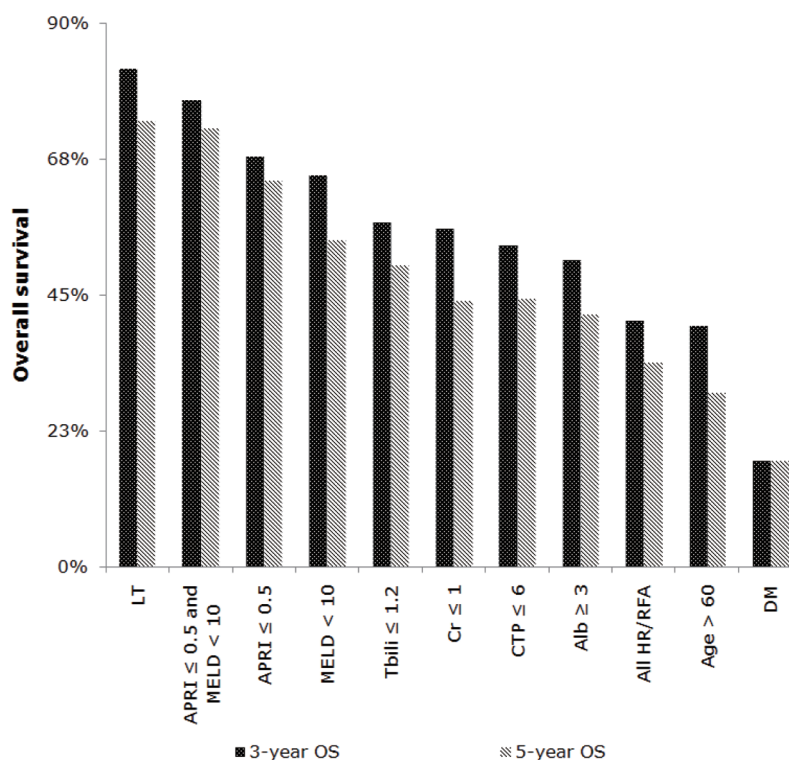


Figure 1: Modified overall 3-year and 5-year survival for selected patient characteristics. LT: liver transplantation; APRI: AST-to-platelet ratio index; MELD: Model for End Stage Liver Disease; Tbili: total bilirubin; Cr: creatinine; CTP: Child-Turcotte-Pugh score; Alb: albumin; HR: hepatic resection; RFA: radiofrequency ablation; DM: diabetes mellitus

limiting factor to transplantation in patients who meet criteria. Prolonged waiting times may lead to tumor progression and/or death from liver failure, and the estimated monthly drop-out rate increases with length of time on the waitlist, reaching 5.6% at 12 months.^[4] Because of limited donors, resection has been recommended for those with better liver function.^[13,15]

Perhaps the biggest dilemma is how to treat the very small HCC, especially those that do not meet minimum transplant criteria. Previous studies have shown good short-term outcome for small HCC whether ablated or resected, however recurrences are more frequent with RFA.^[16] Liu *et al.*^[17] in 237 patients with single HCC < 2.0 cm, concluded that resection provided better overall and recurrence-free survival than RFA and they recommended resection as the first line therapy. Other approaches have included the “wait and not ablate” tactic in small tumors - allowing tumors to progress until patients qualified for liver transplant.^[18,19]

Some patients do have long-term survival after HR or RFA for HCC < 3.0 cm, but few studies identify factors that are predictive of a good outcome in the absence of transplantation. In this study, we demonstrate that although the overall 3-year and 5-year survival rates vary drastically between the HR and RFA groups

(62.5% vs. 34.5% 3-year OS, 56.3% vs. 27.3% 5-year OS), hepatic function is also very different. No patient in the HR group had ascites, vs. 33% of RFA patients, and 75% of HR patients were cirrhotic compared to 91% of RFA patients. We found that MELD < 10, APRI ≤ 0.5 and creatinine < 1.0 were the best factors that predicted survival. Most importantly, when patients had both MELD < 10 and APRI ≤ 0.5 and underwent HR or RFA, the 3- and 5-year survival was similar to those that underwent LT-despite the higher rates of recurrence in the HR/RFA group. The recurrence rates were 42% in the HR/RFA group and 55% in the subset of HR/RFA patients with MELD < 10 and APRI ≤ 0.5, compared to 8.7% in the LT group. The disparity between the higher survival despite a higher recurrence rate in the subset of HR/RFA patients with MELD < 10 and APRI ≤ 0.5 may be partly explained by the timing of recurrences. Most of the recurrences in the HR/RFA group occurred early (within 2 years), while the low MELD and APRI subset tended to have late recurrences (after 2 years). A retrospective study by Portolani *et al.*^[20] which examined intrahepatic recurrence of HCC after resection found that survival was significantly better in patients with late recurrence compared to early recurrence: 61.9% vs. 25.7% at 3 years, and 27.1% vs. 4.5% at 5 years. The authors also found that patients with late recurrences were more likely to be cured by resectional or ablative

therapy of the tumor recurrence, and had survival comparable to those without recurrence. These differences support our hypothesis that careful patient selection, based on characteristics that predict a low level of hepatic parenchyma fibrosis and preserved synthetic function, can identify patients who will have a good long-term outcome after non-transplant therapies.

Although APRI is not widely used in liver transplant literature, we propose that this can be a helpful tool. Liver function can be inferred by prognostic scores such as CTP, MELD or functional tests such as Indocyanine Green. Degree of fibrosis can be assessed more directly by measuring hepatic vein pressures, liver biopsy or transient elastography. These tests are often limited by operator-dependence, biopsy interpretation, sample error, body habitus, and invasiveness. Prognostic scores have been predictive of short-term outcome and survival on a transplant list but these scores were not used specifically to assess fibrosis, longer-term prognosis or predisposition for recurrent cancer. APRI is easy to calculate at the bedside with readily available laboratory parameters and does not require an expensive or invasive test. We found that while an APRI ≤ 0.5 was correlated with a statistically significant OR for both 3-year and 5-year OS in the HR, RFA, and composite HR/RFA groups, an APRI ≤ 1.00 did not predict a survival advantage. APRI is a reasonable surrogate for fibrosis and our study has shown that when used with MELD < 10 , this has prognostic significance.

This study is limited by its retrospective nature and relatively small sample size. Due to the small sample size, we were unable to report on the outcomes following other non-transplant treatments such as TACE and Yttrium-90. It will be necessary to validate these results in a larger prospective study. This analysis also reported only overall survival, as some patients had recurrence of HCC that was treated but died of liver failure or an unrelated problem. It is thus difficult to determine the exact effect of HCC on survival. Because it was a retrospective study, we did not account for patient comorbidities that may have affected candidacy for transplant or overall survival. This is evident by the older age of the patients who underwent RFA who were not likely to be transplant candidates because of comorbidities.

Despite these limitations, the strength of this study is that this represents a single center experience in which patients are referred to a single group of surgeons who perform most of the liver resections and all of the transplants in the state. The surgeons,

hepatologists, oncologists and interventional radiologists are closely associated, so multidisciplinary management allowed equal access to all treatment modalities. Finally, this study was conducted in a small state with a high burden of HCC and has long term follow-up of both transplant and non-transplant patients.

In conclusion, this study suggests that patients with single HCC tumors ≤ 3 cm, with an APRI ≤ 0.5 and MELD score < 10 , have an OS after resection or ablation similar to patients undergoing transplantation. Recurrences are higher in this group than patients who underwent transplantation, however recurrences tended to occur late (> 2 years). While liver transplantation remains the optimal treatment for HCC, perhaps this subset of patients can safely wait until a more urgent reason for transplant arises, in areas where donor livers are limited. Future studies validating this in a larger population could assist in directing patients with good prognosis to non-transplant therapies, and allow allocation of scarce donor livers to patients with a greater need.

Authors' contributions

Concept/design, data acquisition, critical revision: L.L. Wong

Manuscript preparation, data analysis: J. Sarkar

Data analysis: T. DeLeon

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Conflicts of interest

There are no conflicts of interest.

Patient consent

As this was a retrospective study, the IRB did not require patient consent.

Ethics approval

This study received IRB approval at the University of Hawaii.

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First case report of inflammatory myofibroblastic tumor of peritoneal cavity in a living donor liver transplantation recipient

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ABSTRACT

Post-transplantation malignancies are well known complications after liver transplantation. Certain malignancies are more common in pediatric recipients than adults. Inflammatory myofibroblastic tumors (IMTs) are reactive neoplasms with miniscule malignant potential. IMTs are more common after hematopoietic stem cell transplantation. However, there is 1 case reported in the literature after deceased donor liver transplantation. The authors describe a case of IMT after living donor liver transplantation. The patient was a 1-year-old girl who underwent living donor liver transplantation (LDLT) for decompensated cirrhosis secondary to extra hepatic biliary atresia. Six months post LDLT routine ultrasonography revealed multiple solid abdominal masses. Repeated biopsies were inconclusive. Hence surgical excision was carried out. Histopathological examination revealed IMT. Immunohistochemistry was positive for anaplastic-lymphoma kinase activity. Ceritinib, a tyrosine kinase inhibitor, was used as adjuvant chemotherapy for 1 year. At 1.5 years (at the time of writing this paper) of follow-up, the child was disease free on imaging (whole body positron emission tomography-computed tomography). This will be the first case of IMT after LDLT to be reported in the literature.

INTRODUCTION

Post-transplantation malignancies are well known complication of chronic immunosuppression. Certain malignancies are more common in pediatric recipients than in adults. Literature review revealed that inflammatory myofibroblastic tumors (IMTs) generally occur 3 to 6 months post-transplantation and are more common after hematopoietic stem cell transplantation (HSCT). Epstein-Barr virus has been held responsible

in some reported cases. Lungs are the commonest site however they can present anywhere in the body. We describe a case of 6 months old female child who was suffering from end stage liver disease secondary to extra-hepatic biliary atresia (EHBA). She underwent living donor liver transplantation (LDLT). Six months post liver transplantation she was found to have intra-abdominal tumors on routine ultrasonography (USG). She underwent surgical excision of peritoneal neoplasms. Histopathological examination (HPE)



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confirmed IMT with anaplastic lymphoma kinase activity. She received ceritinib chemotherapy for 1 year. At 1.5 years of follow-up, the child is recurrence free.

CASE REPORT

Our patient was a 1-year-old female child from Pakistan. She had decompensated chronic liver disease secondary to EHBA. She underwent primary LDLT at the age of 6 months at our center. Her mother was the donor. She had uneventful post transplantation recovery. At 3 months post liver transplantation (LT), routine USG revealed solid masses in the pelvis and in the sub hepatic region. Trucut biopsy of both the lesions revealed mesenchymal neoplasm. There was no evidence of malignancy. There was gradual increase in size of tumor on follow-up scans over the next 3 months [Figure 1A and B]. She reported back to our center for further evaluation of the intra-abdominal tumors. Review of previous biopsy slides and fresh trucut biopsies done at our center were inconclusive. A tumor board decision was made for surgical excision to get further information about the tumor and also relieve the mass effect in the abdomen. Elective laparotomy was planned. In laparotomy, a 15-cm lesion was seen in right sub diaphragmatic region pushing the hepatic flexure and graft liver which was also adherent to diaphragm [Figure 1C and D]. Another 7-cm firm mass was found adherent to omentum, proximal jejunum and jejunal mesentery. The tumor was removed along with a segment of jejunum. Minimal loculated ascites

was also seen near the mass. Two other small tumors of approximately 2 cm each were seen attached to serosa of small bowel. All the macroscopically visible tumors were completely removed. HPE revealed IMT of the peritoneum with very mild malignant potential. Immunohistochemistry (IHC) was positive for anaplastic lymphoma kinase (ALK) antibodies [Figures 2 and 3]. Postoperative period was uneventful. She was discharged in a hemodynamically stable condition on postoperative day 10. After tumor board discussion, ceritinib was given as adjuvant therapy in view of ALK positivity. At 18 months follow-up, patient is recurrence free with good graft function.

DISCUSSION

The risk of *de novo* malignancy following LT is significantly higher than that of the general population. Skin, hematological, and colon cancers are common *de novo* malignancies after LT. Immunosuppression plays a major role in oncogenesis in the transplant population.^[1] Other risk factors are hepatitis C virus infection, smoking, alcoholic cirrhosis, and sun exposure.^[2]

IMTs are one of the rare groups of post-transplant malignancies. More than 200 cases have been described so far. Majority of them are after HSCT.^[3,4] IMTs occur earlier in the post-transplant period ranging between 3 months and 2.5 years. Presenting complaints of patients are usually fever, weight loss and pain, along

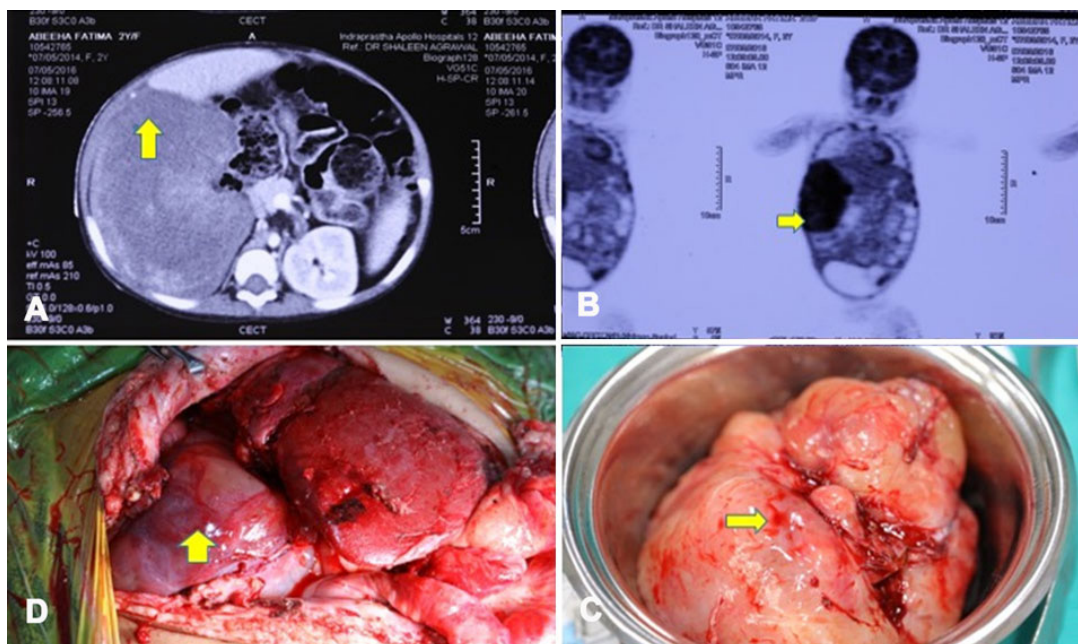


Figure 1: Clinical pictures of the case report. (A) Cross sectional image showing the tumor along the cut surface of the graft liver; (B) coronal section of the PET-CT image showing PET avid lesions; (C) intra-operative picture showing one tumor near the cut surface of graft liver; (D) intra-operative picture showing the tumor masses along with the transplanted liver. PET: positron emission tomography; CT: computed tomography

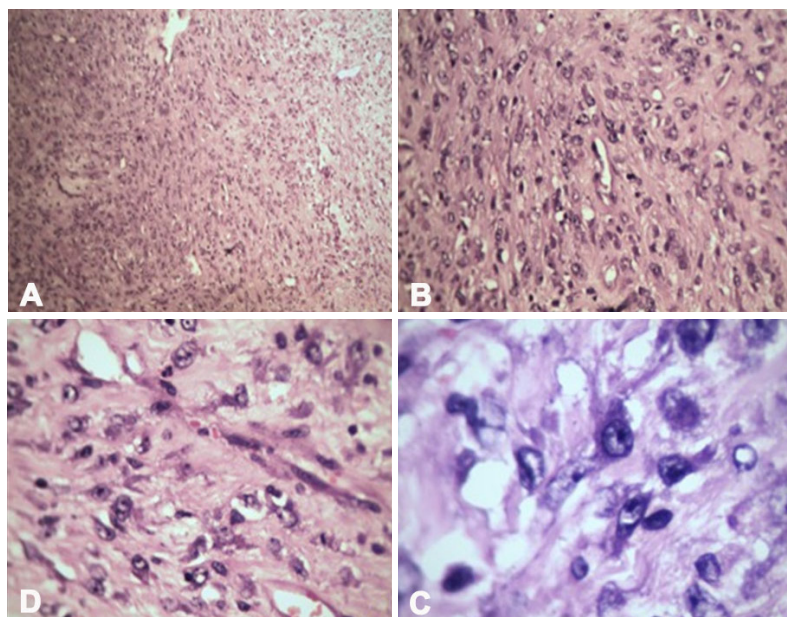


Figure 2: Various magnifications (A: $\times 4$; B: $\times 10$; C: $\times 20$; D: $\times 40$) of haematoxylin and eosin staining showing liver parenchyma along with few malignant spindle cells

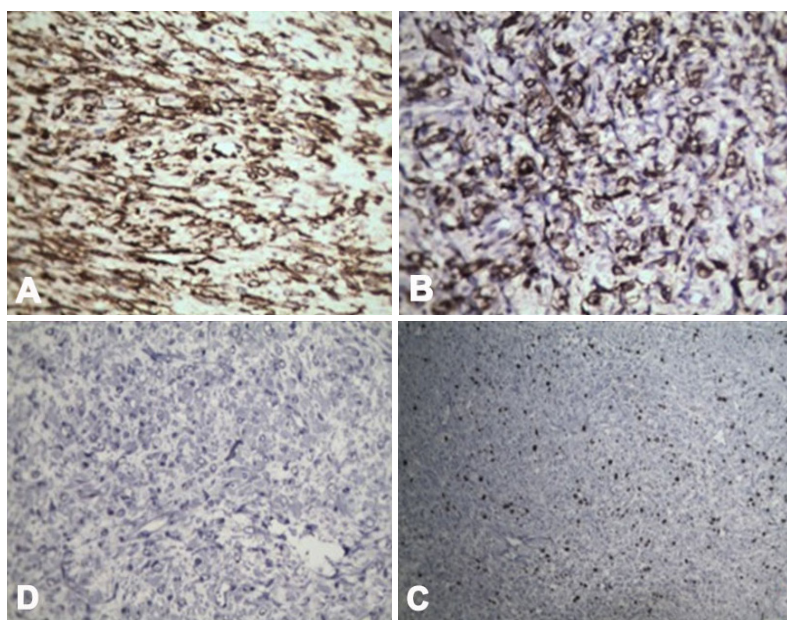


Figure 3: IHC panel. (A) Negative result of IHC for SMA; (B) positive result of IHC for ALK-1; (C) IHC for C-Kit shows tumor cells with mitotic index of less than 5%; (D) IHC for MIB index. IHC: immunohistochemistry; ALK: anaplastic lymphoma kinase; SMA: smooth muscle actin

with mass related symptoms.^[5] Sites of involvement are typically the abdomen, retroperitoneum and pelvis, followed by head, neck, upper respiratory tract, trunk and extremities. Increased morbidity may derive from its site in a vital organ or from aggressive treatment given due to a misdiagnosis of malignancy. Definitive histologic evaluation of the mass must be done to avoid unnecessary treatment-related complications. IMTs may rarely undergo malignant transformation, occasionally with distant metastases. The pathologic feature of IMTs is the proliferation of spindle cells

associated with a variably dense polymorphic infiltrate of mononuclear inflammatory cells (e.g. lymphocytes, plasma cells, histiocytes, and occasional eosinophils). Coffin *et al.*^[6] emphasized their neoplastic nature and proposed the use of the term inflammatory myofibroblastic tumor rather than inflammatory pseudo tumor. Other differential diagnoses include soft tissue sarcomas, gastrointestinal stromal tumors, lymphomas and other miscellaneous tumors. There is only one case report so far after deceased donor liver transplantation (DDLTL).

In our case, multiple preoperative biopsies were not confirmatory and led to delay in treatment. Final excision biopsy was subjected to extensive evaluation with IHC panel which included c-KIT, ALK, CD117, CD 45, CK-7, neuron specific enolase, chromogranin, alpha fetal protein, desmin, actin. The tumor in this case was negative for other IHC markers including those specific for hepatocellular carcinoma, cholangiocarcinoma, neuroendocrine tumors, gastrointestinal stromal tumors and lymphomas. However, the tumor had classic characteristics of IMT along with positive IHC for anti-lymphoma kinase. Complete tumor excision is curative in most cases.^[7] However recurrence at new sites can be prevalent. Ceritinib is a tyrosine kinase inhibitor accepted for treating tumors positive for ALK.^[8] The patient was given ceritinib for 1 year. Follow-up with PET CT at 1 year revealed no recurrence. Hence ceritinib was discontinued. At 2 years, the child is disease free with good graft function.

In conclusion: (1) although IMTs are rare, it is a serious complication after liver transplantation; (2) only one case has been reported in the literature so far after liver transplantation that too after DDLT; (3) in this case report we have described the first case of IMT after LDLT; (4) definitive histologic evidence is essential in their diagnosis and differentiation from other malignant tumors; and (5) IHC based targeted therapy may be helpful as adjuvant.

Authors' contributions

Doing surgery and writing of this manuscript: N. Selvakumar

Preparing the slides for histopathology: P. Saboti

Reporting of HPE: S. Kaul

Chief surgeon in charge of the case: S. Gupta

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None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent was received.

Ethics approval

Since it is not a study and just a report, ethical approval is not needed as per our hospital policies.

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Liver transplantation in acute-on-chronic liver failure

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Over the past five years, we have gained much new knowledge of the cirrhotic patient with liver failure, sick enough to require admission to hospital. In Europe, this has come from the outstanding work of the Chronic Liver Failure (CLIF) Consortium set up by Rajiv Jalan, Vicente Arroyo, and other leading hepatologists to which I will be mainly referring to.^[1] Shiv Sarin and colleagues in Asia Pacific despite using somewhat different definitions have reported similar findings and for their latest views on acute-on-chronic liver failure (ACLF). I would refer you to the excellent review of Sarin and Choudhury^[2] in *Nature Reviews Gastroenterology & Hepatology*, published in 2016. The characterisation of a syndrome of ACLF with defined subgroups has led to an improved prognostic assessment and provides a new basis for determining selection criteria for liver transplantation (LT) and of measures to enhance recovery from ACLF.

Some background first, on the massive clinical problem that hepatology faces from liver failure in Europe: 170,000 European citizens, it is estimated, die of cirrhosis each year - the 5th most common cause of death in individuals aged 45-65 years. Clinical decompensation heralded by ascites, hepatic encephalopathy, gastrointestinal bleeding

or bacterial infection develops in more than 50% of patients within 10 years of the diagnosis of cirrhosis. Most importantly, there is a dramatic worsening of prognosis when this leads to involvement of other organs - multi organ failure (MOF).

Data from a French study as recently as 2014 illustrates how poor the outcome has been and remains so in many hospitals throughout the world for cirrhotic patients treated in the intensive care unit (ICU) and requiring ventilator support.^[3] A third only of 246 consecutive patients became well enough to be discharged from the ICU and of these less than a half were alive at 1 year giving a 11% overall survival, 10 of the 27 survivors having had a liver transplant. The factors found to identify the risk of death after discharge, are measures of severity of the liver damage illness - bilirubin level, high Model for End-stage Liver Disease (MELD) score, on ventilator for > 9 days. Almost all of the patients in this study as they had respiratory failure will have had other organ involvement bringing them within the designation of ACLF.

The characterisation of ACLF by the CLIF Consortium was based on data from the EASL-CLIF Acute-



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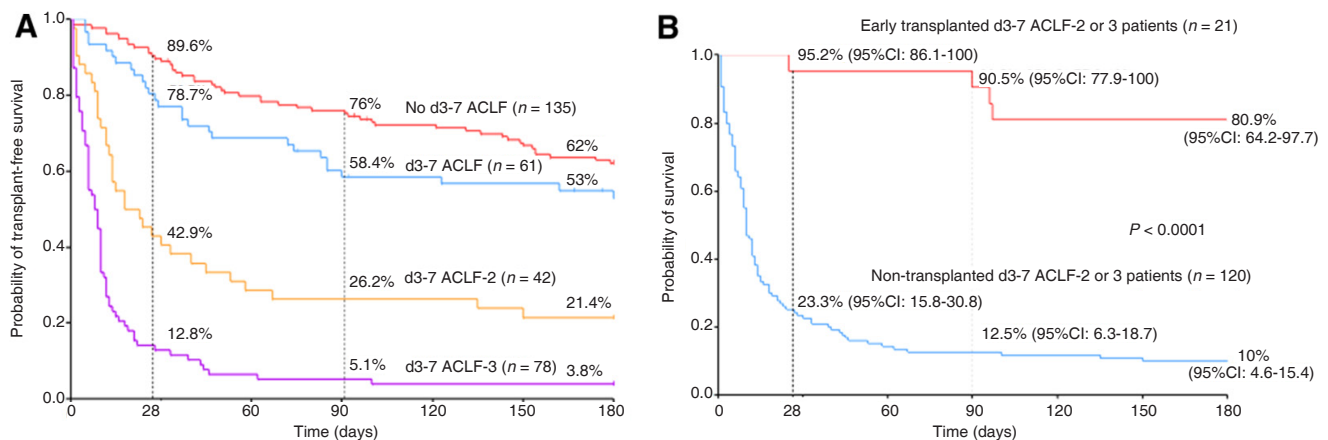


Figure 1: (A) Kaplan-Meier's 180-day transplant-free survival curves of patients based on their acute-on-chronic liver failure (ACLF) Grade at days 3-7 (d3-7 ACLF); (B) probability (180-day) of survival in patients with d3-7 ACLF-2 or -3 not transplanted and in patients undergoing early (28-day) liver transplantation. Kaplan-Meier's curves were compared using log-rank test. (Copyright Permission: Copyright © 2015 by the American Association for the Study of Liver Diseases. Gustot *et al.* Clinical Course of Acute-on-Chronic Liver Failure Syndrome and Effects on Prognosis. *Hepatology*. Publisher: Wiley)

on-Chronic Liver Failure in Cirrhosis (CANONIC) multicentre study of more than 1,300 patients with liver failure from cirrhosis admitted to 29 European hospitals. The subject of an excellent symposium published in the May 2016 issue of *Seminars in Liver Disease* with Rajiv Jalan as Guest Editor.^[1] ACLF is marked by rapid deterioration in liver function in a previously compensated or decompensated cirrhotic patient is accompanied by 1 or more other organ failures - kidney, brain, circulation, lungs and coagulation. Short-term mortality is high, more than 15% at 28 days. There is often a precipitating factor most frequently an exacerbation of liver damage from alcohol excess or HBV reactivation or the effects indirectly on the liver of a variceal bleed or infection. Interestingly, in 40% of cases no clear precipitating factor is identified. ACLF is to be distinguished clinically from acute decompensation in cirrhosis, with similar precipitating factors but which does not lead to failure of other organs apart from that of the liver and some form of non-kidney organ failure, and which has very much better overall prognosis with a < 5% mortality figure. Inflammation and the systemic inflammatory reaction is the driving force in the underlying pathophysiology as further indicated by high white cell and C-reactive protein levels.

It is important to take note of the dynamic nature of ACLF as evidenced by the findings of the CANONIC Study. With ACLF Grade 1 defined by 1 organ failure and mild renal impairment, over 50% of the cases resolve or improve. But with higher grades particularly Grade 3 when there are 3 or more organ failures, the percentage showing improvement is much lower (16%).^[4] These figures give some indication of the scope for LT in ACLF. Changes in clinical status occur rapidly in ACLF and relevant to the consideration of LT

is the observation that the final clinical grade is usually reached by day 7 and at that time the prognosis in the individual case can be reliably predicted.

The development of scoring systems for the quantitation of prognosis in ACLF and for acute decompensation without MOF represent a major step forward. The CLIF-ACLF prognostic score is based on the CLIF organ failure score for 3 categories of severity for the 6 potential organ failures, namely, liver, kidney, brain, coagulation, circulation and respiration is combined with age and the white cell count as independent predictors of outcome.^[5] The scoring ranges from 0 to 100 points. ACLF scores have been shown to have superior prognostic accuracy compared to MELD and other commonly used scores as a result of capturing the markers of inflammation so important in the pathophysiology of the syndrome in addition to the quantitative assessment of organ failure severity. The probability of death for an individual patient at any one time can be determined by calculation of the equation, using an app or through the CLIF Consortium website.

The major influence of the ACLF grade at days 3-7 in determining prognosis by the transplant free survival curve [Figure 1]. The top 2 curves comprising patients with single organ failures and normal or raised serum creatinine values; 62% and 53% are alive at 180 days. Whereas for grade 2 and 3 ACLF survival figures at 180 days are considerably reduced at 21.4% and 3.8% respectively. The other half of the figure shows how well patients with grade 2 or 3 ACLF can do when transplanted; 80.9% of the cohort of 35 patients transplanted within 28 days of diagnosis alive at 180 days and with little fall off in survival at 1 year (77%).^[4]

Excellent survival results in those receiving a liver graft were also shown in the series reported by Finkenstedt *et al.*^[6] from centres in Austria of 144 patients fulfilling ACLF criteria of which 94 (65%) were evaluated and 71 (49%) listed for a transplant. One- and five-year survival figures for the 32 (23%) patients transplanted were 87% and 82% respectively. Less than half of those who had got to the stage of being listed underwent transplantation and deaths on the waiting list were unacceptably high at 50% - a measure of the very short period of time available for these sick patients to obtain a donor organ. Only 10 (7%) of 144 patients in this series survived without a transplant - a similar figure to that for the French series of patients requiring mechanical ventilation shown earlier. At present ACLF is not considered an indication for priority or high urgency organ allocation despite the good outcomes that can be obtained.

An important question to ask is whether some of the deaths on the waiting list could have been prevented by the use of extra corporeal liver support devices, thereby giving more time for an organ to be obtained and allowing more patients in the grade 2 to 3 categories to be considered for LT. Currently the answer has to be "no". With the extracorporeal liver assist device containing a module of cultured hepatocytes (hepatoblastoma cell clone) providing synthetic and detoxifying functions in addition to toxin removal, survival as compared to the control group was improved only in those with a MELD score less than 28 and an age of less than 40 years, indicative perhaps of the potential for regeneration in this group 68.6 vs. 53.6 in controls ($P = 0.077$). In the major molecular adsorbents recirculating system trial of albumin dialysis, there was also no significant benefit overall with figures of 60.7% and 58.9% at 28 days for the treated and control groups despite improvement in some of the organ failures, namely, hepatic encephalopathy and circulatory disturbances.^[7] Possible reasons for this include the failure to correct the systemic reaction which is such an important part of the underlying pathophysiology of ACLF. Furthermore, in neither of the trials were the inclusion criteria based on CLIF diagnostic criteria and scoring. Of the new devices currently under clinical trial, one is based on membrane absorption of endotoxin from the circulation, the other has a more powerful microporous charcoal as the absorbent.

Turning to plasma exchange which is widely used in the Far East for the commonest form of ACLF, namely, hepatitis B reactivation. Replacement of the patients' plasma with its wide range of toxins and mediators by fresh frozen plasma is thought to facilitate liver regeneration and recovery. In each of

the three studies summarised [Table 1],^[8-10] there was a statistically significant improvement either in survival or in the obtaining of a reduced MELD score prior to LT. I would ask you also to take note of the very recently published study of high volume plasma exchange in acute liver failure (ALF) showing in those not transplanted, significant survival improvement.^[11] In ALF, the underlying dysfunctional immune reaction responsible for the multi-organ failure and susceptibility to sepsis is very similar to that which has been demonstrated in ACLF.

It is relevant also in the context of LT for ACLF to mention a number of therapeutic measures which may enhance resolution and improve the survival of patients with hepatic decompensation. These include reduction in bacterial translocation with rifaximin or probiotics and oral carbon for absorption of toxic bacterial products. Currently under clinical trial also is IV human serum albumin with its wide range of anti-oxidant and immunomodulatory effects. Enhancing liver hepatic regeneration through administration of G-CsF is another approach and there is some experience of this use in ACLF. The mechanism is thought to be mobilisation of hematopoietic stem cells to the liver leading to an increased number of CD34 positive progenitor cells stimulating the regeneration process. This was demonstrated in the study of Garg *et al.*^[12] leading to a significant survival benefit. Duan *et al.*^[13] also reported improved survival at 90 days, in ACLF from HBV reactivation in association with a rise in peripheral neutrophil and CD34 positive cell counts. Sarin and Choudhury^[2] from New Delhi have pioneered this exciting new therapeutic approach and more details of later studies are in the reference I gave at the beginning of this presentation.

In addition to the CLIF scoring, a number of biomarkers reflecting the severity of liver injury and of multi-organ failure have been identified which may add to prognostic information of the ACLF score and may be of particular value in early diagnosis and in assessing progression. Hyponatremia has been shown to have an independent predictive effect on 90 days survival and plasma copeptin reflecting changes in vasopressin level have been shown to improve

Table 1: Value of plasma exchange widely used in Far East for ACLF from HBV reactivation

Studies	Changes
Mao <i>et al.</i> ^[8] (2010)	30 days survival 50% vs. 31.7%
Ling <i>et al.</i> ^[9] (2012)	Reduced MELD prior to LT
Wan <i>et al.</i> ^[10] (2015)	12 weeks survival 29% vs. 14%

ACLF: acute-on-chronic liver failure; HBV: hepatitis B virus; MELD: Model for End-stage Liver Disease; LT: liver transplantation

performance of the ACLF score. A very recent 2016 publication showed increased values for urinary neutrophil gelatinase-associated lipocalin (N-GAL) related to stage and severity of cirrhosis as another independent biomarker of ACLF prognosis.^[14] N-GAL is the product of up regulation of the *cn2* gene in the liver driven by the processes of liver cell destruction.

Finally, to return to the selection and prioritisation of patients for transplantation in the clinical setting. Rajiv Jalan, Royal Free Hospital, London, suggests that ACLF scores of up to 30 are consistent with spontaneous recovery and patients should have serial assessments on a regular basis to determine whether this is occurring. With a 30 to 65 score, the patient is unlikely to survive without a transplant and depending on co-morbidity and other criteria should be given priority for an urgent transplant in anticipation of excellent results. A score above 65 raises questions of futility and withdrawal of active treatment measures. The subject is considered in some depth in a recent paper from Putignano and Gustot.^[15]

In summary, 3 points relating to transplantation in ACLF: firstly, improvement or worsening in ACLF grade occur rapidly and likely survival is best predicted at 3-7 days; secondly, transplantation gives good results in those with deteriorating ACLF grades 2 to 3 but timing, priority and selection criteria need to be defined; thirdly, liver support devices, plasma exchange, anti-inflammatory agents and stimulation of regeneration require further evaluation.

Authors' contributions

R. Williams contributed solely.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

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Not applicable.

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Roles of p53 in extrinsic factor-induced liver carcinogenesis

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Dr. Tomoo Iwakuma, M.D., Ph.D., is an associate professor in the Department of Cancer Biology at the University of Kansas Medical Center (KUMC). Dr. Iwakuma received his M.D. at Kyushu University in Japan, majoring in orthopedics in 1991. He also received his Ph.D. at the Department of Biochemistry at the same university in 1997. He spent several years as a research fellow studying gene therapy, pharmacology, and molecular genetics in different laboratories. Following postdoctoral training at the Department of Molecular Genetics at the University of Texas M.D. Anderson Cancer Center, he joined Louisiana State University Health Sciences Center in the Department of Genetics as an assistant professor on August 15, 2005. On August 1, 2011, he transitioned to KUMC as an associate professor. Dr. Iwakuma's primary research focuses on the field of cancer research, specifically on cancer progression and metastasis in bone and soft tissue sarcoma, head and neck squamous cell carcinoma, and liver cancer. Over 50% of human cancer has mutations in the tumor suppressor *p53* which regulates cell cycle progression, cell death, senescence, chromosome integrity, DNA repair, and metastasis. Therefore, understanding of the pathway involved in the regulation of *p53* is essential for discovering novel cancer therapies. With special focus on the tumor suppressor *p53* pathway, Dr. Iwakuma dissects the mechanism of cancer progression using genetically engineered mice, as well as tumor transplantation models, and applies disease models to translational research, to ultimately cure cancer.

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ABSTRACT

Liver cancer remains one of the most common human cancers with a high mortality rate. Therapies for hepatocellular carcinoma (HCC) remain ineffective, due to the heterogeneity of HCC with regard to both the etiology and mutation spectrum, as well as its chemotherapy resistant nature; thus surgical resection and liver transplantation remain the gold standard of patient care. The most common etiologies of HCC are extrinsic factors. Humans have multiple defense mechanisms against extrinsic factor-induced carcinogenesis, of which tumor suppressors play crucial roles in preventing normal cells from becoming cancerous. The tumor suppressor *p53* is one of the most frequently mutated genes in liver cancer. *p53* regulates expression of genes involved in cell cycle progression, cell death, and cellular metabolism to avert tumor development due to carcinogens. This review article mainly summarizes extrinsic factors that induce liver cancer and potentially have etiological association with *p53*, including aflatoxin B1, vinyl chloride, non-alcoholic fatty liver disease, iron overload, and infection of hepatitis viruses.



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INTRODUCTION

Liver cancer is the 6th most common cancer in men and the 9th most common cancer in women with the 3rd highest mortality rate of all cancers globally.^[1,2] The majority of these cases (about 80%) occur in Eastern Asia, South-Eastern Asia, Mid-Africa, and West Africa, within the context of viral hepatitis.^[2-4] Although there are genetic etiologies for hepatocellular carcinoma (HCC) including hereditary hemochromatosis and α 1-antitrypsin deficiency,^[5-7] viral hepatitis, as well as exposure to other extrinsic factors, such as aflatoxin B1 (AFB1), polyvinyl chloride (PVC), a poor diet inducing non-alcoholic fatty liver disease (NAFLD), and excess iron exposure, remain among the most common causes of liver cancer.^[8,9] Despite vaccinations for hepatitis B virus (HBV), new treatments for hepatitis C virus (HCV), regulations governing PVC production, and restrictions preventing AFB1 contamination of food products, countries still struggle to prevent liver cancer.^[9,10]

Surgical resection is currently the preferred treatment, and liver transplantation is ultimately the most effective therapeutic modality of HCC; however, it is limited by the availability of suitable organs.^[11,12] Due to a high

probability of being diagnosed at advanced stages, as well as poor responses to systematic chemotherapy and radiation therapy, prognosis of HCC is particularly bleak with an incidence to mortality ratio of 0.95 and a 5-year survival rate around 17.5%.^[2,13]

Molecular mechanisms involved in liver carcinogenesis remain unclear. The tumor suppressor p53, a transcription factor that regulates many downstream target genes regulating cell cycle progression, apoptosis, DNA repair, senescence, and metabolism,^[14,15] is one of the most commonly mutated genes in HCC.^[16,17] Indeed, p53 is the most commonly mutated human gene, occurring in > 50% of all human cancers.^[18] Additionally, in some HCC cases, proteins such as a 26S proteasome regulatory protein, gankyrin, and a p53-specific ubiquitin ligase, murine double minute 2 (MDM2), are elevated, hence decreasing p53 protein levels.^[19,20] MicroRNAs (miRNAs) can also inhibit p53 activity; specifically, *miRNA-24*, when dysregulated in HCC, is shown to promote invasion and metastasis by decreasing p53 levels.^[21] Thus, p53 activity is impaired by multiple mechanisms in HCC, hence contributing to HCC genesis. In this review article, we focus on HCC-inducing extrinsic factors that are etiologically associated with p53 [Table 1].

Table 1: Extrinsic factors causing liver cancer and their association with p53

Extrinsic factors	Mechanisms of action	Roles of p53	References
AFB1	AFB1 is metabolized to AFB1-8,9-epoxide to form AFB1-N ⁷ -guanine adducts, leading to specific mutation at p53 codon 249 (<i>p53</i> ^{R249S})	AFB1 frequently causes <i>p53</i> ^{R249S} mutation which enhances IGF-2 expression	[25,29,34]
VC	VC activated by CYP2E1 is converted into chloroethylene oxide, which forms bulky DNA adducts, leading to A>T transversions in the genome	It is unclear whether p53 plays protective roles in VC-induced liver cancer	[41,43,44]
NAFLD	NAFLD-induced hepatitis leads to cirrhosis and HCC, and dysregulation of NF- κ B signaling, the PI3K-ATK-PTEN pathway, insulin resistance, and expression of certain miRNAs (e.g. <i>miR-34</i>) is suggested; however, the molecular mechanisms behind NAFLD-mediated HCC remain unclear	The <i>miR-34</i> -SIRT1-p53 pathway plays a role in the progression of NAFLD. However, the direct role of p53 in the NAFLD-mediated HCC is unknown	[49,51-57]
Iron	Excess iron generates ROS and decreases p53 activity, leading to HCC genesis	Chronic iron overload reduces p53 protein levels by heme-mediated degradation or increased MDM2 levels, which can increase intracellular iron levels via a decrease in ISCUC2, thus further promoting HCC development	[64,68-70]
HBV	HBV-induced HCC occurs following repeated inflammation-liver regeneration-cirrhosis process, as well as through oncogenic function of HBx and Ct-HBx in both p53-dependent and -independent manners	Although direct involvement of p53 in HBV-induced HCC is unclear, functional inactivation of p53 by HBx and Ct-HBx may contribute to HCC progression	[76,81,82,85,99,100]
HCV	The majority of HCV-mediated HCC is via cirrhosis. But HCV core protein, NS3, and NS5 are implicated in HCC development in both p53-dependent and -independent manners	There is no direct evidence showing dependency of HCV-induced HCC on p53. However, HCV core protein, NS3, and NS5A inhibit p53 activity by binding to p53, altering subcellular localization, or modulating post-translational modifications	[112,118,119,123-125,131,132]

AFB1: aflatoxin B1; VC: vinyl chloride; NAFLD: non-alcoholic fatty liver disease; HBV: hepatitis B virus; HCV: hepatitis C virus; HCC: hepatocellular carcinoma; ROS: reactive oxygen species; MDM2: murine double minute 2; ISCUC2: iron-sulfur cluster enzyme 2; Ct-HBx: HBx variants with C-terminal truncations

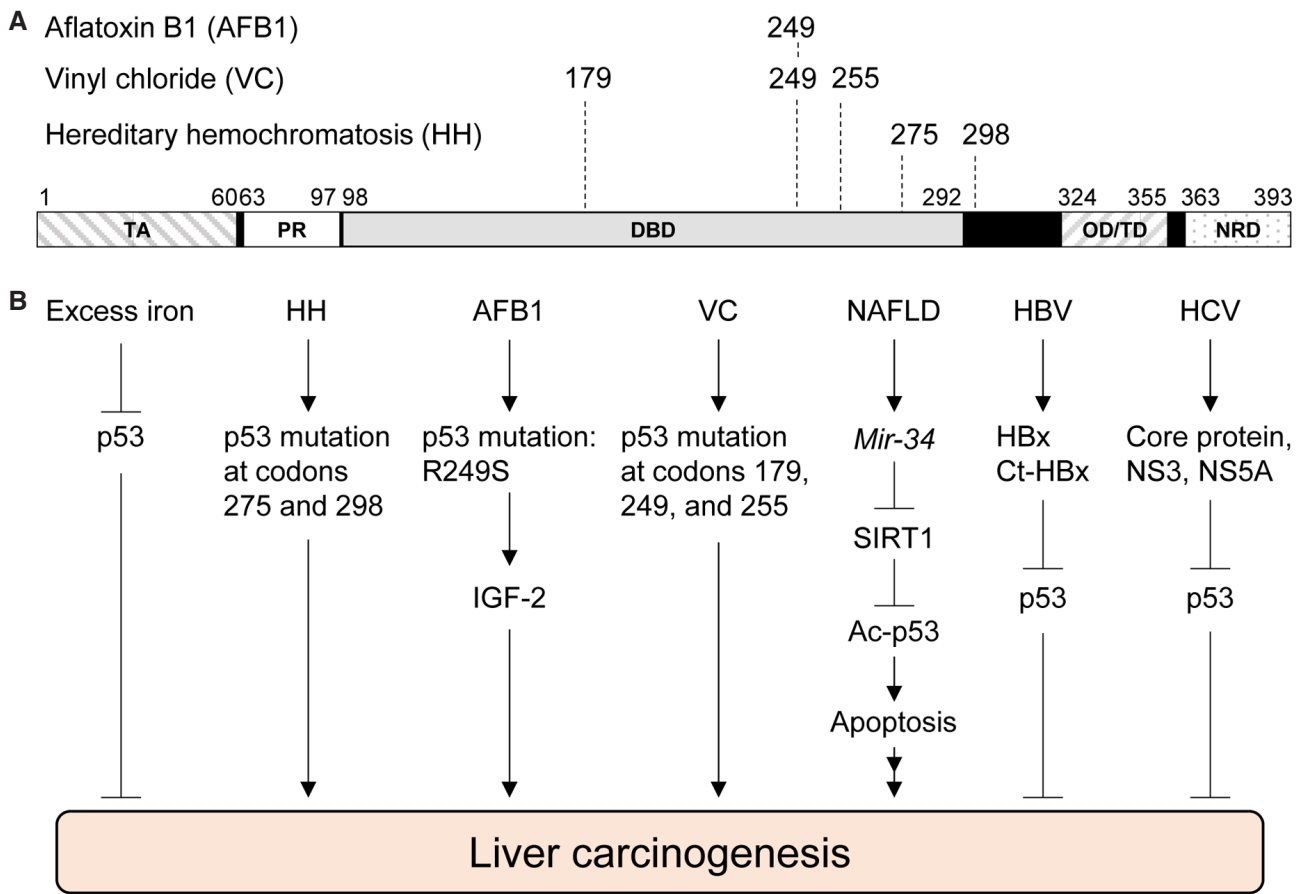


Figure 1: Functional roles of p53 in liver cancer-associated diseases. (A) Functional domains in human p53 and amino acid locations mutated in liver cancer associated with aflatoxin B1 (AFB1), vinyl chloride (VC), and hereditary hemochromatosis (HH). (B) Involvement of p53 in liver carcinogenesis. Multiple hereditary and extrinsic factors cause liver cancer possibly through the p53 pathway. TA: transactivation domain; PR: proline-rich domain; DBD: DNA-binding domain; OD/TD: oligomerization/tetramerization domain; NRD: negative regulatory domain; NAFLD: non-alcoholic fatty liver disease; HBV: hepatitis B virus; HCV: hepatitis C virus; SIRT1: sirtuin 1; IGF-2: insulin-like growth factor 2; Ct-HBx: HBx variants with C-terminal truncations

AFB1

AFB1 is a well-characterized liver mutagen produced by the fungus *Aspergillus*, and can be ingested by humans from contaminated food products.^[22,23] One study estimates the population attributable risk of AFB1-mediated HCC as 17% in some parts of the world.^[24] Mechanistically, AFB1 is activated by CYP40s into AFB1-8,9-epoxide, which reacts with DNA, forming 8,9-dihydro-8-(N⁷-guanyl)-9-hydroxyafatoxin B1 (AFB1-N⁷-guanine) adducts; these adducts, if left unrepaired, induce G>T transversions during DNA replication.^[25,26]

AFB1 is well-known to generate a specific p53 mutation in the DNA binding domain from an arginine to serine missense mutation at codon 249 (R249S), which is caused by a G>T transversion at the third base of codon 249 [Figure 1A].^[27,28] In geographic areas exposed to high levels of AFB1, such as the Qidong City in China, about 50% of HCC cases have the p53^{R249S} mutation,^[29]

suggesting the involvement of p53 in AFB1-induced HCC. AFB1-8,9-epoxide also reacts with guanines of the p53 gene other than those at codon 249, but these guanine adducts do not form cancer-causing mutations as frequently as p53^{R249S}.^[26,28,30] Although AFB1-mediated DNA damages initially activate p53 to induce cell cycle arrest at S to G2/M phases,^[31-33] liver cells that gain p53^{R249S} would escape this cellular defense mechanism with a selective advantage for proliferation, which could further proceed toward liver cancer. Indeed, p53^{R249S} is shown to increase transcription of insulin-like growth factor 2 (IGF-2) in Hep3B (p53^{null}) cells, suggesting a possible gain-of-function activity of p53^{R249S}.^[34] IGF-2 is over-expressed in 16-40% of human HCC and is implicated in promoting HCC progression.^[35] Also, a positive correlation is observed between IGF-2 +3580 AA genotype and the risk of HCC.^[36] Intriguingly, silencing of IGF-2 in HepG2 cells leads to decrease in cell survival and proliferation.^[37] Thus, AFB1-mediated mutation in p53 plays a crucial role in HCC genesis, possibly through enhanced IGF-2 signaling [Figure 1B].

VINYL CHLORIDE

Vinyl chloride (VC) is a carcinogenic gas used in the manufacture of PVC which induces mainly angiosarcomas of the liver (ASL) and rarely HCC, although it remains controversial whether VC can induce HCC in humans.^[38-40] VC is absorbed in the lungs and then metabolized to chloroethylene oxide by CYP2E1 in the liver, which forms bulky DNA adducts, leading to liver cancer.^[41,42] There are four VC-associated DNA adducts detected *in vivo*, including 7-(2-oxoethyl)-deoxyguanosine, 3,N⁴-etheno-deoxycytidine, 1,N⁶-etheno-deoxyadenosine, and N²,3-etheno-deoxyguanosine.^[41]

VC-induced human ASLs are reported to have an increase in A>T transversions at codons 179, 249, and 255 of the *p53* gene [Figure 1A].^[43,44] A study using Sprague Dawley rats also indicates that the majority of *p53* mutations in ASL and HCC following VC exposure are A>T transversions; the A>T transversions in ASLs are detected at codon 253 of rat *p53*, which is equivalent with codon 255 in humans.^[45] Moreover, serum samples from workers exposed to VC have an increase in the levels of p53 protein with mutant conformation, detected by a conformation-specific p53 antibody PAb240, as well as other antibodies for p53.^[44,46] However, it is still unclear whether p53 plays protective roles in VC-induced DNA damages and liver cancer development, and how mutations in *p53* contribute to the VC-induced liver cancer [Figure 1B].

NAFLD

NAFLD represents a range of disorders including non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and HCC. NAFLD is associated with metabolic syndrome, type 2 diabetes mellitus, and obesity.^[47] It is estimated that 20-30% of individuals in the Western world suffer from NAFLD.^[48] However, only 11.5% of patients with NAFLD-induced cirrhosis eventually develop HCC, and about 50% of NASH-induced HCCs occur without cirrhosis.^[49,50] These observations indicate the requirement of additional oncogenic events toward NAFLD-associated HCC. However, the molecular mechanisms behind NAFLD-mediated HCC are not fully understood. Several mediators have been implicated in its genesis, including dysregulation of NF- κ B signaling, the PI3K-ATK-PTEN pathway, insulin resistance, and expression of certain miRNAs (e.g. *miR-34*).^[51,52]

p53 has also been implicated in the progression of NAFLD due to multiple mechanisms. In a mouse

model for NAFLD where *p53*^{+/+} and *p53*^{-/-} mice are fed a methionine- and choline-deficient diet, *p53*^{+/+} mice show increases in histologically observable steatohepatitis, reactive oxygen species (ROS) formation, and fibrosis with increased protein levels of p66Shc, a protein associated with oxidative stress, as compared to *p53*^{-/-} mice.^[53] Human NASH hepatocytes display upregulated p53 activity with increased mRNA levels of *p21* and *p66Shc*, which is positively correlated with fibrosis severity.^[53] The *miR-34*-Sirtuin 1 (SIRT1)-p53 pathway is also implicated in NAFLD pathogenesis; increased *miR-34* expression and subsequent decrease in SIRT1 protein levels are detected in human NAFLD liver tissues with increased acetylation of p53, which is correlated with disease severity.^[54] Activation of the *miR-34a*-SIRT1-p53 axis is also shown to contribute to liver fibrosis or NASH by inducing hepatocyte apoptosis.^[55,56] Moreover, p53 can upregulate *miR-34*, which inhibits *SIRT1* mRNA expression, leading to increased acetylation of p53, thus forming a positive feedback loop [Figure 1B].^[57] These observations indicate that high expression of *miR-34* and p53 is associated with NAFLD. However, it should be noted that *miR-34a*-mediated apoptosis can occur in p53-dependent and p53-independent manners.^[58] Nonetheless, surrounding evidence suggests involvement of p53 in the progression of NAFLD and NASH; however, further studies are required to demonstrate whether p53 directly plays a crucial role in the NAFLD-mediated HCC.

IRON OVERLOAD

Iron is an essential mineral that takes part in numerous metabolic processes, such as heme synthesis, Fe-S cluster biogenesis, and oxygen transport via hemoglobin.^[59] However, when iron homeostasis is perturbed, whether due to genetic or environmental causes, there can be severe consequences including cardiomyopathy, hepatic fibrosis, endocrine disorders, and arthropathy.^[60,61] Importantly, excess iron is a risk factor for many types of neoplasia, including breast cancer, colorectal cancer, and HCC.^[62] In parts of sub-Saharan Africa, dietary iron overload, mainly from beer prepared in iron pots, is strongly associated with an increased risk of HCC.^[63] Experimentally, Wistar rats fed a high-iron diet are shown to develop HCC.^[64] One mechanism implicated in iron overload-mediated HCC genesis is due to ROS-inducing DNA mutations, as multiple rat models and surveys of human HCCs have linked increased iron levels with increases in 8-oxo-2-deoxyguanosine adducts and oxidizing products such as malondialdehyde.^[65-67]

However, there is evidence that iron overload has a

direct effect on p53 activity. C57BL/6 mice fed a high-iron diet show a decrease in p53 protein levels in the liver.^[68] Also, male Sprague-Dawley rats fed a high-iron diet for prolonged periods of time present with an increase of MDM2, and a subsequent decrease of p53 in the liver.^[69] Another molecular mechanism behind decreased levels of p53 due to iron excess includes that p53 is bound by heme, exported to the cytoplasm, and degraded in HepG2 cells via the proteasomal pathway.^[68] Thus, both iron excess and dysregulated heme decrease p53 levels, contributing to HCC development [Figure 1B]. Intriguingly, p53 is also involved in reducing intracellular iron levels by transactivating iron-sulfur cluster enzyme 2 which contributes to reduced iron uptake.^[70,71] Thus, following chronic iron overload, reduced p53 activity leads to increased intracellular iron levels, further promoting HCC genesis. It should be noted that patients with hereditary hemochromatosis show higher rates of p53 mutations (64-71%), as compared with those in sporadic HCC, supporting a role of p53 in iron overload-induced HCC genesis.^[72,73] In HCC tissues from hereditary hemochromatosis, 45% A>C transitions and 33% G>C transversions, including two hotspots at codon 275 and 298, are identified in the p53 gene [Figure 1A].^[73] However, in the study using British families with hereditary hemochromatosis, the p53 mutation spectrum consists of 60% A>G transitions and 40% A>T transversions.^[72] Nonetheless, it remains to be elucidated whether iron overload indeed induces HCC in a p53-dependent manner in animal models.

HBV

Globally, it is estimated that 248 million individuals have chronic HBV infection and are positive for the hepatitis surface antigen.^[74] HBV is the leading cause of HCC, with the majority being attributed to chronic HBV infection.^[75] HBV-mediated HCC tumorigenesis can be caused by repeated bouts of immune-mediated hepatocyte death and subsequent tissue repair, with eventual cirrhosis of the liver.^[76] Importantly, 10-30% of HBV-related HCCs do not occur in the background of cirrhosis, indicating additional oncogenic mechanisms behind HBV-induced HCC genesis.^[77]

HBV, a circular, partially double-stranded DNA virus, consists of four overlapping open reading frames in its genome: a core region, surface region, polymerase region, and X region which produce seven viral proteins named precore, core, polymerase, L, M, HBx, and S.^[78-80] Of these, the HBx protein, which plays a pivotal role in viral replication, is most implicated in HCC genesis.^[80] Indeed, HBx induces HCC by

sequestering p53 to the cytoplasm in transgenic mouse models [Figure 1B].^[81,82]

HBx is also implicated in hepatocyte apoptosis.^[78] In many contexts, HBx inhibits apoptosis not only by increasing levels of anti-apoptotic protein, survivin, but also by binding to and sequestering p53 to the cytoplasm.^[83-86] HBx is also reported to inhibit TGF- β -mediated apoptosis.^[87] Conversely, in some contexts, HBx is shown to induce apoptosis in a p53-independent manner.^[88-90] Hence, the dual roles of HBx in hepatocyte apoptosis and its association with HCC genesis warrant further investigation.

HBx variants with C-terminal truncations (Ct-HBx) are frequently detected in HCC and might also contribute to HCC development, though there is no direct evidence for it.^[91-93] Ct-HBx promotes hepatocyte proliferation and inhibits apoptosis in multiple cell lines.^[94-96] Transcriptional downregulation of ubiquitin specific peptidase 16 (USP16) by Ct-HBx is also shown to enhance tumorigenicity and stem-like properties of HCC cells.^[97] Moreover, Ct-HBx binds to p53 and inhibits p53-mediated apoptosis similar to HBx [Figure 1B].^[85,98,99] Additionally, some Ct-HBx variants have the ability to silence mRNA expression of GAS2, a modulator of p53-mediated apoptosis.^[100] Thus, Ct-HBx may contribute to the pathogenesis of HBV-related HCC by downregulating USP16 and inhibiting p53-mediated apoptosis.

Given that p53 is infrequently mutated in HBV-related HCC, p53 mutations are associated with late stage disease, and both HBx and Ct-HBx bind to and inhibit p53 function [Figure 1B],^[101-103] inactivation of p53 activity may be favorable for HBV-mediated HCC tumorigenesis, rather than p53 mutation. Importantly, HCC patients with wild-type p53 have better overall survival and an increase in recurrence free survival as compared with those having p53 mutations.^[104]

HCV

Hepatitis C is estimated to have a global prevalence of 184 million individuals positive for anti-HCV, and individuals with HCV have a 15 to 20 fold increased risk for HCC.^[105,106] HCV is a 9,600 nucleotide positive sense single-stranded RNA virus with a single open reading.^[107,108] The HCV genome encodes for a polyprotein that is subsequently cleaved into nine viral proteins, including structural proteins (C, E1, E2), and non-structural proteins (p7, NS2, NS3, NS4A, NS5A, NS5B).^[109] Although the vast majority of HCV-related HCCs occur within the context of cirrhosis, there is some evidence showing oncogenic potential

for the HCV viral proteins.^[77,110,111] Specifically, HCV core, NS3, and NS5 proteins have been implicated in HCC development in both p53-dependent and -independent manners.^[112]

Transgenic mice expressing the HCV core protein indeed spontaneously develop HCC, without the background of cirrhosis.^[113,114] HCV core protein also increases ROS, inhibits Fas- and TNF-mediated apoptosis, and upregulates the Wnt- β -catenin pathway.^[115-117] Importantly, the core protein inhibits p53 activity by altering its subcellular localization to the perinuclear region and nuclear granular structures, as well as its post-translational modifications such as phosphorylation and acetylation of p53 in HeLa and HepG2 cell lines [Figure 1B].^[118] Moreover, the core protein upregulates SIRT1, a deacetylation enzyme for p53, leading to impaired p53-dependent apoptosis in HepG2 cells [Figure 1B].^[119] Thus, HCV core protein likely causes HCC in both p53-dependent and -independent manners.

A non-structural HCV protein, NS3, is another HCV protein that can transform human hepatocytes with an increase in cyclooxygenase-2 and activation of mitogen-activated protein kinase.^[120-122] NS3 also complexes with p53 in HeLa and NIH3T3 cells^[123,124] and inhibit p53's transcriptional activity in NIH3T3 and Huh7 cells [Figure 1B].^[124,125] Moreover, NIH3T3 cells transformed by overexpression of NS3 can form tumors in mice.^[126] However, it remains unclear whether transformation by NS3 is p53-dependent or not.

Another non-structural HCV protein, NS5A, can cause steatosis and HCC in transgenic mouse models.^[127] NS5A is shown to inhibit TNF α -mediated apoptosis, transactivate c-fos, and inhibit Bax-mediated apoptosis independent of p53.^[128-130] However, NS5A can also bind to and colocalize with p53 to the perinuclear membrane, leading to inhibition of p53 transcriptional activity [Figure 1B].^[131,132] Moreover, NS5A binds with hTAF_{II}32 at the nucleoplasm membrane and inhibits its ability to stabilize p53, resulting in abrogation of p53-mediated apoptosis in Hep3B cells.^[132] Thus, NS5A contributes to HCC development and progression through p53-dependent and -independent mechanisms.

CONCLUSION

In summary, there is a large body of data indicating p53's involvement in extrinsic factor-induced liver carcinogenesis. Nonetheless, demonstrating *in vivo* evidence for the protective role of p53 in HCC genesis is crucial. While many of the aforementioned

risk factors for liver cancer have become preventable or treatable, efficient therapeutic strategies are still limited. Hence, understanding the role of p53 in the molecular pathogenesis of HCC and restoring p53 activity in tumors would significantly help accelerate the development of new therapies for this therapy-resistant disease.

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Authors' contributions

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Design, writing and revision of the paper: T. Link, T. Iwakuma

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

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Not applicable.

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Detection of urine DNA markers for monitoring recurrent hepatocellular carcinoma

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ABSTRACT

Aim: This study aimed to explore the potential of detecting hepatocellular carcinoma (HCC)-associated DNA markers, *TP53* 249T mutations and aberrant methylation of *RASSF1A* and *GSTP1* genes, for monitoring HCC recurrence. HCC remains a leading cause of death worldwide, with one of the fastest growing incidence rates in the US. While treatment options are available and new ones emerging, there remains a poor prognosis of this disease mostly due to its late diagnosis and high recurrence rate. Although there are no specific guidelines addressing how HCC recurrence should be monitored, recurrence is usually monitored by serum-alpha fetal protein and imaging methods such as magnetic resonance imaging (MRI). However, early detection of recurrent HCC remains limited, particularly at the site of treated lesion. **Methods:** Here, the authors followed 10 patients that were treated for a primary HCC, and monitored for months or years later. At these follow-up visits, urine was collected and tested retrospectively for 3 DNA biomarkers that associate with HCC development. **Results:** This 10-patient study compared detection of urine DNA markers with MRI for monitoring HCC recurrence. Five patients were confirmed by MRI for recurrence, and all 5 had detectable DNA biomarkers up to 9 months before recurrence confirmation by MRI. **Conclusion:** Overall, this suggests that detection of HCC-associated DNA markers in urine could provide a promising tool to complement detection of recurrent HCC by imaging.

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INTRODUCTION

Liver cancer is the sixth most common malignant neoplasm in the world and the second leading cause of cancer death worldwide, with an estimated 782,000 new liver cancer cases and 746,000 deaths during 2012.^[1] Hepatocellular carcinoma (HCC) constitutes 70-85% of all types of liver cancer.^[2] The high mortality rate of HCC (where 85% of patients die

within 5 years) is mainly due to late detection and a high recurrence rate.^[1-5] Rates of recurrence range from 15% for liver transplantation to nearly 100% for surgery or ablation.^[6-10] Recurrence is most common within 2 years.^[11]

Recently, a reduced recurrence rate has been reported for hepatitis B virus (HBV)-associated HCC with concomitant antiviral therapy following initial



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tumor ablation.^[12-16] The high HCC recurrence rate can be attributed to (1) incomplete treatment; (2) micro-metastases within the liver; and (3) *de novo* lesions.^[4,17] With improved assay, combination of alpha fetal protein (AFP), lens culinaris agglutinin-reactive alpha-fetoprotein (AFP-L3%) and des-gamma-carboxyprothrombin (DCP) has been claimed sensitive for HCC surveillance.^[18,19] Nonetheless, early detection of recurrent HCC has been difficult with the currently available diagnostic methods and serial imaging.^[7-9,20-22] Notably, there are no specific guidelines addressing how HCC recurrence should be monitored. Magnetic resonance imaging (MRI)/computed tomography (CT) imaging is the gold standard for diagnosis, although it is expensive and has limited utility in the detection of small tumors (< 2 cm), tumors in the presence of previously treated lesions (especially from local ablation), cirrhosis, obesity, and dysplastic nodules.^[8,9,20] Thus, there is an urgent unmet medical need to have a sensitive test for monitoring HCC recurrence.

Cancer is a disease of the genome and epigenome, and detection of the underlying genetic mutations and epigenetic modifications in the periphery may allow us to detect cancer early.^[23,24] Previously, Su *et al.*^[25-29] demonstrated that fragmented cell-free DNA in urine contains DNA derived from the solid tumors including HCC and colon cancer, if such a tumor is present. They also demonstrated that cancer-related DNA (both mutated and methylated DNA), including HCC-derived DNA modifications, could be detected in the urine of patients with cancer.^[27,29-31]

In this study, we demonstrate the feasibility of early detection of recurrent HCC by detecting three known HCC associated DNA modifications: *TP53* 249T mutation (shortened *TP53m*), and aberrant promoter methylation of Glutathione S-transferase pi 1 (*mGSTP1*) and Ras association domain family 1 isoform A (*mRASSF1A*) genes in urine as compared to the MRI imaging in a small ($n = 10$) blinded prospective study. These three DNA markers were chosen because of the availability of sensitive, cell-free DNA suitable PCR assays that target frequently altered genes in major pathways associated with hepatocarcinogenesis. They were previously demonstrated to be detectable in body fluids such as blood and urine of patients with HCC, regardless the level of serum AFP.^[29,30,32,33] They therefore serve as potential biomarkers for HCC.

METHODS

Patient selection

To explore the potential of the three HCC markers for monitoring HCC recurrence in urine, 10 HCC

patients with a history of HBV infection were studied at the Liver Disease Prevention Center, Division of Gastroenterology and Hepatology, Thomas Jefferson University Hospital, Philadelphia. After curative tumor ablation, patients were monitored for recurrence by MRI and serum AFP. Urine specimens were prospectively obtained when available. The urine was retrospectively examined for the presence of the three HCC DNA biomarkers. All patient samples were obtained with written informed consent and under institutional board approval from Thomas Jefferson University Hospital, Philadelphia, PA.

Urine DNA analysis

Urine collection, storage, and DNA isolation were carried out with written informed consent from patients as described previously.^[34,35] DNA from specimens was isolated and fractionated to obtain low molecular weight (LMW) urine DNA (< 1 kb size). Bisulfite (BS) treatment of DNA was performed using the EZ DNA Methylation-Lightning™ Kit (Zymo Research, Irvine, CA) following manufacturer's guidelines. Three DNA modifications, *TP53* 249T mutation (*TP53m*), aberrant promoter methylation of *GSTP1* (*mGSTP1*), and aberrant promoter methylation of *RASSF1A* (*mRASSF1A*), were quantified in duplicate using assays kits, *TP53* 249T qPCR kit, *mGSTP1* qPCR kit, and *mRASSF1A* qPCR kit (JBS Science Inc., Doylestown, PA), as per manufacturer specification.

RESULTS

To compare the detection of urine DNA markers to the currently available diagnostic methods (serum AFP and MRI imaging) for the diagnosis of HCC recurrence, urine DNA marker values were measured in a blinded fashion and plotted alongside serum AFP at the time of each collection (as shown in **Figure 1** and described in "METHODS"). Briefly, urine samples were collected prospectively from HCC patients (when available) after curative treatment at follow-up visits. The samples were retrospectively analyzed for the HCC DNA biomarkers. For data analysis purposes, we plotted "positive (Pos)" as the time of confirmed recurrence by MRI, and "negative (Neg)" when MRI did not detect recurrence. Of the 10 patients with > 6 months of monitoring with urine DNA markers, cases 1-5 had recurrence of HCC confirmed by MRI.

Recurrent patients had one or more of the three DNA markers examined found in urine before or at the time of MRI diagnosis. One recurrent case (case 5) died of progressive HCC. Case 6 was lost for follow-up during the period of the study. Four patients (cases 7-10) had no recurrence confirmed by MRI. Their urine DNA

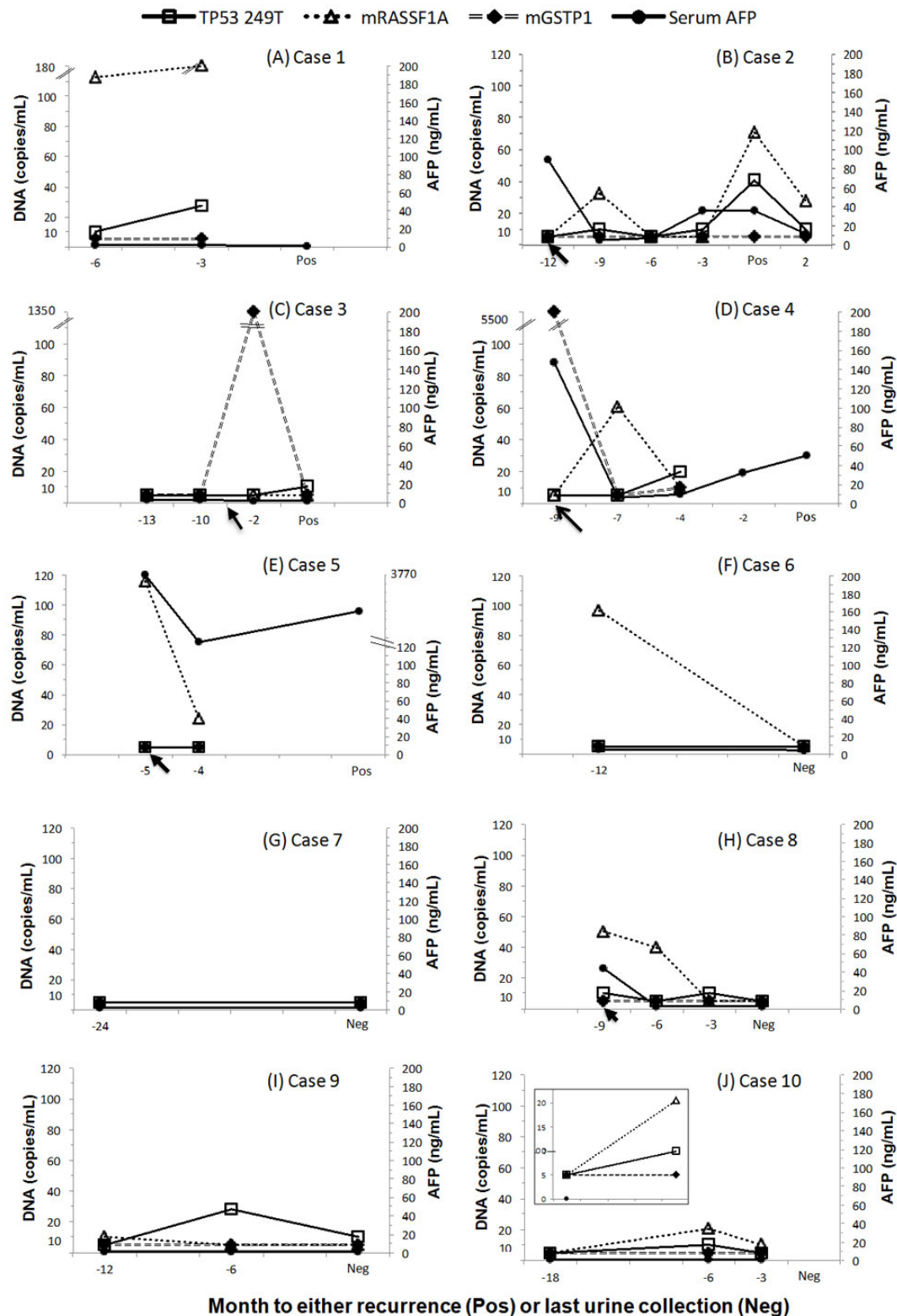


Figure 1: DNA biomarkers levels in serial urine samples from 10 patients. All patients were being monitored for HCC recurrence by MRI and serum AFP. The urine samples were collected prospectively from HCC patients (when available) after curative treatment (indicated by arrows) and at follow-up visits. Samples were retrospectively measured for HCC DNA biomarkers in a blinded fashion, with a follow-up MRI diagnosis of whether or not recurrence was detected. Three DNA biomarker values (copies/mL urine), *TP53* 249T mutation (*TP53m*), methylated *RASSF1A* (*mRASSF1A*) and methylated *GSTP1* (*mGSTP1*), along with serum AFP (ng/mL serum), were plotted at office visits until the last available visit in which an MRI was performed. The “Pos” represents detection of HCC recurrence by MRI and the “Neg” represents no recurrence was detected by MRI at the time of the visit. HCC: hepatocellular carcinoma; MRI: magnetic resonance imaging; AFP: alpha fetal protein

markers were either not detected (case 7), fluctuated (case 8), or detected at low levels (cases 9 and 10).

Case 1

A 68-year-old male underwent transarterial chemoembolization (TACE) for HCC. Six years later, he showed tumor recurrence (Pos) by MRI. Urine specimens were obtained at 6 and 3 months prior to the MRI confirmation of recurrence (indicated as -6, -3 on the X-axis, [Figure 1A](#)). In the urine specimens, *TP53m* and *mRASSF1A* markers were detected at 6 months prior and increased at 3 months before MRI detection of recurrence. Unfortunately urine is missing at the time of MRI imaging. His serum AFP levels remained at 2 ng/mL throughout the study, indicating the tumor was AFP-negative.^[36] He later received liver transplant.

Case 2

A 73-year-old male underwent TACE for HCC. Urine samples were collected after the treatment and during the follow-up period of 12 months when the tumor recurred ([Figure 1B](#)). Three months after the initial TACE treatment (indicated by a black arrow on the X-axis), *TP53m* and *mRASSF1A* levels were elevated while serum AFP had returned to a baseline level of 5.3 ng/mL from 88.9 ng/mL at the time of TACE treatment. These two urine DNA markers dropped to baseline on the next visit 3 months later. The *TP53m* and serum AFP levels rose again about 3 months prior to the detection of recurrence by MRI. At the time of detection of the second recurrence (marked “Pos”), both *TP53m* and *mRASSF1A* levels were elevated. Serum AFP level was at 36.4 ng/mL, indicating a rise from the baseline. Two months after the second treatment, serum AFP, *TP53* and *mRASSF1A* all decreased. The patient did not return after this visit.

Case 3

A 55-year-old male with a 4-cm HCC received TACE. The tumor recurred 5 years later, which was treated with microwave ablation (indicated by the black arrow on the X-axis; [Figure 1C](#)). The tumor recurred again during a follow up appointment 3 months later (marked “Pos”; [Figure 1C](#)). Urine DNA markers at two visits prior to the first recurrence were below the level of detection. However, *mGSTP1* was elevated 1 month after microwave treatment. Interestingly, when the tumor recurred for a second time (1.6 cm) 3 months after treatment, the *mGSTP1* was undetectable while *TP53m* was elevated. This may indicate the heterogeneity of HCC. Note, the serum AFP levels were below 20 ng/mL in the period of study.

Case 4

A 54-year-old male diagnosed with HCC and elevated

AFP. Urine was collected at the time of diagnosis and treatment with microwave ablation ([Figure 1D](#)). The DNA marker *mGSTP1* was highly elevated in urine at the time of HCC diagnosis. Two months after treatment, both urine *mGSTP1* and serum AFP levels decreased to the normal range while urine *mRASSF1A* was elevated. At the next visit 3 months later, *mRASSF1A* decreased but remained detectable while the two other DNA markers, *TP53m* and *mGSTP1* increased. Four months later, an MRI detected a recurrent tumor (solid lesion). Unfortunately, the urine was not collected at “-2” and at the time of diagnosis “Pos”, hence there is no marker data available at these time points.

Case 5

A 56-year-old male underwent TACE for HCC. Urine was collected on the day of treatment and at a follow-up visit 1 month later ([Figure 1E](#)). The *mRASSF1A* marker was detected in the urine on the day of TACE treatment, and the levels of *mRASSF1A* in the urine dropped one month following treatment. Similarly, serum AFP levels decreased nearly 10-fold from 3,770 ng/mL to 323 ng/mL. However, MRI 4 months later detected HCC recurrence and increased levels of serum AFP (1,522 ng/mL). No urine samples were collected at this time point or later. Despite receiving another TACE treatment, the patient passed away 8 months later.

Case 6

A 56-year-old male with HCC underwent TACE. Urine samples were collected at 3 and 4 years after TACE. *mRASSF1A* was found elevated at 3 years and negative at 4 years post TACE. The patient has had no recurrence ([Figure 1F](#)). AFP was in normal range. The patient was lost for follow up.

Case 7

A 58-year-old male with HCC received TACE followed by radiofrequency ablation (RFA). Urine collection started 1 year after RFA. No biomarkers were detected 2 years post RFA, as the patient remained recurrence free ([Figure 1G](#)).

Case 8

A 62-year-old male with HCC received RFA. Urine samples were collected on the day of treatment and every three months after for 9 months ([Figure 1H](#)). Serum AFP, *TP53* mutation, and *mRASSF1A* levels were all elevated on the day of RFA, and decreased 3 and 6 months following the treatment to below the limit of detection. There has been no recurrence by MRI.

Case 9

A 27-year-old female was diagnosed with HCC at age 20 and the original tumor was treated 3 times with

TACE in a 3-year period. Urine was collected every 6 months starting 4 years after the last TACE. *TP53m* mutation was detected in the urine collected on the second visit and decreased, but remained detectable in the third urine sample as indicated in [Figure 1I](#). MRI suggested a mass in the liver, but the mass was not confirmed as recurrent HCC. The serum AFP levels were below 20 ng/mL in the period of study. The patient has been on antiviral treatment since the diagnosis of HCC.

Case 10

A 66-year-old male with HCC underwent RFA followed by resection. He has had no recurrence for the past 10 years. Two urine samples were collected at 8 years (-18) and 9 years (-6) after resection [[Figure 1J](#)]. Serum AFP is normal, and none of the DNA markers were detected until 6 months prior to the MRI, when the *TP53m* and *mRASSF1A* markers were elevated [[Figure 1J](#)]. *TP53m* reverted to baseline and *mRASSF1A* levels declined 3 months later (-3). At the time of MRI testing, there was no HCC recurrence detected from the visit.

DISCUSSION

This study demonstrates the potential applicability of using urine DNA markers in combination with serum AFP for the early detection of HCC recurrence in a small 10-case study. HCC recurrence is known to be the major factor for poor prognosis. In this small 10-case study, MRI identified recurrence in 5 out of 10 patients (cases 1-5). Encouragingly, for all 4 recurrent patients that remain in the study (cases 1-4), urine DNA markers were found to be elevated in urine samples as early as 9 months before MRI confirmation.

Although this is a small longitudinal 10 patient study, the potential of these urine DNA markers for management of HCC recurrence and important characteristics of HCC recurrence is demonstrated. First, for all remaining recurrent cases (cases 1-4), DNA markers were elevated before or at the time of diagnosis by MRI imaging. MRI/CT imaging is the gold standard for diagnosis of recurrent HCC, but has difficulty in detecting early recurrence in the previously treated areas (especially after local ablation). This may explain why the DNA markers were found in urine earlier than MRI diagnosis. Secondly, HCC, like other cancers, is a disease of the genome. Detection of genetic drivers of HCC may provide not only sensitive and earlier detection for monitoring HCC recurrence, but may also provide HCC genetic information to assist in patient management. Furthermore, since collection of urine can potentially be done at home

and then shipped to certified laboratories for testing, the urine screening may result in better compliance while not requiring a doctor's office visit. A larger longitudinal study will be needed to explore the application of urine DNA markers in monitoring HCC recurrence. Lastly, the levels of DNA biomarkers in urine may also be useful to measure effectiveness of cancer treatments that induces apoptosis of tumor cells. We have shown that circulating tumor DNA found in urine was mostly from apoptotic tumor cells.^[28,34] The treatment that induce apoptosis should increase the amount of tumor derived DNA deposited in the blood and secreted into urine. This could be the circumstance for cases 2, 3 and 4 where an elevated *mRASSF1A* or *mGSTP1* marker was detected after the treatment, suggesting the potential to use urine DNA markers to monitor effectiveness of therapy that induces tumor cell apoptosis.

Finally, HCC is often recognized as being multi-clonal. Interestingly, in recurrent case 3, *mGSTP1* levels returned to not detectable in urine while *TP53m* was elevated in the urine collected 3 months later with the MRI report of a 1.6-cm lesion. We speculate that the rising of the *TP53* mutated clone was different from the previously treated tumor nodule and was either not responding to the treatment or was derived from tumor evolution. Furthermore, it is possible that apoptosis of a small tumor nodule through immune system targeting could lead to a temporary rise in DNA markers associating with that tumor. For example, for case 6 the *mRASSF1A* was found elevated at 3 years and negative at 4 years post TACE, where recurrence was not detected.

It is important to note that the levels of urine DNA markers can fluctuate for several reasons including hydration of the patient at time of collection (which can result in diluted DNA in the urine). Therefore, the use of an internal control is important for appropriately setting cutoffs for the urine marker values. While urine protein creatinine is the most used internal control for urine concentration, our pilot study has suggested the concentration of LMW cell-free DNA in urine does not correlate with urine creatinine. Further studies are needed to identify a proper internal control for this work, which is currently in progress.

In conclusion, we have demonstrated that urine DNA biomarker testing may have potential for the early detection of HCC recurrence. A larger longitudinal study design to collect well-annotated serial patient samples is in progress, specifically to monitor for HCC recurrence, to test whether this urine DNA test

can overcome the inherent limitations of imaging technology, and to provide a highly sensitive tool for monitoring HCC recurrence.

Authors' contributions

Concept/design: H.W. Hann, S. Jain, W. Song, Y.H. Su
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Conflicts of interest

W. Song is an employee and shareholder of JBS Science, Inc.; S. Jain and J. Steffen are employees of JBS Science, Inc; all other authors declare they have no other competing interests that exist.

Patient consent

In this prospective study, each patient was informed of this study and gave their consent.

Ethics approval

This study was conducted under the approval of the Institutional Review Board at Thomas Jefferson University Hospital, Philadelphia, PA.

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Management of advanced hepatocellular carcinoma: review of current and potential therapies

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ABSTRACT

Over the past few years, despite improvement in screening and diagnosis of hepatocellular carcinoma (HCC), advanced stage remains the most common presentation at diagnosis, with limited management options, especially options available to patients in limited resource countries. There is currently no effective systemic chemotherapy, targeted, or immunologic therapy for advanced stage HCC. Sorafenib is the only approved front-line molecular-targeted treatment, with slight survival benefit. Regorafenib has recently been approved as second line therapy for HCC after failure of sorafenib. Ongoing research on molecular agents targeting different pathways, combination therapies, and immunotherapy, represent hope for new treatment modalities. This manuscript reviews current treatment, ongoing research, and potential future treatments for advanced HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death globally, with about 800,000 deaths every year.^[1] Unfortunately, considerable number of patients are diagnosed at advanced stage unsuitable for surgery or local treatment with poor prognosis and a median overall survival (OS) of about 6 months. Molecular targeted therapies have demonstrated promising efficacy in the management of cancer. Sorafenib improves survival with median OS rate of 6.5-10.7 months,^[2] with significant benefit in time to progression, despite the absence of objective response. Numerous trials are ongoing in search for other molecular agents that

are more effective than sorafenib, or combinations of therapy that might improve response and survival rates in patients with advanced HCC. Results of trials with lenvatinib as first-line or regorafenib as second line treatments are promising.^[3] Radioembolisation is as safe and effective in advanced-stage HCC as first-line or second-line therapy.^[4] In addition, the use of immunotherapy in clinical trials demonstrated promising results.^[5] This manuscript reviews the current treatments and ongoing research for therapy of advanced HCC.

ADVANCED HCC

Despite advances in screening and diagnosis, most



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patients with HCC are diagnosed with advanced disease [Barcelona Clinic Liver Cancer (BCLC) stage C], unsuitable for surgery or loco-regional curative treatment. Prognosis of advanced HCC is poor, and the 1-, 2- and 3-year survival rates are 29%, 16%, and 8%, with median OS less than 6 months.^[6] This is even less in low and middle income countries, where resources and access to therapy are limited.

In the BRIDGE study that included data for 18,031 patients with HCC from 42 sites in 14 countries in Asia, Europe and North America, more than 50% of cases were diagnosed in advanced and terminal stages.^[7] Whereas 12% of HCC patients in Japan and 17% in Taiwan present in advanced stage HCC (BCLC stage C) and only 1-2% in terminal stage (BCLC D), between 50% and 60% of HCC patients in North America, Europe, China and South Korea present in BCLC stages C and D.^[7]

The proportion of HCC patients presenting in advanced stages is even larger in limited resource countries. A study that included 2,566 HCC patients from 21 tertiary referral centers in 9 African countries showed that only 23% presented in early and intermediate stages (BCLC A and B), 49% in advanced stage (BCLC C) and 28% in BCLC stage D. The proportion of patients diagnosed with HCC while in advanced or terminal stage was much larger in sub-Saharan countries, with 95% of the patients diagnosed in BCLC stages C and D, and 97% of 1,315 patients with HCC did not receive HCC specific therapy because of advanced stage and unavailability of therapy.^[8]

This highlights the importance of developing effective therapies for advanced HCC, and that these therapies should be made available and affordable in limited resource countries where most patients present in advanced stage (especially sub-Saharan Africa). This also emphasizes the importance of screening programs for detection of HCC in early treatable stages.

SYSTEMIC THERAPIES

Molecular targeted therapy

Advanced HCC has experienced the most relevant advancements in research in HCC lately. Hepatocarcinogenesis is associated with genetic and epigenetic alterations that eventually lead to an alteration in the molecular pathways, leading to uncontrolled growth of the hepatocytes.^[9]

Sorafenib

Sorafenib inhibits the serine-threonine kinases Raf-

1 and B-Raf and the activity of vascular endothelial growth factor receptors (VEGFRs) 1, 2, and 3 and platelet-derived growth factor receptor β (PDGFR- β). Also it induces down-regulation of anti-apoptotic proteins, leading to significant enhancement of the cytotoxicity of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) to HCC cells.^[10]

Sorafenib improves survival compared to placebo, with median OS of 6.5-10.7 months, with significant benefit in time to progression (TTP). Many molecular agents have been studied, but only the sorafenib showed efficacy in terms of OS and TTP, based on results of two phase III, randomized controlled studies^[2,11] and confirmed in other clinical trials comparing sorafenib to other molecules,^[12-16] as well as in real life clinical practice.^[17,18]

Despite the approval of sorafenib in advanced-stage HCC, several issues remain not known. An important concern is to identify patients who will most probably benefit from sorafenib, to avoid unnecessary toxicity in patients who will not. Several markers at baseline or during treatment, as vascular endothelial growth factor, angiopoietin-2, hepatocyte growing factor, c-Kit or alfa-feto-protein (AFP) have been shown to predict OS, but not response to sorafenib in patients with advanced HCC.^[19]

Several reports showed that the development of some adverse events as dermatological adverse events,^[20-22] diarrhea,^[23] or arterial hypertension^[24] are associated with favorable outcomes. Patients who develop early dermatological adverse events within the first 2 months after starting sorafenib experienced a longer median OS, comparing to those who did not develop this adverse event (18.2 vs. 10.1 months, respectively).^[20] Hence, it is mandatory to closely follow the patients and to adjust the dose if needed to avoid unnecessary interruption of the drug in a probably responding patient.

Using sorafenib in patients with Child-Pugh B cirrhosis is challenging. Sorafenib was effective in Child-Pugh class B patients as class A patients in terms of progression free survival (PFS), but with lower OS. The median OS was 5.5 months for Child class B patients compared to 11.3 months for Child A patients.^[25] The prospective GIDEON trial confirmed that the median OS was shorter in Child-Pugh class B patients, 5.2 months compared to 13.6 months in Child A, although the TTP and the incidence of adverse events of sorafenib was similar across subgroups, Child-Pugh class B patients experienced more serious adverse events. The liver dysfunction in advanced cirrhosis may

impair the effect of sorafenib on tumor progression and interfere with possible survival improvement.^[18]

Eventually 60-70% of patients with advanced HCC progress on sorafenib. The pattern of progression on sorafenib has been identified as a predictor of post-progression survival.^[26,27] The development of new extra-hepatic lesion, vascular invasion, and worsening performance status on therapy were associated with the poorest prognosis.

For patients in advanced-stage who progress on or cannot tolerate sorafenib, management options are limited, and a large unmet need still exists. However, results of trials with lenvatinib as first line therapy^[28] and regorafenib^[29] and immunotherapy as effective second line treatments are promising.

Other molecular targeted agents

First-line therapy

Sorafenib remains the only approved first line therapy with advanced-stage (BCLC-C) HCC. None of the other targeted agents: the anti-angiogenic tyrosine kinase inhibitors (TKI) sunitinib,^[12] linifanib,^[15] brivanib,^[13] dovitinib^[16] or the combination of sorafenib with erlotinib^[14] were found superior compared to sorafenib in phase II and III trials as first line therapies in patients with advanced HCC, and none have exceeded the benefits of sorafenib [Figure 1, Supplementary Table 1].

