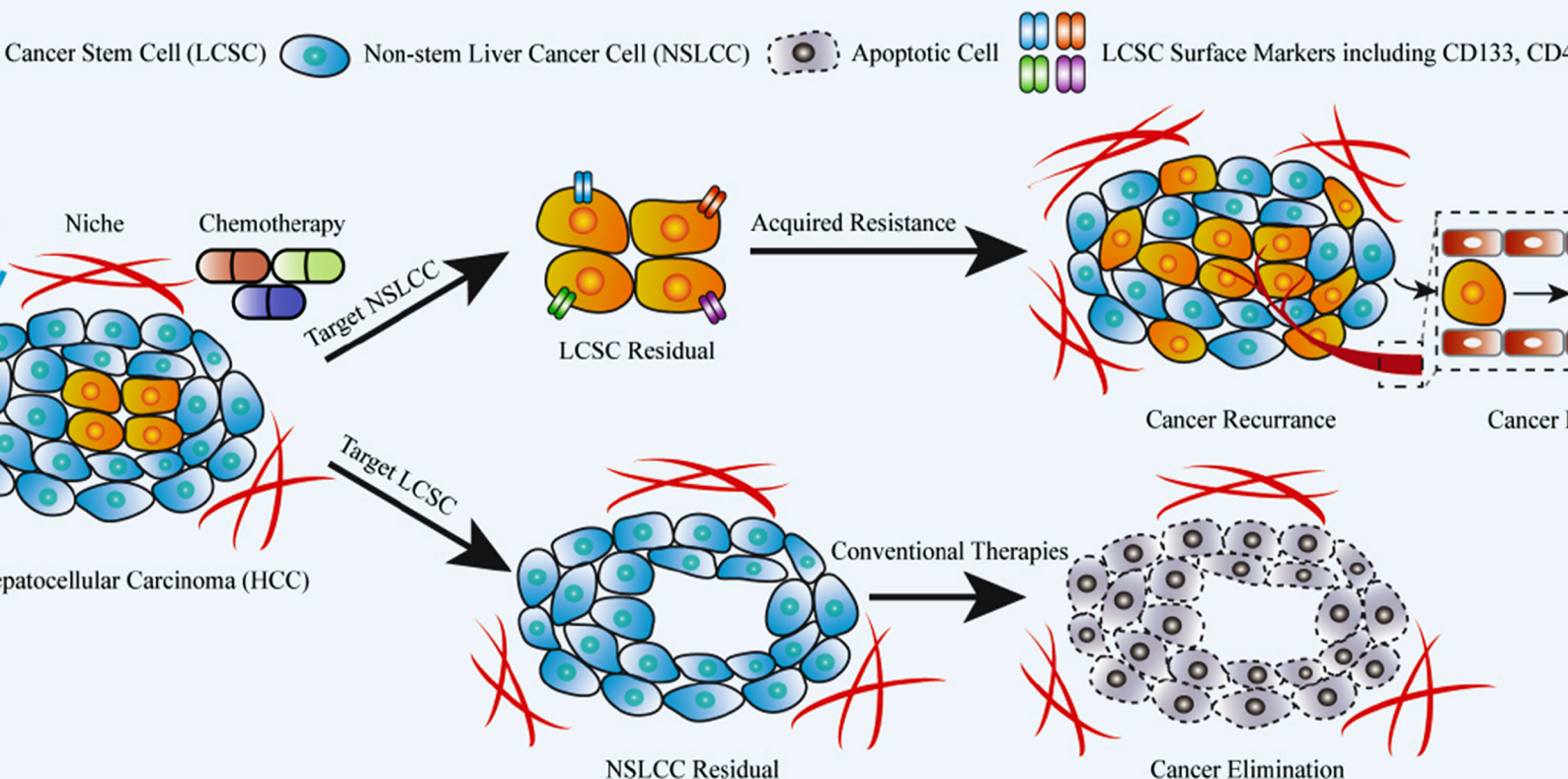
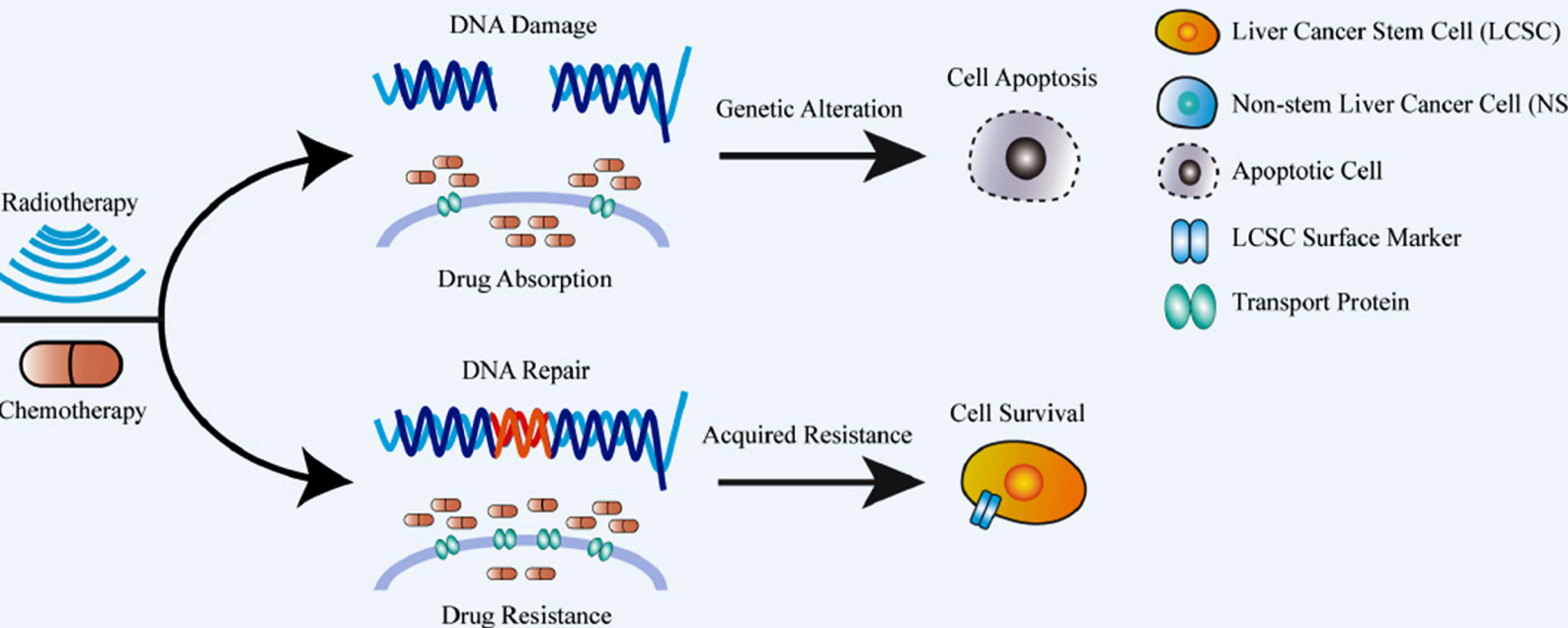


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Review

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# Diagnostic imaging for hepatocellular carcinoma

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## Abstract

Hepatocellular carcinoma (HCC) occurs mostly in individuals with cirrhosis, which is why the guidelines of the most important scientific societies indicate that these patients are included in surveillance programs through the repetition of an ultrasound examination every 6 months. The aim is to achieve early identification of the neoplasia in order to increase the possibility of curative therapies (liver transplantation, surgery or local ablative therapies) and to increase patient survival. HCC nodules arising in cirrhotic livers show characteristic angiographic behavior that can be evaluated with dynamic multidetector computed tomography and dynamic magnetic resonance imaging (MRI). However, the use of these techniques in real life is often hindered by the lack of uniform terminology in reporting and in the interpretation of the exams reflected in the impossibility of comparing examinations performed in different centers and/or at different times. Liver Imaging Reporting and Data System® was created to standardize reporting and data collection of computed tomography and MRI for HCC. In some cases HCC arises in patients with healthy livers and, although there is evidence that angiographic behavior is not different from cirrhotic patients in this clinical situation, the guidelines still indicate the execution of a biopsy. Frequent use of palliative therapeutic techniques such as transarterial chemoembolization, transarterial radioembolization or administration of antiangiogenic drugs (sorafenib) poses problems of interpretation of the therapeutic response with repercussions on the subsequent choices that have been attempted to resolve with the use of stringent criteria such as Modified Response Evaluation Criteria In Solid Tumors.

**Keywords:** Hepatocellular carcinoma, cirrhosis, ultrasonography, magnetic resonance imaging, multidetector computed tomography, Liver Imaging Reporting and Data System, Modified Response Evaluation Criteria In Solid Tumors

## INTRODUCTION

The diagnosis of hepatocellular carcinoma (HCC) can be addressed in two different clinical settings. A first context, rarely, is that of the patient with a healthy liver. In this scenario, patients do not undergo routine



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monitoring and tumors are often large with possible vascular involvement. In these patients, performing a liver biopsy is often necessary for diagnostic confirmation. A second, frequently, is that of patients with chronic liver disease (cirrhosis or advanced fibrosis) under regular ultrasound surveillance<sup>[1]</sup>. The goal of surveillance and screening is to reduce mortality<sup>[2]</sup>. For the aforementioned reasons HCC meets the criteria for developing a surveillance program and the use of ultrasound as a screening tool is accepted by major scientific societies<sup>[3-5]</sup>. In many studies the 3-year survival rate varies between 50.8% and 45.6% in patients under surveillance and 27.9% and 28.8% in those not screened. Even after correction for lead time bias, the three-year survival is better in patients undergoing ultrasound surveillance: 39.7% vs. 29.1%. It is likely that the increased survival in patients undergoing surveillance is linked to an increase in early detection of HCC in patients screened (OR of 2.11; 95% CI, 1.88-2.33) and therefore of the use of curative treatments (61.8% vs. 38.2%)<sup>[5]</sup>.

## ULTRASONOGRAPHY AND CONTRAST-ENHANCED ULTRASONOGRAPHY

Efficacy of detection of HCC with ultrasound varies widely and in cirrhotic patients presents with a sensitivity of 33%-96%<sup>[6]</sup> while specificity reach over 90%<sup>[7]</sup>. The identification of small HCC nodules in cirrhotic liver with a coarse parenchymal pattern is not easy, therefore a skilled operator must work with adequate equipment, preferably in dedicated centers. In gray-scale ultrasound small HCCs typically appear as a hypoechoic lesion. In some cases increased echogenicity may be present due to adipose degeneration. Sometimes the hypoechoic nodule may present a hyperechoic focus which is suggestive of development of HCC within a dysplastic nodule (nodule in nodule phenomenon)<sup>[8]</sup>.

But not all nodules identified with ultrasound in patients with chronic liver disease (cirrhosis or advanced fibrosis) undergoing surveillance are HCCs. In this context, the role of imaging is to differentiate the nodules of HCC from other malignant lesions (intrahepatic cholangiocarcinoma and metastasis) and non-malignant (e.g., regenerative nodules, low and high grade dysplastic nodules, confluent fibrosis, angiomas, *etc.*) that can be found in the cirrhotic liver. In oncology, the diagnosis of cancer generally requires histological assessment; from this point of view HCC is an exception since a non-invasive diagnosis can be achieved with imaging alone in these high-risk populations. The peculiar angiographic behavior of the HCC nodules in a cirrhotic liver characterized by the presence of the wash-in during the arterial phase and by the wash-out during the venous and late phases, represents the diagnostic hallmarks of HCC. These characteristics are able to provide a reliable diagnosis of HCC in high-risk patients affected by liver cirrhosis or with advanced fibrosis, and represent the background for the development, by Western and Asian scientific societies, of different algorithms for non-invasive diagnosis of HCC<sup>[3,4,9,10]</sup>. The recommended imaging methods are computed tomography (CT) and MRI with contrast agents. Diagnostic algorithms of American Association for the Study of Liver Diseases (AASLD) and European Association for the Study of the Liver (EASL) guidelines adopt a strategy dependent on the size of the lesion. Nodules smaller than 1 cm are considered too small to be characterized and both guidelines recommend ultrasound monitoring every three (AASLD) or four months (EASL). As for the diagnosis of nodules with a diameter greater than 2 cm, both guidelines recommend only one imaging method. For nodules with a diameter of 1-2 cm, the statements differ, as the American guidelines recommend the same approach used for lesions larger than 2 cm (only one contrast method is sufficient), while the European guidelines contemplate the concordance of two consecutive images if these cases are not followed in centers with substantial "expertise".

Consequently, in cirrhotic patients, biopsy is indicated only in cases where nodules do not present contrasting features typical of HCC. However, the increasing knowledge about the immunohistochemical and molecular characteristics of HCC may bring biopsy to the forefront in order to select patients who could gain the most benefit from target-driven HCC treatments<sup>[11]</sup>.

In conclusion, the identification of a liver focal lesion greater than 1 cm in the course of surveillance of patients at risk with ultrasonography imposes the study of the nodule vascularization through the use of

multidetector computed tomography (MDCT) or multi-phase nuclear magnetic resonance imaging (MRI) with contrast agents.

Contrast-enhanced ultrasound (CEUS) may be a useful imaging modality for the noninvasive diagnosis of small, newly detected liver nodules during surveillance of cirrhotic patients<sup>[12]</sup>. Ultrasound contrast agents (“microbubbles”) comprise an albumen or phospholipid shell containing a stable perfluorocarbon or sulphur hexafluoride gas. They are predominantly blood-pool agents, as the encapsulated microbubbles are small enough to pass through both pulmonary and systemic circulation after intravenous injection and durable enough to re-circulate for several minutes<sup>[13]</sup>. CEUS can also be utilized in the presence of renal impairment and can be performed at the time in which the lesion is discovered but it does not eliminate the need for CT and/or MRI in order to characterize the lesion and to stage the disease<sup>[14]</sup>. CEUS was inserted as a method for characterizing nodules arising in cirrhotic livers, in the 2005 AASLD guidelines<sup>[15]</sup> but was subsequently eliminated in 2011, partly due to lack of availability of ultrasound contrast in the USA and partly due to false positive diagnoses in patients with intra-hepatic cholangiocarcinoma<sup>[3]</sup>.

## CT AND MRI

As already mentioned, the identification of a liver focal lesion greater than 1 cm in the course of surveillance with ultrasonography of patients at risk imposes the study with higher level image techniques such as MDCT or MRI with an extracellular contrast medium (iodized compound or gadolinium-based compounds: gadoteric acid, gadopentetic acid, gadodiamide, gadoteridol, gadobutrol) that remain in the extracellular space and allows the characterization of blood flow. Multi-phase MRI may be performed also with an hepatospecific contrast agents such as gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid or Gd-EOB-DTPA, gadobenate dimeglumine or Gd-BOPTA, which is captured by “healthy” hepatocytes and excreted in the biliary tract, or by iron oxide particles (SPIO) with superparamagnetic activity, which are captured by Kupffer cells located in the non-neoplastic hepatic parenchyma and in benign lesions but not in malignant ones.

The goals of the evaluation by MRI or MDCT of a hepatic nodule in a patient with liver cirrhosis are not only the determination of the nature of the lesion but also, in the case of an HCC, the estimate of the hepatic extension of the neoplasia and the possible localization in extrahepatic sites in order to propose a treatment based on the exact staging of the disease.

There is universal consensus that the diagnosis of HCC can be achieved without biopsy in a situation where the pre-test probability is very high, as happens in liver cirrhosis, but there is no consensus as to which technique is the best. The angiographic features of HCC are identical in MDCT and MRI, but the latter offers a series of additional imaging sequences such as T2-weighted sequences, diffusion-weighted imaging and in combination with the use of a hepatospecific contrast agent it can improve diagnostic performance<sup>[16-19]</sup>. However, MRI presents greater technical complexity, longer scan times, greater susceptibility to artifacts, a less consistent image quality, higher cost, lower availability, longer scheduling backlogs<sup>[20]</sup> and its diagnostic yield becomes void if the patient is unable to hold his breath, to remain still or presents a high-volume ascites. For these reasons the superiority of one method over the other, especially in real-life contexts remains uncertain.

In a recent meta-analysis in which MDCT was compared with MRI with an extracellular agent, or MRI with gadoxetate disodium, Roberts *et al.*<sup>[20]</sup> concluded that the latter showed significantly higher sensitivity (0.82; 95% CI, 0.75-0.87 vs. 0.66; 95 % CI, 0.60-0.72) and lower negative likelihood ratio (0.20; 95% CI, 0.15-0.28; vs. 0.37; 95% CI, 0.30-0.44) in diagnosis of HCC lesions. Pooled analysis demonstrated that both gadoxetate enhanced MRI and extracellular contrast - enhanced MRI provided significantly higher sensitivity and

lower negative likelihood ratio than MDCT<sup>[20]</sup>. However, the authors do not believe there is enough evidence to provide definitive recommendation for systematic use of gadoxetate-enhanced MRI or extracellular contrast-enhanced MRI over MDCT. In fact, in clinical practice, beyond the diagnostic yield, many other factors may guide the choice between modalities, such as the presence of ascites, the patient's inability to hold his or her breath, the severity of cirrhosis and/or a significant hepatic iron overload, and the presence of contraindications to the use of contrast agents.

## LIVER IMAGING REPORTING AND DATA SYSTEM

The application of the guidelines in real life is often penalized by the lack of uniform terminology in reporting and by the excessive variation in the interpretation of the exams causing the impossibility of comparing examinations performed in different centers and/or at different times. Liver Imaging Reporting and Data System® (LI-RADS®)<sup>[21]</sup> was created to standardize the reporting and data collection of MDCT and MRI for HCC. This method of categorizing liver findings for patients with risk factors for developing HCC allows the radiology community to: (1) apply consistent terminology; (2) reduce imaging interpretation variability and errors; (3) Enhance communication with referring clinicians; and (4) facilitate quality assurance and research.

LI-RADS, was originally released by the American College of Radiology in 2011, and since then revised four times. The system was created to be applied to MDCT and MRI in the context of hepatic diseases at high risk of developing malignant lesions, such as cirrhosis, chronic hepatitis B or history of current or prior HCC. They permit better communication between radiologists and physicians, clearly differentiating between lesions definitively benign (LR1, i.e., LI-RADS 1), probably benign (LR2), with intermediate probability of being malignant (LR3), with high probability of being malignant but not necessarily HCC (LR-M), probably HCC (LR4), and definitively HCC (LR5). The final version of LI-RADS has been published online on the American College of Radiology (ACR) website<sup>[22]</sup>. The assignment to specific categories is obtained considering certain “major features”: (1) arterial phase hyperenhancement; (2) size of the lesion; (3) portal venous phase wash-out; (4) “enhancing capsule” in portal venous/delayed/transitional phases; and (5) speed of growth over a threshold. The “enhancing capsule” is a smooth, uniform, sharp border around the lesion, clearly thicker than the fibrous layers of the background regenerative cirrhotic nodules. The threshold of growth means an increase in size of a mass by a minimum of 5 mm associated with:  $\geq 50\%$  increase in size in  $\leq 6$  months,  $\geq 100\%$  increase in size in  $> 6$  months or a previously unseen nodule on MDCT/MRI, now  $\geq 10$  mm, in  $\leq 24$  months. These “major criteria” must be combined as shown below to ascertain the final category [Figure 1]. The use of “ancillary criteria” is at the discretion of the radiologist, and allows for recategorization [Figure 2]. In fact, one ancillary feature favoring malignancy, allows for upgrading by one category to LR-4 (but can never be used to upgrade to LR-5); on the contrary, one ancillary criterion favoring benignity warrants downgrading by one category; the coexistence of one criterion favoring benignity and another favoring malignity does not modify the current category.

LI-RADS are also applicable in judging the response to treatment: even if there is no description of treatment-specific features, some general indications are given to carry out the categorization, as illustrated in Table 1.

Regarding the category “LR-TR viable”, when the tissue has a thick irregular aspect, the measurement is made by taking the longest diameter of the enhancing area, without traversing the non-enhancing area; when it has a mass like aspect (and possibly more than one mass), the biggest enhancing area is to be measured, by taking its longest diameter.