Lenvatinib, on the other hand, is showing promising results as first line therapy in advanced HCC.^[3] Lenvatinib is an oral TKI that targets VEGFR1-3, fibroblast growth factor receptor (FGFR)1-4, rearranged during transfection (RET), receptor tyrosine kinase (KIT), and PDGFR and is approved for radioactive iodine-refractory thyroid cancer. A multicenter, open-label, phase I/II study of lenvatinib, including 46 patients with advanced HCC. Tumor response and stable disease were found in 37% and 45.7%, respectively,

with median TTP of 12.8 months and median OS of 18.7 months. The most common adverse events observed with lenvatinib were hypertension, diarrhea, anorexia, weight loss, and fatigue.^[30]

Lenvatinib was investigated as first line therapy compared to sorafenib in a multicenter, randomized, open-label, phase III trial that included 954 patients with intermediate or advanced stage HCC. The OS with lenvatinib was non-inferior to sorafenib, and PFS, TTP, and objective response rate (ORR) significantly improved with lenvatinib (NCT01761266).^[31] Lenvatinib thus is the first agent to show results that are equal or better than sorafenib in advanced stage HCC, and might become an alternative to sorafenib as first line treatment.

Second-line therapy

Figure 2 shows OS of second line therapies compared to sorafenib.^[29,32-34] Regorafenib, a multi-kinase inhibitor of VEGFR1-3, tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (TIE2), PDGFR β , FGFR, KIT, RET, rapidly accelerated fibrosarcoma (RAF), is approved for metastatic colorectal cancer and advanced GI stroma tumors.^[35,36] It is the first agent to provide survival benefit after failure of sorafenib in a phase III trial and has recently been approved as second line therapy for HCC. The study included 573 patients who had progressed on sorafenib and it improved OS with a hazard ratio of 0.63 ($P < 0.0001$); the median OS was 10.6 months for regorafenib vs. 7.8 months for placebo and the disease control rate (DCR) was 65.2% vs. 36.1% ($P < 0.001$).^[29]

Ongoing studies are evaluating the efficacies [tyrosine-protein kinase Met or hepatocyte growth factor receptor (HGFR)] (c-MET) inhibitors in advanced HCC. A multicenter, randomized, placebo-controlled phase II trial evaluating tivantinib, a selective c-MET inhibitor, included patients with advanced HCC

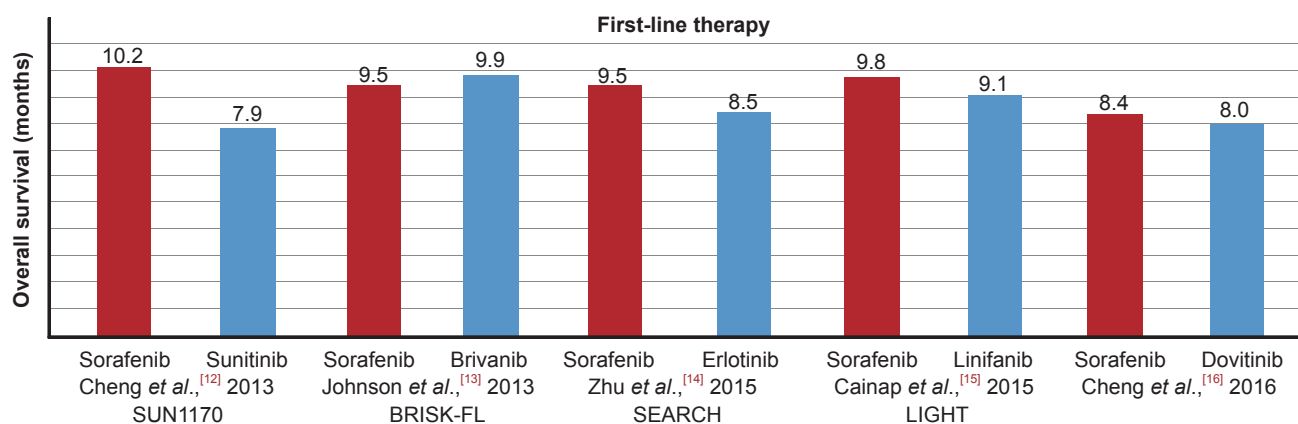


Figure 1: Overall survival in trials of first-line therapy vs. sorafenib for advanced hepatocellular carcinoma

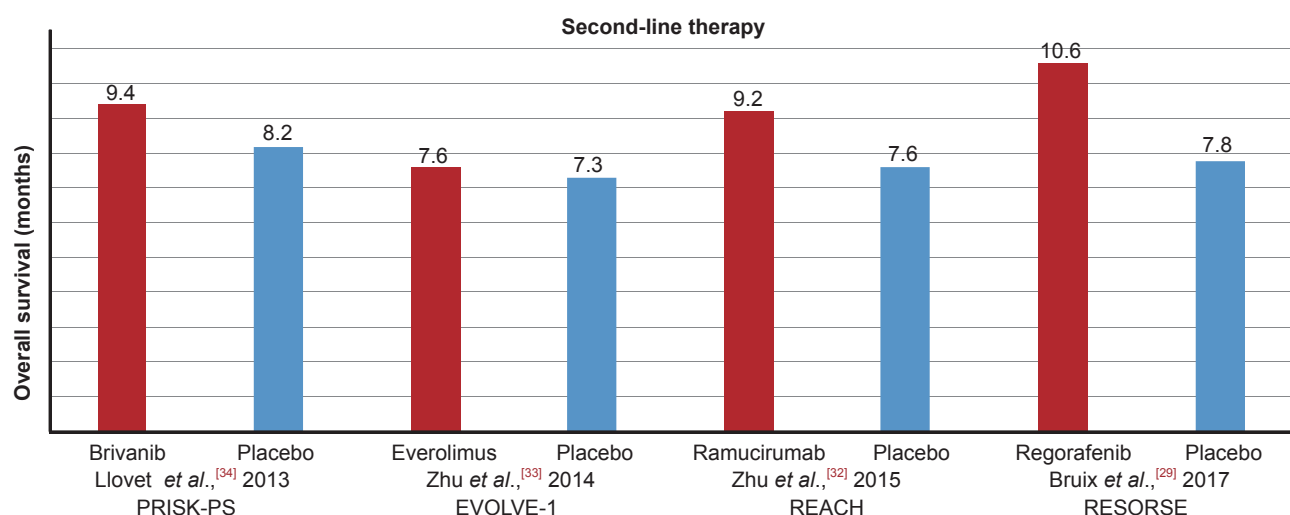


Figure 2: Overall survival in trials of second-line therapy after progression on sorafenib vs. placebo for advanced hepatocellular carcinoma

and compensated cirrhosis, who were refractory or intolerant to sorafenib. Patients treated with tivantinib showed longer TTP than with placebo (1.6 vs. 1.4 months, hazard ratio (HR) 0.64, 90% CI 0.43-0.94, $P = 0.04$) with DCR of 20%.^[37] Patients with high c-met expression showed better OS, and prolonged TTP on tivantinib (OS was 7.2 vs. 3.8 months, $P = 0.01$), and the DCR increased to 50%.^[37] A phase III trial testing tivantinib as second line treatment is ongoing based on better outcome observed in patients with high c-met expression (NCT01755767). Other c-MET inhibitors as foretinib, tepotinib, capmatinib, golvantinib and emibetuzumab are also under investigations.

Ramucirumab, an anti VEGFR monoclonal antibody approved as 2nd line treatment for advanced gastric adenocarcinoma and metastatic lung cancer, was evaluated vs. placebo in a phase III trial as second line treatment in patients with HCC who progressed or were intolerant to sorafenib (REACH study). It demonstrated significant improvement in OS and DCR.^[32] Subgroup analysis showed the survival benefit was limited to patients with baseline AFP ≥ 400 ng/mL (median OS 7.8 months with ramucirumab vs. 4.2 months in the placebo group).^[38] Patients Child-Turcotte-Pugh (CTP) score 5 had better overall response to treatment than patients with CTP scores 6-8, and among those with elevated AFP ≥ 400 ng/mL, improvement with therapy was limited to patients with CTP score 5 and 6 and not in patients with CTP score 7-8 (who experienced higher incident of treatment related grade 3 or higher adverse events).^[39] The phase III REACH-2 trial will evaluate ramucirumab as second-line therapy in patients with advanced HCC and elevated AFP (NCT02435433).

Mammalian target for rapamycin (mTOR) inhibitors in phase I/II studies showed activity in patients with advanced or recurrent HCC.^[40] However, in a phase III

study (EVOLVE-1) everolimus did not improve OS in patients with advanced HCC who had failed or were intolerant to sorafenib (median OS, 7.6 months with everolimus, 7.3 months with placebo; HR 1.05; 95% CI 0.86-1.27; $P < 0.68$).^[33]

Axitinib, a potent and selective inhibitor of VEGFRs 1-3, was investigated as second-line therapy for HCC in a randomized phase II study compared to placebo. Longer PFS ($P = 0.004$) and TTP ($P = 0.006$) was observed in patients treated with axitinib compared to placebo. However, no improvement in OS was detected.^[41]

Apatinib, a selective VEGFR-2 tyrosine kinase inhibitor, in a multicenter, randomized, open-label, phase II trial showed potential survival benefit in patients with advanced stage HCC with Child-Pugh class A liver function at either 850 mg/qd or 750 mg/qd. Apatinib was well tolerated and the main AE were elevated aminotransferases, thrombocytopenia, leukocytopenia, hyperbilirubinemia, hypertension, hand-foot syndrome and fatigue.^[42] Apatinib is being evaluated as second line therapy in patients with advanced HCC who have progressed on, or were intolerant to, sorafenib (NCT027720290), and other anti-angiogenic agents are in very early-stages of development [Supplementary Table 2].

Combination of different molecular targeted therapies

Inhibition of a single signaling pathway may induce feedback activation of other pathways, hence, combination of different molecularly targeted agents possibly induces synergistic beneficial activity.^[43] Molecular targeted agents other than sorafenib, used in combination or with sorafenib, are being

investigated and showed encouraging results in phase II studies. Currently several phase III studies evaluating combination of multiple targeted agents for treatments of advanced HCC are ongoing.

In a phase III study, the combination of sorafenib and the EGFR-TKI erlotinib in advanced HCC (SEARCH study) did not significantly improve survival over sorafenib alone.^[14] Similarly, the combination of the mTOR inhibitor everolimus with sorafenib did not improve the efficacy of sorafenib in patients with unresectable or metastatic HCC.^[44]

Refametinib, an oral mitogen-activated protein kinase (MEK), or mitogen-activated protein kinase (MAPK)/ERK kinase] inhibitor, combined with sorafenib, improved survival in patients with advanced stage HCC (DCR: 43%, TTP: 122 days, OS: 290 days) with patients with rat sarcoma (RAS) mutations experiencing the best clinical response. However, grades 3 and 4 adverse events were reported in about 80% of cases.^[45] An ongoing trial is evaluating the combination in patients with unresectable or metastatic HCC with RAS mutation (NCT01915602).

Clinical trials of sorafenib in combination with the transforming growth factor receptor beta (TGF- β) inhibitor galunisertib (LY2157299), the mTOR inhibitor temsirolimus, and the histone deacetylase (HDAC) inhibitors vorinostat and resminostat are currently ongoing.

Immune therapy for HCC

Programmed cell death ligand 1 (PDL-1) are expressed on HCC tumor cells. These interact with PD-1 receptors on activated T cells, leading to their inactivation, thus enhancing tumor-cell survival. Blocking the PDL-1-PD-1 receptor interaction increases tumor necrosis.^[46]

Nivolumab is a fully human IgG4 monoclonal antibody inhibitor of the programmed death-1 (PD-1) receptor that restores T-cell-mediated antitumor activity. Treatment with nivolumab has extended survival in multiple tumor types, and it is approved for metastatic melanoma, metastatic non-small cell lung cancer, advanced renal cell carcinoma, Hodgkin lymphoma, and recurrent or metastatic squamous cell carcinoma of the head and neck.^[47]

Nivolumab was evaluated in a multiple ascending-dose, phase I/II study in patients with advanced-stage HCC, refusing, intolerant, or progressing on sorafenib with preserved liver function (CTP score up to B7) (Checkmate-040 trial).^[48] Patients received intravenous nivolumab 0.1-10 mg/kg every 2 weeks for up to 2

years, and demonstrated 72% OS rate at 6 months with durable responses across all dose levels and HCC cohorts (ORR:15%, DCR: 65%), with manageable AE profile. These responses were observed regardless of viral hepatitis infection status. In the expansion phase, nivolumab was well tolerated in patients with HBV and HCV related HCC.^[48] OS rates for all patients at 6 and 9 months were 82.5% and 70.8%, respectively. Disease stabilization was observed in patients who previously progressed on sorafenib, 91 of 204 patients (45%) had reduction in tumor burden and 45 (22%) had $\geq 30\%$ reduction in tumor burden compared to baseline.^[48] A phase III trial evaluating nivolumab vs. sorafenib as first-line treatment in patients with advanced HCC is ongoing (Checkmate-459, NCT02576509).

Cytotoxic-T-lymphocyte-antigen (CTLA)-4 blockade could be an efficient alternative in advanced HCC. In a phase II study, the CTLA-4 inhibitor tremelimumab showed partial response rate 17.6%, DCR 76%, TTP 6.5 months.^[49] However, 45% of patients experienced grade-3 aspartate aminotransferase and alanine aminotransferase elevations. Studies exploring combinations of these agents in a randomized, second-line setting are ongoing. A phase II study is currently evaluating the PD-L1 inhibitor durvalumab and tremelimumab alone or in combination for patients with unresectable HCC who progressed on, are intolerant to, or refused treatment with sorafenib (NCT02519348).

Systemic chemotherapy

Cytotoxic agents as 5-fluorouracil, cisplatin, doxorubicin, gemcitabine, capecitabin, epirubicin or combined regimens showed a low response rate (< 10%) with slight improvements in OS.^[50,51] Cisplatin, interferon, doxorubicin, and fluorouracil (PIAF) in combination showed favorable results in a phase II study. In a phase III study, PIAF combination compared to doxorubicin alone demonstrated no significant difference in OS (8.67 vs. 6.83 months) or in ORR (20.9% vs. 10.5%).^[51] Patients treated with the PIAF regimen experienced higher rate of myelotoxicity compared with doxorubicin.

The lower effect of doxorubicin in HCC is assumed to result from multidrug resistance (MDR) mechanisms. Doxorubicin-Transdrug (DT), a nano-particle formulation of doxorubicin, has been shown to enter HCC cells by diffusion, by passing the MDR proteins, and demonstrated higher intracellular concentration and effectiveness than doxorubicin.^[52] A phase III multicenter, randomized, controlled trial comparing the efficacy and safety of IV infusions of doxorubicin-transdrug in patients with advanced HCC after failure or intolerance to sorafenib (ReLive Study) is ongoing

(NCT01655693).

S-1, an oral mixture of tegafur, gimeracil and oteracil, that increases the effect of 5-fluorouracil through increasing its serum concentration while decreasing its gastrointestinal effects, was evaluated as second line therapy in a phase III trial in patients with advanced HCC refractory to sorafenib (S-CUBE). OS was not different from placebo, but PFS was better (80 vs. 42 days).^[53]

In a phase III study, 371 patients with advanced HCC were randomly assigned to receive either FOLFOX4 (infusional fluorouracil, leucovorin, and oxaliplatin) or doxorubicin (EACH trial). OS was higher in patients who received FOLFOX4 compared to doxorubicin (6.4 vs. 4.97 months, $P = 0.07$) and reached statistical significance after extension of follow up 7 more months ($P = 0.04$). FOLFOX4 treatment prolonged the median PFS in comparison to doxorubicin (2.93 vs. 1.77 months, $P < 0.001$), the response rate was 8.15% vs. 2.67% ($P = 0.02$), and the DCR was 31.55% vs. 52.17% ($P < 0.0001$) respectively.^[54] FOLFOX4 was well tolerated, although the incidence of neutropenia and neurotoxicity was slightly higher in the FOLFOX4 group.

Oxaliplatin (OXA)-based chemotherapy may be an effective first line treatment for patients with advanced HCC. In a meta-analysis^[55] that included 13 studies, 6 studies on gemcitabine, 6 studies on 5-fluorouracil or capecitabine and 1 study on doxorubicin in addition to OXA, the PFS was 3.3 and 4 months in capecitabine-based studies and OXA-based studies, respectively. OS was 6.47 months in capecitabine-based studies compared to 11 months in OXA-based studies.^[56]

Combination of sorafenib with systemic chemotherapy

Combination of sorafenib with doxorubicin,^[57] octreotide,^[58] 5-fluorouracil,^[59] tegafur/uracil,^[60] cisplatin and gemcitabine,^[61] gemcitabine/oxaliplatin (GEMOX),^[62,63] and capecitabine/oxaliplatin (SECOX)^[64] have been investigated [Supplementary Table 3]. Currently, modified FOLFOX plus sorafenib is under investigation (NCT01775501).

A randomized, double-blind phase II trial that included 96 patients with advanced HCC evaluated the efficacy of sorafenib in combination with doxorubicin vs. doxorubicin, and resulted in better OS (13.7 vs. 6.5 months). The median TTP was 6.4 vs. 2.8 months, and PFS was 6.0 vs. 2.7 months, respectively.^[57] On the other hand, a phase III randomized study of sorafenib plus doxorubicin compared to sorafenib (CALGB 80802) showed higher toxicity for the combination

without improvement in OS or PFS.^[65]

The phase III SILIUS trial included 210 patients with advanced HCC, and compared sorafenib to sorafenib in combination with low dose cisplatin/fluorouracil hepatic arterial infusion chemotherapy (HAIC). OS was equal in both arms (11.8 months). However, sorafenib plus HAIC significantly improved OS in the subset of patients with major portal-vein invasion (11.4 months vs. 6.5 months).^[66]

LOCOREGIONAL THERAPY

The presence of portal vein thrombosis (PVT) is a relative contraindication for trans-arterial chemo-embolization (TACE) in most international guidelines,^[6,67,68] TACE may be recommended for HCC patients with vascular invasion if radiologic portal invasion is distal to, or in the second-order branches of, the portal vein (Vp1 or Vp2).^[69,70] Real life studies have confirmed the safety and efficacy of TACE in patients with PVT.^[71]

Combination of targeted therapy with loco-regional therapy

Several studies compared sorafenib plus TACE to sorafenib or TACE^[72-78] [Supplementary Table 4]. A meta-analysis of sorafenib in combination with TACE that included data of 1,254 patients found that the combination improved OS and TTP in advanced HCC, but not PFS, with higher rate of severe adverse reaction in the combination group.^[79] This combination is being further evaluated in phase III study (STAH trial, NCT01829035) to evaluate combined sorafenib with conventional TACE vs. sorafenib in patients with TACE-refractory and advanced-stage HCC.

Randomized, controlled studies to evaluate the efficacy and safety of sorafenib combined with TACE in advanced HCC patients compared with sorafenib alone (SELECT) (NCT01906216) or TACE alone (NCT02150317) are ongoing. The safety and efficacy of superselective drug-eluting chemoembolization with hepaspHERE in patients with unresectable advanced HCC is under investigation (SUPER-China, NCT02743065).

A phase II randomized controlled trial (RCT) conducted to explore the efficacy of sorafenib and TACE in advanced HCC patients with major portal vein invasion (NCT01480817) has been terminated and the results are awaited. Combination of TACE with apatinib (NCT03066557) or axitinib (NCT01352728) in patients with advanced HCC are under investigation. Also, comparing TACE plus sunitinib against TACE plus

placebo (SATURNE) (NCT01164202) is ongoing.

Hepatic arterial infusion chemotherapy

The Japanese Society of Hepatology and the Korean National Cancer Center both recommend hepatic arterial infusion chemotherapy (HAIC) in their guidelines for management of patients with advanced HCC and vascular invasion.^[69,80,81]

In a single-center study in Japan, the HAIC using 5-fluorouracil and pegylated interferon $\alpha 2b$ was investigated compared to sorafenib for treatment of advanced HCC. The early ORR was higher in the HAIC group than in the sorafenib group (71.4% vs. 10.5%, $P < 0.01$). The 18-month survival rate was 55.6% vs. 16.2%, $P = 0.03$ for the HAIC and sorafenib groups respectively.^[82]

A multi-center study that included 110 patients with advanced HCC found that HAIC using cisplatin and 5-fluorouracil with or without epirubicin, demonstrated higher treatment response rate (24% vs. 13.3%) and a better median OS (7.1 vs. 5.5 months) compared to sorafenib.^[83] A RCT is recruiting to elucidate the efficacy of HAIC of FOLFOX compared to sorafenib in treatment of advanced HCC (NCT02774187). A phase III randomized open label clinical trial to investigate the efficacy and safety of HAIC (using FOLFOX) compared with TACE in patients with HCC with major portal venous tumor thrombus is recruiting (NCT02856126).

The efficacy and safety of HAIC with cisplatin and 5-fluorouracil in patients who have progressed or were intolerant to sorafenib with non-metastatic HCC is being evaluated further, stratified by expression of biomarker predicting therapeutic response is ongoing (the SHINE study, NCT02967887).

Radioembolisation

Trans-arterial radio-embolization (TARE) using Yttrium-90 spheres is well tolerated with survival rates reported similar to TACE with fewer side effects, better response rate and longer time to progression.^[4,84-86]

TARE is as safe and effective as sorafenib in advanced-stage HCC.^[4,87-90] The median survival with TARE was 13.8 months compared to 10 months with sorafenib ($P > 0.05$), and complete response was only observed in 6.3% of patients in the TARE group.^[91] TARE alone or combined with sorafenib vs. sorafenib in BCLC stage B and C patients are under evaluation (NCT02288507). In a pilot study, sorafenib for 6-8 weeks before Yttrium-90 treatment for patients with unresectable HCC was safe and tolerable. The DCR was 72.4% and tumor necrosis was observed in 82.8% of patients.^[92]

A phase III RCT of Selective Internal Radiation Therapy (SIRT) versus sorafenib in advanced HCC (SIRveNIB) is ongoing (NCT01135056). A study evaluating the monoclonal antibody to PD-1 receptor nivolumab in combination with TARE is under way (NCT02837029). RCTs are ongoing to define the role of TARE as first-line or second-line therapy in advanced HCC.

CONCLUSION

Sorafenib is still the only approved front-line therapy, and several needs are still unmet and need to be addressed: the combination of local with systemic therapies, the optimal timing of molecular targeted agents in relation to loco-regional treatment, the combinations of systemic targeted therapies, and second-line therapies. Results of recent trials point to several promising therapeutic options: lenvatinib as front-line therapy, and regorafenib and nivolumab as second-line therapy. Several other molecules and combinations are in early stages of development, and more effective therapies will evolve over the next few years. However, improving screening and early detection, and improving access to therapy in limited resource settings are as important in improving global outcome of HCC.

Authors' contributions

Reviewed the literature and wrote the manuscript: A. Gomaa, I. Waked

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Conflict of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

Supplementary materials

1. Supplementary Tables 1-4
2. Supplementary References 1-24

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Use of gadobenate dimeglumine dynamic MRI for detection of early hepatocellular carcinoma in atypical hepatic focal lesions

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ABSTRACT

Aim: Gadobenate dimeglumine (multihance) is a contrast medium which can be used not only as an extracellular contrast agent for dynamic imaging of the liver, but also as a liver specific agent for the acquisition of hepatobiliary-phase images which are more helpful in evaluation of small atypical hepatic focal lesions equal or less than 3 cm. The authors tried to evaluate multihance dynamic magnetic resonance imaging (MRI) as a new modality in early detection of hepatocellular carcinoma (HCC). **Methods:** Thirty cirrhotic patients with small hepatic focal lesions (less than 3 cm in diameter), detected by imaging (ultrasound and triphasic computed tomography) were subjected to dynamic MRI with multihance contrast. All patients had a liver biopsy stained with heat shock protein 70, glypican 3, and glutamine synthetase to confirm the diagnosis of HCC. **Results:** Eight out of 30 patients (26.6%) with atypical focal lesions proved to have HCC by histology, whereas 7 out of 8 histologically proven HCC patients (87.5%) were shown to have typical criteria on Multihance imaging. **Conclusion:** Multihance dynamic MRI is a promising diagnostic modality for detection of early HCC, however, future studies on large numbers of patients are warranted to precisely detect the sensitivity and specificity of this new modality.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary tumor of the liver with a global incidence of approximately half a million cases per year.^[1] It is a rapidly fatal cancer that mostly affects persons in

developing countries where hepatitis B and hepatitis C viruses are endemic.^[2] Cirrhosis is the most important risk factor for HCC; overall, one third of cirrhotic patients will develop HCC during their life time.^[3] Portal supply gradually decreases in accordance with higher grades of malignancy of the nodules and finally disappears in



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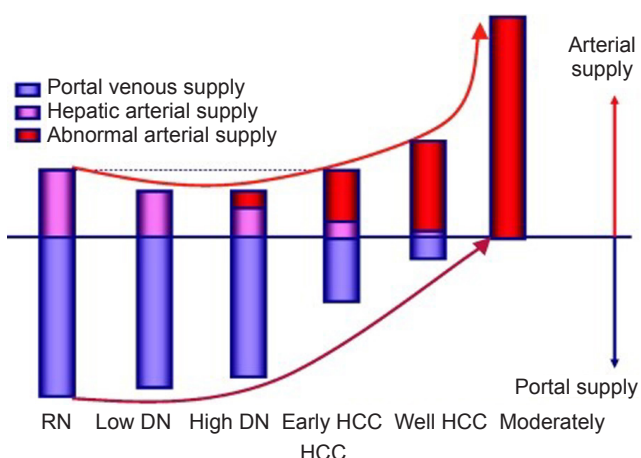


Figure 1: Multistep hepatocarcinogenesis.^[4] HCC: hepatocellular carcinoma; DN:dysplastic nodule, RN: regenerating nodules

moderately differentiated HCCs [Figure 1].^[4]

Nowadays, early diagnosis of HCC is feasible only in 30-60% of cases in developed countries. However, the percentage is much less in developing countries. Early diagnosis enables the application of curative treatments.^[5,6] Thus there is an urgent need to identify better tools to characterize these atypical small lesions.

Diagnosis of small HCC lesions lacking typical hemodynamic criteria could be a challenge. Atypical enhancement patterns and hypovascular HCC lesions seen in a considerable number of HCC patients have led to around 35% false negative results in patients with tumors between 1-2 cm in diameter in triphasic computed tomography (CT) scan.^[7-9]

Extracellular contrast agents in magnetic resonance imaging (MRI) are suited to liver imaging applications that require information obtained during the dynamic contrast-enhanced phase or excellent vascular visualization such as Gadobutrol (Gadovist US, Gadovist EU, Bayer) and Gadoversetamide (Optimark, Covidein).^[10]

Hepatobiliary agents are suited to applications focusing on biliary visualization and distinguishing between hepatocytes and lesions not containing hepatocytes e.g. Gadobenate Dimeglumin (Multihance, Bracco), Gadoxetate Disodium (Eovist US). These agents have extracellular properties but also have affinity for hepatocytes.^[11]

Multihance can be used not only as a non-specific extracellular contrast agent for dynamic imaging of the liver, but also as a liver specific agent for the acquisition of hepatobiliary-phase images. Lesions that contain functioning hepatocytes where hepatobiliary

metabolism is mostly unaltered are expected to uptake multihance and excrete the compound into the bile. Such lesions are typically benign and usually appear isointense or hyperintense as compared to the normal liver parenchyma in the hepatobiliary phase of MRI. In contrast, lesions lacking functioning hepatocytes where hepatobiliary metabolism is blocked or inhibited are generally not able to uptake and excrete multihance into the bile. Such lesions are typically malignant and usually appear hypointense as compared to the normal liver parenchyma on the hepatobiliary phase of MRI.^[10,11] Therefore, multihance dynamic MRI is a potential promising diagnostic modality for detection of early HCC.

METHODS

This prospective study was conducted at National Liver Institute, Menoufia University. The study protocol was approved by the Institutional Review Board (IRB) and local ethical committee. A written informed consent was obtained from all participants in the study.

The study was conducted on 30 adult cirrhotic patients with atypical hepatic focal lesions. Patients were recruited from the outpatient HCC clinic of the National Liver Institute, Menoufia University. Patients were enrolled from October 2014 to June 2015.

Inclusion criteria

Cirrhotic patients with a single hepatic focal lesion not more than 3 cm in diameter detected by ultrasound with atypical enhancement pattern on triphasic CT scan and dynamic MRI and alpha fetoprotein (AFP) level less than 200 ng/mL.

Exclusion criteria

Hepatic focal lesions more than 3 cm, typical HCC lesions on triphasic CT, portal vein thrombosis, extrahepatic lymph node metastasis, metastatic lesions, AFP more than 200 ng/mL or previous HCC treatment. Patients with Child class C decompensated cirrhosis in whom liver biopsy is contraindicated were excluded from the study.

Patient were selected on the basis of clinical presentation, liver function profile, complete blood picture, imaging procedures including ultrasonography, triphasic CT showing atypical enhancement pattern. Dynamic MRI with multihance contrast was used to detect HCC. Histopathological study of biopsy specimens from the lesions was performed using hematoxylin and eosin (HE) and immunohistochemical staining with glypican 3 (GLP3), heat shock protein

Table 1: Patient and tumor characteristics

Variable	Number
Mean diameter of nodules (cm)	
HCC	2.1
Non-HCC	1.2
Laboratory tests	
ALT (U/L)	35 ± 28
AST (U/L)	45 ± 30
Total bilirubin (mg/dL)	1.0 ± 0.5
Albumin (g/dL)	4.0 ± 0.7
INR	1.2 ± 0.2
Platelets(x10 ⁹ /L)	135 ± 80
HCC differentiation	
Well differentiated	6 (75%)
Moderately differentiated	2 (25%)
Poorly differentiation	
AFP (ng/mL)	14.6 ± 27.1

HCC: hepatocellular carcinoma; ALT: alanine transaminase; AST: aspartate transaminase; INR: international normalized ratio; AFP: alpha fetoprotein

70 (HSP70) and glutamine synthetase (GS). All the biopsy specimens were examined by the same pathologist.

RESULTS

A total of 30 patients with liver cirrhosis and atypical hepatic focal lesions were included in the study. The mean age was 56.1 ± 11.6 years (range 39-72 years). The majority of patients were males (24 out of 30 patients, 80%).

Chronic hepatitis C virus infection was the cause of cirrhosis in 25 out of 30 patients (83.3%), whereas chronic hepatitis B was the cause in 5 patients (16.7%). Nineteen patients had Child class A cirrhosis (63.3%) while 11 patients had Child class B cirrhosis (36.7%).

AFP was 8-121 ng/mL with mean 14.619 ± 27.187 [Table 1]. The mean diameter of the nodules was 19 mm. Pathological examination of liver biopsies by HE and immunostaining GLP3, GS and HSP70

Table 2: MRI with multihance findings in all patients

Finding description	HCC patients	Non-HCC patients
Hypointensity on T1	4	5
Hyperintensity on T2	5	6
Arterial hyperintensity	7	7
Delayed hypointensity	0	2
Hepatobiliaryhypointensity	7	5
Rim enhancement	2	1

HCC: hepatocellular carcinoma; MRI: magnetic resonance imaging

showed that only 8 patients (26.6%) had at least 2 immunostaining markers positive for HCC [Figure 2]. Out of 8 patients with histologically proven HCC, 6 (75%) had a well differentiated HCC and 2 (25%) had a moderately differentiated HCC [Table 1].

Out of 30 patients, 6 patients (20%) had regenerating nodules, 9 patients (30%) had high grade dysplastic nodules and 7 patients (23%) had low grade dysplastic nodules.

In triphasic CT, arterial enhancement was seen in 6 HCC and 5 non-HCC patients, portal venous washout was not seen in any HCC patient but seen in 2 non-HCC patients, delayed hypointensity was not seen in any HCC patient but seen in 1 non-HCC patient.

MRI using multihance [Figure 3] showed that 7 patients (23.3% of all atypical hepatic focal lesions; 87.5% of HCC patients) had a hypovascular hepatobiliary phase (HBP) hypointense HCC, 7 patients had non-hyper vascular HBP hypointense focal lesions. Using multihance MRI, 4 HCC patients and 5 non-HCC patients showed hypointensity in T1, 5 HCC patients and 6 non-HCC patients showed hyper intensity in T2 [Table 2]. Arterial enhancement was seen in 7 HCC and 7 non-HCC patients, delayed hypointensity was not seen in any HCC patients but seen in 2 non-HCC patients, HBP hypointensity was seen in 7 HCC and 5 non-HCC patients. Rim enhancement was seen in delayed phase in 2 HCC

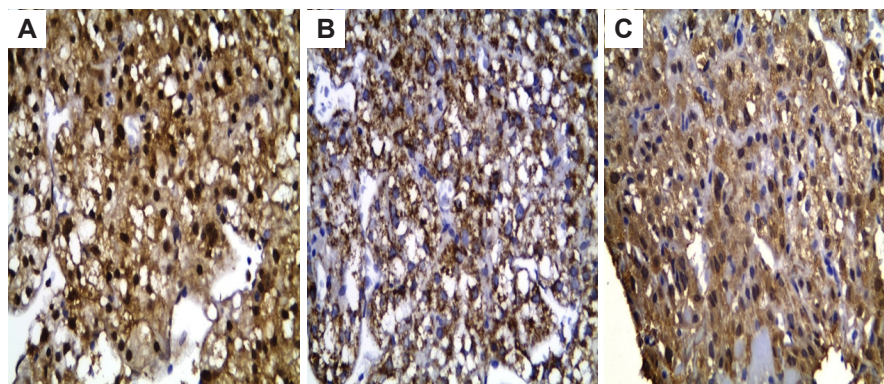


Figure 2: Immunohistochemical staining (x400) in well differentiated HCC showing: (A) high nucleocytoplasmic heat shock protein 70 expression; (B) high cytoplasmic glypican 3 expression; (C) high cytoplasmic glutamine synthetase expression. HCC: hepatocellular carcinoma

Table 3: Combinations of different phases of MRI

Phase	HCC patients	Non-HCC patients
Arterial phase hyperintensity HBP hypointense	7	4
Hypovascular, HBP hypointense	1	7
Arterial phase hyperintensity HBP hypointense, rim enhancement	2	1

HCC: hepatocellular carcinoma; HBP: hepatobiliary phase; MRI: magnetic resonance imaging

patients and 1 non-HCC patient [Table 2].

On combining different phases, it was found that 5 HCC patients showed T2 hyper-intensity and hypervascular HBP hypointense lesions, those findings were not detected in non-HCC patients. Seven non-HCC patients showed hypovascular HBP hypointense lesions versus 1 HCC patient [Table 3]. Two HCC patients showed hypervascular HBP hypointense and rim enhancement at delayed phase versus 1 non-HCC patient [Table 3]. The sensitivity on combining the arterial phase hyperintensity and HBP hypointensity was 87.5% and specificity was 82.8%.

DISCUSSION

Gadobenate dimeglumine is not a novel agent but the

group of atypical HCC selected is a challenging group for diagnosis. Guidelines for diagnosis of atypical cases of HCC are not yet well established and need further studies using different imaging modalities for early diagnosis thereby optimizing treatment outcome.

In our study, 8 out of 30 cirrhotic patients with atypical hepatic focal lesions on triphasic CT, were diagnosed as HCC based on positive immunostaining of at least 2 HCC biomarkers (GLP3, HSP70, or GS) according to the international consensus group for hepatocellular neoplasia. Seven out of these 8 lesions were diagnosed by gadobenate dimeglumine-enhanced MRI (multihance) as HCC, showing hyperintensity in the arterial phase and hypointensity in the HBP (according to the latest guidelines for diagnosis of HCC including those of the Japan Society of Hepatology (JSH),^[12] the Korean Liver Cancer Study Group (KLCSG), the National Cancer Center (NCC),^[13] and the Liver Imaging Reporting and Data System (LI-RADS).^[14]

According to the updated 2014 JSH guidelines, non-invasive diagnosis of HCC can be made using a hepatobiliary contrast if a lesion shows: (1) arterial hypervascularity and venous washout or (2) arterial hypervascularity without venous washout, but with hypointensity on the HBP.^[15]

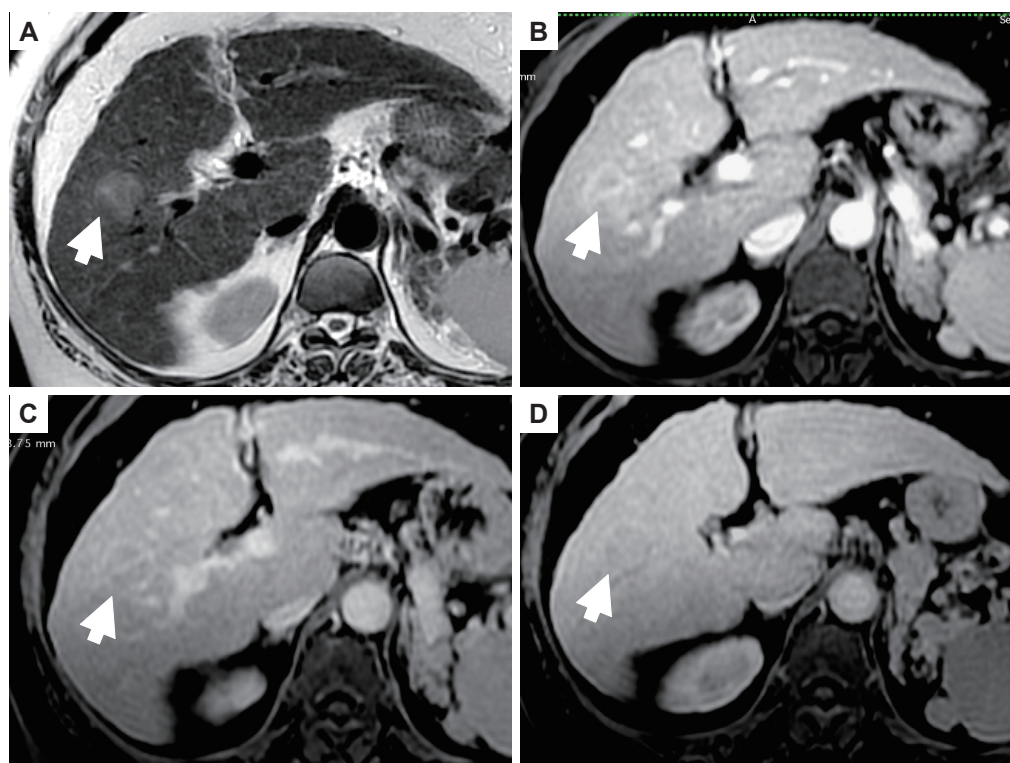


Figure 3: Trans-axial magnetic resonance images of the liver demonstrates right lobe focal lesion (arrow). It appears hyper-intense on T2 image (A). The arterial phase (B) shows faint enhancement in the lesion with washout on the arterio-portal phase (C) and the appearance of capsule. The lesion is hypo-intense to the liver parenchyma on the hepato-biliary phase (D). This lesion proved to hepatocellular carcinoma on histopathology

Most guidelines on the management of HCC recommend four-phase multi-detector CT and/or contrast-enhanced MRI as standard imaging modalities for diagnosis of HCC, based on the typical dynamic pattern of arterial enhancement and washout on the portal/delayed phases.^[16] Some HCC lesions, especially those less than 2-3 cm in diameter, lack typical hemodynamic criteria, making diagnosis of HCC a big challenge. Atypical enhancement patterns were seen in around 30% of HCC patients in many studies and hypovascular HCC lesions have led to around 35% of false negative results in tumors 1-2 cm in diameter.^[9]

HCC is a highly vascular tumor characterized by neoangiogenesis, which contributes to the high rate of metastasis and dismal prognosis. On microscopic examination, HCC displays marked vascular abnormalities, and aberrant microvasculature in the form of arteriogenesis and capillarization. Arteriogenesis is defined as the growth of functional collateral arteries covered with smooth muscle cells from pre-existing arteries. HCC is mainly supplied by hepatic arteries, while in normal liver parenchyma, regenerative and dysplastic nodules are mainly supplied by the portal vein.^[4,17]

MR imaging with hepatobiliary contrasts such as multihance (gadobenate dimeglumine) and primovist (gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid, Gd-EOB-DTPA, or gadoxetic acid) was found to be highly sensitive in HCC diagnosis.^[18] In one study, all HCC lesions were identified with gadoxetic acid, whereas three of 56 HCCs were not identified on dual-contrast MRI. In 59 patients imaged with gadoxetic acid, 10.7% of detected HCCs were detected only on hepatocellular phase images.^[19]

In one study evaluating 86 nodules, the diagnostic ability of gadoxetic acid was significantly higher than that of multi-detector triphasic CT for tumors less than 2 cm in diameter. There was no significant difference in the detection of hypervascular HCCs between hepatobiliary phase images of gadoxetic acid-enhanced MRI (43/45: 96%) and dynamic CT (40/45: 89%), whereas the detection sensitivity of hypovascular tumors by gadoxetic acid-enhanced MRI was significantly higher than that by dynamic CT (39/41: 95% vs. 25/41: 61%, $P = 0.001$). Gadoxetic acid enhancement ratios were decreased in parallel with the degree of differentiation in dysplastic nodules and HCCs.^[20]

In another study, imaging findings of a prospective and consecutive sample of 1-2 cm nodules detected at

surveillance ultrasound suggest that the newly proposed criteria for nodules fitting the American Association for the Study of Liver Diseases practice guidelines or having 3 or more findings in gadobenate dimeglumine MRI can be a useful alternative providing a significant improvement in sensitivity while maintaining high specificity for diagnosis of HCC.^[21] In two other studies using gadobenate dimeglumine-enhanced MR, small nodules that show enhancement on arterial phase and occult on the portal and equilibrium phase images as well as on the T1 and T2-weighted images are more likely to be HCC in patients with hepatitis B-induced cirrhosis^[22,23] which are in accordance with our results.

Triphasic CT and gadolinium MRI are not ideal tools for diagnosis of atypical HCC as they depend only on extracellular contrast. However, gadobenate dimeglumine is a dual extra- and intracellular contrast which can assess not only vascular changes in atypical nodules but also enzymatic activity within hepatocytes that can develop earlier than vascular changes.

The cost of gadobenate dimeglumine contrast is about 30 euros per case, while that of gadolinium (routinely used in dynamic MRI) is around 15 euros per case, and the average cost of triphasic CT contrast, ultravist, is about 10 euros. Unlike triphasic CT and gadolinium-enhanced MRI which are unreliable tools for diagnosis of small atypical HCC (almost one third of HCC cases), gadobenate dimeglumine-enhanced MRI was found to have higher sensitivity (87.5%) and specificity (82.8%) with an accurate diagnosis of more than 80% of atypical HCC in our study. Atypical HCC are usually well differentiated lesions with a better response to treatment, therefore, gadobenate dimeglumine MRI is likely to be more cost effective compared to other imaging modalities which warrants further studies.

In conclusion, gadobenate dimeglumine is a promising hepatobiliary contrast, which can potentially improve the non-invasive diagnosis of early atypical small HCC. The encouraging results from our pilot study warrants further confirmation by larger multi-center studies, as well as cost effective analysis of multihance MRI.

Authors' contributions

Study design: M.F. El-Gazzar, M.A.S. Kohla
Data analysis and manuscript preparation: M.F. El-Gazzar, M.A.S. Kohla, M.M. El-Sakhawy, M.M. Hussein, R.R.H. Yousef, S.H. El-Shorbagy
Critical review of manuscript: M.F. El-Gazzar, M.A.S. Kohla, M.M. El-Sakhawy

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Conflicts of interest

There are no conflicts of interest.

Patient consent

A written informed consent was obtained from all participants in the study.

Ethics approval

The study protocol was approved by the Institutional Review Board (IRB) and local ethical committee of the National Liver Institute, Menoufia University.

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Function of myeloid cell leukaemia-1 and its regulative relations with hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) remains a challenging disease with a high recurrence rate after surgery and there is an imminent need to identify new treatments. Currently, adjuvant therapy like chemotherapeutics arises to counteract the malignant trait escaping from apoptosis of tumors induced by overexpressed anti-apoptotic factors in HCC. Myeloid cell leukaemia-1 (Mcl-1) as an anti-apoptotic member of Bcl-2 is highly expressed in diverse human cancers, which contributes to cancer cell survival and the resistance to diverse chemotherapeutic agents. It is confirmed that Mcl-1 protein expression is quite enhanced in human HCC tissue compared to adjacent non-tumor tissue. Correspondingly, forced Mcl-1 down-regulation leads to prominent apoptosis of HCC cells and a sensitization towards chemotherapeutic drug-induced apoptosis, which indicates Mcl-1 is indeed a crucial regulatory factor of HCC. Hence, this review highlights the function of Mcl-1 on HCC progression, how it is regulated in HCC and the recent anti-hepatoma drug research and development down-regulation of Mcl-1 or targeting on Mcl-1. Meanwhile, the authors discuss Mcl-1 as an essential regulatory factor in HCC can be designed as target for drugs to improve the survival of HCC patients.

INTRODUCTION

Hepatocellular carcinoma (HCC), being the sixth most common cancer worldwide, represents the second most common cause of cancer-related mortality in the world^[1] and the incidence and mortality rates continue to rise all over the world. The main reasons may be late diagnosis and poor treatment options. To date, surgical resection, local treatment and liver transplantation can only cure a small number of patients whose disease

were at early stage, whereas the majority of patients with advanced disease undergone the torture of illness and were not suitable to receive surgery due to various reasons.^[2,3] Although chemotherapy is known as a vital management for advanced HCC, inherent resistance to chemotherapeutics by HCC cells makes it hard to have a good effect on the disease. Therefore, identification of new drugs targeting different signaling pathways is urgently needed to improve the survival of HCC patients.^[4] This review summarizes the current



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advances in the relationship between HCC treatment and anti-apoptotic molecule Mcl-1 and suggests that Mcl-1 is a potential target in abolishing the HCC cells' malignant proliferation.

Bcl-2 is a well established family of proteins and has a significant impact on mitochondrial integrity by influencing the permeability of the mitochondrial membrane. Bcl-2 is localized to the outer membrane of mitochondria, where it plays a part by regulating the progression of apoptosis. According to the structures and the functional contribution, Bcl-2 family members can be divided into two subfamilies: pro-apoptotic members and anti-apoptotic members.^[5] And it is the balance in activity between the two opposing groups which determines a cell's progression towards apoptosis.

Mcl-1 as an antiapoptotic Bcl-2 family protein, is playing a pivotal role in the intrinsic apoptosis pathway and mitotic regulators.^[6] As reported, Mcl-1 expresses extensively in the normal tissue of human and its overexpression is observed in many types of human tumors. In addition, Mcl-1 expression involves in disease grade and survival in human malignancies e.g. in patients with multiple myeloma or B-cell non-Hodgkin's lymphoma.^[7,8] It is also one of the pervasive recognized anti-apoptosis factor in HCC and mainly participate in maintenance of mitochondrial membrane stability and suppresses cytochrome c release from mitochondria to promote cell survival and inhibit cell apoptosis.^[9] In addition, Mcl-1, serves as one of the important antiapoptotic factors in HCC, is involved in the development and progression of HCC. According to a research made by Sieghart *et al.*,^[10] there were 51% liver tumor tissue appeared highly expression of Mcl-1 in 149 HCC patients, while the adjacent normal liver tissue presented a lower expression, which indicates the overexpression of Mcl-1 is one of the characteristics of specific changes of tumor. Additionally, silencing Mcl-1 gene gives rise to apoptosis of tumor cells with no effect to biological character in normal hepatocytes.^[11] Hence, it is indeed escapable and essential to discuss the relationship between Mcl-1 and HCC progression.

STRUCTURAL AND FUNCTIONAL PROPERTIES OF MCL-1

Structure

Mcl-1, one of the antiapoptotic members of the Bcl-2 family protein, was first identified by Kozopas *et al.*^[12] from a human myeloid leukemia cell line in 1993. The human locus of Mcl-1 gene is on chromosome 1q21. With 6502 bp full-length gene, Mcl-1 coding region comprises 3 exons and 2 introns. Bae *et al.*^[13] verified

that alternative splicing occurred in the transcription of Mcl-1 and eventually generated 2 different transcript variants. The one including 3 exons encodes Mcl-1L isoform while the other lack of exon 2 encodes Mcl-1S isoform. Sequence analysis revealed that Mcl-1L contains 350 residues which is larger than Bcl-2 (237 residues) and Bcl like protein X (Bcl-xl) (233 residues) and has 3 homo domains BH1, BH2, BH3 and C-terminal transmembrane (TM) domains but lack the N-terminal BH4 domain compared to Bcl-2 and Bcl-xl. The TM domain could anchor Mcl-1L to the outer mitochondrial membrane (OMM).^[14] By contrast, Mcl-1S comprises 271 residues and retains only BH3 domains just like other BH3-only members of Bcl-2 family and is primarily localized to the cytosol. Surprisingly, Mcl-1L inhibits apoptosis while Mcl-1S exhibits an opposite role and promotes apoptosis.^[13,15] Different from other proteins of Bcl-2 family, the N-terminal region of Mcl-1 (Mcl-1L will be simply called as Mcl-1 hereafter), affecting Mcl-1's function and localization, is larger than that of other Bcl-2 family members which contains PEST sequences rich in proline (P), glutamic acid (E), serine (S), and threonine (T). As characteristic sequences of Mcl-1, the PEST regions are rich in putative regulatory motifs that have been shown to target proteins for degradation, which are thought to be as the main reasons of the short half-life of Mcl-1 protein.^[14,16] There are also multiple phosphorylation sites in Mcl-1 PEST region, and it is likely that multiple proteins resulting in different fates of Mcl-1 mediate the phosphorylation of these sites. Moreover, with a surface-exposed hydrophobic groove formed by BH1, BH2, and BH3, Mcl-1 can integrate with other pro-apoptotic protein containing BH3-domain to impede apoptosis [Figure 1].^[17]

Function

Mainly, Mcl-1 protein is located in OMM, which enables Mcl-1 to interact with other proteins to play a part in anti-apoptosis. Immunoblot analysis revealed that both Mcl-1's C-terminal and N-terminal domains are necessary for its mitochondrial localization. There is a mitochondrial targeting sequence at Mcl-1's amino-terminus which anchors Mcl-1 at outer membrane or matrix. Particularly, the anti-apoptotic activities of Mcl-1 require outer membrane-localized Mcl-1.^[18] Besides, the first 79 amino acids of Mcl-1 regulate its subcellular localization and overexpression of the N terminus of Mcl-1 recruit more Mcl-1 at mitochondria and as a



Figure 1: The structure of myeloid cell leukaemia-1 (Mcl-1) protein sequence. A schematic of the wild-type Mcl-1 protein, highlighting the relative location of functional domains of Mcl-1. PEST: proline, glutamic acid, serine, and threonine domain; BH1, 3, 2: Bcl-2 homology domains 1, 3, 2; TM: transmembrane domain

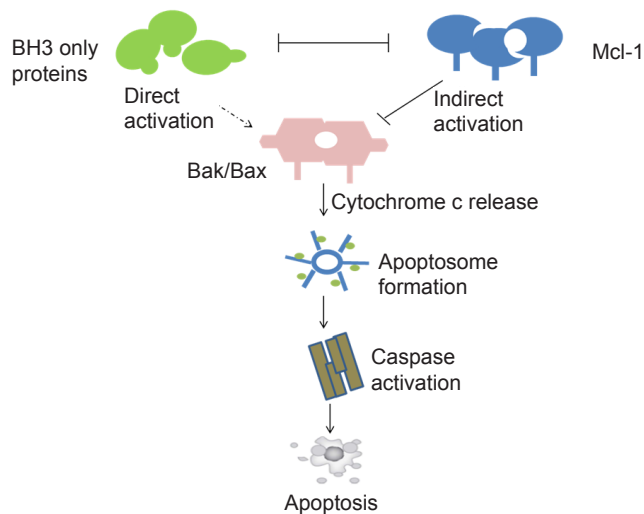


Figure 2: Myeloid cell leukaemia-1 (Mcl-1) regulates initiation of apoptosis through interaction with other Bcl-1 family members. Under normal circumstances, Mcl-1 prevents the activation of Bak and Bax to protect mitochondrial outer membrane integrity and cell survival. Under conditions of stress, the BH3 only proteins are activated and induce apoptosis either by releasing Bak/Bax from Mcl-1 or by BH3 only proteins binding to Mcl-1 directly. Bak/Bax form pores on mitochondrial outer membrane and cytochrome C is released into the cytoplasm. In the cytoplasm, cytochrome C activates a family of cysteine proteases named caspases which subsequently induce cell apoptosis

result, promote cell survival.^[19] Furthermore, Mcl-1 with an internal domain containing an EELD motif (at residue 124-127) interacts with the mitochondrial import receptor Tom70, which facilitates Mcl-1's import onto mitochondrial membrane.^[20] Moreover, the C-terminal transmembrane domain of approximately 20 amino acids is required for targeting Mcl-1 to mitochondria.^[21]

Normally, in order to maintain the inhibition of the pro-apoptotic proteins Bcl-2 homologous antagonist killer (Bak), Mcl-1 often prevents Bak from forming dimer with Bcl-2-associated protein X (Bax) via combination with it. At the same time, Mcl-1 binds to Bax to make sure sequestering Bak and Bax, and then blocks forming pores in the mitochondrial membrane caused by conformational change and homologous oligomerization, and eventually stops the release of cytochrome c into the cytoplasm, which means blocking the subsequent caspase cascade reaction of apoptosis. In addition, Mcl-1 also binds and sequesters BH3 only proteins which act to induce the polymerisation of Bak and Bax to play its antiapoptosis role effectively [Figure 2].^[22] As for the function of Mcl-1's different isoforms induced by alternative splicing, Mcl-1 and Mcl-1S are capable of forming heterodimers and thus neutralize either the pro-apoptotic function of Mcl-1S or the anti-apoptotic function of Mcl-1. Owing to alternative splicing mechanisms and interactions of the resulting Mcl-1 and Mcl-1S proteins, the fate of cells expressing the Mcl-1 gene may be closely

related to the ratio of Mcl-1/Mcl-1S.^[23] It is noteworthy that Mcl-1 plays the leading role in the regulation of apoptosis induced by Mcl-1/Mcl-1S and is expressed at higher levels than Mcl-1S.^[13,24,25] In cancer, Mcl-1S is expressed at much lower levels than Mcl-1 that it was even hardly undetectable.^[26] Some cancer cells such as human lung cancer cell lines A549, Chinese hamster ovary cells and multiple myeloma MOLP-8 cells show high level of Mcl-1S.^[13,15,27] Hence, this review mainly discusses Mcl-1.

Paradoxically, it is possible that Mcl-1 also plays an important role in delaying cell cycle progression for the existence of Mcl-1 in nucleus have been reported as well.^[28] The first 79 amino acids of Mcl-1 promotes its association with mitochondria, the N terminus of Mcl-1 also plays a regulatory role in regulating nuclear (anti-proliferative) functions of Mcl-1 and has an antagonistic effect on proliferation. There seems to be a balance between anti-apoptotic and anti-proliferative functions of Mcl-1 regulated by the N terminus of Mcl-1.^[19] In addition to antiapoptosis, Mcl-1 is capable of interacting with proliferating-cell nuclear antigen (PCNA)^[29] and cyclin dependent kinase 1 (CDK1),^[4] which may inhibit cell cycle progression.^[29,30] On the basis of co-immunoprecipitation experiments, Jamil *et al.*^[30] showed that endogenous Mcl-1 interacted with CDK1. The interaction involved a truncated form of Mcl-1, which was termed snMcl-1 as a result of proteolysis at the C-terminus that regulated cell-cycle progression by an inhibitory effect on CDK1 activity. The snMcl-1 was presented during S and G2 phases. The authors proposed that the Mcl-1-CDK1 interaction associated with a protein containing a nuclear localization signal that mediated rapid translocation to the nucleus.^[30] Mcl-1 can also regulate the S-phase of the cell cycle through interaction with PCNA, and such interaction may be through Mcl-1's binding to PCNA.^[29] Nonetheless, the binding between PCNA and the Mcl-1 can not be detected in solution studied by NMR, which suggests that the interaction occurs very weakly, or with other unidentified factors in cells.^[31] Of note, the interaction with PCNA represses cell cycle progression, but it is not related to Mcl-1's anti-apoptotic activity.^[29] And it's not clear that whether such two kinds of interaction are mechanistically linked.

Mcl-1 is highly expressed in a variety of human hematopoietic, lymphoid cancers and solid tumors including leukemia,^[32,33] lymphoma,^[8] cervical carcinoma,^[34] HCC,^[10,35] breast carcinoma,^[36] lung cancer^[37] and multiple myeloma.^[7,38,39] In addition, its expression is often implicated in the chemotherapeutic resistance and relapse of certain malignancies. For instance, it is crucial for Mcl-1 to survive human

myeloma cells *in vitro* and it has been showed that Mcl-1 is overexpressed *in vivo* in multiple myeloma, which seems to be related to relapse and shorter survival.^[7] Expression of Mcl-1 was also bound up with high tumor grade and reduced survival of patient in human breast cancer samples.^[40] Immunohistochemistry and western blotting analysis showed that Mcl-1 was overexpressed in cervical cancer tissue in comparison with normal tissue and the author confirmed Mcl-1 expression was positively correlated with poor prognosis.^[34] As for acute myeloid leukemia, Mcl-1 served as a critical molecule to develop and maintain malignant tumor.^[41,42] Moreover, Campbell *et al.*^[43] reported that elevated Mcl-1 promotes Myc-induced lymphomagenesis and enhances drug resistance. And also, in human HCC, it has been concluded that Mcl-1 expression was prominently enhanced in diseased tissue as well as in various HCC cell lines.^[10,35] On the contrary, in mice lacking the anti-apoptotic protein Mcl-1 specifically in hepatocytes not only increased hepatocyte apoptosis, but also resulted in hepatocarcinogenesis, which is related to compensatory hyper-proliferation induced by Mcl-1 deficiency.^[44] Besides, another mouse model indicates that Mcl-1 is stabilized by interleukin (IL)-6 and obesity and thus apoptosis of damaged hepatocytes was inhibited, which eventually promoted HCC progression.^[45]

REGULATIVE RELATIONS WITH HCC

Combining unrestrained cell proliferation and damaged apoptosis was found as a main feature of tumor. And as mentioned before, the anti-apoptotic member Mcl-1 was overexpressed in HCC endowing tumor cells with ability to escape from programmed cell death. Consequently, it is of great necessary to make clear the regulation and execution of apoptosis in HCC so that people can find a new way to confront malignant tumor. The regulative relation between Mcl-1 and HCC is listed in Table 1.

Transcriptional regulation

Mcl-1 can be regulated at transcriptional level by a variety of cytokines including signal transducers and

activators of transcription (STAT), cAMP-response element binding protein (CREB), purine-rich nucleic acid binding protein 1 (PU.1), and hypoxia-inducible factor-1 (HIF-1), etc. The STATs, a family of transcription factors, has been shown to bind to Mcl-1 promoter. Al Zaid Siddiquee and Turkson^[46] reported that constitutively activated STAT3 participate in oncogenesis of the liver through up-regulating STAT3-targeted genes encoding apoptosis inhibitors including Mcl-1 and subsequently inhibiting pro-apoptotic molecules such as Bax, Bad, and Bid. Additionally, sorafenib was affirmed for its efficacy against Janus Kinase (JAK)-STAT signaling in HCC cells and downregulation of pSTAT3 and its target genes including Mcl-1 by immunoblotting.^[47] Irophic factor IL-3 also involves in transcriptional upregulation of Mcl-1. Through activation of the PU.1 transcription factor, IL-3 activates Mcl-1 transcription by the P38 mitogen-activated protein kinase (MAPK)-dependent pathway.^[48] On the other hand, Mcl-1 transcription can also be activated by IL-3 increasing of the DNA binding activity of the CRE-2 binding complex through phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway.^[49] HIF-1 is a putative key transcription factor which can regulate cells under hypoxia undergoing different transcriptional adaptations.^[50] Through analysis of the Mcl-1 promoter sequence in hepatoma HepG2 cells incubated under hypoxia, Jean-Pascal Piret *et al.*^[51] demonstrated that there was a hypoxia-responsive element in Mcl-1 promoter fragment that was able to bind HIF-1 *in vitro*. Detailed results revealed that HIF-1 showed a potential anti-apoptotic role and could protect cells against apoptosis as a result of hypoxia by up-regulation of the Mcl-1 protein.^[51] Luciferase reporter assay revealed that overexpression of periostin enhanced HIF-1 α -dependent transcriptional activity and induced multiple HIF-1 α target genes including Mcl-1, and Bcl-xL in HCC cells.^[52] Moreover, the ternary complex factor-serum response factor complex are also involved in regulating Mcl-1 expression and protecting cells from apoptotic cell death.^[53] After activating cells with a variety of cytokines, Mcl-1 expression can be regulated transcriptionally in several signaling pathways. A recent report describes that after treatment of HCC SK-Hep-1

Table 1: Overview of regulation of Mcl-1 in HCC

Transcriptional regulation		Translational regulation	Post-translational regulation		Interacting proteins
			Phosphorylation sites	Kinases	
Cytokines	STATs, IL-3, HIF-1	mir-29b	Thr92	ERK(-)	Mule
			Thr163	ERK(-), JNK(+)	CDK
Signaling ways	PI3K/AKT, P38/MAPK,	mTORC1	Ser121, Thr163	JNK(-)	PCNA
	P53, Wnt/β-actein, Notch		Ser155, Ser159	GSK3(+)	TCTP

The influence that phosphorylation of each residue has on the progression of apoptosis is shown as positive (+) or negative (-) function. HCC: hepatocellular carcinoma; Mcl-1: myeloid cell leukaemia-1

cells with exposure to ursolic acid (UA), western blot results showed decreased expression of the Mcl-1 and that treatment with UA induces apoptosis by inhibition of PI3K/Akt and P38/MAPK signaling pathway.^[54] Yu *et al.*^[55] found that Mcl-1 protein expression was downregulated via inhibition PI3K by LY294002 in HepG2 cells, which indicates that PI3K/Akt signaling pathway regulates Mcl-1 expression. Data from two human HCC cell lines, SMMC7721 and HepG2, indicated that exogenous rhHPPCn (Hepatopoietin Cn) suppressed trichostatin A-induced apoptosis of HCC cells and up-regulated Mcl-1 expression in HCC-derived cells via the MAPK or sphingosine kinase-1.^[56] Real-time polymerase chain reaction analysis and western blot results demonstrated that aspirin induced Mcl-1 expression at mRNA level as well as protein level through Akt/extracellular regulated kinase (ERK) 1/2 and stimulates AMPK-Akt/ERK1/2-Mcl-1 axis in HepG2 cells.^[57] P53 as a tumor suppressor protein also involves the regulation of Mcl-1. It has been reported that mutation in the P53 frequently occurred in HCC and contributed to hepatocarcinogenesis as well as apoptosis resistance.^[58] Additionally, Leu *et al.*^[59] demonstrated that P53 antagonized the interactions between Mcl-1 and Bak. Once mutation happens in HCC, Mcl-1 couldn't be dissociated from Bak and the final result is apoptosis resistance of hepatoma cells. Data from human samples showed that P53 protein was also overexpressed in HCC tissues and its expression was significantly correlated with Mcl-1 expression. Further research indicated that silencing Mcl-1 sensitizes hepatoma cells towards chemotherapy may be attributed to the dysfunction of P53 through Mcl-1/P53 interaction.^[55] According to combination of ICG-001, a small molecule which blocks the interaction of β -catenin with its transcriptional coactivator CREB-binding protein, and sorafenib to treat several HCC cell lines, the effect was a significant downregulation of Mcl-1 which was the most consistent change across tested HCC cell lines. The author concluded that the sorafenib-sensitizing effect of Wnt/ β -catenin pathway inhibition was closely associated with Mcl-1 downregulation in HCC cells.^[60] In addition, recent reports described that Wnt/ β -catenin signaling could regulate Mcl-1 expression indirectly, involving genes regulated by Wnt/ β -catenin pathway or other transcriptional factors.^[61,62] Moreover, a panel of HCC cell lines has been on treatment with Xanthohumol (XN), a prenylated chalcone having anti-proliferative effects in various cancers types *in vitro*, and growth suppression due to apoptosis was evidenced by reduced expression of anti-apoptotic proteins including Mcl-1. Importantly, XN treatment decreased the expression of Notch1 and hairy and enhancer of split-1 proteins while ectopic expression of Notch1 in HCC cells abolished the anti-proliferative

effect of XN. In brief, XN mediated growth suppression of HCC through inhibition of the Notch signaling pathway.^[63]

Translational regulation

The same as Mcl-1 protein, Mcl-1 mRNA have very short half-lives. Translationally, mir-29b binding to the 3'-untranslated region of Mcl-1 mRNA inhibits expression of Mcl-1.^[64] Northern blot and real-time quantitative reverse transcription polymerase chain reaction showed that downregulation of mir-29 was a frequent event in HCC tissues. Further study implicated that mir-29 may promote apoptosis of HCC cells through directly targeting Bcl-2 and Mcl-1. Besides, the ability of HCC cells to form tumor in nude mice was dramatically repressed by induction of mir-29. These results indicated that Bcl-2 and Mcl-1 were predominant mediators of mir-29 promoted apoptosis in HCC cells.^[65] The mammalian target of rapamycin complex 1 (mTORC1) is a protein complex whose role is to activate translation of proteins just like a nutrient sensor controlling protein synthesis and a downstream target of PI3K/Akt.^[66] There was a report that described that activation of mTORC1 was of vital importance to be a potent antiapoptotic signal through Mcl-1 which is a translationally regulated genetic determinant of mTORC1-dependent survival.^[67] And a recent study demonstrated that metformin-induced apoptosis in HCC was mediated by the downstream mTORC1 effectors eukaryotic initiation factor 4E (eIF4E) and eIF4E-binding proteins who were required to induce apoptosis by metformin in HCC and to repress Mcl-1 expression.^[68]

Post-translational regulation

There is variety of modes regulating Mcl-1 at post-translational level. In the preceding part of this review, we have mentioned that the PEST region of Mcl-1 was rich in putative phosphorylation sites which made Mcl-1 different from other Bcl-2 family members. Here, we detail those phosphoresidues of Mcl-1 and the influence of phosphorylation in HCC. Both of the phosphoresidues Threonine 92 and Threonine 163 of Mcl-1 were identified by Ding *et al.*^[69] using ERK-1 kinase assay. ERK-1 phosphorylation of Thr 92 and Thr 163 stabilizes Mcl-1 and then promotes Mcl-1's anti-apoptosis. It has been demonstrated that heat shock protein 90 inhibitor 17-allylaminogeldanamycin (17-AAG) partially reversed (-)-gossypol-induced Mcl-1 accumulation by inhibiting ERK phosphorylation in HCC cells.^[70] Of note, Inoshita *et al.*^[71] concluded that phosphorylation of Thr 163 by JNK destabilized Mcl-1, whereas the results showed by Kodama *et al.*^[72] suggested that C-Jun N-terminal kinase (JNK) as the

kinase contributed to phosphorylation of Thr 163 and Serine 121 of Mcl-1, prolonged the half-life of the Mcl-1 protein and protected hepatocytes against apoptosis induced by tumor necrosis factor alpha (TNF α). And data from Wang *et al.*^[73] demonstrated that the Bcl-2/xL inhibitor ABT-263 increased Mcl-1 stability in HCC cells while activation of ERK and JNK involved in ABT-263-mediated Mcl-1 protein stabilization through phosphorylation of Mcl-1 Thr163. And also it has been reported that the new tubulin inhibitor MT189 (2-(6-fluoro-3-((4-methoxybenzyl)amino)imidazo [1,2- α] pyridin-2-yl) phenol)-mediated JNK activation caused degradation of Mcl-1 protein via facilitating its phosphorylation in the SMMC-7721 cells.^[74]

Glycogen synthase kinase-3 (GSK-3) inactivated by Akt plays a crucial role in the regulation of apoptosis. It has been demonstrated that the control of Mcl-1 stability by GSK-3 is an important mechanism for the regulation of apoptosis by growth factors, PI3K, and Akt.^[75] Deeper research indicated that GSK-3 was conducive to degradation of Mcl-1 by means of phosphorylation of its Serine 155 and Serine 159 and the latter inhibited the interaction of Mcl-1 with the pro-apoptotic protein, Bim, thus impairing its anti-apoptotic function.^[75,76] What's more, Wang *et al.*^[73] indicated that Akt-mediated GSK-3 β inactivation also implicated in ABT-263-induced Mcl-1 stabilization, possibly through regulating the phosphorylation of Mcl-1 Ser159 in HCC cells.

Mcl-1 interacting proteins

The majority of proteins interacting with Mcl-1 belong to the Bcl-2 protein family including multidomain pro-apoptotic members and the BH3-only proteins.^[77-79] In this review, we just discuss other proteins interacting with Mcl-1. The Mcl-1 protein level can be downregulated by adenovirus infection through proteasome-mediated turnover of Mcl-1.^[80] Mcl-1 ubiquitin ligase E3 (Mule) contains a region similar to BH3 domain that enables Mule to interact with Mcl-1. It has been demonstrated that Mule was required for the polyubiquitination of Mcl-1 in the ubiquitin dependent proteasome degradation pathway.^[81] According to a research treatment of HepG2 cells with glycochenodeoxycholate (GCDA), one of the major human bile salts, the author reported that GCDA facilitated Mcl-1 dissociation from E3 ligase Mule and increased the half-life of Mcl-1.^[82]

Cyclin-dependent kinase (CDK) could combine and activate cyclin, and thus lead to phosphorylation of target protein. The phosphoresidue serine 64 of Mcl-1 was identified by Kobayashi *et al.*^[83] through MS analysis of a threonine 163 to alanine mutant of

Mcl-1 and then CDK1 and CDK2, proteins related to cell cycle, and JNK were affirmed to phosphorylate this residue, which plays a negatively role on the progression of apoptosis. Moreover, it was reported that both protein and mRNA levels of Mcl-1 were down-regulated by a novel synthetic CDK inhibitor ibulocyndine in HCC cells.^[84]

Another protein impacting Mcl-1's roles in cell cycle is PCNA. It has been mentioned before that Mcl-1 can bind to PCNA and CDK1 in the nucleus, which participate in repression of cell cycle progression. When transfection of Huh7 and HepG2 cells with glypican 3-specific siRNA, cell proliferation detected by PCNA immunohistochemistry was inhibited, cell cycle was arrested at the G1 phase and anti-apoptotic proteins (Bcl-2, Bcl-xL, and Mcl-1) were down-regulated.^[85] Besides, different from other reports about interaction between PCNA and the Mcl-1 or the CDK2 protein in biochemical assays, De Biasio *et al.*^[31] detected no binding between them and suggested that the interaction, if any, occurs with very low affinity or is mediated by other factors. Lately, a report described that following the inhibition of RNA polymerase II phosphorylation, ibulocyndine caused down-regulation of Mcl-1, survivin, and X-linked inhibitor of apoptosis protein (XIAP), thus inducing apoptosis in HCC cells.^[84]

Owing to the extremely labile nature of Mcl-1, it is as important as those that regulate Mcl-1 synthesis for cellular processes to regulate Mcl-1 stability. Recently, a Mcl-1 interacting protein the translationally controlled tumor protein (TCTP) was identified to upregulate the expression levels of Mcl-1 through modulating Mcl-1 stability and eventually modulate Mcl-1's antiapoptotic activity by the ubiquitin-dependent proteasome degradation pathway. Detailed analysis revealed that TCTP overexpression inhibited apoptosis by binding to Mcl-1 and antagonizing Bax.^[86] It has been well documented that TCTP was implicated in many cellular functions including human allergic response,^[87] apoptosis^[88] and cell growth.^[89] Chen *et al.*^[90] described that Sann-Joong-Kuey-Jian-Tang (SJKJT), a traditional medicinal prescription, could downregulate the protein expression level of Mcl-1 and TCTP in Hep-G2 cells, thus they considered that decreasing TCTP and Mcl-1 expression may be one of the molecular mechanisms by which SJKJT inhibits Hep-G2 cells. It has also been reported that curcumin inhibited the proliferation of human HCC J5 cells and induced mitochondrial dysfunction by decreasing the expressions of TCTP, Mcl-1 and Bcl-2.^[91] Similarly, after treatment of HCC SK-Hep-1 cells with ursolic acid, the western blot results were associated with decreased expression of Mcl-1, TCTP and Bcl-2.^[54]

DRUG R&D FOR REGULATING MCL-1 OF HCC

Drugs that down-regulate Mcl-1

Cyclin-dependent kinase inhibitors

Flavopiridol is a semisynthetic compound that functions as a CDK inhibitor though inhibiting CDKs and thus inducing cell cycle arrest at the G1 or the G2/M transition point.^[92] In a recent study, flavopiridol augmented TNF-related apoptosis-inducing ligand (TRAIL) sensitivity of human HCC cells by up-regulation of TRAIL receptors and down-regulation of survivin, FLICE-inhibitory protein and Bcl-xL.^[93] Flavopiridol has also been shown to induce apoptosis in a P53-independent manner and to down-regulate XIAP, Mcl-1, Bcl-2, survivin in kinds of cancer cells.^[94,95]

Another CDK inhibitor, as mentioned above, is ibulocydine - a novel isobutyrate prodrug inhibitor of CDK7/9. In comparison, ibulocydine inhibited the growth of HCC cells more effectively than other CDK inhibitors via prolonged inhibition of CDK7/9 leading to induction of apoptosis by down-regulating the cellular levels of anti-apoptotic proteins such as Mcl-1 and XIAP. Besides, data from human HCC xenografts indicated that ibulocydine selectively induced apoptosis but has no cytotoxic effects on normal tissues. Consequently, ibulocydine is a strong candidate for the treatment of HCC.^[84]

Deubiquitinase inhibitors

Mcl-1 is degraded rapidly in the cell via a proteasome-dependent pathway, whereas deubiquitinases (DUBs) are capable of removing ubiquitin from ubiquitinated Mcl-1 to rescue Mcl-1 from degradation. WP1130, a small molecule that was initially identified as JAK and STAT inhibitors, can also inhibit activity of DUBs. And it has been demonstrated that DUBs ubiquitin-specific protease 9X (DUB USP9X) was one of the proteins to co-immunoprecipitates with Mcl-1.^[96] In a recent study, it has been found that combined treatment with WP1130 sensitized HCC cells to doxorubicin via USP9X-depended P53 degradation.^[97]

STAT protein inhibitors

As mentioned previously, STAT could regulate Mcl-1 at transcriptional level, thus attenuating the activity of STAT protein by agents is a fine choice to down-regulate the expression of Mcl-1 proteins. Surprisingly, ethanol extracts from *Sedum sarmentosum* have been reported to inhibit STAT-3 signaling, down-regulate Mcl-1 and Bcl-2 expressions, and finally inhibit proliferation of HepG2 cells, and induce HepG2 cells apoptosis.^[98]

PI3K/Akt signaling inhibitors

LY294002 functions as a PI3K/Akt signaling inhibitor which is capable of repressing the activation of AKT-

1 to down-regulate the expression of Mcl-1 protein and to induce the apoptosis of macrophage. In the research of HCC therapies, Yang *et al.*^[99] found that a disintegrin and metalloproteinase 10 (ADAM10) overexpression conferred resistance to doxorubicin-induced apoptosis in HCC, whereas the pretreatment with the PI3K inhibitor LY294002 significantly enhanced doxorubicin-induced apoptosis and diminished the Mcl-1 expression in ADAM10-overexpressing Huh7 cells. And also LY294002 could down-regulate the expression of Mcl-1 rapidly in HCC cells and increase the sensitivity of HCC cells to chemotherapeutics.^[35]

MEK/ERK signaling inhibitors

Sorafenib actually inhibits multiple other kinases. It is the first and only orally administered drug to treat advanced HCC. One of the molecular mechanisms of sorafenib in HCC cells is that sorafenib induces apoptosis by reducing eIF4E phosphorylation and blocking the initiation of Mcl-1 translation.^[100] Chen *et al.*^[47] demonstrated that sorafenib downregulated phospho-STAT3 and subsequently reduced the expression levels of STAT3-related proteins including Mcl-1 in a dose- and time-dependent manner in TRAIL-treated HCC cells.

Antisense oligonucleotide treatment

Antisense oligonucleotide (ASO)^[10] is a kind of synthetic oligonucleotides fragment expressed by antisense expression plasmid. Recently, it has been found that Mcl-1 ASO could downregulate Mcl-1 efficiently in various tumor cells and animal models. According to ASO treatment as monotherapy in the HCC cell lines HepG2 and Snu398, the result showed that ASO targeting Mcl-1 specifically downregulated Mcl-1 protein expression and led to significant dose and time dependent single agent activity in HCC cells characterized by increased apoptosis and decreased cell viability. And Upon combination with cisplatin, Mcl-1 ASO revealed a significant chemosensitizing effect.^[10] However, there is no report about Mcl-1 ASO treatment used in clinic.

BH3 mimetics

BH3 mimetics mainly play a part through the interaction of proteins to inhibit Mcl-1's function. As discussed, there was a surface-exposed hydrophobic groove contributing to the anti-apoptosis function of Mcl-1 [Figure 2].^[17] Consequently, BH3 mimetics were designed to fit into the hydrophobic groove and block Mcl-1's ability to bind pro-apoptotic proteins, inhibiting the anti-apoptosis function of Mcl-1. ABT-737, a small-molecule cell-permeable Bcl-2/Bcl-XL antagonist, is a novel cancer therapeutic agent because it potently induces apoptosis in certain cancer cells. Nevertheless, owing to low affinity with Mcl-1, ABT-

737-mediated apoptosis signaling was inhibited in HCC cells due to the elevated expression of Mcl-1 which may contribute to HCC resistance to ABT737.^[101,102] Consequently, it is unlikely to be effective as a single agent in solid tumors and thereby a great many research about combining ABT-737 with other agents to abolish the resistance has emerged recently such as norcantharidin, celastrol, *etc.*^[103,104]

Polyphenols derivatives

The mother nucleus structure of polyphenols derivatives has polyhydroxyphenol. Gossypol as a typical BH3 mimetic that inhibits the Bcl-2 anti-apoptotic proteins Bcl-2, Bcl-XL, and Mcl-1 by binding to them.^[105] Because of the ability to target Mcl-1, gossypol shows toxicity against variety of cancer types in comparison to ABT-737. Since it was shown that HCC cells were relatively resistant to Bcl-2 inhibitors, then co-treatment of Bcl-2 inhibitor (-)-gossypol and Hsp90 inhibitor 17-AAG attenuated (-)-gossypol-induced protective autophagy by inhibiting ERK-mediated Bcl-2 phosphorylation and downregulated (-)-gossypol-triggered Mcl-1 accumulation by suppressing Mcl-1 Thr163 phosphorylation.^[70] Apogossypolone (ApoG2) was the first derivative of gossypol for the potential non-specific reactivity related to the 2 aldehyde groups in gossypol.^[106] In order to investigate the *in vitro* and *in vivo* activities and related mechanism of ApoG2 against HCC, Mi *et al.*^[107] found that the ApoG2 induced apoptosis in SMMC-7721 cells by downregulation of anti-apoptotic proteins Bcl-2, Mcl-1, and Bcl-XL and up-regulation of pro-apoptotic protein Noxa, which indicated ApoG2 was a potential pan Bcl-2 family protein inhibitor, targeting Bcl-2, Mcl-1, and Bcl-XL, and inducing apoptosis in HCC. Moreover, several gossypol analogues arose to inactivate Mcl-1 such as TM-1206,^[108] BI-33^[109] and TM-179.^[110]

Indole dipyrrole derivatives

Obatoclax is a synthetic indole dipyrrole derivative derived from prodigiosin and acts as a BH3 mimetic which binds the anti-apoptotic Bcl-2 proteins, releases proapoptotic proteins and thus triggers caspase activation. And SC-2001 was originally derived from the Mcl-1 inhibitor obatoclax, that was suggested better antitumor effects than obatoclax in HCC cell lines, including HepG2, PLC5 and Huh-7.^[111]

Acenaphthene heterocyclic derivatives

S-1 (one mixed formulation containing 5-FU prodrug and dihydropyrimidine dehydrogenase inhibitor) inhibits both Bcl-2 and Mcl-1 and is capable of disturbing interaction between Mcl-1 and Bak, resulting in apoptosis.^[112] Furuse *et al.*^[113] reported that S-1 was effective and had an acceptable toxicity profile in

patients with advanced HCC, which indicates S-1 is a potential candidate for antitumor agent.

CONCLUSION

With highly expressed Mcl-1, HCC cells tend to escape from apoptosis and thus proliferate at an increasingly high speed. Mcl-1 is a critical survival factor for malignant tissues of HCC and its expression is regulated via multiple mechanisms. Hence, it is a promising target for HCC treatment. Over the past several decades, there has been significant progress towards relevant molecular interacting with Mcl-1. On the one hand, Mcl-1's expression in HCC is regulated at transcriptional by a variety of cytokines and signaling pathways, including the P38/MAPK, PI3K/AKT, STAT, P53, ERK, JNK, Wnt/ β -catenin, Notch signaling ways. On the other hand, the role of microRNAs in Mcl-1 regulation has been highlighted at the translational level and multiple phosphorylation sites in Mcl-1's PEST region regulate Mcl-1 expression at post-translational level. Other Mcl-1 interacting proteins such as Mule, CDK1, CDK2, PCNA, TCTP, *etc.* also involve in Mcl-1 regulation through interaction with it. According to these molecular mechanisms, numerous of chemotherapeutic agents have been reported to decrease the level of Mcl-1 towards HCC treatment including agents not specifically targeting Mcl-1 but involving downregulation of Mcl-1 and those drugs targeting Mcl-1 directly. Thereinto, BH3 mimetics are the most studied among all the chemotherapies. Of note, HCC with high levels of Mcl-1 are resistant to apoptosis induction by some compounds, posing a major problem for its potential utility. Thus, combination of multiple targets agents for HCC chemotherapy, production of good drug delivery system, and designing novel interventions specifically targeting Mcl-1 will be a major tendency in the future.

Authors' contributions

Design and performing the research, manuscript review: Y.M. Zhang
Manuscript drafting: M. Zhu

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Response rates of hepatocellular carcinoma and hepatic colorectal cancer metastases to drug eluting bead regional liver therapy

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ABSTRACT

Aim: The purpose of this study was to evaluate and compare how hepatocellular carcinoma (HCC) and colorectal metastases respond to LC Bead chemoembolization using doxorubicin and irinotecan. **Methods:** The authors report their experience with doxorubicin and irinotecan eluting beads to treat 13 patients with primary HCC and 25 patients with colorectal metastases over a 1-year period at a single community based oncology practice. Within the colorectal cancer group they compared irinotecan eluting beads to doxorubicin eluting beads. **Results:** Nine of the 11 (81.8%) doxorubicin treated HCC patients had either complete response or partial response. All of the HCC lesions showed reduction in size and tumor enhancement and 10/11 (91%) HCC patients were alive at 24 months post treatment. Fisher's exact test revealed that among the 22 with colorectal metastases for whom follow-up data were available, those 11 who were treated with doxorubicin were significantly more likely to demonstrate complete or partial response compared to the 11 in the irinotecan treated group ($P < 0.001$). **Conclusion:** Overall, HCC and colon metastasis patients clearly demonstrated the effectiveness of drug eluting beads with 91% of the HCC patients alive 24 months after treatment.

INTRODUCTION

Primary and secondary malignancies of the liver are very common accounting for more than 530,000 new cases per year.^[1] Hepatoma and secondary neoplasms of the liver are expected to increase as the incidence of hepatitis C continues to spread throughout the world. Colorectal metastases to the liver and primary malignant hepatic neoplasms have a poor prognosis with dismal survival rates of 31% at 1 year and 26% at

2 years. Surgery is the definitive treatment for isolated lesions while systemic chemotherapy has been the standard treatment for unresectable liver neoplasms.^[1-3] Most lesions are not surgically resectable at the time of diagnosis due to their extensive tumor burden. Treatment strategies for unresectable liver cancer are different for hepatocellular carcinoma (HCC), other primary liver tumors, and metastatic liver cancer. For example, transarterial chemoembolization (TACE) may be the standard for HCC but chemotherapy is still the



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standard of care for colorectal liver metastasis. TACE has been an effective palliative therapy for malignant tumors of the liver for many years.^[4-8] TACE has shown improved patient survival rates compared to conservative treatment for various types of malignant liver tumors.^[6-9] The palliative nature of transcatheter embolizations has shown improved patient survival and quality of life as compared to placebo and systemic based chemotherapy.^[10] TACE is a useful palliative procedure with its ability to simultaneously infuse concentrated dose of chemotherapeutic drug combined with embolization particles.^[5-8] This combination produces elevated local chemotherapeutic drug levels along with vascular occlusion of the feeding vessels killing the tumor resulting in reduced systemic toxicity without causing collateral damage to the surrounding liver parenchyma.

LC Bead drug eluting beads [Biocompatibles UK Ltd, Farnham (a BTG group company)] are approved by the Food and Drug Administration for locoregional embolization. Like conventional TACE, drug eluting beads are available for precision transarterial chemoembolization.^[11-13] However, they are different in the way they deliver the drug to the tumor. The beads are compressible sulphamate modified polyvinyl alcohol hydrogel microspheres.^[14] The drug-eluting beads can be loaded with some positively-charged chemotherapeutic agents such as doxorubicin hydrochloride or irinotecan hydrochloride. There is an ion exchange mechanism which creates the active attraction of the drug to the beads. Just like TACE, the beads are delivered to their exact location with fluoroscopic guided transarterial catheters but this time the drug is loaded into the beads.^[5,15] The mixture of beads with doxorubicin or irinotecan can be easily loaded in the pharmacy 2 h prior to delivering them to the patient. The 2 h of soaking allows the drug and beads to interact effectively according to the manufacturer.^[16] The controlled release of the drug from the drug eluting beads (DEB) demonstrates very little or no post embolization syndrome as compared to conventional TACE procedures. The LC beads maintain a significantly high intratumoral drug concentration in the tumor bed for a 2-week period. This controlled release process may be more effective than conventional TACE. Systemic toxicity is reduced due to a combination of increase late effects and precise arterial deposition of the beads into the tumor as compared to conventional TACE.

LC Bead embolization can utilize both doxorubicin and irinotecan eluting beads for primary hepatomas, colorectal metastasis and a variety of other liver metastases. The purpose of this study is to determine

whether this case series could provide insight into whether treatment methods are associated with treatment response.

METHODS

Computed tomography positron emission tomography (CT-PET) and/or magnetic resonance imaging (MRI) studies were reviewed prior to all procedures to guide endovascular treatment [Figure 1]. Four board certified interventional radiologists reviewed all pre procedure imaging for each patient and all 4 actually performed the LC Bead chemoembolizations. This was a retrospective study and no ethical approval was obtained for this study. Informed consent was obtained prior to all interventional procedures. All patients with metastatic colorectal metastases or HCC over a period of 1 year were included in this study. All of the colorectal metastasis patients were treated with systemic chemotherapy prior to endovascular intervention. All patients were treated with drug eluting beads during the study. The time frame between completing chemotherapy and initiating the endovascular treatment was 3-6 months. Subsequently, a follow-up CT-PET scan demonstrated progressive liver metastasis not improved on intravenous chemotherapy. As for the HCC patients, once deemed unresectable, they were included in this study. The decision to treat was based on a multidisciplinary approach including the patient's oncologist, surgical oncologist and interventional radiologist. The treatment pathway was defined by tumor type and then the appropriate chemotherapeutic agent to be used on that type of liver neoplasm. The treatment pathway included pre-procedural imaging, performing the intra-arterial embolization and then the follow-up CT-PET imaging for evaluation of changes in liver mass. Data were collected and patients were

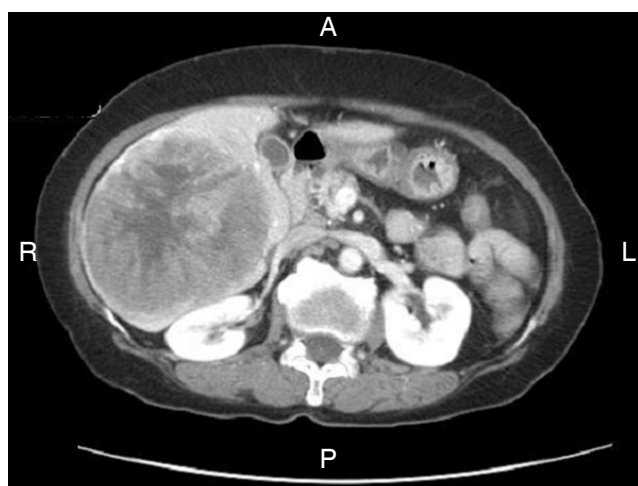


Figure 1: Contrast enhanced computed tomography image of the abdomen demonstrates a large enhancing tumor right hepatic lobe consistent with biopsy proven hepatocellular carcinoma

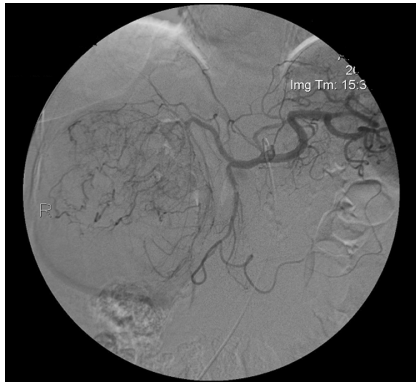


Figure 2: Sub selective angiogram demonstrating an exquisitely vascular hepatocellular carcinoma

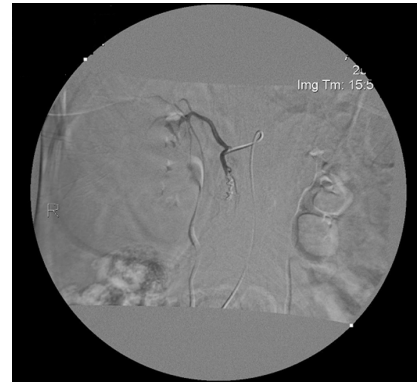


Figure 3: Post doxorubicin embolization angiogram demonstrates no further visualization of the vascular tumor

followed by their attending oncologist at routine oncology clinic visits. Our staff reviewed the follow-up outpatient images and results were included in the patient's electronic medical record for comparison.