Compared to other systems for radiological evaluation of hepatic lesions, LI-RADS has introduced an important innovation that is a program of follow-up for each radiological category. Specifically, benign

## CT/MRI Diagnostic Table

Arterial phase hyperenhancement (APHE)		No APHE		APHE (not rim)		
Observation size (mm)		< 20	≥ 20	< 10	10-19	≥ 20
<b>Count major features:</b> • “Washout” (not peripheral) • Enhancing “capsule” • Threshold growth	None	LR-3	LR-3	LR-3	LR-3	LR-4
	One	LR-3	LR-4	LR-4	LR-4 LR-5	LR-5
	≥ Two	LR-4	LR-4	LR-4	LR-5	LR-5



Observations in this cell are categorized LR-4, except:

- LR-5g, if ≥ 50% diameter increase in < 6 months (equivalent to OPTN 5A-g)
- LR-5us, if “washout” and visibility at screening ultrasound (per AASLD HCC criteria)

*If unsure about the presence of any major feature: characterize that feature as absent*

**Figure 1.** Categories Liver Imaging Reporting and Data System based on the application of major criteria. CT: computed tomography; MRI: magnetic resonance imaging; LR: Liver Imaging Reporting and Data System (LI-RADS); APHE: arterial phase hyperenhancement; OPTN: Organ Procurement and Transplantation Network; AASLD: American Association for the Study of Liver Diseases; HCC: hepatocellular carcinoma

**Table 1. Liver Imaging Reporting and Data System criteria to define the response to treatment**

Responsecategory	Criteria
LR-TR nonviable	No lesional enhancement OR Treatment-specific expected enhancement pattern
LR-TR equivocal	Enhancement atypical for treatment-specific expected enhancement pattern and not meeting criteria for probably or definitely viable
LR-TR viable	Nodular, masslike, or thick irregular tissue in or along the treated lesion with any of the following: Arterial phase hyperenhancement OR Washout appearance OR Enhancement similar to pretreatment

LR-TR: Liver Imaging Reporting and Data System Treatment Response

lesions (LR-1 and LR-2) do not require an adaptation of the normal program of surveillance proposed by the guidelines for patients at risk of HCC, so in this condition a MDCT/MRI with extracellular contrast should be repeated after 6 months. For lesions at intermediate risk of malignancy (LR-3), it is advised to repeat the same imaging examination at 3-6 months; changing the imaging technique is a possible alternative, but is not recommended. For LR-M and LR-4, a multidisciplinary discussion is required to decide whether a biopsy and/or treatment is feasible, otherwise the same imaging examination will be repeated within a maximum of 3 months. A multidisciplinary discussion is also proposed for LR-5 to select the best treatment option. A special category that needs multidisciplinary evaluation is that of LR-TIV (tumor in vein) which is assigned only if the neoplastic nature of the vascular occlusion can unequivocally be determined, combining radiological features with serological biomarkers and (if needed) histological aspect. For treated HCC, independently from the result obtained, a follow-up every 3 months or less using the same imaging modality is suggested. Reporting this last recommendation in clinical practice, a reasonable approach could be that of monitoring the treated lesion with ultrasound as well, in order to better guide the timing for the repetition of MDCT/MRI on the basis of dimensional or aspect modification of HCC.

As mentioned before, ultrasound is the screening method indicated by all guidelines for the surveillance of patients at risk of developing an HCC. In light of the potential importance of a first detection in ultrasound

Ancillary features favoring malignancy	Ancillary features favoring benignity
<b>Favoring malignancy in general, not HCC in particular</b> <ul style="list-style-type: none"> <li>• US visibility as discrete nodule</li> <li>• Subthreshold growth</li> <li>• Restricted diffusion</li> <li>• Mild-moderate T2 hyperintensity</li> <li>• Corona enhancement</li> <li>• Fat sparing in solid mass</li> <li>• Iron sparing in solid mass</li> <li>• Transitional phase hypointensity</li> <li>• Hepatobiliary phase hypointensity</li> </ul>	<ul style="list-style-type: none"> <li>• Size stability &gt; 2 yrs</li> <li>• Size reduction</li> <li>• Parallels blood pool</li> <li>• Undistorted vessels</li> <li>• Iron in mass, more than liver</li> <li>• Marked T2 hyperintensity</li> <li>• Hepatobiliary phase isointensity</li> </ul>
<b>Favoring HCC in particular</b> <ul style="list-style-type: none"> <li>• Nonenhancing “capsule”</li> <li>• Nodule-in-nodule</li> <li>• Mosaic architecture</li> <li>• Blood products in mass</li> <li>• Fat in mass, more than adjacent liver</li> </ul>	

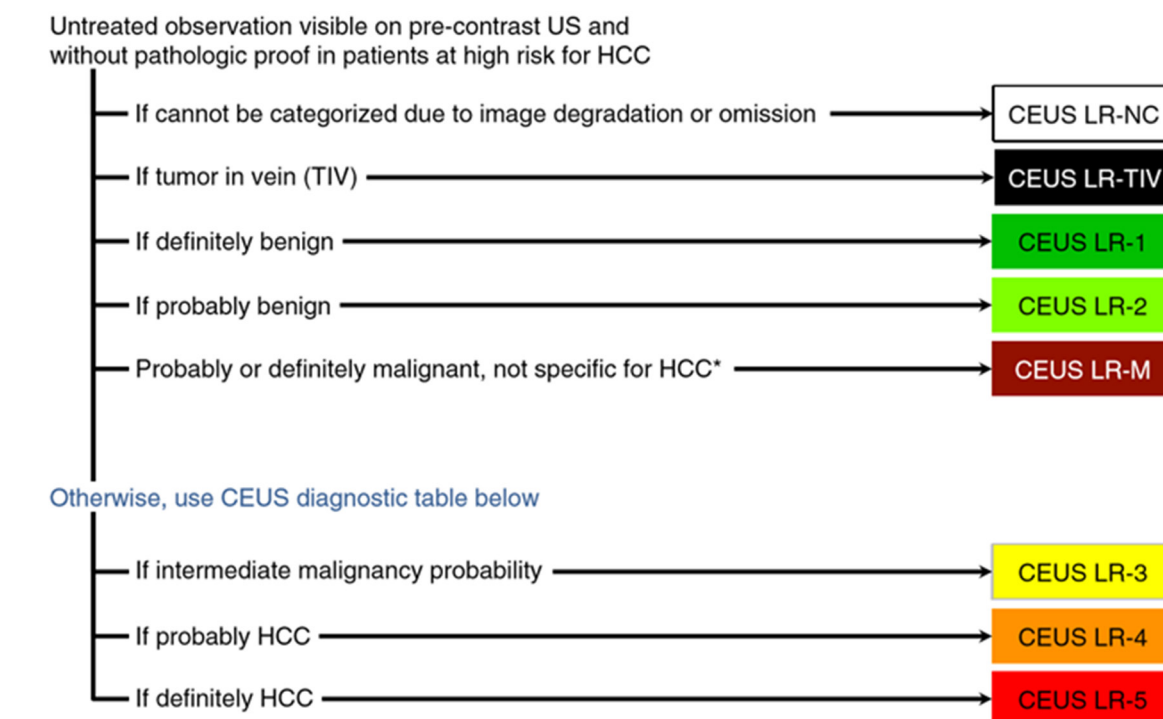
*If unsure about presence of any ancillary feature: characterize that feature as absent*

**Figure 2.** Ancillary features can help the radiologist to upgrade or downgrade of category the hepatic lesions. US: ultrasound; HCC: hepatocellular carcinoma

of nodules suspected of being malignant, ACR has proposed LI-RADS ultrasound v2017<sup>[23]</sup> criteria for the definition of the risk on the basis of the features depicted at the radiological examination of the liver by ultrasound. The first step is to determine the presence/absence of a focal lesion and to subsequently identify the appropriate LI-RADS category: “US-1” (negative) defines the absence of lesions or the presence of clearly benign observations such as cysts, hemangiomas or skip areas around the gallbladder fossa; “US-2” (subthreshold) refers to a solid nodule which is not unequivocally benign, of diameter  $\leq 1$  cm and warrants short-term ultrasound surveillance; “US-3” (positive) takes into account lesions  $\geq 10$  mm in diameter, not unequivocally benign, which may warrant multiphase contrast enhanced imaging. This latter category also comprises new venous thrombosis. Considering the possible limitations of visibility at ultrasound associated with technical difficulties such as large patient body habitus or inability to cooperate, limited acoustic window, parenchymal heterogeneity and/or reduced beam penetration, LI-RADS ultrasound allows for the use of a “visualization score”: (1) no or minimal limitations which are unlikely to meaningfully affect sensitivity; (2) moderate limitations which may obscure small masses; and (3) severe limitations which significantly lower sensitivity for focal liver lesions. The category US-1 requires continuation of screening/surveillance with ultrasound every six months; US-2 demands follow up by ultrasound after 3-6 months; US-3 warrants immediate multiphase contrast-enhanced MDCT/MRI or CEUS<sup>[23]</sup>.

Contrast agents for ultrasound are biodegradable microbubbles that resonate under low-power ultrasound waves and generate harmonic signals. A contrast-specific ultrasound imaging mode, available on the majority of ultrasound scanners, highlights signals from microbubbles while applying specific pulse sequences which suppress signals from tissues. This stimulation of the microbubbles allows for the visualization of arterial hyper-enhancement and venous wash-out. Prospective studies have added evidence that different hepatic malignant lesions appear differently in CEUS and that their post-contrast behaviour is typical and reproducible<sup>[24]</sup>. As such, ACR has included a section dedicated to CEUS firstly in the version of LI-RADS of 2016 and has recently published a new edition<sup>[25,26]</sup>. Similar to those for injected MDCT/MRI, LI-RADS categories for CEUS are: CEUS LR-NC (uncategorizable), CEUS LR-TIV, CEUS LR-1 (definitely





### CEUS Diagnostic Table

Arterial phase hyperenhancement (APHE)	No APHE		APHE **	
Nodule size (mm)	< 20	≥ 20	< 10	≥ 10
No washout of any type	CEUS LR-3	CEUS LR-3	CEUS LR-3	CEUS LR-4
Late and mild washout	CEUS LR-3	CEUS LR-4	CEUS LR-4	CEUS LR-5

**\* CEUS LR-M criteria:**

If rim APHE OR  
early (<60s) washout OR  
marked washout

*If unsure about the presence of any major feature: characterize that feature as absent*

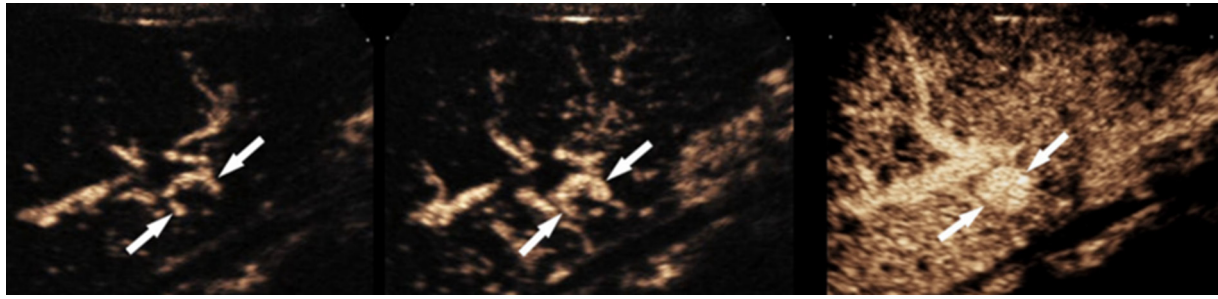
**\*\* APHE:**

- Not rim (indicates LR-M)
- Not peripheral discontinuous globular (indicates hemangioma)

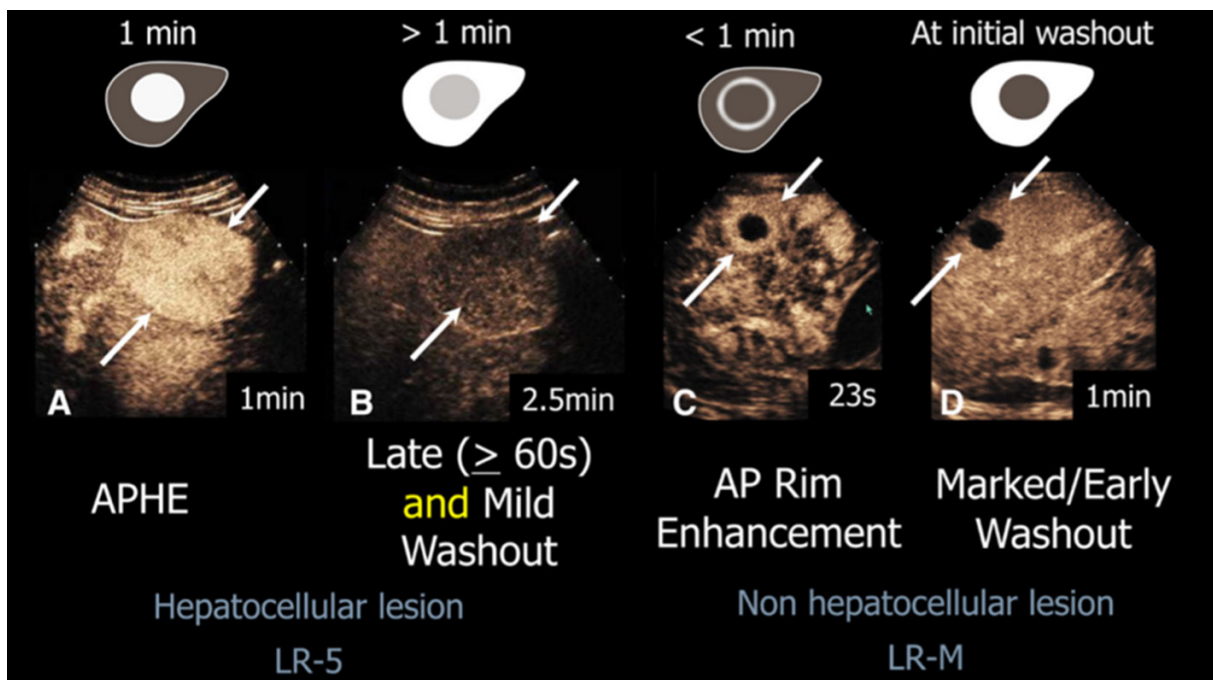
**Figure 3.** Contrast-enhanced ultrasound Liver Imaging Reporting and Data System. LR: Liver Imaging Reporting and Data System (LI-RADS); US: ultrasound; CEUS: contrast-enhanced ultrasound; LR-NC: LI-RADS noncategorizable; TIV: tumor in vein; HCC: hepatocellular carcinoma; APHE: arterial phase hyperenhancement; LR-M: LI-RADS malignancy

benign), CEUS LR-2 (probably benign), CEUS LR-M (probably or definitely malignant but not specific for HCC), CEUS LR-3 (with intermediate risk of being malignant), CEUS LR-4 (probably HCC) and CEUS LR-5 (definitely HCC). The designation of categories CEUS LR-NC, LR-TIV, LR-1, LR-2, LR-M is possible in light of a basal observation of the lesion by ultrasound, whereas the designation of CEUS LR-3, LR-4, LR-5 requires a post-contrast study [Figure 3].

Globally, the post-contrast behaviour of hepatic nodules in CEUS does not greatly differ from that observed in MDCT/MRI in terms of arterial hyperenhancement and venous/delayed washout. However, CEUS offers the possibility of studying the region of interest from a closer point of view and, by temporally monitoring the features of enhancement and of wash-out, it is possible to deduce additional and, sometimes, more



**Figure 4.** The globular-like progressive centripetal arterial filling of hemangioma



**Figure 5.** In A and B, an HCC presents arterial hyperenhancement and late and mild washout with respect to the surrounding liver; on the contrary, in C and D, a malignant lesion of probable metastatic nature shows rim hyperenhancement together with early and marked wash-out. HCC: hepatocellular carcinoma; APHE: arterial phase hyperenhancement; LR: Liver Imaging Reporting and Data System (LI-RADS); LR-M: LI-RADS malignancy

precise information with respect to MDCT/MRI. Obviously, as a counterpart, the global vision of the entire abdomen is lost, such that CEUS is not appropriate for tumor staging. In return, it can clearly depict key details such as the progressively centripetal and globular arterial hyperenhancement typical of hemangiomas (CEUS LR-1) [Figure 4], or the peripheral hyperenhanced rim visible in the early venous phase which is diagnostic of intrahepatic cholangiocarcinoma (CEUS LR-M), the early ( $< 60$  s) and complete wash-out typical of metastases or of intrahepatic cholangiocarcinoma (CEUS LR-M) and which unequivocally differs from late ( $> 60$  s) and partial wash-out which are diagnostic of HCC (CEUS LR-5) [Figure 5].

These are only some examples given in order to emphasize that CEUS has great potential to help adjudicate the right diagnosis, not only in the event of hepatic nodules of uncertain nature and as a complementary diagnostic tool after MDCT/MRI, but also as a first post-contrast examination of an observation of uncertain nature made at ultrasound (i.e., distinction between real nodule *vs.* fat sparing area/accumulation). In many situations CEUS can define the real nature of a lesion with high sensibility and specificity and can avoid an unneeded biopsy or, on the contrary, guide in its realization, in the presence of a hepatic



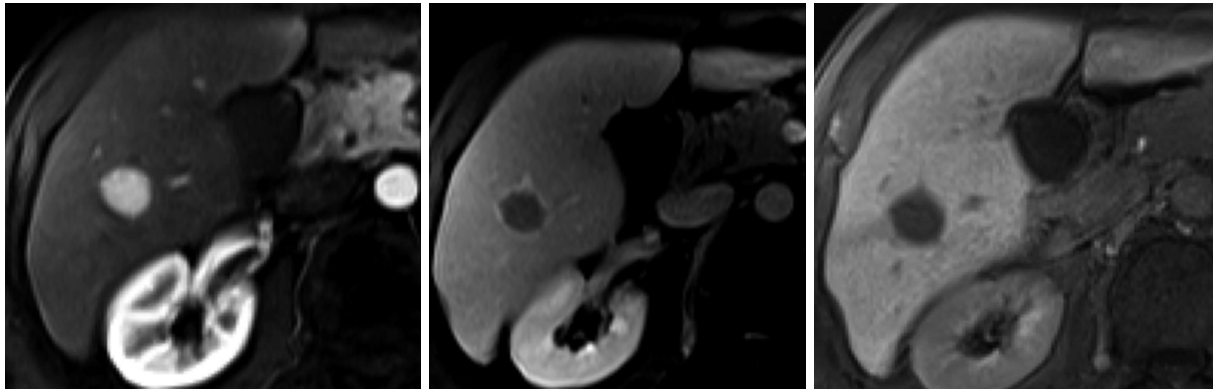
**Figure 6.** From left to right: hepatic arterial phase, venous and late phase on multidetector computed tomography shows no enhancement of the tumor (due to the courtesy of Dr. Michele Di Martino)

lesion that undoubtedly exists but is scarcely visible with a basal ultrasound evaluation. In a recent study including 1,006 nodules, 820 (81%) HCC, 40 (4%) cholangiocarcinoma, 116(11%) regenerative/dysplastic nodules), Terzi *et al.*<sup>[27]</sup> demonstrated that the LR-5 category(52% of all nodules) was 98.5% predictive of HCC, with no risk of misdiagnosis for pure cholangiocarcinoma. Sensitivity for HCC was 62%. All LR-M nodules were malignant and the majority was of non-hepatocellular origin. The LR-3 category included 203 lesions [HCC 96 (47%)] and the LR-4 202 [HCC 173 (87%)]. These and similar results confirm the utility and the great potential of CEUS and justify the re-introduction of CEUS into guidelines. In the latest version of the EASL guidelines, CEUS was introduced in the diagnostic algorithm of HCC in cirrhotic patients but with a moderate degree of evidence and a weak degree of recommendation<sup>[28]</sup>.

### INTERPRETATION OF “NON-HYPERVASCULAR NODULES” IN CIRRHOSIS

The transformation of a regenerative nodule of cirrhosis into a dysplastic lesion involves a progressively reduced portal venous supply and a progressively increased arterial vascularization with sinusoidal capillarization and recruitment of unpaired arterioles; because of this reduced venous drainage, fat content frequently increases in early HCC but regresses in moderately differentiated HCC. Initially, dysplastic nodules show siderosis and copper retention, while during neoplastic transformation, Kupffer cell density decreases, and iron and copper accumulation are gradually lost<sup>[29]</sup>. Injected MDCT and, even better, MRI, can potentially depict all these changes in a rather sensible way and many efforts toward systematization of imaging description and classification have been made and are still made to promote their correct interpretation. In fact, the systems for radiological assessment of hepatic lesions like LI-RADS are based on the analogy between pathological characteristics and specific radiological features. The main limit of LI-RADS is that a diagnosis of HCC is reached only in the presence of arterial hyperenhancement. Thereby, a hepatic nodule that has a non-hypervascular arterial phase, even in the presence of ancillary features suggestive of malignancy, can never be defined as more than a “probable HCC” (LR-4) [Figure 6]<sup>[21]</sup>.

A study that has evaluated the enhancement pattern at multiphasic MDCT of 204 pathologically proven HCC smaller than 3 cm in diameter in cirrhotic patients, has found that the predominant enhancement patterns of HCC differ significantly depending on tumor size and cellular differentiation. Up to 46% of HCCs smaller than 10 mm in diameter do not show arterial hyperenhancement, while it is found in 70% of HCCs measuring 10-19 mm in diameter and in 75% of those measuring 20-29 mm. In line with these results, the association of arterial hyperenhancement and portal venous washout is observed only in 24% of 0-9 mm vs. 28% of 10-19 mm vs. 47% of 20-29 mm HCCs. Cell differentiation also plays an important role: arterial hyperenhancement is found in only 53% of well-differentiated HCCs, whereas the prevalence increases to 79% in moderately differentiated HCCs, and was 60% in poorly differentiated HCCs. In conclusion, this and similar studies confirm that, although large nodules are easily diagnosed, the main difficulty in imaging of cirrhotic patients is the characterization of hepatic nodules smaller than 2 cm in diameter as they frequently do not show the “classical” arterial hyperenhancement<sup>[30]</sup>.



**Figure 7.** From left to right: hepatic arterial phase of gadoxetic acid-enhanced magnetic resonance imaging shows homogeneous marked enhancement of the tumor; transitional phase shows washout of the contrast medium in the tumor with capsular enhancement; hepatobiliary phase shows marked hypointensity of the tumor relative to the liver parenchyma. Modified from Park *et al.*<sup>[31]</sup>

In light of these considerations, reaching a definitive non invasive diagnosis of HCC is still a challenge and MDCT and MRI with injection of extracellular agents (ECA) have proven to be relatively useless in the presence of small hypovascular nodules. The commercialization of gadoxetic acid has represented an important step towards the radiological diagnosis of borderline nodules because, it initially distributes in the extracellular fluid compartment, similar to extracellular contrast agents, and is subsequently taken up by functioning hepatocytes and excreted into the bile. Consequently, it provides both the benefits of dynamic imaging and the delayed hepatobiliary phase during which it is actively picked up by the hepatocytes through the organic anion transporting polypeptide (OATP) B1/8 transporter, a protein that is almost always lost in hepatocarcinogenesis [Figure 7]. A study conducted on surgically resected hepatocellular nodules has found a clear correlation between grade of histological de-differentiation, loss of expression of these transporters and appearance in MRI of a hypointensity in the hepatobiliary phase due to the lost capacity of intracellular uptake of the contrast agent, while the surrounding normal parenchyma remains strongly enhanced. Specifically, the authors evaluated 72 HCCs nodules to determine the correlation among the enhancement ratio on gadoxetic acid-enhanced MRI, the histological grade of tumour differentiation and the intensity of immunohistochemical OATP8 expression. They observed that all of the well, moderately and poorly differentiated HCCs showed a significantly decreased enhancement ratio compared with the background liver, with the exception of 6 moderately differentiated HCC which demonstrated a definitively increased enhancement ratio compared with the background liver. All of these nodules with “atypical behavior” showed increased OATP8 expression compared with the surrounding liver, while in all other HCCs a significant reduction in immunohistochemical OATP8 expression proportional to the grade of de-differentiation was found<sup>[32]</sup>.

These findings, confirmed by other studies, open a new scenario for the non-invasive diagnosis of hypovascular HCCs but the diagnostic role of hepatospecific contrast agents should be endorsed and formalized in international guidelines for radiological diagnosis of HCC, given that the last version of LI-RADS (v2017) still provides a single diagnostic algorithm for multiphase MDCT, MRI with ECA, and MRI with gadoxetate disodium. While initially combined for simplicity, the use of a common algorithm for all three imaging methods has a potentially important drawback: emerging evidence suggests that the assigned categories are modality-dependent, with different modalities assigning different categories to the same observation<sup>[33]</sup>.

## THE ROLE OF POSITRON EMISSION TOMOGRAPHY

The use of PET is still restricted in the field of HCC because of its low sensitivity. In a prospectively conducted study on 99 patients with histologically confirmed HCC who underwent 18F-fluoro-deoxyglucose positron



emission tomography (FDG PET), none of the 7 patients with small tumors (< 2 cm in diameter), 18 of 42 patients (43%) with tumors 2-5 cm in diameter, and 32 of 41 patients (78%) with tumors larger than 5 cm had positive findings for all index lesions. The sensibility increased in Barcelona clinic liver cancer staging system advanced stage, metastatic HCC and in patients with high levels of alphafetoprotein. All indexed lesions with positivity in FDG PET correlated with significantly lower survival with respect to patients with negative or partially positive PET<sup>[34]</sup>. As suggested by these results, other trials have demonstrated that PET positivity correlates with HCC aggressiveness, information which can be used to select, in a non-invasive way, candidates for liver transplantation or major liver resection<sup>[35]</sup>. In a retrospective study conducted on 111 patients with HCC, liver transplantation was performed for 91 of these patients and all underwent PET before the intervention. The tumor recurrence rate after liver transplantation was 3.6% for patients with non- [18F] FDG-avid PET tumors, but it was 54.3% for patients with [18F]FDG-avid PET tumors ( $P < 0.001$ ). The 5-year recurrence-free survival rates were comparable for patients with tumors meeting the Milan criteria (86.2%) and patients with PET negative HCC exceeding the Milan criteria (81%) at liver transplantation, but these rates were significantly higher than the rate for liver recipients with [18F]FDG-avid advanced HCC (21%,  $P < 0.002$ )<sup>[36]</sup>.

Preoperative evaluation of HCC with FDG PET has shown that well-differentiated and some moderately differentiated HCCs do not present FDG uptake exceeding that of the surrounding normal liver, whereas poorly differentiated and undifferentiated HCCs have positive PET findings. The standardized uptake value (SUV) max of sarcomatous HCC is much higher than that of poorly differentiated HCC. The entity of FDG captation of both sarcomatous HCC and combined HCC-cholangiocarcinoma is significantly associated with tumor differentiation, tumor size, microvascular invasion and with poor prognosis after surgery<sup>[37]</sup>.

A recently published study conducted on 207 consecutive patients with monofocal HCC undergoing hepatic resection used pre-operative FDG-PET imaging to stratify tumor aggressiveness and the albumin-bilirubin (ALBI) grade to stratify the hepatic reserve. The ALBI grade is a simple and objective measurement of liver function that uses only serum albumin and bilirubin levels and can be applied to all grades of chronic hepatic diseases, unlike Child Pugh score which is restricted to liver cirrhosis. The study demonstrated a strong correlation between the values of ALBI, the ratio tumorSUV/non-tumorSUV (TNR) and endpoints like overall survival and disease-free survival; whereas tumor size and tumor markers were not significant. Moreover, a high pre-operative TNR showed to be significantly associated with extrahepatic recurrence patterns<sup>[38]</sup>.

The role of PET in the evaluation of tumor response to transarterial treatments has been investigated. Differently from the good sensitivity shown with cholangiocarcinoma and hepatic metastases of colorectal cancer treated with transarterial chemoembolization (TACE), PET has shown to be of little diagnostic value with respect to injected MDCT and MRI for HCC in intermediate stage treated with TACE. Only under specific circumstances, as in the case of strong PET positivity found in pre-treatment evaluation or the presence of a large intrahepatic tumor burden treated with yttrium90-radioembolization has PET shown accuracy in early evaluation of tumor response<sup>[39]</sup>.

In conclusion, even if 18F-FDG-PET does not acquire a definite role in guidelines due to its low sensibility in revealing HCC, it has proved useful in specific instances, such as prior to listing patients with large HCC for liver transplantation, before major resections or when there is suspicion of an extra-hepatic neoplastic diffusion. In the last EASL guidelines FDG PET-scan is not recommended for early diagnosis of HCC because of the high rate of false negative cases but uptake on 18F-FDG-PET seems to be of potential prognostic value. Therefore, it may facilitate the selection of patients for surgical resection or liver transplantation<sup>[28]</sup>.



## HCC IN NON-CIRRHOTIC PATIENTS

HCC occurring in non-cirrhotic livers is uncommon and the clinical presentation is very different from that observed in cirrhosis. Since the tumor occurs in subjects not known to be at risk of HCC, the diagnosis is generally delayed and therefore the tumor is larger than commonly seen in cirrhotic patients. The problem is whether the radiological characteristics that are decisive for the diagnosis of HCC on cirrhosis can be translated in this different clinical situation. Di Martino *et al.*<sup>[40]</sup> retrospectively reviewed histopathological and laboratory findings of 30 non-cirrhotic patients with 32 HCCs. MDCT and gadobenate dimeglumine enhanced MRI were evaluated. Imaging patterns were compared directly with HCC findings in a matched group of cirrhotic patients. The imaging appearance at MDCT and contrast-enhanced MRI was typical in 27 (84.3%) and 28 (87.5%) cases, respectively. Most lesions presented as a well-differentiated large solitary mass, with well-defined margins, areas of necrosis and peripheral capsule. No significant differences in HCC pattern were observed between cirrhotic and non-cirrhotic liver. But in the last EASL guidelines in non-cirrhotic liver, imaging alone is not considered sufficient and histological assessment is required to establish the diagnosis of HCC and has the additional advantage of providing further information regarding the nontumorous liver tissue<sup>[28]</sup>.

## MODIFIED RESPONSE EVALUATION CRITERIA IN SOLID TUMORS FOR THE EVALUATION OF HCC RESPONSE TO TREATMENTS

Radiology plays an important role not only for the diagnosis but also for the evaluation of the response both to locoregional and to systemic treatments of HCC. Until 2010, EASL and AASLD have recommended Response Evaluation Criteria In Solid Tumors (RECIST) and WHO criteria in their guidelines for the management of HCC for the evaluation of response to treatment. The application of these criteria requires the measurement of the major diameter of the HCC nodule/s. However, because of the relevance of the necrotic portion of a treated nodule with respect to its global size, a new version of RECIST modified for HCC, formally taking into account only the vital tissue in each HCC lesion, was published in 2010<sup>[41]</sup>. These criteria have been endorsed in 2012 by EASL and European Organisation for Research and Treatment of Cancer (EORTC) in their guidelines and are currently still the gold standard for the assessment of radiological response, confirmed in the last version of EASL guidelines<sup>[4,28]</sup>. The application of modified RECIST (mRECIST) requires taking into account those HCC nodules which are clearly visible, measurable and showing the typical “hallmark of wash-in and wash-out” as “target lesions”, while all the HCC localizations not definitively measurable or with an atypical post-contrast appearance such as intrahepatic lesions which show infiltrative behavior with poorly defined margins and poorly defined hyperenhancement, malignant thrombosis, neoplastic ascites, adenopathies, very small and/or numerous diffuse lesions, are to be considered “non target lesions”. According to mRECIST, only “target lesions” can undergo a dimensional evaluation of their vital portion (tissue showing arterial hyperenhancement and venous/delayed washout) by the measurement of its longest diameter. The response assessment is to be based on the comparison of this size before and after the treatment. On the contrary, “non target lesions” can be monitored over time on the basis of their absence/presence (i.e., neoplastic ascites) or of their measurement according to RECIST (longest diameter of the lesion as a whole). With respect to “target lesions”, the possible responses are: a complete response (CR) defined by the disappearance of all arterial hyperenhancement in all target lesions; a partial response (PR) in the case of a reduction of at least 30% in the sum of the diameters of the vital portions of the target lesions; progressive disease (PD) if an increase of at least 20% in the sum of the longest diameters of all target lesions is observed; and stable disease (SD) if neither PR nor PD definition criteria can be satisfied [Table 2].

With respect to “non-target lesions”: a CR will correspond to the disappearance of all enhancing tissue in all of them, an incomplete response/SD is defined by the persistence of vital tissue in at least one “non target lesion”; a PD is defined by the appearance of a new lesion or the unequivocal worsening of at least one of the

**Table 2. Comparison between Response Evaluation Criteria In Solid Tumors and Modified Response Evaluation Criteria In Solid Tumors for target lesions<sup>[41]</sup>**

RECIST	mRECIST for HCC
CR = Disappearance of all target lesions	CR = Disappearance of any intratumoral arterial enhancement in all target lesions
PR = At least a 30% decrease in the sum of diameters of all target lesions, taking as reference the baseline sum of the diameters of target lesions	PR = At least 30% decrease in the sum of diameters of viable (enhancement in the arterial phase) target lesions, taking as reference the baseline sum of the diameters of target lesions
SD = Any cases that do not qualify for either partial response or progressive disease	SD = Any cases that do not qualify for either partial response or progressive disease
PD = An increase in 20% in the sum of diameters of target lesions, taking as reference the smallest sum of the diameters of target lesions recorded since treatment started	PD = An increase in 20% in the sum of diameters of viable (enhancing) target lesions, taking as reference the smallest sum of the diameters of target lesions recorded since treatment started

RECIST: Response Evaluation Criteria In Solid Tumors; mRECIST: Modified Response Evaluation Criteria In Solid Tumors; HCC: hepatocellular carcinoma; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease

known “non target lesions”. After having assigned a definition for each category of lesions (target/non target) it will be possible to define the “overall response according to mRECIST” by cross-referencing each response obtained in the two categories of lesions as shown in [Table 3](#).

Subsequent publications have reported a good correlation between the objective response evaluated with mRECIST and overall survival, both after the application of loco-regional treatments<sup>[42]</sup> and also after systemic therapies<sup>[43]</sup>. However, the application of mRECIST criteria in clinical practice is sometimes challenging due to their complexity and also because it is not rare to find an HCC represented exclusively by “non target lesions” or by nodular hepatic lesions which do not exhibit the traditional post-contrast “HCC hallmark”. As such, their use is contraindicated. Finally, it remains to be established that mRECIST is superior to RECIST and a comparative evaluation is therefore required<sup>[44]</sup>. A recent analysis of two phase II trials in patients treated with sorafenib or nintedanib showed that both RECIST and mRECIST response were correlated with overall survival, with similar discriminative abilities in multivariate analysis<sup>[45]</sup>. It was also shown that mRECIST and RECIST are equivalent in the evaluation of progression, which is the most important endpoint in regards to therapeutic decisions.

## IN THE FUTURE

The development of artificial intelligence will probably allow in the future to improve the interpretation of images obtained with the ultrasound, MDTC or MRI with contrast medium limiting diagnostic errors computer-aided diagnosis (CAD) is one of the most important research topics in radiology and medicine. A software based on the recognition of the contrast features of the lesions calculates the probability that they are benign or malignant. Moga *et al.*<sup>[46]</sup> developed a CAD prototype used to analyze 97 videos of good quality CEUS [34% HCC, 12.3% hypervascular metastases, 11.3% hypovascular metastases, 24.7% of hemangiomas, 17.5% of focal nodular hyperplasia (FNH)]. The authors evaluated the diagnostic performance of two young doctors, two experts and CAD in the diagnosis of benign *vs.* malignant lesions. The CAD was useful in improving the diagnostic skills of young doctors, especially when integrated with clinical data, but was lower than the skills of experienced doctors. The most frequently misdiagnosed lesions were FNH and HCC<sup>[46]</sup>.

Kim *et al.*<sup>[47]</sup> developed and evaluated a CAD program for hepatic lesions on MRI for the classification of HCC risk according to the LI-RADS criteria. MRI images of the livers of 41 patients with hyperenhancing liver lesions classified as LR 3, 4 and 5 were evaluated by two radiologists. The agreement on the classification of lesions by radiologists and CAD was 76%-83%, while the agreement between radiologists was 78%.

**Table 3. Overall response according to Modified Response Evaluation Criteria In Solid Tumors for hepatocellular carcinoma**

Target lesions	Nontarget lesions	New lesions	Overall response
CR	CR	No	CR
CR	IR/SD	No	PR
PR	Non-PD	No	PR
SD	Non-PD	No	SD
PD	Any	Yes or no	PD
Any	PD	Yes or no	PD
Any	Any	Yes	PD

CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; IR: incomplete response

## DECLARATIONS

### Authors' contributions

Conception of the work, drafting and revising the work: de Santis A, Gallusi G

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

None.

### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Copyright

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Review

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# Oncogenicity of viral hepatitis B and C in the initiation of hepatic cancer stem cells

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## Abstract

Chronic infection of hepatitis B virus (HBV) or/and hepatitis C virus (HCV) is one of major risk factors in the development of the hepatocellular carcinoma. Recent studies had shown the capacity of viral proteins in inducing the presence of the population of so-called the cancer stem cells (CSC). The integration of HBV *S* and *X* gene in the host genome indicates its direct oncogenicity. In addition, the presence HBV and HCV proteins were shown to modulate intracellular molecular pathways and epigenetic modification. This review summarizes current literature regarding direct oncogenic properties of HBV and HCV in the initiation of CSC both in *in vitro* and *in vivo* studies.

**Keywords:** Cancer stem cells, hepatocellular carcinoma, hepatitis B virus, hepatitis C virus

## INTRODUCTION

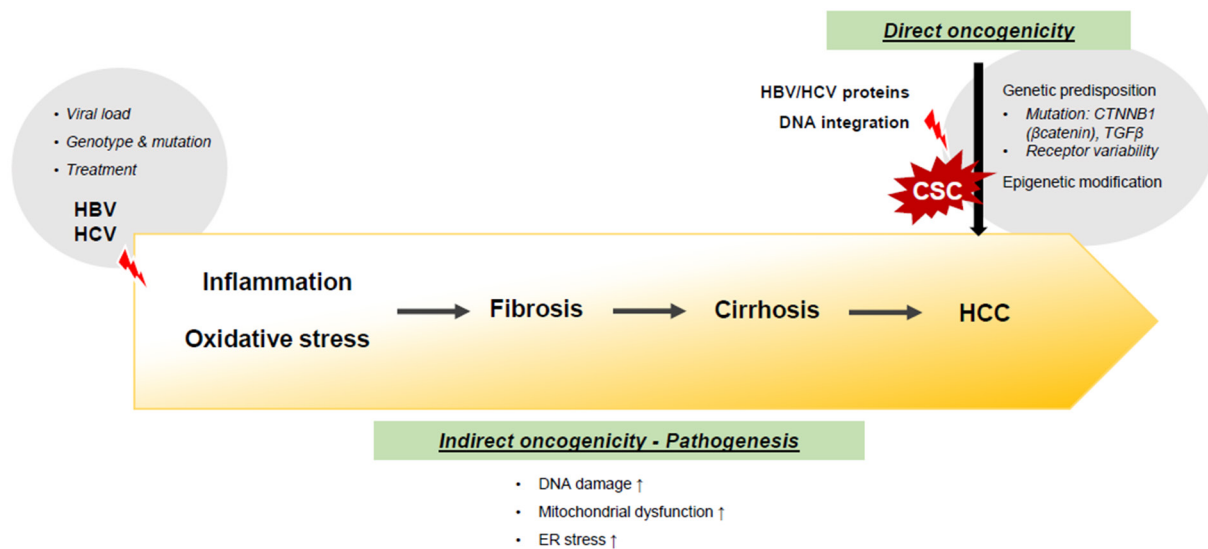
Chronic infection of viral hepatitis B or C is a major risk factor for the development of hepatocellular carcinoma (HCC). In fact, global distribution of HCC is associated with the prevalence of hepatitis viruses: hepatitis B virus (HBV) or hepatitis C virus (HCV). The infection of endemic HBV is the major cause of HCC in eastern Asia and sub-Saharan Africa for around 70%, while in Europe and North American countries, the infection of HCV ranges from 50% to 70% of all cases<sup>[1-3]</sup>. In addition, due to different oncogenic mechanisms of viruses, as well as various genetic host background and long-term development of the disease, viral-related HCCs show high heterogeneity.

Hepatocarcinogenesis is multifactorial, consisting of various steps in a long-term course. At its initiation, disturbance in the molecular and cellular pathways might result in the malignant transformation from



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**Figure 1.** The oncogenicity of viral hepatitis in the development of hepatocellular carcinoma. HBV: hepatitis B virus; HCV: hepatitis C virus; TGF: transforming growth factor; HCC: hepatocellular carcinoma; ER: endoplasmic reticulum

normal to malignant cells. Natural pathogenesis of hepatitis viruses usually involve a sequentially damaging process. It starts with cellular immunological response, triggering DNA damage, mitochondrial dysfunction, and endoplasmic reticulum stress, thus resulting in liver fibrosis, cirrhosis and finally HCC. On the other hand, in the initial step, infection of viral hepatitis can play a significant role in the switch of the fate of the cells by directly triggering the appearance of the cancer stem cells (CSC) [Figure 1].

CSC is the highest-ranking cell population in cancer with the tumorigenic capacity to initiate cancer. It has the capability to divide and differentiate to partially or fully-differentiated cancer cells that comprise the majority of cancer mass. This hierarchy model shows that CSC population is unique, with protective mechanism to be responsible for the maintenance and propagation of the tumor<sup>[4]</sup>. These cells act as the main players in the highest level of the cancer hierarchy and may still have stem cells properties such as self-renewal and ability to multiple cell types. Non-tumorigenic cells are thought to compose the bulk of tumors but have little capacity to contribute to cancer progression<sup>[5,6]</sup>.

The first evidence of CSC in HCC was demonstrated by the isolation of the side population *in vitro*<sup>[7,8]</sup> showing the involvement of CSC in drug resistance. The search and identification method of hepatic CSC progressed by performing sphere colony formation and more commonly, by using CSC markers.

Various markers of CSC from established HCC cell lines and primary tumors had been identified and validated by *in vivo* xenograft assay. Cell protein markers CD133 (PROM1)<sup>[9-11]</sup>, CD90 (THY-1)<sup>[12,13]</sup>, epithelial cell adhesion molecule (EpCAM)<sup>[14,15]</sup>, CD24<sup>[16]</sup>, CD13 (ANPEP)<sup>[7,17]</sup> are the most common method to define a hepatic CSC population. Until now, at least 12 different phenotypical CSC markers had been proposed. The combination of these CSC markers was further used to characterize several subpopulations in a CSC population, resulting in a wide variety of CSC phenotypes.

To understand the mechanism of early initiation of HCC, the oncogenic role of HBV and HCV proteins in hepatic CSC has been started to be explored. They were analyzed by determining the extent of up-regulation and the presence of various hepatic CSC markers, after the exposure of viral proteins into hepatic cells. In addition, these findings were also supported by functional analysis such as cell aggressiveness, migration, and more importantly, by xenograft *in vivo* model, several published data was shown in Table 1.