Pre-procedure images were compared to post treatment images across time to follow response to therapy. Patients were excluded from this study if they had ongoing infection, active gastrointestinal bleeding, liver failure, coagulopathy or allergy to the chemotherapeutic agents. No patients were on Nexavar (Sorafenib) (Bayer HealthCare, Leverkusen, Germany). There was no portal vein invasion in the study patients. No complications due to intra-arterial chemoembolization occurred during the study.

A full angiographic evaluation of all contributing arteries were performed on all patients. A Mariner cobra catheter (Angiodynamics, Latham, NY) was used to perform a pre-embolization angiogram mapping of the hepatic vasculature [Figure 2]. At the discretion of the interventionalist, the gastroduodenal artery was occluded with embolization coils (Target

Medical/Boston Scientific Corp. Natick, MA) of various sizes, shapes and number prior to placement of the drug eluting beads. Subsequently, a Renegade microcatheter (Boston Scientific Corp. Natick, MA) was utilized to select various feeding branches during HCC chemoembolization. A more proximal lobar infusion was used for colorectal metastasis chemoembolization due to their more diffuse presentation. The study was performed with 300-500 μ m LC Bead which were loaded with either doxorubicin (Bedford Laboratories, Bedford, OH) or irinotecan (Pfizer, Inc., New York, NY) in the hospital pharmacy 2 h prior to the procedure. The doses of irinotecan and doxorubicin were 50 mg/mL and 75 mg/mL respectively and did not change during the study.^[1] One 2 mL vial of doxorubicin and irinotecan were mixed with Ominpaque (Iohexol) 350 mg/mL (GE Healthcare Inc, Marlborough, MA) for a total volume of 10 mL. When the beads and drug finished loading, they were deployed through the micro-catheter into the appropriate vascular location. Following deployment of the drug eluted beads, a final angiogram was performed demonstrating no further filling of the neovascular branches to the tumor masses consistent with complete radiographic embolization [Figure 3]. Following the procedure, the patient was monitored overnight for potential discharge the following day. A follow up CT scan was performed the next day to evaluate the embolized tumor [Figure 4]. In 3 months, a follow up PET-CT scan was obtained to evaluate response to the embolization [Figure 5]. In general, all of the pre-treatment images of the HCC patients had similar findings demonstrating significant tumor enhancement on PET-CT. Following LC Bead chemoembolization, there was a significant decrease in size and enhancement of the treated tumor masses exemplified best in Figure 1 and Figure 5. Lesion size, enhancement pattern and metabolic activity were evaluated by the 4 interventionalists on follow-up contrast enhanced CT and/or PET-CT images. Although not included in this manuscript, the HCC patients' images largely demonstrated heterogeneously



Figure 4: Contrast enhanced computed tomography of the abdomen demonstrates gas in tumor the next day post embolization

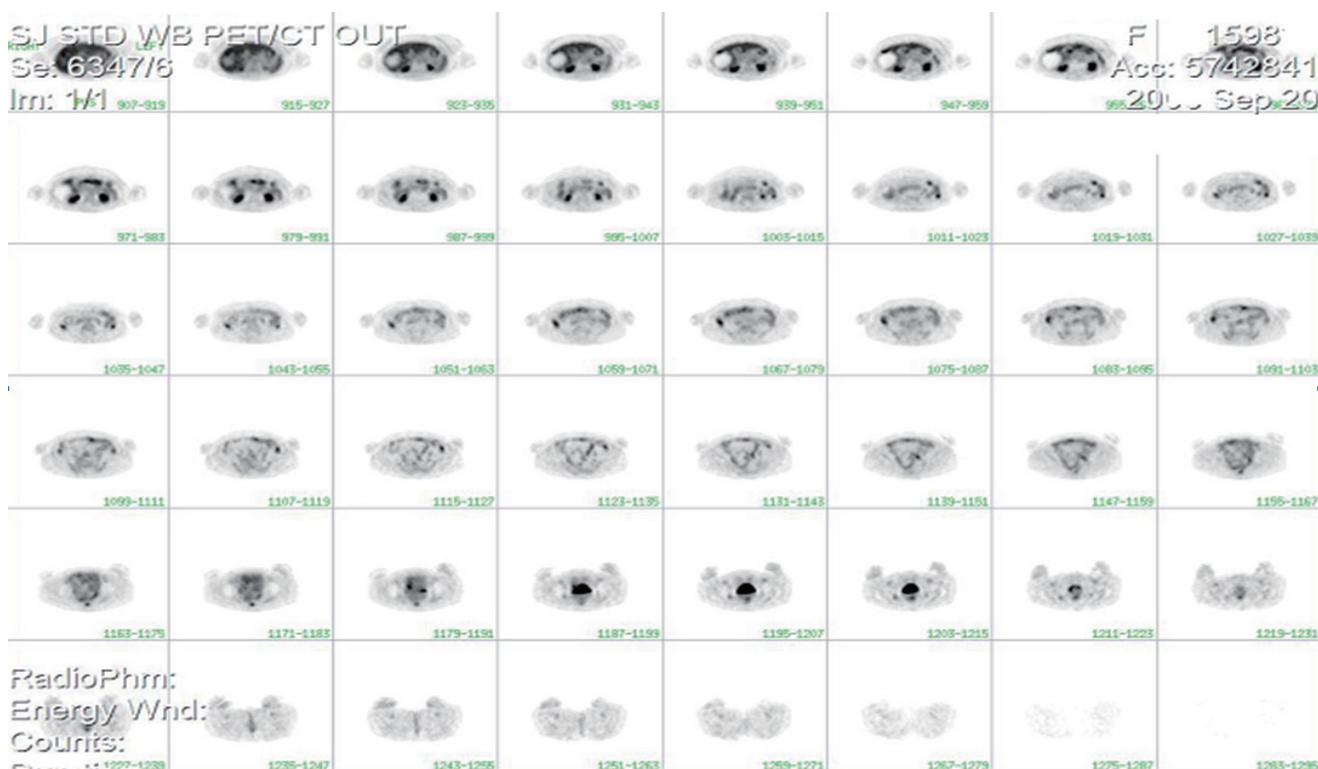


Figure 5: Three months follow-up positron emission tomography scans demonstrating no uptake within the tumor consistent with complete tumor kill

enhancing variable sized tumor masses more common in the right hepatic lobe compared to the left. As in the case presented, 2 of the HCC patients had tumors greater than 10 cm in diameter. Most were single lesions without regional adenopathy or metastasis. Two of the patients had multiple liver masses at time of treatment. The TNM staging for the HCC patients in this study ranged from primary tumor T1, T2 and T3a. There were no primary tumors T3b or T4 lesions. There were no regional lymph nodes N0 and no distant metastasis M0.

In total, 36/48 (75%) of the patients received doxorubicin and 12/48 (25%) patients received irinotecan. The majority (52%) of patients had colon metastasis. Of those, 13/25 (52%) received doxorubicin and 12/25 (48%) received irinotecan. Thirteen patients (27%) had HCC and all 13 received doxorubicin. Of the 10 remaining tumor types, all received doxorubicin. Due to changes in practice over the course of the year when patients were treated, the first 12 colon metastasis patients out of the 25 received irinotecan and all the remaining colon metastasis patients received doxorubicin. Initially, the colon metastasis patients received irinotecan based on the chemotherapy data at that time. However, the initial results of the first 12 patients were statistically poor and the investigators replaced irinotecan with doxorubicin on all the remaining patients in the study. Based on the poor

results with irinotecan eluting beads for the first 12 colorectal metastasis patients, it was clear that this treatment protocol was not effective and needed to be replaced for the benefit of our patients. Certainly, the results were surprising to our researchers especially according to the results documented in the medical oncology literature related to irinotecan treatment of colorectal metastasis. The medical oncologists reviewed all cases with the interventional radiologists and together agreed to replace the protocol in response to the poor irinotecan results. Furthermore, from the onset of the study, all of the HCC patients received doxorubicin based on the chemotherapy data results at that time.

Frequencies and percentages were used to characterize demographic, clinical, and outcomes data from our consecutive case series. Two outcome categories were created for patients with colorectal metastasis by combining those with partial response or stable disease into one category and those with worsened disease into a second category. Due to the small sample size, a Fisher's exact test was used to test the hypothesis that among those with colorectal metastases, outcomes (i.e. partial response or stable disease vs. worsened disease) differed according to the type of treatment received (doxorubicin vs. irinotecan). The difference was considered statistically significant at an alpha of 0.05.

Table 1: Summary of liver neoplasms and treatment type (n = 48)

Type of neoplasm	Number of patients	Treatment type, n (%)		Number deceased
		Doxorubicin	Irinotecan	
Colorectal metastases	25	13 (52)	12 (48)	1
Primary hepatoma (HCC)	13	13 (100)	0	1
Breast metastases	3	3 (100)	0	
Lung metastases	1	1 (100)	0	
Melanoma metastases	1	1 (100)	0	
Sarcoma metastases	1	1 (100)	0	
Pancreatic metastases	1	1 (100)	0	
Neuroendocrine metastases	1	1 (100)	0	
Adrenal metastases	1	1 (100)	0	
Pediatric hepatoblastoma	1	1 (100)	0	1

HCC: hepatocellular carcinoma

Table 2: Treatment responses for patients according to tumor and treatment types

Tumor and treatment types	Complete response	Partial response	Worsened	No follow-up
Hepatocellular carcinoma				
Doxorubicin (n = 13)	8	1	2*	2
Colon metastases				
Doxorubicin (n = 13)	6	4	1	2
Irinotecan (n = 12)	0	1	10*	1

*1 patient died

Table 3: Two-by-two contingency table used to test the hypothesis that among those with colon metastases, those treated with irinotecan had worse outcomes than those treated with doxorubicin

	Complete or partial response	Worsened
Doxorubicin treated (n = 11)	10	1
Irinotecan treated (n = 11)	1	10

RESULTS

A total of 48 patients with unresectable malignant neoplasms of the liver were treated in a 1-year period. There were 28 men (age ranging 34-88 years, with a mean age of 60.5 years) and 20 women (age ranging 34-92 years, with a mean age of 66.2 years). Six patients were lost to follow-up at time of this article. The series includes HCC and colon metastasis [Figure 1]. All of the HCC tumors were hyper-vascular on angiography and became hypo-vascular on follow up scans [Figures 2, 3 and 5]. Many of the remaining tumor types demonstrated hypo-vascular appearance on angiography as compared to HCC. The tumor and treatment types are outlined in Table 1.

Table 2 shows treatment responses according to tumor and treatment types. Nine of the 11 (81.8%) doxorubicin treated HCC patients had either complete response or partial response. All of the HCC lesions showed reduction in size and tumor enhancement and 10/11 (91%) HCC patients were alive at 24 months post treatment [Table 2]. Fisher's exact test revealed that among the 22 with colorectal metastases for

whom follow-up data were available, those 11 who were treated with doxorubicin were significantly more likely to demonstrate complete or partial response compared to the 11 in the irinotecan treated group ($P < 0.001$) [Table 3].

DISCUSSION

Our study compared how HCC and colorectal metastases responded to catheter directed LC Bead embolization with irinotecan and doxorubicin. The results were compelling for a small sample size. Of the 13 colon cancer study patients who were treated with doxorubicin, 46.2% had a complete response and 4/13 (30.8%) had stable disease. The HCC patients on the other hand improved significantly with 81% demonstrating complete or partial response and 91% of them alive at 24 months after treatment.

Overall, the results of this study demonstrated that many patients with unresectable colon metastasis or HCC who were treated with doxorubicin drug eluting beads demonstrated a complete or partial response. All of these patients treated with doxorubicin who showed complete or partial response remained in remission from liver disease for at least 24 months. However, those colon metastasis patients treated with irinotecan eluting beads did poorly and the study investigators stopped using irinotecan on the remaining patient cohort. Only 1 patient out of 12 (8.3%) demonstrated partial response with irinotecan. Even those patients who responded to systemic irinotecan therapy prior

to endovascular treatment did poorly. The irinotecan treated colorectal metastasis patients had poor response rate at 3 months with no reduction in tumor size or tumor enhancement compared to pre-procedural images. The 3 months interval time frame was long enough to account for the post treatment inflammation and edema caused by chemoembolization on the hepatic tumors. Doxorubicin and irinotecan were selected due to the chemotherapy data at that time.

Fiorentini *et al.*^[17] described an 80% response rate following drug eluting bead embolization using irinotecan. However, they used twice the dose of irinotecan (100 mg/mL) compared to this study. Furthermore, their patients were treated once every 3 weeks and subsequently demonstrated improvement in contrast enhancement on all responding patients. In comparison, this article used the standard dosage which may not have been concentrated enough and/or the treatment time may not have been long enough for the embolization to obtain this type of response. Also in their study, the embolization treatments were stopped if findings of progressive disease were noted and subsequently those patients were excluded from the study. On the other hand, our study included all the patients treated with one session of irinotecan bead embolization and none were excluded from the study despite the results.

Along with chemoembolization, combination therapies including radiofrequency ablation, microwave ablation and cryoablation can be used in conjunction with synergistic effects.^[18] The idea of combination therapies is to both embolize the larger tumors decreasing the size with the DEB and then percutaneously ablate the remaining tumor. The DEB treatment prior to percutaneous ablation devascularizes the surface of the tumor which reduces the heat-sink making ablation more effective. Percutaneous ablation of the center of the tumor mass results in a sub lethal temperature experienced at the periphery of the tumor masses allowing these cells to be less resistant to the high concentration of drug.^[18]

The major disadvantage of conventional TACE procedures is the rapid washout of the chemotherapeutic out of the tumor into the systemic circulation. On the other hand, LC Bead chemoembolization has 2 major advantages over conventional TACE. First, the drug is continuously released over a 10-12 days window providing a higher overall intratumoral drug dose over a longer time.^[14] Secondly, with the continuous slow release of drug, there is less systemic toxicity and therefore less post embolization syndrome.^[10,19,20]

Many times peripheral located liver masses that appear successfully embolized can return with increasing size and persistent tumor enhancement on follow-up imaging. Repeat angiograms can demonstrate peripheral tumor vascular recruitment from extrahepatic collateral suppliers prohibiting effective control of the tumor. These angiographic findings were more commonly seen in advanced stages of metastatic liver disease. Those collateral arterial feeders should be separately embolized at that time. Fortunately, a complete vascular assessment during the initial selective angiography eliminated the need for repeat studies attempting to search for new collaterals each time.

There was no intraprocedural discomfort described by the patients during the doxorubicin eluted bead embolization. However, we found 10/12 (83%) of the irinotecan patients described immediate right upper quadrant pain during intraprocedural bead delivery. This phenomenon was rapid in onset, resolved quickly and did not recur following the procedure. This clinical response does not occur with doxorubicin eluted bead placement. If needed, patients were given intravenous analgesia intra-procedurally but no premedication protocol was developed during this study. It may relate to the faster elution of the irinotecan (approximately 4 days) from the beads as compared to doxorubicin.^[21] Also, the amount of liver parenchyma being treated during the embolization frequently is more extensive due to the nature of colonic metastasis.

The study investigators routinely embolized the gastroduodenal artery (GDA) to prevent the embolics from refluxing into the arterial pathways leading into the duodenum and pancreas. In this study, there was no non target duodenal, gallbladder or pancreatic embolization complications. At this institution, GDA embolization is performed in every case because of that small chance of complications related to embolization of non target vascularity. We understand that gastroduodenal artery embolization is not the standard of practice in many centers despite the use of microcatheters for delivery of the embolic material. However, we believe that preserving the gallbladder, duodenum and pancreas from preventable non target embolization is crucial. GDA embolization is a quick and technically easy procedure to perform prior to LC Bead chemoembolization not adding much procedure time to the case.

The study is a retrospective investigation of this institution's LC Bead chemoembolization practice and there are several study limitations. First, doxorubicin was the only chemotherapeutic agent used on HCC.

However, the use of irinotecan for colon metastasis was chosen based on the oncologic data at that time. The literature described irinotecan as very effective to colon metastasis when given intravenously. Therefore, the investigators used this drug initially on all colorectal metastasis patients. Unfortunately, during the early part of the study, this drug was found to be ineffective on the first 12 colon metastasis patients with a poor response given intra-arterially. From that point on, doxorubicin was used exclusively during the remaining part of the study. The reason to switch from irinotecan to doxorubicin was based solely on its poor response in the first 12 patients. Once switched, there were statistically improved results using doxorubicin compared to irinotecan on colon metastasis patients. Secondly, the authors used FDG PET-CT for their follow up imaging. MRI with dedicated liver agents have become readily available and considered sufficiently sensitive for routine use for detection of HCC which may not have been identified on follow-up FDG PET-CT. Lastly, this study consisted of a very small sample size at a single institution and may not be reflective of a larger population. However, these results were compelling and suggest the need for additional systematic or randomized studies that compare these different treatment options.

This retrospective study evaluated and compared how HCC and colorectal metastasis responded to doxorubicin and irinotecan. It demonstrated that doxorubicin eluted bead embolization resulted in longer patient survival as compared to conventional therapies previously reported in the literature.^[6-9] Although the patient sample size was small, 81.8% of the HCC patients and 77% of the colon metastasis patients had either complete or partial response.

Patients receiving irinotecan had a statistically significant poor response as compared to doxorubicin. Furthermore, those patients with colorectal metastases who did not respond to irinotecan initially could be candidates for repeat embolization with doxorubicin and could hopefully improve following repeat treatment outside of this study. Overall, HCC and colon metastasis patients demonstrated the effectiveness of DEB with 91% of the HCC patients alive 24 months after treatment. Prospective randomized trials would be helpful for further evaluation in a large subset of patients. Endovascular specialists should be aware of the benefits LC Bead embolization can bring to the oncology community as malignant neoplasms of the liver continue to increase in the future.

Authors' contributions

Guarantor of integrity of the entire study, study concepts

and design, literature research, clinical studies, and manuscript preparation: G.S. Stambo
Statistical analysis: D. Cragun
Experimental studies/data analysis, and manuscript editing: G.S. Stambo, D. Cragun

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None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Each patient was informed of the study and gave their consent.

Ethics approval

This was a retrospective study and did not require Institutional Review Board approval.

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In vitro antitumor efficacy of *Kochia indica* extract on human hepatocellular carcinoma cell line with or without 5-fluorouracil

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ABSTRACT

Aim: Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and the sixth most common cancer worldwide. The resistance to chemotherapy is a major obstacle in the treatment of HCC, necessitating the discovery of additional agents. There is a growing use of anticancer complementary and alternative medicine worldwide. Therefore, the aim of present study was focused on the confirmation of the suitability and validity of the new markers which would be achieved by demonstrating their significant change and reproducible expression during disease and disease management. **Methods:** HepG₂ cell line was used to provide a source for HCC cells. The cell cultures were divided into 4 groups: control untreated group, 5-fluorouracil (5-FU) treated group as a standard chemotherapy for HCC (positive control) with the following doses (15.625, 31.2, 62.5, 125, 250 µg/mL), *Kochia indica* extract treated group with the following concentration (12.5, 25, 50, 100, 200 µg/mL) and the group treated with a combination of 5-FU and *Kochia indica* in different ratios. **Results:** Treatment with *Kochia indica* extract, 5-FU and the combined treatment showed a significant cytotoxicity to HepG₂ cells, with different IC₅₀ values, when compared to the control. Regarding toxic effect, 5-FU showed IC₅₀ = 237.56 µg/mL which is lower cytotoxic in compared to *Kochia indica* with IC₅₀ = 120.5 µg/mL. The results also revealed that tumor cells were more resistant to 5-FU. Alternatively, the co-treatment with *Kochia indica* extract ameliorated the toxicity induced by 5-FU and enhanced its therapeutic potency, either by synergistic effect of both agents and/or due to its flavonoid components that may enhance the physiological properties of the cell membranes, facilitating 5-FU entrance into tumor cells. This decreased its therapeutic dose to less than 250 µg/mL by combination therapy. **Conclusion:** Present findings assume that *Kochia indica* extract co-therapy can ameliorate the side effects of 5-FU on HepG₂ by enhancing its cellular uptake.



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INTRODUCTION

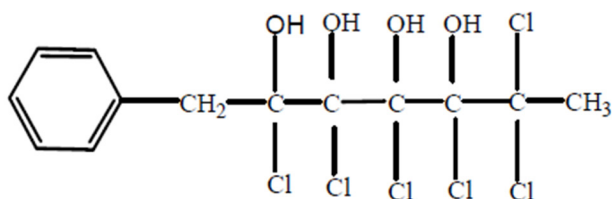
Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and the sixth most common cancer worldwide.^[1] Also, It is an aggressive malignancy with a poor prognosis and is currently the second most common cause of cancer-related mortality.^[2] It was found that about 80% of the estimated new cases of HCC occurred in less developed regions and its incidence is increasing worldwide in more developed countries^[3] including Egypt because of predominant diseases such as hepatitis C virus (HCV) and schistosomiasis.^[4]

It was recognized that the resistance to chemotherapy, 5-fluorouracil (5-FU), is a major obstacle in the treatment of HCC, necessitating the discovery of additional agents.^[5] Thus, the use of natural products in this respect is extensively under investigation.^[6] One of these products is *Kochia indica*, family chenopodiaceae [Figure 1]. It was originally introduced as an ornamental plant in some gardens. In the present time, it has spread and is now presented in both crop and no crop areas.^[7]

The elementary analysis of *Kochia indica* extract was previously carried out by Haroun^[8] using ultraviolet (UV) spectroscopy and infrared spectroscopy, gas chromatography and mass spectrophotometry. As shown in Figure 2, the chemical analysis of *Kochia indica* extract detects an active phenolic compound



Figure 1: A photograph showing *Kochia indica* whole plant



1-phenyl 2,3,4,5,6,6 hexa chloro 2,3,4,5 tetra hydroxy heptan

Figure 2: Phenolic active compound of *Kochia indica* plant extract^[8]

with proposed molecular formula $C_{13}H_{14}Cl_6O_4$, molecular weight 447 and chemical structure, 1-phenyl 2,3,4,5,6 hexachloro 2,3,4,5 tetra hydroxy heptane.

Previous investigators showed that *Kochia indica* has a great medicinal potency. It is used as a heart tonic agent^[9] and it has a strong tumoricidal properties.^[8] Additionally, resinous alkaloid, isolated from alcoholic extract of the plant showed nicotinic action on autonomic ganglia and neuromuscular junctions of voluntary muscles. Fruits and leaves of a related species, *Kochiascoparia* is used as cardiac tonic, anti-dermatitis and diuretic agent.^[10]

Also, ether extract of aerial parts of *Kochia* contains n-alkanes, free alcohols and a mixture of sterols, mainly sitosterol (70.9%). Ether plant extract exhibited antibacterial activity which is attributed to hydrocarbons and sterols present in its parts.

As known, one of the excellent chemotherapeutic drugs is 5-FU. Despite the excellent therapeutic effects of 5-FU, its cytotoxicity and genotoxicity in normal cells remain a major health problem.^[11] Therefore, thinking about herbal use is gradually growing worldwide to reduce the hepatotoxicity induced by 5-FU treatment. The present work was undertaken to test whether *Kochia indica* extract can exhibit antitumor effect by itself and can improve the antitumor efficacy of 5-FU treatment by reducing its probable side effects.

METHODS

Chemicals, cultures and reagents

The anti-cancer 5-FU obtained as an ampoule (10 mL) contains 250 mg of fluorouracil was stored below 30 °C, protected from light and preserved in sealed containers. It is manufactured by ACDIMA International (AiT, S.X. Haipu Pharmaceutical Co., Ltd.).

To prepare the standard anticancer treatment regimen of 5-FU, it was diluted with Dulbecco's modified Eagle medium (DMEM) to desired concentrations ranging from 10 to 250 µg/mL. The final concentration of dimethyl sulfoxide (DMSO) in each cell culture did not exceed 1% v/v, to keep the cytotoxicity of DMSO at less than 10%. However, fluorouracil (25 mg/mL) stock solution was 100 fold diluted (10 mL + 990 mL medium) then it was mixed with the *Kochia indica* extract in various ratios, and each combination ratio was 2 fold serially diluted. The concentration of each agent in the combination was determined according to the following equation:

$N \cdot V$ [half maximal inhibitory concentration (IC_{50}) for agent separately] = $N \cdot V$ (in combination)

$N = IC_{50}$ for the agent, V = volume of the agent separately = 1

N = the concentration of the agent in the combination, V = the volume of the agent in the combination

Cell culture media, including DMEM and fetal bovine serum (FBS) were purchased from GIBCO® (Invitrogen). Penicillin and streptomycin, the culture antibiotics, were purchased from (Aldrich-Sigma Company, CA, USA). DMSO, fluorescence dye, Sodium bicarbonate ($NaHCO_3$), neutral red, 4-(4'-nitrobenzyl) pyridine (NBP) and 5-FU were also purchased from Sigma-Aldrich. All chemicals and reagents used in the experiments are of highly purified grades.

Hepatoma cell line

Hepatoma cell line (HepG₂) was obtained from Holding Company for Biological and Vaccine Production, Cairo, Egypt. HepG₂ was maintained in the cell culture laboratory, Medical Research Institute, Smouha, Alex, Egypt. HepG₂ is a perpetual cell line consisting of human liver carcinoma cells, derived from the liver tissue of a 15-year-old Caucasian male patient who had a well-differentiated HCC.

Natural agents

Kochia indica leaf plant was collected nearby Tanta city, Al-Gharbiya Governorate, Egypt. The plant was authenticated visually in taxonomy laboratory at Botany Department, Faculty of Science, Tanta University, Egypt. The collected plants were washed under running tap water and blotted where they were cut into small pieces and kept for drying in oven at temperature 40 ± 2 °C for 5 days. The dried plant material was ground into powder and stored in air tight container as described by Prayong *et al.*^[12] The crude ethanolic extracts were dissolved in DMSO at 20 mg/mL as stock solutions which were then diluted with DMEM to desired working concentrations ranging from 10 to 250 µg/mL. The final concentration of DMSO in each sample did not exceed 1% v/v, to keep the cytotoxicity of DMSO at less than 10%.

Preparation of HepG₂ culture media

The HepG₂ culture media was prepared according to Van der Bliek *et al.*^[13] Briefly, the vial containing HepG₂ was placed in a 37 °C water bath until the contents were thawed and decontaminated immediately by dipping in or spraying with 70% ethanol. The vial contents were transferred to a centrifuge tube containing 9.0 mL of absolute DMEM. To prepare DMEM growth medium, FBS was added to reach 10% final concentration. Prepared cell culture media were spin at approximately

125 g for 5-7 min. The cell pellet was suspended again in the medium and dispense into a 25 cm² culture flask. The culture was incubated at 37 °C in a 5% CO₂ incubator and thereafter, was ready for sub culturing into working groups.

Experimental cell groups

HepG₂ cells were divided into 4 groups described as follow: (1) first group contained HepG₂ cells cultured in a media and left without any treatments to serve as a negative control; (2) second group contained HepG₂ cells cultured in a media treated with the following doses (15.625, 31.2, 62.5, 125, 250 µg/mL) of 5-FU as a standard chemotherapy for HCC and left to serve as a positive control; (3) third group contained HepG₂ cells cultured in a media and treated with the following doses (12.5, 25, 50, 100, 200 µg/mL) concentrations of *Kochia indica* ethanol extract alone and left as an experimental group; (4) fourth group contained HepG₂ cells cultured in a media treated with a combination of 5-FU and *Kochia indica* extract in different ratios (1:1, 2:1, 1:2, 1:9) and left as an experimental group.

Cell viability determination

The HepG₂ culture was sub-cultured at 37 °C under a humidified environment containing 5% CO₂ incubator. Cytotoxic effect of the tested agents on tumor cells was tested by the neutral red (NR) method described by Fotakis and Timbrell.^[14] In brief, tumor cells were seeded in 96-well plates (100 µL/well at a density of 3×10^5 cells/mL) and treated with various concentrations of the used agents for 24 h. Then, cells were washed twice with 1× phosphate buffer saline and the supernatant was discarded. A total of 100 µL of NR solutions (50 µg/mL) was added to each well and incubated at 37 °C for another hour. The NR dye was then dissolved in 100 µL of 0.33% HCl. Absorbance of NR dye was detected by a dual-wavelength UV spectrometer (Anthos 2010; Biochrom, UK) at 450 nm wavelength. The cytotoxicity was determined against untreated cells by the following equation of Machana *et al.*:^[15] cytotoxicity (%) = $[100 \times (\text{absorbance of untreated group} - \text{absorbance of treated group})] / \text{absorbance of untreated group}$. Therefore, the cell viability (%) = 100 - cytotoxicity.

To calculate the IC_{50} values of the agents under investigation, cytotoxicity (%) was plotted against agent's working concentrations^[14] to give linear equation from which IC_{50} was calculated. Also, the quantitative efficacy of the combination exposure between 5-FU and *Kochia indica* extract was determined as a combination index (CI) according to the following equation:^[16] $CI = (5\text{-FU in combination}) / (5\text{-FU alone} +$

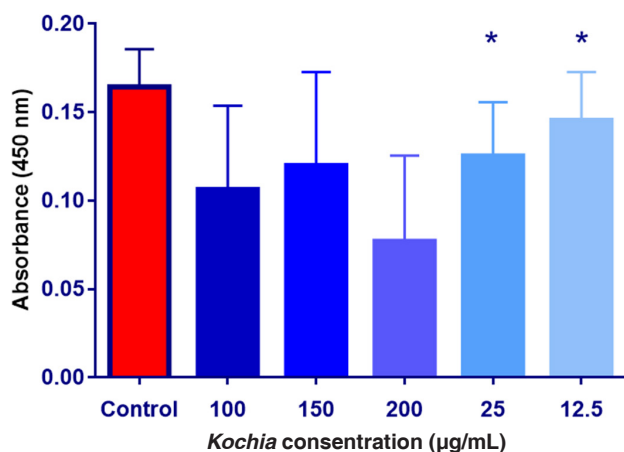


Figure 3: The absorbance values of HepG₂ cultured cells exposed to upgrading concentrations of *Kochia indica* extract. Data are expressed as means \pm SD ($n = 9$); * $P \leq 0.05$ vs. non-treated control group

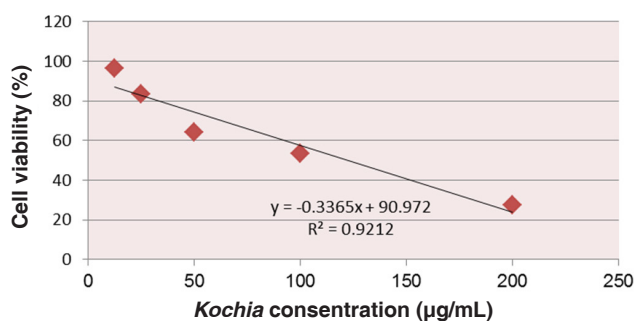


Figure 4: A linear relationship between calculated cell viability of HepG₂ cultured cells and concentrations of *Kochia indica* extract. This linear relationship resulted in $y = -0.3365x + 90.972$ and $R^2 = 0.9212$, from which value of IC₅₀ was obtained as 121.75 $\mu\text{g/mL}$

(*K. indica*) in combination/(*K. indica*) alone. If $CI < 1$, it means antagonistic effect; $CI = 1$, it means synergistic effect; and if $CI > 1$, it means additive effect.

Statistical analysis

Data were expressed as means \pm standard deviation ($n \geq 3$). Data of treated groups were statistically analyzed vs. control group by one way analysis of variance (ANOVA), followed by t -test using Graph Pad prism 6 Software, CA, USA. However, statistical difference was considered significant if $P \leq 0.05$ confidence interval.

RESULTS

Effect of *Kochia indica* extract on HepG₂

As shown on Figure 3, the mean values of absorbance were decreased after the exposure of HepG₂ cells to upgrading concentrations of *Kochia indica* extract compared to the control group. The obtained data were analyzed with ANOVA and showed that the difference among the absorbance values in the control group

Table 1: Effect of upgrading concentrations of *Kochia indica* extract on the HepG₂ cultured cells toxicity and viability ($n = 9$)

<i>Kochia indica</i> concentration	Cytotoxicity (%)	Cell viability (%)	t value	P value
Control	0	100		
12.5 $\mu\text{g/mL}$	3.80435	96.19565	3.761	0.0031
25 $\mu\text{g/mL}$	16.48551	83.51449	1.771	0.104
50 $\mu\text{g/mL}$	36.05072	63.94928	2.555	0.028
100 $\mu\text{g/mL}$	46.73913	53.26087	2.503	0.092
200 $\mu\text{g/mL}$	72.46377	27.53623	1.317	0.215
P value	0.0071			
F value	3.765			

and the other groups treated with *Kochia indica* was significant at $P \leq 0.05$. By application of t -test, the difference between means was significant after the exposure of HepG₂ cells to 12.5 and 25 $\mu\text{g/mL}$ of the plant extract while the differences between means of other groups were not significant.

Data in Table 1 show the values of both cell toxicity and viability percentages after the exposure of HepG₂ cultured cells to all treatments. It was found that the cytotoxicity (%) was increased with increasing concentration of *Kochia indica* extract (12.5-200 $\mu\text{g/mL}$). Oppositely, the cell viability (%) of HepG₂ was gradually decreased with increasing concentration of the plant extract. Therefore, as the concentration of *Kochia indica* extract increases, the cytotoxicity (%) of HepG₂ increases, and vice versa for the % of the cell viability which decreases with the increase of the extract concentration [Figure 4].

Effect of 5-FU concentrations on HepG₂

As shown on Figure 5, the absorbance values were decreased after the exposure of HepG₂ cultured cells exposed to various concentrations of 5-FU compared to the control group. ANOVA showed that the difference among the absorbance values in the control group and the other groups treated with 5-FU was significant at $P \leq 0.05$. To determine the significance, t -test was applied on the obtained data and showed that the difference between means was significant after the exposure of HepG₂ cells to 125 and 250 $\mu\text{g/mL}$ of 5-FU, but not significant with other groups.

Results in Table 2 demonstrates that the percentage of cytotoxicity increased gradually by increasing the concentration of 5-FU, while the viability (%) of HepG₂ cultured cells was decreased. The absorbance means of two groups exposed to both concentrations 125 and 250 $\mu\text{g/mL}$ of 5-FU were significantly different ($P \leq 0.05$) compared to the control.

Data depicted in Figure 6 show the relationship

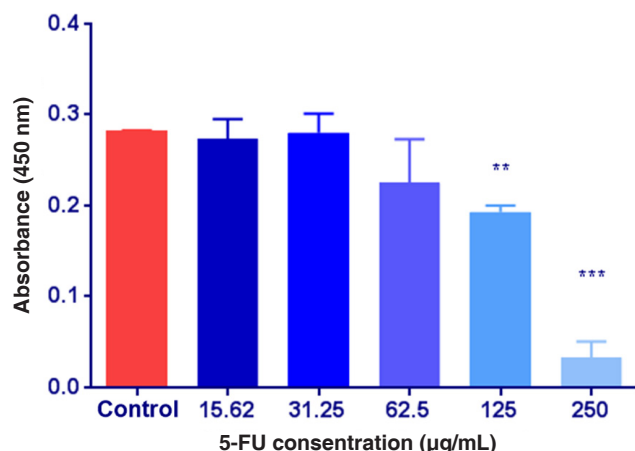


Figure 5: The absorbance values of HepG₂ cultured cells exposed to 5-fluorouracil (5-FU) various concentrations. Data are expressed as means \pm SD ($n = 9$, $**P \leq 0.01$, $***P \leq 0.001$, vs. non-treated control group)

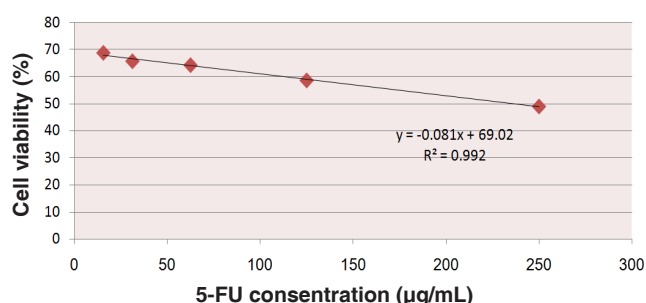


Figure 6: The linear indirect relationship between cell viability percent of HepG₂ cultured cells against upgrading concentrations of 5-fluorouracil (5-FU), value of IC₅₀ that gives 50% viability was obtained as 234.342 µg/mL

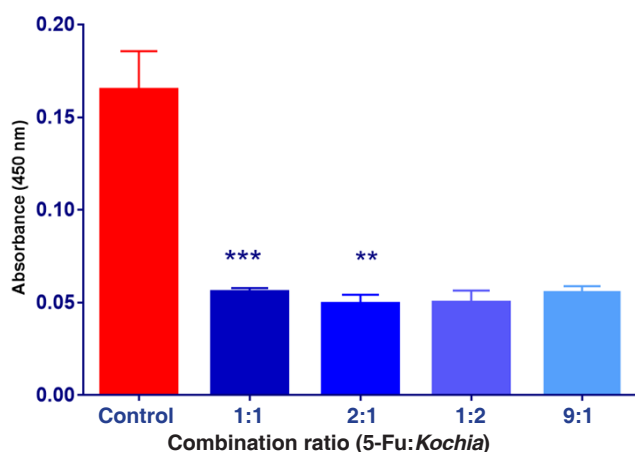


Figure 7: The absorbance values of HepG₂ cultured cells exposed to combination ratios of 5-fluorouracil (5-FU) and *Kochia indica* extract various concentrations. Data are expressed as means \pm SD ($n = 9$, $**P \leq 0.01$, $***P \leq 0.001$, vs. non-treated control group)

between the percentages of the HepG₂ cell viability and upgrading concentrations of 5-FU. This relationship gives rise to a linear equation: $y = (-0.081x + 69.02)$ ($R^2 = 0.992$) from which IC₅₀ be calculated as

Table 2: Effect of upgrading concentrations of 5-FU on the HepG₂ cultured cell toxicity and viability ($n = 9$)

5-FU concentrations	Cytotoxicity (%)	Cell viability (%)	P value (t-test)	t value (t-test)
Control	0	100		
15.625 µg/mL	31.34058	68.65942	< 0.626	0.569
31.25 µg/mL	34.42029	65.57971	< 0.891	0.1549
62.5 µg/mL	35.86957	64.13043	< 0.2339	0.1686
125 µg/mL	41.48551	58.51449	< 0.0031	18.72
250 µg/mL	51.08696	48.91304	< 0.0001	17.8
P value (ANOVA)	< 0.0001			
F value (ANOVA)	78.11			

5-FU: 5-fluorouracil; ANOVA: analysis of variance

Table 3: Effect of combination therapy of 5-FU and *Kochia indica* extract on the HepG₂ cultured cells toxicity and viability ($n = 9$)

Combination ratio 5-FU: <i>Kochia</i> (v:v)	Cytotoxicity (%)	Viability (%)	P value (t-test)	t value (t-test)
Control	0	100		
A ratio of 1:1	42	58	< 0.0005	1.587
A ratio of 2:1	53.2	46.8	< 0.0018	2.321
A ratio of 1:2	52	48	< 0.9319	18.647
A ratio of 9:1	57	43	< 0.736	15.265
P value (ANOVA)	< 0.0001			
F value (ANOVA)	67.08			

5-FU: 5-fluorouracil; ANOVA: analysis of variance

234.342 µg/mL of 5-FU.

Combination effect of 5-FU and *Kochia indica* extract on HepG₂ cells

Data shown on Figure 7 shows means of the absorbance values of HepG₂ cells exposed to various combination ratios of 5-FU and *Kochia indica* extract. It was observed that the obtained absorbance in the experimental groups was reduced compared to the control group. Statistical analysis revealed that means of treatments in ratio of 2:1 was more significant than 1:1, compared to the control.

Results in Table 3 show the combined effect of both 5-FU (250 µg/mL) and *Kochia indica* extract (100 µg/mL) on the cell toxicity and viability of HepG₂ cultured cells. After the exposure of HepG₂ to the combination therapy, data obtained were determined as mean values of the absorbance. Based on the absorbance, the percentage of cytotoxicity was increased while the percentage of the cell viability was decreased by increasing the combination ratios. The most effective ratio was found to be (1:2, 5-FU/*Kochia*) of both agents and this ratio led to cytotoxicity of 52% and cell viability of 48% on HepG₂ cultured cells.

By the application of CI equation on the ratio of 1:2, CI can be calculated as: $CI = 83.33/250 + 66.66/100 = 0.33$

+ 0.6666 = 0.9966 = 1 in approximately (synergistic effect). Accordingly, when 2 v of *Kochia indica* extract was added to 1 v of 5-FU, there is a synergistic effect on the cell viability of HepG₂.

DISCUSSION

The *in vitro* cytotoxicity test was mainly performed to screen potentially toxic compounds that affect basic cellular functions. This toxicity is measured with cellular damage using NR which is a weak cationic dye that penetrates and accumulates in the lysosomes of living cells. Therefore, NR assay was used to determine the cell viability or, in other words, the toxicity of the test compounds.^[14]

Data obtained from *in vitro* study on *Kochia indica* extract, 5-FU and combination treatment showed a significant cytotoxic effect to HepG₂ cultured cells, with different IC₅₀ values, when compared to the control. For example, the value of IC₅₀ of *Kochia indica* extract was found to be 120.5 µg/mL. Also, a previous study showed that *Kochia indica* seeds exerted lethal effect on tumor cells IC₅₀ = 0.147 mg/mL which may indicate that the seeds of this plant have serious toxic side effects than other parts.^[8]

Other studies showed different results between aqueous and alcoholic extracts of *Kochia indica* where the anticancer activity of the aqueous extract was 2.88 and anticancer activity of the ethanolic extract was 1.6. It was observed that the aqueous extract is more potential than the ethanolic one.^[15]

Many studies showed that *Kochia indica* extract is rich in flavonoids^[16] and alkaloids.^[9] Therefore, it has many biological properties about its mode of action such as antioxidant,^[17] heart tonic^[9] and anticancer effect.^[5,9,17] Many cancers develop resistance to chemotherapy, thus lowering its anticancer efficacy. As current treatments become inadequate, higher doses of anticancer therapies such as 5-FU and other novel therapies are used in treating cancers. Such higher dose therapies can lead to cytotoxic and genotoxic side effects in normal cells.^[18]

According to the above information, 5-FU is a widely used anticancer drug, but the response to it shows low efficacy, approximately 20%.^[19] Thus, many studies have investigated 5-FU with other anticancer drugs such as antioxidants.^[20] However, cytotoxicity is nearly inevitable when a combination of 5-FU and antioxidants is used, which remains a major problem in chemotherapy.^[21]

In the present study, 5-FU showed IC₅₀ = 237.56 µg/mL

which is a lower cytotoxic in comparing with *Kochia indica* with IC₅₀ = 120.5 µg/mL which emphasize the resistance of HepG₂ cells to 5-FU as a chemotherapeutic agent. The current work showed also additive synergistic effect when 5-FU combines with *Kochia indica* extract by ratio of 2:1 (v/v) in the treatment of HepG₂ cultured cells. A previous study showed two dual effects when 5-FU enhanced with antioxidant such as, Bupleuri Radix that enhanced 5-FU induced cytotoxicity in HepG₂ hepatoma cells, and protected normal lymphocytes from 5-FU induced cytotoxicity.^[22]

Results of IC₅₀ obtained above showed that the treatment of HCC with either 5-FU or *Kochia indica* extract alone can lead to a cytotoxic effect to the target tumor cells. Instead, the combination between a well-known chemotherapy such as 5-FU and antioxidant such as *Kochia indica* extract can ameliorate the toxicity induced by 5-FU as well as enhance the therapeutic potency of 5-FU toward cancer cells by what is known synergistic effect of both agents. The present results can also suggest that flavonoids found in *Kochia indica* extract enhance the physiological properties of the cell membranes facilitating 5-FU entrance into tumor cells which were resistant to 5-FU, and hence decrease the therapeutic dose to be less than 250 µg/mL as shown in combination therapy. These results are considered as a first line in studying the possible efficacy of *Kochia indica* extract as an anti cancer agent or as adjuvant with 5-FU, however, further *in vivo* studies are suggested at experimental animal sessions.

Authors' contributions

Study design: N.M. Abdel-Hamid

Experimental work: W.O. Zeina, G.A. Tabl

Manuscript drafting: Y.E. El-Bolkainy

Manuscript revision: N.M. Abdel-Hamid, W.O. Zeina

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

University of Tanta approved the whole work prior to any work.

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Advances in the diagnosis and treatment of liver fibrosis

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ABSTRACT

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Liver fibrosis is the center of diagnosis and management of essentially all chronic liver diseases. While liver biopsy examination still has a role in diagnosis and drug development, it is replaced by non-invasive assessments of liver biopsy in majority of the clinical scenarios. Radiological approaches, namely transient elastography, acoustic radiation force impulse imaging, shear wave elastography, magnetic resonance elastography provide accurate diagnosis of advanced fibrosis and cirrhosis. Serum test formulae based on common laboratory parameters or more specialized parameters including those commercially available panels FibroTest[®], FibroMeter[®] and Enhanced Liver Fibrosis are also available. Combining different modalities may further improve the accuracy. The role of all these non-invasive assessments has been further expanded from diagnostic to prognostic, e.g. risk prediction of hepatocellular carcinoma (HCC) by LSM-HCC score. Treatment of liver fibrosis can be achieved by controlling the underlying diseases, with chronic viral hepatitis as the most established disease model. Currently there are multiple clinical trials evaluating different treatment options to improve fibrosis in patients with non-alcoholic fatty liver disease. Specific anti-fibrotic treatment targets e.g. direct downregulation of hepatic stellate cell, collagen synthesis inhibitors and transforming growth factor- β antagonists have been tested in laboratory and pending further studies in clinical settings.

INTRODUCTION

Liver fibrosis is the formation of scar tissue in response to parenchymal injury secondary to chronic liver disease, e.g. chronic hepatitis B and C, non-alcoholic fatty liver disease (NAFLD) or alcoholism. It distorts the normal liver parenchyma.^[1] The continuous and progressive replacement of hepatocytes by extracellular matrix and fibrous tissue leads to liver cirrhosis, which is a key risk factor for hepatocellular carcinoma (HCC).^[2]

Apart from its relationship with HCC, liver fibrosis is also an important treatment indication in various chronic liver diseases. Different international treatment guidelines mentioned that the severity of liver fibrosis should be considered, regardless of the level of ALT, for starting antiviral treatment for chronic hepatitis B (CHB).^[3,4] There are solid evidence supporting the fact that liver fibrosis is potentially reversible.^[5] Therefore, it is important to diagnose and assess the severity of liver fibrosis in order to provide appropriate



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management in order to prevent further liver damage. This article focuses on the up-to-date approaches for diagnosis, both invasive and non-invasive, and latest development in treatments of liver fibrosis, particularly in NAFLD patients for whom a handful of clinical trials are currently ongoing.

DIAGNOSIS OF LIVER FIBROSIS

There are varieties of methods for making the diagnosis of liver fibrosis, which can be classified into invasive and non-invasive approaches.

Invasive approach - liver biopsy examination

For invasive approach, it refers to liver biopsy examination, which provides liver tissue for a histopathological assessment of liver. Liver biopsy examination can be done percutaneously, transvenously (either transjugularly or transfemorally), or surgically (open or laparoscopic operations).^[6] Indications for liver biopsy are for diagnostic and/or prognostic purposes, as well as for treatment planning.^[7]

Liver biopsy is still regarded as the gold standard for liver fibrosis assessment in various chronic liver diseases.^[8,9] Apart from general histological staging, liver biopsy can also provide information concerning morphometry, which can provide additional information on the distribution and the exact quantity of liver fibrosis.^[10] A recent quantitative tool called qFibrosis utilized 87 parameters aiming for combining the results of collagen patterns, collagen architectural features and statistical analysis of features of respective collagen patterns into a single index. This requires an unstained biopsy sample for the automated analysis of liver fibrosis staging.^[11] All these evidences illustrate that liver biopsy plays an important role in the diagnosis of liver fibrosis.

Apart from liver fibrosis staging, liver biopsy can provide different information important for the management of the clinicians. For example, in cases of NAFLD, the degree of necroinflammation and steatosis can be determined by liver biopsy so corresponding management can be provided for this potentially reversible situation.^[12] Liver biopsy is also helpful in diagnosing adverse drug reaction and classification of liver tumors.^[13] Yet, the most common reason for conducting a liver biopsy is for assessing the liver fibrosis in patients with chronic viral hepatitis and NAFLD.

Such a direct and useful method bears quite a few limitations. Sampling error is a major limitation for liver biopsy as only 1/50,000 of the whole liver parenchyma

is obtained. Sampling error can be minimized by either obtaining a specimen of sufficient size (at least 2 cm in length) or from different lobes, which may not be feasible all the time.^[14] Well reported complications from liver biopsy examination include pain,^[15] bleeding such as wound bleeding, intraperitoneal hemorrhage, hemobilia or hemothorax,^[15] transient acute hypotension or vasovagal syncope.^[16] Fatal complications like uncontrolled bleeding, bacteremia and sepsis are rare but still possible.^[17] In patients with HCC, liver biopsy also carries a risk of spreading the cancer cells.^[18]

Non-invasive approach

Radiological assessments are either ultrasonographic-based [e.g. transient elastography, acoustic radiation force impulse (ARFI) imaging and shear wave elastography (SWE)] or magnetic resonance (MR)-based [i.e. MR elastography (MRE)].

Ultrasonographic based

Transient elastography

Transient elastography (Fibroscan®, Echosens, Paris, France) assesses liver stiffness measurement (LSM) by transmitting shear wave followed by ultrasound wave through a probe putting on the skin overlying the liver parenchyma. The velocity of the shear wave passing through the liver parenchyma is calculated by Doppler technique. The higher the velocity, the stiffer the liver parenchyma is. As mentioned by the manufacturer, for an examination to be considered as reliable, it requires at least 10 successful attempts and the ratio of interquartile range to median of those measurements should be less than 0.3.^[19] LSM reflects the degree of liver fibrosis.^[20] It can even identify those with no or minimal fibrosis and differentiate them from those with severe fibrosis or cirrhosis.^[21] It has been proved useful across different liver disease entity (e.g. chronic hepatitis B and C, autoimmune hepatitis).^[22] However, LSM by transient elastography is found to be less reliable in obese patients.^[23,24] It can be less accurate in certain situation, e.g. severe acute exacerbation of hepatitis,^[25] post-treatment fibrosis stages in CHB^[26] or chronic hepatitis C (CHC) patients.^[27]

ARFI imaging

ARFI is another technique for estimating liver fibrosis. It is implemented in current ultrasound scanner, without acquirement of external equipment. The conventional ultrasound probe automatically produces an acoustic "push" pulse for generating shear-wave which passes through the tissue. The wave propagation speed is assessed. Again, higher the speed, higher the liver stiffness measurement is.^[28,29] There are several advantages for ARFI. As it is a function of the ultrasound

scanner, no additional cost is required.^[30] The ARFI not only shows the degree of fibrosis, it also provides external information for disease progression for different chronic liver disease, for example HCV.^[31] Another advantage of this tool is that it can provide real-time results and easy to perform. The measurement results appear to be more accurate in overweight and obese patients, compared with transient elastography.^[32] However, one prominent disadvantage for ARFI is that the range of its measurement is quite narrow (only from 0.5 to 4.4 m/s).^[33] Furthermore, it is quite difficult to match the degree of fibrosis with the wave propagation speed, i.e. a cut-off, which is difficult to be defined.^[34]

SWE

SWE is a 2-dimensional ultrasound technique based on the estimation of shear wave velocity from the radiation force of a focused beam of ultrasound,^[35] and it can be converted results in terms of kPa by an equation.^[36] No extra vibrator or detector is required as it is integrated into a conventional ultrasound system. Besides, elasticity of liver tissues can be shown in both numerical values and color (i.e. higher stiffness is reflected in red color), which can reflect the relative stiffness of the liver tissue quickly. The numerical values can be expressed in either kPa or m/s, which can be comparable with the results from transient elastography or ARFI.^[37] Actually, its accuracy is higher compared to transient elastography or AFRI in assessing the degree of fibrosis, especially in those with early-stage liver fibrosis.^[38] SWE with spleen stiffness index is recommended as the first line assessment for patients with liver fibrosis due to chronic hepatitis C in the latest guidelines.^[39] However, only a few studies validate its clinical application.^[38,40]

MRE

MRE adopts a phase contrast imaging method which depends on mechanical wave propagation to assess the degree of liver stiffness.^[41] Generally, MRE is less operator-dependent and involved in less technical failure. The global picture of the liver can be viewed easily, regardless the obesity or severity of the ascites of the patients. It can also give a comprehensive assessment for the associated complications, for example portal hypertension or associated spleen stiffness.^[42] Meanwhile, it is useful for diagnosis and staging of liver fibrosis, even if the fibrosis is very mild. Another advantage for MRE is that the results are readily reproducible.^[42] However, MRE is more expensive and time-consuming compared to ultrasound-based approach. Respiration creates artifacts on the images. Another important limitation is that it is not applicable on patients with iron overload, or hemochromatosis, because iron might create noise for

the signals received by the MR machine.^[43] There are still limited studies mentioning the clinical significance of MRE results. Even though it is apparently sensitive to mild liver fibrosis, the result may sometimes be unreliable.^[44]

Serum test formulae

Common laboratory parameters

Another commonly adopted non-invasive assessment is based on serum with or without clinical parameters. Examples including common parameters in clinical practice include aspartate aminotransferase (AST) to platelet ratio index (APRI),^[45] Forns index,^[46] Fibrosis-4 (FIB-4),^[47] Fibroindex,^[48] Hui index,^[49] NAFLD fibrosis score (NFS)^[50] and BAAT score^[51] [Table 1]. These parameters are derived from routine liver biochemistry panel, so it is quite convenient. These parameters are also technically easy to obtain and with minimal inter-observer variations. Patients with advanced fibrosis can be identified by these tests.^[52] However, these parameters are often validated in just one or two liver diseases. For example, two scoring systems for CHC patients, namely APRI and FIB-4, are found to be not useful in CHB patients.^[53]

FibroTest®

Some specific biochemical parameters related to fibrinolysis or fibrinogenesis are developed to improve the specificity of liver fibrosis assessment [Table 2]. One example is FibroTest® (BioPredictive, Paris, France; or known as FibroSure® in the United States) consists of 5 components, namely GGT, total bilirubin, α -2 macroglobulin, apolipoprotein A1, and haptoglobin. Sometimes, another test, ActiTest, would also perform together with FibroTest® for assessment for liver activity, with the additional measurement of ALT. The results would be adjusted according to age and gender.^[54] FibroTest® is originally used in patients with CHC.^[55] Nowadays it is recommended by different associations concerning liver studies for evaluation of liver fibrosis in patients with CHB, NAFLD or alcoholic liver disease.^[56-58] It is highly reliable and applicable,^[59] even for patients with obesity.^[60] It performs well for diagnosis of liver cirrhosis for disease entities other than CHC. However, the results are suboptimal for detecting earlier stages before cirrhosis.^[61]

FibroMeter®

FibroMeter® (Echosens, Paris, France) has been validated in patients with CHB, CHC, NAFLD and alcoholic liver disease.^[62] Platelets, prothrombin index, AST, α -2 macroglobulin, hyaluronate, urea and age are taken into accounts.^[63] Second generation (2G) has put age into another important parameter.^[62] FibroMeter® has recently reached its third generation

Table 1: Serum test formulae for liver fibrosis

Parameters or index	Formula
APRI	$\text{AST (ULN)} \times 100 / \text{platelet (} 10^9/\text{L)}$
Forns index	$7.811 - 3.131 \times \ln(\text{platelet count}) + 0.781 \times \ln(\text{GGT}) + 3.467 \times \ln(\text{age}) - 0.014 \times (\text{cholesterol})$
FIB-4	$\text{Age (years)} \times \text{AST [U/L]} / (\text{platelets [} 10^9/\text{L)} \times (\text{ALT [U/L]}^{1/2})$
Fibro index	$1.738 - 0.064 \times \text{platelet [} 10^9/\text{L)} + 0.005 \times \text{AST [IU/L]} + 0.463 \times \text{gamma globulin [g/dL]}$
Hui index	$\exp(3.148 + 0.167 \times \text{BMI} + 0.088 \times \text{bilirubin } [\mu\text{mol/L}] - 0.151 \times \text{albumin [g/L]} - 0.019 \times \text{platelet [} 10^9/\text{L)}) / (1 + \exp(3.148 + 0.167 \times \text{BMI} + 0.088 \times \text{bilirubin } [\mu\text{mol/L}] - 0.151 \times \text{albumin [g/L]} - 0.019 \times \text{platelet [} 10^9/\text{L)}))$
NFS	$-1.675 + 0.037 \times \text{age (years)} + 0.094 \times \text{BMI (kg/m}^2) + 1.13 \times \text{impaired fasting glycaemia or diabetes (yes = 1, no = 0)} + 0.99 \times \text{AST/ALT ratio} - 0.013 \times \text{platelet (} \times 10^9/\text{L)} - 0.66 \times \text{albumin (g/dL)}$
BAAT score	$\text{BMI (} \geq 28 = 1, < 28 = 0) + \text{age at liver biopsy (} \geq 50 \text{ years} = 1, < 50 = 0) + \text{ALT (} \geq 2 \times \text{ULN) = 1, } < 2 \times \text{ULN = 0)} + \text{serum triglycerides (} \geq 1.7 \text{ mmol/L = 1, } < 1.7 = 0)$

ALT: alanine aminotransferase; APRI: aspartate aminotransferase to platelet ratio index; AST: aspartate aminotransferase; BAAT: BMI, age, ALT, triglycerides; BMI: body mass index; FIB-4: fibrosis-4; GGT: gamma-glutamyl transferase; NFS: non-alcoholic fatty liver disease (NAFLD) fibrosis score; ULN: upper limit of normal

Table 2: Different non-invasive approach

Non-invasive tests	Features	Advantages	Disadvantages
Radiological			
Transient elastography	Ultrasound-based liver stiffness measurement by shear wave velocity by a specific probe	Useful across different liver disease entity Special probes designed for different body built Measure liver fat at the same time with CAP Can identify no or minimal fibrosis	Less reliable in obese patients Less reliable in severe acute exacerbation of hepatitis Less reliable in post-treatment fibrosis stages in CHB or CHC patients
Acoustic radiation force impulse imaging	Ultrasound-based wave propagation speed measurement by conventional probe	No additional apparatus except ultrasound machine Can reflect disease progression Real-time results Less technical difficulties Accurate in overweight or obese patients	Narrow range of measurement Difficult to define a cut-off More experienced operators need
Shear wave elastography	Ultrasound measurement of shear wave velocity	No additional apparatus except ultrasound machine Elasticity can be reflected by numbers or colors Sensitive for early-stage fibrosis Results can be expressed into kPa or m/s	Limited studies on its clinical application
Magnetic resonance elastography	Phase contrast imaging depending on mechanical wave propagation	Less operator-dependent and less technical failure Limited effect by obesity or ascites Can assess complications Sensitive for early-stage fibrosis Reproducible results	High cost Limited availability in some countries/regions More time-consuming Not applicable on patients with iron overload or hemochromatosis Limited studies on its clinical application
Serum test formulae			
Common laboratory parameters	Refer to Table 1	Results from routine liver function test, convenient to perform No inter-observer variations	Cannot be used for all chronic liver diseases
FibroTest	Consists of GGT, total bilirubin, α -2 macroglobulin, apolipoprotein A1, and haptoglobin	Useful in different chronic liver disease Reliable Applicable Accurate in overweight or obese patients	Suboptimal for early stage fibrosis
FibroMeter	First 2 generations: consists of platelets, prothrombin index, AST, α -2 macroglobulin, hyaluronate, urea and age 3rd generation (3G): hyaluronate does not take into account	With high fibrosis classification accuracy Good predictive value for severe fibrosis in different liver disease entities	High cost
Enhanced liver fibrosis	Consists of 3 direct blood markers: procollagen III amino terminal peptide, hyaluronic acid and tissue inhibitor of metalloproteinase I	Good prognostic factor for clinical outcomes in patients with chronic liver diseases Similar results by using fresh blood or cryopreserved blood Sensitive for advanced fibrosis or cirrhosis	Not sensitive for early stages of fibrosis Age, low CD4+ T-cell count and other factors can affect ELF results

AST: aspartate aminotransferase; CAP: controlled attenuation parameter; CHB: chronic hepatitis B; CHC: chronic hepatitis C; ELF: enhanced liver fibrosis

(3G), which does not take hyaluronate into account. Therefore, the cost has been reduced but with similar effectiveness.^[64] FibroMeter®, both 2G and 3G, has been shown with high fibrosis classification accuracy.^[65] Besides, it appears to have a good predictive value towards the occurrence of severe fibrosis in those with NAFLD^[66] and chronic hepatitis B or C.^[67] Even though the hyaluronate-free FibroMeter® 3G is in use nowadays, the cost is still high compared to common parameters (e.g. FIB-4 or NFS).^[68]

Enhanced liver fibrosis

Enhanced liver fibrosis (ELF) score is an algorithm consists of 3 direct markers in blood, namely procollagen III amino terminal peptide (PIIINP), hyaluronic acid and tissue inhibitor of metalloproteinase I (TIMP-I).^[69] ELF can be a good prognostic factor for the clinical outcomes of patients with chronic liver disease. The increase in one point in ELF can lead to doubling of the risk of clinical outcomes in patients, especially liver-related clinical outcomes.^[70] ELF results are even similar when using fresh blood or cryopreserved blood. Therefore, it has a high predictive value for identifying patients with risk to develop progressive chronic liver disease at an early stage.^[71] It is sensitive in identifying advanced fibrosis or cirrhosis, but not for lower fibrosis stage.^[72,73] Meanwhile, it is noted that different factors can influence the result of ELF score, with the most significant factor being age.^[74] Other factors include low CD4+ T-cell count, co-existing extra-hepatic fibrosis, etc.^[75] Therefore, the results of ELF should be interpreted with particular clinical context.

Novel serum markers

There are some other novel serum fibrosis markers that raise the attention of the clinicians. Glycosylated Wisteria floribunda agglutinin-positive Mac-2 binding protein (WFA⁺-M2BP) is a marker which is related to fibrosis-related glyco-alteration. It can be measured by a glycan-based immunoassay, FastLec-Hepa. A cut-off index would be calculated based on the measured value.^[76] It is found to be useful for detecting early stages of fibrosis in chronic hepatitis B patients in a recent study.^[77] Another novel marker, YKL-40 (CHI3L1), is an emerging inflammation biomarker which was shown to be related to the early stage of liver fibrosis.^[78] In NAFLD patients, macrophages in liver were showed to express YKL-40. This makes YKL-40 be possible as a biomarker as liver fibrosis.^[79] However, further studies need to be conducted to show the effectiveness and impact of both biomarkers on making the diagnosis or management of patients with liver fibrosis due to any chronic liver diseases.

Combination of different approaches

It is common for using both radiological and

biochemical methods to increase the accuracy in determining the degree of fibrosis. Both types of methods can play a supplementary role to each other. For example, the performance of ELF improves with the assistance of transient elastography.^[80] With the use of ELF-LSM algorithm, a significant proportion of patients can avoid liver biopsy.^[69] Another example is Hui Index and transient elastography. Since LSM result is confounded in patients with elevated ALT, Hui index, a score independent of ALT level, is a good choice for supplementation of transient elastography. Studies have shown that the combinations can help predict hepatic event-free survival in chronic hepatitis B patients.^[81] Another combination for assessment of liver fibrosis in CHB patients is Forns index (another ALT-free index)-LSM algorithm.^[82] FibroMeter® and transient elastography combined together can help improve diagnostic accuracy and avoid liver biopsy in CHC patients.^[83] For the diagnosis of cirrhosis in CHC patients, using the algorithm FibroTest® and transient elastography improves the performance. However, this combination does not show extra benefit for diagnosis of advanced fibrosis compared to the sole use of FibroTest®.^[84]

Non-invasive tests - from diagnostic to prognostic

Portal hypertension and related complications

The role of all these non-invasive tests is moving from diagnostic to prognostic. They are useful to predict liver-related complications and hence the prognosis of patients with chronic liver diseases. For example, a LSM with 13.6 kPa can be a predictive value the presence of portal hypertension.^[85] Combining LSM with APRI or Fibroindex increases the sensitivities for portal hypertension predication.^[85] Liver stiffness with ARFI greater than 2.34 m/s indicates a poor liver reserve function.^[86] Assessment of spleen loss modulus by MRE is a good method for recognizing patients with severe portal hypertension or esophageal varices with high bleeding tendency.^[87] Combining LSM and spleen stiffness measurement (SSM) may exclude the presence of large esophageal varices with high sensitivity^[88] and can be adopted in the risk stratification and variceal screening strategy.^[89]

Survival

Survival for chronic liver disease can be predicted using non-invasive test. LSM^[90,91] or FibroTest® has a high prognostic value for patients with chronic viral hepatitis.^[92,93] The usage of LSM and Hui index for predicting hepatic-event free survival in CHB patients is shown to be accurate.^[81] FibroMeter® is shown to be useful for assessment of liver prognosis in CHC patients with milder disease.^[94] ELF score can be used

to assess transplant-free survival of the patients with primary sclerosing cholangitis,^[95] HCV/HIV co-infected women^[96] and the prognosis of patients with different chronic liver diseases.^[70]

HCC

There is good evidence showing the strong predictive and even diagnostic role of the non-invasive tests for HCC. ARFI is used for differentiating benign and malignant liver tumors by the assessment of virtual touch tissue imaging (VTI) and virtual touch tissue quantification (VTQ), as VTI appears to be stiffer and VTQ is higher in malignant lesion than its benign counterpart.^[97] For MRE, the measurement of loss modulus in liver tumor can help differentiating the benign lesions from the malignant ones, with the former having a lower value.^[98] Non-invasive test is also an important part of some HCC risk score. For example, LSM-HCC score, which is optimized from CU-HCC score with LSM, further increases the negative predictive value to close to 100% for HCC prediction in 3 to 5 years in CHB patients.^[99] Both FibroTest and LSM results can help predict the occurrence of HCC in patients with viral hepatitis.^[100] Patients with ELF higher than 10.4 is known to have higher risk of liver-related events, in which HCC is at the top of the list.^[101] Non-invasive tests can also play some part in prognosis of HCC. For example, in HCC patients receiving partial hepatectomy or transarterial chemoembolization, LSM and APRI is an independent prognostic factor.^[90,91,102]

TREATMENT OF LIVER FIBROSIS

Treatment for underlying diseases

With very potent antiviral agents, patients with chronic viral hepatitis often have liver fibrosis and even cirrhosis regressed after sustained viral suppression or viral clearance.^[103,104]

CHB

There is ample evidence to support the fact that effective antiviral treatment reverses liver fibrosis in majority of CHB patients.^[104,105] Cumulative entecavir therapy for 3 to 7 years regressed liver fibrosis in 88% of 57 CHB patients, including all 10 patients with advanced fibrosis or cirrhosis.^[105] This observation was further confirmed by a larger cohort of 348 patients who tenofovir disoproxil fumarate, in which 176 (51%) had regression of fibrosis at week 240.^[104] More importantly, most (71%) patients with cirrhosis at baseline had regression of cirrhosis. Data from the same trial revealed that body mass index at baseline was the single negative predictor of liver fibrosis regression.^[106]

Importance of metabolic factors on liver fibrosis

regression was also supported by data from Chinese and Korean cohorts established that metabolic syndrome is a risk factor of advanced liver fibrosis and cirrhosis independent of viral factors in CHB.^[107,108] New-onset metabolic syndrome and some of its components (namely central obesity and low high-density lipoprotein cholesterol) were found associated with liver fibrosis progression, independent of change in viral load and ALT level.^[109] Therefore controlling metabolic factors in CHB patients already have good viral suppression with antiviral treatment would be important, not only to enhance liver fibrosis regression and hepatic events, but also to minimize cardiovascular death.^[110]

Indirect evidence of antiviral treatment reversing liver fibrosis also came from two studies using serial LSM results to assess the change in liver fibrosis in large cohorts of asymptomatic CHB patients revealed low incidence rate of liver fibrosis progression, defined as an increase in LSM by 30% or more.^[111,112] It was because patients who had active disease, as evidenced by raised ALT and high HBV DNA, had been started on antiviral treatment.

CHC

Data from last century illustrated the conventional interferon regresses liver fibrosis in CHC patients with sustained virologic response (SVR).^[113] Similar findings have been reported in sustained responders to pegylated interferon.^[114,115] Regression of liver fibrosis, which occurred in 82% of patients, was sustained at 5 years after SVR; more impressively recovery of normal or nearly normal liver architecture is possible.^[103]

Now it is the era of direct-acting antiviral (DAA) agents in treating CHC patients, which leads to an SVR close to 100%.^[116] Studies evaluating liver fibrosis regression in DAA-treated CHC patients often adopted non-invasive assessments like transient elastography. A small study of 54 DAA-treated patients with baseline cirrhosis revealed more pronounced reduction in LSM happened between baseline to end-of-treatment visit, but less obvious in the post-treatment period. Hence the authors concluded that decreased LSM was likely accounted by the reduced necroinflammation and probably to a less extent to regression of cirrhosis.^[117] Another study of larger sample size already made use of serum makers on top of LSM revealed that FIB-4 and APRI improved to the same extent of LSM after SVR.^[118] Yet whether this indicated a true regression of fibrosis or merely resolution of chronic liver inflammation remained to be determined.^[118]

NAFLD

Similar to chronic viral hepatitis, controlling underlying

metabolic risk factors is central in the management to improved liver fibrosis in NAFLD patients. A weight reduction of 10% or more by aggressive lifestyle modification appears to resolve fibrosis in most if not all cases (at least with mild-moderate fibrosis).^[119,120] Thiazolidinediones [peroxisome proliferator-activated receptor (PPAR)- γ agonists] such as pioglitazone and rosiglitazone are insulin sensitizers and were found to be effective to reduce fibrosis in two meta-analyses,^[121,122] but the finding was not confirmed when more recent and bigger studies were included in the analysis.^[123] The largest study of pentoxifylline and also a recent study of obeticholic acid both showed a significant reduction of fibrosis,^[124] the magnitude was not pathologically significant (far less than one fibrosis stage by the non-alcoholic steatohepatitis (NASH) Clinical Research Network system.^[125]

In terms of pharmacological agents, there has been much interest in anti-fibrotic therapy in NAFLD as fibrosis is one of the strongest prognostic markers for NAFLD. Lysyl-oxidase like 2 (LOXL2) is involved in a relatively late step in hepatic fibrogenesis, the crosslinking of extracellular matrix proteins such as collagen and elastin.^[126] Simtuzumab, a humanized monoclonal anti-LOXL2 antibody was once evaluated in Phase 2 trials in nonalcoholic steatohepatitis (NASH) patients with significant fibrosis and cirrhosis.^[127] Nonetheless, the pharmaceutical company developed

this agent announced it discontinued testing of simtuzumab, as it failed to show efficacy in Phase 2 trials of NASH as well as primary sclerosing cholangitis.^[128] More recent data also support that the hepatic expression of the apoptosis signal-regulating kinase 1 (ASK1) marker, phosphorylated-P38 (p-P38), correlates with fibrosis stage in patients with NAFLD.^[129] Therefore, selonsertib, an oral molecule that inhibits ASK1, together with simtuzumab, was found to be effective to regress liver fibrosis in NASH patients with stage 2 or 3 fibrosis. Selonsertib alone is currently evaluated in NASH patients with advanced fibrosis and cirrhosis (Clinicaltrials.gov Identifier NCT03053050 and NCT03053063) [Table 3].

Cenicriviroc is a C-C chemokine receptor type 2 and type 5 (CCR2/CCR5) antagonist, which interrupts the inflammatory cascade in NASH that leads to fibrogenesis. In animal models, the drug has been shown to have anti-inflammatory and anti-fibrotic activity.^[130,131] In an ongoing two-year Phase 2b trial with cenicriviroc, it significantly improved liver fibrosis for at least one stage at 48 weeks when compared to placebo (20% vs. 10%; $P = 0.023$).^[132] Galectins are cell surface glycoproteins that can mediate cell migration, matrix interaction and inflammatory signals. GR-MD-02 and GM-CT-01, two galectin inhibitors, bind to terminal galactose residues in glycoprotein and reduce fibrosis in animal NASH.^[133] GR-MD-02 has favorable

Table 3: Active clinical trials in the clinical trials.gov on anti-fibrotic treatments

Clinicaltrials.gov	Drug	Phase	Disease	Target sample size	Status
NCT01965418	Fufang Biejia Ruangan	4	Chronic hepatitis B	100	Recruiting
NCT02241616	Entecavir + Fuzheng Huayu + TCM Granule	4	Chronic hepatitis B	350	Recruiting
NCT00956098	Oltipraz	2	Chronic hepatitis B or C	81	Completed
NCT02138253 (POLT-HCV-SVR)	IDN-6556	2	Chronic hepatitis C	60	Ongoing, finished recruitment
NCT02744105	Dietary Supplement: Spirulina	N/A	Chronic hepatitis C (in beta-thalassemia)	60	Ongoing, finished recruitment
NCT02217475	Cenicriviroc	2	NASH fibrosis	200	Ongoing, finished recruitment
NCT03059446	Cenicriviroc	2	NASH fibrosis	200	Recruiting by invitation
NCT03028740 (AURORA)	Cenicriviroc	3	NASH fibrosis	2000	Recruiting
NCT02530138	Synbiotic	2/3	NASH fibrosis	42	Recruiting
NCT02686762	Emricasan	2	NASH fibrosis	330	Recruiting
NCT02704403 (RESOLVE-IT)	Elafibranor	3	NASH fibrosis	2000	Recruiting
NCT02548351 (REGENERATE)	Obeticholic Acid	3	NASH fibrosis	2000	Recruiting
NCT03053050 (STELLAR 3)	Selonsertib	3	NASH advanced fibrosis	800	Recruiting
NCT03053063 (STELLAR 4)	Selonsertib	3	NASH cirrhosis	800	Recruiting
NCT01899859	GR-MD-02	1	NASH cirrhosis	31	Completed
NCT02462967	GR-MD-02	2	NASH cirrhosis	156	Ongoing, finished recruitment
NCT02806011	Livercellgram	2	Alcoholic cirrhosis	50	Recruiting by invitation
NCT01452308	Simtuzumab	2a	Any	20	Completed

NASH: non-alcoholic steatohepatitis

safety profile in a phase I study in NASH patients with advanced fibrosis and is now under investigation in patients with NASH cirrhosis (ClinicalTrials.gov Identifier NCT01899859 and NCT02462967; **Table 3**). The pharmaceutical company is going to present the data from this Phase 2 clinical trial by early December 2017.^[134]

Other liver diseases

Ursodeoxycholic acid (UDCA) was found to reduced serum ALT, GGT and PIIIP in an early study.^[135] Candesartan, an angiotensin receptor blocking agent, together with UDCA, when compared to UDCA alone for 6 months, induced more significant improvement of fibrosis in histological and quantitative measurements in patient with compensated alcoholic liver disease.^[136] UDCA combined with budesonide, but not UCDA alone, led to fibrosis regression in patients with primary biliary cholangitis (PBC, previously known as primary biliary cirrhosis). Obeticholic acid (OCA) is a semi-synthetic 6-ethyl analogue of the endogenous bile acid chenodeoxycholic acid (CDCA) that is 100 times more potent than CDCA as a Farnesoid X receptor (FXR) activator. OCA has been shown to have anticholestatic, anti-inflammatory and antifibrotic effects.^[137] OCA is found to be effective to improve liver biochemistries in a Phase 3 trial.^[138]

Specific anti-fibrotic treatment targets

Direct downregulation of hepatic stellate cell

Hepatic stellate cells (HSC) are the main collagen-producing cells in the liver and their activation promotes liver fibrosis. Targeting HSC is a popular strategy for treating liver fibrosis.^[139] Liver fibrosis can be reversed via a few mechanisms, which include inhibition of HSC activation; promotion of HSC phenotypic conversion; immune clearance of HSC; promotion of HSC apoptosis; induction HSC senescence.^[140] Several drugs have been tested to down-regulating HSC activation, which include a few antioxidants (e.g. namely vitamin E, phosphatidylcholine, silymarin, resveratrol), gamma interferon, peroxisome proliferator-activated receptor gamma (PPAR- γ) agonists (e.g. pioglitazone), endothelin receptor antagonists, histone deacetylase (HDAC) inhibitors *etc.*^[139] Yet none of these agents has been approved as anti-fibrotic agents.

Several novel targets have been identified for the treatment of liver fibrosis through suppression of HSC activation. Interleukin (IL)-30 attenuates hepatic fibrosis by inducing natural killer group 2D (NKG2D)/ribonucleic acid export 1 crosstalk between activated HSCs and natural killer T cells and is therefore an ideal therapy for liver fibrosis. Hydrogen peroxide-inducible clone-5 (Hic-5), a transforming growth factor (TGF)-

β 1-inducible focal adhesion protein, facilitates cell proliferation, ECM expansion and vascular restoration and restructuring.^[141] Hic-5 expression also plays a critical role in attenuating fibrosis by enhancing TGF- β 1-induced small mother against decapentaplegic (Smad)2 phosphorylation via the downregulation of Smad7 in both human and mouse activated HSCs.^[142]

Although several drugs show potent anti-fibrotic activities in experimental models of hepatic fibrosis, there is presently no effective pharmaceutical intervention specifically approved for the treatment of liver fibrosis. Targeted delivery systems that bind specifically to receptors solely expressed on activated HSCs or trans-differentiated MFBs are essential to increase treatment efficacy as well as to reduce adverse effects. The applicability and efficacy of sequestering molecules, selective protein carriers, lipid-based drug vehicles, viral vectors, transcriptional targeting approaches, therapeutic liver- and HSC-specific nanoparticles, and miRNA-based strategies are potential and promising treatment strategies.^[143]

Collagen synthesis inhibitors

Continuous accumulation of extracellular matrix (ECM) extremely rich in collagen I and III in response to liver injury leads to scar deposition and liver fibrosis.^[144] Activated HSCs are indeed a major source of collagen in the liver and can abundantly secrete ECM proteins, tissue inhibitors of metalloproteinases, and matrix metalloproteinases (MMPs) that elicit liver architecture remodeling.^[145] Apart from modulating HSC, there are some therapeutic agents directly targeting collagen synthesis.

Halofuginone is an analog of febrifugine - an alkaloid originally isolated from the plant *Dichroa febrifuga*.^[146] Animal model with established liver fibrosis halofuginone elicited reductions in the levels of collagen, collagen α 1 gene expression, and α -smooth-muscle-positive cells, and even complete resolution of liver fibrosis.^[147] Regeneration of the liver, which was blocked in rats with established fibrosis, occurred at an almost normal rate in halofuginone-treated rats.^[148] Nonetheless, there has not been a clinical study specifically that use halofuginone to treat liver fibrosis in human.

TGF- β antagonists

TGF- β 1 is the key pro-fibrogenic cytokine involved in liver fibrosis, as it regulates the production and deposition of ECM.^[149,150] There are several approaches to interfere with TGF- β signaling. TGF- β expression can be down-regulated by applying anti-sense oligonucleotide mRNA. A targeted blocking of a specific

isoform of TGF- β by means of monoclonal antibodies is also feasible. Activation of TGF- β receptors can be inhibited by the use of specific inhibitors, thereby halting downstream signaling. Local activation of TGF- β induced by $\alpha\beta 6$ integrin and by tropomyosin-related kinase (TSP)-1 can be prevented.^[151] The amino acid sequence Leu-Ser-Lys-Leu (LSKL) naturally occurs in the region of the amino terminus of the LAP and that it can hamper the activation of latent TGF- β by TSP-1 through competitive inhibition.^[152] LSKL peptides significantly decrease DMN-induced liver atrophy and fibrosis in an animal model.^[153] Yet LSKL has not been developed clinically. More recently nanoconjugate siRNA against TGF- $\beta 1$ equipped with an N-acetylglucosamin targeting moiety intending to reach HSCs via desmin was reported to colocalize with HSCs and to reduce liver fibrosis.^[154]

Connective tissue growth factor inhibitor

CTGF is a mediator of ECM accumulation and coordinates a late common pathway to fibrosis.^[155] Blocking connective tissue growth factor (CTGF) activity reduces liver fibrosis and preserves liver function.^[156] FG-3019 is a recombinant human anti-CTGF monoclonal immunoglobulin G antibody. FG-3019 reduces collagen deposition in nonclinical models of liver. FG-3019 was tested in CHB patients in a Phase 2 randomized trial; unfortunately the study terminated due to an unexpected prominent effect of entecavir alone in this patient population.^[157]

CONCLUSION

With the wide applicability of non-invasive assessments of liver fibrosis, the management of 2 billion patients with chronic liver diseases worldwide has been revolutionized. While liver biopsy examination still has an important role in the diagnostic process, non-invasive assessments including transient elastography and serum biomarkers have high accurate to diagnose advanced fibrosis and cirrhosis. Transient elastography and serum biomarkers can be used alone or in combination, either simultaneously or in a stepwise approach. Meanwhile, ARFI and SWE are effective for staging liver fibrosis, especially when ultrasound is the first imaging tool for assessment of diffuse liver disease. Treating underlying chronic liver diseases is still the cornerstone of liver fibrosis regression. Potent antiviral treatments for chronic viral hepatitis lead to regression of liver fibrosis and even cirrhosis in majority of patients. Numerous ongoing clinical trials in NAFLD patients will bring us treatment to treat NASH fibrosis and cirrhosis soon. Plentiful therapeutic agents specifically targeting the fibrogenesis pathways, in particular HSC and TGF- $\beta 1$ work well in animal models. We look forward to assess these agents in human and hopefully they

can modify the natural history of chronic liver diseases, and more importantly, to improve patient outcome in the near future.

DECLARATIONS

Authors' contributions

Drafting of the manuscript: J.Y.K. Cheng, G.L.H. Wong
Critical revision of the manuscript for important intellectual content: J.Y.K. Cheng, G.L.H. Wong

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None.

Conflicts of interest

Grace L.H. Wong has served as an advisory committee member for Gilead Sciences. She has also served as a speaker for Abbott, Abbvie, Bristol-Myers Squibb, Echosens, Furui, Gilead Sciences, Janssen and Roche.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Laparoscopic liver resection with lymph node dissection for gallbladder tumors suspected to be T1b/T2 carcinoma

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ABSTRACT

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Aim: The short-term perioperative results of laparoscopic treatment of gallbladder (GB) carcinoma were evaluated to determine whether this technique can be a feasible treatment option. **Methods:** Ten patients with fundus/body GB tumors (GBTs) underwent laparoscopic liver resection (LLR) and lymph node dissection. Additionally, 124 patients underwent LLR for liver tumors. These 124 LLRs included 79 partial resections (PRs), 11 left lateral sectionectomies (LLSs), 25 anatomical resections (ARs), and 9 small ARs (SARs). The operation time (OT), intraoperative blood loss (BL), and postoperative length of hospital stay (LOS) were compared between the GBT and various LLR groups. **Results:** The median (range) OT in the GBT, PR, LLS, AR, and SAR groups was 298 (186-488), 245 (84-700), 328 (150-682), 458 (224-848), and 352 (274-696) min, respectively. The BL was 109 (10-500), 50 (0/uncountable-3,270), 100 (10-516), 375 (25-3,569), and 705 (35-1,920) mL, respectively. The LOS was 16 (8-105), 15 (5-254), 13 (11-52), 22 (8-44), and 15 (8-44) days, respectively. The OT and BL were significantly different between the GBT and AR groups. **Conclusion:** Laparoscopic surgery could be a good treatment option for GBTs suspected to be T1b/T2 GB carcinoma in the GB body/fundus without cystic duct invasion.

INTRODUCTION

Since the development of laparoscopic liver resection (LLR) in the early 1990s,^[1-3] this technique has rapidly expanded from partial LLR of the easily accessible anterolateral segments [segment 2 (S2), S3, S4b, S5, and S6] to left lateral sectionectomy (LLS), hemihepatectomy, other sectionectomies, segmentectomies and resections of S7, S8, and S1,

and more complicated limited or modified anatomical LLRs.^[4] LLR has recently become widely accepted as a less invasive treatment for liver tumors with specific advantages such as less intraoperative bleeding and a shorter postoperative length of hospital stay (LOS).^[5,6] Partial resection (PR) of the anterolateral segments is currently considered a standard procedure.^[5]

Few reports have described intended laparoscopic



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treatments for gallbladder carcinoma (GBC);^[7-9] however, many studies of occult GBC revealed high incidences of port site recurrence and peritoneal dissemination after laparoscopic cholecystectomy.^[10,11] The treatment of T1b/T2 GBC,^[12] which is not in the early stage of intramucosal layer but without serosal invasion, involves a combination of liver resection, lymph node (LN) dissection, and bile duct resection and reconstruction in cases of invasion.^[13,14] Laparoscopic procedures have been less commonly adapted to GBC treatment mainly because of concerns regarding the aggressive features of the malignancy and the technically demanding surgical procedure.^[7-9] However, the liver resection technique performed for treatment of T1b/T2 GBC involves resection of either the GB bed or S4b+5+6a, both of which require resection of the anterolateral segments. LN dissection has also been applied to other more popular procedures.^[15] Because bile duct resection and reconstruction is not necessary during surgical treatment of T1b/T2 GBC of the body/fundus without cystic duct invasion, the operation is a simple combination of anterolateral LLR and limited LN dissection. Furthermore, tumor dissemination and port site recurrence are thought to occur mainly due to bile leakage from intraoperative GB perforation.^[9,11] Theoretically, combined resection of the GB bed liver could prevent these events.^[10,11,16,17] Therefore, we have employed a laparoscopic procedure for treatment of GB tumors (GBTs) suspected to be T1b/T2 GBC located in the GB body/fundus without cystic duct invasion.

In this study, to determine whether laparoscopic treatment of T1b/T2 GBC is a feasible treatment option, we compared the short-term results of patients who underwent this procedure and those of patients who underwent various types of LLR.

METHODS

Among 28 patients who underwent GB resection

for suspected GBC from November 2011 to June 2015, 10 patients with GBTs suspected to be T1b/T2 GBC in the GB fundus/body underwent LLR and LN dissection. The other patients underwent laparoscopic full-thickness cholecystectomy for suspected T1a GBC or open surgery for suspected \geq T3 GBC or possible bile duct resection based on preoperative assessment.

Three patients with T2 GBC underwent LLR of S4b+5+6a with regional LN dissection, and the other seven patients underwent LLR of the GB bed liver with peri-cystic duct LN and peri-bile duct LN dissection. The patients' data are shown in Table 1.

In total, 124 patients underwent LLR for liver tumors (80 hepatocellular carcinomas, 35 metastatic tumors, and 9 others). These 124 LLR procedures included 79 PRs, 11 LLSs, 25 anatomical resections (ARs) (resection of one or more segments, excluding LLS), and 9 small ARs (SARs) (resection of less than a full segment and sometimes combined resection of those).

The conversion, morbidity, and mortality rates were compared between the GBT and various LLR groups. The perioperative short-term results [operation time (OT), intraoperative blood loss (BL), and postoperative LOS] of the 10 patients with GBTs were compared with those of the patients who underwent various types of LLR (PR, LLS, AR, and SAR).

Patients were fully involved in the treatment decision-making process. Informed consent was obtained from each patient for both treatment and use of data in the study. The data obtained through the medical record review were managed according to the privacy policy and ethics code of our institute. The surgeries were performed with the permission of our hospital review board.

Statistical analysis

Results are expressed as median (range) and mean \pm

Table 1: Characteristics of the 10 patients who underwent laparoscopic surgery for suspected T1b/T2 GBC

Gender	Age (years)	Child-Pugh	T-stage (clinical)	T-stage (pathologic)	Ope	Resection margin	OT (min)	BL (mL)	LOS (days)	Comp
Female	57	A	T1b	Benign	GB bed	R0	248	50	10	Bile leakage
Male	63	A	T1b	Benign	GB bed	R0	296	250	15	
Female	72	A	T1b	T1b	GB bed	R0	340	150	105	
Female	38	A	T1b	T1b	GB bed	R0	186	10	11	Bile leakage
Male	82	A	T1b	T1b	GB bed	R0	201	50	17	
Female	39	A	T1b	T1b	GB bed	R0	307	50	8	
Female	63	A	T1b	T1b	GB bed	R0	197	75	10	
Male	65	A	T2	T2	S4b+5+6a	R0	300	500	17	
Male	69	A	T2	T2	S4b+5+6a	R0	442	200	34	
Male	72	A	T2	T2	S4b+5+6a	R0	488	143	24	

GBC: gallbladder carcinoma; LLR: laparoscopic liver resection; Ope: performed operation; OT: operation time; BL: intraoperative blood loss; LOS: postoperative length of hospital stay; Comp: complication; GB bed: LLR of GB bed liver with peri-cystic lymph node and peri-bile duct lymph node dissections; S4b+5+6a: LLR of S4b+5+6a with regional lymph node dissection

standard deviation unless otherwise noted. Differences in each parameter between the GBT and other groups were evaluated using the Mann-Whitney *U* test. All analyses were performed using SPSS, version 22.0 (IBM Corp., Armonk, NY, USA). A *P* value of < 0.05 (two-tailed) was considered statistically significant.