**Table 1. Several studies on the effect of viral hepatitis in the acquisition of cancer stem cells traits**

	Gene	Experimental model	CSC marker	Functional analysis	Ref.
HBV	<i>Pre-S1</i>	Cell lines LO2, HepG2, Huh7	CD133, CD117, CD90	Facilitation of growth and migration; induction of tumorigenesis	[22]
	<i>Pre-S1/Pre-S2/S</i>	Transgenic mouse Tg(Alb1-HBV)Bri44	CD133, EpCAM, CK19, CD34	Follow-up of hepatocarcinogenesis	[21]
	<i>X</i>	Cell line HepG2	OCT4, Nanog, Klf4, EpCAM	Stimulation of cell growth and migration	[25]
		Cell lines 4pX-1 (from AML12), HepAD38	EpCAM	Active DNA demethylation	[29]
HCV	<i>SGR</i>	Cell lines FCA4 (from Huh7), GS5 (from Huh7.5)	CD133, AFP, CK19	Tumorigenicity	[36]
	<i>Core</i>	Cell lines PHH, THH (from IHH)	c-Kit	Sphere formation, tumorigenicity	[35]
	<i>NS5A</i>	Transgenic mice NS5A TG (FVB strain), Tlr4-/-	Nanog, CD133	Liver damage and tumor formation	[38,42]

CSC: cancer stem cells; HBV: hepatitis B virus; HCV: hepatitis C virus; EpCAM: epithelial cell adhesion molecule; OCT4: octamer-binding transcription factor 4; AFP: alpha-fetoprotein

## ONCOGENICITY OF VIRAL HEPATITIS IN HEPATIC CSC ACQUISITION TRAITS

### HBV

HBV, a member of Hepadnaviridae family, is a partially double-stranded DNA virus with 3.2 kb genome size. HBV genome encodes four overlapping open reading frames (ORFs: S, C, P, and X)<sup>[18]</sup>. Up to now, most of the studies in literature focused on the involvement of HBV S and X proteins in the initiation oncogenesis.

ORF S with three translational start sites Pre-S1, Pre-S2, and S, encodes for large, middle and small surface protein (HBs), respectively, which acts as the main factor in the natural pathogenesis of the virus. The accumulation of HBs antigen (HBsAg) in hepatocytes triggers cellular inflammation and oxidative stress driving a sustained prolonged liver injury until the development of HCC.

On the other hand, direct oncogenic effect of HBV in the development of HCC is closely related with the integration of the HBV DNA sequence into the host genome. HBV DNA integration was considered as a strong oncogenic effect in hepatocarcinogenesis. A recent study reported that in HCC patients with occult hepatitis B, HBV DNA integration was found in around 75% of cases, in which the inserted viral genes were mainly X and PreS/S, followed by C and P sequences<sup>[19]</sup>.

A HBV-transgenic mouse model with the insertion of whole S gene region expressed high level of HBsAg, showing inflammation and appearance of glass ground hepatocytes. Interestingly, the damage induced pre-neoplastic lesion and finally HCC in major number of animals<sup>[20]</sup>, indicating a direct oncogenic contribution of this gene. Our time-course study in this HBV-transgenic mouse showed a progressive increase of the expression of CSC and hepatic progenitor marker during the course of hepatocarcinogenesis, up to 18 months. The expression of several markers such as CD133, EpCAM, and CK19 were significantly increased along liver injury. Further, there was a significant correlation between CSC markers and diagnosis<sup>[21]</sup>. Furthermore, it was recently demonstrated that PreS1 of the S gene activated the expressions of CSC markers CD133, CD117, and CD90 in normal hepatocytes and HCC cells. It indicated the new role of PreS1 as a new oncoprotein to play a key role in the appearance and self-renewal of CSC during HCC development<sup>[22]</sup>.

ORF X encodes for HBx, which has pleiotropic functions as an important regulator in viral life cycle, a transcriptional activator, and a stimulator in the cytoplasmic signal transduction pathway. In HCC clinical specimens, high HBx expression was correlated with the expansion of EpCAM or OV6 progenitor cells, aggressive clinicopathological features<sup>[23,24]</sup> and activated  $\beta$ -catenin signalling<sup>[25]</sup>. In depth, a direct *in vitro* model had shown that the insertion of HBx induced the pluripotent stem cell transcription factors Oct4,

Nanog, Klf4, as well as CSC markers EpCAM and  $\beta$ -catenin. The presence of HBx proteins stimulated cell growth and migration<sup>[25]</sup>. This *in vitro* data were then confirmed by using HBx transgenic mice where a high number of EpCAM cells with characteristics of human progenitor cells was observed<sup>[23]</sup>. Transformation of rat oval cells with HBx and the subsequent injection in nude mice treated with aflatoxin B1 *in vivo*, gave rise to tumor that expressed markers of adult hepatocytes as albumin and CK18, undifferentiated marker alpha-fetoprotein (AFP), and oncoprotein c-Myc<sup>[26]</sup>.

The truncation of HBx protein in the C-terminal region (HBx- $\Delta$ C) is a common event because of HBV X sequence integration in the genome. A recent study had shown that HBx- $\Delta$ C promoted the appearance of a CD133 hepatic CSC subset and confer cancer and stem cell-like features in HCC<sup>[27]</sup>. It is associated with cancer cell invasiveness and reduction of apoptotic response, tumorigenicity, chemoresistance, and migration<sup>[27,28]</sup>.

Regardless of the data provided, the exact mechanism by which HBV proteins altered the early fate of the cells was still unclear. Several studies had shown that DNA demethylation could be a major mechanism in the increase of the expression of CSC markers in normal hepatocytes<sup>[29]</sup>, also correlated with HBV DNA integration<sup>[30]</sup> and the axis of HBx-DLL3 (Delta-like 3) of Notch receptor<sup>[31]</sup>. In the last study, the treatment of HBV-transformed cells with a histone deacetylase inhibitor induced DLL3 expression<sup>[31]</sup>. In a recent 2018 study, it was shown that in the very early stage of HCC, the global DNA methylation 5hmC and 5fC contents were decreased significantly. It was found to be correlated with HBV infection, decreased ten-eleven translocation enzyme activity and uncoordinated expression of DNA methylation-related enzymes<sup>[32]</sup>.

## HCV

HCV, a member of flaviviridae family, is a single stranded RNA virus with 9.6 kb genome size. HCV genome is processed into structural proteins core, E1, and E2, and non-structural (NS) proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B<sup>[33]</sup>. Since HCV being an RNA virus cannot integrate into human genome, at the beginning, the mechanism in HCV-related HCC pathogenesis is supposed exclusively to indirect via chronic inflammation and oxidative stress. Subsequently, it leads to fibrosis and eventually cirrhosis as observed in the other HCC etiologies<sup>[34]</sup>. However, current literature in experimental models also showed direct oncogenic effect of the HCV proteins, including on the involvement of the CSC.

A previous study showed that HCV-infected hepatocytes transformed into sphere formation with a number of epithelial-mesenchymal transition (EMT) and CSC markers, including high level of the stem cell factor receptor c-Kit. These spheres were potent in promoting tumor growth in immunodeficient mice. However, these spheres were highly sensitive to cell death from the treatment of sorafenib, a multikinase inhibitor, and stattic, an inhibitor of the Stat3 molecule<sup>[35]</sup>. Furthermore, by inserting HCV sub-genomic replicon in cultured cells, the acquisition of CSC traits, including an enhanced expression of doublecortin and CaM kinase-like-1, Lgr5, CD133, AFP, CK19, Lin28, and c-Myc, was demonstrated. Conversely, curing of the replicon from these cells diminished the expression of these factors. *In vivo* analysis of liver tissues from HCV-positive patients and liver tissue microarrays supported these observations<sup>[36]</sup>.

It had been shown that HCV core and NS proteins, can induce cell transformation *in vitro* and *in vivo* mice transgenic model<sup>[37]</sup>. By using intercross breeding of transgenic mouse models, HCV NS5A protein induced the toll-like receptor 4 (TLR4). This induction mediated liver damage and tumor formation in synergy with alcohol-induced endotoxemia. Consequently, the expression of stem cell marker Nanog and the presence of CD133/Nanog-positive cells were observed<sup>[38]</sup>.

This study was then continued by *in vivo* animal study of NS5A mice fed with high in cholesterol and saturated fat diet (HCFD). Liver tissues of these HCFD mice had increased levels of TLR4, Nanog, phosphorylated signal transducer and activator of transcription (pStat3), and Twist1. Further analysis of

isolated tumor-initiating stem-like cells (TISCs) with the phenotype of CD133<sup>+</sup> CD49f<sup>+</sup> showed that TISCs expressed higher levels of stemness genes and Twist1<sup>[39]</sup>.

It was known that HCV core protein induces the upregulation of transforming growth factor beta 1 (TGFβ1), showing a direct role in fibrogenesis<sup>[40]</sup>. A recent study on HCV core protein demonstrated that the TISCs obtained from the model had the capacity to recruit and activate fibroblasts in a xenograft, exhibited by high expression of fibrogenesis and EMT markers. It showed that in HCV infection, preneoplastic or tumorigenic state of the hepatocytes influenced the network for the tumor environment<sup>[41]</sup>, presumably with the involvement of the hepatic cells stemness. As seen in NS5A transgenic mouse, a study in HCV core transgenic mouse with HCV core insertion showed a corresponding result. The TISCs isolated from this mouse were tumorigenic both *in vitro* and *in vivo* and the TLR4-Nanog pathway was necessary for the maintenance of tumorigenic properties<sup>[42]</sup>.

The Wnt/β-catenin pathway might be the major or one of the major molecular mechanisms involved in the oncogenicity of HCV. The activation of this pathway was noted in transgenic expressions of both HCV core and NS5A proteins. Pharmacological inhibition or loss of the Wnt/β-catenin signal represses TISCs growth *in vitro*, and decreases the accumulation of TISCs *in vivo*<sup>[43]</sup>.

Regarding the core protein, since HCV core is closely related with TGF-β pathway, it is expected that TGF-β is involved in the induced CSC population. A previous study showed that CSC generation by HCV core protein was dependent on the endoglin (CD105), a TGF-β receptor complex. Besides the increase of CSC proteins anti-apoptosis and proliferation are enhanced during infection or ectopic expression of HCV core<sup>[44]</sup>.

As in HBV, epigenetic mechanisms such as DNA methylation could give a hint on the molecular mechanism of the oncogenicity HCV. It was shown that demethylation of CpGs induced Sal-like protein 4, an embryonic stem cell transcriptional regulator. This re-expression was noticed in subgroups of HCC associated with HBV or HCV infection<sup>[45]</sup>.

## RELEVANCE OF CSC MARKERS IN CLINICOPATHOLOGICAL FEATURE OF HCC

The complexity of HCC showed that the heterogeneity is not limited among patients (intertumoral heterogeneity) but also within the same person (intratumoral heterogeneity). Cell morphology, molecular profile, and expression of specific markers can be used to stratify and classify discrete tumor subtypes<sup>[46]</sup>. Consequently, HBV and/or HCV infection contributes to phenotypic and molecular characteristics in hepatic cell populations, including the CSC.

Multiple clinical studies had shown that the high expression of CSC marker CD133 in HCC tissues, in particular in the cytoplasm, is correlated with a poor prognosis<sup>[47-51]</sup>. In addition to prognosis, CD90 is high-expressed in HCC nodule<sup>[52]</sup> and is correlated with HCC differentiation grades<sup>[53,54]</sup>.

Protein analysis showed that the levels of hepatic CD133 were higher in HBV<sup>+</sup> than those in HBV<sup>-</sup> HCC tissues<sup>[22]</sup>, pointing to the oncogenicity of the PreS1. Recent data showed that in HBV-related HCC cases, CD133 in combination with the level of serum AFP, HCC could be subclassified into four subtypes, with different clinicopathological features and various prognosis. A high expressions of both CD133 and serum AFP was associated with a relatively poor prognosis<sup>[55]</sup>.

However, a previous study in endemic HBV area showed that CD133 expression in HCC was negatively associated with the presence of HBsAg<sup>[56]</sup>. A histological analysis of human tissue found a positive correlation between HBV and CD90<sup>[57]</sup> but since co-staining of the CSC markers and the HBV proteins was not performed, it remains unclear if and how HBV alters the physiology of CD90<sup>+</sup> and CD133<sup>+</sup> CSC.



Recent data also showed a correlation between HBV and CSC EpCAM. High expression of HBx in human HBV-related HCC was also correlated with the expansion of EpCAM HCC cells; EpCAM expression was detected more frequently with HBV than with other etiologies. Further, in chemotherapy treated patients, EpCAM was strongly expressed, indicating its association with treatment resistance<sup>[24]</sup>.

In contrast to the clear direct oncogenicity of HBV, the association between clinical and pathological characteristics and CSC markers in HCV-related HCC is still very limited. Recently it was demonstrated that CSC spheres induced by HCV were highly sensitive to cell death from sorafenib. It can be a basis for the development of new targeted therapies against hepatic CSC<sup>[35,58]</sup>.

## PERSPECTIVES

While the pathogenesis of HBV and HCV proteins in the development of HCC has been intensely investigated, information on their significance in the initiation of hepatic CSC is still very limited. It is because the theory of CSC in HCC is still relatively new and further evidence must be demonstrated. Moreover, even though this hypothesis is exciting, CSC theory in HCC is debatable and controversial. Recent studies had indicated that in gastrointestinal cancers, the so-called CSC should be defined as tumor-initiating cells/TISCs. These cells were not pluripotent, but bi- or multipotential to give rise to diverse tumor types and tumor initiation potential in mouse models<sup>[59]</sup>. The complexity of the liver, as well as the limitation in the experimental models, still limit the proof of the CSC concept. Further, genomic diversity and genetic characteristic of the virus (genotypes, subgenotypes, and quasispecies) significantly contribute to different clinical outcome and viral susceptibilities<sup>[33]</sup>.

In addition to the type of the virus, another point to be considered is the state of the hepatic cells during viral exposure. A recent study had shown that the susceptibility of the hepatic cells to HCV was different during cellular maturation course. In this study, an epigenetic transduction by pluripotency factors reprogrammed mature cells into hepatic oval (progenitor) cells. In this progeny stage, cells lost their susceptibility to HCV infection and viral RNA replication. Upon hepatic differentiation, however, a permissiveness to HCV RNA replication was re-obtained. In contrast to HCV, in HBV infection, viral susceptibility was maintained along the course. It indicated that during hepatic maturation process, cells receptor susceptibility are specific to particular virus<sup>[60]</sup>.

Even though basic *in vitro* studies and studies in transgenic animals, as well as clinical data from HCC patients, had shown expanded evidence on “stemness” oncogenicity of HBV and HCV, the mechanism of how viral particle induces hepatocarcinogenesis is still unclear and open for discussion. We presume that there would not be a single answer because of the complexity and heterogeneity of both virus and host factors. Finding strong evidence on this field will keep us busy for some time but the application of potentiality of this trip is intriguing.

## DECLARATIONS

### Authors' contributions

Designed the manuscript: Sukowati CHC, Tiribelli C

Wrote, read and approved the manuscript: Sukowati CHC, Reyes PAC, Tell G, Tiribelli C

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Review

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# Review of therapies for intermediate and advanced stage hepatocellular carcinoma, not suitable for curative therapies: a rapidly changing landscape

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## Abstract

Recent clinical trials and new agents have permitted greater clarity in the choice of effective agents for that majority of patients with hepatocellular carcinoma who have advanced disease at diagnosis and thus cannot be offered potentially curative resection, ablation or liver transplantation. The main treatment for these patients remains chemoembolization, although evidence for selective internal radiation therapy (SIRT) with SIR-Spheres or Theraphere, is beginning to suggest that the results with this may be comparable with less toxicity. Patients who have failed chemoembolization or SIRT or have metastatic disease at presentation are suitable for the multikinase inhibitor sorafenib (nexavar) or newly-approved lenvatinib (lenvima) as first line therapies. The choice between which of them to use first is not currently clear. Patients who have failed sorafenib can be offered a choice of FDA-approved regorafenib (stivarga) or immune checkpoint inhibitor nivolumab (opdivo) as second line agents. For that considerable percent of patients presenting with macroscopic portal vein thrombosis, the choice appears to be between multikinase inhibitor or SIRT, given the potential toxicity of chemoembolization in this setting. However, considering the potency of both nivolumab and regorafenib and the pipeline of new agents such as atezolizumab (tecentriq) in current clinical trials, including new immune checkpoint inhibitors, this landscape may change within a couple of years, especially if new evidence arises for the superior effectiveness of combinations of any of these agents over single agents.

**Keywords:** Hepatocellular carcinoma, advanced, kinase inhibitors, immune checkpoint inhibitors, transarterial chemoembolization, selective internal radiation therapy



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## INTRODUCTION

The prognosis of hepatocellular carcinoma (HCC) depends on multiple factors, hence the large number of staging systems. In particular, it depends on the size and location within the liver of the HCC and the number of HCC nodules, the presence and degree of portal venous invasion, the presence or absence of distant metastases, as well as the degree of liver damage (Child-Pugh class)<sup>[1]</sup>. Patients with a single tumor nodule of < 2 cm have the best prognosis and larger size and number of nodules have worse prognosis<sup>[2]</sup>. T1 lesions are single < 2 cm lesions without portal vein thrombosis (PVT). T2 lesions are > 2 cm to 5 cm, single or multiple, as well as single lesions > 2 cm with vascular invasion. T3 lesions are multiple, with at least one being > 5 cm. The best survival outcomes occur after treatment with ablation, resection or transplantation, in patients having Barcelona Clinic Liver Cancer staging system (BCLC)<sup>[3]</sup> 0 stage (single < 2 cm) or early stage A (1-3 nodules, any < 3 cm with good liver function Child-Pugh class A or early class B cirrhosis). All other patients, who have BCLC stages B (multinodular of any size) or stage C (presence of PVT, lymph nodes or metastases), cannot be offered therapies with curative intent, and constitute the majority of HCC patients who are diagnosed in the absence of a surveillance program and whose treatment is the subject of this review. This constituted at least 65% of newly diagnosed HCC patients in our large series<sup>[4]</sup> [Table 1].

The main treatment modality for these BCLC intermediate stage B patients has been for many years chemoembolization [transarterial chemoembolization (TACE)]. More recently, selective internal radiation therapy (SIRT) or transarterial radioembolization (TARE) has been increasingly seen as a promising treatment approach in this setting, in many institutions. For stage C patients having either or both PVT or metastases, systemic therapy is widely used, involving multikinase inhibitors such as sorafenib, although immune checkpoint inhibitors and newly-approved multikinase inhibitors are changing that landscape. Furthermore, in addition to sorafenib, radioembolization is increasingly considered as both useful and safe (unlike much TACE) in the presence of branch PVT. This review summarizes each of the major treatment modalities for patients who are not suitable for treatments with curative intent and then summarizes current clinical practice and finally evaluates some likely future directions in this rapidly moving field.

## NON-SURGICAL TREATMENT MODALITIES

### Current first line therapies

#### *Chemoembolization or TACE*

Several reviews have been published on the chemotherapy drugs and types of embolization particles that have been used for chemoembolization or TACE<sup>[4,5]</sup>. Objective partial responses have been reported in 30%-60% of patients<sup>[4]</sup>, and an increase in survival was initially reported in 2 randomized placebo-controlled trials, using doxorubicin or cisplatin, respectively<sup>[6-8]</sup>. Due to its relative safety, especially in patients with Child-Pugh A and many with Child-Pugh B cirrhosis and tumors of almost any size and number, it has been a standard of therapy for non surgical and non metastatic HCC for several decades. A wide range of chemotherapeutic agents have been used, but there has not been an analysis of which agents, or combination of agents, nor of which of multiple embolization particle types might be optimal, although doxorubicin, cisplatin or mitomycin C, often mixed with lipiodol, are most commonly used<sup>[9]</sup>, or with defined size embolization particles. Recently, drug-eluting beads have become popular, but their superiority for survival to plain and cheaper particles has been disputed<sup>[10]</sup>, although they may be safer. TACE has been combined with radiofrequency invasion for enhanced results<sup>[11]</sup> and has also been used as bridging therapy to transplant<sup>[12]</sup>. Current trials are in progress to assess improved efficacy of TACE when combined with multikinase inhibitors<sup>[13,14]</sup> or immune checkpoint inhibitors. TACE has been considered the standard therapy for non surgically treatable HCC<sup>[15]</sup>, with SIRT being also widely adopted as an alternative standard.

#### *SIRT*

TARE with SIR-Spheres or Transarterial radiotherapy with Therasphere [Table 1].

**Table 1. Comparison between glass (Therasphere) and resin microspheres (SIR-Spheres)**

	Therasphere	SIR-Spheres
Half-life	64.2 h	64.2 h
Material	Glass	Resin
Size	20-30 $\mu\text{m}$	20-60 $\mu\text{m}$
Activity per sphere	2500 Bq	50 Bq
Number of sphere	1.2-8 milion	40-80 milion
Embolic effect	Minimal	Moderate

Yttrium-90 or  $Y^{90}$  SIRT has gained increased popularity in recent years as a safer alternative to TACE, especially in the setting of PVT. Two non-identical products [Table 1] are available, namely Therasphere and SIR-Spheres. Therasphere contains glass as the carrier and is much more radioactive, but almost not embolic and is FDA-approved for HCC therapy under a humanitarian device exemption (requires individual institutional review board approval). SIR-Spheres are made of resin carrier and are by contrast much less radioactive per dose, but have many more particles per dose and are thus embolic (hence radioembolization). Neither agent seems to induce much post-embolization syndrome, unlike TACE. Therasphere is thus really a pure internal radiation treatment and not radioembolization. There have been few convincing randomized trials with either agent for HCC survival, either against each other (although they are thought to have similar results) or against TACE<sup>[16]</sup>. However, several reports provide evidence for their effectiveness and safety<sup>[16-19]</sup>. Unlike TACE, these radioactive agents need to be received by the institution and handled by radiation safety staff and appropriately monitored. Thus, SIRT therapy requires a special team, including a radiation pharmacy, radiation safety officer, nuclear medicine physician, as well as the interventional radiologist. Unlike for TACE, SIRT patients require a pre-treatment angiogram together with a Technetium  $^{99m}\text{Tc}$  macro aggregated albumin ( $^{99m}\text{Tc}$ -MAA) scan to measure any significant lung shunting. More than 20% lung shunt normally excludes SIRT, as does aberrant gastric or other feeder arteries than cannot be occluded, to prevent gastrointestinal radiation toxicity.

The most remarkable benefit of SIRT is its safety in treating that 30%-40% of HCC patients that have PVT<sup>[20-22]</sup>. However, overall survival (OS) did not differ significantly when SIRT was compared to sorafenib in a phase III trial<sup>[23]</sup>. Nevertheless, the combination of SIRT with sorafenib was associated in one study with enhanced toxicity<sup>[24]</sup>. In the SORAMIC randomized phase II trial, the addition of SIRT (SIR-Spheres) to sorafenib did not add to survival compared with sorafenib alone. When TACE and SIRT were directly compared, they were similar in safety, tumor responses and survival<sup>[25,26]</sup>. Studies are in progress on the uses of SIRT in adjuvant and neo-adjuvant therapy for surgery of HCC, as well as in combinations with several newer therapies.

### *Sorafenib (nexavar)*

Sorafenib is a multikinase inhibitor that is antiangiogenic, inhibits HCC cell growth and induces apoptosis. It is thought to target the Ras/Raf/methyl ethyl ketone (MEK)/extracellular signal-regulated kinase signaling pathway via the vascular endothelial growth factor (VEGFR) and platelet-derived growth factor receptor (PDGFR). For the last 10 years it has been the choice for first line of therapy for patients with HCC metastases, PVT, or those who have failed TACE or SIRT, based on a multi-center, double-blind, placebo-controlled phase III SHARP trial, which reported a 2.8 months increase in median OS with sorafenib (10.7 months) compared with placebo (7.9 months) [hazard ratio (HR) in sorafenib group, 0.69; 95% confidence interval (CI) 0.55-0.87;  $P < 0.007$ ]<sup>[27]</sup>. However, in a similarly designed phase III trial from Asia, results were much worse, with a median OS of 6.5 months (95% CI 5.56-7.56) in patients treated with sorafenib, compared with 4.2 months (3.75-5.46) in those who received placebo (HR 0.68; 95% CI 0.50-0.93;  $P = 0.014$ )<sup>[28]</sup>. The reasons that the OS from Asia after sorafenib treatment was worse than the OS on placebo in the European study are not clear, but point to the need for caution in comparing results of therapies in different ethnic groups, or in patients with differing severity of tumor or cirrhosis. In addition to a significant but only

modest increase in survival in the sorafenib groups compared to placebo controls, the objective response rates of < 2.0% were also very low. However, toxicities have been considerable, with many patients requiring dose reduction, variable drug “holiday” or drug discontinuation. Toxicities include hand-foot syndrome, rash, diarrhea and fatigue, most commonly, but also hypertension, nausea and leukopenia<sup>[29]</sup>.

Several large phase III trials comparing sorafenib with newer agents have failed to successfully meet their planned end-points, including trials of brivanib, linifanib, sunitinib. A randomized phase II trial with sorafenib vs. erlotinib plus bevacizumab likewise failed to show superiority for the comparison arm with respect to sorafenib. The only recent exception thus far, is the recently FDA-approved lenvatinib (below) phase III trial.

Several attempts to improve on sorafenib therapy by combining it with other agents or with TACE or SIRT, have been recently made. However, results have so far been minor at best<sup>[30,31]</sup>. Sorafenib was also evaluated as an adjuvant therapy to resection in the STORM trial, but also without added benefit to surgery alone<sup>[32]</sup>.

#### *Lenvatinib (lenvima)*

FDA has just (Aug 2018) approved lenvatinib for first line therapy of advanced or metastatic HCC, based on a randomized controlled phase III REFLECT trial, comparing lenvatinib 8 or 12 mg daily with sorafenib 400 mg twice daily<sup>[33]</sup>. Median OS was 13.6 months for lenvatinib and 12.3 months for sorafenib. The trial demonstrated that lenvatinib was noninferior (but not statistically superior) to sorafenib for OS, which was the primary endpoint (HR 0.92; 95% CI 0.79-1.06). The overall response rate was higher for lenvatinib than for sorafenib (41% vs. 12% per modified RECIST and 19% vs. 7% per RECIST 1.1). Patients with main trunk PVT were excluded from this trial. The commonest toxicities in the lenvatinib-treated patients ( $\geq 20\%$ ) were hypertension, fatigue, diarrhea, decreased appetite, arthralgia/myalgia, decreased weight, abdominal pain and palmar-plantar erythrodysaesthesia. It is a multi-tyrosine kinase inhibitor of VEGFR1-3, FGFR 1-4, rearranged during transfection (RET), receptor tyrosine kinase (KIT, also called CD117 and stem cell factor receptor) and PDGFR.

Thus, current first-line therapies for previously untreated HCC, include TACE, SIRT, sorafenib and lenvatinib [Table 2]. The initial choice has been conventional chemoembolization (TACE) or more recently SIRT, especially in the presence of PVT and excellent liver function. However, in the presence of 5 or more lesions or bilobar lesions, it is reasonable to consider Sorafenib or Lenvatinib as initial therapy, especially in the presence of serum bilirubin levels > 2.5 mg/dL, in light of the known hepatotoxicity of both TACE and SIRT.

### **Current second line therapies**

#### *Regorafenib (stivarga)*

Regorafenib is a multi-kinase inhibitor of VEGFR1-3, tyrosine kinase with immunoglobulin-like and EGF-like domains 2-unlike sorafenib, PDGFR $\beta$ , FGFR, c-KIT (stronger than sorafenib), RET, BRAF, BRAFV600 and RAF-1. It is the first agent to provide survival benefit in the second line, after failure of sorafenib and has recently been FDA-approved as a second line therapy. The phase III RESORCE study<sup>[34]</sup> was for HCC patients who had progressed on sorafenib, but not failed due to toxicity, and it improved OS with a HR 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 was 65.2% vs. 36.1% ( $P < 0.001$ ). Regorafenib was administered at 160 mg daily for 3 weeks, with a subsequent rest week. The commonest grade 3 or 4 treatment-emergent events were 15% hypertension in the regorafenib group vs. 5% in the placebo group, 13% hand-foot skin reaction/palmar-plantar erythrodysaesthesia for regorafenib vs. 1% in the placebo group, 9% fatigue for regorafenib vs. 5% in the placebo group, with 3% diarrhea for regorafenib vs. none for placebo. Thus, these data differ from the sorafenib SHARP trial results in which few patients had objective responses, suggesting that regorafenib (fluoro-sorafenib) is a more potent agent than sorafenib. Toxicities were similar for regorafenib and sorafenib,

**Table 2. Current therapies for advanced stage hepatocellular carcinoma patients**

First line
A. Chemoembolization or SIRT
B. Sorafenib or Lenvatinib
Second line
A. Regorafenib or Opdivo or Cabozantinib
B. Under FDA review: ramucirumab
Metastasis
Sorafenib or Lenvatinib
PVT
SIRT, Sorafenib, external beam irradiation
Combinations in development
A. Multikinase inhibitors plus chemoembolization or SIRT
B. Immune checkpoint inhibitors plus chemoembolization or SIRT
C. Kinase inhibitors targeting parallel growth pathways
D. Multikinase inhibitors plus immune checkpoint inhibitors

SIRT: selective internal radiation therapy; PVT: portal vein thrombosis; SBRT: stereotactic body radiation therapy

with fatigue, hypertension, hand-foot syndrome, slight elevation of transaminases and bilirubin occurring after both drug treatments. The recommended regorafenib dose is 160 mg per day. The RESORCE trial showed that it is possible to dose-reduce regorafenib and still obtain antitumor effects. Given the remarkable structural similarity between sorafenib and regorafenib - one fluorine atom difference - it is surprising that results for regorafenib were so positive in proven sorafenib-resistant patients. Both sorafenib and regorafenib inhibit the insulin-like growth factor-1 (IGF-1) mediated growth pathway, and their actions *in vitro* are both blocked by IGF-1. By contrast, their actions are augmented by IGF-1 receptor inhibition<sup>[35]</sup> suggesting future directions for enhancing their effects.

#### *Nivolumab (opdivo)*

FDA approved nivolumab, a programmed death receptor 1 (PD-1) immune checkpoint inhibitor, as second line therapy for HCC patients who had failed prior sorafenib due to disease progression or sorafenib intolerance, after tumor response and durability of those responses of the single arm phase Ib/II CheckMate-040 trial<sup>[36]</sup>. Results showed that 22 or 14% of 154 patients responded, regardless of their programmed death receptor ligand 1 (PD-L1) status. Three of these patients had complete responses, with 91% of patients having responses lasting 6 months and 55% of patients having responses for more than a year. Median duration of response was 16.6 months, with a rapid median onset of response at 2.8 months. The 12 months OS rate was 59.9% and the median OS was 16.7 months. Serious adverse events occurred in 49% of patients and included pyrexia, ascites, back pains and abdominal pains and general deterioration. Commonest toxicities were 38% of patients had fatigue, 36% musculoskeletal pain, 34% abdominal pain, 27% pruritus, 27% diarrhea, 26% rash and 23% cough. Thus, the toxicity profile is significant and somewhat different from the multikinase inhibitors. The drug can cause immune-mediated colitis, hepatitis, pneumonitis and endocrinopathies. The toxicity results of long-duration therapy are unknown, but may be of concern. On the positive side, the mechanisms of this class of drugs are so different from TACE, SIRT and other multikinase inhibitors, that they will be very attractive candidates for future drug combination trials. A phase III comparison of nivolumab vs. sorafenib is ongoing.

Two new agents that have met their end-points in phase III trials in the second-line post sorafenib setting. Ramucirumab (cyramza) is awaiting FDA evaluation and cabozantinib (cabometyx) in the Celestial trial has just been FDA approved.

#### *Cabozantinib (cabometyx)*

Cabozantinib is a tyrosine kinase inhibitor, with targets including VEGF receptors 1, 2, and 3, MET, and AXL, which are implicated in the HCC growth and sorafenib resistance. A phase III placebo controlled

trial was reported<sup>[37]</sup>, showing that in sorafenib resistant patients, cabozantinib treatment resulted in longer OS than for placebo patients. Median OS was 10.2 months with cabozantinib and 8.0 months with placebo (HR for death, 0.76; 95% CI 0.63-0.92;  $P = 0.005$ ), and the objective response rates were 4% for cabozantinib, but less than 0.4% for placebo, respectively ( $P = 0.009$ ). 16% of patients discontinued cabozantinib due to treatment-related adverse events (palmar-plantar erythrodysesthesia, hypertension, fatigue, diarrhea and increased aspartate aminotransferase), compared to 3% of patients on placebo. Given that the phase III trial also included patients receiving cabozantinib as third line therapy, this opens the possibility for the potential for third line therapies in patients with resistant HCC or who are intolerant to other therapies.

#### *Ramucirumab (cyramza)*

Ramucirumab is an anti-angiogenic VEGFR-2 antagonist that binds and blocks VEGF-A, VEGF-C and VEGF-D. In a phase III placebo-controlled trial (REACH) in second line on sorafenib failure patients, no significant survival differences were found between ramucirumab and placebo. However, meaningful improvement was observed in a patient subgroup with baseline alpha-fetoprotein (AFP)  $\geq 400$  ng/mL, HR = 0.67,  $P = 0.006$ ; median OS 7.8 months for ramucirumab vs. 4.2 months for placebo controls. Therefore, a subsequent phase III randomized trial was performed (REACH-2), in a biomarker-selected HCC patients, having AFP levels of  $> 400$  ng/mL<sup>[38]</sup>. In patients with baseline AFP  $\geq 400$  ng/mL, a significant survival benefit was found in patients treated with ramucirumab compared with placebo and was coupled with a trend in patient-focused outcome benefits. The only grade 3 toxicity was hypertension and hyponatremia in  $> 5\%$  of the patients. In a Japanese sub-analysis<sup>[39]</sup>, the median OS was 12.9 months for the ramucirumab arm ( $n = 45$ ) and 8.0 months for the placebo arm ( $n = 48$ ) (HR 0.621; 95% CI 0.391-0.986;  $P = 0.0416$ ). In patients with a baseline AFP level of 400 ng/mL or greater, the median OS was 12.9 months for the ramucirumab arm ( $n = 20$ ) and 4.3 months for the placebo arm ( $n = 22$ ) (HR 0.464; 95% CI 0.232-0.926;  $P = 0.0263$ ). Objective response rates were 11% for the ramucirumab arm and 2% for the placebo arm ( $P = 0.0817$ ). Ramucirumab is currently being considered for approval by the FDA.

Thus, 3 agents are currently FDA-approved for second line therapy, namely regorafenib, nivolumab and cabozantinib. However, in 2019 ramucirumab may also be approved in this same setting. How does one choose the optimal sequence for using these agents? In addition, for liver-only HCC patients who have failed chemoembolization and who have preserved liver function, may also be suitably treated with radioembolization. Given the high response rates for regorafenib, this is an attractive agent for use in this setting, but its use is also associated with considerable toxicities. The RESOURCE trial on which its approval was based, did not include patients who were sorafenib-intolerant in the first line setting. Thus, the use of regorafenib in the second line setting may be limited to a subset of patients. Cabozantinib and ramucirumab are also multikinase inhibitors, with similar toxicities to both sorafenib and regorafenib. Therefore, patients whose tumors have failed chemoembolization and/or radioembolization might be most suitably offered nivolumab at the time of writing, due to its different toxicities and even higher responses. New approvals are likely however, for other immune checkpoint inhibitors and/or their combinations with other agents and these recommendations will then need to be reconsidered.

### **EXTRA-HEPATIC METASTASIS AND PVT**

Metastasis is the single most important cause of morbidity and mortality in most solid adult tumors. HCC may be an exception, as patients usually die of their liver failure, either from tumor growth and parenchymal liver destruction, or from the underlying and liver disease that caused the HCC to arise, regardless of the presence or absence of metastasis. HCC with extra-hepatic metastasis may even constitute a distinct HCC subset, and is associated with less cirrhosis than other HCC<sup>[40]</sup>. While systemic therapy is mainly chosen in this circumstance<sup>[41]</sup>, an argument can also be made to initially treat the main disease in the liver. Regardless, several studies with systemic chemotherapy or multikinase therapy have shown no survival benefit in this situation.



Macroscopic PVT (visible on MRI or CT scan) is thought to be present in over 30% of HCC patients and is likely the single worst prognostic factor. In addition to being an important portal for metastases (tumor cells are already in the portal vein), the presence of main stem or major branch PVT impacts the ability to perform liver transplant (high recurrence rates), resection (high recurrence rates and technical surgical difficulties); it is also associated with worse liver function. Many studies have thus focused on the treatment of HCC patients with PVT<sup>[41]</sup>, as well as on treatment of the PVT itself<sup>[41,42]</sup>. Treatments include selective TACE, SIRT<sup>[20-22,43]</sup>, sorafenib and 3-dimensional conformal radiotherapy. However, this is a heterogeneous group of patients<sup>[44,45]</sup>. One consensus suggests hepatic resection when technically feasible for longest survival, otherwise TACE for unresectable patients, followed by external beam radiation<sup>[46]</sup>. Depending on the extent of the PVT, enhanced survival has been reported in a large series for hepatectomy, TACE, TACE plus sorafenib or TACE plus radiotherapy<sup>[47]</sup>. There is currently no standard for therapy for PVT.

## LIKELY PRACTICE SCENARIOS [Table 2]

FDA has approved both sorafenib and lenvatinib as first line therapies. If patients tolerate sorafenib well, then FDA-approved regorafenib or nivolumab will be good second line options. If patients did not tolerate sorafenib, then FDA-approved nivolumab might be an excellent second line option, due to its different mechanisms than sorafenib. But so could ramucirumab, should it get approved by FDA in the second line setting. Furthermore, ramucirumab appears to be attractive for patients in the second line setting with elevated AFP levels.

## NEW AGENTS

A variety of new agents are in current clinical trials and will likely change the clinical landscape again in another 2-5 years. These include particularly a variety of agents inhibiting the immune checkpoint proteins PD-1, PD-L1 and cytotoxic T lymphocyte antigen 4, as well as epigenetic control mechanisms. In addition to further opdivo studies, other agents being tested include ipilimumab, pembrolizumab and durvalumab and tremelimumab, amongst others. Agents against various growth factor targets such as FGF/FGFR (fibroblast growth factor and its receptor, such as BLU 554 or dovatinib) and growth pathways (MEK, signal transducer and activator of transcription 3, AKT-also called protein kinase B), apoptosis induction, epithelial to mesenchymal modulation, and cytolytic viruses, are currently under way. Furthermore, the clinical availability of curative (HCV) or highly effective (HBV) antivirals that are the ultimate cause of HCC and hoped-for contributors to the amelioration of HCC aggressiveness. In this context, the role of inflammatory micro-environment and anti-inflammatory agents in the development of HCC and its modulation, is drawing increased interest.

## WHERE ARE WE HEADING?

The standard of care for patients with advanced, non-curative and non metastatic HCC remains TACE or more recently, TARE [National Comprehensive Cancer Network (NCCN) guidelines version 5.2018, HCC; NCCN.org]. Chemoembolization is associated with 30%-60% objective response rates in various trials and has some minor survival advantage<sup>[6,7]</sup>. Sorafenib has minor response rates, can be given orally and has a proven, but small survival advantage, through quite different mechanisms and different toxicity profiles than for either TACE or TARE. Therefore, it will be rational to evaluate combinations of chemoembolization or SIRT with sorafenib<sup>[48,49]</sup> or the more potent regorafenib or any other multikinase inhibitor. Several trials are under way.

The same reasoning of different mechanisms, applies to combinations of immune checkpoint inhibitor with either: (a) chemoembolization; (b) SIRT; and (c) multikinase inhibitors. In this regard, the combination of VEGF inhibitor bevacizumab (avastin) plus atezolizumab (tecentriq) has just been given (July 2018)

breakthrough therapy designation by FDA, since a phase Ib study presented at American Society of Clinical Oncology (ASCO) 2018 was reported to show objective responses in 32% of patients. More than half of the responders maintained their responses for at least 6 months. A combination trial of nivolumab plus sorafenib (CheckMate-459) for first line therapy is currently in progress. Furthermore, combinations of kinase inhibitors that target different or parallel growth pathways [EGFR, FGFR, hepatocyte growth factor receptor (HGFR)/Met] seems similarly attractive for testing. In addition, it may be that sequencing might show added anti-tumor activity rather than combinations, such as chemoembolization/sorafenib, SIRT/sorafenib, sorafenib/regorafenib, immune checkpoint inhibitor (high responses)/multikinase inhibitor. Thus, the field may look quite differently in 3 years than currently. The role of anti-viral or anti-inflammatory agents (above section) may also turn out to be beneficial in selected patient subsets, with greater inflammatory characteristics<sup>[50]</sup>. Either way, HCC sub-phenotype identification may be important in matching individuals to selected treatments. However, a final word of caution may be useful. A major phase III trial recently failed to meet its end-points, even though patients were selected, based on their tumors having the putative target (Met) for the agent being tested<sup>[51]</sup>. However, given the high responses and their durability for immune checkpoint inhibitors such as Nivolumab, one of them may become a preferred first line therapy, if ongoing clinical trials support this idea.

## DECLARATIONS

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The author contributed solely to the article.

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All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

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Review

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# Stereotactic ablative radiotherapy for hepatocellular carcinoma

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## Abstract

Patients with hepatocellular carcinoma (HCC) often present with underlying liver disease and significant comorbidities, limiting treatment tolerance. With the development of improved toxicity models and highly conformal radiation delivery systems, external beam radiotherapy has become a valuable treatment option for liver cancer. Using cutting edge technology, stereotactic ablative radiotherapy (SABR) allows for the delivery of ablative doses in few fractions while sparing uninvolved liver tissue. This approach permits dose escalation and precise tumor targeting with minimal risk of radiation induced liver disease. This review clarifies SABR's role alongside liver-directed treatments such as radiofrequency ablation, transarterial radioembolization, and transarterial chemoembolization in the management of HCC. It also examines the promising potential of SABR combined with immunotherapy to treat advanced HCC.

**Keywords:** Hepatocellular carcinoma, stereotactic ablative body radiation therapy, image guided radiation therapy, adaptive radiation therapy, radiation toxicity, multidisciplinary cancer treatment

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer worldwide and the second leading cancer-related cause of mortality<sup>[1]</sup>. Incidence in the United States (US) has risen dramatically over the past two decades and is now estimated at 25,000 new cases each year<sup>[2]</sup>. In US, patients diagnosed with HCC have a poor prognosis, with mortality nearly doubling in recent decades and a 5-year survival rate less than 30%<sup>[3]</sup>.