Operative procedure for GBTs of the fundus/body suspected to be T1b/T2 GBC

The patients underwent general anesthesia and were placed in the reverse trendelenburg position. The operating table was tilted to the left or right as necessary to acquire an adequate operative field of view.

The first trocar port was introduced with a mini-laparotomy on the umbilicus, and 8- to 12-mmHg carbon dioxide pneumoperitoneum was established through this port. This port was also mainly used for the laparoscope. Three other 12-mm ports and one 8-mm port were placed in the upper middle to right abdomen and used to introduce the surgeons' forceps, energy devices (SonoSurg, BiClamp bipolar forceps, and irrigation monopolar electric cautery with soft-mode coagulation), clips, and Cavitron ultrasonic surgical aspirator (CUSA) as well as the assistant's forceps. The Pringle maneuver was not applied.

S4b+5+6a LLR

For S4b+5+6a LLR, the operation was started with liver parenchymal transection on the right edge of the umbilical Glissonian pedicle [Figure 1] after confirming the locations of the GBT and major vessels by intraoperative laparoscopic ultrasonography. If needed, adhesions from a previous surgery were dissected before the ultrasonographic examination and transection. The liver parenchymal transection started with the use of the SonoSurg on the shallow surface of the liver. The BiClamp bipolar forceps, used in a clamp-and-crush manner, and the CUSA were used for deep parenchymal transection far from and near the major vessels, respectively. Small vessels were exposed and sealed with energy devices, clipped or ligated, and finally divided. Hemostasis of bleeding from the transection surface was accomplished by irrigation monopolar electric cautery with soft-mode coagulation or suturing by hand. During the transection on the umbilical line, two or three Glissonian pedicles to S4b (G4b) were dissected, encircled, ligated, and divided [Figure 2]. The ischemic demarcation line appeared on the liver surface after division of G4b [Figure 3], showing the left part of the transection line of the resected liver (S4b of S4b+5+6a). According to this line, liver transection was performed from left to right, exposing the hilar plate at the bottom.

The peripheral part of the middle hepatic vein was revealed and divided on the transection plane between S4b and S5 [Figure 4]. When the bottom of the transection line reached the right edge of the hilar plate, the LNs around the bile duct were dissected and the root of the cystic duct was exposed and divided [Figure 5]. Intraoperative frozen section pathology of the stump of the cystic duct confirmed the absence of tumor invasion. The cystic plate including the cystic duct, artery, and LNs was attached to the resected liver. Dissection was then performed from the hepatic duct to right Glissonian pedicle.

During dissection of the right Glissonian pedicle, G5a, G6a, and G5b were exposed and divided [Figure 6]. Liver parenchyma transection was performed according to

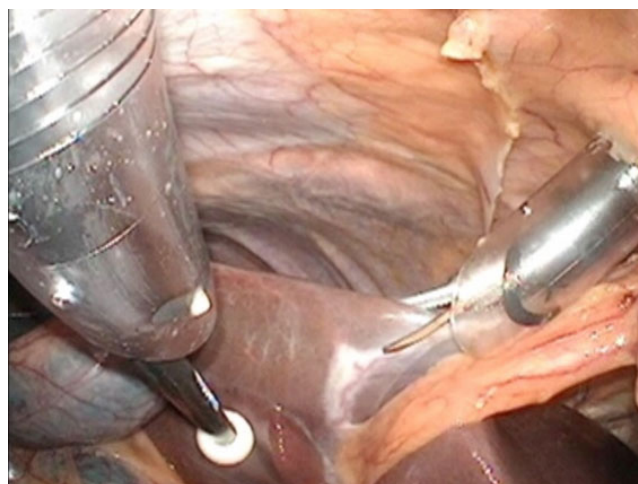


Figure 1: Operative procedure for gallbladder tumor of the fundus/body suspected to be T1b/T2 gallbladder carcinoma-1 (liver parenchymal transection on right edge of the umbilical plate). For S4b+S5+S6a LLR, the operation was started from the liver parenchymal transection on the right edge of the umbilical plate

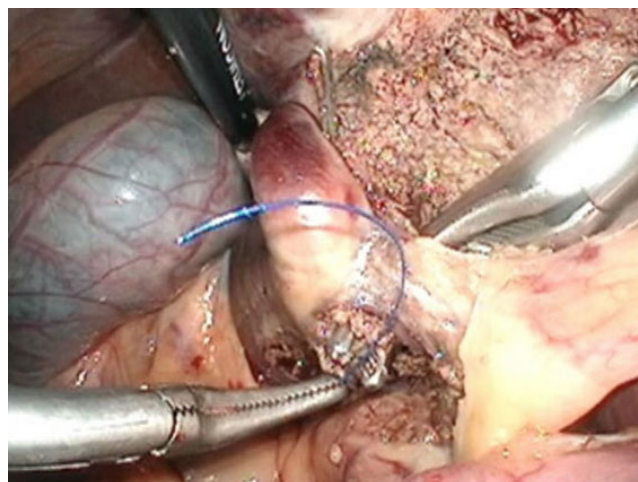


Figure 2: Operative procedure for gallbladder tumor of the fundus/body suspected to be T1b/T2 gallbladder carcinoma-2 (Glissonian pedicles to S4b). During the transection, the Glissonian pedicles to S4b were dissected, encircled, ligated, and divided

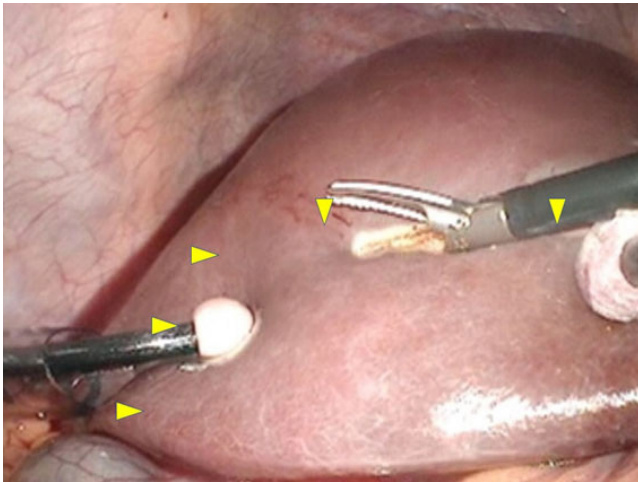


Figure 3: Operative procedure for gallbladder tumor of the fundus/body suspected to be T1b/T2 gallbladder carcinoma-3 (ischemic demarcation line of S4b). The ischemic demarcation line (arrowheads) was observed on the liver surface after division of the Glissonian pedicles to S4b. According to this line, liver transection was performed from left to right, exposing the hilar plate

the ischemic demarcation line on the liver surface that appeared after division of these Glissonian pedicles and exposure of the right part of the transection line of the resected liver (S5+6a of S4b+5+6a) [Figure 7]. The resected liver was extracted in a plastic bag through the umbilical port. Abdominal drainage catheters were routinely placed in the operative area.

GB bed LLR

For GB bed LLR, the operation started with liver parenchymal transection from the left anterior side (in S4) with a 1-cm surgical margin from the GB after confirming the locations of the GB bottom in the liver bed, GBT, and major vessels by intraoperative laparoscopic ultrasonography. If needed, adhesions from a previous surgery were dissected before the ultrasonographic examination and transection. The liver parenchymal transection started with use of the SonoSurg on the shallow surface of the liver. The BiClamp bipolar forceps, used in a clamp-and-crush manner, and the CUSA were employed for deep parenchymal transection far from and near the major vessels, respectively. Small vessels were exposed and sealed with energy devices, clipped or ligated, and finally divided. Hemostasis of bleeding from the transection surface was accomplished by irrigation monopolar electric cautery with soft-mode coagulation or suturing by hand. During the transection, small peripheral branches of G4b, middle hepatic vein, G5, and G6a were dissected, ligated, and divided. The liver transection was performed from left to right and ventral to dorsal, reaching the right corner of the hilar plate.

When the transection line reached the right corner of the hilar plate, the LNs around the bile duct were

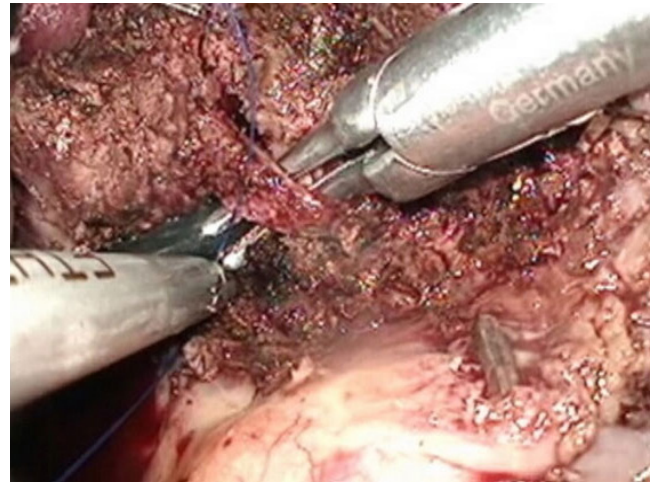


Figure 4: Operative procedure for gallbladder tumor of the fundus/body suspected to be T1b/T2 gallbladder carcinoma-4 (middle hepatic vein). The peripheral part of the middle hepatic vein was divided on the transection plane

dissected and the root of the cystic duct was exposed and divided. Intraoperative frozen section pathology of the stump of the cystic duct confirmed the absence of tumor invasion. The cystic plate including the cystic duct, artery, and LNs was attached to the resected liver and removed *en bloc*. The resected liver was extracted in a plastic bag through the umbilical port. Abdominal drainage catheters were routinely placed in the operative area.

Regional LN dissection

Additional regional LN dissection was performed after the liver resection when the tumor was confirmed to be T2 GBC. The common bile duct, proper and right hepatic arteries, and portal vein were dissected and taped [Figure 8]. The surrounding tissue including the LNs was resected with the tissues of the common hepatic artery, splenic vein, and posterosuperior surface of the pancreas after performing the Kocher maneuver.

RESULTS

Conversion, morbidity, and mortality in each group

Pathological R0 resection was achieved in all 10 patients with GBTs. One patient (10%) developed a Clavien-Dindo grade 3 complication (bile leakage) and had a long postoperative LOS (105 days), although no conversions to open procedures or mortality occurred in this group.

Among the 79 patients who underwent PR, 2 (2.5%) underwent conversions to open procedures and 4 (5.0%) developed grade 3 postoperative complications (postoperative ascites, bile leakage, cholecystitis, and

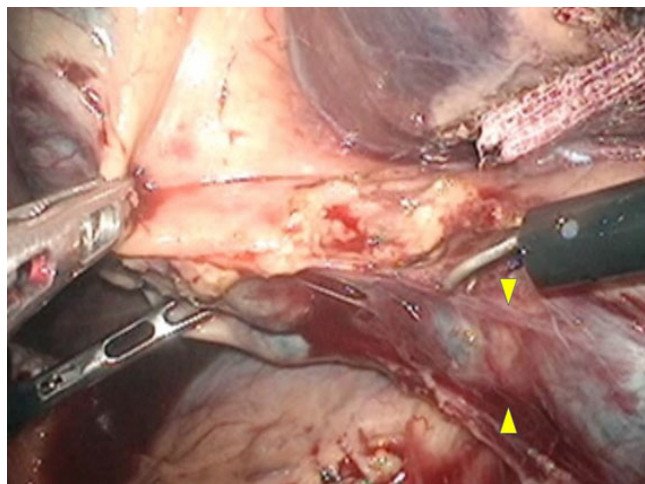


Figure 5: Operative procedure for gallbladder tumor of the fundus/body suspected to be T1b/T2 gallbladder carcinoma-5 (cystic plate). When the transection line reached the right side of the hilar plate, the lymph nodes around the bile duct were dissected and the root of the cystic duct (arrowhead) was exposed and divided. The cystic plate including the cystic duct and artery was attached to the resected liver, and dissection from the hepatic duct to the right Glissonian pedicle was performed

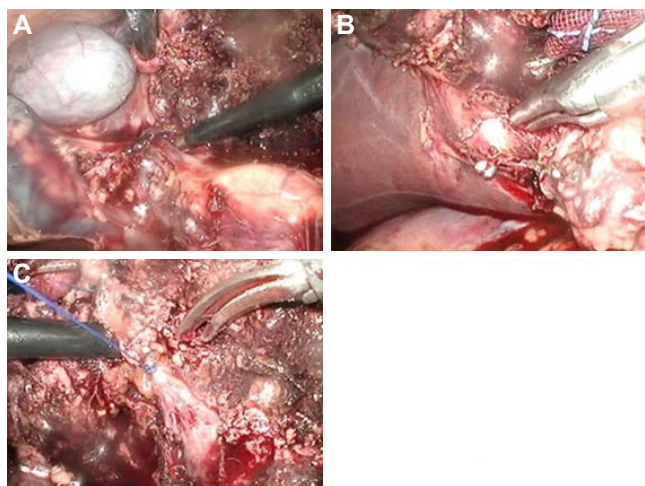


Figure 6: Operative procedure for gallbladder tumor of the fundus/body suspected to be T1b/T2 gallbladder carcinoma-6 [Glissonian pedicles, (A) S5a, (B) S6a, and (C) S5b]. During dissection of the right Glissonian pedicles, S5a, S6a, and S5b were exposed and divided

ileus). No mortality occurred.

No conversions or mortality occurred in the LLS, AR, or SAR groups. Two (18.2%) of 11 patients in the LLS group developed grade 3 postoperative complications (pancreatic juice leakage after pancreaticoduodenectomy in one patient, and postoperative intra-abdominal infectious hematoma after gastrectomy in another patient with protein S deficiency). Two (8.0%) of 25 patients in the AR group developed grade 3 postoperative complications (ascites and pleural effusion). Two (22.2%) of 9 patients in the SAR group developed grade 3 postoperative

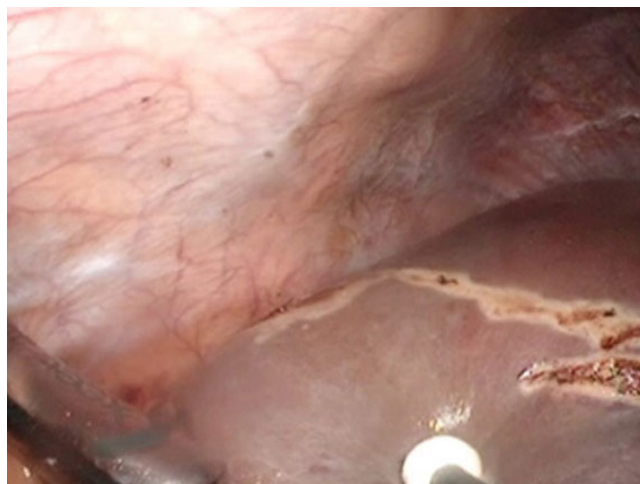


Figure 7: Operative procedure for gallbladder tumor of the fundus/body suspected to be T1b/T2 gallbladder carcinoma-7 (demarcation line after division of Glissonian pedicles, S5a, S6a, and S5b). According to the ischemic demarcation line that appeared after division of the Glissonian pedicles, S5a, S6a, and S5b, liver parenchymal transection was performed. The resected liver was extracted in a plastic bag through the umbilical port

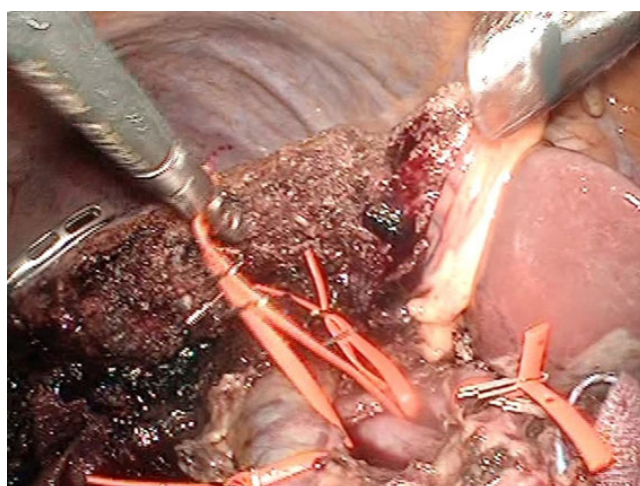


Figure 8: Operative procedure for gallbladder tumor of the fundus/body suspected to be T1b/T2 gallbladder carcinoma (GBC)-8 [lymph node (LN) dissection]. Regional LN dissection was performed after liver resection when the tumor was pathologically confirmed to be T2 GBC (taped vessels from left to right are the common bile duct, portal vein, right hepatic artery, and proper hepatic artery)

complications (postoperative liver failure for a patient who underwent surgery immediately after the treatment of ruptured esophageal varices, and anastomotic failure of concomitant high anterior rectal resection in the other patient).

No statistically significant differences in the conversion, mortality, or morbidity rates were found among the groups.

OT in each group

The median OT among all 10 patients with GBTs was 298 min (range 186–488 min), and the mean \pm standard

deviation was 301 ± 102 min.

The OT among the 79 patients in the PR group, 11 in the LLS group, 25 in the AR group, and 9 in the SAR group was 245 (84-700) and 292 ± 140 min, 328 (150-682) and 343 ± 152 min, 458 (224-848) and 504 ± 161 min, and 352 (274-696) and 415 ± 159 min, respectively.

In the comparison of the OT between patients with GBTs and patients in the other laparoscopic surgery groups (PR, LLS, AR, and SAR) a significant difference was found between the GBT and AR groups [Table 2].

Intraoperative BL in each group

The BL in patients with GBTs was 109 (10-500) and 148 ± 145 mL. The BL in patients who underwent PR, LLS, AR, and SAR was 50 (0/uncountable-3,270) and 278 ± 556 mL, 100 (10-516) and 166 ± 182 mL, 375 (25-3,569) and 758 ± 911 mL, and 705 (35-1,920) and 821 ± 794 mL, respectively.

In the comparison of BL between patients with GBTs and patients in the other laparoscopic surgery groups (PR, LLS, AR, and SAR), a significant difference was found between the GBT and AR groups [Table 2].

Postoperative LOS in each group

The LOS in patients with GBTs was 16 (8-105) and 25 ± 29 days. The LOS in the PR, LLS, AR, and SAR groups was 15 (5-254) and 20 ± 30 days, 13 (11-52) and 19 ± 64 days, 22 (8-44) and 24 ± 12 days, and 15 (8-44) and 21 ± 15 days, respectively.

No significant differences were found in the LOS between patients with GBTs and patients in the other laparoscopic surgery groups (PR, LLS, AR, and SAR) [Table 2].

Short-term results of LLR of S4b+5+6a (with regional LN dissection) and LLR of GB bed (with peri-cystic LN and peri-bile duct LN dissection)

Of the 10 patients with GBTs, 3 patients with T2 GBC who underwent LLR of S4b+5+6a with regional LN

dissection had an OT of 300 , 442 , and 488 min; BL of 500 , 200 , and 143 mL; and LOS of 17 , 34 , and 24 days, respectively. The third patient underwent the surgery, 2 weeks after the first cholecystectomy of severe cholecystitis, for the T2 GBC revealed in the postoperative pathological examination [Table 1].

The other seven patients who underwent LLR of the GB bed with peri-cystic LN and peri-bile duct LN dissections had an OT of 248 (186-340) and 254 ± 61 min, BL of 50 (10-250) and 91 ± 82 mL, and LOS of 11 (8-105) and 25 ± 35 days [Table 1].

DISCUSSION

Although no differences in LOS were observed, the BL and OT were significantly lower in the GBT than AR group. Additionally, no differences were observed in the conversion, morbidity, or mortality rate between laparoscopic GBT surgery and conventional LLR of any type. When compared with other types of conventional LLR, the short-term results (OT, BL, and LOS) of all 10 patients with GBTs were comparable with those in the LLS group. Three patients with T2 GBC who underwent LLR of S4b+5+6a with regional LN dissection had perioperative short-term results comparable with those of patients who underwent AR, although the number of patients was small. The perioperative short-term results of the other 7 patients who underwent LLR of the GB bed with peri-cystic duct LN and peri-bile duct LN dissections were comparable even with those of patients who underwent PR. LLR of the GB bed or S4b+5+6a with LN dissection was feasible for treatment of GBTs of the body/fundus suspected to be T1b/T2 GBC without cystic duct invasion. Itano *et al.*^[8] reported that laparoscopic surgery for T2 GBC had a comparable OT (368 vs. 352 min), significantly smaller BL volume (152 vs. 777 mL), shorter LOS (9.1 vs. 21.6 days), and similar morbidity rate (1/15 vs. 3/11 patients) compared with open surgery. Our results are similar to those from their laparoscopic surgeries. LLR has the advantages of a smaller BL volume and shorter LOS in some conditions, such as minor resections of the anterolateral segments.^[5] LLR of S4b+5+6a

Table 2: Perioperative short-term outcomes of different types of laparoscopic liver resections

	OT (min)		BL (mL)		LOS (days)	
GBT ($n = 10$)	298 (186-488)		109 (10-500)		16 (8-105)	
PR ($n = 79$)	245 (84-700)	NS	50 (NC -3,270)	NS	15 (5-254)	NS
LLS ($n = 11$)	328 (150-682)	NS	100 (10-516)	NS	13 (11-52)	NS
AR ($n = 25$)	458(224-848)	$P < 0.001$	375 (25-3,569)	$P < 0.05$	22 (8-44)	NS
SAR ($n = 9$)	352 (274-696)	NS	705 (35-1,920)	NS	15 (8-44)	NS

Data are shown as median (range). OT: operation time; BL: intraoperative blood loss; LOS: postoperative length of hospital stay; GBT: laparoscopic liver resection with lymph node dissection for gallbladder tumor; PR: laparoscopic partial liver resection; LLS: laparoscopic left lateral sectionectomy of the liver; AR: laparoscopic anatomical resection of the liver (resection of one or more sections, excluding LLS); SAR: laparoscopic small anatomical resection of the liver (resection of less than a full segment); NC: not countable; NS: not significantly different from GBT data; $P < 0.001/P < 0.05$: significantly different from GBT data

or GB bed with limited LN dissection and no bile duct resection for treatment of GBC is thought to have similar advantages, although such a procedure also has potential disadvantages (risk of tumor cell dissemination and port site recurrence).^[11]

Liver resection for treatment of T1b/T2 GBC involves PR of the anterolateral segments, where laparoscopic approaches are easily applied,^[4,5] and the techniques for LN dissection have also been applied in other established procedures.^[15,18] Although dissection of the posterosuperior pancreatic and peri-splenic vein LNs is difficult, this dissection can reportedly be easier with the Kocher maneuver.^[8,9] However, bile duct resection and reconstruction is still a demanding technique with limited reports.^[9,16,19,20] It is often required for bile duct invasion by the tumor in patients with T3 GBC or GBC in the neck. Therefore, in the present series, only patients with GBTs suspected to be T1b/T2 GBC in the body/fundus were selected for laparoscopic surgeries with intraoperative pathological examination for confirmation of negative cystic duct tumor invasion. No cases of mismatch of the intraoperative and postoperative pathological results of cystic duct tumor invasion were encountered. Furthermore, an accurate preoperative diagnosis of the tumor depth (T stages 1a, 1b, 2, and 3^[12]) is needed for application of this technique. Itano *et al.*^[8] reported that precise preoperative endoscopic ultrasonography led to no underestimation of the preoperative diagnosis regarding tumor invasion into the muscular or subserosal layer in their patients with T1/T2 cancer. We also attempted to avoid underestimation, which leads to the need for a second operation and/or carcinoma recurrence, rather than overestimation in our series. We observed no cases of underestimation; however, 2 patients had benign (overestimated) lesions, including 1 xanthogranuloma. Overestimation and overapplication of this procedure for benign or Tis/T1a GBC is also a potential problem. However, the drawbacks of laparoscopic cholecystectomy for Tis/T1a GBC include the risk of GB wall perforation and bile leakage contaminated with tumor cells, which may lead to port site recurrence and peritoneal dissemination.^[21] These risks could be overcome by combined GB bed resection. Given the fact that this procedure was performed with short-term outcomes comparable with those of laparoscopic LLS or PR, overestimation and overapplication of this procedure might be justified. However, LLR of S4b+5+6a with regional LN dissection, which we applied to the patients with T2 GBC, is a more complicated and demanding procedure and was associated with a longer OT and larger BL volume comparable with AR. The application of this procedure is currently limited

to patients with proven T2 GBC in our institute.

Itano *et al.*^[8] reported that the disease-free and overall survival rates of patients with T2 GBC tended to be superior, although not significantly so, among patients who underwent laparoscopic than open surgery. However, they also mentioned that this observation may have been due to selection bias because their study was a semi-historical control study (the period for the laparoscopic group was from December 2007 to December 2013, and that for the control open group was from June 2003 to May 2011), and patients with more advanced disease might have been selected for the open surgery group before the advent of precise endoscopic ultrasonography examination. They still concluded that the laparoscopic approach for suspected T2 GBC was at least comparable with open surgery in terms of both the surgical and oncological outcomes.

The sample size of the present study was too small to perform a definitive statistical analysis of the short-term outcomes, and concerns regarding tumor dissemination and port site recurrence are still valid when performing laparoscopic procedures with restricted manipulation and instruments. Further studies of laparoscopic surgery for GBC are needed. Nevertheless, this technique could be a good treatment option for GBTs suspected to be T1b/T2 GBC in the GB body/fundus without invasion of the cystic duct.

DECLARATIONS

Authors' contributions

Performed the treatments and wrote this manuscript: M. Isetani

Planned and performed the treatments: Z. Morise
Supervised planning and writing this manuscript: Z. Morise, A. Horiguchi

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None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Patients were fully involved in the treatment decision-making process. Informed consent was obtained

from each patient for both treatment and use of data in the study.

Ethics approval

The data obtained through the medical record review were managed according to the privacy policy and ethics code of our institute. The surgeries were performed with the permission of our hospital review board.

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Hepatocellular carcinoma following direct anti-viral for hepatitis C treatment: a report of an Egyptian case series

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ABSTRACT

Egypt had been vexed by the highest load of chronic hepatitis C in the world. It represents a vast market of the new direct-acting anti-viral drugs (DAAs); effectively treating chronic hepatitis C virus (HCV) infection. Eradication of HCV in Egypt has been challenged by the observed increased diagnosis of hepatocellular carcinoma (HCC) in relation to DAAs therapy. This is the first Egyptian report annotating to a series of sixteen chronic HCV infected cases without a diagnosis of HCC before DAAs therapy and unexpected development of HCC during or after completion of DAAs therapy.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most dreadful sequels of hepatitis C virus (HCV)-related cirrhosis.^[1] New direct-acting antivirals (DAA) had successfully created a new era of HCV elimination.^[2] However, their role in moderating the incidence of HCC in those patients is still questionable. Beyond the several observations of the proximity between DAAs therapy, and emerging HCC, many systematic reports have been sequentially reported.^[3-5] The first

one is from Barcelona reported that HCC recurrence in 27.6% of the studied patients after a median follow-up of 5.7 months. Notably, they achieved viral eradication and had no pretreatment evidence of residual HCC.^[3] In the Italian cohort that included 59 patients with earlier HCC and 295 patients negative for HCC, the HCC recurred at a rate of 28.8%, while *de novo* HCC showed a lower rate (3.16%).^[4] The French report that included 3 studies and 6,000 patients who received interferon (IFN)-free regimens had refuted the Spanish and Italian data. The researchers found no increased



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risk of developing *de novo* HCC and a relatively low risk for HCC recurrence.^[5]

Amelioration of HCV natural history is the anticipated post treatment target. Sustained virological responses (SVR) and their link to lessened HCV-related morbidity and mortality, including HCC had been interrogated since the era of IFN-containing regimens.^[6] This conception has been already recalled in the new era of DAAs with evolving comparable perspectives.^[7] This is the first report from Egypt; registering 16 primary HCC cases respective to DAAs therapy.

CASE REPORT

This report includes a series of 16 patients who were diagnosed as Child A HCV-related cirrhosis. They presented to National Liver Institute Hospital, Menofia University, Egypt, to receive care and management as inpatients in the Clinical Hepato-Gastroenterology Department.

The patients were males except for 1 female and at their late fifties. They were diagnosed as having HCV infection during the least 4 years. Pre DAAs treatment evaluation, laboratory, endoscopic as well as tedious professional abdominal imaging [either abdominal ultrasound or computerized tomography (CT) scan] were available for all patients.

All patients received IFN-free, sofosbuvir-based regimens. Sofosbuvir plus ribavirin was prescribed to 11 cases (68.8%), sofosbuvir/daclatasvir plus ribavirin were given to 3 patients (18.8%), 1 patient was given sofosbuvir plus daclatasvir (6.2%), and 1 patient (6.2%) had received sofosbuvir plus simeprevir. SVR at week 12 post treatment was achieved in 13 cases (81.25%).

The patients had completed their treatment regimens, except 2 cases that developed drug-related complications, and stopped the treatment. Only 1 relapse was reported in this study group.

The newer sonography and CT imaging in 2 cases as well as the remaining 14 patients had surprisingly unveiled presence of predominantly small HCC. The small-sized lesions added to the mean timing for HCC detection (4.19 ± 3.48 months post-treatment), and the pre-treatment compensated liver disease have suggested HCC occurrence rather than a continuation of pre-treatment lesions.

Most of these new lesions were small; less than 3 cm in 12 patients (81%), 3-5 cm in 3 cases (18.8%), while 1 patient who was diagnosed with a lesion more than 5 cm. All these patients presented less than 1 year post-

treatment (4.19 ± 3.48 months). The focal lesions were mainly cited in the right hepatic lobe (62.5%), 12.5% in the left lobe while multi-focal lesions were detected in 4 cases (25%) [Table 1].

Malignant portal vein thrombosis was radiologically documented in 1 patient (6.25%). Significant biochemical derangements were reported following revelation of HCC. They were significant enough to transfer most of the affected patients from Child class A to Child C cirrhosis [Table 2].

Statistical analysis

Statistical analysis was carried out using SPSS (Statistical Package for Social Science) program. Data was entered as numerical or categorical, as appropriate. Quantitative data was shown as mean, and SD, while qualitative data has been expressed as frequency and percent.

DISCUSSION

Obviously, sofosbuvir is the principal DAA in the current case series and all published reports of HCC connected to DAAs.^[8] However, the alleged link between DAAs in general, sofosbuvir or sofosbuvir related metabolites and carcinogenesis needs to be analyzed. Several theories were hypothesized to explain this proposed linkage; however, none of them had a robust proof

Table 1: Descriptive demographic and bibliographic data of the studied patients (n = 16)

Studied variables	Mean	SD
Age (years)	56.63	6.79
Duration of HCV infection (years)	8.69	4.64
Timing of HCC presentation post treatment (months)	4.19	3.48
Gender	Number	Percent
Males	15	93.8
Females	1	6.2
HCV genotyping	All cases were genotype 4a	
Site of lesion(s) by ultrasound		
Right	10	62.5
Left	2	12.5
Multifocal	4	25.0
Size of the lesion(s) (cm)		
Less than 3	12	75.0
3-5	3	18.8
More than 5	1	6.2
Virological responses		
End of treatment	14	87.5
Sustained responders	13	81.3
Relapsers	1	6.2
Incomplete course	2	12.5
The DAAs' regimens		
Sofosbuvir + ribvirin	11	68.8
Sofosbuvir + simeprevir	1	6.2
Sofosbuvir + daclatasvir	3	18.8
Sofosbuvir + daclatasvir + ribvirin	1	6.2

HCV: hepatitis C virus; HCC: hepatocellular carcinoma; DAA: direct-acting antiviral

Table 2: Descriptive laboratory data of the studied patients (n = 16)

Laboratory investigations	Before treatment	On HCC diagnosis
Total bilirubin (mg/dL)	0.80 ± 0.66	4.84 ± 2.14
Direct bilirubin (mg/dL)	0.50 ± 0.20	1.82 ± 1.68
AST (IU)	88.40 ± 34.34	79.00 ± 75.42
ALT (IU)	74.30 ± 23.60	74.63 ± 52.12
ALK (IU)	45.00 ± 17.25	172.25 ± 156.19
GGT (IU)	36.00 ± 12.00	69.06 ± 72.15
Serum albumin (mg/dL)	3.40 ± 0.50	2.20 ± 0.88
Hemoglobin (gm/L)	12.30 ± 2.20	11.81 ± 2.37
WBCs (10 ³ /L)	4.60 ± 6.10	8.63 ± 4.32
Platelet (10 ³ /L)	123.00 ± 32.50	102.50 ± 45.10
Prothrombin concentration (%)	87.20 ± 12.40	44.94 ± 25.47
INR(s)	1.20 ± 0.30	1.50 ± 0.46
Serum HCV-RNA average levels (IU)	517,229.10	
Serum AFP (ng/mL)	20.00 ± 12.46	479.46 ± 588.96

HCC: hepatocellular carcinoma; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALK: alkaline; GGT: gamma glutamyl transpeptidase; WBC: white blood cell; INR: international normalized ratio; AFP: alpha fetoprotein

of concept. DAAs induced HCV elimination with subsequent disturbance of immune functions and less anti-tumoral potency is the most proposed explanation for developing HCC. Also, deprivation of the hepatic microenvironment from the inflammatory scene containing endogenous IFN-inducible natural killer cell/cytotoxic T lymphocytes and many other antiviral tumor molecules; definitely has a pro-oncogenic effect.^[9]

The reported downregulation of IFN and IFN stimulated genes following dual sofosbuvir-ribavirin induced viral eradication might add another explanation.^[10] In pre-clinical studies, IFN alpha had demonstrated activity against several tumor types including HCC. Many reports had demonstrated the beneficial effects of IFN alpha in reducing incidence of HCC in cirrhotic patients who achieved sustained virological response. van der Meer *et al.*^[11] in their sizeable multinational study, with longstanding follow-up periods had proved the positive effect of post IFN SVR on reducing morbidity and mortality and in diminishing HCC incidence rates in HCV-related cirrhosis patients. They reported that only 4% of those who achieved SVR had experienced HCC development against 76% in those who didn't.^[11]

A recent systematic review had examined the HCC incidence in 5 randomized controlled trials (RCTs) including 1,926 chronic hepatitis C (CHC) patients

with cirrhosis or advanced fibrosis has concluded that IFN-treated CHC cirrhotic patients showed a lower HCC incidence than non-IFN-treated controls after 5-years follow-up.^[12] The same review examined the outcome of antiviral treatments in 6 RCTs with a total of 374 HCV-related HCC patients who had received curative therapy for HCC. After a more than 25 months (median) follow-up, IFN-treated patients showed a lower recurrence rate of HCC, than non-IFN-treated controls.^[12]

Although the exact mechanism behind the anti-tumor properties of IFN has not been yet fully elucidated, it has been widely used for the treatment of numerous types of cancer, including certain hematological malignancies and solid tumors.^[13] A recent *in vivo* study reported the IFN's ability to synergize the apoptotic, autophagic as well as the anti-proliferative action of cisplatin.^[14] Autophagy has been shown to be induced in HCC cell lines when treated with IFN-α2b in a dose-dependent manner.^[15]

Of note, autophagic cell death had been suggested as one of the anti-cancer actions of anti-cancer therapeutics.^[16] Supporting these postulations was the recent study by Liang *et al.*^[17] who concluded that treatment by pegylated IFN was associated with a lower HCC incidence than nucleos(t)ide analogues in chronic HBV infection. They described the oncogenic surface antigen truncation mutations to be detected in entecavir-treated patients with HCC but not in pegylated IFN-treated patients.^[17]

Unlike IFN, DAAs have neither anti-angiogenic nor anti-proliferative properties and have no effect on oncogenic buds that already would reside cirrhotic livers.

For the time being, risk assessment for HCC should be rigorously undertaken before DAAs, and those at risk should have attentive surveillance during treatment and afterward. For people at risk, it is noteworthy to explain the importance of continued surveillance after HCV eradication. Also, physicians in the outreach clinics should know by heart that in HCV-positive patients, the risk of HCC is reaching higher figures compared with those eliminated the virus, yet sustained responders having advanced fibrosis are still at high HCC risk.

Liver fibrosis has been proven to be regressive in some patients who eliminated the virus;^[18] hence post treatment transient elastography would be beneficial in defining patients within the surveillance program. Moreover, surveillance programs had to be strengthened by predictive genetic as well as

angiogenic HCC bio-markers.

In conclusion, surveillance programs should be widely endorsed during and after DAAs therapy for patients at HCC risk, even for those who had been achieved HCV cure. Perhaps IFN still has a role -- using it as a backbone therapy might benefit patients at the highest risk of HCC.

DECLARATIONS

Authors' contributions

Idea and study design: E.A. Rewisha

Collection of literature data: O. Elshaarawy, D.M. Elsabaawy

Data analysis: A. Abdelah

Clinical data collection, manuscript writing and critical revision: O.M. Alhaddad, M.M. Elsabaawy

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None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

A written informed consent was obtained from all participants in the study.

Ethics approval

The study protocol was approved by the Institutional Review Board (IRB) and local ethical committee of the National Liver Institute, Menoufia University.

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Transcatheter arterial chemoembolization in recurrent unresectable hepatocellular carcinoma after orthotopic liver transplantation

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ABSTRACT

Aim: To investigate the survivals and efficacy of the doxorubicin drug eluting beads transcatheter arterial chemoembolization (TACE) in patients with recurrent hepatocellular carcinoma (HCC) status post orthotopic liver transplantation. **Methods:** Consecutive patients with HCC who underwent orthotopic liver transplantation from 2005 to 2012 were reviewed. Patients who developed recurrent HCC after orthotopic liver transplantation and received doxorubicin drug eluting beads TACE therapy were identified and included in the study. Survivals were calculated from the time of 1st doxorubicin drug eluting beads TACE of recurrent HCC. Kaplan Meier estimator with log rank test was used for survival analysis. **Results:** Eight patients had recurrent HCC after orthotopic liver transplantation and received doxorubicin drug eluting beads TACE. The overall median survival of these patients was 15.6 months. Two patients had significantly poorer overall median survival from doxorubicin drug eluting beads TACE (3.4 months) and both showed elevated serum alpha-fetoprotein levels (> 400 ng/mL) and extra-hepatic metastases ($P = 0.03$). Patients with poorly differentiated HCC in explant liver had the poor median overall survival (3.6 months) compared to the patients with well-to-moderately differentiated HCC (21.7 months, $P = 0.004$). **Conclusion:** Doxorubicin drug eluting beads TACE appears to be an effective treatment option for patients with recurrent HCC after orthotopic liver transplantation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading cause of cancer related death globally.^[1] Among all the

treatment options for HCC and cirrhosis, orthotopic liver transplantation (OLT) is considered the curative treatment option, especially for patients with end-stage liver disease. Unfortunately, recurrence of HCC



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occurs after OLT.^[2] Many studies have reported on the patterns and prognostic factors for recurrence of HCC after liver transplantation.^[3-6] However, the reported prognostic factors investigated have been focused more on histopathologic and postoperative clinical data after HCC who did not receive chemoembolization.^[3-6] Several studies have been reported on the efficacy of conventional transcatheter arterial chemoembolization (cTACE) in recurrent HCC after OLT.^[7,8] Little is known about the survivals, efficacy and prognostic factors following doxorubicin drug eluting beads transcatheter arterial chemoembolization (DEB TACE) in patients with recurrent HCC status post OLT.

The purposes of this study were, first, to investigate the survivals and efficacy following DEB TACE in patients with recurrent HCC status post OLT and second, to identify the prognostic factors of survivals among these patients with recurrent tumors and to report the review of the literature.

METHODS

This is a single institutional retrospective analysis of prospective database with the patient's consent, approved by the Local Institutional Review Board and is Health Insurance Portability and Accountability Act compliant.

Study objective

The primary objective of the study was to assess the survivals and efficacy following DEB TACE in patients with recurrent HCC status post OLT. And the second objective was to identify the prognostic factors of survival among these patients with recurrent HCC after OLT who were treated with DEB TACE and to report the review of the literature on the similar studies.

Patient selection

There were 420 consecutive patients with unresectable HCC who received DEB TACE therapies from December 2005 to September 2012. A total of 56 patients underwent OLT after downstaging of HCC from DEB TACE. Patients who developed recurrent HCC after OLT were identified. Those patients who underwent DEB TACE for recurrent HCC were included in the study. A total of 8 patients met the inclusion criteria and included in the study. None of the HCC tumor was feasible for surgery or ablation treatment due to size or close proximity with liver capsule or hepatic vasculature. All patients had cirrhosis before OLT. One patient was alive at the end of the study. The patients who received treatment with sorafenib were also included in the study. All patients had an initial outpatient clinical evaluation, including pertinent medical and physical

evaluations. The eastern cooperative oncology group (ECOG) performance status (PS) of each patient was documented before the DEB TACE procedure. The functional liver status was determined by using the Child-Pugh criteria. The American Association for the Study of Liver Disease-Journal of the National Cancer Institute guidelines^[9] were used to diagnose HCC. HCC was diagnosed if magnetic resonance imaging (MRI) showed a mass with the typical vascular pattern of arterial enhancement and portal venous "washout". For the index lesions between 1 and 2 cm, two different studies were used to detect the typical pattern and for lesions > 2 cm in diameter, only one study was used. Here, index lesion means the largest lesion in the liver. Lesions with inconclusive features on imaging were biopsied for pathologic confirmation.

DEB TACE procedure

There were 18 DEB TACE procedures performed in 8 patients. The detail techniques of the procedure were mentioned elsewhere.^[10] The third or fourth order branches of feeding vessels supplying the tumor were catheterized with a 2.8 F (Renegade Hi-Flo; Boston Scientific, Natick, MA, USA) or a 2.1 F microcatheter (STC Renegade Hi-Flo; Boston Scientific, Natick, MA, USA). Then, the tumors were treated with a slow fluoroscopy-guided injection of iodinated contrast mixed 100-300 μ m low compression beads impregnated with 50 mg of doxorubicin in each vial. The first and second order branches of the right or left hepatic arteries were kept patent and documented on post-embolization completion angiogram. The endpoint for treatment included the administration of the 2 vials of DEB or sluggish flow in the subsegmental branches of the hepatic artery to the region of the tumor, without an effect on the flow in the main or lobar hepatic artery. After 2 vials of DEB TACE, no additional embolization was performed despite persistent high flow within the tumor.

Follow-up

Patients with large tumors of more than 5 cm or multifocal disease were re-treated in 4 weeks and the remainders of the patients were followed up in the clinic in 4 weeks with liver function tests and an MRI of the liver. Follow-up cross-sectional imaging was performed at 4 weeks from the last single or repeat DEB TACE treatment. Further treatments were based on clinical evaluation, laboratory values, and imaging response. If there was a progressive disease on follow up MRI at 4 weeks, then the patients were assessed for systemic therapy. Simultaneously, these patients were re-treated with DEB TACE unless the disease progressed to the Barcelona-Clinic Liver Cancer D stage. If follow up MRI demonstrated residual or recurrent HCC, then the patients were retreated with DEB TACE. If patients

responded completely, then they were followed-up every 3-6 months with MRI.

Statistical analysis

Survivals were also stratified on the basis of age, gender, etiology, tumor burden, Okuda staging, ECOG PS, Child-Pugh class and Cancer of the Italian Liver Program staging. A *P*-value of 0.05 was held as significant. Survival was calculated from the time of first transcatheter therapy. The Kaplan-Meier method with the long rank test was used to estimate survival and difference. A patient was censored if he/she was alive at the end of the study period. SPSS software, version 21.0 (IBM, Somers, NY) was used to perform the statistical analyses.

RESULTS

Patient population

Eight patients had recurrence of HCC after OLT and received 18 DEB TACE treatments (range 1 to 4) after recurrence. The demographics, clinical, pathology and imaging characteristics of the patients are shown in Table 1. The mean age of the patients was 53.4 years (SD 4.6 years). The 5 patients had Child Pugh class A disease and 3 patients had Child Pugh class B disease at the time of presentation of recurrent HCC. Cirrhosis was present in all patients, diagnosed by imaging. The 7 patients had hepatitis C and 1 patient had hepatitis B. The portal venous hypertension (PHT) was present in 50% of patients. The PHT was

diagnosed on MRI. Clinically, ascites was present in 1 patient. The mean size of the index tumor was 3.3 cm (SD 0.85 cm). Portal vein thrombosis or invasion was not present in any of the patients and extra-hepatic metastases were present in 25% of the patients (*n* = 2) at the time of initial presentation. The 1 patient has T11 vertebral body metastasis and showed mildly increased activity on computed tomography (CT) positron emission tomography examination. The other patient had a single 9 mm lung metastasis on CT chest and it was surgically resected. These both patients had alpha-fetoprotein (AFP) of greater than 2,400 ng/dL. During DEB TACE therapies, 25% (*n* = 2) of patients received concurrent sorafenib systemic chemotherapy. The 6 patients (75%) had solitary HCC and unilobar involvement after OLT. The 30-day mortality was zero.

Survival analysis

The overall median and mean survivals from the time of 1st DEB TACE were 15.6 and 19.6 months accordingly. The mean recurrence free survival from the time of OLT was 50.5 months. The mean survival from the time of the OLT was 72.1 months. One year and 2-year survivals from the time of 1st DEB TACE were 62.5% (5/8) and 50% (4/8) respectively. The univariate survival analyses were performed for different categories as shown in Table 2. Two patients had significantly poor overall survivals from DEB TACE (3.27 and 3.4 months) as compared to other patients and both showed elevated serum AFP levels (> 2,400 ng/mL) and extra-hepatic metastases [Table 2]. The

Table 1: Demographics, clinical, imaging, staging and survival characteristics of recurrent HCC patients after OLT treated with DEB TACE

Variables	P 1	P 2	P 3	P 4	P 5	P 6	P 7	P 8
Age (years)	51.7	44.5	54.0	59.5	55.0	51.4	52.7	58.3
Living status	Alive	Dead	Dead	Dead	Dead	Dead	Dead	Dead
Gender	Male	Male	Male	Male	Male	Male	Female	Female
Race	White	Other	White	White	White	Black	White	Other
Etiology	Hepatitis C	Hepatitis B	Hepatitis C	Hepatitis C	Hepatitis C	Hepatitis C	Hepatitis C	Hepatitis C
Index tumor size (cm)	2.2	2.2	2.8	3.1	3.3	3.9	4.1	4.7
Number of the tumor	1	12	1	9	1	1	1	1
Histology grading of the explant liver HCC	Well or moderately differentiated	Poorly differentiated	Poorly differentiated	Well or moderately differentiated	Well or moderately differentiated	Well or moderately differentiated	Well or moderately differentiated	Well or moderately differentiated
Metastases at time of recurrent HCC presentation before DEB TACE	No	Yes	No	No	No	Yes	No	No
Alfa-fetoprotein (ng/dL) of recurrent HCC	5	>2,400	<5	10.6	11.8	>2,400	40.9	9.8
Child-Pugh class of recurrent HCC	A	B	A	A	B	A	A	B
Tumor free survivals from OLT (months)	32.9	25	13.7	83.1	117	40.3	28.4	63.6
Concurrent sorafenib treatment	No	Yes	No	No	No	Yes	No	No

P: patient number; DEB TACE: doxorubicin drug eluting beads transcatheter arterial chemoembolization; OLT: orthotopic liver transplantation; HCC: hepatocellular carcinoma

shortest survival from DEB TACE was 3.4 months and the patient had hepatitis B and more than 12 HCC tumors with extra-hepatic metastasis and AFP > 2,400 ng/mL. Survival curves generated by Kaplan Meier analysis according to the status of AFP and metastases are shown in the Figure 1A and B. The histology grading of HCCs of the explant liver was correlated with the survivals. The patients with poorly differentiated HCC had the poor overall survivals (3.4 months) compared

Table 2: Median survivals (from 1st DEB TACE) HCC patients after OLT treated with DEB TACE

Demographics	Number of patients	Median survival (months)	P value
Total number of patients	8	15.6	
Child-Pugh class			
A	5	21.7	0.45
B	3	15.6	
Okuda staging			
I	6	9.3	0.9
II	2	15.6	
CLIP staging			
Early	3	39.4	
Intermediate	5	15.6	0.2
ECOG performance status			
0	4	39.4	
1	3	21.7	0.3
2	1	7.7	
Imaging findings			
Ascites			
Present	1	26.8	0.7
Absent	7	15.6	
Portal hypertension			
Present	4	7.8	0.2
Absent	4	21.7	
Tumor morphology			
Tumor locations			
Unilobar	6	15.6	0.27
Bilobar	2	3.4	
Number of tumors			
Solitary	6	15.6	0.14
Multiple	2	3.4	
Size of index tumor			
< 3 cm	6	15.6	
3 cm or more	2	7.8	0.87
Extrahepatic metastasis			
Present	2	3.4	0.03
Absent	6	21.7	
Laboratory data			
Serum alpha-fetoprotein level (ng/dL) of recurrent HCC			
< 400	6	21.7	0.03
≥ 400	2	3.4	
Histology grading of the explant liver HCC			
Well or moderately differentiated	6	21.7	0.004
Poorly differentiated	2	3.6	

HCC: hepatocellular carcinoma; DEB TACE: doxorubicin drug eluting beads transcatheter arterial chemoembolization; OLT: orthotopic liver transplantation; CLIP: Cancer of the Italian Liver Program; ECOG: eastern cooperative oncology group

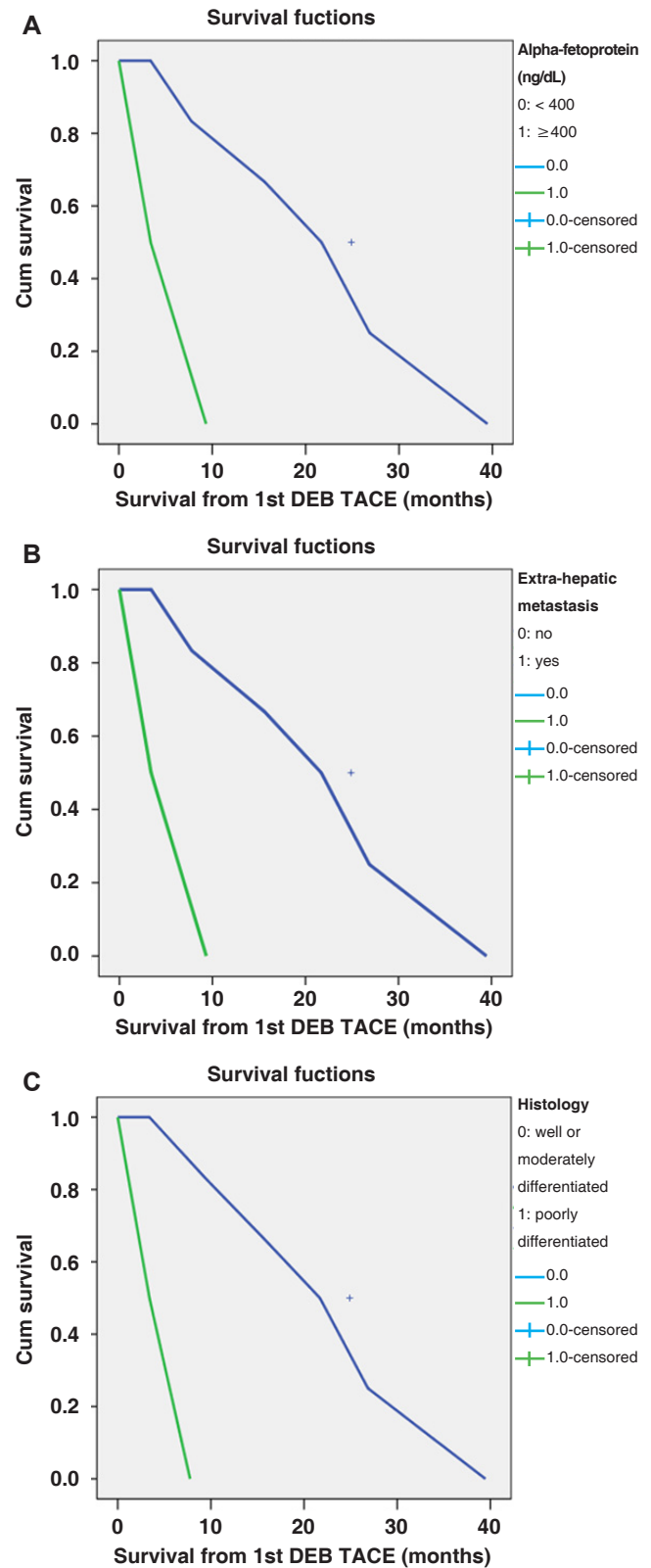


Figure 1: Survival curves generated by Kaplan Meier analysis according to the status of alpha-fetoprotein level (A), presence or absence of the extra-hepatic metastasis (B) and histology grading of the explant liver (C) before DEB TACE in patient with recurrent HCC after orthotopic liver transplantation. HCC: hepatocellular carcinoma; DEB TACE: doxorubicin drug eluting beads transcatheter arterial chemoembolization

to the patients with well or moderately differentiated HCC of the explant liver (21.7 months, $P = 0.004$). The survival curve generated by Kaplan Meier analysis according to the status of histology grading of the explant liver is shown in the **Figure 1C**. Although, there was a survival difference in the patients between Child A and B disease, statistically it was found nonsignificant. This can likely be due to small sample size. In this study, 25% patients received concurrent treatment with sorafenib with a median survival of 7.7 months compared to a median survival of 21.7 months in patients who did not receive sorafenib ($P = 0.19$).

DISCUSSION

Recurrence of HCC after liver transplantation has a major effect on reducing patient's overall survival.^[11] In general, all treatment options currently available for advanced HCC are also potentially feasible after OLT. Treatments include resection, ablation, transarterial embolization or radioembolization, and systemic treatment with sorafenib. The 5-year posttransplant survival was 47% for patients who underwent surgical resection to treat recurrence.^[11] The ability for surgical treatment and a late onset (> 24 months) of recurrence are factors associated with long-term survival.^[12] Local ablative techniques, such as radiofrequency ablation, cryoablation, or percutaneous ethanol ablation, also yield favorable survival outcomes in patients with small unresectable recurrent HCC.^[13,14] In our study, none of the HCC tumor was feasible for surgery or ablation treatment due to size or close proximity with liver capsule or hepatic vasculature. All patients had cirrhosis before OLT. In 2007, sorafenib was the first agent to demonstrate a significant improvement in the overall survival of patients with advanced HCC.^[15,16] The survival benefits from sorafenib ranged from 2 to 3 months in advanced HCC patients.^[15,16] Since these two landmark studies, sorafenib has become the standard of care for advanced HCC patients. It has also shown improved survival benefits in patients with recurrent HCC after OLT as compared to best supportive care.^[17] Yttrium-90 radioembolization has shown benefits in HCC patients.^[18] However, no specific radioembolization study was found in patients with recurrence of HCC after OLT.

DEB TACE is a well-known locoregional treatment for HCC evaluated by multiple randomized controlled studies. Recently, numerous studies have been reported favorable outcomes with the use of DEB TACE for HCC.^[10,19-22] DEB TACE has demonstrated improved survival, better tolerability, and fewer side effects as compared to conventional TACE.^[19,21-23] In these reported DEB TACE studies, the survivals in

patients with unresectable HCC, ranged from 13.5 to 24.5 months.^[10,19-22] In the current study, the overall median and mean survivals from the time of 1st DEB TACE were 15.6 and 19.6 months accordingly, which is comparable with the reported DEB TACE studies.

Little is known on the survivals, efficacy and prognostic factors of survivals following DEB TACE in patients with recurrent HCC status post OLT. Few similar studies were found from English literature.^[7,8] Zhou *et al.*^[7] reported that conventional TACE is safe following in patients with recurrent HCC status post OLT. Their study indicated that TACE treatment seems to produce an effective tumor response for targeted recurrent HCC after liver transplantation. The Child Pugh Class of HCC patient is considered to be the one of the main prognostic factors for survival following TACE in HCC patients.^[24-26] In our study, there was a survival difference in the patients between Child A and B disease. However, statistically it was found nonsignificant. This can likely be due to small sample size.

Recurrence of HCC ranged from 10% to as high as 40%.^[2,27,28] Therefore, surveillance with MRI of the abdomen is very important in these patients. Patients with early recurrence had much worse overall survival than those with late recurrence.^[2,27,28] In our studies, 2 patients had shortest tumor free survival of 13.3 and 25 months and had worst overall median survivals of 3.4 and 7.7 months respectively. Both patients had poorly differentiated HCC of the explant liver. The patients with poorly differentiated HCC had the poor overall survivals (3.4 months) compared to the patients with well to moderately differentiated HCC of the explant liver (21.7 months, $P = 0.004$). A histological grade of HCC is an important prognostic factor affecting patient survival after OLT. The importance of the grade of the histology of the explant liver HCC in patient's prognosis has previously reported.^[5,6,29]

The prognostic factors for poor survivals other than the histology grading, the number and size of the tumors have been reported by many investigators. These factors include microscopic vascular invasion by the HCC,^[30,31] presence of partial necrosis of the nodule in the explanted specimen,^[32] presence of microscopic satellite nodules in the explanted specimen,^[33] specific type of lymphocytic infiltrate to the tumor as immune response,^[34] high preoperative level of serum AFP,^[35] and advanced tumour-node-metastasis stage and extra-hepatic metastases.^[4,5] In this study, 2 patients had elevated AFP and extra-hepatic metastases had the poorest survivals. As these facts help in identifying the patients who will get the most benefit from the DEB TACE treatment.

We acknowledge, this study has several limitations. First, the sample size of the study is small and so results should be taken as preliminary data. Second, this is a single institution non-randomized study, so selection bias and late look bias may be inherent. Third, patients who were treated with sorafenib (25%) were also included in this study, so outcomes after DEB TACE may be confounded. However, concomitant therapy with sorafenib did not significantly affect survival in univariate analysis. Therefore, we believe that survival advantage in this study is largely from the effect of DEB TACE therapy.

In conclusion, recurrence of HCC after OLT is not uncommon. DEB TACE could help to extend the survival of the patients with recurrent HCC after OLT. As the sample size of the study is small, the results should be taken as preliminary. Further, multi-institutional prospective trial is needed to explore its benefit on these patients with recurrence of HCC after OLT. Patients with poorly differentiated HCC of explant liver, > 400 ng/dL AFP and metastases at the time of TACE had a poor overall prognosis.

DECLARATIONS

Authors' contributions

All three authors were involved with study concept, design, acquisition of data, analysis and interpretation of data, drafting of the manuscript and critical revision of the manuscript for important intellectual content.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Obtained.

Ethics approval

The study is approved by the Local Institutional Review Board and is Health Insurance Portability and Accountability Act compliant.

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Surgical strategy for huge and advanced hepatocellular carcinoma in Hong Kong

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ABSTRACT

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Key words:

Associating liver partition and portal vein ligation, staged hepatectomy, hepatocellular carcinoma, liver resection, vascular resection and reconstruction, radiofrequency ablation

In Hong Kong, surgical resection is the core curative treatment for huge and advanced hepatocellular carcinoma (HCC). For tumors measuring 10 cm or above, major hepatectomy is usually required, but a future liver remnant not large enough will preclude the operation. Hypertrophy of future liver remnant is a way to render more patients operable, and measures include portal vein embolization and associating liver partition and portal vein ligation for staged hepatectomy. For HCC that has invaded a major vessel, *en bloc* resection with immediate vessel reconstruction is necessary if thrombectomy would not suffice. In case of bilobar involvement, radiofrequency ablation is a useful adjuvant therapy. In the treatment of extrahepatic metastasis, metastasectomy offers a cure to properly selected patients.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and is the most common primary liver malignancy worldwide.^[1] Like hepatitis B, it is most prevalent in Asia; at the same time, most cases of HCC on the continent are related to hepatitis B, and Hong Kong is no exception.^[2] Diagnoses of HCC are mostly made at a late stage as regular screening for the disease is uncommon, and the disease often develops in a multifocal manner and infiltrates into major vessels. As such, surgical resection is a common

curative treatment. Fan *et al.*^[3] reported 5-year survival rates of 73% and 81% achieved by partial hepatectomy and living donor liver transplantation respectively in patients within the Milan criteria.

In the case of huge and advanced HCC, treatment is more limited. Only transarterial chemoembolization (TACE) and systemic therapy are recommended in Western countries,^[4-7] but more aggressive management is adopted in Hong Kong. A newly developed Hong Kong liver cancer (HKLC) staging is now in use. In the study by Yau *et al.*,^[8] surgery had



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a significant survival benefit over TACE in treating HKLC-2 HCC, with a 5-year survival of 49% vs. 0% ($P < 0.001$); on the other hand, TACE had a significant survival benefit over systemic therapy in treating HKLC-3 HCC, with a 3-year survival of 10% vs. 2% ($P < 0.001$). If the patients are young, fit and properly selected, aggressive resection may still be beneficial despite large or multiple nodules or intrahepatic venous invasion.^[8]

Disease treatment should be individualized. In general, surgical resection is the core curative treatment for huge and advanced HCC in Hong Kong.

HCCS OF 10 CM OR BIGGER

Hepatectomy is the first-line HCC treatment for tumor clearance and a cure for patients with preserved liver function.^[3,9,10] For HCCs ≥ 10 cm, major hepatectomy is usually needed. Measures to ensure safe major hepatectomy with acceptable complication and perioperative mortality rates include careful patient selection (patients should be fit for surgery and with preserved liver function),^[9,11] adoption of the anterior approach to avoid mobilization and rupture of large tumors,^[12] close liaison with the anaesthesiologist to ensure a low central venous pressure in order to reduce blood loss,^[13] and use of surgical instruments (such as Cavitron Ultrasonic Surgical Aspirator).^[9,14-16] Major hepatectomy may not be possible for patients who have marginal liver function or a relatively small future liver remnant (FLR). At our center, we use Indocyanine green (ICG) clearance test to assess preoperative liver function.^[17] For consideration for major hepatectomy, an ICG retention rate $\leq 14\%$ at 15 min is required. Besides ICG test result, other factors are also taken into account. A low platelet count, poor renal function test results and the presence of significant morbidity can mean a risky major hepatectomy. An adequate FLR with preservation or reconstruction of major hepatic veins and meticulous surgical skills to avoid massive bleeding and vascular insult to the liver are essential to a successful major hepatectomy.^[18] FLR is assessed by calculation of the liver volume measured by tracing the liver contour on the cross sectional image on computed tomographic volumetry, and the University of Hong Kong formula is used at our center.^[19,20] A patient's estimated standard liver volume (ESLV) can be derived from the patient's weight, height, and body surface area.^[20,21] Patients with liver cirrhosis and relatively poor liver function need a bigger FLR.^[22-25] At our center, we use a ratio of FLR/ESLV of $> 35\%$ for major hepatectomy for patients who have Child-Pugh A cirrhosis and an ICG retention rate $\leq 14\%$ at 15 min.^[26] Liver cirrhosis and inadequate FLR are risk factors for postoperative liver failure.^[25,27]

METHODS TO INCREASE FLR

In order to increase the chance and safety of major hepatectomy for HCC patients, preoperative portal vein embolization has been used to increase FLR. The idea of portal vein embolization is to embolize (in an open or percutaneous manner) the portal vein ipsilateral to the liver lobe harboring the tumor, so as to induce hypertrophy of the FLR.^[28,29] However, it usually takes at least four weeks for the FLR to hypertrophy enough.^[29] During the time, disease progression may occur. If there is tumor invasion of a major vessel (e.g. the ipsilateral portal vein), the disease can progress in terms of weeks. If contralateral propagation and metastasis develop, the tumor will be inoperable.^[30-32] And sometimes hypertrophy does not occur as anticipated.

Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) is a relatively new method of increasing FLR and is gaining popularity. It features two open operations. In the first operation, liver partition and portal vein ligation are performed to induce hypertrophy of the FLR while no resection is done. When the FLR has hypertrophied enough, the second operation is conducted for tumor resection. ALPPS is particularly useful if there is ipsilateral portal vein tumor thrombosis (PVTT) because the first operation also prevents further propagation of the thrombus into the main and contralateral portal veins. ALPPS was initially applied to relatively normal liver, such as that in the case of colorectal liver metastasis.^[33-36] Subsequently its application was extended to steatotic liver and cirrhotic liver.^[37-39] With ALPPS, the increase of FLR between the two operations can be as high as 70%,^[40] and it usually takes only one week to achieve enough hypertrophy. ALPPS outperforms conventional portal vein embolization when it comes to time and extent of hypertrophy.^[41,42] As the interval between the two operations is not long, adhesion formation resulting from the first operation is relatively immature when the second operation takes place, thereby allowing continuation of dissection and resection of the liver with ease.

However, there is no guarantee that adequate hypertrophy always occur, and liver failure might result from the portal vein ligation. The Pringle maneuver is not advisable as it poses further risk of liver injury. Our center has simplified the ALPPS procedure by using an anterior approach to allow liver transection without mobilization of the right lobe, and as such the amount of adhesion is decreased, thereby streamlining the second operation.^[39] The hilar plate and the right hepatic duct are left untouched in the first operation

to minimize the chance of bile leakage, a complication that might lead to biloma, infection and sepsis and thus prohibit the second operation. ALPPS is a technically demanding and challenging procedure that should not be performed by inexperienced surgeons.

ALPPS should be offered with curative intent when a large tumor load is encountered and a marginal FLR is anticipated.^[41] Major vascular invasion, such as portal vein involvement, does not preclude its application.^[35] Many patients who would otherwise be unsuitable for major hepatectomy are rendered eligible by ALPPS; the operation rate is thus raised. Nonetheless, the procedure entails higher rates of surgical complication and mortality when compared with conventional major hepatectomy. The reported perioperative mortality rates range from 12% to 28%^[40,41,43,44] and the complication rate can be as high as 50%.^[43,45] Liver insufficiency (e.g. ascites, persisting cholestasis, sepsis), bile leakage, septic complications and failure to proceed to the second operation have been reported. The long-term outcomes of ALPPS are still pending. Since 2014, 21 patients have undergone ALPPS with curative intent at our center (unpublished data). All of them had R0 resection. No hospital mortality occurred. Three (14%) patients developed major complications. The overall survival was 89% and the disease-free survival was 58% at one year. With time goes by, more data will be available.

INVASION OF THE MAJOR PORTAL VEIN, HEPATIC VEINS, OR THE INFERIOR VENA CAVA

In the case of ipsilateral PVTT, the thrombus is confined to the liver lobe harboring the HCC and is usually resected when hepatectomy is conducted to remove the HCC. For the management of PVTT extending to the portal vein bifurcation or farther to the main or contralateral portal vein, different approaches have been advocated. It is believed that *en bloc* resection (resection of tumor together with all affected parts of the portal vein) can achieve good oncological outcomes with residual microscopic foci removed. Nonetheless, this is a challenging approach since subsequent portal vein reconstruction is required. On the other hand, it has been documented that thrombectomy can yield similar survival outcomes with lower operative mortality and morbidity.^[46-48]

In a previous study trying to address the controversy about *en bloc* resection versus thrombectomy, we compared 3 groups of patients: group 1 ($n = 71$), with ipsilateral PVTT resected in a hepatectomy; group 2 ($n = 10$), with PVTT extending to or beyond the

bifurcation, treated by *en bloc* resection with portal vein reconstruction; group 3 ($n = 7$), with PVTT extending to or beyond the bifurcation, treated by thrombectomy.^[48] The median survival duration was 10.9 months in group 1, 9.4 months in group 2, and 8.6 months in group 3. No significant differences were found in terms of hospital mortality and morbidity between *en bloc* resection and thrombectomy. The practice of living donor liver transplantation at our center certainly had contributed to the low morbidity after portal vein resection.^[49] The 1-, 3- and 5-year survival rates were 50%, 13% and 13% respectively in group 2, and 29%, 14% and 14% respectively in group 3. The two approaches again showed no significant differences in terms of overall survival and disease-free survival, and patients with ipsilateral PVTT also had similar survival to patients with PVTT extending to or beyond the bifurcation. These survival outcomes are superior when compared with a median of 2.7 months of survival of patients with PVTT not treated.^[1]

Patients with advanced PVTT may not be suitable for resection due to underlying medical conditions and main portal vein involvement, and non-surgical treatment is their chance. The combination therapy using sorafenib and TACE appears to provide a survival benefit for patients with PVTT and adequate liver function. This benefit seems to be more pronounced in patients whose first-order or more distal branches of the portal vein are involved^[50] than in patients with main portal vein involvement.^[51] Head-to-head comparison between surgical and non-surgical treatments is warranted.

One point to note is that patients may have falsely elevated preoperative ICG retention rates due to PVTT. Exploration should be offered to patients who fail their ICG test but otherwise show normal liver function. With accumulation of experience from living donor liver transplantation, resection of major vessels such as portal and hepatic veins should yield satisfactory results.

If the tumor thrombus in the inferior vena cava (IVC) or hepatic vein is non-adhering, thrombectomy should suffice [Figure 1]. Sometimes IVC resection with immediate reconstruction should be considered, especially for young patients. Some technical issues need to be considered when IVC resection with immediate reconstruction is required. First, if the lesion is above the hepatic vein confluence, total vascular exclusion with the Pringle maneuver and re-implantation of the hepatic veins are necessary. Second, it is the lesion's relation to the lower level of the IVC resection (i.e. the renal vein level). In fact, the chance of renal vein invasion is very low. If there is

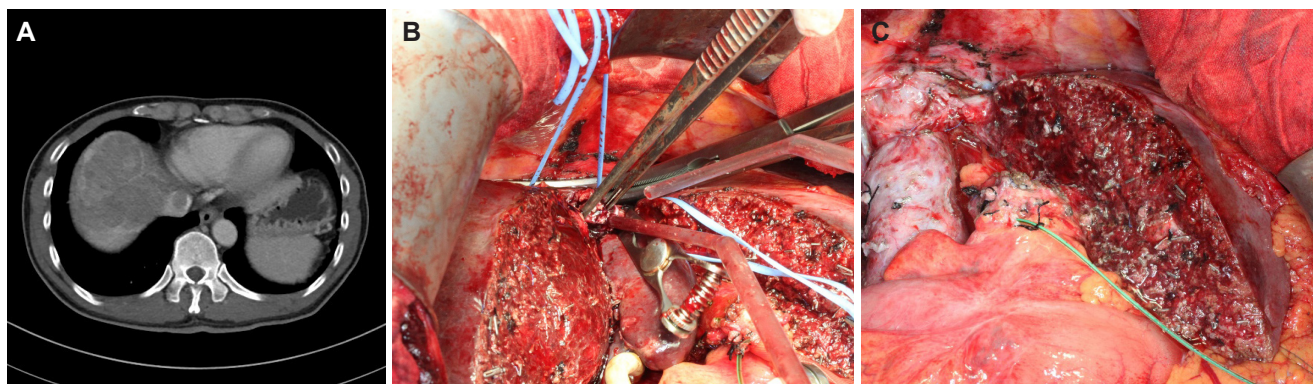


Figure 1: (A) Hepatocellular carcinoma invasion of the suprahepatic inferior vena cava; (B) tumor thrombectomy; (C) closure of the venotomy

invasion of a single renal vein, its resection without reconstruction will not affect normal kidney function. Third, it is the choice of reconstruction conduit. Choices include cadaveric vein graft, autologous vein graft (e.g. renal vein, internal jugular vein) and synthetic graft (e.g. ringed Gore-Tex). At our center, we prefer cadaveric vein graft for it is less rigid and therefore anastomosis will be easier. Nonetheless, its use is limited by availability, blood group compatibility, and length. Length is dictated by donor body size. Usually bench-table work can be done to lengthen a cadaveric IVC graft by incorporating donor bilateral iliac veins. To

avoid creating an additional surgical wound, we prefer not to use autologous vein graft. So, if cadaveric vein graft is not available, a ringed Gore-Tex graft is used [Figure 2].

If the tumor thrombus extends above the diaphragm, a cardiopulmonary bypass by cardiac surgeon may be necessary for its complete removal. However, before considering this high-risk procedure, aggressive workup must be done to rule out other extrahepatic spread of disease, and the treatment approach should be thoroughly discussed with the patient.

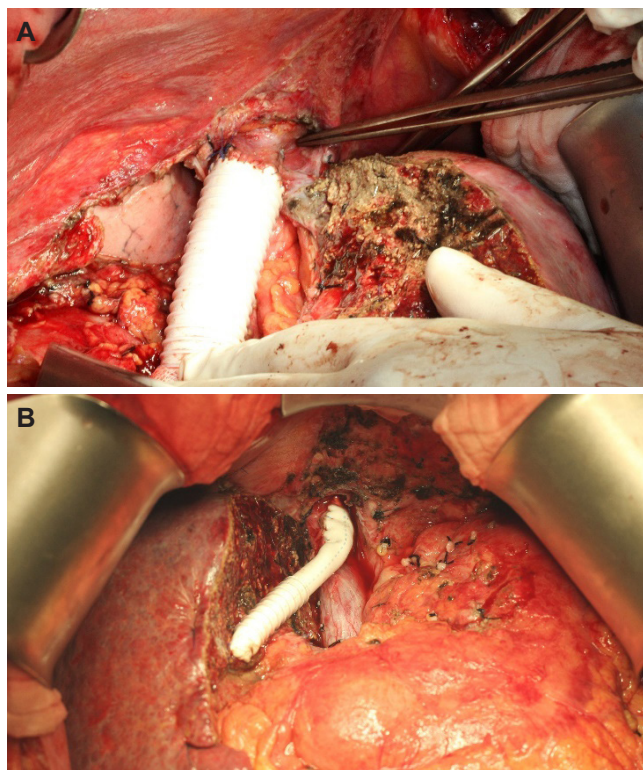


Figure 2: (A) Inferior vena cava reconstruction with a ringed Gore-Tex graft; (B) middle hepatic vein reconstruction with a ringed Gore-Tex graft

BILOBAR INVOLVEMENT

For selected patients with bilobar HCC, the combination of resection and radiofrequency ablation can offer a cure. Cheung *et al.*^[52] compared 19 patients having such a combination of treatments with 54 patients having resection only. Fourteen (74%) patients in the combination group and 3 (6%) patients in the resection group had bilobar involvement ($P = 0.04$). Major resection was performed in 6 (32%) patients in the combination group and 35 (65%) patients in the resection group, whereas minor resection was performed in 13 (68%) and 19 (35%) patients in the combination group and resection group respectively ($P = 0.012$). The combination group had less blood loss (400 vs. 657 mL, $P = 0.007$), shorter operation (270 vs. 400 min, $P = 0.001$), and shorter hospital stay (7 vs. 8.5 days, $P = 0.042$). The two groups were comparable in hospital mortality (5% vs. 6%, $P = 1$), surgical complication (16% vs. 32%, $P = 0.24$), disease recurrence (63% vs. 50%, $P = 0.673$), and overall survival (53 vs. 44.5 months, $P = 0.496$). Thorough intraoperative assessment backed by a sound understanding of the liver anatomy helps to maximize the chance of cure for patients with bilobar HCC.

DOWNSTAGING

Both TACE and radioembolization are safe and effective in highly selected patients. Radioembolization may confer a survival benefit over sorafenib on advanced-stage patients. Radioembolization is preferable to TACE for advanced-stage patients, especially those with macrovascular invasion, since TACE might induce liver failure.^[53] However, the effectiveness of downstaging is not conclusive, as most of the cases reported were limited by poor underlying liver function.

EXTRAHEPATIC METASTASIS

Lung is the most common site for extrahepatic metastasis of HCC.^[54] A previous study by our center reported that metastasectomy conferred a survival benefit on HCC patients who developed lung metastasis after hepatectomy.^[55] Overall survival was compared in patients with resectable and unresectable lung metastases and in two periods (Era 1: 1989-1995, Era 2: 1996-2010). The median survival duration of patients with resectable and unresectable disease was 40.4 and 7.5 months respectively ($P < 0.0001$). In Era 1, the median survival duration of patients with resectable and unresectable disease was 43.2 and 5.6 months respectively ($P < 0.0001$). The corresponding figures in Era 2 were 32.9 and 8.4 months ($P < 0.0001$). Survival of patients with resectable disease did not differ significantly in the two periods but there was a significant improvement in survival of patients with unresectable disease in Era 2. Their 1-, 3- and 5-year survival rates in Era 1 vs. Era 2 were 11% vs. 38%, 6% vs. 9%, and 3% vs. 4%, respectively ($P = 0.041$). The corresponding figures in their counterparts were 90% vs. 86%, 80% vs. 46%, and 40% vs. 30%, respectively ($P = 0.443$). Whenever possible, metastasectomy for pulmonary metastases of HCC should be offered to medically fit patients.

CONCLUSION

Although huge and advanced HCC is deadly, surgical treatment in properly selected patients is still feasible with acceptable risks. In recent years, there are revolutionary changes in surgical techniques together with new strategies to enhance the resectability of this fatal disease. Ways to increase FLR and improvements in surgical techniques allow more patients to benefit from surgical resection even in the presence of cirrhosis and major vascular invasion. Adjunctive use of radiofrequency ablation for bilobar involvement and selective use of metastasectomy for extrahepatic metastasis have been shown to be effective. Optimal

treatment modalities are still evolving. ALPPS will continue to be developed and more long-term results will be available in the near future.

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Authors' contributions

K.S.H. Chok contributed solely to this paper.

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Conflicts of interest

The author has no conflicts of interest with regard to the study or its publication.

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Not applicable.

Ethics approval

Not applicable.

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Outcomes of emergency and interval hepatectomy for ruptured resectable hepatocellular carcinoma: a single tertiary referral centre experience

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ABSTRACT

Article history:

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Key words:

Hepatocellular carcinoma,
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Aim: The short and long term outcomes of patients who underwent emergency and interval hepatectomy for ruptured and resectable hepatocellular carcinoma (HCC) were analysed. **Methods:** The data of patients with ruptured HCC presenting between April 2004 and October 2015 were analysed. Emergency hepatectomy was defined as hepatectomy within 48 h of the clinico-radiological diagnosis of HCC rupture. **Results:** Thirty patients underwent hepatectomy for ruptured HCC. Nine (30%) patients underwent emergency hepatectomy. The median age was 56 and 54 years ($P = 0.13$) with a similar gender distribution. The mean HCC size (10.5 vs. 8.3 cm, $P = 0.17$), total blood loss (3,000 vs. 850 mL, $P = 0.002$) and total units of red blood cell transfusion (1.9 vs. 0.5 units, $P = 0.27$) were greater in the emergency hepatectomy group. The complication rate was 44% and 38% ($P = 0.53$), with median length of hospital stay of 10 and 12 days ($P = 0.07$) in the emergency and interval hepatectomy groups, respectively, and no 30-day mortality in both groups. The median overall survival was 29 and 15.7 months ($P = 0.25$), with survival rates of 78%, 45%, 0% and 85%, 43% and 5% at 1, 3 and 5 years in the emergency and interval hepatectomy groups, respectively. **Conclusion:** Hepatectomy should be considered for ruptured HCC provided the patient could tolerate curative resection.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth commonest malignancy globally.^[1] Rupture of HCC is the third commonest presentation of this condition, with an incidence of 3-15% and an associated in-hospital mortality of up to 75%.^[2-4] The pathogenesis of HCC

rupture includes increased pressure within the tumour, rapid tumour growth or necrosis. This situation might be exacerbated by the presence of liver cirrhosis with concurrent thrombocytopenia and coagulopathy.^[5]

The treatment for ruptured HCC is determined by the haemodynamic stability of the patient.^[6] In the



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presence of haemodynamic stability, non-operative management with close monitoring is gold standard care.^[7] However, when there is haemodynamic instability, several treatment options are available. These include non-operative procedures such as transarterial embolisation or absolute alcohol injection, and surgical intervention (perihepatic packing, hepatic artery ligation, suture ligation, radiofrequency ablation or hepatic resection).^[8-12] Despite the multiple treatment options for ruptured HCC, there remains no consensus on the optimal approach for these patients.

The advantage of one stage emergency liver resection is the spontaneous control of haemorrhage with definitive management of the HCC. Emergent operation can also reduce the duration of peritoneal seeding of ruptured HCC tumour cells by lavage with water at operation.^[13,14]

However, emergent operative intervention must be balanced against the high mortality rate of up to 40%^[15] consequent to the lack of pre-operative objective assessment of functional liver reserve and extent of disease burden, hypovolaemic shock condition and coagulopathy.^[16]

As a result, the alternative option of staged liver resection after initial haemorrhage control with trans-arterial embolisation (TAE) is offered in some centres. This allows for subsequent assessment of functional liver reserve and operation under elective circumstances. The success of TAE haemostasis is 50-100%, with a risk of liver failure of up to 33%. Additionally, the 30-day mortality after TAE is lower compared to emergent hepatectomy (0-9% vs. 0-37%).^[16]

The survival benefits of two-stage liver resection over emergent hepatectomy remain controversial. Liu *et al.*^[15] concluded that survival after two-stage liver resection post-HCC rupture was inferior compared to patients who did not have this complication, whereas Yeh *et al.*^[17] found that ruptured HCC had similar overall survival rates compared to non-ruptured HCC but inferior disease-free survival rates. Mizuno *et al.*^[18] noted that there was no difference in overall survival between ruptured and non-ruptured HCC.

In this retrospective single-centre study, the short and long term outcomes of patients who underwent emergency and interval hepatectomy for ruptured and resectable HCC were analysed.

METHODS

Patients with a diagnosis of ruptured HCC presenting between April 2004 and October 2015 to our hospital

were retrieved from the in-house prospectively maintained hepatectomy database. The clinical data of these patients were collected and analysed retrospectively. In addition, the hepatectomy histopathology results were reviewed to confirm HCC rupture.

Due to the prospective nature of the database, some patients had just undergone hepatectomy and had not had sufficient follow-up period so were excluded for data analysis. Patients with intra-operative findings of incidental peri-tumoural haematoma suggestive of previously ruptured HCC were excluded. Emergency hepatectomy was defined as liver resection within 48 h of the clinical or radiological diagnosis of HCC rupture. Some patients were referred to our hospital after haemodynamic stabilisation at the parent hospital using TAE. These patients were included in the emergency hepatectomy group if they proceeded to liver resection within 48 h of first presentation of HCC rupture.

TAE was performed by experienced interventional radiologists with selective cannulation and then embolisation of the tumour-feeding artery with gel-foam particles. Surgical intervention was indicated when TAE failed to achieve adequate haemostasis. An experienced team of hepato-biliary surgeons performed hepatectomy. Hepatic parenchymal transection was undertaken using an ultrasonic dissector and TissueLink (Medtronic, Ireland) radiofrequency dissector. Intermittent Pringle manoeuvre might be applied during hepatectomy. The clinical decision algorithm for ruptured HCC as utilised in the author's institution is shown in **Figure 1**.

Post-operative follow-up of hepatectomy included ultrasound at 3 months and contrast triphasic computed tomography (CT) at 6 months with 3-monthly monitoring of serum alpha-fetoprotein and liver function test for 2 years, then 6-monthly thereafter. Supplementary CT was done in the presence of raised serum alpha-fetoprotein or suspicion of HCC recurrence on ultrasound. Recurrent HCC was diagnosed with radiological imaging (CT or positron emission tomography CT) to identify the location of intra-hepatic recurrence, tumour disease burden and the presence of extra-hepatic disease recurrence. Treatment options for recurrent HCC included further liver resection, local ablation therapies, transarterial chemo-embolisation (TACE), external beam radiotherapy, systemic chemotherapy or targeted immunotherapy. A multi-disciplinary team decided on treatment, taking into account the patients' liver functional status, recurrence pattern and comorbidities.

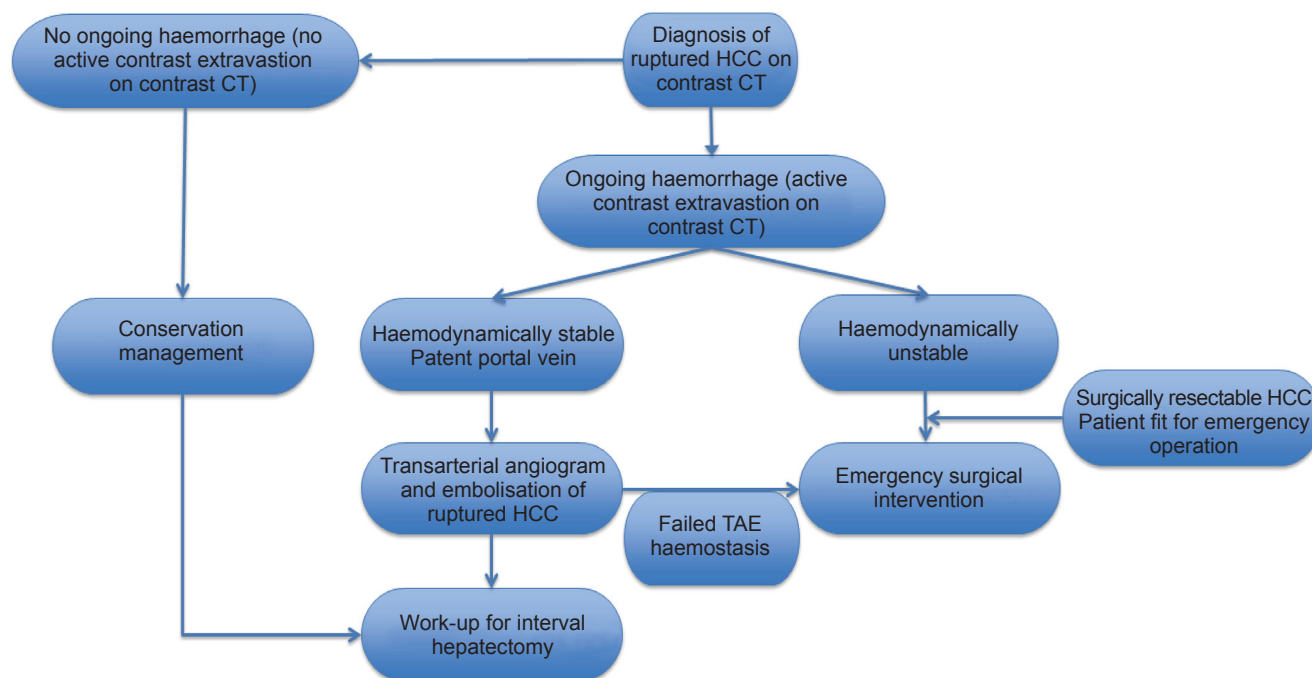


Figure 1: Algorithm for the management of ruptured HCC. HCC: hepatocellular carcinoma; TAE: trans-arterial embolisation; CT: computed tomography

Statistical analysis was performed with independent *t* test for continuous variables and chi-square test was used to compare discrete variables. Kaplan-Meier analysis was used to estimate overall survival between emergency and interval hepatectomy groups. Overall survival was defined as the time from hepatectomy until death from any cause, or until the observation period was completed. Survival data were censored on November 7th, 2015. Statistical significance was defined as a *P* value < 0.05 and statistical calculations were performed on SPSS 22 software (IBM).

RESULTS

Preoperative demographics

Thirty patients underwent hepatectomy for ruptured resectable HCC. Nine (30%) patients underwent emergency hepatectomy with a median time to operation of 0 days (range 0-2). For interval hepatectomy, median time to operation was 19 days (range 3-49). The median age for patients who underwent emergency hepatectomy was 56 years compared to 54 years in the interval hepatectomy group (*P* = 0.13). There was a similar distribution of male patients in both groups (89% vs. 90%, *P* = 0.66). The pre-operative haemoglobin (10.1 vs. 12.0, *P* = 0.07) and platelet count (171 vs. 220, *P* = 0.11) were lower and creatinine was worse (102 vs. 87, *P* = 0.32) in the emergency hepatectomy group but this did not reach statistical significance. There were no significant

differences in pre-operative international normalized ratio and bilirubin levels between the two groups.

Eight (89%) and 18 (90%) patients in the emergency and interval hepatectomy groups were hepatitis B virus positive, respectively (*P* = 0.66). There was more severe liver dysfunction in the emergency hepatectomy group, with higher pre-operative Child-Pugh grade (*P* = 0.04, Table 1).

Five (56%) patients underwent pre-operative TAE in the emergency hepatectomy group compared to 10 patients (48%) in the interval hepatectomy group (*P* = 0.5). Two patients in the emergency group had failed embolisation due to small collateral vessels, whereas 4 patients had unresponsive shock despite adequate fluid resuscitation and proceeded to emergency hepatectomy without prior TAE.

Operative characteristics

In the emergency hepatectomy group, all patients underwent anatomical resection (5 left lateral sectionectomies, 2 left hepatectomies and 2 right hepatectomies) compared to 15 (76%) in the interval group (3 left lateral sectionectomies, 2 left hepatectomies, 9 right hepatectomies and 1 caudate lobectomy) (*P* = 0.07). The mean HCC tumour size was larger (10.5 vs. 8.3 cm, *P* = 0.17) in the emergency hepatectomy group.