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Patients with HCC often present with a large tumor burden on a background of cirrhosis and hepatic decompensation, complicating treatment tolerance<sup>[4,5]</sup>. Prognosis of HCC depends on stage at presentation as well as overall liver function<sup>[6]</sup>.

Surgical resection is considered the first-line treatment for non-cirrhotic patients. Preoperative criteria such as Child-Pugh (CP) classification have been developed for risk stratification to minimize postoperative hepatic decompensation and prevent futile interventions<sup>[7]</sup>. Contraindications to resection include major vascular invasion, portal hypertension, large multifocal lesions, extrahepatic disease, CP class B/C (CP-B/C) or inadequate liver remnant. Predicted liver remnant must be in the range of 40% of preoperative total liver volume or 700 cm<sup>3</sup> for a patient to be considered eligible for resection<sup>[7]</sup>.

More than 70% of HCC patients have portal hypertension and cirrhosis at diagnosis, making them ineligible for liver resection<sup>[8]</sup>. Orthotopic liver transplant (OLT) is an alternative for patients who meet the Milan criteria (a single tumor < 5 cm or up to three tumors < 3 cm without vascular invasion or extrahepatic manifestation)<sup>[9]</sup>.

Patients who are not candidates for tumor resection or OLT may be candidates for liver-directed therapy. Liver-directed therapies can be grouped into the following broad categories: intra-arterial treatments (radioembolization, chemoembolization, bland embolization), percutaneous approaches [radiofrequency ablation (RFA), microwave ablation, focused ultrasound, ethanol ablation, electroporation] and external beam radiation therapy (EBRT). EBRT can use three-dimensional (3-D) conformal techniques for palliation or more advanced strategies such as stereotactic ablative radiotherapy (SABR) or particle beam therapy for definitive treatment<sup>[10]</sup>.

Historically, EBRT (delivered mostly by 3-D conformal technique) had been considered ineffective in the treatment of HCC since the dose required to cure HCC far exceeded liver tissue tolerance to radiation therapy. Advances in EBRT techniques with SABR and particle beam therapy in the past two decades have allowed clinicians to deliver much higher doses with significant sparing of uninvolved liver, increasing local control while minimizing the risk of radiation induced liver disease (RILD). The major advantages of EBRT are non-invasiveness and the ability to treat the majority of patients with localized liver disease who are not candidates for surgery/transplant, arterial-directed therapy or ablative therapy. Multiple centers around the world have reported long-term outcomes with excellent local control, survival and acceptable toxicity profiles. [Table 1](#) summarizes prospective trials showing that SABR is an excellent option for HCC tumor control with limited toxicity. Recent National Comprehensive Cancer Network (NCCN) guidelines list EBRT as a locoregional treatment option for patients who are not candidates for surgery/transplant or who are waiting for transplantation (bridge to transplant)<sup>[10]</sup>.

## WHAT IS SABR?

SABR, also called stereotactic body radiation therapy, is an advanced form of EBRT that combines tumor/organ motion management and multiple beams of high energy photons to deliver very high doses of radiation precisely to a small target volume over a short treatment course. In US, SABR is delivered in one to five fractions but can be more fractionated in other countries.

SABR effectively treats primary and secondary malignancies in the liver, lung, bone, spine, and pancreas. When applied to malignant and benign disease of the central nervous system it is also referred to as stereotactic radiosurgery.

Radiation treatment for liver cancer can be challenging because (1) tumors tend to be large and complex, requiring high doses for control; (2) underlying liver is usually compromised from liver disease and

**Table 1. Stereotactic ablative radiotherapy disease control and toxicity**

Author, year	Patients/tumors CP score	Tumor size (cm)	Study design	Dose/fractions	Local control % (1-year/ 2-year/3-year)	Overall survival % (1-year/2-year/3- year)	Toxicity % ≥ grade 3
Scorsetti <i>et al.</i> <sup>[43]</sup> , 2015	43/63 23 CP-A, 20 CP-B	4.8	Observational	36-75 Gy/3-6	94/86/-	78/45/-	16
Lasley <i>et al.</i> <sup>[52]</sup> , 2015	59/65 38 CP-A, 21 CP-B	4	Phase I/II	36-48 Gy/3-5	CP-A: 91/91/91 CP-B: 82/82/82	CP-A: 94/72/61 CP-B: 57/33/26	CP-A: 11 CP-B: 38
Bujold <i>et al.</i> <sup>[41]</sup> , 2013	102/164 102 CP-A	7.2	Phase I/II trial	36 Gy (30-54)/6	87/-/-	55/34/-	36
Kang <i>et al.</i> <sup>[42]</sup> , 2012	47/56 41 CP-A, 6 CP-B	2.9	Phase II (TACE + SABR)	42-60 Gy/3	-/95/-	-/69/-	11
Cárdenes <i>et al.</i> <sup>[68]</sup> , 2010	17/25 6 CP-A, 11 CP-B	4	Phase I trial	40-48 Gy/3-5	100/100/-	75/60/-	18

SABR: stereotactic ablative radiotherapy; TACE: trans arterial chemoembolization; CP: Child-Pugh

vulnerable to decompensation from radiation toxicity; (3) nearby organs such as small bowel, heart, stomach and gallbladder cannot tolerate high-dose radiation; and (4) inter-fraction (day-to-day) variation of tumor size and intra-fraction (during treatment) tumor and organ movement with respiration can be significant. SABR uses a variety of strategies to overcome these challenges during the simulation, planning and radiation delivery phases of treatment.

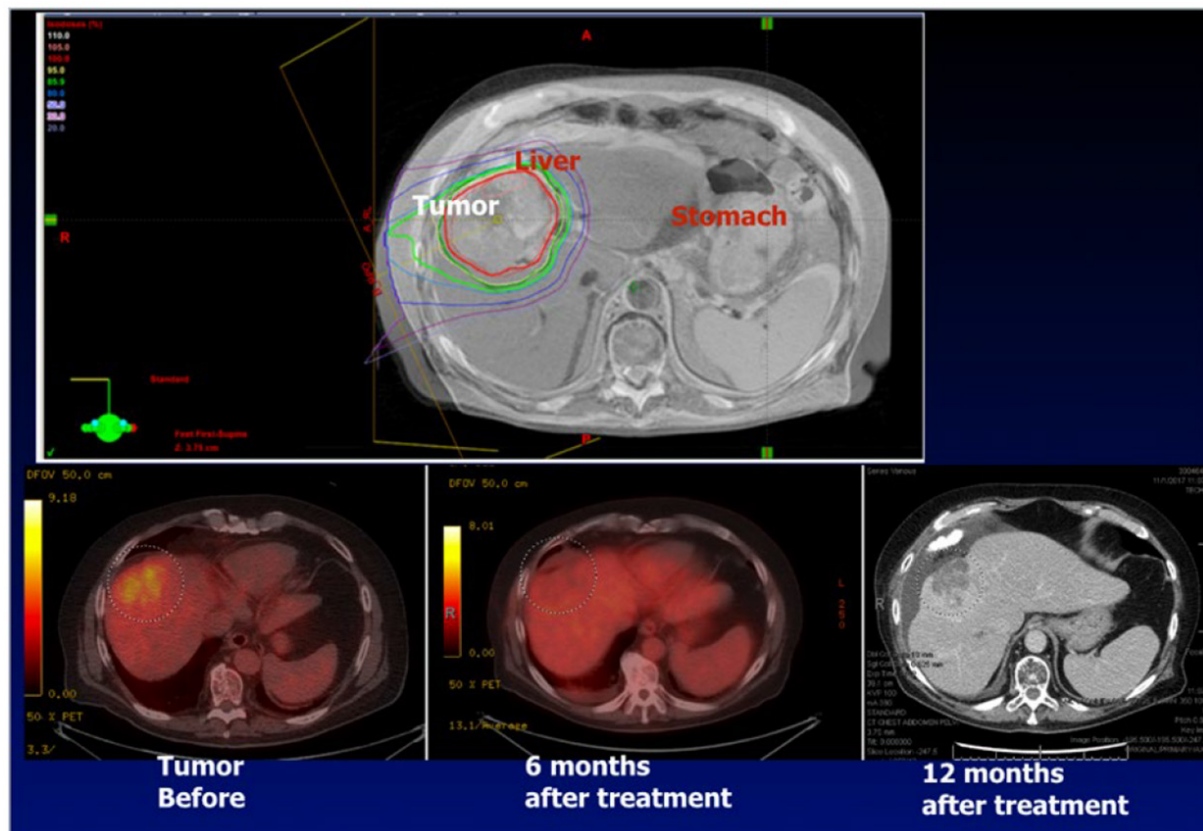
During simulation, the reference conditions for future treatment are determined. The patient is positioned supine on a computed tomography (CT) tabletop similar to the treatment couch surface used in radiation delivery. Immobilization aides, such as an alpha cradle or vacuum lock, help to ensure the most precise positional reproducibility consistent with patient comfort. Motion management starts with acquisition of high resolution 3-D and 4-D CT scans during simulation to quantify tumor and organ intra-fraction movement.

Intrahepatic fiducial markers are sometimes used to assist in localization of tumors poorly visualized on diagnostic CT scans. These small metallic radiopaque markers are inserted in the vicinity of tumor, placed percutaneously under local anesthesia at least three days prior to the simulation<sup>[11]</sup>.

In selected centers, where latest generation approaches allow for magnetic resonance imaging-guided (MRI-g) SABR, a planning MRI scan is performed in addition to the planning CT [Figure 1]<sup>[12]</sup>. When MRI-g is available, patients forego fiducial placement. While MRI-g SABR represents a promising advance, most patients treated for HCC with radiation receive photon therapy using CT-based techniques.

The simulation scan is transferred to a computer planning system and fused with available diagnostic imaging such as positron emission tomography, CT or MRI. The radiation oncologist then delineates tumor, uninvolved liver parenchyma and adjacent normal organs in the computer planning system. Due to the liver's proximity to the diaphragm, this process must account for liver and organ motion across the breathing cycle<sup>[13]</sup>. One option is to add a security margin around the tumor equal to its cephalo-caudal motion measured on the 4-D CT simulation scan. The downside of this approach is that a larger volume of surrounding normal tissue will receive radiation during treatment delivery. Motion management strategies such as abdominal compression, breath-hold technique and respiratory gating enable sparing of liver parenchyma, an important end-point among HCC patients with cirrhosis<sup>[14,15]</sup>. Abdominal compression restricts physiologic organ motion, while breath-hold and respiratory gating permit radiation treatment in only a single phase of the breathing cycle.

Prior to each treatment delivery, an X-ray or CT scan is performed to align patients to the simulated treatment position. Fiducials, which can be seen on these images, assist in target localization. This process



**Figure 1.** Stereotactic ablative radiotherapy (SABR) dose distribution and post-treatment tumor response. Top left shows an example of SABR tumor and dose distribution. The image is an axial view of the planning target volume (red) with planned dose distribution extending from the interior (orange isodose line, 105% of prescribed dose) to the periphery (purple isodose line, 30% of prescribed dose) of the tumor. The bottom left image shows the same tumor on positron emission tomography (PET) computed tomography (CT) prior to SABR, with high avidity within the tumor volume. The bottom middle shows the same tumor on PET 6 months after treatment, with resolution of PET avidity. The bottom right shows the same tumor on CT 12 months after treatment, with tissue necrosis in the previously treated volume

of alignment is called image-guided radiation therapy (IGRT). Once alignment is complete with millimeter accuracy, the radiation is delivered. Currently most IGRT is performed using X-ray images or CT scans with or without fiducial markers. Together, custom immobilization, respiratory management and IGRT help to minimize the tumor's security margin<sup>[16-18]</sup>.

The latest generation of IGRT allows for the use of MRI, which offers continuous and high-resolution 3-D images of tumor and normal organs during treatment. IGRT with MRI, currently available at a handful of centers, offers much higher accuracy compared with traditional IGRT approaches. Treatment units equipped with on-board MRI permit real-time tracking of the tumor (on-board monitoring with four MRI images per second). Target visualization can be further improved using gadoxetate contrast<sup>[19]</sup>. Safety mechanisms turn the beam off when the target transgresses the tracking volume, making it very safe to deliver high doses to tumor with tight margins, sparing adjacent organs-at-risk [Video 1].

Proton beam therapy offers theoretical advantages over photon therapy due to sharp dose fall-off at a specific depth (Bragg peak). Due to this beam characteristic, proton SABR could lead to improved normal liver sparing compared to conventional photon treatments. This comparative reduction in mean liver dose gives proton SABR the potential to escalate dose or increase target size<sup>[20,21]</sup>. Dosimetric studies suggest that proton SABR is more effective than photon SABR for dome and central tumors  $\geq 3$  cm, and for tumors  $> 5$  cm

when photon therapy cannot meet dose constraint objectives<sup>[22]</sup>. Such advantages have yet to be validated clinically. A national cancer institute-sponsored phase 3 prospective randomized trial (NRG GI-003) is underway, comparing proton vs. photon SABR for unresectable HCC using either 5 or 15 fractions. While few radiation oncology centers in the world currently have the ability to treat patients with proton therapy, 80 proton facilities are in development in US. As the number of proton centers equipped with respiratory gating continues to increase, proton SABR will become more widely available.

The safety of SABR allows the radiation oncologist to prescribe a very high dose per fraction. Prescriptions in the range of 50 Gy in five fractions can be delivered safely to the target. A high radiation dose delivered in few fractions produces much greater biological effect than the same dose delivered over a protracted regimen. For this reason, 50 Gy delivered in 5 fractions has an ablative, tumoricidal effect while 50 Gy in 25 fractions is associated with low tumor control probability for HCC<sup>[23,24]</sup>.

Compared to other liver directed therapies, SABR has the additional advantage of being minimally-invasive. It can be delivered to lesions regardless of adjacent vascular structures, vascularity of the tumor, associated venous thrombus, or location within the liver. In contrast to more invasive liver-directed therapies, SABR can be used to treat patients at high risk of bleeding, a clinical situation frequently encountered in the cirrhotic patient population. It can also be used to simultaneously target enlarged portal nodes or portal vein tumor thrombus. An example of SABR target and dose distribution, as well as tumor response on post-treatment imaging, is shown in [Figure 1](#).

### SABR CLINICAL INDICATIONS

SABR is often used to treat liver lesions beyond the capabilities of other local, ablative techniques: large volume tumors; lesions near the liver capsule, major vessels, or diaphragm; and disease complicated by portal vein tumor thrombus (PVTT).

HCC tends to invade the portal vein causing PVTT, especially in patients with advanced disease at presentation. If untreated, overall survival (OS) after PVTT diagnosis is 2.7-4 months<sup>[25]</sup>. SABR can recannulate the portal vein, facilitating subsequent embolic therapies for which the presence of PVTT is a relative contraindication. In this setting, SABR used in combination with embolic therapies increases patient OS<sup>[26]</sup>.

Although SABR is a liver directed therapy most frequently used for patients who are not candidates for surgery or OLT, it can also be used as a bridge procedure to downstage lesions that do not meet the Milan criteria or to prevent disease progression while patients are on a waiting list for OLT.

Published clinical series demonstrate that SABR is a safe and well-tolerated procedure when used as a bridge to transplant. In 2011 O'Connor *et al.*<sup>[27]</sup> evaluated a clinical series of 10 patients with 11 HCC lesions treated with SABR while on the OLT waiting list. Local control over this period was 100% and all patients underwent OLT without increased surgical complications. In another phase I study, a 27-patient subgroup treated with SABR as a bridge to transplant had a 100% local control rate<sup>[28]</sup>.

Alternatives techniques used as bridge procedures to transplant include RFA and transarterial chemoembolization (TACE). RFA, a commonly employed percutaneous technique in HCC treatment, involves the insertion of a monopolar or bipolar probe into targeted liver tissue, using frictional heat generated by alternating current to destroy tumor via coagulative necrosis<sup>[29]</sup>. With RFA, best outcomes occur when the lesion is less than 3 cm in diameter, distant from large hepatic vessels that divert heat from the intended target, and with at least a 1 cm margin from adjacent organs such as bowel to avoid injury to critical structures<sup>[30]</sup>. RFA is an invasive procedure often requiring general anesthesia.

Intra-arterial embolizations such as TACE are primary treatments for HCC patients with unresectable tumors and CP-A or B hepatic function who do not meet transplant criteria and cannot receive local ablation. In bland transarterial embolization, micron-sized particles are delivered into the tumor vasculature to decrease blood supply to the tumor and induce necrosis through hypoxia; in TACE, a chemotherapy agent infused into the region of interest remains sequestered due to subsequent microparticle embolization, potentiating cytotoxic effects<sup>[31]</sup>. Absolute contraindications to TACE include tumor involving more than half the liver, renal insufficiency, extrahepatic disease, reduced portal flow, or poor prognosis indicated by hepatic encephalopathy and jaundice<sup>[32]</sup>.

In 2017 Sapisochin *et al.*<sup>[33]</sup> first compared SABR ( $n = 36$ ) with TACE ( $n = 99$ ) and RFA ( $n = 244$ ) as bridges to OLT in patient with HCC. The study found that SABR, while treating a greater tumor burden than RFA, demonstrated similar post-transplant survival and recurrence rates as the other techniques<sup>[33]</sup>.

In cases of borderline ineligibility for transplant, SABR is a logical option for downstaging HCC, as it is less invasive than surgery, RFA or TACE and provides comparable survival outcomes<sup>[33-35]</sup>. A recent study comparing SABR to resection in patients with CP-A disease and lesions  $\leq 5$  cm in greatest dimension reported comparable OS with fewer complications in the SABR group<sup>[34]</sup>. A 2015 University of California San Francisco study recommended an individualized approach to the choice of locoregional therapy for downstaging, determined case-by-case at a multidisciplinary tumor board<sup>[36]</sup>. The multidisciplinary model has been shown to improve HCC patient outcomes<sup>[37]</sup>.

## SABR FOR PATIENTS WHO ARE NOT SURGICAL CANDIDATES

Treatment algorithms corresponding to clinical stages of HCC continue to evolve. There is increasing recognition that spatial cooperation with combination therapies can improve patient survival<sup>[38]</sup>. Among all treatments for HCC, only palliative systemic agents sorafenib and regorafenib are supported by category 1 evidence. Surgical, ablative, intra-arterial and external beam approaches rely on consensus support from oncologists based on category 2 evidence<sup>[10]</sup>.

The 2017 US NCCN guidelines emphasize the ability of SABR to treat HCC at any location in the liver<sup>[10]</sup>. NCCN considers SABR an appropriate alternative to ablation/embolization techniques. This recommendation is supported by a bulk of published data including the retrospective studies comparing SABR with RFA and TACE. A 2016 retrospective study by Wahl *et al.*<sup>[39]</sup> compared SABR (63 patients treated with 27-60 Gy in 3-5 fractions) to RFA (161 patients), showing these two modalities to be equally effective for the treatment of inoperable HCC  $< 2$  cm, with SABR providing better local control than RFA for lesions  $\geq 2$  cm. Another retrospective series that compared TACE and SABR reported that 2-year local control was significantly better for SABR, 91.3%-22.9% with no significant difference in OS<sup>[40]</sup>.

While SABR is most often used to treat tumors  $\leq 5$  cm and 1-3 liver lesions, it can ablate more extensive disease provided radiation constraints and liver remnant limits are met. In Princess Margaret Hospital (PMH) phase I and II trials, Bujold *et al.*<sup>[41]</sup> reported the ability of SABR to accommodate an increased tumor burden. While the PMH multivariate analysis revealed that gross tumor volume was unrelated to treatment outcome<sup>[41]</sup>, other studies report significantly better local control when using SABR on tumors  $< 5$  cm<sup>[42,43]</sup>.

SABR also can serve as second-line therapy when alternatives are contraindicated or have already failed. In the PMH trial, SABR was used to treat 102 patients with advanced HCC, ineligible for surgery, TACE or RFA. Median tumor diameter was 7.2 cm, more than half the cohort had PVT and 12% had distant metastases. Despite this heavy disease burden, patients receiving a median dose of 36 Gy (range 30-54 Gy) in 6 fractions had a 17-month median OS and one year local control rate of 87%, superior to historical controls for sorafenib (6.5-10.7 months OS) and supportive care (4.2-7.9 months OS)<sup>[41]</sup>.



Andolino *et al.*<sup>[28]</sup> reported results of a phase I dose escalation study in which 36 patients with CP-A disease received 48 Gy in 3 fractions while 24 patients with CP-B disease received 40 Gy in 5 fractions. Two-year local control was 90%, 2-year OS was 67%, and median time to progression was 47.8 months<sup>[28]</sup>.

## SABR VS. TRANSARTERIAL RADIOEMBOLIZATION

While SABR is a minimally invasive, external beam radiation platform with precise dosimetry able to reliably target subsegmental lesions, transarterial radioembolization (TARE), also known as selective internal radiation therapy is often used to treat large multifocal disease impossible to address with SABR techniques. TARE can deliver very high local doses of radiation to HCC involving entire segments of the liver with a single invasive procedure. Published data support the use of both modalities, and no direct comparison has been attempted through clinical trials. The decision to use TARE or SABR is institution specific, based on disease distribution, co-morbidities and multidisciplinary tumor board consensus.