The mean operative time for liver resection in the

Table 1: Patient demographics and intraoperative characteristics, expressed as means with standard deviation or median with range

	Emergency hepatectomy (n = 9)	Interval hepatectomy (n = 21)	P value
Male	8 (89%)	18 (90%)	0.66
Age (years)	56 ± 15	54 ± 10	0.13
ASA at time of operation			0.19
1	-	1	
2	4	13	
3	2	6	
4	3	1	
Pre-operative haemoglobin (g/L)	10.1 ± 3.5	12.0 ± 2.0	0.07
Platelet count	171 ± 67	220 ± 71	0.11
INR	1.3 ± 0.5	1.1 ± 0.2	0.10
Bilirubin	16 ± 13	15 ± 11	0.78
Creatinine	102 ± 43	87 ± 33	0.32
Hepatitis B carrier	8 (89%)	18 (90%)	0.66
Hepatitis C carrier	1 (11%)	1 (5%)	0.93
AFP	2,790 (2-23,400)	3,220 (2-32,500)	0.89
Child-Pugh A cirrhosis	4	17	0.04
Child-Pugh B cirrhosis	3	4	
Child-Pugh C cirrhosis	2	0	
Prior trans-arterial angiogram and embolisation	5 (56%)	10 (48%)	0.50
Failed embolisation of ruptured HCC	2 (22%)	-	
Time from diagnosis of rupture HCC to liver resection (days)	0 (0-2)	25 (3-49)	< 0.05
Anatomical resection	9 (100%)	15 (71%)	0.07
Operative time (min)	200 ± 71	276 ± 83	0.02
Blood loss: skin incision to start of hepatectomy (mL)	1,900 ± 1,130	390 ± 250	0.012
Start to finish of hepatectomy (mL)	630 ± 490	305 ± 250	0.06
Total operative blood loss (mL)	3,000 ± 1,500	850 ± 440	0.002
Blood transfusion post-op (units)	1.3 ± 0.5	1.3 ± 0.5	0.86
Total blood transfusion (units)	1.9 ± 3.6 (0-11)	0.5 ± 0.8 (0-2)	0.27
Duration of drain placement (days)	5 (3-10)	4 (3-11)	0.83
Cirrhosis	6 (67%)	15 (71%)	0.10
Ishak liver cirrhosis scores (0-6)	4 ± 2 (1-6)	5 ± 2 (0-6)	0.31
Tumour size (cm)	10.5 ± 4.3	8.3 ± 3.8	0.17
Number of tumour lesions			0.58
1	6	14	
2	-	3	
3	1	1	
4	-	1	
> 5	2	2	
Micro-vascular invasion	6 (67%)	11 (52%)	0.52
Resection margin (cm)	1.3 ± 1.1 (0.5-4.0)	1.7 ± 1.1 (0-4.5)	0.47

ASA: American Society of Anesthesiologists; INR: international normalized ratio; AFP: alpha-fetoprotein; HCC: hepatocellular carcinoma

emergency and interval hepatectomy groups were 200 ± 71 and 276 ± 83 min respectively ($P = 0.02$). Total blood loss (3,000 vs. 850 mL, $P = 0.002$) and the mean total units of red blood cell transfusion (1.9 vs. 0.5 units, $P = 0.27$) were greater in the emergency hepatectomy group [Table 2].

Post-operative outcomes

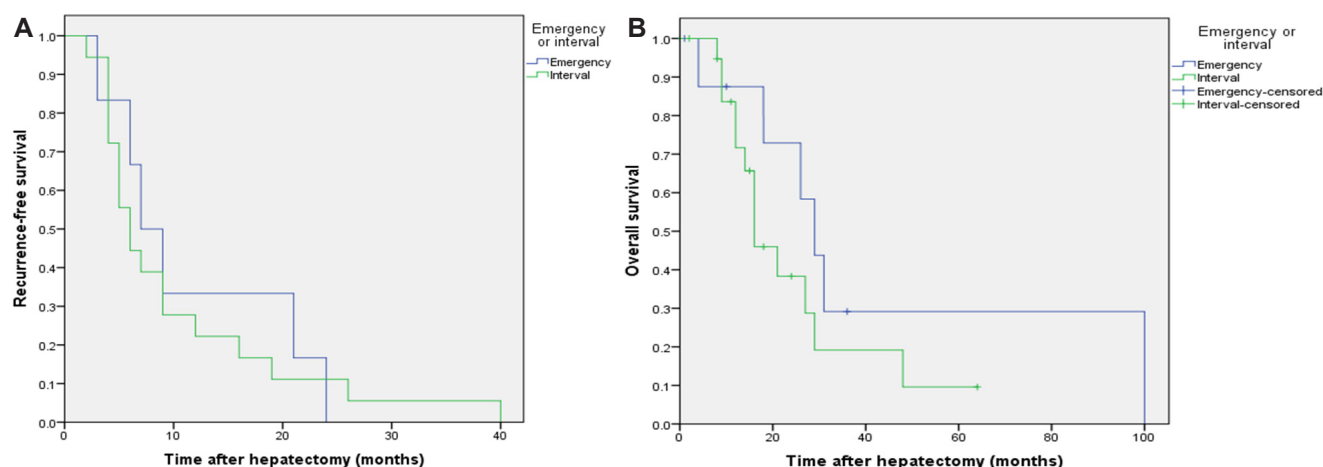
The post-operative complication rate was 44% and 38% in the emergency and interval hepatectomy groups respectively ($P = 0.53$). One patient in the interval hepatectomy group required pigtail drainage of pleural effusion. The median total length of hospital stay was 10 and 12 days respectively ($P = 0.07$) with no 30-day mortality in both groups [Table 2].

The median time to intra-hepatic recurrence was 7.8 months in the emergency hepatectomy group and 5.0 months in the interval hepatectomy group ($P = 0.12$). The median time to extra-hepatic recurrence was 6.8 and 9.7 months ($P = 0.59$), to earliest recurrence was 6.8 and 5.6 months ($P = 0.74$, Figure 2A) and overall survival was 29 and 15.7 months ($P = 0.25$, Figure 2B) respectively. Survival rates were 78%, 45%, 0% and 85%, 43% and 5% at 1, 3 and 5 years in the emergency and interval hepatectomy groups respectively [Table 2].

In the present study, patients who underwent emergency hepatectomy had more pulmonary recurrence (33% vs. 19%) compared to the interval group at follow-up. Additionally, the time to intra-hepatic recurrence

Table 2: Post-operative outcomes and long-term follow-up data for post-hepatectomy patients, expressed as medians with range

	Emergency hepatectomy (n = 9)	Interval hepatectomy (n = 21)	P value
Complications			
Wound infection	2	2	0.35
Pleural effusion	2	3	0.60
Pleural effusion requiring drainage	0	1	0.51
Confusion	0	1	0.12
Ascites	0	1	0.51
Total	4 (44%)	8 (38%)	0.53
Median hospital stay after hepatectomy (days)	10 (5-17)	12 (6-32)	0.07
30 day mortality rate	0	0	-
Time to intra-hepatic recurrence (months)	7.8 (2.6-100)	5.0 (1.1-39.5)	0.12
Time to extra-hepatic recurrence (months)	6.8 (6.4-8.9)	9.7 (4.0-47.9)	0.59
Peritoneal recurrence	1 (11%)	6 (29%)	0.27
Time to peritoneal recurrence (months)	6.4	6.4 (4.0-10.1)	0.55
Pulmonary recurrence	3 (33%)	4 (19%)	0.44
Time to pulmonary recurrence (months)	6.8 (6.4-8.9)	7.9 (4.0-12.2)	0.06
Recurrence in other location	0	4 (33%)	0.15
Time to other location recurrence (months)	-	11.7 (10.1-47.8)	
Time to earliest recurrence (months)	6.8	5.6	0.74
Overall survival (months)	29 (4-100)	15.7 (8-49)	0.25
1-year overall survival	7/9 (78%)	18/21 (85%)	0.59
3-year overall survival	4/9 (45%)	9/21 (43%)	0.94
5-year overall survival	0/9 (0%)	1/21 (5%)	0.51

**Figure 2: (A) Recurrence-free survival after emergency and interval hepatectomy for ruptured hepatocellular carcinoma ($P = 0.74$, log rank test); (B) overall survival after emergency and interval hepatectomy for ruptured hepatocellular carcinoma ($P = 0.25$, log rank test)**

was longer, but extra-hepatic recurrence shorter in the emergency hepatectomy group. Overall median survival time was longer in the emergency group (29 vs. 15.7 months, $P = 0.26$) but overall 1-, 3- and 5-year survival rates were similar in both groups.

DISCUSSION

Rupture of hepatocellular carcinoma (HCC) is a rare but life-threatening complication of HCC, and is associated with a high mortality rate (up to 75%) in the acute phase due to a combination of hypovolemic shock, coagulopathy and subsequent hepatic failure.^[19,20]

The risk factors for HCC rupture are multifactorial, and include rapid tumour growth with necrosis, vessel erosion or venous thrombosis by tumour cells.^[16,21] Additionally, left lobe tumours might be more inclined to rupture due to the smaller anatomical span of the left lobe.^[22]

Bassi *et al.*^[23] commented that rupture of HCC which were located at the free surfaces of the liver can result in bleeding into the peritoneal cavity due to the lack of hepatic parenchyma covering the tumour. Kanematsu *et al.*^[24] showed that tumour protrusion was a risk factor for its subsequent rupture, whereas

Li *et al.*^[25] identified tumours located in segments II, III and VI to be associated with its rupture. Furthermore, tumour rupture can occur in both large and small HCCs.^[8] Chan *et al.*^[26] found that ruptured HCC was associated with more aggressive disease compared to non-ruptured HCC as evidenced by higher tumour marker titres, higher rates of micro-vascular invasion and tumour multifocality. Zhu *et al.*^[5] found that tumour size > 5 cm, hypertension, liver cirrhosis, vascular thrombus and extra-hepatic invasion were predictive of spontaneous HCC rupture on multivariate analysis.

In the present study, emergency hepatectomy was defined as liver resection within 48 h of the clinical or radiological diagnosis of HCC rupture. In the published literature, there are no guidelines on the optimal time for emergency operative intervention for ruptured HCC. Whilst an arbitrary method to distinguish hepatectomy into same admission liver resection (emergency group), and hepatectomy during second hospitalization (elective) is valid and clinically practical, we undertook this subgroup analysis and found that there was considerable overlap between emergency and interval hepatectomy groups in terms of the time interval from onset of ruptured HCC to liver resection (data not shown). However, the use of the 48-h time interval resulted in eliminated this overlap.

The indications for emergency hepatectomy comprised of patients with CT confirmed ruptured HCC that presented with hypovolaemic shock, which was refractory to adequate fluid resuscitation and with failed trans-arterial angiogram and embolization of the ruptured HCC. Patients who remained haemodynamically unstable for angiogram were transferred to the operating room. The liver function and CT were assessed for feasibility of safe and curative hepatectomy prior to proceeding with emergency operation.

The patients who underwent emergency hepatectomy had worse preoperative Child-Pugh grade, larger tumour size, greater operative blood loss and blood transfusion requirements and higher rates of anatomical resection but shorter operative times compared to the elective hepatectomy group. In the post-operative period, the complication rate was higher in the emergency group (44% vs. 38%) but there were no 30-day mortality or requirement for re-operative intervention in both groups. Emergency hepatectomy for ruptured HCC in patients with Child-Pugh C cirrhosis is associated with significant peri-operative mortality as reported in other case series.^[20,23,27] but in this present study, 2 patients with Child-Pugh C cirrhosis underwent emergency hepatectomy without 30-day mortality. The favourable

post-operative outcomes might be related to the short operation time (mean 146 min), small transection area (mean 35 cm²) and no pre-operative angiogram and embolisation.

The main objective of ruptured HCC treatment is haemorrhage control whilst preserving as much functional liver tissue as possible.^[6,28,29] The management of ruptured HCC is challenging and multiple treatment options are available, dependent on the clinical condition and haemodynamic stability of the patient.^[7]

TAE is the preferred method for non-operative haemostasis of ruptured HCC.^[20,30] TAE can function as definitive palliative therapy or act as a bridge to interval hepatectomy.^[23,27,31] However, whilst TAE may achieve haemostasis of the tumour haemorrhage, there are risks of re-bleeding, liver abscess and this intervention cannot treat the tumour cells that have seeded the peritoneal cavity.^[32] Surgical intervention for ruptured HCC is indicated when haemostasis with TAE has been unsuccessful.^[16]

Yang *et al.*^[33] reviewed the outcomes of 132 patients with ruptured HCC, of which 17 patients underwent emergency hepatectomy and 11 patients had TAE then interval hepatectomy. There were no 30-day mortality and 1-year survival rates were 56.3% and 63.6% respectively. The median overall survival was 13.0 and 14.6 months. In the present series, 1-year survival was 78% and 85%, with overall median survival of 29 months in the emergency hepatectomy group compared to 15.7 months in the interval group ($P = 0.25$).

Zhang *et al.*^[29] reported on the impact of interval hepatectomy or repeat TACE after successful TACE for ruptured HCC. One hundred and twenty-six cases of ruptured HCC underwent TAE for haemostasis of which 74 had interval hepatectomy. The 90-day mortality rate was 6.8% in the hepatectomy group and 7.7% in the TACE group ($P = 0.84$), all of whom died from tumour recurrence. The 1-, 3-, 5-year survival rates were 85.1%, 63.5% and 37.8% in the hepatectomy group compared to 69.2%, 46.2% and 17.3% in the TACE group ($P = 0.004$).

Dissemination of ruptured HCC tumour cells into the peritoneal cavity is one argument for proponents of emergency hepatectomy for ruptured HCC.^[34,35] Zhang *et al.*^[29] reported an 11.8% incidence of peritoneal disease in their series of ruptured HCCs. In the present study, there was an 11% peritoneal recurrence rate in the emergency hepatectomy group compared to 29%

in the interval hepatectomy group ($P = 0.27$). The mean time to peritoneal recurrence was 6.4 and 6.4 months ($P = 0.55$) in the emergency and interval hepatectomy groups respectively. This 11% peritoneal recurrence rate was similar to that of hepatectomy for non-ruptured HCC as reported by Jianyong *et al.*^[36] In Chan *et al.*^[26] of interval hepatectomy for ruptured HCC, they found an intra-hepatic recurrence rate of 23.8% and extra-hepatic recurrence rate of 17.9% ($n = 77$). Additionally, peritoneal recurrence was 14.9% compared to 9.9% in a matched non-ruptured HCC group ($P = 0.5$). Hiraoka *et al.*^[37] found a peritoneal recurrence rate of 7.7% in their case series. Other researchers have also noted no increase in the incidence of peritoneal metastases after ruptured HCC.^[18,38] Moreover, there are reports to suggest that patients with peritoneal recurrence after hepatectomy for HCC have no prior evidence of HCC rupture.^[39] These results suggested that intra-peritoneal tumour cell implantation might not be a common event. Although peritoneal recurrence of HCC can be managed by radical surgical resection, in the present case series, all the patients with resectable peritoneal recurrence opted for non-surgical treatments.

In this study, the median time to extrahepatic recurrence was shorter in the emergency hepatectomy group, with no statistical difference in overall survival. There were no statistical differences in the tumour size, vascular involvement, resection margins or degree of cirrhosis, to explain the mechanisms for earlier extrahepatic recurrence in the emergency hepatectomy group (data not shown). Whether there is increased haematogenous spread of HCC tumour cells at the time of emergency compared to interval hepatectomy with subsequent extrahepatic seeding and HCC recurrence is a concept that this study cannot answer.

Yang *et al.*^[40] reported on the outcomes of 143 patients who underwent emergency ($n = 28$) or interval hepatectomy ($n = 115$) for ruptured HCC. Interestingly, they found that the recurrence-free survival (23%, 9% and 9% vs. 45%, 26% and 16% at 1, 3 and 5 years, $P = 0.025$) and overall survival (50%, 8% and 8% vs. 70.3%, 29.2% and 19.4% at 1, 3 and 5 years, $P = 0.016$) were worse in the emergency group. This data suggested that the ruptured HCC tumours were advanced at the time of presentation with probable micro-metastases. Although the median overall survival time was longer in the emergency group, the absolute numbers in this group were small which might skew the data and give a false survival advantage in the emergency group.

There were several limitations in this study. This was

a retrospective analysis of patients with ruptured and resectable HCC managed at a single tertiary referral centre. The absolute number of patients was low given the rarity of rupture HCCs, although all eligible patients for analysis were included. There was selection bias in determining which patients should proceed to interval hepatectomy for ruptured HCC with the prerequisite of satisfactory liver functional reserve and resectable HCCs with curative intent. The heterogeneous nature of patient and tumour characteristics was another potential source of bias. Furthermore, the departmental database focussed on patients who underwent hepatectomy, and consequently, the data and clinical outcomes for patients who had ruptured HCC but were not subjected to hepatectomy (i.e. managed with TAE only or best supportive care) cannot be retrieved for analysis.

In conclusion, this study showed the feasibility of emergency or interval hepatectomy for highly selected patients with ruptured and resectable HCC. Although patients in the emergency hepatectomy group had larger tumours, worse pre-operative Child's grading and greater intra-operative blood loss, the recurrence-free and overall survival rates were similar in both groups. Hepatectomy should be considered for ruptured HCC provided the patient could tolerate curative resection and have surgically resectable tumours.

DECLARATIONS

Authors' contributions

Data collection, compiling results, writing and producing the final manuscript: A.K.Y. Fung
Editing the manuscript drafts: C.C.N. Chong, K.F. Lee, J. Wong, Y.S. Cheung, A.K.W. Fong, P.B.S. Lai
Approved the final manuscript for submission: A.K.Y. Fung, C.C.N. Chong, K.F. Lee, J. Wong, Y.S. Cheung, A.K.W. Fong, P.B.S. Lai

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Conflicts of interest

There are no conflicts of interest.

Patient consent

The data obtained through the medical record review were managed according to the privacy policy and ethics code of our institute.

Ethics approval

This was a retrospective study and did not require Institutional Review Board approval.

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Total regression of hepatocellular carcinoma bone metastases, after liver transplantation, with sorafenib-everolimus

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ABSTRACT

Hepatocellular carcinoma (HCC) represents the 5th commonest malignancy worldwide. Liver transplantation consist a radical and most efficient treatment for HCC. Tumor recurrence or metastases after liver transplantation is not uncommon. Hereby is presented a case of a patient transplanted for alcoholic liver disease and HCC and presented with bone metastases a few months later. Treatment with sorafenib and everolimus showed full regression of the metastases. In conclusion, the point of this report is to advertise a single case of total regression of bone lesions due to HCC recurrence, with the combination of mammalian target of rapamycin and sorafenib, along with radiation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) consists a health-care problem with rising incidence rates.^[1] Liver transplantation (LT) is a radical treatment for HCC. In order a patient with HCC to receive a liver graft, he must fulfill certain enlisting criteria^[2] and no extrahepatic disease. Tumor recurrence or metastases after liver transplantation for HCC is not uncommon. Sorafenib has been used as rescue therapy in patients with

recurrent HCC after LT.^[3] We hereby present a case of a patient transplanted for alcoholic liver disease (ALD) and HCC that presented with bone metastases a few months later. He was treated with sorafenib and everolimus, showed full regression of the metastases and he is still alive 9 years after LT.

CASE REPORT

A 63-year-old male patient was enlisted for LT for



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liver cirrhosis due to ALD. He was presenting portal hypertension, ascites and episodes of encephalopathy. His model for end-stage liver disease (MELD) score was 21. He was transplanted with piggy-back technique, from a heart-beating donor. Cold ischemia time was 9 h. He was put on triple immunosuppression maintenance therapy with prednisolone, mycophenate mofetil and cyclosporine.

The explant's pathology report revealed the presence of two incidental HCC lesions measuring 15 and 20 mm, with no portal involvement, of medium differentiation, with pseudocapsule, clear-cell type, without extrahepatic nodules or other findings. The post-operative course was uneventful. His immunosuppression therapy was changed to tacrolimus and everolimus, along with tapering of prednisolone. Tacrolimus and everolimus levels were monitored.

Two months post transplantation the patient complained of back pain. Bone Scanning 99m Technetium helix destabilizing protein (99mTc-HDP) revealed 2 osteoblastic lesions on the T8 and T11, possibly secondary-HCC lesions. Prednisolone was ceased and sorafenib 400 mg bid was initiated, along with ibandronic acid (diphosphonic acid) qd. Radiotherapy was induced, photons 60Co. He received a total of 2,300 centigray (cGy), in doses of 46 cGy, 5 times/week.

Otherwise the patient was in good condition. His kidney function with radioisotope renography with 99m Technetium diethylene triamine pentaacetic acid (99mTc-DTPA) was 52 mL/min/1.73 m². Alpha fetoprotein (AFP) level was 6.6 ng/mL.

Seven months after the first discovery of the spinal osteoblastic lesions, the repetition of the 99mTc-HDP, revealed further progress of the disease [Figure 1]. New lesions were being detected at the 5th, 6th, 7th,

and 8th left ribs. Magnetic resonance imaging (MRI) scan failed to reveal any additional findings. Therapy remained the same.

Another 99mTc-HDP bone scanning 18 months post LT showed, for the first time, regression of the rib lesions, while the known 2 spinal lesions were significantly minimized. Therapy remained unaltered. Patient's clinical condition was excellent.

Finally, 28 months post LT, a new bone scanning certified the complete regression of all the osteolytic lesions [Figure 2].

DISCUSSION

HCC is the third cause of cancer related mortality nowadays, according to World Health Organization (WHO). The primary etiologic factor is liver cirrhosis. To the present case, HCC was incidental finding in the explant. A prior transplantation computed tomography (CT) failed to detect the presence of liver or extrahepatic lesions. Additionally, AFP levels were low [Table 1], failing to justify a position emission tomography (PET) scan preoperatively. Even if the patient was evaluated for Milan Criteria (MC), according to the explants' pathology, the patient would be inside MC. Moreover, piggy-back technique is the standard LT procedure performed by our center, like many other centers universally. It does not consist a risk factor for HCC recurrence, compared to the classic technique.

The induction of sorafenib, an oral multi-kinase inhibitor, targeting HCC control, demands compensated liver function, and applies to patients with advanced HCC.^[4] Regarding HCC recurrence post LT, the current strategy remains controversial. Recurrence can be

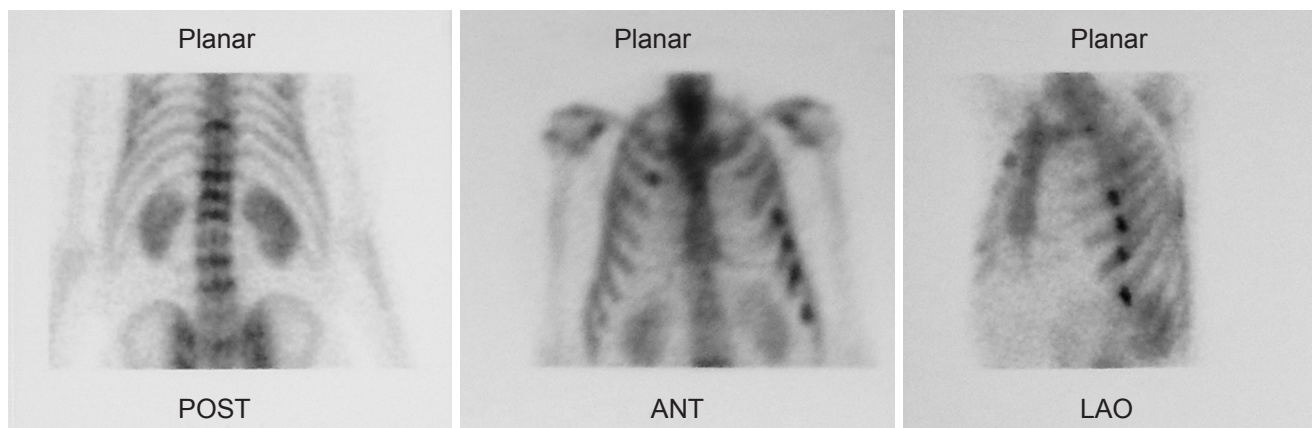


Figure 1: Initial 99m Technetium helix destabilizing protein scintigraphy showing metastatic lesions in the spine and ribs. POST: posterior; ANT: anterior; LAO: left anterior oblique

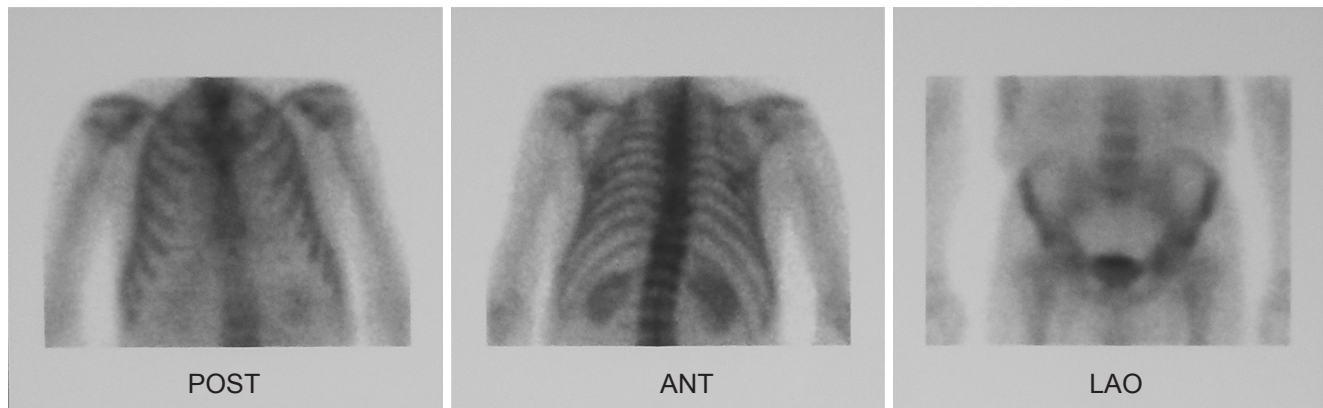


Figure 2: 99m Technetium helix destabilizing protein scintigraphy post sorafenib, immunosuppression modification and radiation, showing regression of metastatic lesions in the spine and ribs. POST: posterior; ANT: anterior; LAO: left anterior oblique

located to liver graft, lung, bone, abdominal lymph nodes, adrenal glands and peritoneum. According to De Angelis *et al.*,^[5] on a total of 61 studies selected, the median time recurrence presented was 13 months post transplantation, while 67% of patients presented with extra-hepatic lesions.^[5] Overall survival was 12.97 months.

A classification of management according to the location of the recurrence has been attempted, recently. Toso *et al.*^[6] when HCC reappear, underline the importance of immunosuppression change to mTOR and propose initiation of sorafenib only for non-resectable multiple lesions, or cases that cannot profit of less invasive strategies, like radio frequency ablation or transarterial chemo embolization. If the recurrence is limited in the liver, without extrahepatic spread, local excision should be attempted.^[7] The initiation of sorafenib finds place in cases of advanced HCC, when no other approach is plausible.

Table 1: Patient data, before and after liver transplantation

Characteristics	Preoperative	30 days post-liver transplantation
MELD score	21	-
Child-Pough stage	C (10 points)	-
AFP (ng/mL)	2.8	5.2
CA 19-9 (ng/mL)	25.47	5.2
CEA (ng/mL)	3.46	1.58
PLT (K/ μ L)	66,000	109,000
INR	1.96	0.89
AST (U/L)	36	22
ALT (U/L)	20	29
ALP (U/L)	88	64
BIL (mg/dL), total/direct	7.0/3.3	1.0/0.23
γ GT (U/L)	20	46

MELD: model for end-stage liver disease; AFP: alpha fetoprotein; CA: carbohydrate antigen; CEA: carcinoembryonic antigen; PLT: platelets; INR: international normalized ratio; AST: aspartate transaminase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; BIL: bilirubin; GT: glutamyl transpeptidase

This plan though, is not free of complications. Discontinuation of the sorafenib treatment due to adverse side effects is not uncommon.^[8] The induction of radiation as an only and palliative treatment, is not improving the survival rates. In the study of Seong *et al.*,^[9] 51 patients received radiation therapy for 77 osteolytic metastatic lesions. Though there was pain relief in 56 lesions (73%), 1-year survival was only 15%. In the review of He *et al.*,^[10] radiation therapy for bone lesions, due to HCC post LT, improves patients' quality of life. It has the same impact for transplanted patients, as in non-transplanted.

Vanishing of osteolytic lesions, to a patient under treatment with sorafenib-mToR, after radiation, has not been reported so far, to our knowledge. Though no biopsy was being done, we are convinced on the malignancy: the pain and imaging spread was distinctive, and they disappeared after treated like bone recurrence.

The patient had an interesting natural history of the disease. HCC was an incidental finding of explants' pathology. Metastasis to the bones was an unexpected event, since tumors were small and AFP was low. Nevertheless the patient presented with spine and later with ribs metastases. Oncologically he was treated primary with sorafenib and secondary with mTOR drug inhibitor everolimus, along with radiation. His immunosuppression therapy was revised accordingly.

Our patient discontinued sorafenib 4 years ago, due to coronary disease. He remains under mTOR treatment, without new HCC recurrence [Figure 3].

DECLARATIONS

Authors' contributions

Concept: D. Giakoustidis, V. Papanikolaou

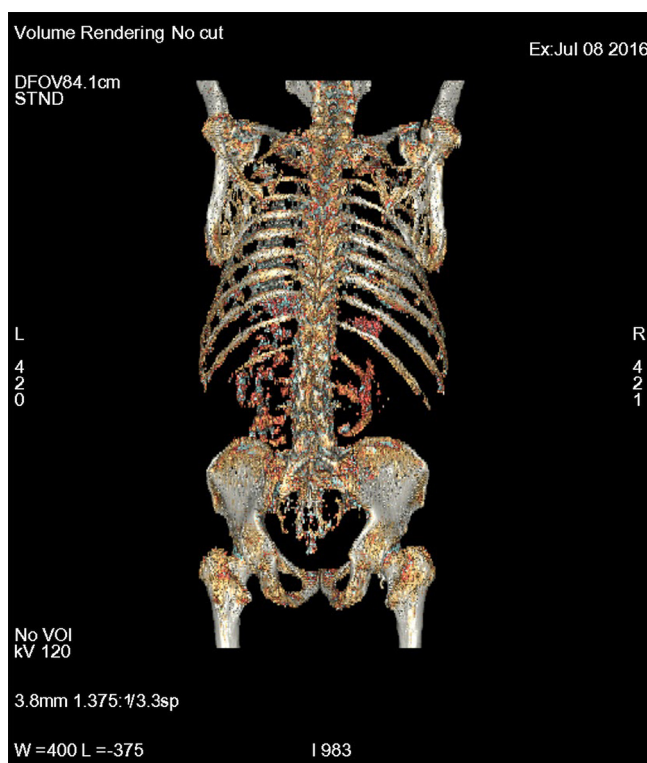


Figure 3: Recent 18-FDG-PET/CT (Volume rendering) showing no lesions in ribs or spine. FDG: fluorodeoxyglucose; PET: position emission tomography; CT: computed tomography

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Conflicts of interest

There are no conflicts of interest.

Patient consent

The patient has provided us an informed written consent, available upon request.

Ethics approval

An ethics approval is not necessary for a case report; the informed written consent is sufficient.

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Clinical outcomes of direct-acting antiviral therapy in patients with compensated hepatitis C virus-related cirrhosis

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Direct-acting antiviral therapy, compensated cirrhosis, hepatocellular carcinoma, clinical decompensation

ABSTRACT

Aim: The aim was to assess the clinical impact of direct-acting antiviral treatment in patients with compensated hepatitis C virus-related cirrhosis after one year of follow-up. **Methods:** An observational retrospective study was conducted on 129 consecutive patients with compensated cirrhosis treated in 2015, analyzing the evolution of liver function and the development of hepatocellular carcinoma and clinical decompensations. **Results:** The median follow-up time was 16 months. Most patients were males (73%), the mean age was 58.1 years and the most frequent genotype was 1b (52.2%). All participants were Child-Pugh A class at the start of the treatment and the median model for end-stage liver disease (MELD) score was 7. Four patients (4.4%) suffered a decompensation: three episodes of ascites and one acute on chronic liver failure. The incidence of *de novo* hepatocellular carcinoma during the follow-up was 3.6%. Seven patients (7.8%) improved MELD score more than one point and in 11 patients (12.2%) it worsened more than one point. There was a significant improvement in the mean platelets count [$P < 0.001$, 95% confidence interval (CI): -26,360, -12,096] and in the mean albumin levels ($P < 0.001$, 95% CI: -322, -130) after treatment. **Conclusion:** Direct-acting antiviral treatment is not associated in the short term with a decrease in the development of hepatic decompensation or hepatocellular carcinoma compared to what it was reported for untreated compensated cirrhotic patients. There is an improvement in pre and post-treatment platelet counts and albumin levels showing a probable improvement of the hepatic function.

INTRODUCTION

Chronic hepatitis C virus (HCV) infection affects around 160 million people worldwide^[1]. Around 16% of patients will develop cirrhosis after 20 years of infection^[2], although fibrosis progression can vary due

to several factors such as age, alcohol consumption or hepatitis B or human immunodeficiency virus (HIV) co-infection^[3]. Once cirrhosis is established, a yearly incidence of hepatocellular carcinoma of 1.4-3.4%^[4-6], and a yearly incidence of hepatic decompensation (including episodes of ascites, jaundice, hepatic



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encephalopathy or variceal bleeding) of 3.9-5.7%^[4,6] has been reported.

Before 2011, the best potentially curative treatment option for chronic HCV infection was pegylated interferon in combination with ribavirin. However, sustained virological response rates were reported to be as low as 33% in cirrhotic patients^[7], with an significant number of side effects^[8]. However, it has been demonstrated that the achievement of a sustained virological response after interferon-based therapy is associated with lower rates of hepatocellular carcinoma and with lower rates of hepatic decompensation^[9,10]. A regression of fibrosis after viral eradication has also been reported^[11].

The arrival of second-generation direct-acting antivirals improved the sustained virological response rates to more than 90%, even in compensated cirrhotic patients, with fewer side effects^[12,13]. However, the achievement of sustained virological response with this treatment does not appear to be associated with a decrease in the occurrence of hepatocellular carcinoma in the short term^[14]. It may even be associated with a higher rate of tumor recurrence than what it is expected^[15]. A recent prospective multicenter study did not find any evidence of an increased risk of hepatocellular carcinoma recurrence in patients treated with direct-acting antivirals^[16]. One report additionally demonstrated an improvement in liver function tests among patients with decompensated liver disease after treatment with oral antiviral therapy^[11].

The aim of our study was to assess the clinical impact of direct-acting antiviral treatment in terms of the evolution of liver function and in terms of the development of clinical decompensations and hepatocellular carcinoma in patients with compensated HCV-related cirrhosis after one year of follow-up.

METHODS

Data from all the patients with compensated HCV-related cirrhosis, without co-existent HIV or hepatitis B infection, who were treated at our center with direct-acting antivirals between January and October 2015, were retrospectively collected. At the end of October 2016, the database included 129 patients. We excluded 39 patients because they did not complete a follow-up of 12 months, which included at least physical examination, hepatic ultrasound, and blood tests every 6 months. Fourteen patients were lost to follow-up during the first year and 25 patients had the last clinic or ultrasound appointment after October 2016.

The diagnosis of cirrhosis was previously established

by biopsy, transient elastography (> 14.5 Kpa) or unequivocal clinical diagnosis (chronic HCV with previous episodes of decompensation or with imaging tests showing portal hypertension signs).

Sustained virological response 12 weeks post-treatment [sustained virological response 12 (SVR12)] was defined as undetectable HCV RNA at week 12 after the end of therapy. For HCV RNA detection we used real time polymerase chain reaction, with a limit of detection of 15 IU/mL.

Follow-up started the first day of treatment, which was defined as time 0. We analyzed: (1) the development of hepatocellular carcinoma. We performed an ultrasound every 6 months and, when it showed a suspicious focal lesion, the diagnosis of hepatocellular carcinoma was completed with a triple-phase computerized tomography scan and/or with a contrast enhanced magnetic resonance imaging; (2) the development of hepatic decompensation, which included jaundice, variceal bleeding, ascites and/or encephalopathy; (3) the evolution of liver function, using Child-Pugh and MELD scores, which were calculated on the first day of treatment and on the last clinic visit, at least one year later. We also performed a brief statistical analysis, using paired *t* test to compare means of baseline and follow-up platelet counts and bilirubin and albumin levels. A *P* value below 0.05 was considered statistically significant. The analysis was performed using IBM statistical product and service solutions statistics for Macintosh, version 21.0 (Armonk, NY, IBM Corp).

RESULTS

Baseline characteristics of patients

We analyzed data from 90 consecutive patients with compensated HCV-related cirrhosis who were treated with direct-acting antivirals between January and October 2015 and completed a follow up of at least one year after initiation of therapy.

The median follow-up time after initiation of direct-acting antiviral treatment was 16 months (12-21 months). Seventy-three percent of participants were males, the mean age was 58.1 years and the most frequent genotype was 1b (52.2%). Only 37.8% of patients were naïve, and 11% had liver graft cirrhosis. All patients were Child-Pugh A class at the start of the treatment and the median MELD score was 7 (6-16). At the initiation of therapy, mean bilirubin level was 1.06 ± 0.27 mg/dL, mean platelet count was $117,788 \pm 50,546/\text{mm}^3$, and mean albumin level was $4,140 \pm 424$ mg/dL. The baseline characteristics of the study population are shown in Table 1.

In terms of treatment, the most frequent combination was sofosbuvir/ledipasvir (35.5%) and 66.7% of patients also received ribavirin. Most participants (78.9%) were treated for 12 weeks. Six patients (6.7%) had relevant adverse effects: 3 patients treated with ribavirin developed moderate anaemia, which improved after lowering the dose; 1 patient referred severe asthenia; 1 patient developed a purpura which required corticosteroids and 1 patient suffered an acute on chronic liver failure which required discontinuation of therapy during the third week of treatment. Eighty-six out of 90 patients (94.6%) achieved SVR12. One patient died during the follow-up due to a metastatic hepatocellular carcinoma. There were no deaths due to unrelated liver causes.

Five patients had a history of hepatocellular carcinoma: 2 patients had one nodule smaller than 5 cm, 2 patients had 3 nodules smaller than 3 cm, and 1 patient had 2 nodules smaller than 2 cm. Three of them underwent liver transplantation between 2004 and 2013 and developed graft cirrhosis. The other 2 patients were treated first with direct-acting antivirals and underwent liver transplantation during the follow-up period.

Hepatocellular carcinoma

Five patients (5.5%) developed hepatocellular

carcinoma [Figure 1]. In one case, a suspicious lesion was detected before treatment and, during the follow-up, 2 nodules were confirmed with a triple-phase computerized scan, one of 4.8 cm and one of 1.5 cm, compatible with hepatocellular carcinoma. Another patient had a post-transplant recurrence in the form of lymphatic metastasis 15 months after initiation of therapy (without hepatocellular carcinoma in the liver graft). One patient developed a 4-cm nodule with portal vein thrombosis, one patient had a 3.3-cm nodule and the last patient developed multiple hepatic nodules and bone metastasis. Thus, 3 patients out of 84 (3.6%) developed *de novo* hepatocellular carcinoma. The median time between initiation of treatment and the diagnosis of liver cancer was 12 months.

Clinical decompensations

Four patients (4.4%) suffered an episode of hepatic decompensation during the year of follow-up [Figure 2]: 1 patient with non-malignant portal thrombosis developed ascites, 1 patient with a history of ascites developed an acute on chronic liver failure during the treatment, and 2 patients developed ascites coinciding with the diagnosis of hepatocellular carcinoma.

Evolution of liver function

Seven patients (7.8%) improved MELD score more than one point, 63 patients (70%) showed no differences or \pm one point and in 11 patients (12.2%) MELD score worsened more than one point. Two patients (2.2%) underwent liver transplantation during the follow-up and there was insufficient data to calculate MELD score in 7 patients (7.8%).

Table 1: Baseline characteristics of the study population

Characteristic	Data, n (%)
Follow-up (months), median (range)	16 (12-21)
Age (years), mean \pm SD	58 \pm 8.57
Gender: male	66 (73.3)
Treatment	
Naive	34 (37.8)
Experienced	56 (62.2)
Liver graft cirrhosis	10 (11.1)
Genotype	
1a	27 (30)
1b	47 (52.2)
2	1 (1.1)
3	11 (12.2)
4	4 (4.5)
Treatment	
Sofosbuvir/ledipasvir	32 (35.5)
Sofosbuvir/daclatasvir	24 (26.7)
Sofosbuvir/simeprevir	16 (17.8)
Paritaprevir/ritonavir/ombitasvir/dasabuvir	11 (12.2)
Simeprevir/interferon	4 (4.4)
Sofosbuvir/interferon	1 (1.1)
Ribavirin: yes	60 (66.7)
Weeks of treatment	
2	1 (1.1)
12	71 (78.9)
24	18 (20)
MELD score, median (range)	7 (6-16)
History of previous hepatocellular carcinoma	5 (5.6)
Albumin (mg/dL), mean \pm SD	4,140 \pm 424
Platelets (mm ³), mean \pm SD	117,788 \pm 50,546
Bilirubin (mg/dL), mean \pm SD	1.06 \pm 0.27

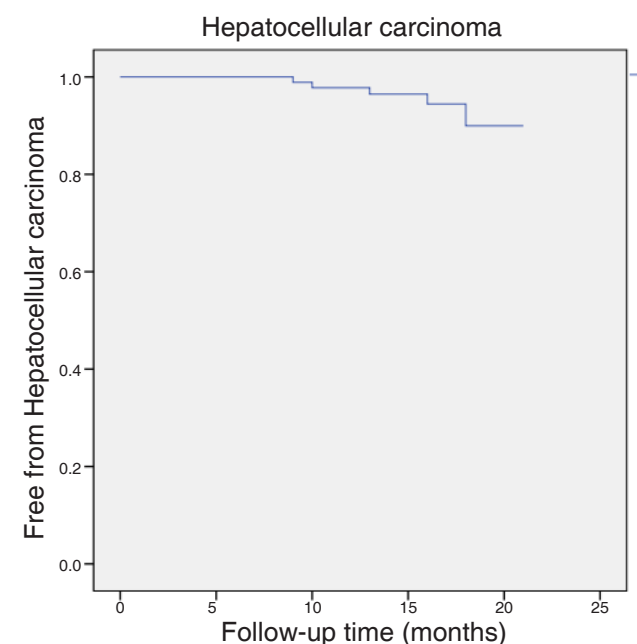


Figure 1: Kaplan Meier estimates of staying free of hepatocellular carcinoma after direct-acting antiviral treatment

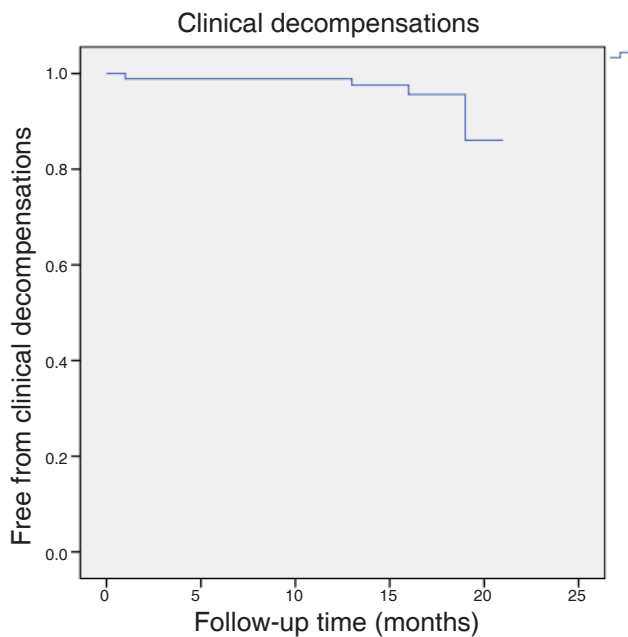


Figure 2: Kaplan Meier estimates of staying free of clinical decompensation after direct-acting antiviral treatment

Seventy-eight patients (86.7%) stayed in stage A of Child-Pugh score at the end of the follow-up and 3 patients (3.3%) worsened to Child-Pugh B class. Two patients (2.2%) underwent liver transplantation during the follow-up and there was insufficient data to calculate Child-Pugh stage in 7 patients (7.8%).

The statistical analysis showed a significant improvement in the mean platelet counts ($P < 0.001$, 95% CI: -26,360, -12,096) and in the mean albumin levels ($P < 0.001$, 95% CI: -322, -130) after antiviral treatment but not in the mean bilirubin level ($P = 0.74$, 95% CI: -0.70, 0.97).

DISCUSSION

It has been described that patients with advanced chronic liver disease who achieved sustained virological response with interferon-based treatments have a hepatocellular carcinoma annual rate as low as 1% [6], while for untreated patients it is around 3% [5,6]. In terms of hepatic decompensation, it has been described an annual rate of 1.4% for patients treated with interferon, in opposition to a 5.7% for untreated cirrhotic patients [6]. Although it is known that direct-acting antiviral therapy has changed the history of chronic HCV infection, achieving very high cure rates and an excellent safety profile [12], a recent prospective study has shown that the resolution of the infection with this treatment in cirrhotic patients does not seem to reduce the incidence of hepatocellular carcinoma in 24 weeks of follow-up [14]. Also, a recent

publication has suggested that there is a higher risk of tumor recurrence in patients with hepatocellular carcinoma treated with direct-acting antivirals [15], while this association was not found in another prospective study [16]. It is important to continue investigating this relationship, developing long-term follow-up studies, in order to clarify the effect that direct-acting antivirals may have on the development of hepatocellular carcinoma and clinical decompensations so that we can better understand and explain to our patients what they can expect after achieving sustained virological response.

In our study, the development of hepatocellular carcinoma in a median of 16 months of follow-up is 3.6%, as high as what it is expected for untreated cirrhotic patients [4,5]. Thus, there seems to be no benefits in the short term in this aspect. Undoubtedly, the study has many limitations as it is retrospective, it was performed in a single center, and it has a relatively small sample size. However, not much data have been published to date and the follow-up period is longer than in previous publications.

It is known that the risk of hepatocellular carcinoma increases in advanced stage of fibrosis, in patients with comorbidities such as diabetes mellitus, and in older age [17,18]. The reason why there are different outcomes in terms of cancer development depending on the treatment received could be explained by the fact that patients with more advanced liver disease and with comorbidities are now being treated with direct acting antivirals, as they were not considered suitable for interferon based therapies before due to the possibility of dangerous side effects. However, in our study the mean age of patients was only 58-year-old, so we cannot justify the high incidence of hepatocellular carcinoma as a result of the old age of patients treated with direct-acting antivirals. On the other hand, it has been described that alpha-interferon can activate natural killer cells, which are part of innate immunity and play a role in the control of viral infections and tumors [19], while interferon-free regimes produce a rapid decrease of HCV RNA levels which is followed by a rapid decrease in natural killer cells activation [20]. This hypothesis could explain the different outcomes between patients receiving one or the other treatment.

In any case, neither direct-acting antivirals nor interferon-based treatments eliminate the risk of hepatocellular carcinoma and patients should continue screening every 6 months after the achievement of sustained virological response.

In terms of clinical decompensation, we also did

not see a clinical benefit as our study showed a similar risk than what it is expected for untreated cirrhotic patients^[4,6]. In a recent study it has been found that hepatic venous pressure gradient (HVPG) decreased after interferon-free treatment in patients with HCV-related cirrhosis, but in patients with a pre-treatment HVPG of 10-15 mmHg, clinically significant portal hypertension was only decreased in 43%^[21]. In our study, 4 out of 4 patients who developed clinical decompensation had a previous history of ascitis or were diagnosed with hepatocellular carcinoma during the follow-up, suggesting a more advanced disease. This may explain why no clinical benefit was observed in these patients, and suggests that a longer follow-up period may be needed.

In terms of the hepatic function, our study did not show a significant improvement in the Child-Pugh and MELD scores. This may be because pre-treatment scores were already low. We also had no control group, making it difficult to determine if the outcomes were better or worse than expected. We therefore performed a statistic analysis comparing the mean platelet counts and bilirubin and albumin levels pre- and post-treatment which reflects the hepatic function. We found that there is a statistically significant improvement in platelet counts and in albumin levels showing some benefit in direct-acting antiviral treatment in the short term.

In conclusion, direct-acting antiviral treatment is not associated with a decrease in the development of hepatic decompensations or hepatocellular carcinoma in the first year of follow-up compared to literature reports for untreated compensated cirrhotic patients. On the other hand, there is a statistically significant improvement in platelet counts and in albumin levels, showing a possible improvement of the hepatic function in the short term. More studies are needed to determine the benefits of direct-acting antiviral therapy in the long term.

DECLARATIONS

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Authors' contributions

Concept and design: E. Berge, A. Arencibia, F. Pérez
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Data analysis: E. Berge, F. Pérez

Manuscript preparation: E. Berge, E. Otón, L. Cejas, S. Acosta

Critical revision: A. Arencibia, E. Otón, F. Pérez

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None.

Conflicts of interest

E. Berge, A. Arencibia, E. Otón, L. Cejas and S. Acosta declare that they do not have anything to disclose with respect to this manuscript. F. Pérez: Advisory board for Abbvie, BMS, Gilead, Janssen and MSD.

Patient consent

The data obtained through the medical record review were managed according to the privacy policy and ethics code of our institute.

Ethics approval

This was a retrospective study and did not require Institutional Review Board approval.

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Efficacy and HCC development after DAA therapy for patients with chronic hepatitis C: a single center retrospective cohort study

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sarcoidosis

ABSTRACT

Aim: The development of hepatocellular carcinoma (HCC) is reduced after interferon based treatment in patients with chronic hepatitis C (CHC). A new therapy using direct-acting antiviral agents (DAA) has been widely applied since 2014 for CHC. The purpose of this study is to investigate the efficacy, safety and development of HCC after DAA treatment. **Methods:** The authors enrolled 33 consecutive patients who were treated with DAA for CHC at the hospital between January 2015 and March 2016. The laboratory data were collected at the start and 24 weeks after DAA therapy. **Results:** The authors analyzed 33 patients (18 male, 15 female, mean age of 68-year-old). The hepatic C virus genotypes were type 1 (27 patients) and type 2 (6 patients). The number of patients treated with sofosbuvir (SOF) + ledipasvir, daclatasvir + asunaprevir and SOF + ribavirin was 14, 13 and 6, respectively. The sustained virological response (SVR24) rate was 100%. Aspartate aminotransferase, alanine aminotransferase and FIB4-index were significantly decreased after SVR24. Adverse effects were observed in 9 patients (anemia, 5; liver function test disorder, 2; sarcoidosis, 1; pruritus, 1). With regard to HCC development, one elderly patient (3.0%) had multiple HCC recurrence after SVR24. **Conclusion:** DAA therapy achieved a high SVR24 rate with a good serological response. However, one patient had multiple HCC recurrence. These findings indicate that careful follow-up may be essential after DAA therapy.

INTRODUCTION

During the previous decades, Pegylated interferon (PEG-IFN) plus ribavirin therapy for patients with chronic hepatitis C (CHC) cured hepatic C virus (HCV) infection in approximately 50% of treated patients^[1]. Emerging treatments with IFN-free direct-acting antiviral agents

(DAA) for patients with chronic CHC directly target HCV replication and have been widely used globally since 2014. Compared to conventional IFN-therapy, the sustained virological response (SVR) rate is higher and the side effects are reduced with DAA therapy. Previously, it was reported that IFN-based therapy reduced the risk of liver complications, including the



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occurrence of hepatocellular carcinoma (HCC)^[2-4]. However, these results were supported by studies with only IFN-based regimens. Thus, it is unclear whether DAA therapy reduces the occurrence of HCC.

The aim of this study was to evaluate the safety, efficacy and HCC development after DAA therapy in patients with CHC.

METHODS

Patients

In this retrospective cohort study, we analyzed data from consecutive patients with CHC who were treated with DAA therapy at our hospital in the middle south area of Nara prefecture, Japan, between January 2015 and March 2016 [Figure 1]. All data were obtained from individual patient records at our hospital. The eligibility of each patient for the treatment of HCV with DAA therapy was assessed following the criteria established by the Japan society of hepatology. The criteria for treatment included: patients with chronic hepatitis or Child-Pugh class A liver cirrhosis with no evidence of HCC as confirmed by ultrasound sonography (US) and/or contrast-enhanced computed tomography (CT)/magnetic resonance imaging (MRI). At the end of therapy and at 24 weeks off therapy, liver function was estimated again. The virological response to DAA therapy was assessed by quantitative HCV-RNA detection using real-time polymerase chain reaction. At 24 weeks off therapy, patients underwent another abdominal ultrasound evaluation. If focal lesions of the liver were detected by US, patients were re-evaluated with CE-CT/MRI to assess the occurrence or recurrence of HCC.

Statistics

Bivariate analyses of continuous variables were

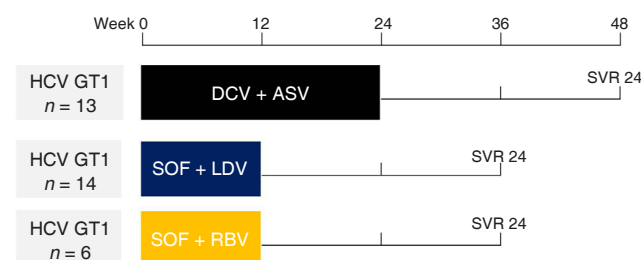


Figure 1: A total of 33 consecutive patients were treated with DAA for CHC at our hospital between January 2015 and March 2016. Patients were divided into a DCV + ASV group, an SOF + LDV group and an SOF + RBV group consisting of 13, 14 and 6 patients, respectively. The laboratory data were collected at the start and after 24 weeks of DAA therapy. HCC development was estimated by ultrasound sonography and/or CE-CT; DAA: direct-acting antiviral agents; CHC: chronic hepatitis C; DCV: daclatasvir; ASV: asunaprevir; SOF: sofosbuvir; LDV: ledipasvir; RBV: ribavirin; CE-CT: contrast-enhanced computed tomography

performed using the Wilcoxon rank-sum test. A *P* value of < 0.05 was deemed statistically significant. All statistical analyses were performed using IBM SPSS statistics version 22 (IBM Corp., Armonk, NY, USA).

RESULTS

Baseline characteristics of patients

Between January 2015 and March 2016, a total of 33 patients received DAA therapy. All patients were followed for 24 weeks after DAA treatment. Table 1 shows the principle baseline characteristics of this study.

Virological response

Sustained virological response after 24 weeks (SVR24) was achieved in all patients (100%), regardless of genotype 1 or 2 [Figure 2].

Serological response

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) indicated significantly decreased liver inflammation after SVR24. The FIB4-index, which is a calculated hepatic fibrosis marker, was also significantly decreased. Moreover, alpha (α)-fetoprotein, which showed both liver inflammation and tumor marker, was significantly decreased. However, total bilirubin (T-bil), albumin and platelet count were not significantly changed after SVR24 [Table 2].

Adverse events

Liver function disorder was only observed in the daclatasvir (DCV) + Asunaprevir (ASV) group. Anemia was found only in the sofosbuvir (SOF) + ribavirin (RBV) group [Table 3]. One patient treated with SOF +

Table 1: The principle baseline characteristics of the study, *n* = 33

Characteristics	<i>n</i> (%)
Mean age, year (range)	68 (41-83)
Male	18 (54.5)
Japanese	33 (100)
History of IFN therapy	20 (60.6)
HCV-RNA, log IU/mL (range)	6.0 (4.5-7.1)
HCV GT1	27 (81.8)
GT2	6 (18.2)
Cirrhosis	8 (24.2)
Platelet (10^4 mm ³)	14.4 \pm 4.5
Aspartate aminotransferase (IU/L)	43 \pm 20
Alanine aminotransferase (IU/L)	46 \pm 29
T-bil (mg/dL)	0.8 \pm 0.3
Albumin (g/dL)	4.1 \pm 0.3
AFP (ng/mL)	11.1 \pm 19.0
FIB4 Index	3.6 \pm 2.6

Data are reported as the mean \pm SD. HCV: hepatic C virus; T-bil: total bilirubin; AFP: Alpha-fetoprotein; IFN: interferon

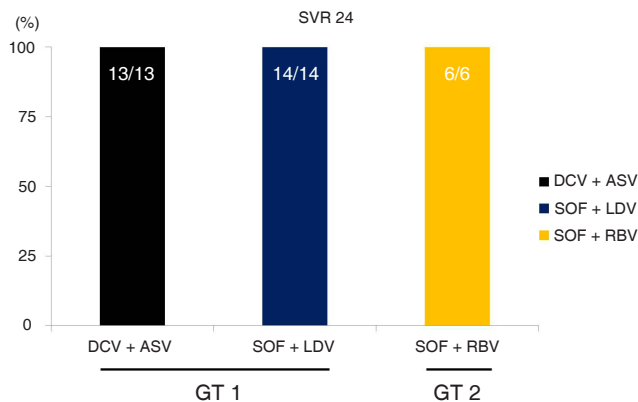


Figure 2: SVR24 rate with GT1 or GT2. All patients achieved SVR24 regardless of GT1 or GT2. SVR: sustained virological response; DCV: daclatasvir; ASV: asunaprevir; SOF: sofosbuvir; LDV: ledipasvir; RBV: ribavirin

Table 2: Virological response before and 24 weeks following therapy

Characteristics	Baseline	24 weeks off therapy	P
AST (IU/L)	43 ± 20	22 ± 5	0.000
ALT (IU/L)	46 ± 29	17 ± 5	0.000
T-bil (mg/dL)	0.8 ± 0.3	0.8 ± 0.3	0.094
Albumin (g/dL)	4.1 ± 0.3	4.3 ± 0.2	0.081
Platelet (10 ⁴ × mm ³)	14.4 ± 4.5	15.1 ± 4.3	0.125
AFP (ng/mL)	11.1 ± 19.0	6.0 ± 13.1	0.000
FIB4-index	3.6 ± 2.6	2.6 ± 1.3	0.000

AST, ALT, AFP and FIB4-index were significantly decreased. However, T-bil, albumin and platelet count were not significantly changed after SVR24. AST: aspartate aminotransferase; ALT: alanine aminotransferase; T-bil: total bilirubin; AFP: alpha-fetoprotein

ledipasvir (LDV) developed sarcoidosis after SVR24 [Figure 3]; after the end of DAA treatment, renal dysfunction occurred. Renal biopsy revealed renal sarcoidosis. Moreover, chest X-P showed bilateral hilar lymphadenopathy while the ophthalmologic examination showed iritis. Eradication of HCV or DAA treatment itself might trigger the onset of sarcoidosis.

HCC development

With regard to HCC development, patients without an HCC history did not develop HCC in these observed periods [Table 4]. One elderly patient (3.0%) had multiple

Table 4: HCC development after DAA therapy, n = 33

HCC development	n (%)
HCC pre-treatment (+)	
HCC occurrence (+)	1 (3.0)
HCC occurrence (-)	0
HCC history(-)	
HCC occurrence (+)	0
HCC occurrence (-)	32 (97)
Observed periods after DAA, month	10.4 ± 3.7

Patients without HCC history did not develop HCC in these observed periods. One elderly patient (3.0%) had multiple HCC recurrence after SVR24. HCC: hepatocellular carcinoma; DAA: direct-acting antiviral agents

HCC recurrence after SVR24 [Figure 4]. Before the start of DAA treatment, transarterial chemoembolization (TACE) was performed twice. Then, DAA treatment was initiated after a complete response was achieved. CE-CT after 3 months from the end of DAA treatment showed local and distant HCC recurrence. Common hepatic artery angiography showed multiple HCC. Thus, a 3rd TACE was performed for HCC recurrence.

DISCUSSION

IFN-based therapy for CHC should not be used for elderly patients and autoimmune diseases because of adverse effects and the mechanism of IFN. On the other hand, DAA therapy can be used for these patients with relative safety. However, as DAA therapy is relatively new, it is unclear whether DAA therapy ameliorates hepatic fibrosis and suppresses the development of HCC. Thus, the purpose of this retrospective cohort study was to elucidate changes in liver function and fibrotic markers before and after DAA therapy using SVR24, adverse events and HCC development.

The effect for SVR24 was very high in this cohort study. The SVR rate of DCV + ASV is known to be slightly lower than SOF + LDV. We checked the resistance associated substitution (RAS) before DCV + ASV administration. All DCV + ASV patients didn't have Y93 mutation, which was key mutation involving for non-SVR. In our speculation, the reasons of the high SVR24 rate of DCV + ASV group may be wild type of RAS and the small number of patients (DCV + ASV, n = 13).

Table 3: Adverse events during and after DAA therapy

Adverse events	DCV + ASV n = 13	SOF + LDV n = 14	SOF + RBV n = 6
Anemia	0	0	Grade1, 2 (33.3%) Grade 2, 3 (50%)
Liver function test disorder	Grade1, 1 (7.7%) Grade3, 1 (7.7%)	0	0
Pruritus	1 (7.7%)	0	0
Sarcoidosis	0	1 (7.1%)	0

Liver function disorder was found in only the DCV + ASV group. Anemia was found in only the SOF + RBV group. One patient treated with SOF + LDV developed sarcoidosis after SVR24. DCV: daclatasvir; ASV: asunaprevir; SOF: sofosbuvir; RBV: ribavirin; LDV: ledipasvir; SVR: sustained virological response

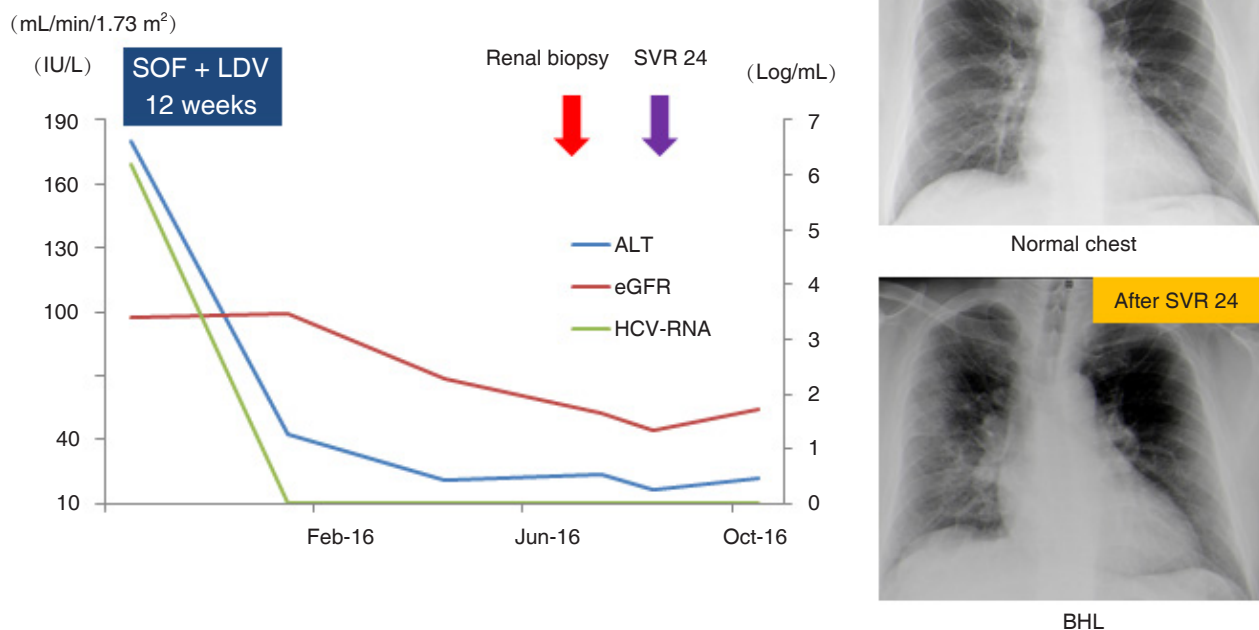


Figure 3: A case of sarcoidosis after DAA treatment. A 46-year-old male. After the end of DAA treatment, renal dysfunction occurred. Renal biopsy revealed renal sarcoidosis. Moreover, chest X-P showed BHL while the ophthalmologic examination showed iritis. The eradication of HCV or the DAA treatment itself might have triggered the onset of sarcoidosis. DAA: direct-acting antiviral; BHL: bilateral hilar lymphadenopathy; HCV: hepatic C virus; SOF: sofosbuvir; LDV: ledipasvir; SVR: sustained virological response

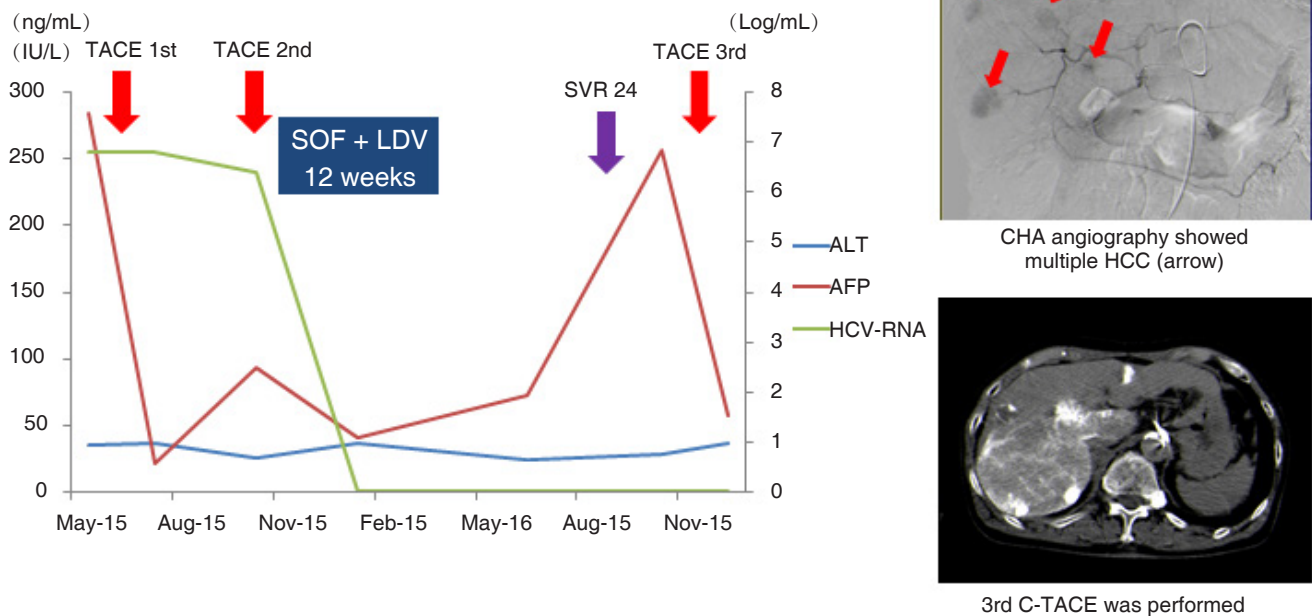


Figure 4: A case of HCC development after DAA treatment. A 81-year-old male. Before the start of DAA treatment, TACE was performed twice. Then, DAA treatment was initiated after a complete response was achieved. CE-CT after 3 months from the end of DAA treatment showed local and distant HCC recurrence. CHA angiography showed multiple HCC. Thus, a 3rd TACE was performed for HCC recurrence. HCC: hepatocellular carcinoma; DAA: direct-acting antiviral; TACE: transarterial chemoembolization; CT: computed tomography; SOF: sofosbuvir; LDV: ledipasvir; ALT: alanine aminotransferase; AFP: alpha-fetoprotein; HCV: hepatic C virus; SVR: sustained virological response

In the IFN era, liver fibrosis was improved after SVR in patients with CHC^[4-6]. Shiratori *et al.*^[3] reported that the resolution of hepatic fibrosis was achieved in 0.283 stages per year. However, whether anti-fibrotic effects occurred with DAA therapy was unclear. In our study, platelet count, T-bil and albumin were unchanged, likely because the observed period was only six month after the end of DAA treatment. However, AST and ALT, which showed that liver inflammation was significantly decreased, as well as the FIB4-index, which is a calculated hepatic fibrosis marker, were significantly decreased after SVR24.

IFN-based treatment for CHC has multiple adverse events, such as influenza like syndrome, interstitial pneumonia, cytopenia, depression, *etc.* Conversely, DAA therapy rarely has adverse events compared to IFN-based treatment. ASV, an NS3/4 protease inhibitor, is more likely to result in liver function test disorder^[7]. In our study, 2 patients in the DCV + ASV group ($n = 13$) had liver function disorder [Grade 1, $n = 1$ (7.7%); Grade 3, $n = 1$ (7.7%)]. One patient withdrew from DAA therapy. Another patient decreased their dosage of ASV. Fortunately, these two patients achieved SVR24.

RBV + PEG-IFN therapy was used for patients with CHC. It is known that ribavirin triggers hemolytic anemia^[8]. In our study, the SOF + RBV group ($n = 6$) had anemia in 5 patients [Grade 1, $n = 2$ (33.3%); Grade 2, $n = 3$ (50%)]. If anemia developed, we decreased the dosage of RBV. After the end of DAA treatment, anemia was naturally improved without blood transfusion or iron pill administration.

IFN is one of the cytokines in response to several pathogens, such as virus, bacteria, parasite and tumor. So, IFN therapy activated immune systems that help to eradicate HCV. The previous report indicated that IFN therapy for CHC triggered sarcoidosis via activating immune system^[9-11]. Sarcoidosis is a granulomatous autoimmune disease of unknown etiology that may affect many organs. Until now, IFN was thought to be involved in the onset of sarcoidosis. In the other hands, DAA itself don't influence immune system. Moreover, HCV is known to have several systemic autoimmune disorder, such as cryoglobulinemia, Sjögren's syndrome, diabetes mellitus, thyroiditis, membranoproliferative glomerulonephritis, *etc.* Interestingly, in our study, the SOF + LDV group ($n = 14$) had a patient with lung and renal sarcoidosis that was induced after DAA therapy. Our case indicated that the eradication of HCV itself might have induced sarcoidosis via an acute change in immune status. In speculation, HCV decrease with the very short periods may trigger acute change in immune status.

With regard to the effect on HCC reoccurrence, the PEG-IFN + RBV combination therapy reduced HCC occurrence if SVR was achieved^[12-14]. In Italy, Bruno *et al.*^[15] reported that the SVR to IFN- α was associated with improved outcomes in HCV-related cirrhosis. Moreover, IFN itself has an anti-tumor effect^[16], and low-dose and long-term maintenance administration of PEG-IFN α -2 α decreased the incidence of HCC in non-SVR patients^[17]. On the other hand, DAA has no direct anti-tumor effect, and the suppressive effect of DAA on HCC occurrence remains controversial in western countries^[18-21]. In a Japanese retrospective cohort study, the HCC risk rate after SVR was similar regardless of whether it was achieved by DAA or IFN-based regimens^[22]. In our study, during a median follow-up period of 10.4 ± 3.7 months, one elderly patient (3.0%) with an HCC history developed multiple HCC recurrence after SVR24. Patients without HCC history did not develop HCC in this observed period.

This study has several limitations. First, this was a single-center study with a limited number of patients. Therefore, the statistical power was low. Second, all patients in this study were Japanese. Thus, applying these results to other ethnic groups is difficult. Third, the criteria of liver function for DAA therapy in Japan are only CPS grade A. Therefore, the efficacy and safety of DAA therapy in patients with CPS grades from B to C are unknown.

In conclusion, DAA therapy achieved a high SVR rate and a good serological response. However, one patient had multiple HCC recurrence in our small cohort study. These findings indicate that careful follow-up may be essential after DAA therapy.

DECLARATIONS

Authors' contributions

Designed the report: A. Douhara
 Attending doctors for patients: A. Douhara, H. Ogawa, E. Shioyama, M. Yoshikawa, S. Ueda
 Discussed the pathogenesis: A. Douhara, H. Ogawa, S. Nakatani, T. Ozutsumi, E. Shioyama, M. Yoshikawa, S. Ueda
 Organized the report: S. Ueda
 Wrote the paper: A. Douhara

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

In our institution, we obtained informed consent from

each case as medical care.

Ethics approval

This study conforms to the ethical guidelines of the Helsinki Declaration. The approved number of the institutional review board (IRB) is 72.

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Interventional radiology for post-transplant anastomotic complications

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ABSTRACT

The effectiveness of percutaneous interventional radiology for anastomotic stricture in hepatic vein, portal vein, and biliary tract after living donor liver transplantation (LDLT) is described. As a number of patients with LDLT are infants < 10-year-old in the study, the first treatment option was balloon dilatation, not primary stenting. However, stent placement was performed in patients with recurrent, repeated stenosis.

INTRODUCTION

Liver transplantation is an established treatment for end-stage liver disease^[1]. The recent advances in surgical techniques and immunosuppression have led to improvements of post-transplant outcomes but various complications including bleeding, infections, rejection, vascular complications at the anastomotic site, and biliary complication will occur after liver transplantations^[2,3]. Although deceased donor liver transplantation is considered a standard procedure, living donor liver transplantation (LDLT) has been widely performed owing to the shortage of donors^[4]. LDLT is technically demanding because of the use of short vascular pedicles, which are more likely to cause

postoperative vascular complications, such as hepatic venous outflow obstruction (HVOO) at the anastomotic site and anastomotic portal vein stenosis (PVS)^[5-8]. Moreover, biliary complications remain common after LDLT, and some studies suggested that biliary stricture at the anastomotic site occurs more frequently in post-LDLT patients than in deceased liver transplantation. This is because of the small diameter of the anastomotic portion of the bile duct, anatomical diversity of the bile ducts or the complicated nature of the surgical procedure^[9,10].

In this study, the effectiveness of interventional radiology (IR) for anastomotic complications after LDLT mainly in pediatric patients was described.



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IR FOR HVOO

Vascular complications after liver transplantation include occlusion/stenosis at the site of anastomosis of hepatic artery, portal vein and hepatic vein. Although HVOO is an uncommon complication after liver transplantation, it is still an important cause of graft failures after liver transplantation^[2]. The incidence of HVOO after orthotopic liver transplantation is reported to be about 1% and that after LDLT is reported to be about 2-4%^[11,12]. This is because an anastomotic orifice is small and the grafts grow in LDLT. The causes of HVOO were stretching, twist and compression of hepatic vein with graft growing and adhesion change at anastomotic site^[13].

HVOO are suspected with the findings of intractable ascites, abnormal venous flow patterns at Doppler ultrasonography (US), histologic findings suggesting venous congestion, or deterioration of liver function not otherwise explained. Doppler US is a useful modality for diagnosing HVOO whose findings is disappearance of pulsatile hepatic venous flow or flatness of the hepatic venous wave.

Percutaneous balloon dilatation is a safe and effective method of treating HVOO. In our study balloon dilatation is performed for patients with initial HVOO after LDLT, and expandable metallic stent placement is tried in patients with repeated HVOO after the balloon dilatation. This strategy is based on three our concepts. First, routine primary stenting may result in unnecessary placement of an expandable metallic stent. Second, long-term patency for metallic stent for decades is unknown in pediatric patients. Because infant and young patients grow, it is unknown whether their growth can match to the unchanged size of implanted expandable metallic stent. Third, implanted expandable metallic stent may disturb re-transplantation. At re-transplantation, the presence of expandable metallic stent in the wall of the suprahepatic inferior vena cava might be technically a

challenge for surgeons.

Procedures

The approach to the hepatic vein is made through transjugular or transhepatic method. After passage of the catheter through the stenotic segment of the hepatic vein, venography and manometry; measurement of venous pressure of proximal and distal sides of the stenosis and the pressure gradient across the stricture is performed. Patients with a pressure gradient of more than 3 mmHg are considered to have significant outflow obstruction and are candidates for balloon dilatation.

Balloon dilatation [Figure 1] is performed following venography with a 7.0-Fr percutaneous transluminal angioplasty catheter with a balloon diameter of 6-12 mm. The balloon is inflated three times for 60 s with an atmospheric pressure of 10 atm. The diameter of the balloon is the same as the vein on the mesenteric side of the stenosis. The balloon is routinely inflated 3 times for 60 s with an atmospheric pressure of 10 atm. In patients showing recurrent HVOO, the stent placement [Figure 2] is performed. We used a self-expanding metallic stent with a diameter 20-30% larger than that of the hepatic vein.

Results

In our reported study^[14], the rates of technical success, primary patency and primary-assisted patency were evaluated. Technical success is defined as success in interventional procedures. Primary patency is defined as the interval between the initial balloon angioplasty and recurrent HVOO necessitating percutaneous intervention. Primary-assisted patency is defined as patency following the initial angioplasty until repeated percutaneous intervention therapy is discontinued.

We performed IR for 48 patients with HVOO after LDLT whose follow-up periods ranged from 1 to 182 months (median, 51.5 months). Technical success was achieved in 92 of 93 sessions (99%) and in 47 of 48 patients



Figure 1: A 6-year-old boy with biliary atresia underwent left-lobe LDLT, HVOO was diagnosed 5.1 years after LDLT, and hepatic venography was performed. (A) preoperative venogram showing an anastomotic stricture. As to the manometry finding, the pressure gradient, HV-RA was 12 mmHg; (B) fluoroscopic view during balloon dilatation showing the notch of the balloon at the stenosis; (C) preoperative venogram after the balloon dilatation showing improvement of the stenosis. The pressure gradient improved; HV-RA was 2 mmHg. LDLT: living donor liver transplantation; HVOO: hepatic venous outflow obstruction; HV: hepatic vein; RA: right atrium

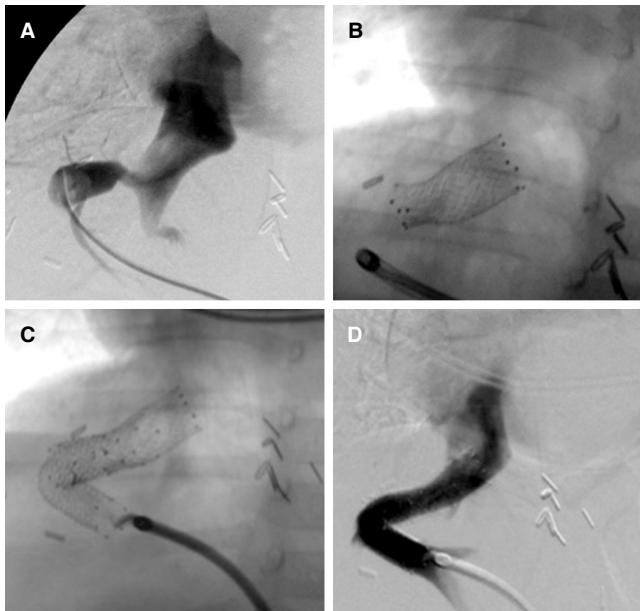


Figure 2: A 1-year-old girl with biliary atresia underwent left-lobe LDLT, HVOO repeated after 3-sessions of balloon dilatation, and stent placement was performed. (A) preoperative hepatic venogram showing an anastomotic stricture; (B) fluoroscopic view after stent placement. However, HVOO repeated, and additional stent placement was performed twice. After the 3rd stent placement, HV was patent, and no HVOO was noted for 5 years. (C) fluoroscopic view the 3rd after stent placement; (D) hepatic venogram showing no anastomotic stricture. LDLT: living donor liver transplantation; HVOO: hepatic venous outflow obstruction

(98%). The primary and primary assisted patency at 1, 3, 5, 10 years after the initial privacy threshold analysis (PTA) were 64%, 57%, 57%, 52% and 98%, 95%, 95%, and 95% respectively.

IR FOR PVS

The rate of PV complications after deceased donor liver transplantation has been reported to be <3%^[7]. However,

in patients with reduced-size liver transplantation or LDLT, the rate of PV complication can be higher (9-14%) than in patients with conventional deceased donor liver transplantation^[7,15]. PV complications are divided mainly into anastomotic PVS and portal vein thrombosis (PVT)^[16]. Anastomotic PVS can lead to graft failure if not properly treated. The treatment options for PVS after liver transplantation are surgical treatment and percutaneous interventions, including percutaneous balloon dilatation and stent placement. However, surgical treatment of these complications has been limited owing to technical difficulties or invasiveness. Currently, the surgical treatment of PVS after liver transplantation has been replaced by percutaneous balloon dilatation and stent placement, because of lower invasiveness and greater effectiveness.

PVS was clinically suspected with the following findings: (1) clinical symptoms of portal hypertension, such as ascites, splenomegaly, gastrointestinal tract bleeding from varices, and thrombocytopenia; and (2) US findings, including greater than 50% stenosis (the diameter of stenosis/the diameter of a main PV on the mesenteric side) or no flow in the PV; or the presence of an acceleration of flow at the stenosis or a post-stenotic jet flow or minimal flow in the intrahepatic PV on Doppler US. Our inclusion criteria for PVS were: (1) greater than 50% stenosis (the diameter of the stenosis/the diameter of a PV on the distal side); or (2) > 5 mmHg pressure gradient across the stenosis between the proximal and distal PV.

Procedures

The approach to the intrahepatic PV is transhepatic at the first session of percutaneous intervention. Balloon dilatation [Figure 3] is performed following portography with a 7.0-Fr percutaneous transluminal angioplasty catheter with a balloon diameter of 6-12 mm. The balloon

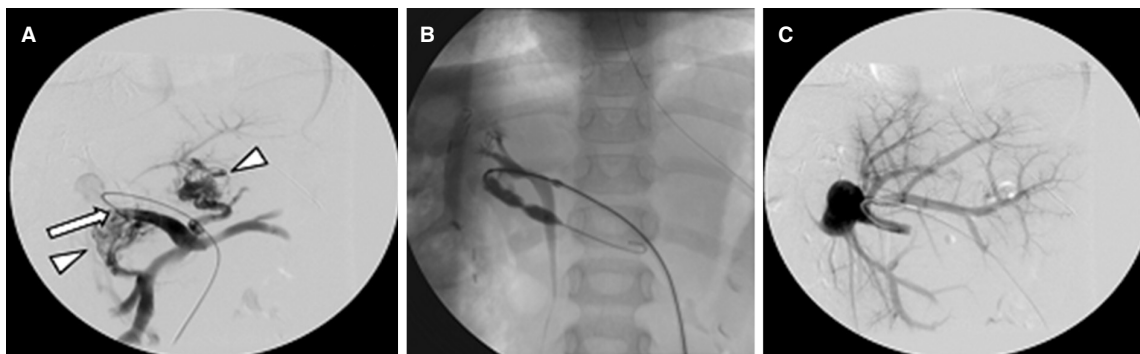


Figure 3: A 7-year-old girl with biliary atresia underwent left-lobe LDLT, PVS was suspected 5 years after LDLT, and portography was performed. (A) pretreatment portogram showing an anastomotic stricture (arrow), collateral vessels (arrowhead), and poor flow through the intrahepatic portal vein; (B) fluoroscopic view during balloon angioplasty showing the notch of the balloon at the stenosis; (C) portogram after the balloon angioplasty showing improved blood flow through the portal vein and disappearance of collateral vessels. PVS did not recur after the balloon angioplasty; LDLT: living donor liver transplantation; PVS: portal venous stenosis

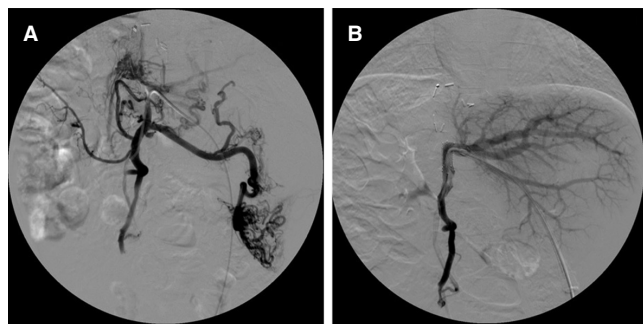


Figure 4: A 2-year-old girl with biliary atresia had undergone left-lobe LDLT and seven sessions of balloon angioplasty for PVS, because recurrent PVS was suspected, portography was performed. (A) pretreatment portogram showing a severe anastomotic stricture and no flow into the intrahepatic portal vein; (B) portogram after stent placement showing improved blood flow into the portal vein, PVS did not recur after stent placement; LDLT: living donor liver transplantation; PVS: portal venous stenosis

is inflated three times for 60 s with an atmospheric pressure of 10 atm. The diameter of the balloon is the same as the vein on the mesenteric side of the stenosis. The balloon is routinely inflated three times for 60 s with an atmospheric pressure of 10 atm. Stent placement [Figure 4] is performed in patients who developed recurrent PVS. We used a self-expanding metallic stent with a diameter 20-30% larger than that of the PV proximal to the stenosis and with sufficient length to cover the stricture. In patients where the percutaneous transhepatic approach to the PV is unsuccessful, or where placing a metallic stent with the percutaneous transhepatic approach might be technically difficult owing to a severely curved PV, a transileocecal approach is chosen following laparotomy.

Results

In our reported study^[17], the rates of technical success, primary patency and primary-assisted patency were evaluated. Technical success is defined as success in interventional procedures. Primary patency is defined as the interval between the initial balloon angioplasty and recurrent PVS necessitating percutaneous intervention. Primary-assisted patency is defined as patency following the initial angioplasty until repeated percutaneous intervention therapy is discontinued.

We performed IR for the 43 patients with PVS after LDLT, whose follow-up periods ranged from 5 to 169 months (mean, 119 months). Technical success was achieved in 65 of 66 sessions (98%) and in 42 of 43 patients (98%). The primary and primary assisted patency at 1, 3, 5, 10 years after the initial PTA were 83%, 78%, 76%, and 70%, respectively, and 100%, 100%, 100%, and 96%, respectively [Figure 5].

IR FOR ANASTOMOTIC BILIARY STENOSIS

Anastomotic biliary stricture is the most common biliary complication. Some studies have suggested that biliary stricture occurs more frequently in post-LDLT patients than in deceased liver transplantation because of the small diameter of the anastomotic portion of the bile duct, anatomical diversity of the bile ducts, or the complicated nature of the surgical procedure^[9,10,18]. There are two strategies for treating anastomotic strictures: via the endoscopic retrograde approach^[19] or the percutaneous transhepatic approach^[20]. The endoscopic retrograde approach is feasible for post-transplant patients with a duct-to-duct anastomosis, and endoscopic stent placement has been reported to be effective for biliary strictures in post-transplant patients^[21]. Because the most common disease in pediatric patients with LDLT has been biliary atresia, most of them have undergone Kasai's surgery and Roux-en-Y hepaticojejunostomy (RYHJ). Thus, percutaneous transhepatic biliary drainage (PTBD) is believed to be a first-line treatment for biliary strictures in pediatric patients who underwent LDLT with RYHJ.

An anastomotic biliary stricture is suspected based on laboratory, US, cholescintigraphic findings, and liver biopsy results. Liver function tests show increases in total bilirubin, direct bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyl transpeptidase (γ -GTP), and/or alkaline phosphatase (ALP). US findings that suggest anastomotic stricture are dilatation of intrahepatic bile ducts that appeared during the follow-up. Cholescintigraphy shows delayed visualization of the bowel (> 10 min after injection of the radiotracer ^{99m}Tc-N-pyridoxyl-5-methyltryptophan). Liver biopsy reveals cholestasis.

Procedures

Access to the biliary duct was made under US guidance. After puncture of a biliary duct with a 21-gauge needle under US guidance and opacifying the biliary duct (percutaneous transhepatic cholangiography), PTBD is performed using a 0.018-inch guidewire and a 5-Fr catheter [Figure 6]. Then, passage through the anastomotic biliary stricture is attempted with a 0.035-inch hydrophilic guidewire and a 5-Fr catheter. After successful passage of the catheter and exchange of a 7-Fr interventional sheath introducer [Figure 6], dilatation was performed with a balloon catheter (diameter; 4-10 mm). The diameter of the balloon was matched to the diameter of the intrahepatic bile duct on the hepatic side of stricture. The balloon was placed across the stricture and inflated for 180 s with

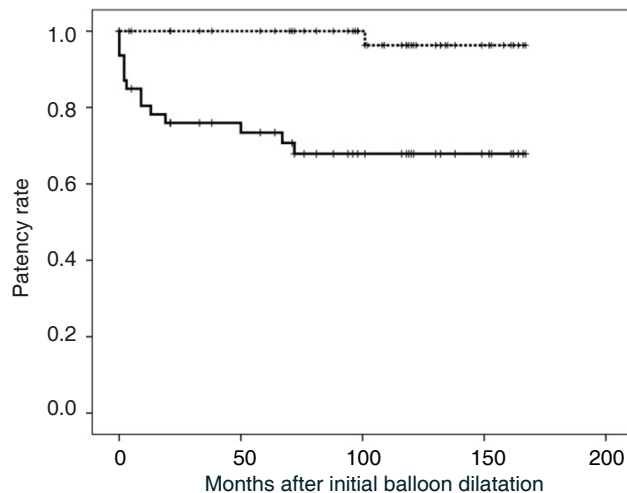


Figure 5: Kaplan-Meier curve showing primary- and primary-assisted patency rates. Solid and dotted lines indicate primary patency and primary-assisted patency, respectively. Vertical lines on both lines indicate censored observations. At 1, 3, 5, and 10 years after the first balloon angioplasty the primary patency rates were 80%, 76%, 73%, and 67%, respectively, and the primary-assisted patency rates were 100%, 100%, 100%, and 96% respectively

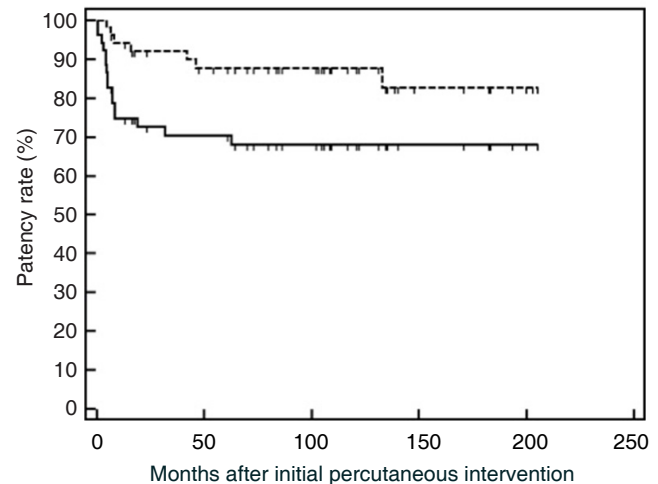


Figure 7: Kaplan-Meier curve showing primary- and primary-assisted patency rates. Solid and dotted lines indicate primary patency and primary-assisted patency, respectively. Vertical lines on both lines indicate censored observations. The primary patency rates at 1, 3, 5, and 10 years after the initial drainage tube placement were 75%, 70%, 70%, and 68%, respectively. The primary-assisted patency rates at 1, 3, 5, and 10 years after the initial drainage tube placement were 94%, 92%, 88%, 88%, respectively

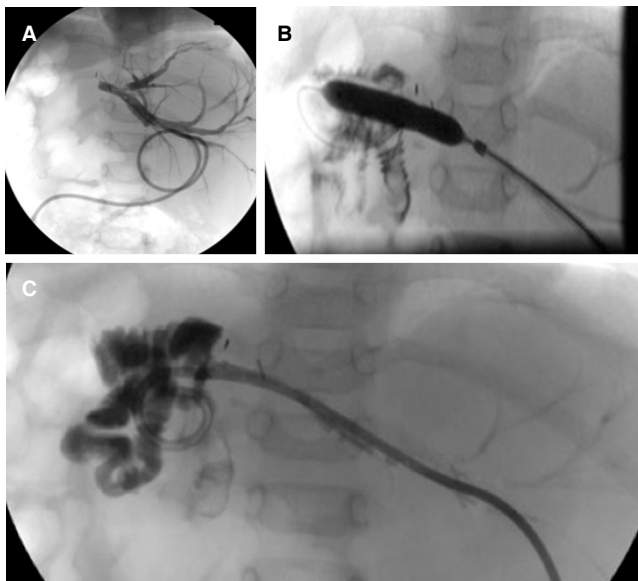


Figure 6: A 2-year-old boy who had undergone LDLT 20 months ago was suspected of having a biliary anastomotic stricture. (A) PTC showing an anastomotic stricture; (B) fluoroscopic view during balloon dilatation. Balloon dilatation was performed at 10 atm for 3 min using a 6-mm-diameter balloon catheter; (C) fluoroscopic view shows an 8.5-Fr. internal-external drainage tube placed across the anastomotic stricture. After serial exchange with a larger diameter catheter (16-Fr), the drainage tube was removed. No recurrent stricture was noted for 117 months after the biliary interventions. LDLT: living donor liver transplantation; PTC: percutaneous transhepatic cholangiography

an atmospheric pressure of 10 atm. After balloon dilatation, cholangiography was repeated to evaluate the effectiveness. Then, an 8.5-Fr internal-external drainage tube (Pig-tail catheter, Cook; IN, USA) was

placed, covering the Roux-Y jejunum and intrahepatic bile ducts across the anastomotic stricture.

Serial exchanges for a larger 14-Fr or 16-Fr drainage tube with or without balloon dilations were routinely performed at 1- to 6-week intervals. At a follow-up session, cholangiography was performed to evaluate the persistence of stricture. If the stricture had widened and the laboratory data had resolved, the tube was removed.

Results

In our reported study^[22], clinical success, tube independent rate, and patency rate were evaluated. Clinical success is defined as resolution or marked improvement of clinical symptoms including fever, and improvement of laboratory findings, including the serum levels of AST, ALT, total bilirubin, direct bilirubin, r-GTP, and ALP. Tube independent rate is defined as the rate at which the patient can undergo tube removal after symptoms are diminished and laboratory findings have improved. Patency rate is estimated by the Kaplan-Meier analysis. Primary patency is defined as the interval between placement of an internal drainage tube and appearance of a recurrent biliary stricture necessitating percutaneous biliary interventions. Primary-assisted patency is defined as the interval between placement of an internal drainage tube and when treatment with repeated percutaneous interventions is discontinued.

We performed IR for the 52 patients with anastomotic biliary stenosis after LDLT, whose follow-up periods

ranged from 5 to 206 months (median, 100 months). Clinical success was noted in 43 of 52 patients (83%). Removal of the drainage tube was achieved in 49 of 52 patients (94%). Of the three patients having a drainage tube, two underwent surgical reanastomosis, and one had a drainage tube implanted subcutaneously. The primary patency rates at 1, 3, 5, and 10 years after the initial drainage tube placement were 75%, 70%, 70%, and 68%, respectively. The primary-assisted patency rates at 1, 3, 5, and 10 years after the initial drainage tube placement were 94%, 92%, 88%, and 88%, respectively [Figure 7].

CONCLUSION

In conclusion, percutaneous IR is a minimally invasive, effective treatment for HVOO, PVS, and anastomotic biliary stricture after LDLT.

DECLARATIONS

Authors' contributions

The author contributed solely to this paper.

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

This review paper is waived for ethics approval.

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Reducing liver cancer risk beginning at birth: experiences of preventing chronic hepatitis B virus infection in China

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ABSTRACT

In China, the death numbers due to primary liver cancer every year account for more than half of this disease burden worldwide. Hepatocellular carcinoma (HCC) represents the major histological type of primary liver cancer. In the Chinese population, at least 85% HCC cases are due to chronic infection with hepatitis B virus (HBV), most of which were acquired in the perinatal period or in early life. As of January 1992, HBV immunization of newborns was introduced to the national Expanded Program of Immunization of China. Prior to this program, the Qidong County in China conducted an hepatitis B intervention study, which was a population-based, cluster randomized, controlled trial of HBV vaccination in neonates. The study demonstrated that among young adults < 30 years old, neonatal HBV immunization decreased around 84% risk of HBV-related liver cancer, and 70% risk of mortality due to severe end-stage chronic liver diseases. More than 72% efficacy of neonatal vaccination against chronic HBV infection in adulthood was achieved; however, when catch-up HBV vaccination was given to children at age 10-14 years, the protection efficacy was only 21%. No difference in mortality of HBV-related liver diseases was observed among the young adults < 30 years who received and those who did not receive the catch-up HBV vaccination. These results highlight the crucial importance of HBV vaccination of neonates in reducing the liver cancer risk beginning at birth in highly HBV endemic regions. Due to large numbers of HBV-infected pregnant women with high viremia in China, clinical studies in which antiviral therapy with the nucleot(s)ide analogues was given to HBV-infected pregnant women have provided important evidence that such therapy can reduce the risk of mother-to-child HBV transmission. These clinical data based on cohort studies, randomized clinical trials, and clinical practices in the Chinese population provide important information on prevention of liver cancer, particularly HCC, by preventing chronic HBV infection starting from birth for other populations.



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INTRODUCTION

Primary liver cancer (PLC) is one of leading causes of cancer deaths in China. Worldwide, hepatocellular carcinoma (HCC) represents the major histological type of liver cancer and likely accounts for 70-85% of cases, followed by intrahepatic cholangiocarcinoma (iCCA) which accounts for approximately 10-25% of all hepatobiliary malignancies^[1]. Other risk factors for PLC include exposure to aflatoxin, algal hepatotoxins in drinking water, betel nut chewing, diabetes mellitus, alcohol consumption, and tobacco use^[2]. Approximately 80% of HCC worldwide was estimated to be associated with chronic infections with hepatitis B virus (HBV) and hepatitis C virus (HCV)^[3]. Some studies also found the relationship between iCCA and HBV or HCV^[4], although their causal effects need to be further confirmed.

The contribution of HBV or HCV to HCC differs in different geographical areas, mainly due to the varied prevalence of HBV or HCV in different populations^[1,3]. A meta-analysis including 39 studies in China from 1954 to 2010, based on the seroprevalence of hepatitis B surface antigen (HBsAg), and/or antibodies against HCV (anti-HCV) in HCC patients, reported that about 70% of HCC was associated with HBV infection alone, 5% with HCV infection alone, and 6% with HBV & HCV co-infection. The analysis also suggested that around 19% of HCC was unrelated to HBV or HCV^[3].

HBV occult infection, which is indicated by undetectable serum HBsAg and low level of serum HBV DNA (sometimes HBV was detected only in the liver), has been frequently reported in different liver diseases^[5,6]. In recent years, a substantial HBsAg-negative HCC patients were found to be serum HBV-DNA positive by using nucleic acid testing^[7]. To understand the impact of HBV and/or HCV on PLC, Wang *et al.*^[8] in National Cancer Center of China recently analyzed a total of 2,172 liver cancer cases, which were confirmed by histology. In this report, a total of 5,988 patients with PLC were identified from Northern regions of China (from January 1, 2003 to December 31, 2014) based on clinical diagnosis criteria. The analysis found no differences in the distributions of age, gender, ethnicity, and serological virus markers between the cases with and the cases without histological confirmation. Therefore, the proportion of HBV and/or HCV markers among the 2,172 histologically confirmed liver cancer cases could well represent the general cases^[8]. Although the data were from one single medical center, the patients in this study were from diverse regions throughout all the provinces in the Northern regions of China. HBsAg seroprevalence was 4-6% in the Northern regions, lower than that of the overall population (7.18%), while anti-HCV prevalence was 0.53%, higher than that of the overall population

(0.43%)^[9,10]. The seroprevalence of HBV and/or HCV serological markers among the liver cancer patients from these regions is helpful to understand the impact of HBV and/or HCV on this disease in China.

The study by Wang *et al.*^[8] reported that 83.9% of liver cancer patients with histological confirmation were HCC and 11.0% were iCCA. Among the 1,823 HCC patients, 1,567 (86.0%) cases had HBV markers alone, indicated by HBsAg(-)/(+) & anti-HBc(+). Remarkably, 18.2% of them were HBsAg(-) & anti-HBc(+) and serum HBV-DNA positive. HCV infection alone, indicated by presence of anti-HCV(+), was found in 2.5% of HCC cases, and HBV & HCV co-infection were found in 6.7% of HCC cases. Altogether, the contribution of HBV infection to HCC was at least 85-90%^[8]. This study indicated that the contribution of HBV infection to HCC in China had been under-estimated previously, most probably due to the unrecognized status of occult HBV infection among the HBsAg-negative HCC cases. The role of chronic HBV infection in HCC in China is clearly dominant. Therefore, controlling chronic HBV infection is crucial for reducing the risk of liver cancer, particularly HCC.

Although some perinatal infections from maternal HBV transmission may cause fulminant hepatitis in infancy^[11], a fatal disease of acute hepatocyte necrosis leading to hepatic encephalopathy and coagulopathy, HBV infection in infancy or early childhood leads to a high rate of persistent infection^[12]. It was reported that among infected neonates born to mothers with positive hepatitis B e antigen (HBeAg), the chronicity of HBV infection was 80-90%^[12]. Of children infected before 6 years of age, chronic infection was reported to develop in approximately 30%^[13,14]. It had been documented that the majority of persons with chronic HBV infection in China acquired it at birth or in early childhood^[15].

Long-term major adverse outcomes of chronic HBV infection are liver cancer and cirrhosis. Longitudinal studies of untreated persons with chronic HBV infection showed that there is about 8-20% of cumulative risk of developing cirrhosis over five years. In those with cirrhosis, there is an approximately 20% annual risk of hepatic decompensation and the annual incidence of HCC could be as high as to 5%^[16]. Therefore, it is important to reduce the risk of liver cancer beginning at birth or in early childhood by preventing chronic HBV infection.

Brief history of HBV vaccination program and its effect in reducing HBsAg seroprevalence in China

The relationship of chronic HBV infection and HCC development was well established based on a

prospective study of 22,707 men in Taiwan^[17]. In 1992, before the national HBV vaccination program was implemented, HBsAg seropositive rate in the 1-4 age group was 9.67%, as high as in the general population (9.75%)^[15], reflecting the fact that most of the chronic infections in Chinese population were acquired in the perinatal period or in early life. It is instrumental to reduce the incidences and mortalities of HCC and other liver diseases through universal HBV vaccination for infants and children. The Chinese government therefore developed a substantial number of policies to promote and implement the vaccination program^[18,19]. A brief history of the HBV vaccination program is summarized in Figure 1.

The plasma-derived HBV vaccine was firstly manufactured and found to be effective in humans in clinical trials^[20-22]. In 1982 two plasma-derived HBV vaccines, which were prepared from plasma of chronic HBsAg carriers, from France and from the United States were licensed^[23]. A World Health Organization (WHO) Scientific Group meeting was convened from Jan 30 to Feb 4, 1983 to discuss HBV vaccination for the prevention of PLC^[24]. Millions of the first-generation plasma-derived vaccines were administered worldwide to neonates, infants, children, and adults at high-risk, and the effectiveness and safety records are excellent^[18,23,25]. Due to the large population and high prevalence of HBV in China, the most important technical issue was to provide a safe and effective HBV vaccine sufficient to meet the requirement of immunizing 20 million newborns each year as well as other high risk groups. Through technical transfer from Merck, China manufactured both plasma-derived and recombinant HB vaccines domestically in late 1980s^[18]. With the maturation of recombinant DNA technology, the recombinant vaccines, prepared from yeasts, or from mammalian cells, were manufactured in early 1993, and entirely replaced the plasma-derived vaccine in 1997^[18,19,26].

Beginning in January 1992 universal immunization to newborns was integrated into the national Expanded Program of Immunization (EPI) of China with the family paying for all the costs^[18,19]. Due to the relative high vaccine cost during that period, the immunization was mainly carried out in some urban areas and the wealthier Eastern provinces of China. From 1 January 2002, the vaccines were provided for free and the family paid only for user fee^[18,19]. Between 2002-2007, with the support from the Global Alliance for Vaccines and Immunization and the central government of China, HBV vaccination was fully integrated into routine immunization to all infants in Western region and in poverty-affected counties in the Central regions of China, and from 2008 the central government of China took over the cost^[19].

In 2006 (14 years after universal HBV immunization to newborns), the Chinese government conducted a national hepatitis serosurvey in the same areas as did in 1992 by measuring the prevalence of HBV markers among the population aged 1-59 years to evaluate the impact of the HBV vaccination program. Overall, 82,078 persons were surveyed, from whom 82,008 blood samples were collected. Among the general population aged 1-59 years, the prevalence of HBsAg, anti-HBs, and anti-HBc were 7.2%, 50.1%, 34.1%, respectively. However, the HBsAg prevalence was greatly reduced among those age < 15 years compared to that found in the 1992 national serosurvey. The HBsAg seroprevalence in the 1-4 age group was 0.96%^[27], which was significantly reduced compared to the same group studied in 1992^[15]. The HBsAg seroprevalence was also reduced in the 5-59 age group, with a 2.32% seroprevalence in the 5-14 age group, and a 5.4% seroprevalence in the 15-19 age group, and more than 8.0% in individuals aged 20-59 years^[27]. Further investigation was done in the same areas in 2014 among the 1-29 years age group. The HBsAg seroprevalence was 2.64% (95% CI: 2.28-3.06%) in 2014 and decreased by 73.92% as compared with the

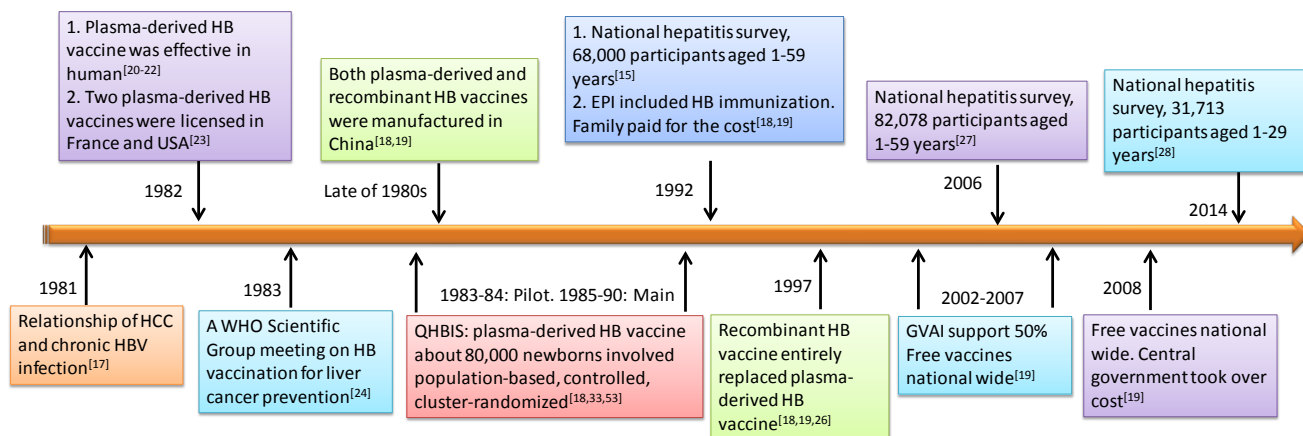


Figure 1: Brief history of HBV vaccination program in China. HBV: hepatitis B virus; HCC: hepatocellular carcinoma; WHO: World Health Organization; QHBIS: Qidong Hepatitis B Intervention Study; EPI: Expanded Program of Immunization

rate of 10.13% (95% CI: 9.81-10.45%) in 1992. Further analysis found that HBsAg prevalence was only 0.64% (95% CI: 0.54-0.75%) in 2014 among those (1-12 years of age) who were born between 2002-2013 after HBV vaccination was integrated into national EPI program^[28]. China has already reached the national goal of reducing HBsAg prevalence to less than 1% among children under 5 years, and an estimated 16-20 million HBV carriers were prevented through the HBV vaccination program^[27].

Reduced liver cancer incidence in general population by historical comparison cannot be entirely attributed to HBV vaccination

Some perinatal infection from maternal HBV transmission may cause fulminant hepatitis in infancy^[11], a very rare condition that develops in about 0.5-1% of cases^[29]. Mortality rate of fulminant hepatitis can be as high as 67%^[30]. Reports from several countries and areas have documented the dramatic decrease of the incidence of fulminant hepatitis after HBV immunization to newborns was implemented^[31-34]. However, the infection in the perinatal period and early life mainly resulted in chronic HBV infection, which could be as high as 90%^[12,14].

Long-term major adverse outcomes of chronic HBV infection are liver cancer and cirrhosis. Studies from Taiwan, which analyzed data based on cancer registry in birth cohorts born after the universal vaccination program as compared to the birth cohorts born before the program, documented that HBV vaccination was effective in reducing the incidence and mortality of liver cancer^[35]. A study among the Alaskan Native of the United States reported the elimination of HCC and acute hepatitis B in children 25 years after a HBV immunization program^[36]. Studies based on the cancer registry data of Korea^[37] and Japan^[38] also reported a decreased incidence of liver cancer after the implementation of HBV vaccination programs. In China, the time trend analysis of liver cancer incidence during 1988-2005 also showed a gradual decrease based on the cancer registry data in 11 cities and counties that covered a population of 401,506,812 (male patients 204,475,147; female patients 197,031,665, sex ratio 1.04). The annual percent change was -1.44%^[39]. Recent analysis showed that the age-standardized liver cancer incidence rate during 2000-2011 was further decreased with an average annual percentage change of -1.8%^[40], reflecting the effect of HBV vaccination in reducing liver cancer risk.

Chronic HBV infection is the most important risk factor for liver cancer in humans, which is endemic in the regions of Africa and Asia, especially in China. The other established etiological factors also include heavy exposure to aflatoxin, algal hepatotoxins in contaminated water, betel nut chewing, diabetes mellitus, and alcohol

abuse leading to liver cirrhosis^[2]. In addition, cohort studies showed that the intakes of vitamin C^[41] and vitamin E^[42], from both diet and supplements could potentially reduce the risk of liver cancer. Reducing the dietary aflatoxin exposure to non-detectable levels could also reduce HCC cases in high risk areas by about 23%^[43]. An observational study in a rural population indicated that decline of liver cancer incidence in the younger generation was not fully attributed to controlling chronic HBV infection alone. Changes in their staple food and drinking water were also important in reducing liver cancer risk^[44]. A study conducted in an urban area of China reported the positive roles of vegetable-based dietary pattern in decreasing liver cancer risk^[45]. All studies based on cancer registry data historically comparing the immunized and unimmunized cohorts at either the national or community level, support the hypothesis that HB vaccination is associated with a reduced risk of liver cancer. Nevertheless, because of potential differences in baseline characteristics and in exposures to other risk factors^[2] between the immunized and historical comparison (unimmunized) birth cohorts, it is difficult to make the inference that the observed reduced liver cancer risk was entirely attributed to HBV vaccination^[35,44,46]. The Qidong Hepatitis B Intervention Study (QHBIS) addressed the causal link between HBV vaccination and the observed benefits^[33,47,48].

Efficacies of HBV vaccination in preventing liver cancer and other liver diseases in rural China: experience from Qidong Hepatitis B Intervention Study

Qidong County, China, is a rural area with high liver cancer incidence and mortality compared to China as a whole. The incidence of PLC in Qidong was 79.6/10⁵ for man and 23.1/10⁵ for woman during 1978-2002^[49], and it was 28.15/10⁵ for man, 9.31/10⁵ for woman in China cancer registry which covered 11 cities and counties during 1988-2005^[39]. Two major risk factors identified in Qidong were high prevalence of chronic HBV infection and appreciable dietary aflatoxin exposure, with the HBV infection greatly sensitizing hepatocytes to the mutagenic effects of aflatoxin^[50-52]. Therefore, the neonatal HB vaccination began in a large controlled clinical trial on 1 September 1983 in Qidong (World Health Organization, Prevention of liver cancer. Technical Report Series 691, World Health Organization, Geneva.1983). It was later registered with Clinical Trials.gov number NCT00222664^[33].

QHBIS is a population-based, cluster randomized, controlled trial of HBV vaccination conducted between 1983-1990 in Qidong. During that time, Qidong had a population of 1.1 million and approximately 13,000 births each year. Approximately 80,000 newborns were randomly assigned into the vaccination or control groups^[18,33,53]. The study was conducted during a time

period when HBV vaccine was not available in any rural areas of China. The neonates in the control group did not receive the HBV vaccine at birth, and the neonates in the vaccination group received the three-dose plasma-derived HB vaccine, which were manufactured and donated by the Merck Company through the WHO. The first dose (5 µg) of HBV vaccine was administered within 24 h after birth, followed by two doses (5 µg/dose) at 1 and 6 months of age, respectively^[18,33]. Due to the shortage of hepatitis B immunoglobulin (HBIG), all the newborns were given the vaccine only regardless of their maternal HBsAg status. The protocol can be found via <https://doi.org/10.1371/journal.pmed.1001774.s003>.

During that time, it was considered ethically justifiable (to have neonates in a control group that did not receive the HBV vaccine) given that the plasma-derived HBV vaccine and recombinant vaccine was not universally available in China. However, in June 2000, the Qidong Center for Disease Control and Prevention (CDC) issued a Notification (File No. 2000-010) regarding HBV catch-up vaccination and booster (<https://doi.org/10.1371/journal.pmed.1001774.s004>). Children born in 1986-1990 and randomized into the control group of QHBIS received 3 doses of recombinant HBV vaccine at age 10-14 years, and those in the vaccination group received 1 dose of recombinant HBV vaccine at age 10-14 years^[33].

This cohort provides us a unique opportunity to examine the efficacy of HBV vaccination administered at different ages against liver cancer and other liver diseases. To reflect the real world situation of HBV vaccination, participants who were involved in the pilot study were no longer included in our analysis^[33,54]. The 30-year follow-up study demonstrated that more than 72% efficacy of neonatal vaccination against chronic HBV infection in adulthood was achieved. However, when catch-up HBV vaccination was administered to the control group at age 10-14, the protection efficacy was only 21% (95% CI: 10-30%), substantially weaker compared to neonatal vaccination, and highlighting the crucial importance of HBV vaccination on neonates against chronic HBV infection in highly HBV endemic regions^[33].

Using brain tumor as the negative control, the incidence rates between the vaccination group (0.52/10⁵) and the control group (0.71/10⁵) were similar. However, liver cancer cases were only diagnosed in the control group when the participants reached 20 years or older. The incidence rate in the vaccination group was 0.71/10⁵ person-year, and it was 1.41/10⁵ person-year in the control group. The protective efficacy was 84% against HBV-related liver cancer development in young adults < 30 years. The mortality rate of severe end-stage

chronic liver diseases, including liver cancer and chronic liver failure, was significantly lower in the neonatal vaccination group than in the control group (HR = 0.30, 95% CI: 0.11-0.85), and the efficacy was 70% (95% CI: 15-89%)^[33]. The study population was still young and incidence and deaths were very low. Remarkably, in the QHBIS participants, no differences in liver cancer incidence and mortality of chronic liver failure were observed by age 30 years between the individuals who received and those who did not receive the catch-up vaccination^[33]. These results further addressed the importance of HBV vaccination on neonates in highly HBV endemic regions.

Risk factors related to vaccination failures in children and HBV vaccination guideline by Chinese Center for Disease Control

HBV vaccination has been recommended universally for prevention of HBV infection and liver diseases. As of 2012, 183 (94%) of the 193 member states have initiated a HBV vaccination program, with an average of 79% for the third dose vaccine coverage in infants worldwide (www.who.int/mediacentre/factsheets/fs378/en). Current data from different areas demonstrated that vaccination is very efficacious in decreasing the HBsAg seroprevalence in children and in preventing the devastating complications of HBV infection in young adults^[32,33,55-57].

The vaccination failure in children was found mainly due to being born to HBeAg-positive HBsAg carrier mothers, the mother-to-child transmission (MTCT)^[23,58-60]. It had been reported that the protective efficacy of HBV vaccination was enhanced by co-administration of HBIG among the neonates who were born to HBsAg-positive mothers. Current recommendations strongly suggest screening pregnant women, and adding on HBIG to the newborns if the mother is positive for HBsAg, regardless of the HBeAg status^[23]. For neonates born to the HBeAg-positive HBsAg carrier mothers, another important issue affecting the efficacy of HBV vaccination is the timely birth dose of the vaccine. The delay in this initial dose showed an increased risk of infection in children^[61]. Premature gestation ages or low birth weights also affected the vaccination efficacy^[6,63]. The administration of a recombinant HBV vaccine shortly after birth is less immunogenic in preterm infants weighing < 1,800 g at birth than in full term infants. Therefore, it was suggested that the first dose of the vaccine in HBsAg-negative mother's infants weighing < 2,000 g might be administrated until they reached 2,000 g, or alternatively, until one month old^[23].

Based on the practices and experiences conducted in different areas, the National Health and Family Planning Commission of the People's Republic of China issued

a recommendation/guideline of HBV vaccination in children in December 2016 (<http://www.nhfpc.gov.cn/jkj/s3581/201701/a91fa2f3f9264cc186e1dee4b1f24084.shtml>). For general infants, it recommends three doses of HBV vaccines, with the first dose vaccine to be given within 24 h after delivery. The second and third doses are to be given at 1 month and 6 months of age. Each dose contains 10 µg vaccine from recombinant yeast or from Chinese hamster oocyte. For infants born to HBsAg-positive mothers, it recommends the administration of 100 IU HBIG within 12 h after birth combined with a full course of HBV vaccination. The administration dose is 10 µg vaccine from recombinant yeast or 20 µg vaccine from Chinese hamster oocyte. The vaccinated infants should be tested for the presence of HBsAg and the anti-HBs titer 1-2 months after completing the third dose of vaccine. If he/she is seronegative for HBsAg and has anti-HBs < 10 mIU/mL, the fourth dose of HBV vaccine should be given. If the neonates are born to HBsAg-positive mothers at premature gestation ages or low birth weights, the first dose of the vaccine should be administered when they are one month old.

Anti-viral therapy and vaccination for prevention of mother-to-child transmission

Despite the increased efficacy of passive-active immunization given to infants born to HBsAg-positive mothers, some infants still become infected with HBV, especially those born to HBeAg-positive and highly viremic mothers^[59]. Many different studies have identified that mothers with significant viremia are at a much higher risk of MTCT. HBV can be vertically transmitted to their infants, indicated by HBV-DNA positivity in the cord blood^[64,65]. Experience in reducing the risk of perinatal transmission of human immunodeficiency virus with lamivudine-zidovudine combination therapy drove the physicians to follow a similar rationale to prevent the perinatal transmission of HBV-infection. Lamivudine, which strongly inhibits HBV replication, was used to treat eight highly viraemic HBV-positive Caucasian or Negroid pregnant women (HBV-DNA levels $\geq 1.2 \times 10^9$ geq/mL) starting from week 34. The results suggest that reduction of viremia by antiviral therapy may be an effective and safe measure to reduce the risk of MTCT^[66]. Therefore, several studies were conducted to evaluate the risk of MTCT with different maternal levels of HBV DNA. Many studies have identified that serum HBV DNA level in pregnant women was the single most important predictor and independent risk factor for immunoprophylaxis failure in different populations^[59,65]. Some studies reported that maternal HBV DNA level of > 8 log₁₀ IU/mL was associated with increased likelihood of immunoprophylaxis failure^[67,68]. A large scale study on more than 1000 Chinese mother-infant pairs found that maternal HBV DNA levels (OR

= 1.88, 95% CI: 1.07-3.30) and detectable HBV DNA in the cord blood (OR = 39.67, 95% CI: 14.22-110.64) are independent risk factors for immunoprophylaxis failure^[65]. Maternal HBV-DNA level > 6 log₁₀ copies/mL (200,000 IU/mL) in Chinese women is still associated with the risk of immunoprophylaxis failure^[65].

Treatments aimed at lowering HBV-DNA levels during pregnancy may be helpful to ultimately decrease global disease burden. The exact threshold for treatment remains controversial. However most studies have accepted levels greater than 200,000 IU/mL as significant viremia^[69]. A Systematic Review and Meta-Analysis found that the use of any antiviral therapy compared to control reduced the MTCT risk as defined by infant HBsAg seropositivity (RR = 0.3; 95% CI: 0.2-0.4) or infant HBV-DNA seropositivity (RR = 0.3; 95% CI: 0.2-0.5) at 6-12 months. Notably, no significant differences were found in congenital malformation rate, prematurity rate, and Apgar scores^[70].

Lamivudine has been used the longest as antiviral therapy of chronic HBV approved by the Chinese Food and Drug Administration, and is currently classified as a pregnancy Category C drug. A meta-analysis and systematic review of 14 lamivudine studies showed that lamivudine reduced maternal HBV DNA levels, reduced infant HBsAg seropositivity by 11.7% and reduced infant HBV DNA seropositivity by 21.2%, respectively^[70]. During the period of January 2009 to March 2011, a prospective, open-label, interventional trial was conducted in Beijing Youan Hospital of China (ClinicalTrials.gov Number: NCT01743079). In the enrolled 700 pregnant women who were HBsAg-positive and HBeAg positivity, with HBV-DNA levels > 6 log₁₀ copies/mL (200,000 IU/mL), and alanine aminotransferase (ALT) < 40 IU/mL, 55 women were treated with lamivudine, 263 were treated with telbivudine, and the rest (374 women) received no antiviral therapy (the control group). Based on the data from 661 infants who completed the HBV vaccination series and the 52-week follow-up, the study found that mothers who received lamivudine or telbivudine had significantly lower HBV DNA levels than those who received no antiviral therapy. At birth, HBsAg was detected in 20% of treated and 24% of untreated newborns; however, by week 52, an intention-to-treat analysis indicated 2.2% (95% CI: 0.6-3.8) of HBsAg-positive infants from the treated versus 7.6% (95% CI: 4.9-10.3) in the untreated group ($P = 0.001$), and no difference in HBsAg-positive rate between infants in the lamivudine or telbivudine groups. On-treatment analysis indicated 0% of HBsAg-positive infants in the treated group versus 2.84% in the untreated group ($P = 0.002$). The study concluded that lamivudine or telbivudine in late pregnancy for highly viremic mothers was equally effective in reducing MTCT^[71].

Telbivudine and tenofovir are both classified as pregnancy Category B drugs. A large prospective, controlled trial included 229 HBeAg-positive patients with HBV DNA levels $> 7 \log_{10}$ copies/mL found that the telbivudine given to 135 pregnant women from weeks 20 to 32 of gestation safely reduce perinatal HBV transmission compared to the 94 untreated pregnant women. MTCT was markedly lower in the treatment group (0% vs. 8%) and the immunized infant had a higher proportion of detectable HBsAb (100% vs. 92%). A short-term follow-up showed that telbivudine was well-tolerated with no safety concerns in the mothers and/or their infants^[72].

Tenofovir Disoproxil Fumarate (TDF) currently remains the first-line therapy for chronic HBV infection based on its safety and resistance profile as well as its potency and efficacy. It is currently rated as pregnancy Category B. To evaluate the efficacy and safety of maternal TDF in reducing HBV MTCT, a prospective, multi-center trial was conducted that enrolled 118 HBeAg-positive and HBeAg-positive pregnant women with HBV DNA $\geq 7.5 \log_{10}$ IU/mL. In this trial, 56 mothers received no medication and 62 mothers received TDF 300 mg daily from 30-32 weeks of gestation until 1 month postpartum, respectively. Treatment with TDF for highly viremic mothers decreased infant HBV DNA at birth and infant HBsAg positivity at 6 months. However, TDF did not affect the MTCT rate in the per-protocol analysis at the 12-month follow-up^[73]. To verify the effect and safety of TDF, the China Study Group for the Mother-to-Child Transmission of Hepatitis B included 200 mothers who were positive for HBeAg and had an HBV DNA level higher than 200,000 IU/mL (ClinicalTrials.gov number NCT01488526)^[74]. The participants were randomly assigned in a 1:1 ratio to receive usual care without antiviral therapy or to receive TDF from 30 to 32 weeks of gestation until postpartum week 4. At delivery, 68% of the mothers in the TDF group (66 of 97 women) had an HBV DNA level $< 200,000$ IU/mL, while only 2% of the mothers had an HBV DNA level $< 200,000$ IU/mL in the control group. The TDF effect in reducing mothers' HBV DNA level was significant ($P < 0.001$). All the infants received immunoprophylaxis. At postpartum week 28, the MTCT rate was found to be significantly lower in the TDF group than in the control group, both in the intention-to-treat analysis [with transmission of virus to 5% of the infants (5 of 97) vs. 18% (18 of 100), $P = 0.007$] and the per-protocol analysis [with transmission of virus to 0 vs. 7% (6 of 88), $P = 0.01$]. Notably, there was no significant difference in the fetal safety profiles between the two groups^[75]. The results indicated that it might be too late to prevent the MTCT to commence the TDF treatment at 30 to 32 weeks of gestation if the pregnant women had higher viremic HBV^[73]. As the participants were

different, antiviral therapy for pregnant women with high HBV DNA levels is recommended in various guidelines including the AASLD, EASL and APASL. A long term follow-up should be conducted regarding the safety of the mothers and/or their infants. This work was funded by the State Key Projects Specialized on Infection Diseases of the Thirteenth 5-year Plan of China.

Status of immunological memory after neonatal vaccination in young adults

In children, HBV vaccination proves to be very efficacious in decreasing HBsAg seroprevalence^[23,25,27,28,33,58]. After 10-15 years of the vaccination, neutralizing antibodies (anti-HBs) waned or reached undetectable levels in many individuals, and some became seropositive with anti-HBV core antigen (anti-HBc)^[75,76]. Although HBV primary infection in the perinatal period and early life has a high rate of chronicity, the possibility of becoming chronically infected in unprotected young adulthood was reported to be about 2.7-7.7% after horizontal transmission^[12,14,77]. It is necessary to confirm the efficacy of neonatal vaccination in protecting young adults who later had low or absent levels of anti-HBs.

In order to understand the long-lasting immunity in preventing chronic HBV infection, Zanetti and colleagues introduced a booster test to assess the presence of anamnestic responses against HBV infection based on the principle that re-exposure of HBsAg in recombinant vaccine should mimic the response to wildtype HBV infection^[78]. The enrolled population in this study consisted of children born to HBsAg-negative mothers and vaccinated adolescents (Air Force recruits), who were all vaccinated 10 years before the enrollment. Serum anti-HBs levels of each individual was firstly quantified, followed by giving 10 μ g recombinant HBV vaccine if they were seronegative for anti-HBc. Serum anti-HBs level in each of the participants was quantified again two weeks later. Presence of an anamnestic response was defined as those with prebooster anti-HBs titers < 10 mIU/mL and post-booster titers ≥ 10 mIU/mL, a representation of protection conferred through immunologic memory. Later studies that used similar or modified methods, which also determined the presence of HBsAg-specific T cells, were conducted in many different populations worldwide^[79]. These results revealed that HBV vaccine does elicit immunologic memory, in which memory B cells could appropriately generate a robust anamnestic response to HBsAg even if the anti-HBs titer falls below 10 mIU/mL.

Nevertheless, the conclusion is controversial regarding the immune protection of the uninfected children/adolescents who had serum anti-HBs < 10 mIU/mL, based on the studies conducted in the vaccinated population who lived in areas with different HBV

prevalence and different maternal HBV status^[79]. Results from the investigations conducted in Taiwan^[80,81], in Thailand^[82], and in Qidong of China^[83] indicated that a notable proportion of fully vaccinated adolescents had no or low immunological memories against HBsAg. All enrolled populations in these studies received infantile HBV vaccination and lived in high HBV endemic areas. Because the duration and uniformity of this immunologic memory after primary vaccination at infancy is uncertain, these studies would suggest a necessary consideration of 1 or 2 booster doses. However, other studies conducted in Italy^[78], that enrolled participants who were all born to HBsAg-negative mothers, and in Hong Kong^[84], that enrolled children who received primary HBV vaccination at ages 3 months to 11 years, found significant anamnestic response among the vaccinated populations. Based on the percentage of the anamnestic responders, these investigators suggested that the primary vaccination confers lifelong protection against HBV infection and no booster is needed^[78,79,84,85].

HBV breakthrough infection in young adults may occur if the immunologic memory to HBsAg is absent upon sexual, or horizontal exposure including household HBV exposure^[86]. The setting of booster tests conducted in different studies was different. It is still questionable whether all the adolescents uniformly remain protected against HBV infection when they were engaged in more social activities. The duration and immunologic memory status after primary vaccination might be different when they were born to mothers with different maternal status and the ages of vaccination received. In the last decade, reports of HBV infection among the vaccinated young adults have been documented^[87,88]. A study conducted in Qidong of China, that enrolled a total of 2,919 young adults aged 19-21 years who received plasma-derived neonatal HBV vaccination found a total of 124 (4.2%, 124/2,289) participants were HBsAg negative, but double positive for anti-HBs and anti-HBc [HBsAg(-) & anti-HBs(+) & anti-HBc(+)]. None of them were positive for HBeAg or for anti-HBe or for anti-HCV. Notably, 7/124 (5.65%) individuals with seromarkers of HBsAg(-) & anti-HBs(+) & anti-HBc(+) had serum ALT ≥ 40 U/mL^[87]. Serum levels of HBV DNA were quantified among the 124 individuals, and 14/124 (11.3%) of them had > 10,000 copies/mL, 37/124 (29.8%) of them had 500-10,000 copies/mL, and 73/124 (58.9%) were below the detection limitation. The longitudinal follow-up studies found that some of the vaccinated children became infected with HBV in adulthood when they lost anti-HBs at childhood^[83,87].

Adolescent booster to children born to HBsAg-positive mothers decreased the risk of HBV infection

Although investigators worldwide have determined

and accumulated evidence regarding the presence of immunologic memory by the booster test^[79], HBV breakthrough infection still happened. For precise prevention against chronic HBV infection, it is necessary to understand the human immune responses to HBV vaccine in different individuals with distinct HBV exposure status in the prenatal period. Recently, it has been demonstrated that the immunological response pattern to microbes/microbial products in the HBV-exposed neonates was very different from the healthy ones. Prenatal exposure to HBV induced complex changes in the newborn's immune system^[89]. Follow-up studies worldwide have demonstrated that children were well protected after HBV vaccination. More evidence is needed regarding the adolescent booster effect against HBV infection based on different maternal HBsAg-carrying status.

HBsAg has been detected in amniotic fluid, cord blood, breast milk, vaginal fluids, and infant gastric content^[64,90]. According to the immune selection theory, T cells that recognize the epitopes in HBsAg with high-affinity receptors (TCR) might be deleted during immune system development^[91]. Basic immunology studies have revealed that the differentiation and proliferation of specific antibody producing B cells was regulated by a distinct T cell subset, the follicular helper T cells (Tfh)^[92]. Although the murine immune system is different from that of humans, we can understand the potential implication from the murine immune responses to model antigen. Experimental data by using the I-Ek-restricted helper T cell response of B10.BR mice to pigeon cytochrome c, the tractable protein vaccination model for studying different TCR affinities, demonstrated that significantly more T cells with high affinity TCR developed into "resident" Tfh cells *in vivo* than the T cells with low affinity TCR, and the low affinity clonotypes of T cells failed to form memory^[93,94]. The experimental data revealed that Tfh function was regulated by the strength of T cell antigen receptor binding, i.e. TCR affinity. Therefore, the function of Tfh and the B cell memory after primary vaccination in the individuals born to healthy mothers should not be the same as those born to HBsAg-positive mothers and those born to HBeAg- & HBsAg-positive mothers. Currently, no data is documented about the difference.

Sexual contact is an important pathway for HBV transmission in low HBV endemic areas^[86]. Universal neonatal HBV vaccination significantly reduces the HBsAg seroprevalence, and horizontal exposure will be the major route of HBV infection. Because of the controversial conclusion regarding immune protection of the uninfected children/adolescents who had serum anti-HBs < 10 mIU/mL, it is still uncertain whether all the children who were protected by primary vaccination

are uniformly resistant to chronic HBV infection when they grow up and engage in risky behaviors that might increase HBV exposure.

Studies were done to examine the different responses to booster doses conducted in different areas, to determine if this might be due to enrolling populations with different or unknown maternal HBsAg status. Recently, by enrolling the participants of the vaccination group in the QHBIS, Wang *et al.*^[54] addressed the question of adolescent booster effect against HBV infection in individuals born to mothers with different HBsAg-carrying status^[54]. In this study, a total of 9,793 vaccinated individuals, who were HBsAg(-) at childhood (10-11 years of age), re-donated their blood samples for HBV serological surveys in mature adulthood (23-28 years of age). Among them, a total of 7,414 children received one dose (10 µg) of recombinant HBV vaccine booster and 2,379 did not receive the adolescent booster. Although the booster was not randomized, the distributions between the participants who received the booster and those who did not receive the booster were similar in age, gender, maternal HBsAg status, maternal childbearing age, and family income per capita. Their results showed that HBV breakthrough infection occurred in the vaccinated individuals, who had been protected at childhood by a neonatal vaccination series. Some of the infection developed chronicity in adulthood, especially among the individuals who were born to HBsAg-positive mothers and lost anti-HBs or anti-HBs < 10 mIU/mL in the childhood. Hence, some of the adolescents born to HBsAg-positive mothers might be susceptible to chronic HBV infection if serum anti-HBs is < 10 mIU/mL^[95,96].

Further analysis showed that one dose of adolescent booster provided protection (against chronic HBV infection in adulthood) to these high-risk individuals who were born to HBsAg-positive mothers and had lost anti-HBs or had anti-HBs < 10 mIU/mL. Nevertheless, no booster effect was observed in those who were born to HBsAg-negative mothers, regardless of their anti-HBs status at 10-11 years of age when they completed the neonatal three-dose HBV vaccine series. The difference in immunity in the HBV-exposed neonates was proven to be very different from the healthy ones^[89]. When they grow up, their immunity and the immunological memory in the prenatal HBV-exposed individuals seem to be also different in their response against chronic HBV infection. The presence of immunological memory and effect of adolescent booster should be re-visited^[95,96]. A recent study reported that the HBsAg prevalence was 6.35-6.47% in men aged 25-39 years living in the rural areas of China^[97], indicating the high risk of HBV infection upon sexual, parenteral, or horizontal (household) HBV exposure. Following the documentation of HBV

infection among vaccinated young adults^[87,88], it might be appropriate to receive the adolescent boosters, especially for the high-risk individuals^[33,56,80,82,88].

Experience and recommendation to reduce liver cancer risk beginning at birth by preventing chronic HBV infection

To control chronic HBV infection, plasma-derived HBV vaccine, the first generation vaccine, had been administered to millions of infants. In addition, due to various reasons, the HBIG was not administered to high-risk infants who were born to HBsAg-positive mothers in many low- and middle-income countries and areas^[23]. The plasma-derived HBV vaccine has now been totally replaced by recombinant HBV vaccines, and most of the high-risk infants were immunized together with HBIG administration.

As the population who was vaccinated with plasma-derived HBV vaccine stepped into their third decades, we can get further insights from them by monitoring their HBV serological markers, and disease development. The studies based on QHBIS provided some information on HBV vaccination strategies in controlling chronic HBV infection, liver cancer, and chronic liver failure. Our accumulated clinical data on using antiviral therapies in HBV-infected Chinese pregnant women with higher viremia have shown efficacy in blocking MTCT transmission of HBV. All these experiences will be helpful for better controlling PLC, especially HCC, by eradicating HBV beginning at birth.

The experiences and recommendations are as follows [Table 1]: (1) in endemic regions, HBV vaccination in neonates is crucial against chronic HBV infection. Although catch-up vaccination given after age 10 was useful, the protection efficacy was substantially weaker compared to neonatal vaccination; (2) children/adolescents born to mothers with different HBsAg-status had distinct immunity against chronic HBV infection, even after initial protection offered by HBV vaccination. The adolescents/young adults seem to be susceptible to chronic HBV infection when they were born to HBsAg-positive mothers when they have lost anti-HBs or when their anti-HBs is < 10 mIU/mL. It is recommended to have at least one booster dose given during adolescence to those who were born to HBsAg-positive mothers and had lost anti-HBs or when their anti-HBs is < 10 mIU/mL, to ensure the immunity against chronic HBV infection; (3) to prevent MTCT transmission of HBV, screening for HBsAg in the first trimester of pregnancy is strongly recommended; (4) For pregnant women with serum HBV DNA levels > 200,000 IU/mL, antiviral treatment is recommended. Therapy with tenofovir or telbivudine should start at

Table 1: Summary of recommendations for HBV vaccinations

Setting	Recommendation
Pregnancy HBsAg-negative	Screening for HBsAg in the first trimester; Receive three doses of HBV vaccine: the first dose to be given within 24 h after delivery, the second and third doses to be given at 1 month and 6 months of age
Pregnancy HBsAg-positive HBV DNA < 200,000 IU/mL	Screening for HBsAg in the first trimester and determine the serum levels of HBV DNA; Administration of 100 IU HBIG within 12 h after birth in combination with a full course of HBV vaccination. The administration dose is 10 µg vaccine from recombinant yeast or 20 µg vaccine from Chinese hamster oocyte; The vaccinated infants should be tested for the presence of HBsAg and the anti-HBs titer 1-2 month after completing the third dose of vaccine; The fourth dose of HB vaccine should be given if he/she is seronegative for HBsAg and had anti-HBs < 10 mIU/mL
Pregnancy HBsAg-positive HBV DNA > 200,000 IU/mL	Screening for HBsAg in the first trimester and determine the serum levels of HBV DNA. Antiviral treatment is recommended; Antiviral therapy using tenofovir or telbivudine should start at week 24-28 of gestation to reduce the risk of perinatal transmission of HBV; For newborns: administration of 100 IU HBIG within 12 h after birth in combination with a full course of HBV vaccination. The administration dose is 10 µg vaccine from recombinant yeast or 20 µg vaccine from Chinese hamster oocyte; The vaccinated infants should be tested for the presence of HBsAg and the anti-HBs titer 1-2 month after completing the third dose of vaccine; The fourth dose of HB vaccine should be given if he/she is seronegative for HBsAg and had anti-HBs < 10 mIU/mL For mothers, the antiviral therapy could be discontinued at birth to 1 month postpartum. With discontinuation of treatment, women should be monitored for ALT flares every 3 months for 6 months
Vaccinated adolescents completed neonatal HBV vaccination series without HBIG administration	If born to HBsAg-positive mother, determine the HBsAg and anti-HBs levels. Receive the HBV vaccine booster to generate anti-HBs > 10 mIU/mL; If born to HBsAg-negative mother, no HBV vaccine booster necessary in general setting

HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; HBIG: hepatitis B immunoglobulin

week 24-28 of gestation to reduce the risk of perinatal transmission of HBV. The oral antiviral drugs currently are pregnancy Class C (lamivudine, entecavir, and adefovir dipivoxil) or Class B (telbivudine and tenofovir). Telbivudine or tenofovir is recommended based on the studies conducted in multiple medical centers and the recommendations from AASLD, EASL and APASL; (5) for the mothers, antiviral therapy could be discontinued at delivery to 1 month postpartum. With discontinuation of treatment, women should be monitored for ALT flares every 3 months for 6 months; (6) for infants born to HBsAg-positive mothers regardless of the serum HBV DNA levels, the administration of 100 IU HBIG within 12 h after birth should be combined with a full course of HBV vaccination. The administration dose is 10 µg vaccine from recombinant yeast or 20 µg vaccine from Chinese hamster oocyte. The vaccinated infants should be tested for the presence of HBsAg and the anti-HBs titer 1-2 months after completing the third dose of vaccine. If he/she is seronegative for HBsAg and has anti-HBs < 10 mIU/mL, the fourth dose of HBV vaccine should be given.

DECLARATIONS

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Authors' contributions

Drafted the outline of this review: C. Qu
 Drafted the parts regarding on the hepatitis B immunization: C. Qu, K. Chen
 Drafted the parts regarding on the antiviral therapy to pregnant women and prevention of mother to child transmission: Z. Duan, H. Zou
 Finalized the manuscript: C. Qu

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Conflicts of interest

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the manuscript. The findings and conclusion in this review do not represent the official position of Chinese Center of Disease Control and Prevention. The official document can be found via the link of <http://www.nhfpc.gov.cn/jkj/s3581/201701/a91fa2f3f9264cc186e1dee4b1f24084.shtml>.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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***Cancer Evo-Dev*, a novel hypothesis derived from studies on hepatitis B virus-induced carcinogenesis**

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ABSTRACT

Non-resolving inflammation, which may be maintained by infection, pollution, and metabolic stimulants and their interactions with immunogenetic predisposition, provides a fertile field for cancer development. This is strongly evident in hepatocellular carcinoma. Here, the framework of a hypothesis called *Cancer Evo-Dev* is presented, based on the advances in hepatitis B virus-induced hepatocarcinogenesis. Several aspects central to this theory are as follows: (1) immune imbalance caused by the interaction of immunogenetic predispositions and hepatitis B virus infection maintains non-resolving inflammation; (2) active inflammation executors promote mutations in viral and host genomes via disbalancing mutagenic forces including cytidine deaminases and mutation-repairing forces including uracil-DNA glycosylases, thus promoting cancer-related somatic mutations and viral mutations; (3) a small percentage of the cells whose somatic mutations alter the survival signalling adapt to the inflammatory microenvironment, de-differentiate via demethylating role of cytidine deaminases, and reversely develop into tumor-initiating cells (TICs); (4) under the cultivation of some factors like POSTN from tumor-infiltrating fibroblasts and M2 macrophages, TICs acquire the stemness, cancer-stem cells obtain distinct metastatic and drug-resistant potentials under the selection pressure from distinct microenvironments; (5) glycolysis persistence in the presence of oxygen provides essential energy for cell survival and the raw material for DNA synthesis. Thus, cancer development is characterized by an evolutionary process of “mutation-selection-adaptation”. The framework of *Cancer Evo-Dev* can be verified in other cancers. *Cancer Evo-Dev* lays theoretical foundation for understanding the mechanisms by which inflammation promotes cancer development, and it also plays a role in specific prophylaxis, prediction, and targeted treatment of cancers.

NON-RESOLVING INFLAMMATION AND HEPATOCELLULAR CARCINOMA

Inflammation, firstly characterized as “heat, redness,

pain, and swelling” by a Roman physician Cornelius Celsus, is a complex biological response to harmful stimuli such as infections and tissue damage. Inflammation can be classified into acute inflammation and chronic inflammation. Acute inflammation, also



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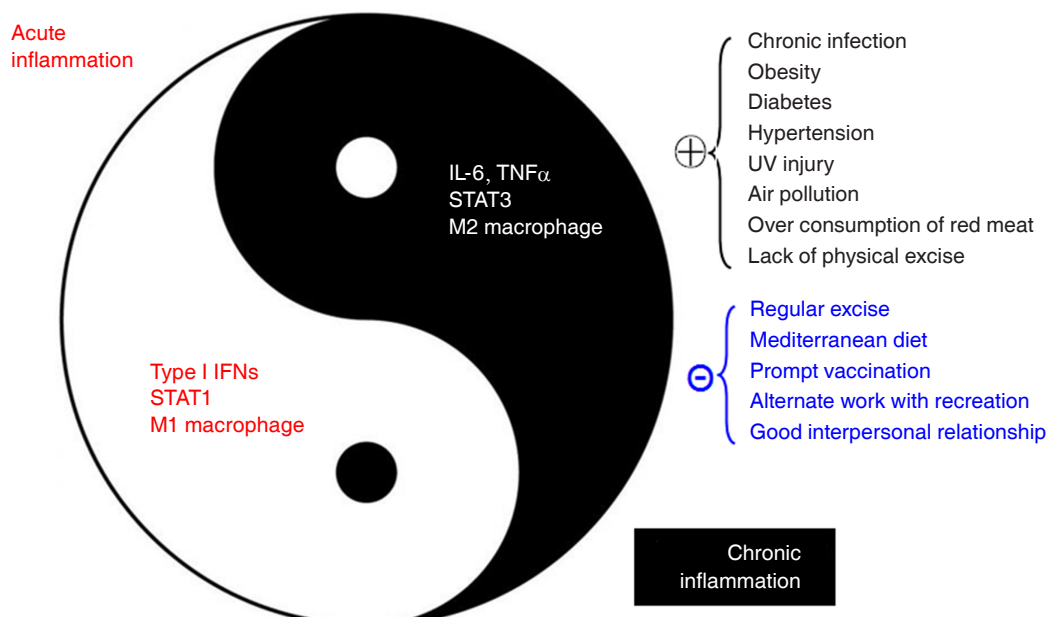


Figure 1: Factors affecting chronic inflammations and their associations with acute inflammations. IFN: interferon; STAT: signal transducers and activators of transcription; IL: interleukin; TNF: tumor necrosis factor; UV: ultraviolet

termed as resolving inflammation, is an initial stage of inflammation mediated through activation of innate immunity; it lasts for short period. Chronic inflammation, also termed as non-resolving inflammation, is the second stage of inflammation that persists for a long period of time. Chronic inflammation may develop from acute inflammation if the stimuli are not eradicated or inflammation appears with a chronic process, reflecting the weak but active nature of host immunity. Although the two kinds of inflammation are closely linked to form a correlative antagonistic unity, inherent mechanisms regarding proinflammatory molecules, types of infiltrating macrophages, and inflammatory pathways are distinct, as shown in Figure 1^[1]. Acute inflammation is often regarded as therapeutic inflammation to ward off infections and/or to repair the tissue damage; whereas chronic inflammation is now considered as pathogenic, being closely linked with most chronic illnesses, such as cancer, cardiovascular diseases, diabetes, obesity, pulmonary diseases, neurologic disorders, and even depression^[2]. Non-resolving inflammation is the prerequisite for the development of most cancers. For examples, chronic hepatitis B or C, chronic bronchitis, chronic colitis including ulcerative colitis, chronic cervicitis, chronic atrophic gastritis, and chronic esophagitis (gastroesophageal reflux disease - caused Barrett's esophagus) often precedes liver cancer, lung cancer, colorectal cancer, cervical cancer, gastric cancer, and esophageal cancer, respectively. Non-resolving inflammation is clearly evident in the development of hepatitis B virus (HBV)-induced hepatocellular carcinoma (HCC). It is generally believed that oral-administered antiviral therapy decreases the risk of developing HCC in patients with chronic hepatitis

B (CHB)^[3]. However, the risk of developing HCC is significantly higher in the oral nucleos(t)ide analogues-complete responder group compared with the inactive CHB group, regardless of the presence of baseline liver cirrhosis^[4], indicating that continuous active inflammation in liver facilitates the development of HCC. Although surgical technologies for the treatment of liver cancer have been improved, postoperative prognosis remains to be precisely evaluated^[5-7]. Active inflammation on chronic inflammation background, as reflected by an Ishak hepatic inflammation score (> 6), a higher neutrophil-to-lymphocyte ratio (> 5), and a higher C-reactive protein in sera (> 0.3 mg/dL), etc., also indicate a poor postoperative prognosis such as postoperative recurrence and shorter recurrence-free survival in HBV-related HCC (HBV-HCC) patients^[8,9]. Nuclear factor- κ B (NF- κ B) and signal transducers and activators of transcription 3 (STAT3) are two most important transcription factors involved in inflammatory pathways that play predominant roles in carcinogenesis, especially in HBV-induced hepatocarcinogenesis^[10,11]. Thus, inflammatory microenvironment including proinflammatory molecules, tumor-associated fibroblasts, and tumor-associated immune cells with altered expression of the inflammatory pathways facilitates the evolution and development of cancers.

Maintenance of chronic HBV infection and hepatic inflammation

Chronic transformation of HBV infection relies on three aspects: infection occasion, the characteristics of HBV genotypes, and genetic predisposition of the key immune molecules. HBV infection in early childhood

is generally believed to be one of the major causes of chronic HBV infection in adulthood. Of the infants born to hepatitis B surface antigen (HBsAg)-positive mothers globally, 42.1% who did not receive HBV passive-active immunoprophylaxis and 2.9% of infants who received the immunoprophylaxis acquired HBV infection perinatally. The perinatal infection occurred in 84.2% and 8.7% of infants born to hepatitis B e-antigen (HBeAg)-positive mothers who did not and did receive immunoprophylaxis, respectively. The infection rates were 6.7% and 0.4% for infants born to HBeAg-negative-carrier mothers, respectively. Moreover, the chronicity rates of HBV infection acquired perinatally were 28.2% in infants born to HBeAg-negative mothers and 64.5% in infants born to HBeAg-positive mothers^[12]. This is possible due to the fact that the immaturity of immune system in infants make it unable to recognize HBV as an external antigen, thus establishing chronic HBV infection although the immune response can be aroused thereafter. Clearly, perinatal HBV infection is an important but not the predominant cause of chronic HBV infection in adulthood. Chronic transformation of acute hepatitis B caused by horizontal transmission among adolescents and adults contributes to the remaining proportion of chronic HBV infection. Approximately 8.5% of acute hepatitis B in adults in Shanghai, China, develops into chronic HBV infection 6 months after acute infection^[13]. In mainland China where genotypes B (B2) and C (C2 and C1) are endemic, HBV subgenotype B2 is more apt to causing acute infection because of higher viral load in the virus-providing chronic carriers whereas HBV subgenotype C2 is more apt to causing chronic transformation following an acute course^[13,14]. HBV C2 is more likely to develop liver cirrhosis and HCC than does HBV B2, the two major HBV subgenotypes endemic in China, possibly because of the stickiness nature of HBV subgenotype C2^[15-17]. The third most important cause of chronic HBV infection and active inflammation is the genetic predispositions of some key immune and proinflammatory molecules. Genome-wide association study in the eastern Asian populations have shown that a total of 11 single nucleotide polymorphisms (SNPs) in genetic loci including HLA-DPA1 and HLA-DPB1, some SNPs in genetic loci including HLA-DQ and -DR, and a locus near HLA-Care significantly associated with CHB^[18-22]. Interestingly, these SNPs in the loci encoding human leukocyte antigen-class II (HLA-II) are also significantly associated with vaccine response as well as the risks of acute-on-chronic liver failure, HBV-related liver cirrhosis, and HBV-associated HCC^[23-27]. Interestingly, different human races have different allelic frequencies of SNPs that affect the expression of HLA-DP, HLA-DQ, and the inhibitory component of NF- κ B complex I κ Ba gene *NFKBIA*. These genetic loci whose dominant alleles are significantly association with increased risks of chronic progression of HBV infection

(or whose rare alleles are significantly associated with decreased risks of chronic HBV infection) include rs3138053 (affecting *NFKBIA*), rs2856718, rs7453920, and rs9275319 (affecting HLA-DQ), and rs9277378, rs2395309, rs2301220, and rs9277341 (affecting HLA-DP)^[12]. The polymorphic genotypes that are significantly association with increased risks of chronic progression of HBV infection as well as immune selection of the end-stage liver diseases-associated HBV mutations are more frequent in the Han Chinese than in European populations. These data indicate that the Han Chinese are inherently more apt to progressing into chronic infection once exposed to HBV infection than European, whereas European tend to recover from HBV infection spontaneously^[12]. This might be one of the reasons why chronic HBV infection and the HBV-induced end-stage liver diseases are more frequent in Chinese than in European populations. The HLA-II genetic polymorphisms may predispose immune imbalance upon HBV infection, impair immune function for HBV clearance, and maintain chronic HBV infection and hepatic inflammation, and thus facilitating the progression of CHB into liver cirrhosis and HCC.

“Dead-end” evolution of HBV

During HBV-induced hepatocarcinogenesis, both of hepatic cells and the viruses experience the process of evolution. Viral evolution serves as a valuable clue to investigate the mechanism underlying HBV-induced hepatocarcinogenesis. HBV belongs to the Hepadnaviridae family found in both mammals (orthohepadnaviruses) and birds (avihepadnaviruses) and is highly conserved in their host species during the long-term evolutionary process. Although primate hepadnaviruses are indigenous to their hosts, hepadnaviruses isolated from apes are grouped as HBV genotypes in phylogenetic analyses. With only 5% divergence from the chimpanzee viral isolates, the isolates from gorilla are categorized in the HBV genotypes. Avihepdnaviruses are the most distant relatives of HBV with a nucleic acid homology of only 40%. Compared to avihepdnaviruses, Woodchuck hepatitis virus and ground squirrel hepatitis virus as mammalian hepadnaviruses are more closely related to HBV and differ by only 17%. Genetic evolution analysis indicates that HBV and orthohepadnaviruses from non-human primates are phylogenetically clustered in the same evolutionary branch^[28]. These evidences indicate that members of Hepadnaviridae family are highly conserved in their evolutionary history. However, HBV experiences a relative rapid evolution in their genome since chronic HBV infection is established in a subset of infected populations.

Previous researches by our group identified the wild-type HBV sequences (so-called the standard

sequences) of HBV subgenotypes B2 and C2, based on the whole HBV genome sequenced using approximately 1,000 asymptomatic HBsAg carriers from community-based epidemiological surveys in the Yangtze river delta region of mainland China. Based on the wild-type HBV sequences, we subsequently characterized the end-stage liver diseases-related mutations and their development patterns in HBV subgenotypes B2 and C2. We found that the HBV mutations posing a significant risk of HCC or liver cirrhosis were mainly located within the enhancer II/basal core promoter/precore (EnhII/BCP/PreC) and preS regions of HBV genome^[29-31]. We summarized the data concerning the association of the HBV mutations and HCC risk published up to 2009, and found that the frequencies and locations of the HBV mutations accumulate consecutively during the “trilogy” of HBV-induced carcinogenesis (CHB, liver cirrhosis, and HCC) and that these HBV mutations can be applied to predict the occurrence of liver cirrhosis and HCC^[32]. In our prospective cohort study, we have identified the baseline HBV mutations (C1653T, A1762T/G1764A, and T1753V) increase the risk of HCC both independently and “dose-dependently”. The so-called “dose-dependently” is referred to that the HCC risk is significantly higher in the CHB patients carrying one of the three mutations than in those without the mutation, is significantly higher in those with two of the three mutation than in those with one of the three mutation, and is also significantly higher in those with all the three mutation than in those with two of the three mutations. Thus, the baseline HBV mutations in combination are able to predict the occurrence of HCC in CHB patients^[33]. Several longitudinal studies carried in China have also demonstrated that baseline A1762T/G1764A mutation increases the risk of HCC in chronic HBV carriers or CHB patients^[34-37]. Among the HCC-risk HBV mutations, the A1762T/G1764A is usually detected in the early stage in young adolescents, while other mutations including T1753V, C1653T, G1899A, and preS deletion appear only at the late stage of chronic HBV infection^[12,38]. Reaction to chronic HBV infection, as characterized by immune response-induced hepatocyte damage and release of transaminase, facilitates the generation of the HBV mutations, indicating active immune selection of the HBV mutants during HBeAg seroconversion from HBeAg-positive to HBeAg-negative. One of the main features of HBV mutations is a deficiency of the CD8⁺ T-cell epitope, a consequence of immune selection. Reduction of CD8⁺ T cell epitopes of HBV is one of the common strategies to evade immune eradication. HBV that has a low density of CD8⁺ T cell epitope in their core and X proteins are selected during long-term evolution^[39], thus CD8⁺ T cells play an important role in the immune selection of HCC-related HBV mutants [Figure 2].

HBV acquired during infancy or early childhood, or at early infection stage in adults, is usually in the form of wild-type^[12,38]. In the initial immune tolerant phase of chronic HBV infection, HBeAg is positive, viral load is high, and immune pressure is weak. With the progression of chronic infection, especially during HBeAg seroconversion, the proportion of HBV mutants gradually increases^[40]. Although the HBV strains carrying the HCC-related mutations are present in the cord blood of infants, neonatal infection is usually caused by wild-type HBV strain rather than the mutant ones. In the HBV-infected children, the frequencies and locations of HCC-related mutations increase with increasing age. However, compared with their mothers who have been exposed to chronic infection for approximately 25 years, children have fewer HCC-related HBV mutations^[38]. In individuals with chronic HBV infection, HBV is synthesized and packaged in hepatocytes and released into the circulation at a pace of up to 1011 viral particles daily. HBV is regularly cleared from the circulation by the host immune system, with a half-life of approximately 1.2 days. Thus, hepatocytes and their surrounding immune cells are responsible for the generation of HBV mutants^[41]. HBV reverse transcriptase lacks proofreading activity, resulting in an estimated mutation rate of 4.57×10^5 nucleotide substitutions per site per year and this rate will increase after HBeAg seroconversion^[42]. Inflammatory factors in the microenvironment of inflammatory liver promote the generation of HBV mutations, at least partially, via activating the human apolipoprotein B mRNA-editing enzyme catalytic polypeptide (APOBEC) family of cytidine deaminases^[43,44]. Although many HBV genome fragments including the PreC/BCP/EnhII region and the S region are generally sensitive to editing by members of APOBEC3, the sequence encoding HBV X protein (HBx) is more vulnerable. APOBEC3 prefers the HBx region as its editing target and generates carboxylic acid-terminal truncated HBx (Ct-HBx)^[44], the main form of HBV DNA integrated into the host genome. Insufficient immune responses elicited by HBV antigens select disease-related HBV mutations during the long-term infection process. Only the HBV variants best adapted to the host immune system will survive and thrive in liver. HBV accumulates mutations via minimizing the total number of epitopes recognized by CD8⁺ T cells, particularly in the HBx and the preS1/preS2/S regions, to avoid immune clearance^[39]. These HBV mutations are probably selected via virus-immune interactions in the inflammatory microenvironment. Because of overlapping open reading frames, HBV mutations altering the genes necessary for viral replication are unlikely transferred into their progeny viruses. Natural selection ensures only the fittest survive to pass their genes on to the next generation. Thus, the random

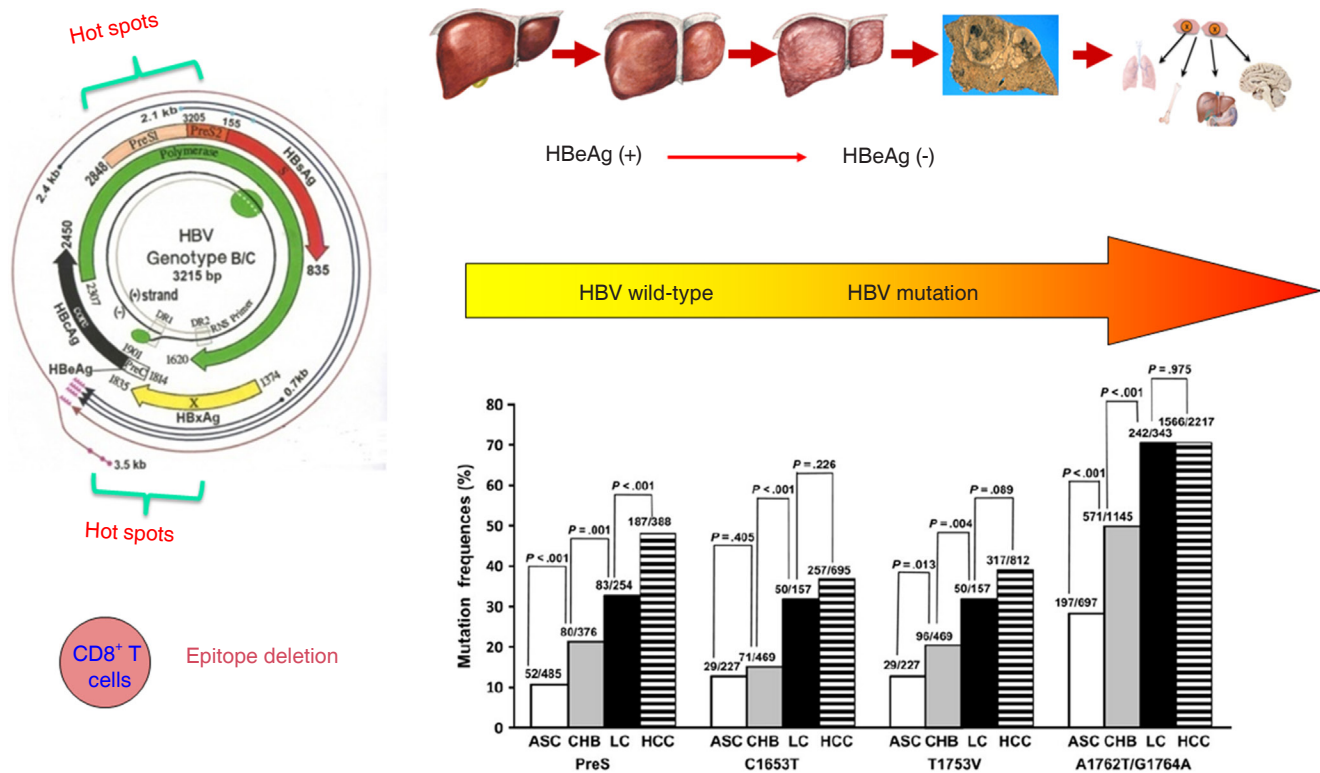


Figure 2: The HCC-related HBV mutations are hallmark molecular events during HBV-induced carcinogenesis. ASC: asymptomatic HBeAg carrier; CHB: chronic hepatitis B; LC: liver cirrhosis; HBeAg: hepatitis B e antigen; HCC: hepatocellular carcinoma

natural mutations are therefore constrained to special regions of the HBV genome.

The inflammatory microenvironment of liver tissue is therefore necessary for the co-evolution of HBV and the host genome^[45]. Although tumor-adjacent tissues are pathologically categorized as inflammatory hepatic tissues, they are typical precancerous lesions and have already reached the middle stage of HCC evolutionary process because somatic mutations might occur in the inflammatory liver tissues. Somatic mutations in given genomic locations will become the driving mutations if they confer growth advantage of the mutated hepatocytes. The HCC that relapses more than 2 years after curative resection is believed to be mostly recurrent HCC and not from a spread of the initial HCC cell diffusion into the remnant liver tissue. The species and frequencies of certain HBV mutations in adjacent tissues are different in HBV-infected patients with HCC (HBV-HCC patients) with distinct postoperative prognosis. Together with immune markers and expression levels of inflammatory genes in adjacent hepatic tissues, the HBV mutations can be applied to predict postoperative prognosis in HCC patients^[46]. The HBV mutations in the EnhI/BCP/PreC region such as A1762T/G1764A in the remnant liver after curative surgery or in the circulation before liver transplantation have been proven to be predictive markers for postoperative survival and HCC

recurrence, although this result has not been repeated by some research groups^[47-49]. This indicates that HBV evolution in adjacent tissues continues until the patient dies. Antiviral therapy that can attenuate viral replication and subsequent hepatic inflammation notably promotes postoperative prognosis of HBV-HCC patients^[50-53], possibly because antiviral treatment can block HBV evolution in adjacent hepatic tissues and also likely in remaining HCC tissues.

Thus, Hepadnaviridae family members, including HBV, are highly conservative across species with distinct but connected evolutionary background. Wild-type HBV has the advantage of infecting hepatocytes of new hosts, facilitating viral spread from one individual to another, and contributing to the maintenance of viral species. The HCC-related HBV mutants can cause or promote malignant transformation, but might have lost the advantage of person-to-person transmission. Those mutants are therefore eliminated if their host individuals died of the end-stage liver diseases including HCC, which is termed “dead-end” evolution of HBV.

The HCC-related HBV mutated X or large S fragments promote the malignant phenotypes

As described above, the baseline HBV mutations including A1762T/G1764A, C1653T, and T1753V in the 3' terminal of HBx gene in sera “dose-dependently”

predict the occurrence of HCC in longitudinal studies, especially cohort studies^[33-37]. The frequency of HBV preS deletion also increases consecutively from ASCs to HCC and preS mutation is associated with a 3.77-fold increased risk of HCC^[32]. The predictive value of HBV preS deletion on the occurrence of HCC in patients chronically infected with HBV has been confirmed in a prospective study^[54]. Recent deep sequencing analysis has demonstrated that the preS deletions involving a specific fragment (nt2977-3013) in HBV genotype C are significantly associated with HCC^[55]. These epidemiological evidences indicate that the HBV mutations including preS deletion, A1762T/G1764A, C1653T, and T1753V are the etiological factors of HBV-induced HCC. Experimentally, *in vitro* transfection with the HBx mutants with changes that correspond to A1762T/G1764A, T1753A, T1768A, or a combination of these (combo) showed that the combo mutant decreased levels of p21, increased cyclin E expression, and increased expression of S-phase kinase-associated protein 2 (SKP2) in primary human hepatocytes and HepG2 cells. The combo mutant accelerated p21 degradation and cell cycle progression in HepG2 cells. Thus, HBx mutants with changes that correspond to a combination of the core promoter mutations up-regulate SKP2, which then down-regulates p21 via ubiquitin-mediated proteasomal degradation. The core promoter mutations might increase the risk of HCC by this pathway^[56]. Transfection of full-length HBV genome with the core promoter mutations in combination also upregulated SKP2 expression via activating the E2F1 transcription factor and in turn downregulate cell cycle inhibitors, thereby accelerating cellular proliferation^[57]. Mutations in the preS and S regions also notably facilitate carcinogenesis. Transfection of Huh7 cells with the large S region with preS deletion has shown that HCC-associated single-nucleotide variants (SNVs) in the small surface region of HBV genome influence carcinogenesis pathways, including endoplasmic reticulum-stress and DNA repair systems^[55]. The HBV large envelope protein gene fragment (preS1/preS2/S), with F141L mutation in the preS2 region, can significantly promote the proliferation of hepatocytes by downregulating the p53 and p21 pathways and upregulating the expression of cyclin-dependent kinase 4 and cyclin A. The colony-forming rates of hepatocytes expressing F141L-large envelope protein are about twice as high as those expressing the wild-type HBV large envelope protein^[58]. Random integration of HBV DNA into the host genome is present in HBV-infected subjects. If the integration events endow the hepatocytes with growth advantage, the integration might facilitate the development of HCC, therefore, have the opportunity of being recorded. HBV integration is common in HBV-HCC, leading to the truncation of

the HBV genome, particularly at the C terminus of HBx (Ct-HBx)^[59]. Ct-HBx can enhance cell invasiveness and metastasis of HCC in a manner that is more potent than that evoked by full-length HBx and often predict the poor postoperative prognosis and ineffectiveness of antiviral prophylaxis for HCC recurrence^[51,60]. These evidence indicates that some HBV X mutants and large S mutants can promote the development and progression of HCC.

Interaction of genetic predispositions of immune or inflammatory molecules with the HBV mutations in HBV-induced hepatocarcinogenesis

As described above, the HLA-II genetic polymorphisms are statistically associated with the outcomes of exposure to HBV^[18-27]. This genetic background may predispose immune imbalance upon HBV infection. In our previous epidemiological studies, we found that the HLA-II genetic polymorphisms are statistically associated with the generation of the liver diseases-associated HBV mutations. The HLA-DP polymorphisms rs3077 (CT + TT vs. CC), rs3135021 (GA + AA vs. GG), rs9277535 (GA + AA vs. GG), and rs2281388 (CC vs. CT + TT) significantly decrease HBV persistence in genotype B HBV-infected subjects; HLA-DP genotypes that promote HBV clearance are associated with a lower prevalence of HBV mutations increasing HCC risk (C1653T, T1674C/G, A1846T, G1896A, preS2 mutations, and preS deletion in genotype C) and a higher prevalence of HBV mutations decreasing HCC risk (G1652A, T1673C, T1674C, G1719T, G1730C, and G1799C in genotype B and A1727T in genotype C); furthermore, significant effects of HBV mutations on cirrhosis and HCC are selectively evident in those with the HLA-DP genotypes that promote HBV persistence^[61]. Thus, the HLA-DP polymorphisms affect genotype B HBV clearance, regulate immune selection of viral mutations, and influence cirrhosis and HCC risks contributed by the HBV mutations. In addition, HLA-DQ genetic polymorphisms rs2856718 variant genotypes are significantly associated with an increased frequency of HBV A1726C mutation, a cirrhosis-risk, HCC-protective mutation, in genotype C; a rs9275319 variant genotype (GG) is significantly associated with an increased frequency of preS1 start codon mutation, an HCC-risk mutation, in genotype C. Thus, the HLA-DQ polymorphisms affect the risks of cirrhosis and HCC differently in chronic HBV-infected subjects, possibly via interacting with the HBV mutations^[62]. As NF- κ B and STAT3 are two most important inflammatory pathways^[10,11], their genetic predispositions affecting the expression of both signaling pathways may play roles in HBV-induced hepatocarcinogenesis. We have demonstrated that STAT3 SNP rs2293152 (GG vs. CC) is significantly associated with HCC risk compared with the

subjects without HCC. Compared with HCC-free HBV-infected subjects, rs2293152 GG is solely associated with HCC in women. This genotype is significantly associated with high viral load ($\geq 1 \times 10^4$ copies/mL) and increased frequencies of T1674C/G and A1762T/G1764A. Multiplicative interaction of STAT3 rs1053004 with T1674C/G significantly increases HCC risk, whereas rs2293152 and A1726C interaction reduces it, adjusting for covariates including HBV mutations in the EnhII/BCP/PreC region; the interaction of rs4796793 with preS2 start codon mutation significantly increases HCC risk, adjusting for covariates including HBV mutations in the preS region. Thus, STAT3 SNPs appear to predispose the host with HBV mutations to hepatocarcinogenesis^[63]. We have also demonstrated that genetic polymorphisms improving NF- κ B activity contribute to genotype B HBV clearance. In the genotype C HBV-infected subjects, variant genotypes of rs2233406 (NFKBIA-826C>T) are significantly associated with an increased risk of HCC compared with HCC-free HBV-infected subjects and significantly increase the frequencies of HCC-related HBV mutations including A1762T/G1764A, T1753V, preS1 start codon mutation, and preS deletion; Del allele of rs28362491 (NFKB1-94Ins>Del) significantly increase the frequency of A1762T/G1764A and reduce the frequency of preS2 start codon mutation. The variant genotypes impair NFKBIA promoter activity in hepatic cells. The interaction of rs2233406 variant genotypes (CT + TT vs. CC) with A1762T/G1764A significantly increase the risk of HCC in genotype C HBV-infected subjects^[64,65]. These lines of evidence imply that immunogenetic polymorphisms may predispose chronic transformation of HBV infection, increase the frequencies of viral mutations via activating cytosine deaminases, and facilitate immune selection of HCC-causing HBV mutations via arousing active but not effective immune response against the pathogen.

DEVELOPMENT AND EVOLUTION (*DEV-EVO*), A NOVEL HYPOTHESIS RELATED TO CARCINOGENESIS

Development is referred to the process that a fertilized egg develops into an individual. In humans, the fertilized diploid cell composing of paternal haploid and maternal haploid differentiates into various functional and/or structural cells to form different organs and tissues of an infant in mother's uterus within 40 weeks. The developmental process is a succession of functional and morphologic changes from a single cell form (fertilized egg) to a multicellular form (blastocyst), from an aquatic state (living in amniotic fluid) to a terrestrial state (pulmonary respiration). During this process, the founding diploid cell, a progenitor, divides rapidly and

gives many other different types of cells via altering their gene expression profiling. The changes in gene expression profiling are achieved by epigenetic modifications such as methylation in the upstream regulatory regions of given genes, rather than altering the primary sequences of these genes. After born, lung takes over the responsibility of gas exchange, some genes only expressed in the embryonic stage are silenced and other genes solely expressed in adult cells are activated, most possibly by some epigenetic modifications. Surprisingly, the developmental process of humans resembles the process of long-term organic evolution morphologically, e.g. from single cell creatures to multicellular creatures, and from aquatic creatures and amphibian to terrestrial mammals [Figure 3]. Furthermore, some evolutionarily conserved molecular networks such as HOX, Hedgehog, and Myc play important roles in the developmental process^[66-72], indicating development and evolution share some inherent mechanisms. During the past 20 years, the discovery of conserved gene networks that control embryonic development and the ability to examine genomic records has revolutionized Darwinian evolutionism that animal relationships had to be deduced by observation of external morphological characteristics. The integration between developmental biology and evolution has been named *Evo-Devo*^[73-77]. Dr. Raff pointed out that the evolution of complex organisms such as animals and plants had involved marked changes in morphology and new features had appeared; but evolutionary change occurred not by the direct transformation of adult ancestors into adult descendants but rather when developmental processes produced the features of each generation in an evolving lineage. Thus, evolution cannot be understood if do not understand the evolution of development, and how the process of development itself biases or constrains evolution^[75]. Based on these previous work, in combination with previous observations, I like to define *Evo-Dev* as follows: *Evo-Dev* is a discipline to investigate the inherent mechanisms by which the short-term developmental process resembles the long-term evolutionary process and to characterize the role of developmental process on the evolution of complex organisms.

Carcinogenesis represents an evolutionary process. It was firstly proposed by Dr. Nowell in 1976 that most neoplasms arise from a single cell of origin, and tumor progression results from acquired genetic variability within the original clone allowing sequential selection of more aggressive sublines; tumor cell populations are apparently more genetically unstable than normal cells, perhaps from activation of specific gene loci in the neoplasm^[78]. Cancer clone genetic diversification and sub-clonal selection occurs within tissue ecosystems.

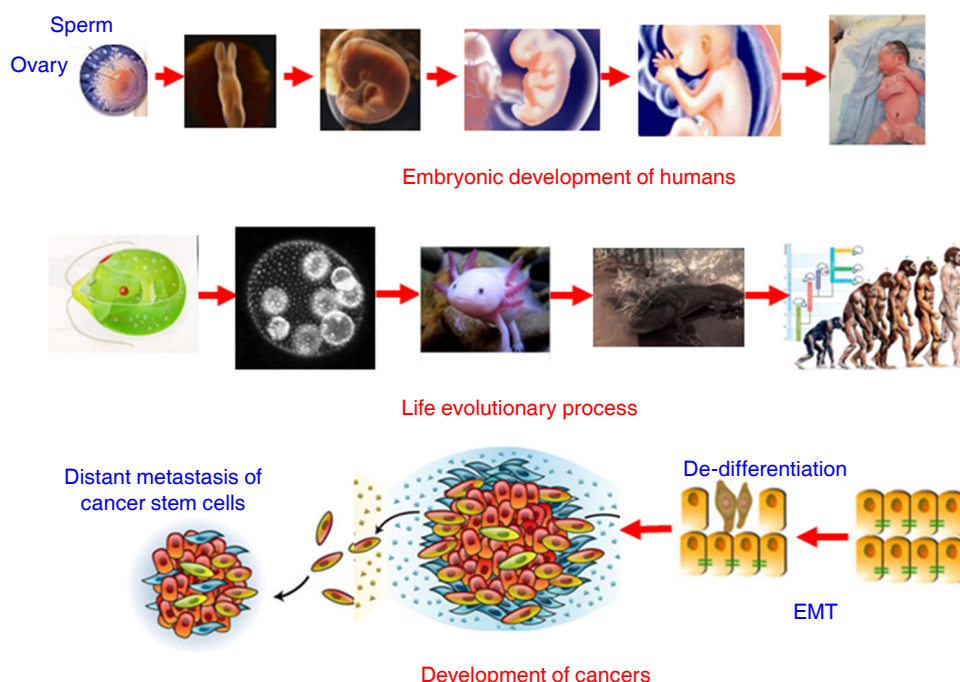


Figure 3: Synthesis of *Dev-Evo* and its potential link to carcinogenesis. *Dev-Evo*: synthesis of development and evolution; EMT: epithelial-mesenchymal transition

The implicit parallel was to Darwinian natural selection with cancer equivalent to an asexually reproducing, unicellular, quasi-species^[79]. In the past decades, especially after widespread application of new generation sequencing, cancer evolution including a reiterative process of clonal expansion, genetic diversification, and clonal selection within the tissue ecosystems have been extensively investigated. Drs. Greaves and Maley^[80] outlined key contents of cancer evolution as follows: (1) cancers are characterised by divergent cells of origin and mutational spectra; (2) cancers evolve over variable time frames (about 1-50 years) and tempos and the clonal structure, genotype and phenotype shifts over time; (3) the number of mutations found in any cancer can vary from 10-20 to hundreds of thousands, the great majority are “passengers” and a few are “drivers”; (4) cancers acquire, via mutational (and epigenetic) changes, a variety of critical phenotype traits that compound to empower territorial expansion, via proliferative self-renewal, migration, and invasion; (5) advanced, disseminated or very malignant cancers appear to be almost uniquely competent to evade therapy; and (6) this complexity can be explained by classical evolutionary principles. Most mutational processes have biases at the DNA sequence level and mutational spectra in cancers can reflect or implicate particular error-prone repair processes or particular genotoxic exposures, e.g. cigarette carcinogens, UV light, and chemotherapeutics^[81]. We believe that somatic mutations increase with increasing age for

two major reasons: (1) accumulation of exposures to various mutagens increases with increasing age; and (2) “mistake” mutations can be spontaneously generated or introduced in each cell cycle. However, the most of somatic mutations do not alter gene expression-defined key functions related to cell growth advantage or resistance to environmental insults. These mutations can be “passenger mutations”. If a somatic mutation endows the cell a growth advantage in a hostile inflammatory microenvironment, it is termed as “driver mutation”. Cells carrying “driver mutation” are positively selected in inflammatory microenvironment, facilitate the cross-talks with proinflammatory cells including tumor-associated M2 macrophages and neutrophils, promote a process termed as epithelial-mesenchymal transition (EMT) and stepwise de-differentiation into tumor-initiating cells (TICs), and adapt to new inflammatory microenvironment [Figure 3]. In our previous study, we found that expression of periostin (POSTN) from tumor-infiltrating fibroblasts significantly promoted the proliferation, anchorage independent growth, invasion, and chemo-resistance of cancer cells; whereas these effects were counteracted via targeting to PI3K/Akt or Wnt/ β -catenin signaling pathway. These evidence indicates that POSTN generated in the microenvironment nurtures the cancer-stemness via activating PI3K/Akt or Wnt/ β -catenin signaling pathway^[82]. Thus, cancer development represents “mutation-selection-adaptation” evolutionary process in proinflammatory microenvironment, which is quite in accordance with

Darwinian evolutionism.

During human embryo development, most genes expressing at this stage will be silenced after birth, but some genes including that encoding α -fetoprotein (AFP), will re-express for a short period of time when liver or testis is injured. Importantly, cancer is characterized as a reverse-developmental process, that is, develop from differentiated cells into undifferentiated cells. Oncofetal proteins are mostly referred to those that normally express at embryonic stage are silenced after birth, and then re-expressed persistently in the circulations of cancer patients. AFP serves as a diagnostic biomarker of HCC. The human homologue of the *Drosophila* spalt homeotic gene, SALL4, encoding an oncofetal protein Sall4, is one of the key factors for self-renewal and maintenance of embryo stem-cell pluripotency. SALL4 is expressed in the human fetal liver and silenced in the adult liver, but can be detected in a subgroup of HCC. The re-expression of SALL4 is related to the “stem function” of HCC cells and indicates invasion and unfavourable prognosis^[83-86]. As a matter of fact, some cells with stem-cell-like characteristics become the main malignant subgroup in tumor tissues; embryonic or stem-like gene expression signatures expressed in cancers of distinct histotypes including HCC are robustly associated with cancer cell self-renewal, EMT, increased aggressiveness, and poor postoperative prognosis^[87-92]. Furthermore, the cell aging process is accompanied by the shortening of telomeres, which does not seem to occur in cancer cells. Telomerase activation occurs through telomerase reverse transcriptase (hTERT) induction. hTERT and ZEB1 form a complex, which directly binds to the E-cadherin promoter, and then inhibits E-cadherin expression and promotes EMT^[93]. Sirtuin 1 has been implicated in telomere maintenance and HCC growth. Sirtuin 2, another member of the sirtuin family, plays a role in maintaining the motility, invasiveness, and EMT phenotypes of HCC^[94]. In terms of morphology, EMT is the process in which epithelial cells lose their epithelial characteristics and acquire mesenchymal characteristics, structure, and biologic function. An EMT usually occurs at a critical stage of embryonic development, and it is equally important for cancer metastasis^[95]. In the process of cancer invasion through EMT, epithelial cells acquire “stemness”, including self-renewal and antiapoptotic capacities. Most tumor cells are differentiated, with limited amplification ability. However, a small proportion of tumor cells with the “stemness” feature becomes the main malignant cell subsets in tumors and is known as cancer stem cells, responsible for the disease’s malignant nature and chemo-resistance. Thus, retro-differentiation or reverse-development is the hallmark in cancer development.