TARE is an ablative radiation technique that involves injection of radiolabeled yttrium-90 (Y-90) microspheres into the hepatic artery by guided catheterization. Isotope-containing microspheres lodge in arterioles feeding liver tumors, embolize the small vasculature and deliver very high, tumoricidal doses (estimated to be 85-120 Gy and even higher in cases of TARE segmentectomy 300-400 Gy)<sup>[44]</sup>. TARE selectively targets disease by exploiting the liver's dual blood supply: tumors greater than 3 cm in size are fed primarily by the hepatic artery while the liver parenchyma's main source of blood supply is through the portal vein.

Y-90, a beta-emitting isotope with a half-life of 2.67 days, is packaged in glass (Theraspheres, BTG Canada) or resin (SIR-Spheres, Sirtex Australia) particles. Spheres with diameters between 20 and 60 microns occlude arteries feeding the tumor proximal to arteriovenous anastomoses, sparing central venules from toxic doses. Central vein obliteration is characteristic of RILD, so precapillary entrapment combined with short-range activity accounts for low rates of radioembolization induced liver disease (REILD) in TARE studies<sup>[45]</sup>.

For HCC patients with CP-A liver function treated with TARE, multiple studies report OS greater than 15 months<sup>[46-48]</sup>. Many patients in these studies had significant tumor burdens, with multifocal disease, PVTT and median tumor diameters greater than 5 cm. Table 2 summarizes prospective trials showing TARE as an excellent option for tumor control in high-volume and multifocal HCC.

In the treatment of unresectable primary liver cancer, TARE's clinical applications range from palliation to transplant bridging<sup>[10]</sup>. TARE can treat HCC in the setting of PVTT, whereas reduced main portal vein flow is a contraindication to TACE<sup>[47,49]</sup>.

Mild TARE-related syndromes are commonly reported after treatment of HCC, such as fatigue, abdominal pain, nausea, vomiting, and low-grade fever. Adverse events grade 3 or greater include 10% biliary toxicity 2%-13% REILD and 5.8%-23% bilirubin elevation<sup>[50,51]</sup>. While randomized studies show that Y-90 radioembolization significantly prolongs time to HCC progression compared with TACE, grade 3 or higher toxicity rates are comparable between TARE and TACE<sup>[46-49]</sup>.

Dosimetric software, providing accurate assessment of the dose delivered to tumor and adjacent normal liver tissue during TARE procedures, has recently been FDA approved (Hermes Medical Solution). Better assessment of dose delivered to tumor tissue and uninvolved liver may permit strategies combining both SABR and TARE in selected situations or may allow for better comparisons and selection between the two techniques for individual patients.



**Table 2. Transarterial radioembolization disease control and toxicity**

Author, year	Patients/ treatments CP score	Tumor size (cm)	Study design	Solitary/ multifocal	Time to progression, in months	Median overall survival, in months	Toxicity % $\geq$ grade 3
Salem <i>et al.</i> <sup>[47]</sup> , 2016	24/- CP-A 10, CP-B 13, CP-C 1	3.0	Randomized phase II	13/11	> 26	18.6	Clinical: 17 (Ascites: 13, bacterial peritonitis: 6)
El Fouly <i>et al.</i> <sup>[69]</sup> , 2015	44 CP-B 44	6.4	Two-center	44	13.3	16.4	Clinical: 45 (Fatigue 40, abdominal pain 5, ascites 2)
Salem <i>et al.</i> <sup>[70]</sup> , 2010	291/526 CP-A 131, CP-B 152, CP-C 8	7	Single-center	78/213	CP-A 10.8 CP-B 8.4	CP-A 17.2 CP-B 7.7	Biochemical: bilirubin (19) albumin (18) ALT (14), AST (19), ALK (4)
Hilgard <i>et al.</i> <sup>[46]</sup> , 2010	108/159 CP-A 84, CP-B 24	-	Single-center	2/106	10.0	CP-A 17.2 CP-B 6.0	Biochemical: bilirubin (23) lymphopenia (71) platelets (4)

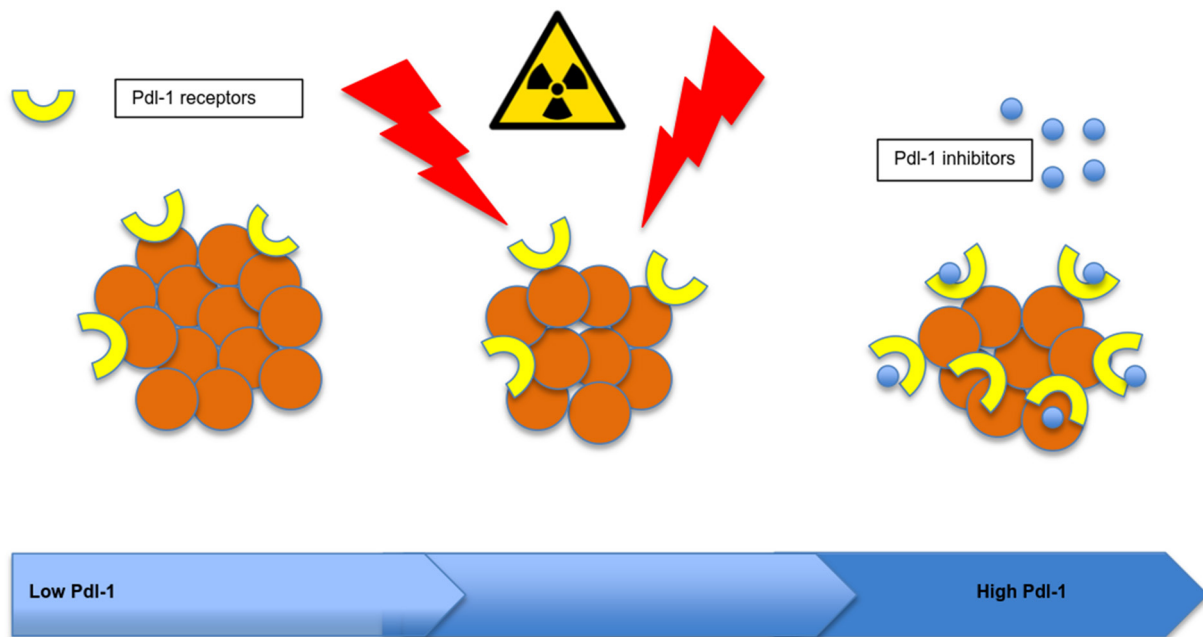
CP: Child-Pugh; AST: aspartate transaminase; ALT: alanine transaminase; ALK: alkaline phosphatase

## SABR, SYSTEMIC TREATMENT AND IMMUNOTHERAPY

HCC tumors are somewhat resistant to cytotoxic and targeted therapies due to compromised metabolism caused by underlying liver disease<sup>[52]</sup>. For patients with advanced HCC, sorafenib is the first line agent, with a partial response rate of 2% and a rate of stable disease driving prolonged survival in the multicenter European SHARP trial<sup>[53]</sup>. For previously untreated patients receiving sorafenib, clinical trials have shown a median OS of 10.7 months *vs.* 7.9 months with placebo. The REFLECT study comparing lenvatinib to sorafenib demonstrated lenvatinib to be non-inferior as a first line agent in the treatment of HCC, with an overall response rate of 24.1% for lenvatinib *vs.* 9.1 % for sorafenib<sup>[54]</sup>. Lenvatinib was approved by the FDA for frontline HCC in August 2018. Oncologists managing HCC continue to look for alternative treatments and there is growing interest in immune based therapies.

The host immune system's inability to reject tumor during cancer development may represent failure at any step in the immune regulatory process. As a result, any host immune system regulatory element is a potential target for systemic treatment. Checkpoint antibodies such as pembrolizumab, ipilimumab and nivolumab have demonstrated clinical activity against melanoma, non-small cell lung cancer, Hodgkin's lymphoma, and renal cell carcinoma<sup>[55]</sup>. Checkpoint antibodies also demonstrate antitumor effects in the treatment of advanced HCC<sup>[56,57]</sup>. In November 2018, the FDA granted accelerated approval to pembrolizumab for patients with HCC previously treated with sorafenib, based on results of the KEYNOTE-224 trial<sup>[58]</sup>. In that single arm multicenter trial, 104 CP-A patients who had already received or were intolerant to sorafenib were treated with pembrolizumab and had an overall response rate of 17%<sup>[59]</sup>. While duration of response may be prolonged, the response rates with checkpoint antibodies are generally 20% or less, contributing to growing interest in strategies that combine local treatments to amplify tumor immunogenicity. Cancer cell apoptosis induced by the delivery of high dose per fraction radiation releases tumor fragments into the tumor microenvironment and can stimulate the host immune response. Over 25 ongoing clinical trials are evaluating the combined use of SABR and systemic immunotherapy agents for different disease sites<sup>[55]</sup>.

Conventional EBRT is known to be immunosuppressive. Large treatments fields can damage adjacent bone marrow stem cells and kill circulating blood cells. SABR is directed to a much smaller field, minimizing normal cell exposure while inducing proinflammatory tumor cell death. Cell death by apoptosis exposes tumor antigen and stimulates innate and adaptive immune responses<sup>[60]</sup>. Studies show increased myeloid and lymphocytic infiltration of tumor following dose-escalated radiation<sup>[61]</sup>. The hypothesis that ablated tissue



**Figure 2.** Hypofractionated radiation therapy increases tumor cell programmed death ligand 1 receptor expression

can act as an *in-situ* vaccine through tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and T-cell stimulation matured from case reports describing an abscopal effect, where tumor regression was observed outside of the treatment field following local irradiation<sup>[62]</sup>. The clinical significance of the abscopal effect remains hypothetical with limited supportive clinical data to date.

In HCC, preclinical models report positive results for combined radiation and checkpoint blockade. Radiation upregulates programmed death ligand 1 (PD-L1) expression, and tumors treated with combination radiation plus anti-PD-L1 inhibitors had significantly greater results than radiation or immunotherapy alone<sup>[63]</sup>.

Gustafson *et al.*<sup>[64]</sup> in 2017 reviewed peripheral blood immunophenotypes in a series of patients with liver cancer before and after the administration of SABR. A 50% drop in peripheral CD3+ T-cells was observed, suggesting that T-cells were trafficking to tumor and lymph nodes both at the target site and possibly to disease outside of the treatment field<sup>[64]</sup>.

A recent clinical study by Kim *et al.*<sup>[63]</sup> shows that PD-L1 expression is elevated following SABR treatment of HCC, similar to effects identified in murine models. This phenomenon points to potential therapeutic benefit from combination treatment with a PD-L1 checkpoint inhibitor such as atezolizumab<sup>[63]</sup>. Phase I/II clinical trials are underway evaluating SABR plus ipilimumab, a cytotoxic T-lymphocyte associated protein 4 (CTLA-4) inhibitor [Figure 2]<sup>[55]</sup>.

In summary, the combination of immunotherapy with SABR to treat advanced HCC is a novel strategy with promising potential.

## SABR TOXICITY

Historically, the risk of hepatic decompensation due to RILD has discouraged the use of radiotherapy to treat liver cancer. RILD triggers a fibrotic process leading to the obliteration of central venules and widespread

venous congestion. Signs and symptoms can present in classical or non-classical patterns, developing between 2 weeks to 8 months after treatment. Outcomes vary from full recovery with supportive care to rare cases of liver failure and death.

Improvements in normal tissue complications probabilities (NTCP) modeling and awareness of the liver's parallel physiology provide the rationale for partial-liver irradiation to minimize the risk of RILD. A phase I trial of SABR in the treatment of liver metastases used partial hepatectomy outcome data to set volume parameters for normal tissue sparing<sup>[65]</sup>. The trial reported no cases of RILD when 700 cm<sup>3</sup> of uninvolved liver tissue were protected from doses exceeding 15 Gy in 3 fractions. Since then, NTCP modeling has established mean liver dose constraints reducing the risk of RILD to less than 5% in selected patients with CP-A hepatic function<sup>[66]</sup>.

In CP-A patients, prospective studies show a range of grade 3 or higher toxicities in 11%-30% of patients, almost all gastro-intestinal related [Table 1]. The highest number is from the PMH trial, in which patients had a greater than typical disease burden (average tumor size > 7 cm, 55% with PVTT, 12% with metastatic disease, all patients deemed untreatable by RFA, TACE or surgery)<sup>[41]</sup>. Patients most commonly complain of increased fatigue and poor appetite, usually resolving by 3 weeks after completion of their radiation course. Non-RILD toxicities, such as gradual liver decompensation, moderately elevated liver enzymes or virus reactivation can also occur. For patients with advanced cirrhosis, tissue-sparing volumetrics and dose constraints may require reduction of the total dose prescribed.

It is difficult to distinguish RILD from progressive liver disease, which can be multi-focal and out of the radiotherapy treatment field. For CP-B patients it may be reasonable to offer SABR when patients have no other option, though it must be done with caution. Non-critical use in inexperienced hands may result in toxicity. In 2015, a phase I/II trial reported 38% grade 3 or higher toxicities for CP-B HCC patients treated with SABR<sup>[52]</sup>. Ablative dose escalation should be applied carefully among CP-B patients as RILD rates increase in this population and limited safety data exists. SABR is not recommended for patients with CP-C disease. Proper commissioning of all equipment involved in SABR treatment, comprehensive quality assurance programs and specialized training for all staff involved in planning and delivery are essential safeguards<sup>[67]</sup>.

## CONCLUSION

SABR is a minimally-invasive treatment option for patients with non-metastatic HCC who are not candidate for resection or liver transplant. Published series show that this treatment approach is associated with excellent tumor control and can be done safely when NTCP guidelines are applied.

In addition, SABR may have some immunomodulation effects. Many ongoing clinical trials are looking at innovative ways to combine hypofractionated radiation therapy with immunotherapy to potentiate the systemic treatment response.

## DECLARATIONS

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Original Article

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# A dimethylbromobenzene-cysteine stapled peptide dual inhibitor of the p53-MDM2/MDMX interactions

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## Abstract

**Aim:** Hepatocellular carcinoma (HCC) has emerged as one of the most commonly diagnosed forms of human cancer; yet, the current treatment for HCC is less effective than those used against other cancers. Transcription factor p53 induces cell cycle arrest and apoptosis in response to DNA damage and cellular stress, thereby playing a critical role in protecting cells from malignant transformation. The oncoproteins MDM2 and MDMX negatively regulate the activity and stability of the tumor suppressor protein p53, conferring tumor development and survival.

**Methods:** In this work, we firstly explored the feasibility of antagonists targeting the p53-binding domains of MDM2 and MDMX as a potential method for HCC therapy via the survival rate analysis in The Cancer Genome Atlas. Moreover, we developed a novel stapling strategy for peptide drug design using the reaction between mercapto group and bromine to crosslink the side chains of the two Cys at (i, i+4) positions, and apply it to a series of peptides derived from a dodecameric peptide antagonist of both MDM2 and MDMX, termed p53-MDM2/MDMX inhibitor (PMI).

**Results:** Notably, all of these stapled peptides can compete with p53 for MDM2 or MDMX binding as the similar affinity



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as PMI. More importantly, this stapling functionally rescued PMI that, on its own, failed to activate p53 because of its poor membrane permeability and susceptibility to proteolytic degradation.

**Conclusion:** Taken together, this work not only illustrates that the restoration of p53 is a potentially feasible program for HCC therapy, but promises an important new tool for peptide drug discovery and development for a variety of human diseases.

**Keywords:** Hepatocellular carcinoma, p53, stapled peptide, dimethylbromobenzene-cysteine

## INTRODUCTION

p53 acts as a tumor suppressor by initiating cell-cycle arrest, apoptosis, and senescence in response to cellular stress to maintain the integrity of the genome<sup>[1]</sup>. In a substantial proportion of cancers, p53 is wild type but the protein is inactivated; this offers an attractive strategy for cancer therapy based on p53 reactivation<sup>[2,3]</sup>. Recent studies in cancer patients have provided proof-of-concept for this approach<sup>[2]</sup>. Such activators are the product of basic research conducted over the past 20 years that has led to the appreciation of MDM2 and MDMX as the two major negative regulators of p53, which now seem to be “druggable” using a variety of strategies<sup>[4]</sup>.

Of all human cancers, hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed cancer worldwide and is the third leading cause of cancer death globally<sup>[5]</sup>. Yet, the current most common treatment for HCC is surgical resection, which is less effective than those used against other cancers<sup>[1]</sup>. Because the loss of p53 function plays a critical role in multistage hepatocarcinogenesis, the p53 gene has been regarded as a good candidate for modulating HCC risk<sup>[6]</sup>. Furthermore, the top two risk factors of HCC are metabolic disease (such as fatty liver) and viral infection (such as hepatitis B and C), both of which cause cirrhosis before HCC<sup>[7,8]</sup>. As one of the hallmarks of cancer, the changes observed in cancer cell metabolism and bioenergetics are also regulated by p53<sup>[9,10]</sup>. Therefore, the connection between p53 stress response and the disordered metabolic process leading to HCC is a potential avenue for HCC therapies.

Several classes of molecules that inhibit this interaction between p53/MDM2 (MDMX) have been developed (e.g., Nutlin and MI-219)<sup>[11,12]</sup>. They mimic the conserved residues from a region of the p53 N-terminal that are functional for the interaction with the N-terminal p53 binding domain of MDMX or MDMX<sup>[4]</sup>. This region forms an  $\alpha$ -helix upon binding, enabling the three conserved hydrophobic residues of the MDM2 binding motif (F19, W23, and L26) to optimally embed into the hydrophobic binding groove located on MDM2 and its homologous MDMX protein<sup>[13,14]</sup>. Except for small molecules, it has been proved that the p53 peptide is appropriate as a biological tool and prototype therapeutic by enforcing its R-helical structure while preserving the key interacting residues that enable specific MDM2 and MDMX engagement<sup>[15]</sup>.

As the wild-type p53 peptide (ETFSDLWKLLPE) has a low affinity for MDM2/MDMX and comes from a region of p53 that interacts with many other proteins<sup>[15]</sup>, we explored the effects of stapling a peptide derived from phage selection experiments<sup>[14]</sup>. Phage display and rational design methods have been used to isolate linear peptides that bind MDM2 with high affinity<sup>[13]</sup>. The most avid of these published peptides, described by Pazgier *et al.*<sup>[14]</sup>, named p53-MDM2/MDMX inhibitor (PMI), was used as the template for this study. Besides, when PMI helices are taken out of protein context and placed into aqueous buffer in isolation, it usually adopts random coil conformations, leading to a drastic reduction in biological activity and thus diminishing therapeutic potential<sup>[14,16]</sup>. To overcome it, numerous strategies have been developed to stabilize or mimic peptide helices<sup>[17-19]</sup>. Among these, the most straightforward, yet effective, strategy is sidechain cross-linking (“peptide stapling”)<sup>[16]</sup>. Since peptide stapling necessitates macrocyclization, an entropically unfavorable process, very few reactions are known to date that give rise to good yields along with the

reinforced structures. These include disulfide bond formation<sup>[20]</sup>, lactam formation<sup>[21]</sup>, ruthenium-catalyzed ring closing metathesis<sup>[15]</sup>, and copper-catalyzed azide-acetylene cycloaddition<sup>[22]</sup>. While these reactions have enabled the synthesis of stapled peptide helices, the development of additional stapling reactions with high yields and predictable structural effect is still highly desirable. Herein, we report the first synthesis of stapled PMI helices using 1,2(1,3 or 1,4)-dimethylbromobenzene reacting with the sulfhydryl of cysteine and the subsequent structural, protein chemistry and *in vitro* anticancer activity studies of the stapled PMI.

## METHODS

### Patient data

The data of p53, MDM2 and MDMX expression at mRNA level in HCC patients were collected and obtained from The Cancer Genome Atlas (TCGA) project<sup>[23,24]</sup> via the data portal on 03/24/2018.

### General remarks

All synthetic peptide sources were obtained from CS Bio (Shanghai) Ltd. All other chemicals used in this study were purchased from Sigma-Aldrich unless otherwise specified. Acetonitrile and water (HPLC grade) were purchased from Fisher Scientific Ltd. All products were used as received without further purification.

### Synthesis of peptides

All peptides were synthesized on appropriate resins on an CS bio 336X automated peptide synthesizer using the optimized HBTU activation/DIEA in situ neutralization protocol developed by an HBTU/HOBt protocol for Fmoc-chemistry SPPS.2 After cleavage and deprotection in a reagent cocktail containing 88% TFA, 5% phenol, 5% H<sub>2</sub>O and 2% TIPS, crude products were precipitated with cold ether and purified to homogeneity by preparative C18 reversed-phase HPLC. The molecular masses were ascertained by electrospray ionization mass spectrometry (ESI-MS).

### Reversed phase analytical and preparative HPLC

Analytical HPLC was run on a Waters instrument using an analytical C18 column purchased from Waters at a flow rate of 1.0 mL/min. Solution A was ultrapure water containing 0.1% trifluoroacetic acid (TFA), and solution B was acetonitrile containing 0.1% TFA. The gradient is linear from 5% B to 65% B in 30 min. Preparative HPLC was run on a Preparative Waters instrument using an analytical C4 column purchased from Waters at a flow rate of 15.0 mL/min. Eluent A and B were same as the solution used in analytical HPLC. The gradient is linear from 25% B to 50% B in 60 min.