THEORETICAL FRAMEWORK OF CANCER EVO-DEV

Cancers are caused by the accumulation of somatic mutations - a process that abides by the Darwinian evolutionism: mutation-selection-adaptation. Somatic mutation patterns related to chronic inflammation have been identified in most cancers^[96]. In some cancers, the inflammation-related somatic mutations increase with time, accompanied by a decline in the mutations related to the initial exposure^[97]. Those distribution characteristics and the switch in mutation domination can be analyzed from an evolutionary perspective, suggesting that inflammation sometimes induced by chronic infection might not only cause somatic mutations, but also play an important role in selecting TICs as a cancer-supportive niche. We ever proposed the scientific hypothesis of Cancer Evolution-Development (“*Cancer Evo-Dev*”) and summarize the basic concepts and theoretical framework^[98-100]. Here, some further evidence are presented to optimize the theoretical framework of *Cancer Evo-Dev*. This novel scientific hypothesis may help in elucidating the mechanisms by which cancer develops and optimizing the most cost-effective ways to control these life-threatening diseases.

Figure 4 depicts the framework of *Cancer Evo-Dev* exemplified by HBV-induced hepatocarcinogenesis. Evolutionary process of HCC is a succession of important molecular events-from inflammatory precancerous lesions to carcinogenesis, postoperative recurrence, and metastasis. Those events include, but are not limited to viral mutation, epigenetic modification, somatic mutations, and alteration of signaling pathway networks. The synergetic effects of genetic and environmental factors contribute to imbalance of the host immune system, resulting in the activation and maintenance of non-resolving inflammation, thus providing a microenvironment for the process of cancer evolution and development. Under conditions of non-resolving inflammation, activated NF- κ B complex and proinflammatory molecules can trans-activate the expression of nucleic acid editing enzymes including APOBEC family of cytidine deaminases, rather than uracil DNA glycosylases (UNG), thus promoting viral and somatic mutations. Actually, the imbalance between mutation-promoting forces like AID/APOBECs and mutation-repairing forces like UNGs is responsible for the generation of somatic and viral mutations^[99]. Viral mutants facilitate the malignant transformation of normal hepatic cells via inducing EMT. Most mutant cells are eliminated by selective pressures imposed by the weak but active immune response. Although a small proportion of mutant cells survive in the hostile

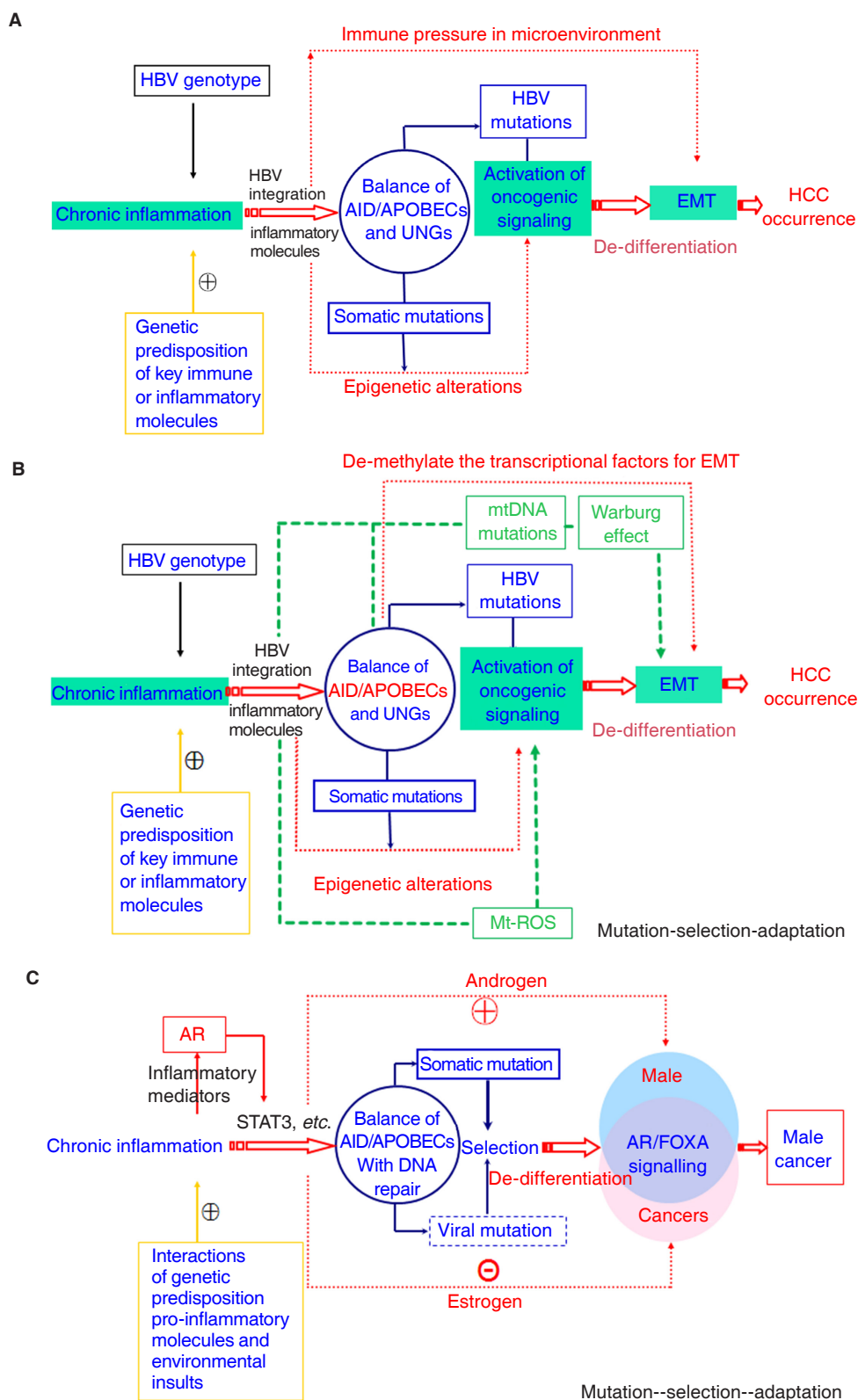


Figure 4: Theoretical framework of *Cancer Evo-Dev*, as exemplified by HBV-induced hepatocarcinogenesis. A: The classic diagram of *Cancer Evo-Dev*. The imbalance between mutation-promoting forces like AID/APOBECs and mutation-repairing forces like UNGs is responsible for the generation of somatic and viral mutations; B: exposures to some mutagens and HBV infection lead to mitochondria DNA mutations, thus promoting Warburg effect. In addition, AID/APOBECs also demethylates the promoters regions of some transcriptional factors including, thus directly promoting EMT; C: the diagram of *Cancer Evo-Dev* explaining why HCC is a male sex-predominant cancer. mtDNA: mitochondria DNA; AID/APOBECs: activation-induced cytidine deaminases/the human apolipoprotein B mRNA-editing enzyme catalytic polypeptide; UNG: uracil DNA glycosylase; EMT: epithelial-mesenchymal transition; HBV: hepatitis B virus; AR: androgen receptor; ROS: reactive oxygen species; Mt: mitochondria; HCC: hepatocellular carcinoma

inflammatory microenvironment in precancerous lesions. Those surviving mutant clones later evolve into TICs by altering the original cell signal patterns and promoting EMT through epigenetic regulation possibly by APOBECs. Some established cancer markers such as AFP and SALL4 are usually expressed at the embryonic stage, silenced after birth, and re-expressed in cancer patients. The process of cancer development can be characterized as “backward evolution” and “retro-differentiation”.

KEY ISSUES REGARDING *CANCER EVO-DEV*

Indispensable role of non-resolving inflammation

It is widely accepted that most solid tumours and some hematologic malignancies are associated with non-resolving inflammation. According to the Darwinian evolutionism and the origin of species, the process of cancer evolution is based on two conditions: the continuous acquisition of somatic mutations and natural selection acting on the resultant phenotypic diversity^[101]. A chronic inflammatory microenvironment serves as a niche for that process by inducing endogenous mutagenic factors such as APOBECs and provides selection pressure. During carcinogenesis, cancer cells must overcome four barriers: (1) the cell-cycle checkpoint that regulates cell division; (2) apoptosis, which limits cell proliferation; (3) telomere length, which determines the total number of cell divisions; and (4) the cell adhesion barrier that prevents cell migration. The non-resolving inflammation can alter the “ecologic” conditions in local and/or systematic tissues, weaken the functions of the above barriers, cause genomic instability via inducing the overexpression of AID/APOBECs, and provide opportunities for backward evolution into cancer stem cells in mesenchymal tissues. In inflammatory microenvironment, inflammatory mediators such as prostaglandin E2, leukotrienes, cytokines, and chemokines are highly induced via autocrine or paracrine modes of action^[102], resulting in abnormal transformation of the tissue microenvironment, infiltration of dysfunctional immune cells, and decreased epithelial integrity, thus promoting cancer evolution. Non-resolving inflammation not only promote the occurrence of cancers of the most histotypes, but also facilitate distant metastasis and the recurrence after the treatment^[46,103,104], indicating that non-resolving inflammation promotes the development of cancers in the entire course of cancer evolution.

APOBECs bridge inflammation and cancer

The APOBECs, a family of cytidine deaminases, are powerful endogenous mutagenic factors that play critical roles in many biologic processes, especially in innate immunity and humoral immunity. This group of

enzymes can specifically catalyze irreversible cytidine and deoxycytidine deamination to convert bases from cytosine to uracil, creating a cytosine-to-uracil mismatch in minus-strand and reverse-transcript guanosine-to-adenosine (G-to-A) transitions in plus-stranded DNA. Activation-induced cytidine deaminase (AID) and APOBEC3 cytidine deaminases were found in the pathways of both the acquired and innate immunities^[99]. APOBEC3 cytidine deaminases can also hyper-edit HBV DNA and inhibit HBV replication. APOBEC3 proteins are present at low levels in normal liver, but its gene expression is highly stimulated by both IFN- α and IFN- γ . APOBEC3 cleave amino groups from cytidine bases converting them to uracil in newly synthesized DNA following reverse transcription of pregenomic RNA. This modified HBV DNA is susceptible to degradation, or alternatively, numerous G-to-A nucleotide mutations are incorporated into positive-strand viral DNA^[99,105]. This process is counteracted by UNG^[106]. Accordingly, APOBECs family members can also increase somatic mutations to a threshold that exceeds the host's repair ability, thus initiating the cancer evolutionary process. In AID transgenic mouse models, mutations induced in the TP53 and β -catenin genes by constitutive expression of AID can generate HCC (13.75%), lung cancer (8.75%), and gastric cancer (1.25%)^[107]. In humans, genetic susceptibility, viral infection, and their interaction contribute to an unbalanced immune system, resulting in chronic inflammation. In the inflammatory microenvironment, the proinflammatory cytokine/chemokine and NF- κ B complex are persistently activated, which can significantly increase the expression of APOBECs at the transcription level^[108]. The high levels of APOBECs expression can overcome the strength of UNG, APOBECs get the advantage to edit the single-stranded DNAs that are temporarily generated during the transcription and replication process, consequently promoting somatic mutations^[109]. If the overall metabolic level exceeds the reserve capacity of the downstream repair pathways, somatic mutations will be further increased. An APOBECs-directed mutagenesis pattern is widespread in human cancers. Significant presence of the APOBEC mutation pattern are evident in bladder, cervical, breast, head and neck, and lung cancers, reaching 68% of all mutations in enrolled tumor samples. The APOBEC mutation pattern also extends to cancer-associated genes, implying that APOBECs-induced mutagenesis is carcinogenic^[110]. The spontaneous rate of somatic mutations is not high enough to trigger the evolution process. There must be some mutagenesis-driving forces including defective DNA repair capacity, exogenous or endogenous mutagen exposures, and intrinsic mistakes of DNA replication, which increases the mutation rates in cancer genomes. A distinct mutagenic process generates various mutation combinations

termed as “signature”. The APOBECs-related mutation signature is widely prevalent in more than half of all cancer types under investigations, suggesting that the inflammatory response is the common mechanism by which mutations are generated. Even though, the frequencies of somatic mutations in a single gene are not high in the patient population. For example, the rates of mutation in the coding regions of ARID1A and ARID2, two genes with classic HCC-related genetic variations, are 16.8% and 5.6% respectively^[111]. Such a low detection rate of each mutation makes it unable to be applied for the prediction, prevention, early diagnosis, and treatment of cancers. However, somatic mutations in different genes with a similar function can alter a specific signal pathway that is related to the stem characteristic of cancers and therefore promote carcinogenesis. For the most malignancies, so-called driver somatic mutations, mostly at low frequencies in tumor tissues, alter a limited number of cellular signaling pathways through which a growth advantage can be incurred. All of the known driver genes can be classified into one or more of 12 pathways of three major functions: cell survival, cell fate, and genome maintenance. Cell survival contains “cell cycle/apoptosis”, “RAS”, “phosphoinositide 3-kinase (PI3K)”, “STAT”, “mitogen-activated protein kinase (MAPK)”, and “transforming growth factor β (TGF- β)”. “NOTCH”, “Hedgehog”, “APC”, “chromatin modification”, and “transcriptional regulation” contribute to cell fate function. Genome maintenance is governed by “DNA damage control”^[112]. The combined mutations-affected critical molecules in the signalling pathway networks can be developed as novel diagnostic biomarkers and therapeutic targets. A series of somatic mutations in HBV-induced HCC mainly affect the chromatin remodelling pathways (ARID1A, ARID1B, and ARID2), the p53/RB tumor suppression pathway (IRF2, TP53, and CDKN2A), the Wnt/ β -catenin signal pathway (RPS6KA3-AXIN1, NFE2L2-CTNNB1), and the Ras/PI3K pathway (PTEN, PIK3CA, KRAS, NRAS)^[111,113-115]. Key genes affecting epigenetic activities including ARID2, encoding a subunit of the polybromo- and BRG1-associated factor (PBAF) chromatin remodeling complex and ARID1A, encoding a component of the SWI/SNF chromatin remodeling complex are most frequent ones. In addition, cell invasion-related factors-coding genes *VCAM1* and *CDK14*, and gene encoding androgen receptor (AR)^[113,116]. Both C:G>A:T and T:A>A:T transversions are frequent among the non-silent mutations^[114], indicating AID/APOBECs-induced somatic mutation is one of the major mutation patterns. These mutations facilitate the development of HCC via activating some evolutionarily conserved signal pathways, such as PI3K/Akt/mammalian target of rapamycin, NF- κ B/TNF- α , Raf/MAPK/ERK, TGF- β 1, Jak, Wnt/ β -catenin, and STAT3/interleukin 6 (IL-6)^[117,118], and also indicate that Wnt/ β -catenin signaling

may cooperate with both oxidative stress metabolism and Ras/MAPK pathways in hepatocarcinogenesis^[113]. Activated STAT3/IL-6 and NF- κ B/TNF- α can induce hepatocytes to lose their epithelial characteristics (EMT) and initiate backward evolution. TGF- β 1 can facilitate EMT, which can be enhanced by IL-6 and TNF- α . The synergistic effect of those three cytokines can promote the transformation of normal hepatocytes into stem-like cells. Antiviral therapy can significantly reduce the risks of occurrence and postoperative recurrence by HCC via relieving hepatic inflammation^[50-53,119], possibly because termination of inflammation can destroy the fertile environment for cancer evolution.

AID/APOBECs-regulated demethylation and EMT are important in malignant transformation

AID/APOBECs not only promote somatic hypermutation but also regulate gene expression epigenetically by directly deaminating 5-methylcytosine (5mC) or 5-hydroxymethylcytosine (5hmC) in concert with base-excision repair to exchange cytosine, thus promoting gene demethylation and removing epigenetic memory to stabilize the pluripotent state in embryonic stem cells^[120,121]. EMT, a driving force behind the development of cancers, in its various forms is driven by the transcription factors Snail (SNAI1), Slug (SNAI2), ZEB1 (ZEB1), and ZEB2 (ZEB2). Expression of AID is induced by inflammatory signals that induce the EMT in nontransformed epithelial cells and in cancer cells. AID regulates expression of master regulators (SNAI1, SNAI2, ZEB1, and ZEB2) in the EMT. Knockdown of AID blocks induction of the EMT and prevents cells from acquiring invasive properties, suppresses expression of several key EMT transcriptional regulators and is associated with increased methylation of CpG islands proximal to the promoters of SNAI1, SNAI2, ZEB1, and ZEB2^[122]. AID-mediated, CpG-methylation dependent mutagenesis is proven to be a common feature of carcinogenesis^[123]. Thus, we have reasons to postulate that re-expression of embryonic factors in cancers as cancer biomarkers might result from epigenetic reprogramming caused by AID/APOBECs, whose expression is induced by proinflammatory factors.

AID/APOBECs promote tumor heterogeneity

There are two kinds of tumor heterogeneity: intertumor heterogeneity and intratumor heterogeneity. First, patients with tumors of the same pathologic type show distinct clinical manifestations, including occurrence, metastasis, therapeutic response to chemo- and radiation-therapies, and postoperative prognosis. This heterogeneity is the basis for the development of biomarkers and therapeutic targets that can predict cancer occurrence, metastasis, and therapeutic

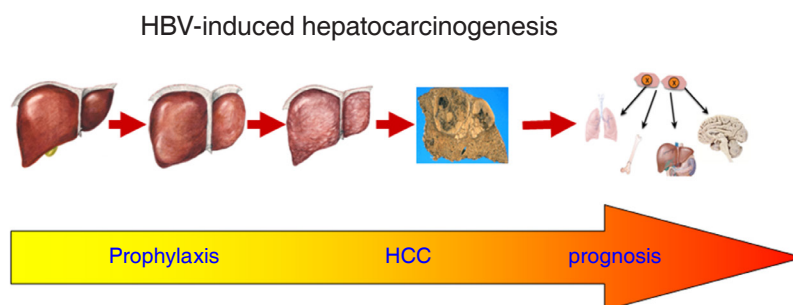
response, thus, contributing to personalized medicine. Second, different tumor cells or masses in an individual show significant differences in genomic mutation profile, evolution pathways, and gene expression. This intratumor heterogeneity was discovered and subsequently validated by the application of next-generation sequencing^[124,125]. They found that heterogeneous driver alterations that occurred later in evolution were found in more than 75% of the tumors and were common in PIK3CA and NF1 and in genes that are involved in chromatin modification and DNA damage response and repair^[125]. An important emerging mechanism fueling tumor diversity and subclonal evolution is genomic DNA cytosine deamination catalyzed by APOBEC3B and at least one other APOBEC family member. Deregulation of APOBEC3 enzymes by different microenvironment causes a general mutator phenotype that manifests as diverse and heterogeneous tumorsubclones. APOBEC mutational signatures may be enriched in tumorsubclones, indicating APOBECs fuel subclonal expansions and tumor heterogeneity. APOBEC family members might represent a new class of drug target aimed at restricting tumor evolution, adaptation, and even chemo-resistance^[126]. APOBEC3B-catalysed deamination provides a chronic source of DNA damage in cancers, thus explaining how some tumors evolve rapidly and manifest heterogeneity^[127]. Thus, APOBEC3B-catalysed somatic mutations serve as potential drivers in promoting the formation and progression of tumor heterogeneity.

A small proportion of somatic mutations can lead to advantageous phenotypes that are positively selected during the evolutionary process and thus are called “driver” mutations. The remaining mutations are “passengers” that contribute very little to carcinogenesis. Driver mutations are selected at certain phases of carcinogenesis, but might not be detectable at all stages. At the early stage of carcinogenesis, cells with initial driver mutations can survive and multiply rapidly. However, at the later stages, cells with other driver mutations can gain more advantages in the survival competition. They can replace the cells that have only initial mutations and become the dominant subset. For example, in lung cancer patients who continue to be exposed to tobacco smoking, the signatures of the tobacco-related mutations decline over time, accompanied by an increase in the APOBEC-related mutations^[128]. Tracing the positive selection of drivers and the patterns of cancer genomic alteration can help in demonstrating the lineage of the malignancy clones and the major mutagenic factors. Exome-sequencing data from solid tumours and hematologic neoplasms confirmed the clonal heterogeneity of primary tumours and metastases, supporting the evolution model at the

genetic level^[129]. Thus, the APOBEC-related mutations are more likely to be drivers. Tumors in different microenvironments and at different treatment stages might have distinct mutation spectra, thus demonstrating, within a solid tumour, an obvious heterogeneity that is the result of continuously imbalanced evolution that persists under the selection pressure of the microenvironment. Therapies can also serve as selection pressure, bring their own changes in malignant clones, and that evolution-induced heterogeneity will complicate cancer therapeutic regimes. Cancer therapy should therefore be designed as sequential treatments with the specific purpose of targeting critical pathways during cancer evolutionary process.

Energy metabolism and Cancer Evo-Dev

In the 1920s, Otto Warburg and co-workers showed that tumor tissues metabolize approximately tenfold more glucose to lactate in a given time than normal tissues under aerobic conditions, that is, a preferential use of glycolysis for energy production, even in the presence of oxygen, to support rapid growth of cancer cells, a phenomenon known as the Warburg effect^[130]. Warburg hypothesized that this phenomenon occurs due to the malfunction of mitochondria in cancer cells. Up to now, there are two conflicting points of view on effects of mitochondria DNA mutations on the Warburg effect. First, the genetic events that drive aberrant cancer cell proliferation also alter biochemical metabolism, including promoting aerobic glycolysis, but do not typically impair mitochondrial function. Mitochondrial biogenesis and quality control are often upregulated in cancers and mitochondria play a central and multifunctional role in malignant tumor progression^[131]. Second, mitochondrial mutations could be the origin of the Warburg phenotype by way of hypoxia-inducible factor activation^[132]. Pyruvate kinase M2 (PKM2), an alternatively spliced variant of the pyruvate kinase gene that is preferentially expressed during embryonic development and in cancer cells, alters the final rate-limiting step of glycolysis, resulting in the cancer-specific Warburg effect. PKM2 also mediates EMT via interacting with the transcriptional factor TGF- β -induced factor homeobox 2 to induce the deacetylation of histone H3, thus, resulting in repressed E-cadherin expression^[133]. In addition, Warburg effect in tumor-associated macrophages (TAMs) promotes vascular network formation, augments extravasation of tumor cells out of blood vessels, and induces higher levels of EMT at inflammatory foci within the tumor^[134]. In microenvironment with both hypoxia and hypoglycemia, stem cell-, angiogenic-, and EMT-biomarkers, as well as glycoprotein-P content and invasiveness of cancer cells are enhanced^[135]. Thus, we believe that the Warburg effect promotes the evolutionary process of



- For HBV-HCC, a fatal disease, prophylaxis is the hope of reducing HCC and death;
- “Cancer Evo-Dev” in HBV-induced HCC pave the way for prophylaxis, prediction, as well as targeted treatment;
 - To identify what kind of HBV-infected subjects will develop HCC
 - To testify what kind of prophylactic treatment will reduce the risk of HCC
 - To specifically target key pathways that drive the evolution of HCC

Figure 5: HBV-induced hepatocarcinogenesis. HBV: hepatitis B virus; HCC: hepatocellular carcinoma

cancer under both hypoxia and hypoglycemia condition. The Warburg effect can provide essential energy for cell survival in hostile microenvironment, furthermore, glycolysis generates the raw material for DNA synthesis of progeny cells.

ROLES OF *CANCER EVO-DEV* ON SPECIFIC PROPHYLAXIS AND TARGETED THERAPY OF MALIGNANCIES

Based on studies of HBV-induced hepatocarcinogenesis, we present the hypothesis of *Cancer Evo-Dev* to elucidate the critical steps of a common evolutionary and developmental process for most malignancies. The framework of *Cancer Evo-Dev* can be verified in other cancers such as breast cancer, cervical cancer, head and neck cancer, colorectal cancer, gastric cancer, and lung cancers. Key molecular events occur in the critical steps during *Cancer Evo-Dev* process can be applied for the occurrence and prognosis prediction and specific prophylaxis of malignant diseases. Furthermore, the core molecules in a functional subnetwork that maintains cancer stemness and promotes *Cancer Evo-Dev* process can be efficiently targeted by the high-efficiency inhibitors to block corresponding signal pathways, thus providing a powerful treatment strategy for advanced cancers. Thus, *Cancer Evo-Dev* has three major roles in cancer prophylaxis and treatment: first, to identify what kind of precancerous changes or lesions will develop into cancers; second, to testify what kind of prophylactic option or treatment will reduce the cancer incidence and delay its occurrence; and third, to specifically target key pathways that drive the evolution and development of cancer to reduce morbidity and

mortality rates. For the control of HBV-induced HCC, a highly fatal malignancy, active prophylaxis should be of top priority [Figure 5]. Our hypothesis can therefore contribute to the realization of “P4 pattern” medicine (predictive, preventive, personalized, and participatory)^[2], therefore promote the prophylaxis and control of cancer.

DECLARATIONS

Authors' contributions

G.W. Cao contributed solely to the paper.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Neoplastic macrovascular invasion represents an independent risk factor for dismal survival in sorafenib treatment for hepatocellular carcinoma

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ABSTRACT

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Aim: Sorafenib efficacy and safety in advanced hepatocellular carcinoma (HCC) have been demonstrated in two randomized international clinical trials and in clinical practice studies. Because of poor survival advantage, to identify clinical and biological parameters remains an unmet clinical need. **Methods:** Eighty-four patients treated with sorafenib were evaluated for response to therapy and prognostic factors possibly associated with survival. **Results:** Median overall survival was 8.5 months. Median duration of therapy was 2.5 months with a median daily dose of 800 mg (IQR 600-800). Dose was adjusted in 52% of patients. Radiological response to therapy showed a significant impact on survival. Child-Pugh score and neoplastic invasion of the portal system were negatively associated with survival. Continuation of sorafenib even at lower dose was positively correlated with survival. The multivariate analysis identified vascular invasion as the only independent variable: median survival of 5.5 months for neoplastic portal vein thrombosis compared to 12 months in the remaining subjects. **Conclusion:** A lower sorafenib daily dose is advantageous, even though the reason of this association cannot be explained at present. Neoplastic portal vein thrombosis is strongly associated with dismal survival. Alternative or complementary treatment approaches should be studied in order to improve outcome in this subgroup of patients.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary solid tumor of the liver and occurs predominantly in patients with underlying chronic liver disease and cirrhosis. It is the third leading cause of cancer deaths worldwide,

with over 570,000 people affected^[1,2]. The incidence of HCC is higher in Asia and Africa, where the endemic high prevalence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections strongly predisposes to the development of chronic liver disease and consequently HCC^[3,4]. In developed countries there is the growing



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problem of cirrhosis developing in the setting of non-alcoholic fatty liver disease in patients with obesity, type 2 diabetes, dyslipidemia and hypertension^[5-8]. Programs of surveillance with upper abdomen ultrasound examination and characterization of focal liver lesions with computed tomography (CT) scan or magnetic resonance imaging (MRI) increase the rate of early diagnosis and curative treatments such as surgical resection, liver transplantation and locoregional ablative treatments^[9-13] with improved survival. In the advanced stage, Barcelona Clinic Liver Cancer (BCLC) stage C, systemic therapy with sorafenib^[14] represents the first line treatment for these patients, while regorafenib is available for second line as well as anti-PD-1 that has been recently approved by the Food and Drug Administration for HCC.

Sorafenib is an oral multi-kinase inhibitor that acts both on tumor cells by inhibiting cytoplasmic cascades RAS-RAF and MEK-ERK, involved in cells proliferation, and also on endothelial cells by blocking plasmatic receptors implicated mainly in neo-angiogenesis (VEGFR and PDGFR)^[15-19]. A correct patient management can increase drug tolerability and seems to improve significantly quality of life and survival^[20-24]. The opportunity to continue treatment also in patients with radiological progressive disease or when tolerance is poor despite dose adaptation remains controversial^[25,26]. However, in clinical practice, progression is not always a clear indication to stop sorafenib, especially if there isn't a second-line trial available and in patients with a good Performance Status (PS) with a reasonable life expectancy, an excellent drug tolerance and slow tumor progression. Sorafenib, compared to other target therapies, shows low frequency of radiological responses, but stable disease can be achieved frequently as shown in registration trials^[27].

The aim of the present study was to evaluate prognostic relevance of clinical, epidemiological and tumor characteristics on survival. Reported results confirmed that dose reduction is associated with longer survival underlining relevance of drug management to increase tolerability. On the other hand, neoplastic portal vein thrombosis, a condition associated with fast liver decompensation and disease progression, was independently associated with poor clinical outcome.

METHODS

Patient characteristics

This is an observational monocentric retrospective study conducted on 84 consecutive subjects starting sorafenib treatment at the Unit of Infectious

Diseases and Hepatology, Azienda Ospedaliero-Universitaria di Parma. Data were obtained from the analysis of medical charts and a dedicated database. Inclusion criteria were: radiological or histological diagnosis of HCC not amenable to surgical resection or locoregional treatment, BCLC stage C, PS < 2 according to the Eastern Cooperative Oncology Group system, measurable lesions in CT or MRI scans. Patients with an impaired liver function and a Child-Pugh score ≥ 10 were excluded. Eighty-four patients were considered, 63 males (75%) and 21 females (25%), with a median age of 73 years (range 32-81) [Table 1]. Of these patients, 45% had comorbidities: the most frequent was hypertension (29 subjects), followed by diabetes mellitus (16 subjects), previous ischemic vascular events like heart attacks and stroke (11 subjects) and chronic obstructive pulmonary disease (COPD, 9 subjects). Eight subjects had a history of tumors other than HCC [Table 1]. The etiology of chronic liver disease underlying HCC was HCV infection in 46 patients (54.5%), nonalcoholic steatohepatitis or alcohol in 21 patients (25%), HBV infection in 7 patients (8.5%), HBV-HCV infection in 3 patients, while in 7 patients (8.5%) the cause of liver disease was unknown [Table 1]. Most of subjects (91.5%) was on a Child-Pugh score A, seven were scored B7 [Table 1]. Majority of patients (82%) was previously treated: 72.5% underwent loco-regional therapies, 33% surgical resection and 18% both [Table 1]. Regarding the anatomical characteristics of HCC, it appeared multifocal in 96.5% of cases and was interested in only one lobe of the liver in 77.5% of cases, most frequently the right [Table 2]. In 47 patients (56%) HCC showed signs of neoplastic vascular invasion and 20 subjects (24%) presented both vascular invasion and extrahepatic spread [Table 2]. Treatment was stopped at radiological evaluation at 8 weeks of treatment in case of disease progression.

The study was approved by the local ethical committee [Comitato Etico Indipendente (IRB/IEC) of the Azienda Ospedaliero-Universitaria di Parma, Italy].

Treatment with sorafenib and evaluation of response rate

Sorafenib was administered at a dose of 400 mg bid continuously, equivalent to a total daily dose of 800 mg, without food or with a low or moderate fatty meal. Therapy was continuous, but by convention was codified in cycles of 28 days. Patients had to measure their blood pressure at least twice daily and use skin lotions to prevent or reduce any hand-foot syndrome manifestation. Every 4 weeks a reevaluation of treatment was planned through a detailed physical examination of patients, the correction of possible adverse effects

Table 1: Clinical and epidemiological characteristics of patient population at baseline

Characteristics	Data
Gender	
Male	63 (74%)
Female	21 (26%)
Age (median years, IQR)	73 (67-75)
BMI (median, IQR)	25 (23-28)
Comorbidities (yes/no)	39 (46.5%)/45 (53.5%)
Hypertension	29/39
Diabetes mellitus	16/39
Cardiovascular events	11/39
COPD	9/39
Other tumours	8/39
Kidney disease	0/39
Etiology	
HCV	46/84 (54.5%)
HBV	3/84 (3.5%)
HBV + HCV	7/84 (8.5%)
Alcohol and/or dysmetabolic	21/84 (25%)
Other or unknown	7/84 (8.5%)
Child-Pugh score	
A5	21/84 (25%)
A6	56/84 (66.5%)
B7	7/84 (8.5%)
Previous treatments (yes/no)	69 (82%)/15 (18%)
Resection	23/69
Loco-regional treatments	60/69
RFTA	46/60
TACE	39/60
PEI	31/60
Resection + loco-regional treatments	14/69

IQR: interquartile range; BMI: body mass index; COPD: chronic obstructive pulmonary disease; HCV: hepatitis C virus; HBV: hepatitis B virus; RFTA: radiofrequency thermal ablation; TACE: transarterial chemoembolization; PEI: percutaneous ethanol injection

(diarrhea, skin rash, high blood pressure, edema), the evaluation of blood tests examinations such as liver function tests (transaminases, albumin, bilirubin), renal function (creatinine, urea, electrolytes), coagulation parameters (prothrombin time), lipase, creatine-phosphokinase and the alpha-fetoprotein dosage. It was allowed to reduce sorafenib dose to limit adverse effects of treatment. A thorax-abdomen CT scan with contrast was scheduled at 8 weeks of treatment. The instrumental response to treatment was evaluated according to Modified Response Evaluation Criteria in Solid Tumors criteria^[11,12]: complete response (CR) was defined as the disappearance of intra-tumoral arterial enhancement in all target lesions, partial response (PR) as a reduction > 30% of the sum of the diameters of the vital areas in the parameter lesions and progressive disease (PD) as an increase of > 20% of the sum of the diameters of the vital areas in the parameter lesions, compared to the baseline size. Stable disease (SD) included all the other cases not classified as PR or PD. In patients classified as not applied, therapy was interrupted before 8 weeks because of liver failure, adverse events or poor performance status.

Table 2: Anatomical and functional characteristics of hepatocellular carcinoma

Characteristics	Data
Location of tumour	
Monolobar	65/84 (77.5%)
Bilobar	19/84 (22.5%)
Extrahepatic spread	
Absent	52/84 (62%)
Present	32/84 (38%)
Macroscopic vascular invasion	
Absent	37/84 (44%)
Present	47/84 (56%)
Metastasis and macroscopic vascular invasion	
Both present	20/84 (24%)
Both absent	27/84 (32%)
Tumour marker at the beginning of therapy	
Alpha-fetoprotein (median ng/mL)	130.5 (range 1-65,671)

Sorafenib management and toxicity

Toxicity was evaluated according to National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0^[20] every 4 weeks. According to the grade of the event, a dose reduction or suspension of the treatment was planned. For grade 1 adverse events it was advised to institute supportive measures and continue sorafenib treatment; at first appearance of grade 2 adverse events it was suggested to establish support measures and reduce sorafenib at 400 mg/day for 28 days: if toxicity regressed to grade 1, it was indicated to re-increase the dose at 400 mg twice daily, otherwise it was recommended to discontinue sorafenib for at least 7 days then 400 mg/day, finally the full dose. At the appearance of the second or third potential grade 2 toxicity, sorafenib was permanently administered at the reduced dose of 400 mg/day. In case of the fourth appearance of grade 2 adverse event it was considered the definitive suspension of treatment. At the occurrence of grade 3 toxicity, sorafenib was interrupted for at least 7 days or until the decrease to grade 0-1, then prescribed at a low dose (400 mg/day) and further increased to 400 mg twice a day. At the second appearance of grade 3 adverse event, the conduct was the same, but at the time of resumption sorafenib was definitely prescribed a low dose (400 mg/day). In some cases, it was performed a treatment with lower doses than indicated above, up to a minimum of 200 mg/day.

Statistical analysis

Survival curves are expressed by Kaplan-Meier curves and compared with log-rank test. Cox proportional hazards model was used for multivariate analysis of survival. The variables associated with survival showing a *P* value < 0.1 in the univariate analysis were included in the multivariate analysis model, except response rate that was not available for all patients. Prism (Graph Pad) and StatPlus (AnalystSoft Inc.)

Table 3: Dose, duration and response of treatment

Characteristics	Data
Treatment duration (median months)	2.5
Response rate	
PR	5/84 (6%)
SD	27/84 (32%)
PD	26/84 (31%)
NA	26/84 (31%)
Median daily dose mg (IQR)	800 (600-800)
Patients treated with median dose of 800 mg	53/84 (63%)
Patients treated with median dose < 800 mg	31/84 (37%)
Dose reduction	
Yes	44/84 (52%)
No	40/84 (48%)
Adverse events (yes/no)	77 (91.5%)/7 (8.5%)
Asthenia	72/77 (93.5%)
Gastro-intestinal symptoms	63/77 (82%)
Rash, peeling, itchy (general)	48/77 (62%)
Hypertension	36/77 (47%)
HFSR	27/77 (35%)
Alopecia	21/77 (27%)
Bleeding	15/77 (19.5%)
Cardiovascular events	0/77 (0%)
Reason of treatment suspension	
Progressive disease	25/84 (30%)
Adverse events	23/84 (27%)
Liver failure	22/84 (26%)
Other reasons	7/84 (9%)

PR: partial response; SD: stable disease; PD: progressive disease; NA: not applied; IQR: interquartile range; HFSR: hand-foot skin reaction

were used to perform the statistical analysis. The comparison between mean values was performed with Student *t* test for unpaired data. Statistical significance was considered for values $P < 0.05$.

RESULTS

Overall picture

Results on response rate, treatment duration, sorafenib dose and side effects are reported in Table 3: PR was achieved in 5 patients (6%), SD in 32% and PD in 31% of patients. None of patients achieved a CR. Treatment was discontinued for adverse events or clinical worsening before radiological evaluation in 26 patients (31%). Median treatment duration was 2.5 months. Forty patients (48%) received full sorafenib dose (800 mg/day) during all the treatment, while 44 subjects (52%) reduced sorafenib dose. Median daily dose was 800 mg. Thirty-seven percent of patients received a median dose of 800 mg, while the remaining (63%) a minor dose (range 200-600 mg) because of adverse events. Dose reductions ranged between 5% and 90% of the time on treatment. Most of patients (92.5%) developed adverse events: gastro-intestinal symptoms, asthenia, rash and skin peeling and high blood pressure; the most common adverse event was severe weight loss associated with asthenia and diarrhea. Finally, 20 patients after sorafenib discontinuation

received other treatments: percutaneous ablative treatments (2 patients) or other systemic treatments such as capecitabine or tivantinib (6 patients).

Survival analysis based on epidemiological and clinical data and previous treatments

Median overall survival was 8.5 months [Figure 1A]. The epidemiological and clinical parameters shown in Table 1 were assessed as factors that could have an effect on survival. Only Child-Pugh score (A vs. B; $P = 0.0289$) showed an impact on survival, while the remaining epidemiological and clinical characteristics did not show significant differences. History of previous treatment for HCC was a positive factor, however not achieving statistical significance [Table 4], in particular also considering independently locoregional treatments, that represented the most frequent treatment, there was no significant impact on survival (not shown). Eight patients with history of different tumors showed comparable survival to the remaining subjects (not shown).

Impact on survival of HCC characteristics

Tumor parameters [Table 2] were evaluated as factors potentially influencing survival. Unexpectedly, alpha-fetoprotein levels, multifocal tumor extended to both lobes as well as extrahepatic spread didn't influence survival significantly. Macroscopic vascular invasion was found to be a strong predictor for survival ($P = 0.0141$) [Figure 1B], while the association of metastasis and vascular invasion did not worsen patient outcome.

Survival analysis based on response rate, sorafenib dose and treatment duration

All data related to therapy reported in Table 3 were analyzed as parameters that could influence clinical outcome. As expected, longer duration of therapy (beyond median time of treatment) was positively associated with survival ($P < 0.0001$) [Figure 1C], even though this may not represent an effect of treatment, since other factors like progressive disease or adverse events, could have influenced time on treatment. Response rate showed a significant impact on survival ($P = 0.0237$) [Figure 1D], with median survival of 12.5 months in patients with SD or PR compared to 9.5 months for patients with PD. Dose reduction was a favorable parameter ($P = 0.004$) as well as drug regimen below median daily dose ($P = 0.04$) [Figure 1E and F].

Adverse events and tolerability

Sorafenib appeared well-tolerated as in previous studies and registration trials, however adverse events were reported, also in this study. Overall incidence of adverse effects was 91.5% of this cohort [Table 3]. Asthenia, fatigue and gastro-intestinal symptoms

Table 4: Univariate and multivariate analysis of variables potentially related with survival

Variables	Univariate		Multivariate	
	P	HR (95% CI)	P	HR (95% CI)
Median age (< 73 vs. > 73 years)	0.94	0.98 (0.60-1.59)		
Gender (male vs. female)	0.96	0.98 (0.55-1.75)		
Comorbidities (yes vs. no)	0.16	0.72 (0.44-1.15)		
Etiology (only HCV vs. no HCV)	0.76	1.08 (0.64-1.82)		
Child-Pugh score (A vs. B)	0.0289	0.44 (0.09-0.88)	0.093	0.48 (0.20-1.13)
AFP levels (< 130.5 vs. > 130.5 ng)	0.28	0.78 (0.47-1.25)		
Response rate (PD vs. PR + SD)	0.0237	2.08 (1.10-3.92)		
Localitation (mono vs. bilobar)	0.17	1.54 (0.84-2.66)		
Extrahepatic spread (yes vs. no)	0.42	1.20 (0.75-2.00)		
Macrovascular invasion (yes vs. no)	0.0141	1.73 (1.14-3.14)	0.016	1.84 (1.11-3.05)
Previous therapies (yes vs. no)	0.06	0.59 (0.25-1.04)	0.52	0.81 (0.42-1.55)
Dose reduction (yes vs. no)	0.004	0.52 (0.29-0.79)	0.45	0.73 (0.31-1.66)
Median daily dose (< 800 vs. 800 mg)	0.041	0.60 (0.37-0.98)	0.35	0.73 (0.37-1.42)

HCV: hepatitis C virus; AFP: alpha-fetoprotein; PD: progressive disease; PR: partial response; SD: stable disease; HR: hazard ratio; CI: confidence interval

(mainly moderate to serious diarrhea) were the most common adverse events that required patient hospitalization in some cases; rash, itch, hypertension, hand-foot skin reaction (HFSR), alopecia and bleeding were reported in some cases. Cardiovascular events linked to sorafenib treatment were not observed.

Univariate and multivariate analysis of survival according to clinical and anatomical-functional characteristics of cancer at baseline

All studied parameters were evaluated for their impact on survival. As shown in Table 4 by univariate analysis: Child-Pugh score, neoplastic vascular invasion, dose reduction and median daily dose showed a significant effect. In particular, Child A, absence of vascular invasion, dose reduction and daily dose lower than median were associated with improved survival. Multivariate analysis showed that neoplastic vascular invasion was the only independent condition correlated with a worse outcome [$P = 0.0166$; hazard ratio (HR) = 1.846, 95% confidence interval (CI) = 1.118-3.050].

DISCUSSION

The aim of this study was to analyze the role of epidemiological, clinical, tumor parameters and treatment dose on clinical outcome in a cohort of 84 patients from a single clinical center. Outcome was measured as overall survival. Sorafenib effectiveness was confirmed by response rate, that was significantly associated with survival ($P = 0.0237$). In particular, PR was achieved in 5 patients (6%), while SD in 27 patients (32%).

Metastasis were negatively associated with rate response while there was no significant association

with portal thrombosis and intrahepatic tumor burden. Our patients were all in BCLC stage C with majority (91.5%) of subjects with compensated liver disease (Child-A) and the remaining patients with Child-B cirrhosis. If compared to previous studies, our patient cohort was characterized by a more advanced tumor stage. In fact, the 2 registration trials included 18%^[28] and 5%^[14] of patients with intermediate HCC stage (BCLC-B), similarly to real-life studies including 19-25% of patients that could be classified in the intermediate stage while all our patients were in BCLC-C stage. Even if stage was more advanced, median survival was 8.5 months, comparable to what observed in registration trials^[14,28], ranging between 6.5 and 10.7 months and real-life studies^[28,29]. Median time on treatment was 2.5 months that is indeed less than what reported in other studies ranging between 3.75 and 5.1 months^[14,28-30]. This may be explained by the more advanced tumor stage of these patients characterized by early disease progression in many cases leading to early discontinuation.

Neoplastic portal thrombosis was present in 56% of the cases while it ranged between 22% and 39% in previous studies^[14,28-30]. Major causes of early stop of treatment were premature death, hepatic failure, other complications as systemic infections and sorafenib intolerance.

Then we evaluated parameters significantly associated with longer overall survival. Child-Pugh score A, absence of macroscopic vascular invasion and reduced sorafenib daily dose (below median value) were identified by univariate analysis while only absence of neoplastic portal vein thrombosis was independently associated with survival by Cox regression analysis. Multivariate analysis, showed that macroscopic vascular invasion almost doubled the risk of death (HR = 1.846),

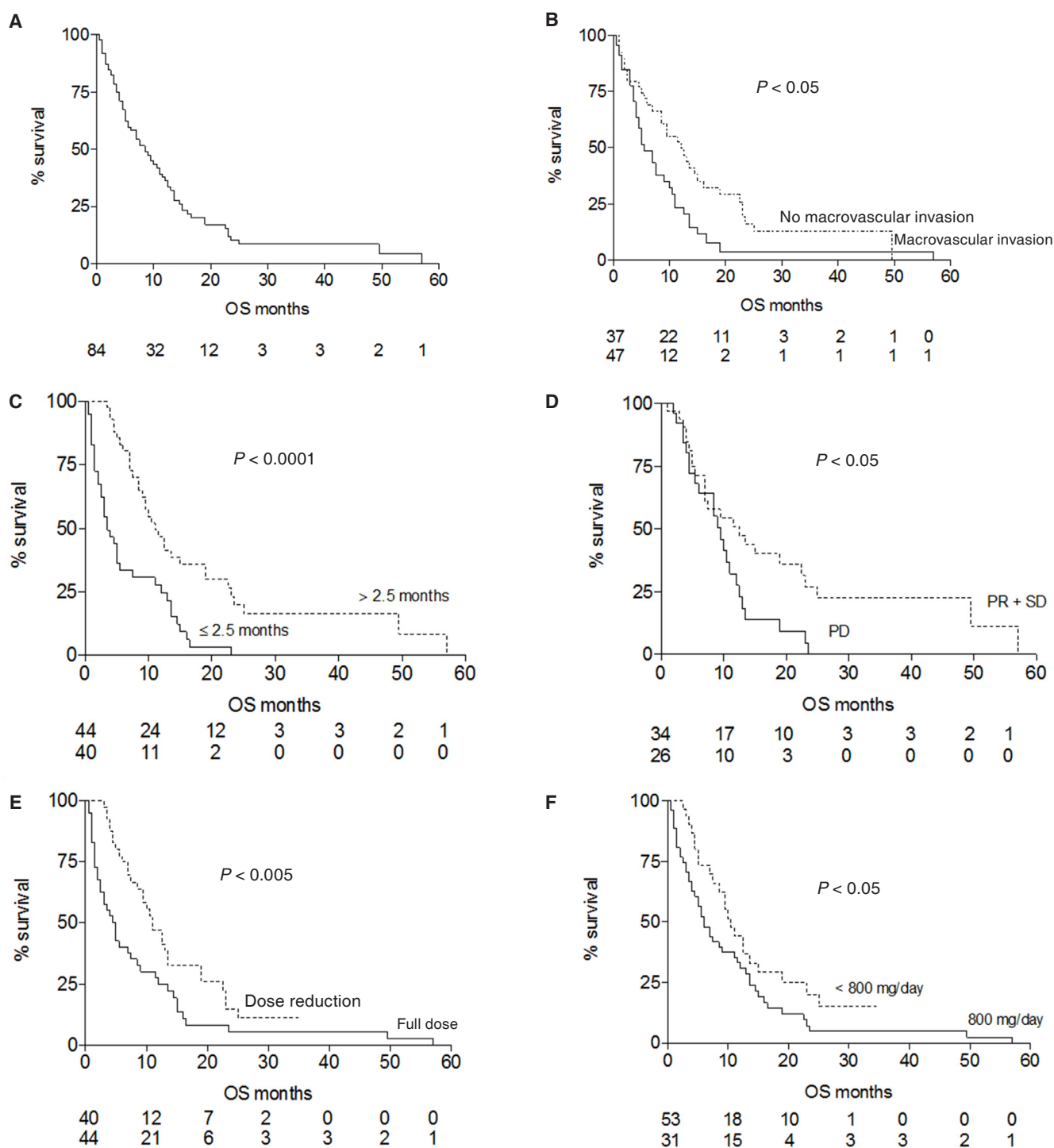


Figure 1: Overall survival (OS) of the whole patient population and survival according to risk factors. (A) Median OS for sorafenib treated patients was 8.5 months; (B) presence of macroscopic neoplastic vascular invasion of the portal venous system, present in 56% of subjects, was a strong negative predictor on survival, with a median OS of 5.5 vs. 12 months observed in patients without neoplastic thrombosis; (C) a duration of sorafenib treatment beyond median time of 2.5 months positively influenced outcome (median OS 11 vs. 3.5 months); (D) analysis of radiological response rate at 8 weeks of treatment showed a significant impact on survival: median OS was 12.5 months in subjects with stable disease or partial response and 9.5 months in progressive disease patients; (E) dose reduction showed a benefit on survival (median OS 11 vs. 5 months); (F) sorafenib daily dose below median (800 mg) was associated with better survival (median OS 10.5 vs. 6 months)

similarly to what previously reported^[28,29] (HR = 1.7), thus confirming that the presence of portal neoplastic thrombosis is a very negative prognostic factor on

survival. Indeed, this condition severely impacts on the natural history of the disease, characterized by an aggressive disease course, because of fast spread

of cancer cells, worsening of portal hypertension and liver function and poorer tolerance to treatment. As evidence of this, majority of patients (71%) stopping treatment before radiologic evaluation presented this complication. Neoplastic macrovascular invasion was associated with a survival expectancy less than half, suggesting the usefulness to investigate alternative treatments like combination of different therapies modalities such as external radiotherapy or selective internal radiation therapy^[24]. Whether best supportive care may represent the best medical option may not be concluded on the base of our findings however it could be considered in selected cases.

Interestingly, sorafenib dose reduction and median daily dose less than 800 mg were positively associated with survival, in fact patients that reduced dose during treatment showed a median survival of 11 months compared to 5 months of the remaining patients. Similarly, it has been reported a survival of 21.6 months compared to 9.6 months for patients treated for more than 70% of the time at half dose^[29]. Therefore, a lower dose may be advantageous, enabling a more prolonged treatment, with no reduction of therapeutic effect. In other studies^[30,31], starting dose, was analyzed as a variable that could influence management and efficacy of sorafenib showing longer time on treatment and better survival for patients starting with full dose. However, in this study^[31] median daily dose was not reported and is not clear if dose reductions allowed longer time on treatment and better outcome.

Treatment adverse events were not significantly different compared to previous reports, registering at least one adverse effects in 91.5% of our patients. The most common effects didn't differ to what previously reported^[14,28-30], represented by asthenia, gastrointestinal symptoms, in particular moderate or severe diarrhea, hypertension and dermatological lesions as systemic rash or HFSR.

In conclusion, portal neoplastic thrombosis is the most important prognostic factor being associated with a rapid clinical deterioration leading to death. Finally, we confirm the importance of clinical management for individualized treatment dose in order to provide longer treatment periods, that seems to be crucial to improve survival of our patients.

DECLARATIONS

Authors' contributions

Concept and design: G. Missale, C. Schianchi

Data acquisition: M. Lecchini, E. Biasini

Data analysis: M. Lecchini, A. Olivani, E. Biasini, R.

Dalla Valle, G. Missale

Statistical analysis: A. Olivani, G. Missale

Literature search and manuscript preparation: M. Lecchini

Manuscript editing: A. Olivani, C. Ferrari, G. Missale, C. Schianchi

Manuscript review: C. Ferrari, G. Missale, C. Schianchi

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Consent was obtained from patients still alive at the time of data collection and analysis.

Ethics approval

The study was approved by the local ethical committee (Comitato Etico Indipendente (IRB/IEC) of the Azienda Ospedaliero-Universitaria of Parma, Italy).

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Role of early growth response 1 in liver metabolism and liver cancer

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ABSTRACT

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The liver is an essential organ for nutrient and drug metabolism - possessing the remarkable ability to sense environmental and metabolic stimuli and provide an optimally adaptive response. Early growth response 1 (Egr1), an immediate early transcriptional factor which acts as a coordinator of the complex response to stress, is induced during liver injury and controls the expression of a wide range of genes involved in metabolism, cell proliferation, and inflammation. In support of an important role of Egr1 in liver injury and repair, deficiency of Egr1 delays liver regeneration process. The known upstream regulators of Egr1 include, but are not limited to, growth factors (e.g. transforming growth factor β 1, platelet-derived growth factor, epidermal growth factor, hepatocyte growth factor), nuclear receptors (e.g. hepatocyte nuclear factor 4 α , small heterodimer partner, peroxisome proliferator-activated receptor- γ), and other transcription factors (e.g. Sp1, E2F transcription factor 1). Research efforts using various animal models such as fatty liver, liver injury, and liver fibrosis contribute greatly to the elucidation of Egr1 function in the liver. Hepatocellular carcinoma (HCC) represents the second leading cause of cancer mortality worldwide due to the heterogeneity and the late stage at which cancer is generally diagnosed. Recent studies highlight the involvement of Egr1 in HCC development. The purpose of this review is to summarize current studies pertaining to the role of Egr1 in liver metabolism and liver diseases including liver cancer.

INTRODUCTION

Early growth response 1 (Egr1) is an immediate early, zinc finger transcription factor that was first identified based upon its induction by nerve growth factor (NGF) in rat PC12 cells, which is why it was initially known as nerve growth factor inducible protein A (NGFI-A)^[1]. Egr1 is one of four family members that also include Egr2, Egr3, and Egr4^[2]. Also known as *Krox24*, *zif268*, and *TIS8*, *Egr1* encodes a protein of 80-82 kDa that consists of three zinc finger DNA-binding motifs [Figure 1]. Thus,

it is not elusive that zinc metal is crucial to the function of Egr1, such as nuclear localization^[3]. Specifically, two of three zinc fingers interact with the nuclear localization sequence to promote Egr1 nuclear localization^[3]. Depletion of the zinc metal reduces Egr1 promoter activity^[4]. Transcriptional co-repressors NGFI-A binding protein 1 and 2 (NAB1 and NAB2, respectively) repress Egr1, Egr2, and Egr3 transcriptional activity by binding to the respective repressor domains upstream of the zinc finger motifs and could potentially co-regulate Egr1 target genes^[5-7].



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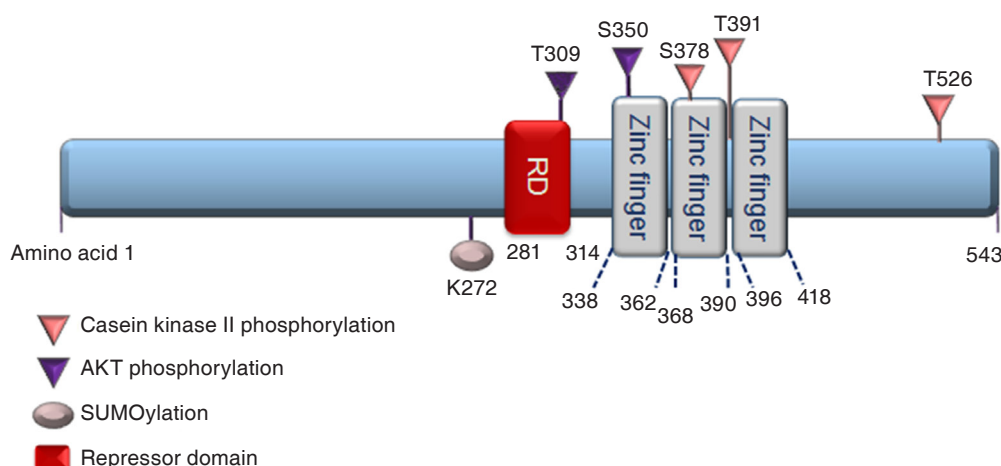


Figure 1: Schematic representation of EGR1 protein structure and post-translational modifications. EGR1 is a 543-amino acid (aa) protein consisting of three Cysteine 2-Histidine 2 (C_2H_2) zinc fingers DNA-binding domains, approximately 23 aa each. Zinc fingers 2 and 3 (amino acids 361-419) interact with amino acids 315-330 for EGR1 nuclear localization. The T309 and S350 sites are phosphorylated by protein kinase B (PKB, also known as AKT); whereas, S378, T391, and T526 sites are phosphorylated by casein kinase II. EGR1 protein can be SUMOylated by SUMO1 at K272. Transcriptional co-repressors NGFI-A binding protein 1 and 2 (NAB1 and NAB2, respectively) inhibit Egr1 transcriptional activity by binding to the repressor domain (RD). EGR1: early growth response 1

Egr1 expression can be induced by growth factors, ionizing radiation^[8], and insulin signaling^[9]. Upstream regulators of Egr1 include transforming growth factor β 1 (TGF- β 1)^[10], mitogen-activated kinase kinase-1, hepatocyte nuclear factor 4 α , and E2F transcription factor 1 (E2F1); whereas small heterodimer partner and peroxisome proliferator-activated receptor- γ agonist are negative regulators of Egr1^[11-14]. Egr1 recognizes a highly conserved G-C-rich consensus nucleotide sequences (GCGGGGGCG)^[15] and either activates or represses the transcription of genes in a zinc-dependent manner. The presence of this specific Egr1 response element on its target gene promoter could thus be a good indication of direct transcriptional regulation by Egr1.

The expression of Egr1 has been described in liver, heart, brain, spleen, skeletal muscle, kidney, ovary and prostate^[16]. Accordingly, important roles of Egr1 has been implicated in various cell types and pertain to embryogenesis^[17], cell growth and differentiation^[18], neurogenesis^[19], adipogenesis^[20], apoptosis^[21], fibrogenesis^[22], and tumorigenesis^[23]. Egr1 is one of the predominantly expressed EGR family members in the liver and liver-derived cell lines^[24,25]. Extensive research has been conducted in animal models to elucidate Egr1 function in various liver diseases. In this review article, we begin by discussing the role of Egr1 in liver metabolism, and then focus on Egr1 in pathological states of liver with a particular interest in hepatocellular carcinoma (HCC). An unbiased discussion of what additional studies are necessary to aid in developing possible therapeutic interventions is also included.

EGR1 AND LIVER METABOLISM

Liver is a major site for synthesis, metabolism, storage and redistribution of glucose and lipids^[26]. In the postprandial state, insulin is secreted from pancreatic beta cells in response to a high blood-sugar level. Circulating glucose is taken up by the hepatocyte via the glucose transporter type 2 - regulated by the serine/threonine kinase PI3K/AKT pathway in response to insulin signaling - and is phosphorylated to glucose-6-phosphate by liver glucokinase (Gck). Glucose-6-phosphate is either further processed for fuel via glycolysis, for nucleotide biosynthesis via pentose phosphate pathway or utilized for glycogen synthesis via glycogen synthase, depending on the systemic metabolic state. In addition, insulin further promotes *de novo* lipogenesis of fatty acids from acetyl-CoA or malonyl-CoA. In the fasting state, glucagon is secreted by the alpha cells of pancreas in response to a low blood-sugar level. Upon glucagon stimulation, the liver synthesizes glucose *de novo* as well as catabolizes glycogen to release glucose for other organs to use for energy. During this time, lipolysis in adipose tissues is increased and results in the production of free fatty acids, which is taken up by hepatocytes. Depending on the metabolic state, fatty acids are then either processed to triglycerides (TAGs) for storage or rapidly metabolized for the generation of ketone bodies that are, in part, oxidized by hepatic mitochondria. In the event of excess lipid accumulation in hepatocytes that exceeds 5% of liver weight, whether due to over nutrition or hyperglycemia, non-alcoholic fatty liver disease can develop. Thus, hepatic lipids can either derive from endogenous

lipogenesis (*de novo* lipogenesis), which may account for up to 30% of TAGs in steatotic livers^[27], or derive from the active uptake of circulating fatty acids into the hepatocytes.

Glucose and insulin regulate Egr1 expression

The contributions of glucose and insulin to Egr1 expression have been extensively studied in a variety of tissues and cell types. One earlier study showed that glucose rapidly and transiently induces Egr1 mRNA in SV40-transformed murine pancreatic beta-cell line MIN6 cells that is accompanied with an induction of insulin^[28]. This study also demonstrated that the induction of Egr1 by glucose was unique to beta cells since glucose couldn't induce Egr1 expression in NIH-3T3 fibroblasts or hepatocytes^[28]. The results raise a question whether glucose regulates Egr1 expression requires insulin signaling activation. Later, another study showed that in vascular endothelial cells, glucose and insulin independently regulated Egr1 expression and they had an additive effect to induce Egr1 in the co-treatment^[29]. Specifically, glucose mediates its effects through activation of PKC while insulin acts through the extracellular signal-regulated kinase (ERK1/2) pathway^[29]. Collectively, these studies suggest that glucose or insulin differentially regulates Egr1 expression in a cell-type dependent manner.

Insulin regulates Egr1 expression in hepatoma cells^[9] and in non-liver-derived cells overexpressed with insulin receptors^[30,31]. Keeton *et al.*^[9] showed that in rat hepatoma H4IIE cells, insulin treatment rapidly and transiently induced Egr1 mRNA, reaching its maximum levels by 15 min, which was coordinately regulated by a regulatory network involving MAPK kinase (MEK)-ERK, p38 MAPK, and PI3-kinase (PI3K). In addition, the authors found that the activation of ERK1/2 was essential for the induction of Egr1 in response to insulin that could be further modulated by alterations in the activity of the p38 MAPK pathway^[9]. By contrast, inhibition of the PI3K pathway augmented insulin's effect on Egr1 expression, suggesting that some factor downstream of PI3K may partially inhibit induction of Egr1. Of particular interests, Egr1 has been implicated to mediate the regulation of insulin on genes in liver metabolism, including hepatic malic enzyme (ME)^[32,33] and apolipoprotein A-I gene (ApoA1)^[34]. Taken together, these studies suggest that induction of Egr1 in response to insulin is vital to insulin's action on liver metabolism.

Egr1, insulin resistance, and obesity

Insulin resistance is a central defect in type 2 diabetes mellitus (T2DM). The link between Egr1 and insulin resistance is originally from the observation that Egr1

mRNA is highly increased in adipocytes from diabetic mice^[35]. PI3K/Akt pathway is activated upon insulin stimulation, which is required for glucose uptake and glycogenesis to lower circulating glucose level^[36]. Meanwhile, insulin stimulates the activation of MAPK (ERK1 and 2) that promotes insulin resistance^[37]. Thus, the balance between PI3K/Akt and MAPK signaling pathway is critical to maintain insulin sensitivity. Egr1 transcriptionally regulates phosphatase and tensin homologue (PTEN), a suppressor of PI3K/Akt signaling^[38]. Meanwhile, Egr1 regulates geranylgeranyl pyrophosphate synthase (GGPPS), an activator of ERK/MAPK signaling^[39]. Thus, inhibiting Egr1 in adipocyte simultaneously blocks MAPK signaling and augments PI3K/Akt signaling, and subsequently improves insulin sensitivity^[40]. Collectively, these studies suggest that pharmacological targeting adipocyte Egr1 could be potentially applied for developing novel treatment for T2DM.

Obesity commonly coexists with Insulin resistance. The link of Egr1 to obesity and obesity-associated fatty liver has been reported in mouse studies. For example, whole body *Egr1*-deficient mice fed a high fat diet are less susceptible to diet-induced obesity and obesity-associated disorders such as insulin resistance, hyperinsulinemia, hyperlipidemia, and fatty liver, which largely depends on the increase of energy expenditure in the adipose tissue of *Egr1*-null mice^[20]. These studies suggest that the upregulation of Egr1 in adipocytes is involved in promoting metabolic disorders and that targeting Egr1 in adipocyte could be useful for the obesity treatment.

The report of Egr1 function in liver steatosis is somehow contradictory. One earlier study showed that Egr1 expression levels in the liver are positively correlated to high caloric intake in mice, humans, and non-human primates^[41]. In addition, whole-body *Egr1*^{-/-} mice are protected from chronic ethanol-induced fatty liver due to the decreased expression and release of TNF α from macrophages^[42]. However, recent studies highlight that increasing Egr1 levels in the liver ameliorates diet-induced fatty liver disease. For example, the white pitaya (*hylocereus undatus*) juice attenuates diet-induced liver steatosis and improves insulin sensitivity in C57BL/6J mice, which is accompanied by an increase in hepatic Egr1 mRNA level^[43]. Thus, future research focusing on hepatocyte-specific Egr1 function in liver metabolism will be very valuable.

Egr1 and cholesterol biosynthesis

Cholesterol is an essential component for cell membrane and serves as the precursor to all steroid

hormones. However, high intracellular cholesterol is toxic to cells and high blood levels of cholesterol increase the risk for atherosclerosis development^[44]. Therefore, the overall cholesterol level is tightly controlled in the body. The liver plays a central role in this regulation by balancing multiple pathways involved in *de novo* cholesterol biosynthesis, cholesterol conversion to bile acids, biliary cholesterol excretion, and reverse cholesterol transport^[45]. Sterol response element binding proteins (SREBPs) are important transcription factors that regulate expression of genes in lipid metabolism including fatty acids and cholesterol synthesis. Three isoforms (SREBP-1a, SREBP-1c, and SREBP-2) have been identified in mammals. SREBP-1 mainly regulates genes required for fatty acid biosynthesis and SREBP-2 is responsible for the induction of genes involved in cholesterol biosynthesis and uptake, including HMG-CoA synthase (*Hmgcs*) and low-density lipoprotein receptor (*Ldlr*)^[46].

Egr1 regulates the expression of cholesterol biosynthetic genes, such as *Hmgcs*, farnesyl-diphosphate synthase (*Fdps*), farnesyl-diphosphate farnesyltransferase 1 (*Fdft1*), lanosterol synthase (*Lss*), sterol-4 α -carboxylate 3-dehydrogenase (*Nsdhl*), and malic enzyme (*Me1*), in rat hepatomaH4IIE cells^[24]. Additionally, Egr1 acts in concert with SREBP-2 to mediate insulin-induced cholesterol biosynthesis in the liver^[24]. Oncostatin M (OM) is a gp130 family member produced by the F4/80-positive macrophages^[47]. In human hepatoblastoma HepG2 cells, Egr1 is induced by OM and binds to the sterol-independent regulatory element (SIRE) in LDLR promoter region with co-activator CCAAT/enhancer binding protein-beta (C/EBP β) and activates LDLR transcription^[48,49]. Together, these studies point to Egr1 as an important modulator of cholesterol metabolism in the liver.

EGR1 AND LIVER REGENERATION

The liver has a tremendous capacity to regenerate after injury, which is a highly coordinated process involving both liver parenchymal and non-parenchymal cells. During liver regeneration, adult hepatocytes enter the cell cycle (G0 to G1) and progress through the cell cycle (G1 to M) until liver mass is restored^[50]. Many signals regulate the process of liver regeneration^[51]. For example, lipopolysaccharide and cytokines are important mediators of the initiation phase^[52]. Growth factors such as hepatocyte growth factor (HGF) and epidermal growth factor (EGF) regulate the progression phase^[53]. TGF- β 1 signals later terminate hepatocyte proliferation^[54]. Additionally, growth arrest-specific 1 (Gas1), a cell proliferation inhibitor, is induced during liver regeneration at the cycle G1/

S transition, contributing to the final termination of regeneration^[55]. Perturbations in the liver-regenerative response cause prolonged liver injury and delayed liver recovery.

The role of Egr1 in liver regeneration was first suggested by animal studies demonstrating that Egr1 was immediately induced during the initiation phase of liver regeneration^[56,57]. Using a transgenic Egr1 luciferase (Egr1-luc) mouse model, Dussmann et al.^[14] demonstrated that Egr1 expression was increased at the site of wound healing in partial hepatectomy. Another earlier study showed that Egr1 expression significantly increased after 15 min and subsided within 60 min after partial hepatectomy in rat livers^[56]. More recent studies in mice have extended the peak of Egr1 induction to 12 h in partial hepatectomy-induced liver regeneration^[58] and to 2 h in carbon tetrachloride (CCl₄) exposure-induced liver regeneration^[18]. The specific signals that regulate Egr1 expression during liver regeneration are not quite understood, a number of candidates are worthy of consideration. For example, extracellular ATP has been implicated as a potent stimulus for Egr1 expression^[59]. P2Y purinoceptor 2 (P2Y2) is a G protein coupled receptor that is activated by ATP in hepatocytes. The fact that the induction of Egr1 is impaired in P2Y2^{-/-} liver subjected to partial hepatectomy indicates that P2Y2 may regulate Egr1 expression during liver regeneration^[60]. Additional candidates that regulate Egr1 expression are likely to include interleukin-6 (IL-6) and C/EBP β , because the induction of Egr1 has been shown to be impaired in IL-6^{-/-} or C/EBP β ^{-/-} liver subjected to partial hepatectomy^[61,62].

EGR1 is essential for cell-cycle entry and progression during liver regeneration as Egr1 directly regulates cell cycle mediators. Lai et al.^[63] found that *Egr1*-deficient mouse livers had a substantially lower recovery rate after liver injury, which was accompanied with the reduced expression of cell cycle mediators such as Cyclin D1, Cyclin E, and proliferating cell nuclear antigen. After subcutaneous administration of CCl₄, *Egr1*-deficient mice exhibited increased liver injury and delayed cell cycle progression^[18,58]. Acute ethanol dosing of *Egr1*^{-/-} mice also resulted in exacerbated liver injury associated with impaired liver repair^[64]. Collectively, these studies suggest that Egr1 and its regulated cell-cycle entry and progression is critical for liver regeneration. Additionally, Egr1 contributes to the regulation of a large number of genes required for the regenerative response, including cell division cycle 20 (*cdc20*), a key regulator of the mitotic anaphase-promoting complex, and cytokines necrosis factor-alpha (TNF α), IL-6, and lymphotoxin-beta^[14,18,57,65,66].

Therefore, Egr1 plays a critical role in liver regeneration after injury.

EGR1 IN LIVER FIBROSIS AND ACETAMINOPHEN-INDUCED HEPATOTOXICITY

Liver fibrosis is the wound-healing response of the liver to chronic injury that entails cell proliferation, inflammation, angiogenesis, as well as synthesis and remodeling of extracellular matrix^[67-70]. Prolonged tissue injury can lead to excessive accumulation of extracellular matrix in the organ, a hallmark of fibrosis. Egr1 has been shown to induce transcription of growth factors and stimulate collagen production in human fibroblasts and fibrosarcoma cells, suggesting the contribution of Egr1 to fibrogenesis^[22,71]. TGF- β 1, a key regulator of fibrogenesis, is an upstream regulator of Egr1^[10]; however, Egr1 also regulates the expression of TGF- β 1 in response to the hepatitis B virus^[72], which hints to the existence of a possible feedback regulation between TGF- β 1 and Egr1 during fibrogenesis.

Acetaminophen (APAP) is widely used to treat pain and reduce fever. APAP is mainly metabolized by the liver, undergoing glucuronidation, sulfation, or N-hydroxylation. The sulfate product is the primary, non-toxic metabolite in children; whereas, the glucuronide metabolite is the primary, non-toxic metabolite in adults. The hydroxylated product is the bioactivation of APAP by cytochrome 2E1 (Cyp2E1) that leads to the toxic, reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI). The final attempt to prevent toxicity is to conjugate NAPQI to glutathione^[73]. In the event of APAP overdose, the glutathione stores are depleted; the reactive metabolite binds to hepatic proteins, leading to hepatic necrosis. In western countries, acute liver injury due to APAP overdose is the main cause for drug-induced acute liver failure^[74]. In addition, long-term application of APAP has been linked to the increased hepatic inflammation and liver fibrosis in patients^[75].

The report of Egr1 function in acute or chronic APAP-induced hepatotoxicity is contradictory. In an acute APAP-induced liver injury mouse model, both Egr1 mRNA level and transcriptional activity in hepatocytes are increased^[76]. Inhibition on ERK1/2-mediated Egr1 transcriptional activation by caffeic acid (an organic compound found in coffee, fruit, and herbs) attenuates APAP-induced hepatotoxicity^[76], suggesting that inhibiting Egr1 activation is beneficial to protect against APAP-overdose induced acute hepatotoxicity. By

contrast, a recent study using WT and *Egr1*^{-/-} mice in chronic APAP-induced liver injury has demonstrated that *Egr1*^{-/-} livers exhibited a more severe hepatotoxicity and fibrotic response compared to WT mice under APAP overdose^[77]. Collectively, these data support Egr1 as an important mediator in APAP-induced hepatotoxicity and liver fibrosis; however, whether Egr1 could act as an inducer or protector against APAP-induced liver injury has remained elusive. Additional studies using cell-type specific *Egr1*-deficient animals to determine the involvement of Egr1 in acute and chronic APAP-induced liver injury would be highly beneficial for a more clear definition of cell-type specific role of Egr1 in liver injury and fibrosis.

EGR1 AND LIVER CANCER

Egr1 is demonstrated to act as both a tumor suppressor and a tumor promoter in cancers. The tumorigenic role of Egr1 was described in prostate, skin and kidney cancers^[78]. By contrast, tumor suppressor activity of Egr1 was reported in fibrosarcoma, glioblastoma, lung and breast cancers^[79,80]. The role of Egr1 in liver cancers remains elusive, as studies evaluating the role of Egr1 in liver cancer development and progression have reported contradicting conclusions.

Accumulating studies suggest Egr1 as a tumor suppressor in HCC. Egr1 is commonly downregulated in HCC tissues from humans and murine, indicating that the downregulation of Egr1 is related to HCC development^[81]. However, mechanisms responsible for the downregulation of Egr1 in liver cancer remain unknown. A recent study has described that EGR1 carries mutational intratumoral heterogeneity and frameshift mutations in colorectal and gastric cancers which have high microsatellite instability^[82]. Thus, it could be interesting to know whether the same mechanism could exist in liver cancer and contribute to the decrease of EGR1. Aberrant MAPK signaling activation is a key player in driving tumor proliferation^[83-85]. Inhibition of P42/44MAPK in HepG2 cells leads to suppression on cell growth, proliferation, and survival, accompanied by an induction of Egr1 in tumor cells^[86]. Recently, (125)I-UdR radionuclide therapy combined with Egr1-promoter-based interferon gamma (*IFN* γ) gene therapy was described to efficiently reduce tumor proliferation and promote animal survivals in mice bearing H22 hepatomas^[87]. Overexpression of Egr1 decreases the growth rate and tumorigenicity of the HCC cell line HHCC cells^[88]. Furthermore, Egr1 induces apoptosis in human hepatoma cells (HepG2 and Hep3B) that can be enhanced by synthetic chenodeoxycholic acid

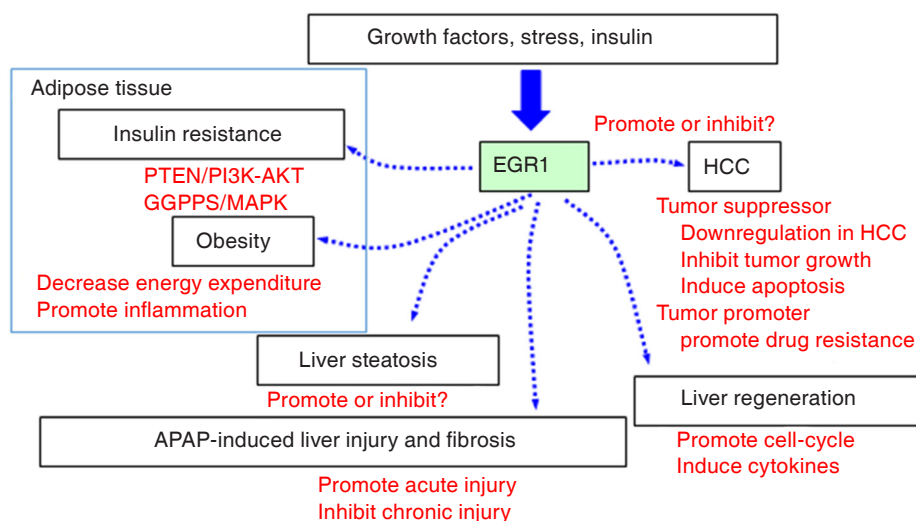


Figure 2: Model of EGR1 function in metabolic diseases and liver diseases. EGR1 is induced in response to various stimuli such as growth factors, stress, and insulin signal. EGR1 regulates a wide array of transcriptional targets involved in multiple biological functions related to lipid and glucose metabolism. In particular, increase of EGR1 in adipose tissue is associated with insulin resistance and obesity. In the liver, dysregulation of EGR1 is associated with liver steatosis. EGR1 promotes acute acetaminophen (APAP)-induced liver injury while attenuates chronic APAP-induced liver fibrosis. EGR1 is important for liver regeneration as it promotes cell-cycle entry and progression, as well as stimulates production of cytokines required for tissue repair. Finally, dysregulation of EGR1 associates with HCC development. EGR1 regulates HCC tumor growth and apoptosis, and is involved in hypoxia-induced drug resistance. EGR1: early growth response 1; HCC: hepatocellular carcinoma

derivative, HS-1200^[89]. Collectively, these studies have demonstrated that Egr1 functions as a tumor suppressor in HCC via inhibiting tumor proliferation and promoting apoptosis.

In addition, Egr1 regulates the expression of a large number of genes required for suppressing HCC growth, including PTEN^[38], a very well known tumor suppressor that inhibits PI3K signaling pathway in HCC. EGR1 protein sumoylation is required for activation of PTEN transcription, in which the phosphorylation of EGR1 by AKT at S350 and T309 allows EGR1 protein sumoylation^[90]. In addition, Egr1/PTEN axis is essential for ribonucleotide reductase regulatory TP53 inducible subunit M2B (RRM2B) inhibition on HCC cell migration^[91]. Recently, Wang *et al.*^[92] has described a cascade, involving Egr1, microRNA-203a (miR-203a), and homeobox D3 (HOXD3), inhibits HCC tumorigenesis. Through both *in vitro* and *in vivo* studies, the authors have demonstrated that Egr1 directly activates miR-203a expression by binding to the miR-203a promoter that results in suppression on HOXD3^[92]. Taken together, these studies support an anti-tumor role of Egr1 in HCC.

Contrasting the anti-tumorigenic role of Egr1 is study indicating that Egr1 is associated with HCC tumorigenesis. In a study using cDNA microarray and chromatin immunoprecipitation (ChIP) assay to assess the genes associated with tumor angiogenesis, Egr1 is identified as a key player to mediate HGF-induced

upregulation of vascular endothelial growth factor and IL-8^[93]. In an attempt to identify early biomarkers of HCC, Archer *et al.*^[94] has performed gene expression microarray analyses in HCC tissues and revealed that Egr1 and vesicle associated membrane protein-2 are positively correlated to hepatitis virus-induced HCC. Additionally, G protein-coupled receptor kinase2 overexpression reduces insulin-like growth factor 1-induced HCC cell proliferation and migration that is mediated by decreasing Egr1^[95]. All these studies suggest that activation of Egr1 might promote HCC development.

Additionally, Egr1 is described to contribute to hypoxia-induced HCC cells' resistance against anticancer drugs^[74,96]. One of the proposed mechanisms behind such phenomenon connects Egr1, hypoxia, and microtubules. Egr1 is co-localized with microtubules and mediates hypoxia-induced stabilization of microtubules from disassembly^[96]. Expected, knockdown of Egr1 improves drug effectiveness under hypoxic conditions^[96]. Another mechanism connects Egr1, hypoxia, and autophagy to HCC drug resistance. Autophagy contributes to the HCC cells resistance against chemotherapeutic agents under hypoxic conditions^[97-99]. Egr1 transcriptionally regulates hypoxia-induced autophagy by binding to the promoter of microtubule-associated protein 1 light chain 3 and promotes autophagosomes formation in HCC cells^[74]. Collectively, these studies suggest that inhibiting Egr1 expression or function to increase tumor cells'

sensitivity to chemotherapeutics could be applied as a novel approach for HCC therapy. In addition, whether the current discrepancies on Egr1 function in HCC could be due to a dual role of Egr1 during HCC development, first acting as an activator and then as a repressor, still remains elusive and requires further investigation.

CONCLUSION

As a Zinc-finger transcription factor, Egr1 has a diverse range of functions implicated in various cell types. The major roles of Egr1 in liver diseases are summarized and depicted in **Figure 2**. Research efforts using various animal models such as fatty liver, liver injury and fibrosis have contributed greatly to the elucidation of Egr1 liver-specific function. However, in some instances, such as in insulin signaling as well as HCC studies, the data regarding the role of Egr1 are contradictory. Hence, much progress is required to uncover and characterize the role of Egr1 in various types of cells in the regulation of normal liver function. For example, studying the effects of insulin signaling, APAP, ethanol, or CCL₄ in hepatocyte-specific or macrophage-specific *Egr1* knockout models are greatly appreciated. Utilization of primary cell cultures (such as hepatocytes, stellate cells, and macrophages) from normal liver to assess Egr1 functions may also aid in elucidation of liver-specific Egr1 regulation. On the other hand, due to its regulation of key fibrotic mediators, Egr1 may be a promising target for anti-fibrotic therapy. Overall, much progress is required to uncover and characterize the cell-type specific role of Egr1 in the liver. Improving our understanding of Egr1 in liver metabolism and liver cancer may provide new insights to facilitate developing novel treatments or prevention strategies for liver diseases.

DECLARATIONS

Authors' contributions

Reviewed the literature and wrote the manuscript: N. Magee, Y. Zhang

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Conflicts of Interest

The authors declare no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Robotic hepatectomy for hepatocellular carcinoma: a clinical review

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ABSTRACT

The robotic surgical system was developed to overcome the disadvantages of conventional laparoscopic surgery. The use of robots in liver surgery was not well evaluated. This article aimed at reviewing robotic partial hepatectomy to conventional laparoscopic or open partial hepatectomy in terms of perioperative, oncologic, and healthcare costs for hepatocellular carcinoma (HCC). Studies were identified by searching MEDLINE and PubMed databases for articles from January 2004 to June 2017 using the keywords "laparoscopic hepatectomy", "robotic surgery", "robotic hepatectomy", and "hepatocellular carcinoma". Case reports were not included. The open conversion rate, overall morbidity rate, and mortality rate of robotic partial hepatectomy were reported as 0-14.3%, 0-27%, and 0-3%, respectively. Although little data regarding robotic approach for HCC have been reported, it appears to be better than open approach, particularly blood loss and hospital stay, and similar to conventional laparoscopic approach in terms of short term outcomes. The oncological outcomes were comparable to open or laparoscopic approach. Well-known advantages of the robotic system allow resection of tumor location over posterior and superior segments or major hepatectomy with more ease. The main disadvantage of robotic approach was its high cost. In conclusion, oncological data from homogenous series of HCC after robotic partial hepatectomy was needed. Robotic approach was safe to be an alternative option of minimally invasive hepatectomy for HCC. Its future implementation will depend on the advantages that it can provide over open or conventional laparoscopy approach.

INTRODUCTION

The introduction of minimally invasive surgery (MIS) has revolutionized surgical practice in the past 3 decades. MIS benefits patients in terms of better pain control, shorter hospital stay, earlier recovery, and better cosmesis [Table 1]. Traditionally, liver surgery is considered as one of the most challenging surgeries among the abdominal procedures. Its MIS

development is also lag behind other gastrointestinal organs' development. These advanced techniques also require highly experienced laparoscopic skills. Increasing understanding of liver anatomy and advancements in technology have facilitated the development of MIS approach of hepatectomy^[1,2]. Two international expert consensus conferences on laparoscopic partial hepatectomy were held in Louisville, KY, USA, in 2008 and in Morioka, Japan,



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Table 1: Potential advantages of MIS approach of hepatectomy

Operation	Recovery
Improved visualization	Less postoperative pain
Reduced blood loss	Earlier mobilization
Reduced blood transfusion requirement	Improved perioperative lung function
Less intra-abdominal adhesion formation	Fewer wound complications
	Reduced perioperative immune suppression
	Improved cosmetic outcome
	Shorter recovery time
	Shorter hospital stay
	Decreased ascites in patients with portal hypertension

MIS: minimally invasive surgery

in 2014, respectively^[3,4]. The jury in the second consensus meeting concluded that minor laparoscopic hepatectomy should be a standard practice, and major laparoscopic hepatectomy is still in exploration phase. Continued cautious introduction of laparoscopic major hepatectomy was recommended. In a recent review, over 9,000 cases of laparoscopic hepatectomies were performed worldwide, and 65% of cases were performed for malignant pathologies^[5].

The recent introduction of robotic surgical systems has given a new face of MIS. It was developed to overcome the disadvantages of conventional laparoscopic surgery. Well-known advantages of the robotic system such as improved vision via three-dimensional view, magnification, tremor suppression, and the flexibility of the instruments have allowed precise operating techniques in a variety of procedures in general surgery. These features allow the surgeons to perform delicate tissue dissection and precise intra-corporeal suturing. The main drawback of robotic system is the associated cost.

Hepatocellular carcinoma (HCC) is the sixth most common malignancy worldwide and the most common primary liver cancer. Over 80% of cases HCC grown in a cirrhotic liver^[6,7]. In view of the benefit of MIS, minimally invasive approach for HCC treatment is increasing continuously adopted^[8-11]. The postoperative course after MIS approach of partial hepatectomy may also be improved in patients with liver cirrhosis because the abdominal wall is preserved, kinetics of the diaphragm is improved, collateral venous drainage is better and there is less postoperative ascites. Systematic reviews or meta-analyses suggests that laparoscopic partial hepatectomy of HCC is safe and can provide improved patient outcomes when compared to the open approach^[12-14]. Herein, we review the literature to compare robotic partial hepatectomy to conventional laparoscopic or open partial hepatectomy in terms of perioperative, oncologic, and healthcare

costs for HCC.

Studies were identified by searching MEDLINE and PubMed databases for articles from January 2004 to June 2017 using the keywords "laparoscopic hepatectomy", "robotic surgery", "robotic hepatectomy", and "hepatocellular carcinoma". Case reports were not included.

PERIOPERATIVE OUTCOMES

Robotic vs. open partial hepatectomy

Three nonrandomized comparative studies compared robotic and open partial hepatectomy^[15-17]. Patriiti *et al.*^[15] from Italy compared outcomes between robotic partial hepatectomy ($n = 19$) and open ($n = 69$) partial hepatectomy at 2 centers for lesions in the right posterior section between January 2007 and June 2012. Matched patients undergoing robotic and open partial hepatectomy showed no significant differences in blood loss (376.3 vs. 457.5 mL), intraoperative transfusion rate (31.6% vs. 15%), postoperative transfusion rate (10.5% vs. 7%), mean hospital stay (6.7 vs. 7.9 days), overall complication rate (15.8% vs. 13%) and mortality rate (0% vs. 0%). According to the Clavien-Dindo classification, major (grades 2-4) complications were not significantly different between the 2 groups (5.3% vs. 1.4%). Robotic group had significantly longer mean operative time (303 vs. 233 min) and inflow occlusion time (75 vs. 29 min) compared with open group. In malignancies, tumor-free margin rates were similar in both groups (R1 resections, 10.5% vs. 9%). Kingham *et al.*^[16] from United States compared outcomes between robotic partial hepatectomy ($n = 64$) during 2010-2014 and open ($n = 64$) partial hepatectomy during 2004-2012. In the robotic group, 41% were segmental and 34% were wedge resections. There was a 6% open conversion rate. There was a significant shorter median operating time (163 vs. 210 min), lower median estimated blood loss (100 vs. 300 mL), and shorter median hospital stay (4 vs. 7 days) in robotic group. The complications rates (10.9% vs. 14.1%) and mortality rates (3% vs. 1.6%) were similar in both groups. Eleven of the robotic operations were isolated resections of tumors in segments 2, 7, and 8. The resection margins of the malignant tumors were similar using both groups. Margins > 10 mm were found in 16% of robotic group and 17% of open group. Daskalaki *et al.*^[17] from United States compared robotic ($n = 68$) and open partial hepatectomies ($n = 55$) during 2009-2013. There was an 8.8% open conversion rate. Mean estimated blood loss was significantly less in the robotic group (438 vs. 727.8 mL). Overall morbidity was significantly lower in the robotic group (22% vs. 40%). Clavien-Dindo

Table 2: Advantages and disadvantages of conventional laparoscopic and robotic approaches

	Conventional laparoscopic approach	Robotic approach
Advantages	Well-developed technology Less start-up cost Less maintenance cost	3-dimensional magnified view Good dexterity 7 degrees of freedom in movement Elimination of fulcrum effect Elimination of physiologic tremors Good in suturing Tele-surgery More ergonomic in working position
Disadvantages	Loss of tactile feedback Compromised dexterity Limited degrees of motion Fulcrum effect Magnification of physiologic tremors	Total absence of tactile feedback High start-up cost Very expensive in maintenance New technology with limited evidence

grade 3/4 complications were also significantly lower (4.4% vs. 16.3%). The length of stay in the intensive care unit (ICU) was significantly shorter for patients who underwent a robotic procedure (2.1 vs. 3.3 days). The mean operating time (293.4 vs. 256 min), 30-day mortality (0% vs. 1.8%) and mean hospital stay (6.8 vs. 9.2 days) were similar in both groups. Robotic group had less overall morbidity, ICU, and hospital stay. This translates into decreased average costs for robotic surgery. The mean total cost, including readmissions, was \$37,518 for robotic approach and \$41,948 for open approach.

Based on current limited nonrandomized comparative studies, robotic approach has better perioperative outcomes, particularly blood loss and hospital stay, than open approach.

Robotic vs. conventional laparoscopic partial hepatectomy

Traditionally, conventional laparoscopic partial hepatectomy can either be pure laparoscopic or hand-assisted laparoscopic approach. Techniques of hand-assisted laparoscopic approach has been attempted to bridge the gap between open and pure laparoscopic approach. The benefits of hand-assisted laparoscopic approach in hepatectomy are: (1) facilitation in manual retraction, which may be the best atraumatic tool; (2) feasibility in assessing margins of resection with the use of tactile sensation; (3) safety in parenchymal dissection laparoscopically; and (4) possibility of immediate hemostasis and prevents air embolism in case the hepatic vein is severed. Obviously, pure laparoscopic procedure is superior to hand-assisted approach in terms of wound pain, and cosmetic outcome as hand-assisted laparoscopic hepatectomy usually required a 6-8 cm incision for the placement of the hand-port. Another possible disadvantage of hand-assisted laparoscopic approach includes possible obstruction of the visual field by the surgeon's hand during the operation. Based on the platform of the development and experiences of conventional

laparoscopic hepatectomy, robotic surgical system was developed to overcome the disadvantages of conventional laparoscopic approach and hand-assisted laparoscopic approach. When robotic system compared to conventional laparoscopic approach, the pros and cons of each approach were shown in Table 2. Robot-assisted laparoscopic partial hepatectomy was increasingly studied in recent years. Up till now, no randomized trials are available for robotic hepatectomy. All data have been reported as case series or nonrandomized comparative studies. Most data were obtained from prospectively maintained databases. Tables 3 and 4 showed the results of nonrandomized comparative studies comparing robotic and laparoscopic partial hepatectomy in patients with minor hepatectomies^[18-25] and in patients with minor and major hepatectomies^[26-29]. Although the perioperative outcomes seemed to be similar in both groups, the benefit of robotic approach has been shown in several studies. The potential benefits included less open conversion rate, higher proportion of major hepatectomies and easier for resection of those tumours located over superior and posterior segments^[22,26,28,30-33].

Based on current nonrandomized comparative studies, robot-assisted laparoscopic partial hepatectomy appears to be similar to conventional laparoscopic approach in terms of blood loss, morbidity, mortality rate and hospital stay. Robot-assisted laparoscopic hepatectomy may have longer operation time. However, the definition of operation time was variable. Some authors refer to a "total operation time" and specify an included "robot set-up and docking time", whereas others refer to a "procedure time" with a separate "system time" (from positioning the robot over the patient to disconnection of the robot) and "dissection time" (surgeon's active time at the console); others calculate the time from "induction of anesthesia to incision" or from "incision to extubation". However, robotic approach is more expensive than laparoscopic approach.

Table 3: Nonrandomized comparative studies comparing robotic and laparoscopic minor hepatectomy

Studies	<i>n</i>	Operating time (min)	Blood loss (mL)	Conversion (%)	Complication (%)	Mortality (%)	Hospital stay (days)	R0 resection (%)	Cost
Berber <i>et al.</i> ^[18] (2010)	9 vs. 23	258.5 vs. 233.6	136 vs. 155	11.1 vs. 0	11 vs. 17	\	\	\	\
Packiam <i>et al.</i> ^[19] (2012)	11 vs. 18	175 vs. 188	30 vs. 30	0 vs. 0	27 vs. 0*	0 vs. 0	4 vs. 3*	\	\$6,553 vs. \$4,408*
Lai <i>et al.</i> ^[20] (2013)	33 vs. 33	202.7 vs. 133.4*	373.4 vs. 347.7	\	3 vs. 9	0 vs. 0	\	90.9 vs. 90.9	\
Tranchart <i>et al.</i> ^[21] (2014)	28 vs. 28	210 vs. 176 (median)	200 vs. 150	14.3 vs. 7.1	17.9 vs. 17.9	0 vs. 0	4.5 vs. 3	\	\
Yu <i>et al.</i> ^[22] (2014)	13 vs. 17	291.5 vs. 240.9*	388.5 vs. 342.6	0 vs. 0	0 vs. 11.8	0 vs. 0	7.8 vs. 9.5	\	\$11,475 vs. \$6,762*
Kim <i>et al.</i> ^[23] (2016)	12 vs. 31	337.4 vs. 216.4*	225 vs. 150 (median)	0 vs. 3.2	25 vs. 22.6	0 vs. 0	7 vs. 7	\	\$8,183 vs. \$5,190 *
Montalti <i>et al.</i> ^[24] (2016)	36 vs. 72	306 vs. 295	415 vs. 437	13.9 vs. 9.7	19.4 vs. 19.4	2.8 vs. 0	6 vs. 4.9	88.9 vs. 87.5	\
Salloum <i>et al.</i> ^[25] (2017)	16 vs. 80	190 vs. 162	247 vs. 206	13 vs. 3	13 vs. 11	0 vs. 1	6 vs. 7	100 vs. 98	€5,522 vs. €6,035

P* < 0.05Table 4: Nonrandomized comparative studies comparing robotic and laparoscopic minor and major hepatectomy**

Studies	<i>n</i>	Operating time (min)	Blood loss (mL)	Conversion (%)	Complication (%)	Mortality (%)	Hospital stay (days)	R0 resection (%)	Cost
Tsung <i>et al.</i> ^[26] (2014)	57 vs. 114	253 vs. 198.5*	200 vs. 100	7 vs. 8.8	19.3 vs. 26	0 vs. 1.8	4 vs. 4 (median)	95 vs. 92	\
Spampinato <i>et al.</i> ^[27] (2014)	25 vs. 25	430 vs. 360	250 vs. 400	4 vs. 4	16 vs. 36	0 vs. 4	8 vs. 7	100 vs. 91	\
Wu <i>et al.</i> ^[28] (2014)	38 vs. 41	380 vs. 227*	325 vs. 173*	5 vs. 12.2	8 vs. 10	0 vs. 0	7.9 vs. 7.2	\	\
Lee <i>et al.</i> ^[29] (2016)	70 vs. 66	251.5 vs. 215*	100 vs. 100 (median)	5.7 vs. 12.1	11.4 vs. 4.5	0 vs. 0	5 vs. 5	\	\

**P* < 0.05

ONCOLOGICAL OUTCOMES

At present, available survival data about robotic partial hepatectomy for HCC in the literature are limited still. Difficult learning curves, adequate resection margins, tumor seeding, metastases of the wounds, and the long-term outcome are the major concerns. No port-site recurrence was reported. However, specific survival data in homogenous group of pathology was very limited. The majority of the papers included deals with patients undergoing robotic partial hepatectomy for different diseases, whereas HCC represent a variable (often small) proportion of the total. Therefore, a meaningful analysis of survival data for HCC after robotic surgery was difficult still.

Robotic vs. open approach for HCC

In Chen *et al.*^[33], a total of 183 patients underwent robotic partial hepatectomy and 275 patients underwent open partial hepatectomy by the same surgical team between January 2012 and October 2015. Eighty-one newly diagnosed HCC cases in each group were compared under propensity score matching in a 1:1 ratio. With robotic partial hepatectomy, the conversion

rate was 1.6% and the complication rate was 4.4%. The two groups had a comparable percentage of major partial hepatectomy (41.9% vs. 39.5%) and liver cirrhosis (45.7% vs. 46.9%). Compared with the open group, the robotic group required longer operating times (343 vs. 220 min), shorter hospital stay (7.5 vs. 10.1 days), and lower dosages of postoperative patient-controlled analgesia (350 vs. 554 ng/kg). The 3-year disease-free survival of the robotic group was comparable with that of the open group (72.2% vs. 58.0%), and also similar in the 3-year overall survival (92.6% vs. 93.7%).

Robotic vs. conventional laparoscopic approach for HCC

In 2013, the short-term survival outcome after robotic partial hepatectomies for 41 consecutive patients with HCC was reported by Lai *et al.*^[20]. The mean operation time and blood loss was 229.4 min and 412.6 mL, respectively. The R0 resection rate was 93%. The hospital mortality and morbidity rates were 0% and 7.1%, respectively. The mean hospital stay was 6.2 days. The 2-year overall and disease-free survival rates were 94% and 74%, respectively. In the subgroup

analysis of minor hepatectomies, when compared with the conventional laparoscopic approach, the robotic group had similar blood loss (mean, 373.4 vs. 347.7 mL), morbidity rate (3% vs. 9%), mortality rate (0% vs. 0%), and R0 resection rate (90.9% vs. 90.9%). However, the robotic group had a significantly longer operative time (202.7 vs. 133.4 min). Recently, Lai and Tang^[34] also compared the long-term oncological outcomes of robotic ($n = 100$) and conventional laparoscopic partial hepatectomy ($n = 35$) for HCC. Robotic group had a significant higher proportion of major hepatectomies (27% vs. 2.9%) and tumors located at or across posterosuperior segments (29% vs. 0%) than conventional laparoscopic group. For the perioperative outcomes, robotic group had a significant longer mean operating time (207.4 vs. 134.2 min). Both groups had similar blood loss (334.6 vs. 336 mL). There was no difference in morbidity (14% vs. 20%) and mortality rate (0% vs. 0%). Concerning oncological outcomes, there was no difference between 2 groups in R0 resection rate (96% vs. 91.4%), 5-year overall survival (65% vs. 48%), and disease-free survival (42% vs. 38%). Recently, Magistri et al.^[35] also reported the short-term outcomes of patients who had underwent robotic resections ($n = 22$) and laparoscopic ($n = 24$) resections for HCC. In the robotic group, there were 6 left lateral sectionectomies, 2 right hepatectomies, and 14 minor resections, including 9 segmentectomies and 5 wedge resections. In the laparoscopic group, there were 14 segmentectomies and 10 wedge resections, but no major hepatectomies. Operating time was significantly longer in the robotic group (318 vs. 211 min), whereas estimated blood loss was comparable between the two groups (400 vs. 320 mL), with one case needed blood transfusion in each group. In the robotic group, Clavien-Dindo classes I and II complication was significantly less frequent than in the laparoscopic group ($n = 13$ vs. $n = 22$). During analyzing specific complications, pleural effusion was significantly less frequent in the robotic group ($n = 2$ vs. $n = 10$). Regarding major complications, there were no differences of incidence among the two cohorts ($n = 2$ vs. $n = 3$). In both the groups, one case of R1 resection was observed. They also found that robotic surgery allowed the surgeon to safely deal with liver segments that are difficult to resect in laparoscopic approach, such as segments I-VII-VIII.

CONCLUSION

Although little data regarding robotic liver surgery have been reported, it appears to be superior to open approach, particularly blood loss and hospital stay, and similar to conventional laparoscopic approach in terms of operative time, blood loss, morbidity rate, mortality

rate and hospital stay. However, robotic surgery is more expensive than conventional laparoscopic approach. It should be emphasized that considering robot-assisted laparoscopic partial hepatectomy requires 4 conditions: (1) appropriate selection of patients; (2) follow the principle of open liver surgery; (3) specific expertise and training, in both liver and laparoscopic surgery; and (4) familiarization with the robotic machine and pay precaution of its potential dangers, such as visceral injury by robotic arm, total loss of tactile feedback. For the oncological outcome for robotic resection of HCC, the data are very limited. Oncological data from homogenous series of HCC after robotic partial hepatectomy was needed. Its future implementation and clinical value will depend on the advantages that it can provide over conventional laparoscopy or open surgery.

DECLARATIONS

Authors' contributions

Proposed the idea, structure, and content: E.C.H. Lai
Literature search: E.C.H. Lai, D.T.M. Chung, O.C.Y. Chan

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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The role of oral antiviral therapy in hepatitis B-related hepatocellular carcinoma

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ABSTRACT

Hepatitis B virus (HBV) is the leading cause of hepatocellular carcinoma (HCC) in places where chronic hepatitis B infection is endemic. Oral nucleos(t)ide analog (NA) therapy can reduce the risk of HCC, but cannot completely prevent its development. For HBV-related HCCs, viral inhibition by NAs can preserve or improve liver function, thereby increasing the chance of therapeutic intervention. After surgical resection, NAs can prevent reactivation of HBV, and also reduce the chance of *de novo* development of HCC in the remnant liver. For those who undergo liver transplantation, NAs are essential to prevent reactivation and graft hepatitis, but is not likely to prevent HCC recurrence, which is due to metastatic disease. The role of NAs for non-curable advanced HCC is less well defined. These include patients undergoing locoregional therapy, chemotherapy, or palliation. Although antiviral therapy can preserve liver function, which may be compromised by HBV, it is unable to prevent disease progression from HCC. At the time of HCC diagnosis, most patients will already be receiving NAs, and these patients should be maintained on therapy. For patients not on antiviral therapy at the time of HCC diagnosis, the decision to commence therapy is often determined by the stage of HCC and life expectancy. Patients undergoing curative therapy, or locoregional therapy/chemotherapy with reasonable life expectancy, should be commenced on antiviral therapy.

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INTRODUCTION

An estimated 240 million worldwide are currently infected with the hepatitis B virus (HBV) and have chronic hepatitis B (CHB)^[1]. In regions where CHB infection remains endemic, HBV remains the leading cause of hepatocellular carcinoma (HCC)^[2]. Although the exact mechanism of hepatocarcinogenesis

remains unclear, it is likely that HBV can promote the oncogenic process both directly and indirectly^[3]. Direct mechanisms include the integration of HBV DNA into the host genome, leading to genomic instability and malignant transformation^[4]. The integration of HBV DNA into genes responsible for cellular proliferation and differentiation may lead to uncontrolled cellular proliferation via altered expressions of oncogenes and tumor suppressor genes. In fact, integrated HBV



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sequences can be observed early in the course of HBV infection, and can be detected in approximately 80% of HBV-related HCCs^[5].

Other wildtype/truncated HBV proteins (HBx, HBc, PreS) may also contribute directly towards the development of HCC. HBx is a regulatory protein that acts as a transcription activator by interacting with viral and host regulatory elements. HBx can interfere with the hepatocyte DNA repair system, cell cycle regulation, and apoptosis^[6]. Due to the process of DNA integration into the host genome, the HBx gene can be maintained even in the absence of HBV replication^[7]. The preS1/preS2/S region encodes a transcriptional activator, which may promote hepatocyte proliferation in the presence of preS mutations. Mutations in the HBV surface proteins can also lead to unfolded proteins accumulating in the cytoplasm and subsequent heightened oxidative stress in the endoplasmic reticulum, contributing to hepatocarcinogenesis^[8].

Similar to other chronic liver diseases, HBV can also cause HCC indirectly via chronic necro-inflammation, induced apoptosis, and regenerative activity, with subsequent accumulation of mutations, which may be responsible for malignant transformation. During repeated episodes of chronic inflammation and hepatitic flares, activation and interaction between different cytokines may promote immune escape and alter apoptosis. Inflammation-mediated T cell dysfunction may also impair the immune response against neoplastic cells^[9].

From the clinical standpoint, older age, male gender, high viral load, and the presence of cirrhosis are the commonly associated factors for HCC development in CHB patients^[10,11]. Of these factors, only viral load can be easily modifiable, and emphasizes the importance of antiviral therapy and its ability to induce complete viral suppression. The REVEAL study demonstrated a linear relationship between serum HBV DNA levels and the risk of developing HCC^[10]. This is not surprising, as a high viral load may increase the risk of HCC both directly and indirectly by increasing the chance of oncogenesis with higher rates of HBV integration, and by increasing inflammatory activity respectively. This highlights the importance of viral suppression in preventing HCC development. As hepatitis B e-antigen (HBeAg) is a marker of viral replication, its presence has been associated with the development of HCC^[12]. More recently, HBeAg and its precore precursors have been shown to interact with NUMB, leading to reduction of tumor suppressor p53 activity^[13]. Other HBV serological markers have also been shown to be associated with HCC development, including hepatitis

B core related antigen and hepatitis B surface antigen (HBsAg)^[14].

HBV REPLICATION

During the initial stage of infection, the HBV enters the hepatocyte via a host receptor. Once inside the cytoplasm, the relaxed circular DNA (rcDNA) enters the nucleus to form covalently closed circular DNA (cccDNA)^[15]. The cccDNA functions as a template for mRNA transcription, which is then transported into the cytoplasm for translation of viral proteins and genomic replication via reverse transcription to form negative-strand DNA. This is followed by formation of positive-strand DNA and rcDNA within the nucleocapsid, which can then undergo further assembly and exported as mature virions, or be recycled back into the nucleus to form cccDNA.

The lack of proofreading mechanism by the HBV polymerase enzyme combined with the high replicative rate leads to high genomic variability with quasi-species containing various mutations. Some of these mutations may be associated with HCC development. These include mutations in the PreS regions as described previously, and drug resistant mutations as result of antiviral therapy. Other mutations associated with higher rate of HCC include the basal core promoter (BCP) mutation (T1762/A1764)^[16,17]. The exact mechanism for hepatocarcinogenesis is unclear, although BCP mutations can be associated with disease progression and development of cirrhosis, thereby conferring a higher risk of HCC.

ANTIVIRAL THERAPY FOR CHB

Presently, the only oral antiviral therapy approved for the treatment of CHB infection are nucleos(t)ide analogs (NAs). These are HBV polymerase inhibitors which compete with natural nucleotide substrates that target DNA elongation by acting as chain terminators^[18]. NAs may also target other synthetic functions of HBV polymerase, including priming activity, reverse transcription, and the synthesis of DNA^[19]. Although interferon- α 2b and peginterferon- α 2a are approved for CHB infection, it is not used in the setting of cirrhosis or HCC. The currently approved NAs for CHB are lamivudine (LAM), adefovir (ADV), telbivudine, entecavir (ETV), tenofovir disoproxil fumarate (TDF), and most recently, tenofovir alafenamide (TAF). All NAs are formulated as fixed dose tablets to be taken once daily. For patients with HCC, the duration of antiviral therapy is usually life-long. Due to the risk of the development of drug-resistant strains, only compounds

with high potency and high barriers to resistance, such as ETV, TDF, and TAF, should be used^[20-22]. A high barrier to resistance ensures that long-term use of these drugs is associated with minimal risk of developing drug resistance. The development of drug resistance leads to virological rebound and subsequent hepatitis flares, leading to higher viral load and increase in inflammatory activity respectively, resulting in higher rates of disease progression^[23]. As a result, the risk of developing HCC may be increased. In a meta-analysis of 14 observational studies with 1,284 patients, the one year overall survival and HCC recurrence were significantly reduced and increased respectively with LAM use when compared with ETV^[24]. Several studies have also demonstrated a link between the presence of *de novo* drug resistance mutation and the development of HCC, although the mechanism of tumor development remains unclear^[25,26].

Long-term oral antiviral therapy has been shown to be effective in preventing and even reversing cirrhosis^[27,28]. However, the evidence for preventing HCC is less robust. Although it is likely that antiviral therapy can reduce the incidence of HCC, complete elimination of the risk is not possible^[23,29-31]. The paradoxical effect of survival of CHB patients to an older age may increase the risk of development of HCC by allowing time for detrimental effects caused by HBV carriage and HBV DNA integration. The risk is likely highest for those with established cirrhosis, whereby the liver is already at a carcinogenic stage. This may also explain in part why antiviral therapy is unable to fully prevent the development of HCC. To this end, CHB patients are at a lifelong risk of HCC, and should receive appropriate surveillance to enable earlier diagnosis.

For CHB patients who develop HCC, the role of antiviral therapy is even less well defined. Despite this, most patients will receive antiviral therapy, even though the evidence for its use may not be apparent to the prescriber. Given that antiviral therapy is unable to fully prevent HCC occurrence, a proportion of patients will already be on antiviral therapy at the time of tumor diagnosis. For these patients, it is likely that antiviral therapy will be continued irrespective of the therapeutic approach adopted for management of HCC. For patients not on treatment at the time of HCC diagnosis, most will be commenced on antiviral therapy. However this will often be dependent on the stage and treatability of the HCC. Although the clinical scenario may differ depending upon the stage of HCC and the treatment offered, the general indications of antiviral therapy include preserving liver function and prevention of *de novo* or recurrent HCCs.

ANTIVIRAL THERAPY FOR HCC PATIENTS UNDERGOING SURGICAL RESECTION

For patients with preserved liver synthetic function, absence of significant portal hypertension, and resectable tumors, surgical resection remains the best curative option^[32]. Compared to HCV, HBV may be associated with less risk of recurrence after resection, although the reason for this is unclear^[33]. However, another study has shown a worse prognosis after resection for HBV-related HCCs compared to non-HBV disease^[34]. There is evidence to suggest that patients with high viral load at the time of resection are associated with post-resection liver failure and recurrence of HCC^[35-37]. Active HBV replication may also be associated with an increased risk of vascular invasion^[38]. Given that sufficient remnant liver function is a prerequisite for survival after partial hepatectomy, it would be important to preserve or improve liver function by inhibiting HBV, and to prevent ongoing inflammation or damage which may worsen liver function. For these reasons, all CHB patients with HCC and planning for resection should receive antiviral therapy prior to surgery.

After resection, patients should remain on long-term antiviral therapy. Surgery itself may predispose patients to HBV reactivation after resection, and is a significant cause of hepatitis and liver failure^[39,40]. Although the exact mechanism for reactivation is unclear, the stress of partial hepatectomy itself may represent a physiological immunosuppressed state, thereby increasing the risk of reactivation^[41]. Factors that may increase this risk include general anesthesia, the use of blood transfusion, and intraoperative ischemic injury. Studies in animal models have also documented that duck HBV (DHBV) reactivation occurs following partial hepatectomy in ducks^[42]. It is possible in this case that hepatocytes remaining in the liver after partial hepatectomy will divide to increase the mass of the liver to preoperative levels and these newly divided hepatocytes provide targets for high levels of DHBV infection and replication, which may be detected as postoperative reactivation.

The highest risk for reactivation is likely observed in patients who are not on antiviral therapy^[43]. Even for patients with low HBV DNA levels, there is still a risk of postoperative reactivation^[44,45]. HBV reactivation may worsen liver function, but has also been associated with recurrence of HCC for those with low viral load at baseline^[46].

Recurrence of HCC can occur early (within 2 years) or

late (beyond 2 years) after resection. Early recurrence is usually due to intrahepatic metastasis and is related more to the characteristics of the primary HCC. In contrast, late recurrence is usually as a result of new primary HCC from *de novo* carcinogenesis arising from a premalignant liver. Therefore, the latter is more related to the characteristics of the remnant liver, including the presence of cirrhosis, inflammatory activity, and viral load^[47,48]. The fibrotic burden and the presence of cirrhosis may increase the chance of recurrence and reduce disease-free and overall survival after resection^[49]. Hence, the use of antiviral therapy after liver resection may also potentially reduce the risk of HCC recurrence. However, this only applies to new primary HCCs, and antiviral therapy is unlikely able to prevent intra- or extra- hepatic disease due to metastasis. To this end, it is likely that antiviral therapy can help to prevent late rather than early HCC recurrences^[50,51]. HBV replication and high viral load has been associated with vascular invasion, although this has not been consistently shown^[52]. Even for patients with low viral load, those with high levels of HBsAg may be at increased risk of HCC recurrence^[53,54].

In fact, the use of antiviral therapy has been shown to be independently associated with reduced risk of HCC recurrence. As expected, the benefits were mainly seen with late rather than early recurrences^[55-59]. In a territory-wide study of 2198 CHB patients with HCC from Hong Kong, NAs reduced the risk of HCC recurrence after surgical resection^[60]. A meta-analysis of 7,619 postoperative HBV-HCC patients showed more favorable 1-, 3-, and 5-year recurrence-free survival with antiviral therapy compared with no treatment^[61]. In another meta-analysis of 12 studies involving 8,204 HBV-related HCC patients, NA therapy significantly reduced the risk of recurrence and improved both disease-free and overall survival^[62]. For those with HCC recurrence, a preserved liver function at the time of recurrence via the use of antiviral therapy increased the proportion of patients that can receive curative treatment^[63,64]. For patients with repeat hepatectomy for recurrent HCC, antiviral therapy was also associated with better long-term prognosis^[65].

ANTIVIRAL THERAPY FOR HCC PATIENTS UNDERGOING LIVER TRANSPLANTATION

For patients who are eligible for liver transplantation, antiviral therapy should be commenced at the time of diagnosis and while they are on the waiting list^[66]. The use of antiviral therapy in this setting can prevent acute flares and chronic inflammation, and thus may prevent liver decompensation^[67]. The improvement in liver

function may also increase the likelihood of patients being able to receive loco-regional bridging therapy. In addition, viral inhibition prior to liver transplantation may reduce the likelihood of recurrence of HBV infection after transplantation. Lifelong antiviral therapy is required after transplantation to prevent graft hepatitis and graft loss. Although liver transplantation is curative for cirrhosis and HCC, it does not eradicate HBV from the host, likely due to the existence of extrahepatic sites of HBV infection. Prior to the availability of effective antiviral prophylaxis, liver transplantation for CHB was a relative contraindication due to the high rate of graft hepatitis and subsequent graft loss. The availability of hepatitis B immune globulin (HBIG) together with LAM was a major milestone in preventing HBV recurrence^[68]. HBIG may bind to HBV surface protein to prevent uptake of HBV into the hepatocytes by host receptors, and may neutralize viral particles through the formation of immune complexes^[69]. As a form of passive immunoprophylaxis, HBIG has to be administered parenterally at regular intervals to maintain sufficient levels to be effective. Since then, studies have also demonstrated the efficacy of using lower doses, and also replacing HBIG with combination oral antiviral therapy^[70,71]. With the introduction of more potent NAs with minimal drug resistance, oral antiviral therapy alone without HBIG has also been shown to be highly effective in preventing graft hepatitis together with excellent long-term outcome^[72-75].

The re-appearance of HBsAg and HBV DNA after liver transplantation has been associated with HCC recurrence^[76]. Previous studies have shown a temporal relationship between the development of post-transplant HCC recurrence and the re-appearance of HBsAg and HBV DNA^[77]. This suggests an association rather than viral factors being a causative factor for recurrence. Despite adequate antiviral therapy in this setting, the re-appearance of HBsAg and HBV DNA suggests that the source is possibly tumor in origin, where the antiviral penetrance may be reduced.

ANTIVIRAL THERAPY FOR HCC PATIENTS UNDERGOING LOCOREGIONAL THERAPY

For patients ineligible for surgical resection or transplantation, locoregional therapy (LRT) can be potentially curative, and can offer palliative control for inoperable tumors. The effect of LRT on HBV replication, and the effect of antiviral therapy in this setting are not well defined. Transarterial chemoembolization (TACE) is widely used, and can be effective in reducing tumor progression, with improvement in survival^[78]. The delivery of highly concentrated chemotherapy using LRT results in a high

intra-tumor concentration of cytotoxic drugs. Although systemic chemotherapy can be associated with HBV reactivation, it is likely that chemotherapy delivered by TACE poses a far less risk. The lipiodol that is widely used to deliver the drug to the tumor allows for the drug to remain concentrated in the tumor for longer periods, thereby reducing the systemic effect. In addition, the risk of HBV reactivation is dependent on the type of chemotherapeutic agent used. Although doxorubicin can cause HBV reactivation, the risk is likely relatively lower than chemotherapy regimens that contain rituximab and high dose steroid^[79].

Several risk factors have been identified for HBV reactivation. These include HBeAg status, viral load, baseline liver function, age, gender, and the intensity of LRT and the use of anthracyclines^[80]. However, the data for HBV reactivation following TACE remains somewhat inconclusive, with some studies suggesting increase risk, whereas other studies have demonstrated no changes, or even decline in HBV DNA after chemoembolization^[81,82]. The mechanism underlying the decline in viral load remains unclear, and may be due to the natural fluctuation that is independent of the TACE, or possibly from a reduction in tumor load, which may support HBV replication or impair the host immunity. On the other hand, patients with low viral load are still at risk of HBV reactivation after TACE^[83]. For patients receiving TACE, prophylactic oral antiviral therapy significantly decreased virological events and hepatitis flares due to reactivation^[39,84,85]. Achieving undetectable HBV DNA with antiviral therapy has been shown to significantly improve the progression-free survival in patients receiving TACE^[86].

For LRT that does not involve chemotherapy, the data is even sparser. HBV reactivation for CHB patients receiving radiofrequency ablation (RFA) is significantly lower than those undergoing surgical resection, although it still can occur^[87]. The pre-RFA viral load has been shown to be associated with HCC recurrence after RFA^[88], and the use of antiviral therapy after curative RFA was associated with better outcomes regarding HCC recurrence and overall survival^[89]. In a case control study of 399 post-RFA patients, antiviral therapy was shown to be an independent factor associated with a decreased risk of HCC recurrence^[90].

Therefore, antiviral therapy should be recommended for those receiving LRT with HBV-related HCC. The likely benefits of antiviral therapy are most likely those that can be observed in the short term. These include improving and preserving liver function, suppressing viral load, prevention of reactivation, and subsequently decrease the risk of hepatic failure after LRT^[91]. The

longer-term benefits of antiviral therapy are more difficult to assess, given that a significant proportion will succumb to their underlying malignancy independent of the HBV status. However, viral suppression may potentially improve long-term survival by reducing HBV reactivation and HCC recurrence^[92]. In a systematic review of 994 patients with unresectable HCC receiving LRT, there were significant improvements for progression-free and overall survival in the NA treated group compared with the control group^[93].

ANTIVIRAL THERAPY FOR HCC PATIENTS AFTER CHEMOTHERAPY/IMMUNOTHERAPY

Unlike other solid organ tumors, chemotherapeutic options for HCC remain limited. Sorafenib was approved for the treatment of advanced HCC in 2007. In contrast to the traditional chemotherapy agents, which are associated with immunosuppression, sorafenib may have immunomodulatory function through its effect on T cells, thereby augmenting the immune system^[94]. Therefore one would anticipate a low risk for HBV reactivation, although there is currently limited data regarding HBV reactivation with the use of sorafenib. A high baseline viral load has been shown to be an adverse prognostic factor for HBV reactivation and survival in patients with advanced HCC receiving sorafenib^[95,96]. In this setting, antiviral therapy may be associated with improve survival, and is a cost-effective approach^[95,97,98]. However, in a recent meta-analysis of 3,256 patients receiving sorafenib for advanced HCC, improvement in survival was only observed in HCV patients and not those with HBV^[99]. Whether these patients were on antiviral therapy, and its effect on survival, was not studied. In 2017, regorafenib, a multikinase inhibitor, was approved for HCC previously treated by sorafenib. The effect of regorafenib on HBV replication is unknown, although it is likely to be similar to sorafenib. It is likely that the long-term outcome for patients with advanced HCC and receiving palliative chemotherapy/immunotherapy will be unchanged by antiviral therapy, as the survival is limited by the advanced nature of the tumor.

ANTIVIRAL THERAPY FOR PATIENTS WITH UNTREATABLE HCC

For patients with advanced HCC not amenable to treatment, the role of antiviral therapy is limited. Patients will succumb to disease progression arising from the tumor rather than from HBV infection. Therefore, the life expectancy and quality of life is unlikely to be improved with antiviral therapy.

Those already on antiviral therapy should remain on treatment, as there may still be chance of severe flare with cessation of therapy. For those not on antiviral therapies with advanced HCC for palliation, commencing antiviral therapy at this juncture will be futile for the overwhelming majority. Even in the setting of high viral load and elevated transaminases, it may be difficult to confirm that it is due to HBV-related hepatitis rather than locally advanced infiltrative disease. The decision for antiviral therapy in this setting should be made on a case-by-case basis, taking into account the tumor stage and life expectancy of the patient.

SUMMARY

Although direct evidence is sparse, there is a general consensus that antiviral therapy can reduce the risk of HCC in CHB patients. To date, only one randomized placebo-controlled study has been published, showing a reduction in HCC and cirrhosis for advanced CHB patients treated with lamivudine^[23]. It is unlikely that future placebo-controlled studies will be performed due to ethical reasons. However, there is increasing circumstantial evidence to suggest that long term antiviral therapy will reduce or delay HCC^[100,101]. The key to antiviral therapy therefore is starting early, as the presence of advanced fibrosis and cirrhosis at the time of starting therapy is already associated with higher risk of HCC^[102,103].

Once HCC occurs, antiviral therapy is likely still beneficial. The goals of therapy in this instance include HBV DNA inhibition, preservation of liver function, prevention of further disease progression, reduction in the risk of flares, reduction in the risk of HCC recurrence, and hopefully improvement in survival^[104]. The choice of antiviral therapy will be dependent on the availability, but in general, a highly potent agent with high barriers to resistance should be used. For HBV-related HCC, ETV has been shown to have better overall survival, decompensation-free survival, and recurrence-free survival compared to LAM^[105].

A meta-analysis of 15 studies totaling 8,060 patients with HBV-related HCC after curative therapy showed a better 1-, 3-, and 5-year overall and disease-free survival for those that received NAs^[106]. In another meta-analysis of 21 studies including 8,072 similar patients, NA therapy significantly improved recurrence-free and overall survival^[58]. Other systematic reviews of HBV-related HCC patients also demonstrated improve survival and reduced early recurrence after curative treatment^[107,108]. However, the most important determinant factors for short-term recurrence are likely those related to the tumor. These include the tumor

size, number, differentiation, and the presence of lymphovascular permeation. In a large study of 3,855 HBV-related HCC patients, antiviral therapy did not reduce the risk of progressive disease or mortality after adjusting for the tumor status^[109].

For those undergoing liver transplantation, recurrence of HCC after transplantation is likely related to pre-transplant tumor factors, rather than from HBV-related factors. Despite this, antiviral therapy is essential for CHB patients to prevent graft loss from reactivation of hepatitis B.

The role of antiviral therapy for those undergoing palliation is less clear, and is likely determined by the stage of HCC and the life expectancy of the patient. It will be prudent to ensure that all HBV-related HCC patients be considered for antiviral therapy, especially with current NAs being extremely safe with minimal side effects and risks. For those with extensive disease and limited life expectancy, where quality of life and survival is determined by HCC rather than HBV infection, the use of NAs is unlikely to be of benefit. For those with less advanced disease and reasonable short-term survival, NAs may preserve underlying liver function and prevent hepatitis flares.

Currently, there are numerous novel agents undergoing development in clinical trials for both HCC and HBV infection. It is likely that NAs will continue to have an important role with viral inhibition. Newer agents will target different sites of the HBV replication cycle, including viral entry, the formation of cccDNA, transcription, viral packaging and assembly, and the release of mature virions^[110]. These novel therapies may increase the chance of HBsAg and cccDNA clearance, thereby reducing the production of oncogenic proteins, and potentially reducing the risk of developing HCC.

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Authors' contributions

Concept, literature search, manuscript preparation, manuscript editing, manuscript review: J. Fung
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There are no conflicts of interest.

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Not applicable.

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Association of serum levels of transforming growth factor β 1 with disease severity in patients with hepatocellular carcinoma

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ABSTRACT

Aim: Transforming growth factor (TGF) is overexpressed by tumor cells like other proteins and growth factors. TGF- β 1 is then activated in the extracellular compartment but is unable to control cell proliferation because of the absence or low level of TGF- β 1 receptors on the plasma membrane of malignant hepatocytes. This potential mechanism might interrupt the autocrine regulation loop of TGF- β 1 and its blocking effect on cell proliferation. TGF- β 1 is a multifunctional cytokine involved in the regulation of growth and differentiation of both normal and transformed cells. This study aimed to evaluate the association of serum levels of TGF- β 1 with disease severity. **Methods:** A total of 180 subjects were classified into 6 groups according to Barcelona clinic liver cancer (BCLC) classification, 30 patients each: early (BCLC 0 and A), intermediate (BCLC B), advanced stage (BCLC C), and terminal stage (BCLC D) of hepatocellular carcinoma as well as 1 group of patients with cirrhosis only and 1 control group. Serum levels of TGF β 1 were measured. **Results:** Serum levels of TGF- β 1 were significantly higher in patients with HCC ($1,687.47 \pm 1,462.81$ pg/mL) than cirrhotics (487.98 ± 344.23 pg/mL, $P < 0.001$) and controls (250.16 ± 284.61 pg/mL, $P < 0.001$). **Conclusion:** TGF- β 1 may have a role in tumor growth and progression.

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INTRODUCTION

Liver cancer is the sixth most common cancer, the third cause of cancer related deaths, and accounts for 7% of all cancers^[1]. The main risk factors for hepatocellular carcinoma (HCC) are chronic infections with either hepatitis B virus (HBV) or

hepatitis C virus (HCV), making up approximately 75%-85% of all cases, as well as excessive alcohol consumption, which is responsible for about 40% of HCC development in Western countries^[2]. Chronic inflammation and tissue damage by these agents leads to cirrhosis which is the underlying condition for the majority of HCC cases^[3]. Early detection and



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appropriate treatment remain the best strategy for reducing mortality.

Transforming growth factor β (TGF- β) superfamily is known to be involved in embryonic development, adult tissue homeostasis, and disease pathogenesis. Specifically, it has been shown to control proliferation, differentiation, apoptosis, migration, extracellular matrix remodeling, immune functions, and tumor invasion/metastasis^[4]. TGF- β enhances hepatic stellate cell activation, stimulates collagen gene transcription and suppresses matrix metalloproteinases expression. Thus, TGF- β , as well as its intracellular mediators; Smad proteins, can be potential therapeutic targets for liver fibrosis. TGF- β inhibits hepatocyte proliferation, but it also promotes HCC. TGF- β has been shown to play both tumor-suppressive at early stage and tumor-promoting roles at later stage^[5]. At the early stage of tumorigenesis, TGF- β 1 inhibited normal cell growth and tumorigenesis by suppressing G1/S phase transition^[6], in later stages; malignant cells become resistant to suppressive effects of TGF- β either through mutation and/or functional inactivation of TGF- β receptors or by downstream alterations in the SMAD-signaling pathway^[7]. Mutations in downstream TGF- β signaling components cause variable attenuations or complete loss of expression; these mutations, which have been detected in many common tumors, affect TGF- β signal transmission that potentially results in human cancer development and progression^[8]. TGF- β 1 expression was related to tumor grade and pathological stage. Furthermore, overexpression of plasma TGF- β 1 was associated with invasiveness of HCC and worse prognosis^[9].

The aim of this study was to evaluate the association between serum level of TGF- β 1 and disease severity in Egyptian patients with HCC.

METHODS

This cross sectional study was conducted at National Liver Institute, Menoufia University. The study protocol was approved by institute Ethics Committee. A written informed consent was obtained from all participants in the study.

The study was performed on 180 subjects attending HCC and cirrhosis clinics, 120 HCC patients, 30 cirrhotic patients and 30 matched apparently healthy subjects served as control group. HCC patients were classified according to Barcelona clinic liver cancer (BCLC) classification into 6 groups: group 1 comprised 30 patients with an early HCC stage (BCLC 0 and A); group 2 comprised 30 patients with HCC

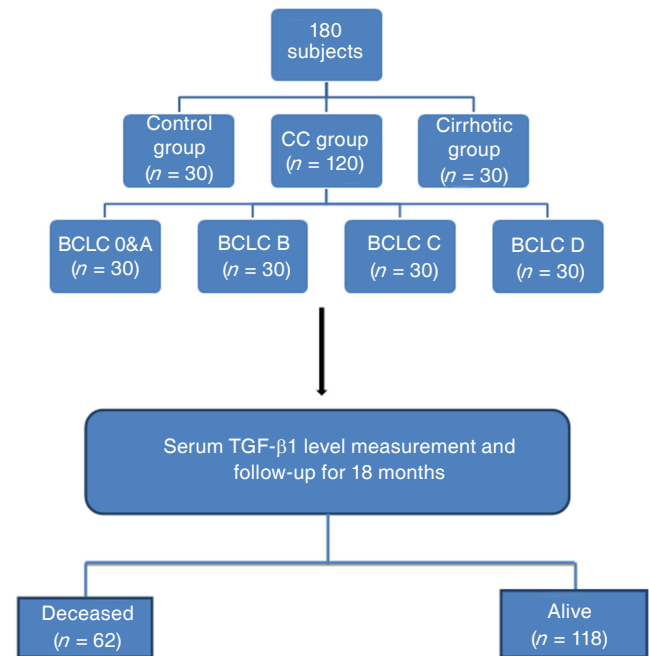


Figure 1: Flow chart of the study. BCLC: Barcelona clinic liver cancer; TGF- β 1: transforming growth factor beta 1; HCC: hepatocellular carcinoma

intermediate stage (BCLC B); group 3 comprised 30 patients with an advanced HCC stage (BCLC C); group 4 comprised 30 patients with a terminal HCC stage (BCLC D); group 5 comprised 30 patients with cirrhosis without evidence of HCC; and group 6 comprised 30 healthy subjects as a control group [Figure 1].

The diagnosis of HCC was based on non-invasive criteria using multi-slice triphasic spiral computed tomography or contrast enhanced dynamic magnetic resonance imaging. The presence of typical features of arterial enhancement and rapid portal or delayed washout on one imaging technique was diagnostic of HCC for nodules > 2 cm in diameter in cirrhotic patients. In cases of uncertainty or atypical radiological findings, diagnosis was confirmed by biopsy^[10]. Liver cirrhosis was diagnosed by ultrasonographical findings (shrunken liver, coarse echo pattern, attenuated hepatic veins and nodular surface) and biochemical indication of parenchymal harm.

Laboratory investigations

Venous blood (10 mL) were drawn from all participants and divided into 3 parts: the 1st part, 2 mL was collected in EDTA containing tube for complete blood picture using Sysmex K-21, (Sysmex Corporation, Kobe, Japan); the 2nd part, 5 mL for serum which was used for assessment of liver function tests using fully automated autoanalyzer SYNCHRON CX9ALX (Beckman Coulter Inc., CA, USA), for immunoassay

Table 1: Demographic criteria in all subjects

	Control		Cirrhotic		HCC		χ^2	P value
	n	%	n	%	n	%		
Gender								
Male	16	53.3	20	66.7	99	82.5	12.222	0.003
Female	14	46.7	10	33.3	21	17.5		
Age, years, mean \pm SD	47.67 \pm 3.387		52.29 \pm 2.997		50.20 \pm 3.916		26.2	0.001
Residence								
Rural	15	50	10	33.3	95	79.1	27.19	0.001
Urban	15	50	20	66.6	25	20.8		
Occupation								
House wife	8	26.6	15	50	17	14.1	20.28	0.004
Farmer	15	50	10	33.3	55	45.8		
Employee	7	23.3	5	16.6	48	40		
Smoking:								
Non	19	63.3	20	66.7	56	46.7	9.240	0.055
EX	0	0	4	13.3	16	13.3		
Yes	11	36.7	6	20.0	48	40.0		
Pesticidal exposure								
No	15	50	14	46.7	65	54.2	0.612	0.736
Yes	15	50	16	53.3	55	45.8		
Bilharziasis or anti bilharzial								
No	22	73.3	17	56.7	23	19.2	39.058	0.001
Yes	8	26.7	13	43.3	97	80.8		
Antiviral treatment								
No	30	100	22	73.3	109	90.8		
Yes	0	0	8	26.7	11	9.2	12.033	0.002
DM								
No	22	73.3	22	73.3	91	75.8	0.133	0.936
Yes	8	26.7	8	26.7	29	24.2		
BCLC	NA	NA	NA	NA			NA	NA
A					30	25.0		
B					30	25.0		
C					30	25.0		
D					30	25.0		
Child								
A	NA	NA	29	96.7	50	41.7	29.1	0.001
B			1	3.3	40	33.3		
C			0	0.0	30	25.0		

HCC: hepatocellular carcinoma; DM: diabetes mellitus; BCLC: Barcelona clinic liver cancer; NA: not applicable

HBs Ag and HCV Ab (Abbott Laboratories, Abbott Park, IL, USA), and for serum AFP level measurement using the automated chemiluminescence system ACS 180 (Siemens Medical Solutions, USA); and the 3rd part, 3 mL for serum and was used for measurement of TGF- β 1 using human TGF- β 1 ELISA Kit (New York, NY 10123) according to the manufacturer's instructions.

Statistical analysis

Data was statistically analyzed using SPSS (Statistical Package for Social Science) Program version 13 for Windows and for all the analysis a P value < 0.05 was considered statistically significant.

RESULTS

The demographic criteria of all subjects included in the study are shown in Table 1.

Table 2 shows the tumor characteristics in 120 patients with HCC included in the study.

Serum levels of TGF- β 1 was significantly higher in HCC groups compared to cirrhotic and control groups ($P = 0.000$) as shown in Table 3 and Figure 2.

More advanced BCLC stage was generally associated with higher serum levels of TGF- β 1, as shown in Table 4 and Figure 3, patients at an early stage HCC (BCLC stage A), had significantly lower serum levels of TGF- β 1 compared to BCLC stage C and D ($P = 0.004$ and 0.038 respectively) but not to BCLC stage B ($P = 0.267$). Similarly, serum levels of TGF- β 1 were significantly higher with more advanced liver disease assessed by Child Pugh classification [Table 4 and Figure 4].

Table 5 shows that higher serum levels of TGF- β 1 were significantly associated with vascular and invasion and tumor size ($P = 0.001$ and 0.02 respectively) rather than number of nodules ($P = 0.964$).

Lower serum levels of TGF- β 1 was associated with a higher probability of survival, using a cut-off value of 301.9 pg/mL, the median survival of patients

Table 2: Tumor characteristics in HCC patients

Variable	Number	Percent (%)
Site of nodule(s)		
Bi-lobar	54	45
Uni-lobar	66	55
Right lobe	48	40
Left lobe	18	15
Number of nodules		
Single	58	48.3
Multiple	62	51.6
2	18	15
3	6	5
> 3	38	31.6
Size of nodule(s)		
Largest diameter for single or sum of largest diameter (2-3 nodules)	< 5 cm	47
	5-8 cm	18
	> 8 cm	17
Size of largest (> 3 nodules)	5-8 cm	6
	> 8 cm	32

HCC: hepatocellular carcinoma

Table 3: Serum levels of TGF- β 1 in all subjects

Variables	The studied groups (mean \pm SD)			Kruskal Wallis test	P-value
	HCC (n = 120)	Cirrhotic (n = 30)	Control (n = 30)		
TGF	1,687.47 \pm 1,462.81	487.98 \pm 344.23	250.16 \pm 284.61	33.990	0.000

HCC: hepatocellular carcinoma; TGF- β 1: transforming growth factor beta 1**Table 4: Serum levels of TGF- β 1 according to BCLC and Child-Pugh score**

Variable	TGF (mean \pm SD)	Kruskal Wallis test	P value
BCLC stage			
A	652.83 \pm 1084.60	12.100	0.007
B	1378.95 \pm 1660.50		
C	2150.68 \pm 1970.01		
D	1668.78 \pm 1628.15		
Child classification			
A	1079.45 \pm 1491.016	6.729	0.035
B	1232.30 \pm 1717.276		
C	1668.78 \pm 1628.15		

BCLC: Barcelona clinic liver cancer; TGF- β 1: transforming growth factor beta 1

with levels < 301.9 pg/mL was not reached with a probability of survival of 71.9%, the median survival for patients with level \geq 301.9 pg/mL was only 13 months, the difference was statistically significant using log rank test ($P = 0.04$), as shown in Tables 6 and 7 and Figure 5.

Analysis of the receiver operating characteristic (ROC) curve of TGF- β 1 showed that, at cut-off value 301.9 pg/mL of TGF- β 1; area under the curve for the prediction of HCC was 0.765 and 95%CI 0.694-0.885, with a sensitivity of 72% and a specificity of 65%.

Table 5: Serum levels of TGF- β 1 and tumor burden (vascular invasion, tumor size and number)

Tumor burden	TGF (mean \pm SD)	Statistical test	P-value
Vascular invasion		Mann Whitney test	0.001
Yes	1909.29 \pm 1872.17	3.32	0.02
No	1019.65 \pm 1425.38		
Tumor size		Kruskal Wallis Test	0.02
< 5 cm	1106.06 \pm 1541.75	7.2	0.964
5-8 cm	1217.14 \pm 1522.95		
> 8	1925.32 \pm 1815.78	0.002	0.964
Tumor Number			
Single	1427.55 \pm 1669.07	Mann Whitney test	0.002
Multiple	1495.79 \pm 1717.45		

TGF- β 1: transforming growth factor beta 1**Table 6: Number of cases**

TGF	Total number	No. of events	Censored n	Percent
Negative < 301	73	20	53	72.6%
Positive \geq 301	107	42	65	60.7%
Overall	180	62	118	65.6%

TGF: transforming growth factor

Analysis of the ROC curve of alpha-fetoprotein (AFP) showed that, at cut off value 20 μ g/L area of AFP; area under the curve for the prediction of HCC was 0.86 with 95%CI 0.815-0.930 at this cut-off; the sensitivity was 72%, while the specificity was 43%.

Combining both TGF- β 1 (at a cut-off value of 301.9 pg/mL) and AFP (at a cut-off value of 20 μ g/L) would raise the sensitivity to 90%, but decreasing the specificity to 32%.

DISCUSSION

TGF- β 1 acts as a growth inhibitor in normal cells, whereas in tumor cells, it loses the ability to mediate growth inhibition and instead promotes tumor progression by enhancing migration, invasion, and survival of tumor cells^[11].

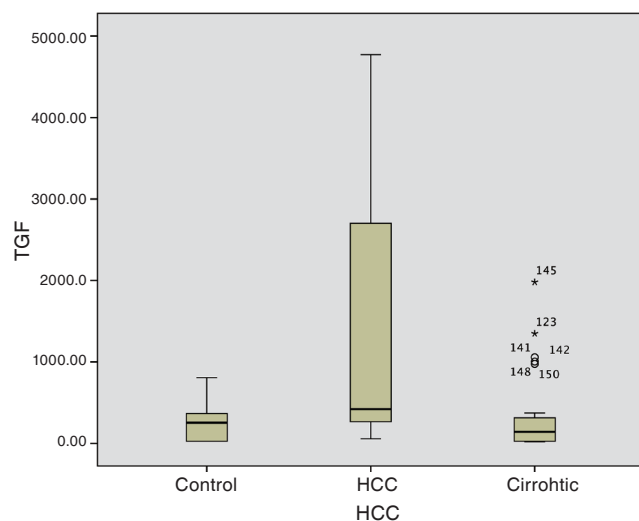
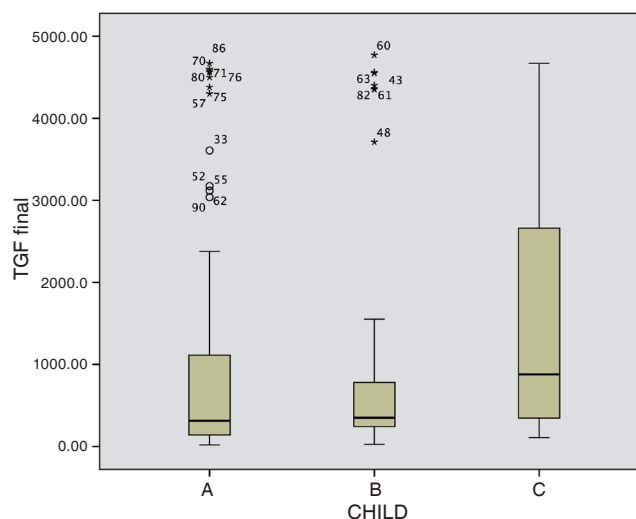
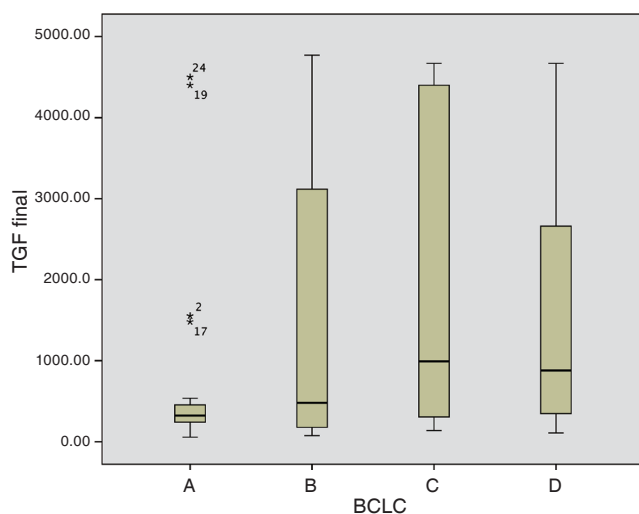
In liver diseases, the persistence of chronic inflammation, as observed in chronic viral hepatitis, plays a major role in determining the shift in the TGF- β 1 signaling pathway from tumor suppression to increasing the risk of HCC^[12].

Our study evaluated the serum levels of TGF- β 1 in HCC patients, cirrhotic patient and normal subjects. Its aim was to determine the specific contribution of TGF- β 1 over-expression to progression of HCC.

In our study, we demonstrated significantly

Table 7: Means and medians for survival time

TGF score	Mean				Median			
	Estimate	Std. Error	95% CI		Estimate	Std. Error	95% CI	
			Lower bound	Upper bound			Lower bound	Upper bound
Negative < 301	11.599	0.501	10.617	12.582
Positive > 301	9.715	0.527	8.681	10.748	13.000	2.136	8.813	17.187
Overall	10.498	0.385	9.743	11.252	13.000	.	.	.

**Figure 2: TGF level among the studied groups. TGF- $\beta 1$:** transforming growth factor beta 1; HCC: hepatocellular carcinoma**Figure 4: Serum TGF level according to child score. TGF- $\beta 1$:** transforming growth factor beta 1**Figure 3: Serum TGF level according to BCLC classification. BCLC:** Barcelona clinic liver cancer; TGF- $\beta 1$: transforming growth factor beta 1

higher levels of TGF- $\beta 1$ in HCC patients ($1,687.47 \pm 1,462.81$ pg/mL) compared to the other two groups (cirrhotic 487.98 ± 344.23 pg/mL and healthy control 250.16 ± 284.61 pg/mL), with no significant difference between control and cirrhotic groups. These findings signified the role of TGF- $\beta 1$ in tumor growth and progression, implicating its potential use as novel

marker for risk prediction of HCC development in cirrhotic patients.

These results are in agreement with Shehata *et al.*^[13], who found that TGF- $\beta 1$ levels were also significantly higher in patients with HCC group compared to chronic hepatitis C patients and control groups.

Similarly, Lee *et al.*^[9] found that plasma TGF- $\beta 1$ levels were significantly higher in patients with HCC than in cirrhotic patients and normal controls. However, serum levels of TGF- $\beta 1$ in the cirrhotic patients were significantly lower than those in normal controls and explained that by decreased synthetic function in patients with advanced cirrhosis, resulting in a lower production of TGF- $\beta 1$ from hepatocytes themselves.

Our study showed that serum levels of TGF- $\beta 1$ in HCC patients were associated with more advanced BCLC stages. These findings signified the role of TGF- $\beta 1$ in tumor growth and progression, implicating its utility as a potential novel marker for risk prediction of HCC progression.

These results are in agreement with Shehata *et al.*^[13], who found that there was a significant difference regarding TGF- $\beta 1$ between early stage ($421.9 \pm$

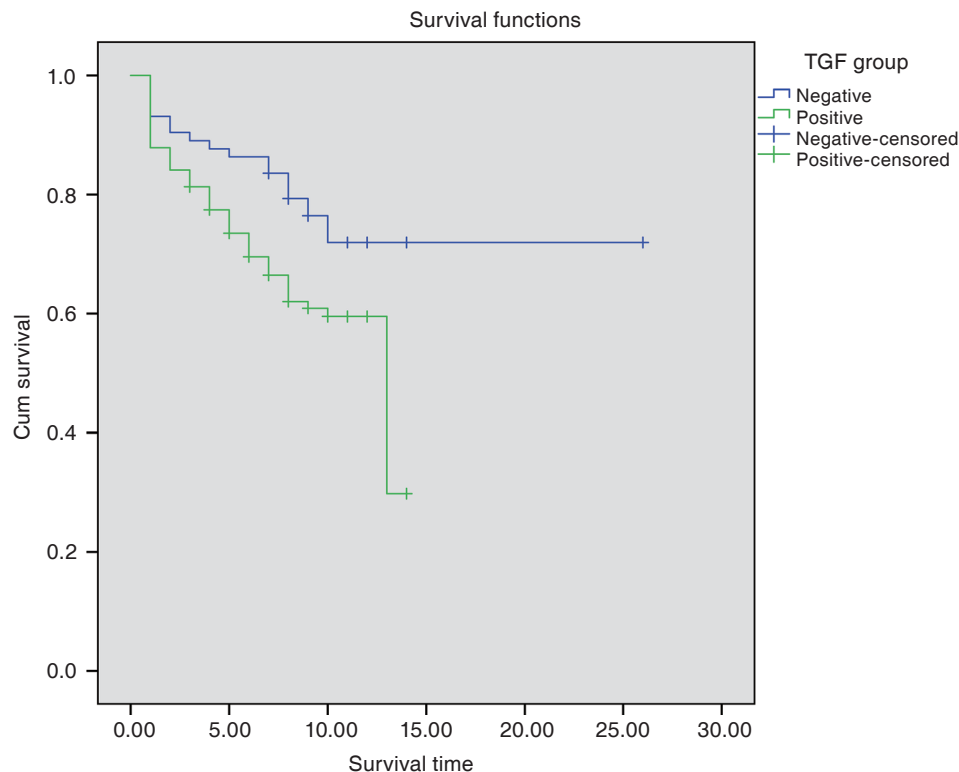


Figure 5: Survival according to TGF- $\beta 1$. TGF- $\beta 1$: transforming growth factor beta 1

105.5 pg/mL) and late stage (769.9 ± 115.8 pg/mL) of HCC patients ($P = 0.001$).

In our study, serum levels of TGF- $\beta 1$ were significantly higher in patients with larger tumors. Moreover, higher serum levels of TGF- $\beta 1$ were associated with vascular invasion, the mean value for tumors without vascular invasion was ($1,019.65 \pm 1,425.38$), while the mean value for tumors with vascular invasion was ($1,909.29 \pm 1,872.17$) ($P = 0.001$).

Similar results were shown by Lee *et al.*^[9], who found that there was a positive correlation between plasma TGF- $\beta 1$ concentration and tumor size. These findings suggest that plasma TGF- $\beta 1$ concentration increases with the invasiveness of HCC making it a novel biomarker for risk prediction of HCC progression.

As cancer develops, cancer cells become more resistant to the growth inhibitory properties of TGF- $\beta 1$ and both the cancer cells and the stromal cells often increase the production of TGF- $\beta 1$ which stimulates angiogenesis and cell motility. Also, it suppresses immune response with the extracellular matrix and increases the interaction of tumor cell leading to greater invasiveness and metastatic potential of the cancer^[14] acting as a promoter of malignancy during tumor progression^[15].

In this study, the serum levels of TGF- $\beta 1$ was significantly higher in patients with more advanced liver disease, being highest in patients with decompensated Child C cirrhosis and lowest in patients with compensated Child A cirrhosis.

These findings are in agreement with the results of Hussein *et al.*^[16] and Flisiak and Prokopowicz^[17], who reported that plasma TGF- $\beta 1$ was elevated in patients with a higher Child score and also stated that elevated plasma TGF- $\beta 1$ levels in patients with chronic liver disease might be caused by decreased clearance.

But these results disagreed with Mayoral *et al.*^[18] and Lee *et al.*^[9], who found that the TGF- $\beta 1$ values decrease significantly with progression of liver dysfunction as assessed by Child-Pugh Score.

The follow-up of our HCC patients for 18 months revealed that: the overall mortality was 51.6% with a median survival of 9 months.

In comparison of the survival rate with plasma TGF- $\beta 1$ levels, patients with a higher plasma TGF- $\beta 1$ level (≥ 301 pg/mL) showed significantly lower survival rates than those with a lower plasma TGF- $\beta 1$ level (< 301 pg/mL) (higher group vs. lower group, 29.8% vs. 71.9% at 18 months). This result in agreement with

the result of Lee *et al.*^[9], who found that patients with a higher plasma TGF- β 1 levels showed significantly lower survival rates than those with a lower plasma TGF- β 1 level (higher group vs. lower group 47% vs. 60% at 12 months).

The association between high TGF- β 1 levels and poor treatment outcomes in advanced HCC patients was anticipated because activation of the TGF- β pathway was linked to angiogenesis and the progression, invasion, and metastasis of cancer cells in late stage malignancies^[19].

In this study, ROC curve analysis of TGF- β 1 in HCC showed that the best cut-off value of TGF- β 1 for detection of HCC patients was 301.9 pg/mL with area under the curve of 0.765 and 95%CI 0.694-0.885, the sensitivity and specificity were 72% and 65% respectively. These results were found to be slightly different from the published reports by Shehata *et al.*^[13], who reported that with a cut off value of TGF- β 1 (370 pg/mL); the sensitivity and specificity for differentiation of HCC patients were 86.7% and 100% respectively, whereas area under the curve was found to be 0.97. This difference is mostly due to differences in the study population.

In a systematic review of literature, when AFP of \geq 20 mcg/L is used as a cut off, the sensitivity of detecting early HCC is reported to be 25%-65% and specificity to be 80%-94%^[20]. But here in this study at cut-off point of 20 mcg/L the sensitivity was 72%, while specificity was 43%.

Aiming to increase the sensitivity for early detection of HCC, combination of TGF- β 1 and AFP will raise sensitivity to 90% but decreasing specificity to 32%. So TGF- β 1 could be complementary to AFP in the diagnosis of HCC, particularly for the cases at an early stage.

DECLARATIONS

Authors' contributions

Concept and design: M.A.S. Kohla, H. Taha

Data acquisition: M.A.S. Kohla, A. Attia, N. Darwesh

Laboratory work: M. Obada

Data analysis: M.A.S. Kohla, A. Attia, N. Darwesh, M. Obada, H. Taha, M.F. Youssef

Statistical analysis: M.F. Youssef

Literature search and manuscript preparation: M.A.S. Kohla, A. Attia, N. Darwesh

Manuscript editing: M.A.S. Kohla, A. Attia, N. Darwesh, H. Taha

Manuscript review: M.A.S. Kohla, A. Attia, N. Darwesh, H. Taha

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Conflicts of interest

There are no conflicts of interest.

Patient consent

A written informed consent was obtained from all participants in the study.

Ethics approval

The study protocol was approved by institute Ethics Committee.

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The impact of nucleos(t)ide analog therapy in hepatitis B on the incidence of hepatocellular carcinoma: an update including recent literature findings

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ABSTRACT

Worldwide, hepatocellular carcinoma (HCC) is a significant cause of morbidity and mortality. In men, it is the fifth most common cancer and seventh most common in women; HCC is the second highest cause of cancer-related death worldwide. It is less prevalent in the USA and Northern Europe and more prevalent in Eastern and South-Eastern Asia. Over 700,000 cases are diagnosed each year - half of which occur in China - and result in roughly the same number of deaths per year. HCC significantly impairs quality of life and is associated with great costs to society. It is estimated that half of the deaths from HCC are associated with hepatitis B virus (HBV). Fortunately, HBV vaccination and antiviral therapy have shown excellent efficacy in decreasing the incidence of HCC. We will discuss the relationship of HBV to HCC, address available treatments for HBV and the impact of treatment on the development of HCC.

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nucleos(t)ides,
entecavir;
tenofovir,
lamivudine

INTRODUCTION

Hepatitis B virus (HBV) is a DNA virus that incorporates into the host genome and thereby increases the risk of developing hepatocellular

carcinoma (HCC). This risk of HCC is increased even in patients with HBV without cirrhosis; the risk of developing HCC is up to 100 fold higher in persons infected with hepatitis B compared to uninfected persons^[1]. An effective strategy shown to decrease



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the incidence of HCC is vaccination against HBV. A recent analysis of two Taiwanese HCC registries of 1,509 patients diagnosed with HCC from 1983-2011 demonstrated an incidence per 10⁵ person-years of 0.92 in the unvaccinated cohort and 0.23 in the vaccinated cohort^[2]. Another appealing strategy to decrease the incidence of HCC in patients with chronic hepatitis B is inhibition of viral replication. In the seminal study by Liaw *et al.*^[3], the chemopreventive effect of nucleos(t)ides was first suggested as the suppression of HBV replication led to decreased rates of cirrhosis, liver failure, and the development of HCC.

THE RELATIONSHIP BETWEEN HBV AND HCC

Chronic hepatitis B (CHB) stimulates the immune system to release cytokines and reactive oxygen species, which cause damage to genes, results in cell death and initiates a cascade of fibrosis. As a result, the hepatocyte cell cycle is accelerated and leads to accumulation of genetic alterations, which leads to malignant transformation of hepatocytes^[4]. In addition, HBV integrates into the host DNA where it modifies the expression of certain oncogenes. Certain mutations have been implicated in contributing to a higher incidence of HCC. These include the HBV protein known as HBx, infection with HBV genotype C, the hepatitis B genome mutations pre-S deletions and core promoter mutations (V1735, T1762 and A1764)^[4,5]. Another risk factor is the level of the hepatitis B surface antigen (HBsAg) titer. Levels of HBsAg that are greater than 1,000 IU/mL may independently predict increased risk for developing HCC in Asians in HBeAg negative patients with low HBV viral load^[6]. One retrospective study examined the cumulative probability of HCC development over time despite long-term nucleos(t)ide analog (NA) therapy. The study included treatment-naïve CHB patients (*n* = 524) who received treatment with NAs between January 2003 and December 2007 for longer than 48 weeks. The study revealed a cumulative probability of developing HCC at 1, 2, 3, 4 and 5 years of 0.2%, 1.8%, 3.6%, 5.8%, and 9.3% respectively. In multivariate analysis, age greater than 50 years [hazard ratio (HR) 1.05], family history of HCC (HR 5.48), and the presence of cirrhosis (HR 17.16) were significant predictors of HCC development. Importantly, maintaining a virologic response or HBV DNA < 20 IU/mL for longer than 12 months reduced the risk of HCC development (HR 0.09)^[7]. These studies suggest that persistent HBV viral replication and subsequent liver injury are major risk factors for developing HCC.

The incidence of HBV-related HCC varies between

the western world and Asia; the 5-year cumulative incidences of HCC in Asia among inactive carriers and those with compensated cirrhosis are 1% and 17%, respectively. In Europe and the United States, those incidences are 0.1% and 10%^[8]. A recent meta-analysis evaluated 66 studies with a total of 347,859 patients using multivariate regression analysis, and after adjusting for age, there were no significant differences in HCC incidence between Western and European studies. The analysis showed that age, symptomatic carrier status, chronic hepatitis, or compensated cirrhosis were the greatest risk factors for development of HCC when compared to inactive carriers^[9].

GOALS OF HBV THERAPY

There are 7 drugs currently approved for the treatment of CHB and they can be divided into 2 groups. The immune-modulators include pegylated interferon alfa-2a and interferon alfa-2b. The NA are oral medications, which include lamivudine, telbivudine, adefovir, tenofovir and entecavir. The oral agents have a better side effect profile and thus, most patients are treated with oral therapy. Goals of treating CHB in the short term include suppressing replication with induction of hepatitis B e-antigen (HBeAg) seroconversion in patients with HBeAg-positive CHB and normalization of alanine aminotransferase. In the long term, the goal is to achieve seroconversion of HBsAg to hepatitis B surface antibody. However, HBsAg seroconversion is not common with currently available therapies. It is seen in 1% and 1.5% of patients after 52 weeks of lamivudine or telbivudine therapy respectively. Furthermore, 5 years of adefovir therapy results in HBsAg loss in only 3% of patients. The rates of HBsAg seroconversion are slightly better with entecavir and tenofovir. Ninety-six weeks of entecavir results in 5% seroconversion rate and 4 years of tenofovir yields a 10% seroconversion rate. The best HBsAg seroconversion rate (15%) is seen after 72 weeks of treatment with pegylated interferon alfa-2a and lamivudine^[10-12]. Although seroconversion of HBsAg doesn't occur frequently, multiple studies show that treatment favorably impacts fibrosis, survival and reduces HCC development in patients who are treated for CHB.

The first nucleoside approved for the treatment of HBV was lamivudine. However, development of resistance with prolonged treatment has limited its use. After 5 years of therapy, resistance is reported to be as high as 75%^[13]. Telbivudine and adefovir have a moderate genetic barrier to resistance and are considered to be second line therapies. Currently, entecavir and

tenofovir are first line agents for treating CHB because they have such a high barrier to resistance. Many studies with nucleos(t)ide therapy have confirmed a decrease in the rate of HCC in treated patients, regardless of the strength of the proposed treatment's barrier to resistance.

TREATMENT OF HBV AND HCC

Antiviral therapy with NAs and interferon can improve liver fibrosis and suppress HBV viral replication, which leads to decreased HCC incidence in patients with CHB^[14]. Most of the studies describing the impact of treating CHB on the incidence of liver cancer evaluated the first generation drugs, specifically lamivudine and adefovir. There is less available data regarding the effect of the 3rd generation drugs, tenofovir and entecavir. One recent meta-analysis of patients with HBsAg seroclearance ($n = 34,952$) showed a significantly decreased risk for developing HCC in comparison to those who did not seroconvert [risk ratio (RR) 0.34, 95% confidence interval (CI): 0.20-0.56, $P < 0.001$], but among those who seroconverted, 2.29% (95%CI: 1.19-4.37) still developed HCC^[15].

Adefovir and lamivudine

Liaw *et al.*^[3] published the only randomized clinical trial that addresses the benefits of using lamivudine in CHB patients with cirrhosis or advanced fibrosis proven by biopsy. Compared to the placebo group, the lamivudine group had a significant reduction in HCC, 7.4% vs. 3.9% respectively (HR 0.49, $P = 0.047$). Additionally, the group treated with lamivudine had a nearly 50% reduction in progression of disease (7.8% vs. 17.7%, HR 0.45, $P = 0.001$). As a result of the significant difference found between the 2 arms, the study was stopped prematurely after a mean duration of 32.4 months.

The advantages of using the first-generation NAs to reduce HCC risk has since been supported in meta-analyses and systematic reviews. In a meta-analysis evaluating 5 studies that compared oral treatment to placebo, treatment with NAs was associated with 78% reduced incidence of HCC (RR 0.22, $P < 0.001$) irrespective of cirrhosis. Treatment with NAs has also been shown to benefit patients who developed treatment resistance (NA 3.3% vs. control 6.4%, RR 0.52, $P = 0.04$)^[16]. Similar results were reported in a systematic review that assesses adefovir, lamivudine, and the combination of both vs. placebo in 3,881 CHB patients naive to treatment with NAs. Over a period of 42 months, HCC incidence was lower in treated patients (2.8%) compared to patients who were not treated (6.4%; $P = 0.003$)^[17]. Another meta-analysis

reported rates of HCC of 3.5% in lamivudine-treated CHB patients compared to 9.6% in CHB patients who were not treated, over a period of 4 years^[18].

Entecavir and tenofovir

The introduction of the third generation NAs, tenofovir and entecavir, which both have a high genetic barrier to resistance, has led to further decreases in HCC incidence. A retrospective study comparing the incidence of HCC in entecavir-treated patients to a historical cohort of lamivudine-treated patients without rescue therapy in the event of resistance development was conducted in Japan. Propensity score matching was used to eliminate baseline differences and the authors found that entecavir-treated patients had a lower 5-year cumulative incidence of HCC compared to historical controls (3.7% vs. 13.7%, $P < 0.001$). The benefit of treatment was seen mainly in cirrhotic patients, 7% in the entecavir group vs. 39% in historic controls ($P = 0.049$) compared to the non-cirrhotic group, and 3.3% in the entecavir vs. 3% in controls ($P > 0.05$)^[19]. In an observational study conducted by Wong *et al.*^[20], there was also decreased incidence of HCC with entecavir treatment compared to historical controls, also significant only in cirrhotic patients (13.8% vs. 26.4%, $P = 0.049$). A similar observational study by Su *et al.*^[21] of patients with cirrhosis demonstrated 5 year cumulative HCC incidence of 26.4% in the untreated historical cohort and 11.3% in the treated cohort with entecavir resulting in reduction of HCC risk by approximately 60% (HR 0.40, 95%CI 0.28-0.57). In another propensity score-matched study of Japanese patients ($n = 234$), Kumada *et al.*^[22] determined that entecavir therapy significantly reduced HCC incidence; the 5- and 10-year cumulative incidence of HCC were 11.3% and 40% in untreated controls, respectively, compared to 2.7% and 3.3% in patients treated with entecavir. Long-term entecavir treatment has been shown to reduce fibrosis by more than 1 point by the Ishak fibrosis score in 88% of patients who were treated for 6 years^[23]. A large retrospective study of Taiwanese patients ($n = 21,595$), assessed a cohort of NA-treated patients and a cohort of patients receiving hepatoprotective agents, but no NA treatment matched by propensity score. The 7-year incidence of HCC was significantly lower in the cohort treated with NA (7.3%), compared to the non-NA treated cohort (22.7%) (adjusted HR 0.37; $P < 0.001$). In this study, the benefits of NA therapy were noted among patients without (HR 0.27) cirrhosis in addition to patients with cirrhosis (HR 0.72)^[24].

A recent retrospective study conducted in Canada utilized the REACH-B scoring system to evaluate the risk of developing HCC among patients treated

with NAs. A total of 322 patients were followed for a median of 3.2 years; median treatment duration with NAs was 3.4 years (interquartile range 1.6-5.9) and 80% of the patients were treated with tenofovir or entecavir. During the study period, 11 patients, 3.2%, developed HCC; 9 of these were Asian men. Cirrhosis was the strongest risk factor for HCC development (unadjusted risk 22-fold); patients with cirrhosis had an annual HCC incidence rate of 4.3% vs. 0.2% in patients without cirrhosis. Use of NAs reduced the risk of HCC development; based on the REACH-B model, there was a 50% relative reduction in HCC incidence with NA use, noted as early as 4 years after initiation of treatment^[25]. The Chronic Hepatitis Cohort Study, a longitudinal study in the United States, recently evaluated the relationship between CHB therapy and HCC incidence in 2,671 patients. Patients were diagnosed with CHB between 1992 to 2011 and data were analyzed and collected over a 5-year period; 49% of the sample was Asian. Using propensity score matching and Cox regression analysis, the authors found that patients treated with antivirals had a lower risk of HCC than those who were not treated with antivirals (adjusted HR 0.39; 95%CI 0.27-0.56; $P < 0.001$), after adjusting for abnormal level of alanine aminotransferase (ALT). Like the Canadian study above, the observational, retrospective, multicenter cohort study ENUMERATE conducted in the United States used the REACH-B system to assess HCC risk in NA-treated patients. The study included 841 treatment-naïve CHB patients over an 8-year period who had received > 12 months of entecavir with a median follow-up of 4 years. Overall, HCC was diagnosed in 17 patients (2.6%): 8 patients had cirrhosis (13.1%) and developed HCC and 9 patients without cirrhosis (1.5%) developed HCC. In comparison to those who did not develop HCC, the patients with HCC were more likely to have cirrhosis (47.1% vs. 8.4%) and to be older (53 years vs. 47 years). Among patients who did not have cirrhosis, the observed HCC incidence was lower than the predicted incidence by the fourth year [standardized incidence ratio (SIR) 0.37; 95%CI 0.166-0.82]. By 8.2 years, the maximum follow-up time, the observed incidence of HCC was significantly lower than predicted for all patients (SIR 0.56; 95%CI 0.35-0.905)^[26].

In addition to reversing fibrosis, tenofovir therapy has been shown to decrease HCC risk. In the seminal study by Marcellin *et al.*^[27], treatment with tenofovir for 5 years led to improvement in histology and regression of fibrosis regression (≥ 1 point decrease by Ishak scoring system) in 87% and 51% of the patients, respectively. Kim *et al.*^[28] compared the observed HCC incidence among the 641 patients enrolled in 2 tenofovir registration trials to the incidence of HCC

estimated by the REACH-B risk calculator. Starting at 3.3 years, divergence emerged and progressively widened between the predicted and observed incidence of HCC between the 2 groups. Furthermore, at latest follow-up (median of 5.52 years), the SIR between observed and predicted supporting that treatment with tenofovir is beneficial. A recent study conducted in Taiwan examined the efficacy and safety of treatment in NA-naïve and NA-experienced patients with CHB; after 3 years of therapy, cumulative HCC incidence at 12, 24 and 36 months were 0%, 1.2%, and 4.8%, respectively, and no significant differences were found between NA-naïve and NA-experienced patients in regards to HCC development^[29].

IMPACT OF NA CHOICE ON HCC INCIDENCE

In a study conducted in Korea, patients with compensated cirrhosis secondary to CHB, hepatitis B DNA < 2,000 IU/mL, and normal ALT had HCC incidence of nearly 10% over 5 years, but NA therapy reduced incidence to 5.9% for HBV patients treated with NAs; longer duration of treatment and virological response were associated with lower risk of HCC^[30]. A recent multicenter study demonstrated a reduction of 77% in HCC incidence in those treated with NAs treatment compared to those who were untreated; this was adjusted for age, gender, ALT, and HBV DNA and was independent of the presence of cirrhosis^[31].

Several studies have also evaluated whether the choice of NA affects risk reduction of HCC. In a retrospective study of CHB patients with cirrhosis ($n = 227$, 104 with decompensated cirrhosis) who were followed over 21-36 months, Koklu *et al.*^[32] showed the incidence of HCC to be 3%, 5%, and 8%, respectively, in the tenofovir, entecavir, and lamivudine groups. There was no significant difference found between the NA in the prevention of HCC. In a study of 355 treatment-naïve patients with CHB, 39.2% of whom had cirrhosis, who received entecavir or tenofovir, Idilman *et al.*^[33] found that the cumulative incidence of HCC at 1 year was 3.3% and at 4 years was 7.3%. No significant difference was found between the 2 groups. A multicenter European study evaluated 1,756 Caucasian patients in an attempt to evaluate the impact of treatment with entecavir and/or tenofovir for 39 months on HCC occurrence. Overall, the 5-year cumulative probability of HCC was 8.7%. In patients without cirrhosis, the cumulative 5-year HCC rate was 3.7% compared to 17.5% in patients with cirrhosis and 36.3% in patients with decompensated cirrhosis^[34]. In a recent review of NAs including lamivudine, tenofovir, and entecavir, Papatheodoridis *et al.*^[35] concluded

that no significant difference exists between agents in preventing HCC even in patients who were rescued after development of lamivudine resistance.

A recent Greek analysis compared a cohort of patients treated with entecavir ($n = 321$), for a median duration of 40 months to a matched cohort of patients ($n = 818$), initially treated with lamivudine for a median duration of 60 months. Using multivariable Cox regression analysis, risk of HCC was independently associated with male gender ($P = 0.011$), older age ($P < 0.001$), and cirrhosis ($P = 0.025$); HCC risk was not associated with the choice of agent used, at least for the first 5 years^[36]. In a Taiwanese population-based cohort study, 1,544 patients with active hepatitis due to HBV taking lamivudine, entecavir, tenofovir, or telbivudine over an 8-year period were evaluated for HCC risk and risk of mortality. For the propensity score matching, patients not treated with NAs ($n = 1,544$), were selected as the comparison group. As mentioned previously, the treated cohort had a significantly lower rate of HCC occurrence (6.0%; 95%CI 4.4%-7.9%) compared to the cohort not treated with NAs (8.5%; 95%CI 6.6%-10.6%; $P = 0.0025$). Overall mortality rate for the treated cohort was 6.9% (95%CI 5.3%-8.7%) compared to 9.4% for the untreated cohort (95%CI 7.7%-11.3%) ($P = 0.0003$). Cox regression analyses demonstrated that use of NAs use significantly reduced the risk of HCC (HR 0.64; 95%CI 0.45-0.93; $P = 0.017$) and overall mortality (HR 0.58; 95%CI 0.43-0.79; $P < 0.001$)^[37].

Finally, there is new evidence that treatment of CHB reduces mortality related to HCC and HCC recurrence in patients undergoing curative treatments^[38]. Huang *et al.*^[38] demonstrated antiviral therapy after liver resection to be an independent protective factor of late tumor recurrence (HR 0.348). Similar results were reported by Yin *et al.*^[39] In a randomized controlled trial, antiviral therapy reduced both tumor recurrence (HR 0.48) and HCC-related death (0.26). In a study of Taiwanese patients undergoing resection ($n = 4,569$), those who received NA had significantly lower recurrence rate at 6 years compared to patients not treated with NAs (45.6% vs. 54.6% respectively) ($P < 0.001$). Additionally, the NA-treated group had lower mortality overall at 6 years (29% vs. 42.4%) ($P < 0.001$)^[40]. In a recent meta-analysis including 8,204 patients status-post curative resection of HCC, high viral load was significantly associated with increased risk of recurrence, poorer disease-free survival and overall survival of HBV-related HCC after surgical resection. However, NA therapy significantly decreased the recurrence risk (RR 0.69; 95%CI 0.59-0.80; $P < 0.001$) and improved both disease-free (RR 0.70; 95%CI 0.58-0.83; $P < 0.001$) and overall survival

(RR 0.46; 95%CI 0.32-0.68; $P < 0.001$) in these patients^[41]. Clearly, surgical and medical treatment of CHB improves mortality due to HCC and reduces its recurrence.

LIMITATIONS OF THE HCC PREDICTOR MODELS

Several HCC risk calculators have been proposed including the REACH-B based on a Taiwanese population, the Chinese-University-Hepatocellular carcinoma score (CU-HCC) score^[42], and the GAG-HCC score, which incorporates age, gender, HBV DNA, presence of core promoter mutations and cirrhosis^[43]. These models were developed in Asians and the application to other populations is unclear, though one study showed good performance in non-Asians^[44]. The platelet, age, gender (PAGE-B score is based on platelet, age and gender and was developed to assess risk of HCC in Caucasians. Another limitation of these models is that they do not include a liver fibrosis assessment such as transient elastography. In addition, some models like the CU-HCC included 15% of HBV treated patients rather than all treatment naïve patients. It is questionable whether the HCC risk predictor models can be used in patients on HBV therapy, as therapy leads to viral suppression and may lead to fibrosis regression. In addition, the absence of the degree of HBV viral suppression in some models is a major limitation of the risk calculators^[35].

CONCLUSION

In patients with CHB, successful treatment can reduce but not eliminate the risk of developing HCC, regardless of the presence or absence of cirrhosis. Treatment of CHB can reverse fibrosis as demonstrated by studies involving the third-generation NAs tenofovir and entecavir, which have a high genetic barrier to resistance. Additionally, growing evidence supports that treatment of CHB reduces recurrence rates of HCC and HCC-related mortality in CHB patients who received curative treatments for HCC.

Most data regarding chemoprevention is derived from studies using lamivudine and this significantly limits interpretation of the data. It is possible that the chemopreventative effect is more pronounced with the long term use of entecavir and tenofovir, which have a much lower risk of resistance with prolonged use when compared to lamivudine. Most of the studies evaluating the effect of chemoprevention are retrospective in nature, which is another major limitation. In other

studies, the reduction of HCC incidence was not the primary outcome measured. Despite these limitations, results from medium-length follow up studies with entecavir and tenofovir and analyses of registration trials already suggest that treatment with these NAs have chemopreventive effects and reduce risk of HCC.

Continued viral suppression is critical to minimize the risk of HCC development, although achieving viral suppression will not eliminate the risk of HCC, specifically in high-risk patients with advanced fibrosis or cirrhosis. In these situations, continuous surveillance for HCC is essential. Prospective studies which address the confounding factors such as gender, age, fibrosis stage. Finally, HCC screening algorithms are necessary to better elucidate the impact of chemoprevention on HCC development in HBV patients treated with the newer nucleos(t)ide agents.

In summary, treatment of hepatitis B leads to decreased incidence of hepatocellular carcinoma in Asians and Caucasians regardless of the nucleos(t)ide used. Also, decreasing the HBV viral load, regardless of achieving seroconversion, results in decreased HCC incidence. Despite this reduction in HCC incidence, patients treated with nucleos(t)ides still need to undergo liver cancer screening. Several HCC predictor models have been developed, but as of now, there are limitations in applicability.

DECLARATIONS

Authors' contributions

Project conception: W.S. Ayoub, P. Martin, P.D. Jones
Literature review, manuscript drafting and critical revision: W.S. Ayoub, F. Dailey, P. Martin, P.D. Jones

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None.

Conflicts of interest

Walid S. Ayoub, Francis Dailey, Paul Martin, Patricia D. Jones declare that they have no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

This article does not contain any studies with human or animal subjects performed by any of the authors.

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AUTHOR INSTRUCTIONS

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In the first paragraph: include the title and type (e.g., Original Article, Review, Case Report, *etc.*) of the manuscript, a brief on the background of the study, the question the author sought out to answer and why;

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Manuscript Type	Definition	Abstract	Keywords	Main Text Structure
Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Review	A Review paper summarizes the literature on previous studies. It usually does not present any new information on a subject.	Unstructured abstract. No more than 250 words.	3-8 keywords	The main text may consist of several sections with unfixed section titles. We suggest that the author includes an "Introduction" section at the beginning, several sections with unfixed titles in the middle part, and a "Conclusion" section in the end.
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Systematic Review	A Systematic Review collects and critically analyzes multiple research studies, using methods selected before one or more research questions are formulated, and then finding and analyzing related studies and answering those questions in a structured methodology.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
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2.3 Manuscript Structure

2.3.1 Front Matter

2.3.1.1 Title

The title of the manuscript should be concise, specific and relevant, with no more than 16 words if possible. When gene or protein names are included, the abbreviated name rather than full name should be used.

2.3.1.2 Authors and Affiliations

Authors' full names should be listed. The initials of middle names can be provided. Institutional addresses and email addresses for all authors should be listed. At least one author should be designated as corresponding author. In addition, corresponding authors are suggested to provide their Open Researcher and Contributor ID upon submission. Please note that any change to authorship is not allowed after manuscript acceptance.

2.3.1.3 Abstract

The abstract should be a single paragraph with word limitation and specific structure requirements (for more details please refer to Types of Manuscripts). It usually describes the main objective(s) of the study, explains how the study was done, including any model organisms used, without methodological detail, and summarizes the most important results and their significance. The abstract must be an objective representation of the study: it is not allowed to contain results which are not presented and substantiated in the manuscript, or exaggerate the main conclusions. Citations should not be included in the abstract.

2.3.1.4 Keywords

Three to eight keywords should be provided, which are specific to the article, yet reasonably common within the subject discipline.

2.3.2 Main Text

Manuscripts of different types are structured with different sections of content. Please refer to Types of Manuscripts to make sure which sections should be included in the manuscripts.

2.3.2.1 Introduction

The introduction should contain background that puts the manuscript into context, allow readers to understand why the study is important, include a brief review of key literature, and conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved. Relevant controversies or disagreements in the field should be introduced as well.

2.3.2.2 Methods

Methods should contain sufficient details to allow others to fully replicate the study. New methods and protocols should be described in detail while well-established methods can be briefly described or appropriately cited. Experimental participants selected, the drugs and chemicals used, the statistical methods taken, and the computer software used should be identified precisely. Statistical terms, abbreviations, and all symbols used should be defined clearly. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supplementary materials.

2.3.2.3 Results

This section contains the findings of the study. Results of statistical analysis should also be included either as text or as tables or figures if appropriate. Authors should emphasize and summarize only the most important observations. Data on all primary and secondary outcomes identified in the section Methods should also be provided. Extra or supplementary materials and technical details can be placed in supplementary documents.

2.3.2.4 Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

2.3.2.5 Conclusion

It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

2.3.3 Back Matter

2.3.3.1 Acknowledgments

Anyone who contributed towards the article but does not meet the criteria for authorship, including those who provided professional writing services or materials, should be acknowledged. Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgments section. This section is not added if the author does not have anyone to acknowledge.

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Each author is expected to have made substantial contributions to the conception or design of the work, or the acquisition, analysis, or interpretation of data, or the creation of new software used in the work, or have drafted the work or substantively revised it.

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In order to maintain the integrity, transparency and reproducibility of research records, authors should include this section in their manuscripts, detailing where the data supporting their findings can be found. Data can be deposited into data repositories or published as supplementary information in the journal. Authors who cannot share their data should state that the data will not be shared and explain it. If a manuscript does not involve such issue, please state "Not applicable." in this section.

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References should be numbered in order of appearance at the end of manuscripts. In the text, reference numbers should be placed in square brackets and the corresponding references are cited thereafter. Only the first five authors' names are required to be listed in the references, other authors' names should be omitted and replaced with "et al.". Abbreviations of the journals should be provided on the basis of Index Medicus. Information from manuscripts accepted but not published should be cited in the text as "Unpublished material" with written permission from the source.

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Books	Sherlock S, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. <i>The genetic basis of human cancer</i> . New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm . [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. <i>Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming</i> ; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
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