### Preparation of stapled PMI

To prepare stapled PMI, the peptide was firstly dissolved in reaction buffer [80% 10 mmol/L PBS (pH 7.4) and 20% acetonitrile] at a concentration of 100 µmol/L, meanwhile 1,2(1,3 or 1,4)-dimethylbromobenzene were dissolved in DMSO at a concentration of 10 mmol/L. After the preparation of reaction fluid, 10 mL peptide buffer were magnetic stirred in a beaker at room temperature, and then 50 µL 1,2 (1,3 or 1,4)-dimethylbromobenzene buffers were mixed into the buffer in four times every 10 min. After the reaction, pure stapled PMI can be collected by preparative HPLC.

### CD spectroscopy

CD spectra of variants at a concentration of 20 µmol/L in 10 mmol/L phosphate buffer (pH 7.4) were obtained at room temperature on a J-810 spectropolarimeter (Jasco, Easton, MD) using a 1-mm quartz cuvette as previous reports<sup>[25-27]</sup>. Scanned area was from 250 nm to 190 nm, and the scanning speed was 50 nm/min. Every curve was the average of three independent detections.

### Fluorescence polarization-based competitive binding assay

As for fluorescence polarization assay, Fluorescein (FITC) was conjugated to <sup>15-29</sup>p53 *via* its N-terminal amino group in DMF, and the resultant product <sup>15-29</sup>p53-FITC were HPLC-purified and lyophilized. The

Fluorescence polarization-based competitive binding assays were performed in Microfluor® 2, 96-well black plates (Thermo Fisher Scientific) and readings were taken using a Tecan Infinite M2000 fluorescence plate reader. Serially diluted Lupbin or corresponding peptide were prepared in Tris-HCl buffered saline (10 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, pH 7.0) and incubated with 200 nmol/L <sup>15-29</sup>p53-FITC/MDM2 or 50 nmol/L <sup>15-29</sup>p53-FITC/MDMX in a total volume of 150 µL per well. After 2 h incubation at room temperature, fluorescence polarization was measured at  $\lambda_{\text{ex}} = 470 \text{ nm}$  and  $\lambda_{\text{em}} = 530 \text{ nm}$ . Nonlinear regression analyses were performed to give rise to IC<sub>50</sub> values.

### Cell culture and cell viability analysis

Human colon cancer cell line HCT116<sup>+/+</sup> (wild-type p53) was purchased from ATCC, and maintained in McCoy's 5A medium with 10% FBS. The isogenic HCT116<sup>-/-</sup> (p53 deletion) cells were presented by Prof. Bert Vogelstein of Johns Hopkins University (Baltimore, MD), and maintained in McCoy's 5A medium with 10% FBS. human hepatoma cell line SK-Hep-1 was also purchased by ATCC, and maintained in DMEM with 10% FBS. All cells were maintained at 37 °C in an atmosphere of 5% CO<sub>2</sub>. For cell viability test, three cell lines were plated in 96-well plates at a density of 2,500 cells/well (100 µL). After 24 h, cells were treated with drug sample at the indicated concentrations and times in FBS-free mediums, respectively. The *in vitro* cytotoxicity was then measured by using a standard MTT (Thermo Fisher scientific) assay after 72 h drug treatment.

### Apoptosis analysis

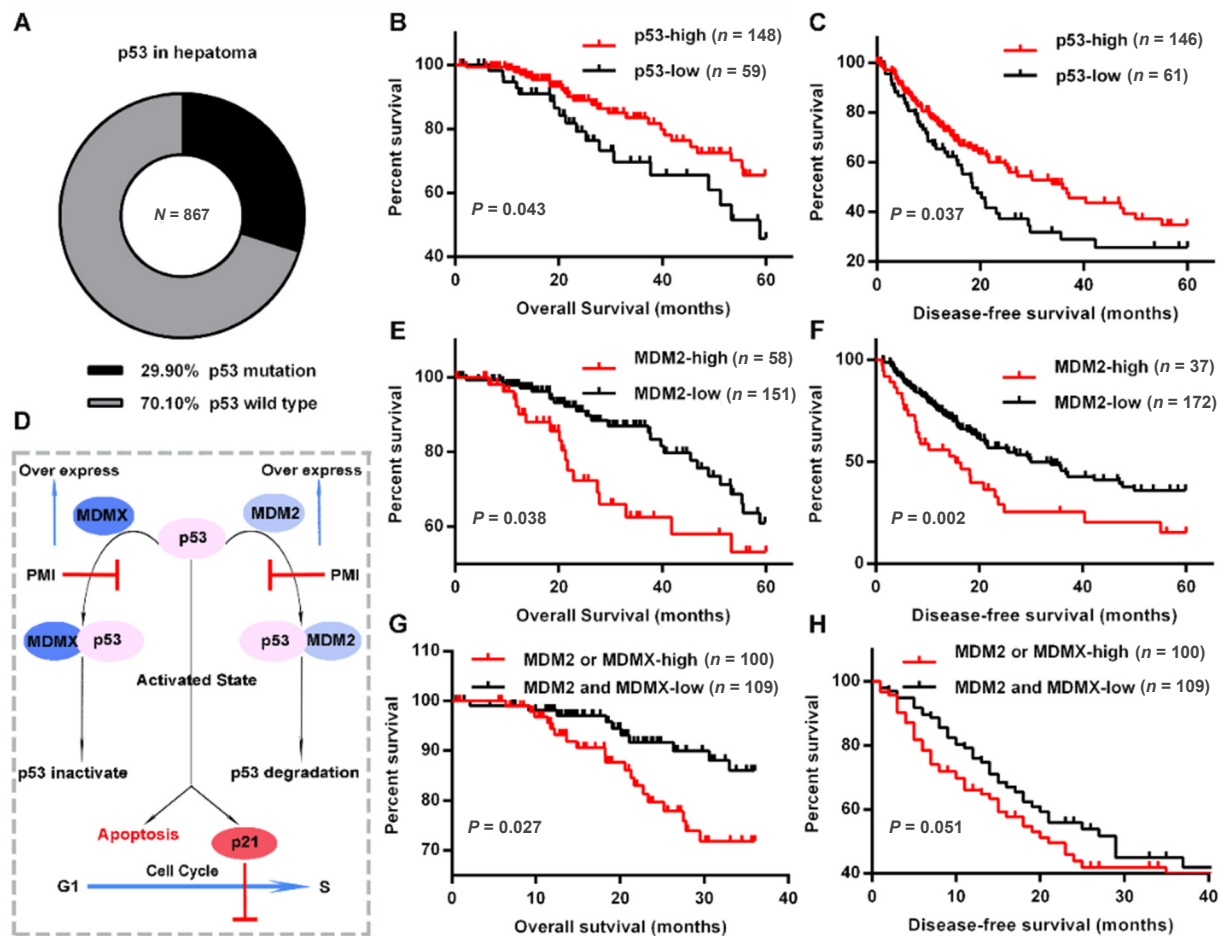
Necrosis/apoptosis was evaluated by flow cytometric analysis using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences). Briefly, cells were treated with samples for 48 h. Cells were then harvested, washed twice with cold PBS, and re-suspended in 1× binding buffer at a concentration of  $1 \times 10^6$  cells/mL. One hundred microliters of the solution ( $1 \times 10^5$  cells) was transferred to a 5 mL culture tube, followed by addition of 5 µL of FITC Annexin V and 5 µL of PI. After gentle vortexing and a 15-min incubation in the dark at room temperature, 400 µL of 1× binding buffer was added to the tube, and cells were analyzed by fluorescence-activated cell sorting (FACS).

## RESULTS

### Wild-type p53 is a feasible target for HCC therapy

Research has shown that the tumor suppressor p53 has an important role in tumor progression, and that it is mutated or functionally inactivated in most human cancers<sup>[2]</sup>. As for HCC, p53 was nonsynonymously mutated in 259 (29.9%) of 867 hepatoma cases in TCGA [Figure 1A], suggesting that in a substantial proportion of HCC, TP53 (which encodes p53) is wild type but the protein is inactivated. To explore the importance of p53 and its two agonists- MDM2 and MDMX- in the HCC process, we analyzed the relationship between these protein expression and survival of HCC patients carried wild-type p53. As shown in Figure 1B and C, decreased expression of p53 was significantly associated with poor patient overall survival ( $P = 0.043$ ) and disease-free survival ( $P = 0.037$ ). It is well-known that the tumor suppressor activity and *in vivo* stability of p53 are abrogated by regulatory molecules such as the E3 ubiquitin ligase MDM2 and its homologue MDMX (also known as HDMX and MDM4) [Figure 1D]<sup>[28,29]</sup>. This offers an attractive strategy for cancer therapy based on p53 reactivation by blocking the interaction between p53 and MDM2 (MDMX). In this case, the two major negative regulators of p53 now seem to be “druggable”, and recent studies in cancer patients have provided proof-of-concept for this approach<sup>[2]</sup>. To further verify the feasibility of p53 restoration via MDM2 and MDMX blocking for HCC therapy, we attempted to evaluate the association of MDM2 and MDMX expression with survival in 209 HCC patients carried wild-type p53. As expected, the 5-year overall survival [Figure 1E] and disease-free survival [Figure 1F] rates of MDM2 high-expressed cases are significantly higher than that of MDM2 low-expressed cases. Meanwhile, MDMX showed the same tendency as MDM2 [Figure 1G and H]. Collectively, all these results demonstrated that high-level p53 is beneficial to the survival of HCC patient, thus p53 restoration was a potentially feasible program for HCC therapy in p53-wild-type patients.



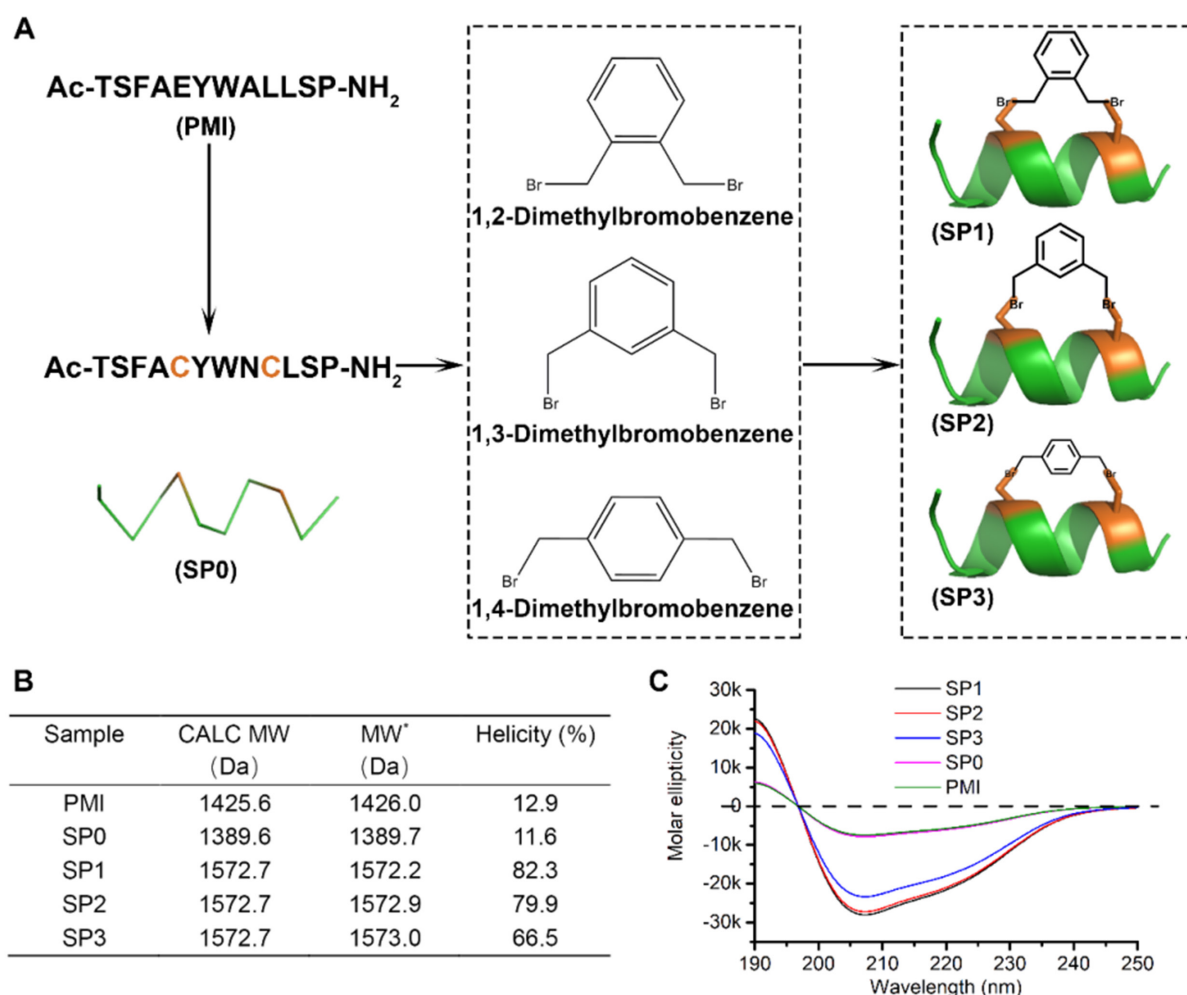


**Figure 1.** Wild-type p53 is a potential target for HCC therapy. A: The percentage of wild-type p53 in 867 HCC patients in The Cancer Genome Atlas; B and C: the Kaplan-Meier survival curves of overall survival and disease-free survival duration based on p53 expression in the mRNA level; D: the Schematic diagram for the mechanism of p53 function and its connection with MDM2 and MDMX; E-H: the Kaplan-Meier survival curves of overall survival and disease-free duration based on MDM2 and MDMX expression in the mRNA level. The receiver operating characteristic curve was used to define the cutoff, and log-rank analysis was used to test for significance. HCC: Hepatocellular carcinoma; PMI: p53-MDM2/MDMX inhibitor

### Preparation of dimethylbromobenzene-cysteine stapled peptide

In this study, we firstly used PMI-a potent dodecameric peptide antagonist of MDM2 and MDMX that, despite its high affinity for both proteins<sup>[14]</sup>, fails to activate p53 and kill p53<sup>+/+</sup> tumor cells due presumably to its inability to traverse the cell membrane and susceptibility to proteolytic degradation<sup>[30]</sup>. Our new chemistry for stapling peptide entails an efficient click reaction between the bromine in dimethylbromobenzene and the mercapto group in peptide Cys. Due to the fact that the effective concentration in the molecule was much higher than the intermolecular concentration, dimethylbromobenzene would specifically be conjugated to the two Cys in one peptide rather than the two intermolecular Cys [Figure 2A]. Previous structural and functional studies of PMI (TSFAEYWNLLSP) identified Phe3, Tyr6, Trp7 and Leu10 as the most critical residues for MDM2/MDMX binding<sup>[14]</sup>. Thus, we maintained those four residues in the design of stapled peptides and introduced Cys-Cys pairs into (5,9) positions of PMI (Figure 2A, SP0 TSFACYWNCLSP). This N-acetylated and C-amidated peptides were synthesized using Fmoc-chemistry for solid phase peptide synthesis as our previous reports<sup>[31,32]</sup>, and purified by HPLC to homogeneity. Crosslinking two Cys side chains was readily accomplished in 2 h in PBS/acetonitrile (4:1) buffer containing 100 μmol/L SP0 peptide and 150 μmol/L dimethylbromobenzene, as verified by ESI-MS [Figure 2B], resulting in 3 stapled constructs termed SP1, SP2 and SP3 [Figure 1A and B]. Not surprisingly, SP1, SP2 and SP3 partially adopted an α-helical structure in aqueous solution according to CD analyses, whereas SP0 and PMI showed very limited





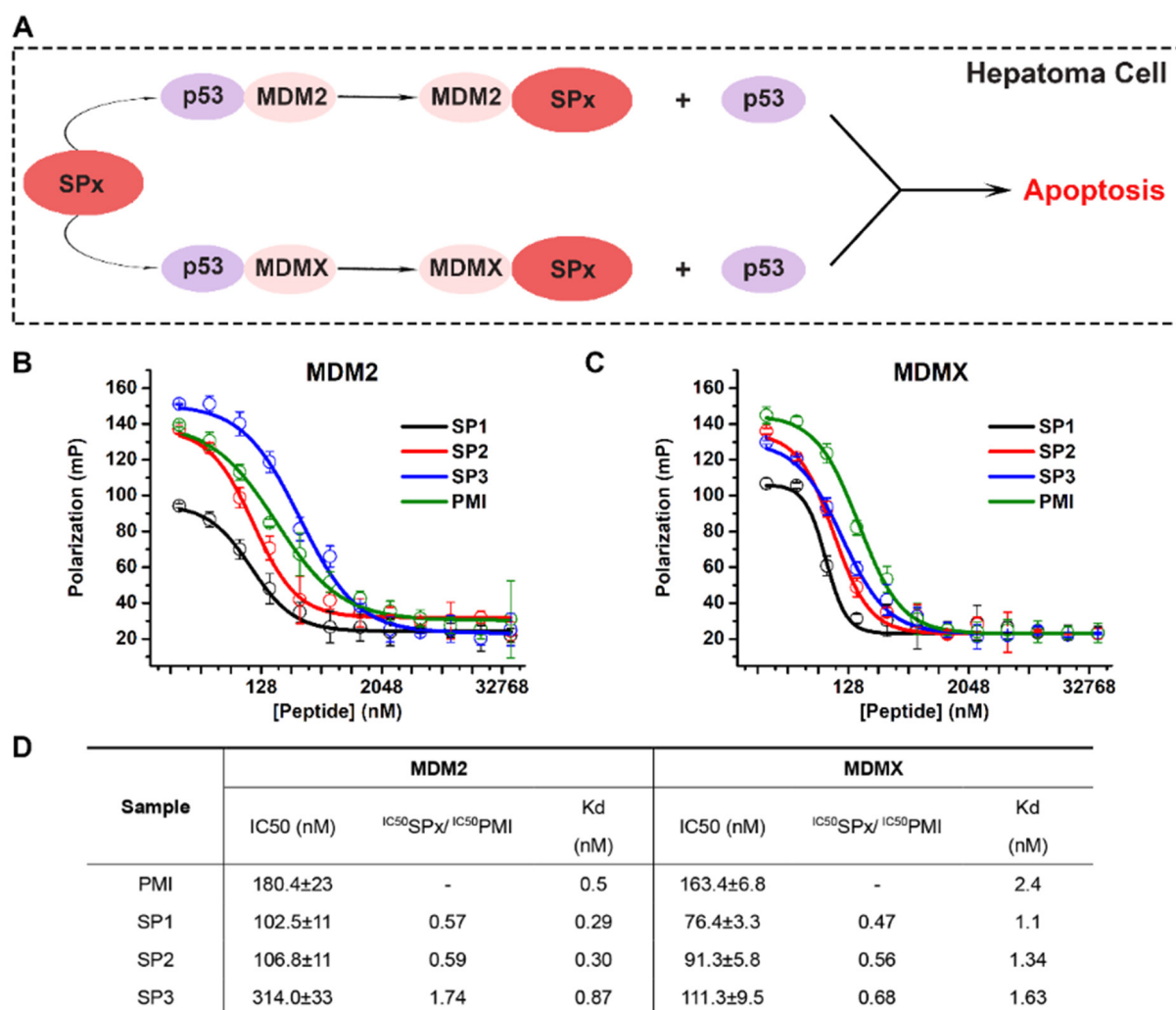
**Figure 2.** Characterization of dimethylbromobenzene-cysteine stapled peptide. A: Schematic diagram for the preparation of dimethylbromobenzene-cysteine stapled peptide; B: the table for the molecular weight and helicity of the p53-MDM2/MDMX inhibitor (PMI) and stapled peptides. CALC MW stands for the theoretical molecular weight of peptides. \*Stand for that the molecular weight was measured by ESI-MASS; C: circular dichroism spectra of PMI, SP0, SP1, SP2 and SP3. The experiment was repeated independently for 3 times with similar results

topological structure [Figure 2B and C], suggesting that crosslinking Cys-Cys side chains stabilized peptide conformation productive for targets binding.

### Dimethylbromobenzene-cysteine stapled peptide specifically targets intracellular complexes of p53/MDM2 and p53/MDMX

Dubbed the “guardian of the genome”<sup>[33]</sup>, p53 is critical for maintaining genetic stability and preventing tumor development<sup>[4]</sup>. MDM2 binds the N-terminal transactivation domain of p53 with high affinity to block p53 regulating responsive gene expression, resulting in the p53 inactivation<sup>[34]</sup>. Moreover, MDM2 controls p53 stability by targeting the tumor suppressor protein for ubiquitin-mediated constitutive degradation<sup>[35]</sup>. Although MDMX lacks E3 ubiquitin ligase activity, the MDM2 homologue acts as an effective transcriptional antagonist of p53, and impedes p53-induced growth inhibitory and apoptotic responses<sup>[36]</sup>. Thus, the ideal p53 activators are dual specific inhibitors to target both MDM2 and MDMX, and SPx (SP1, SP2 or SP3) may well be one of them.

For verification, the inhibitory effects of stapled PMI SPx on the interaction between p53 and MDM2/MDMX were measured by fluorescence polarization-based competition assays, in which different

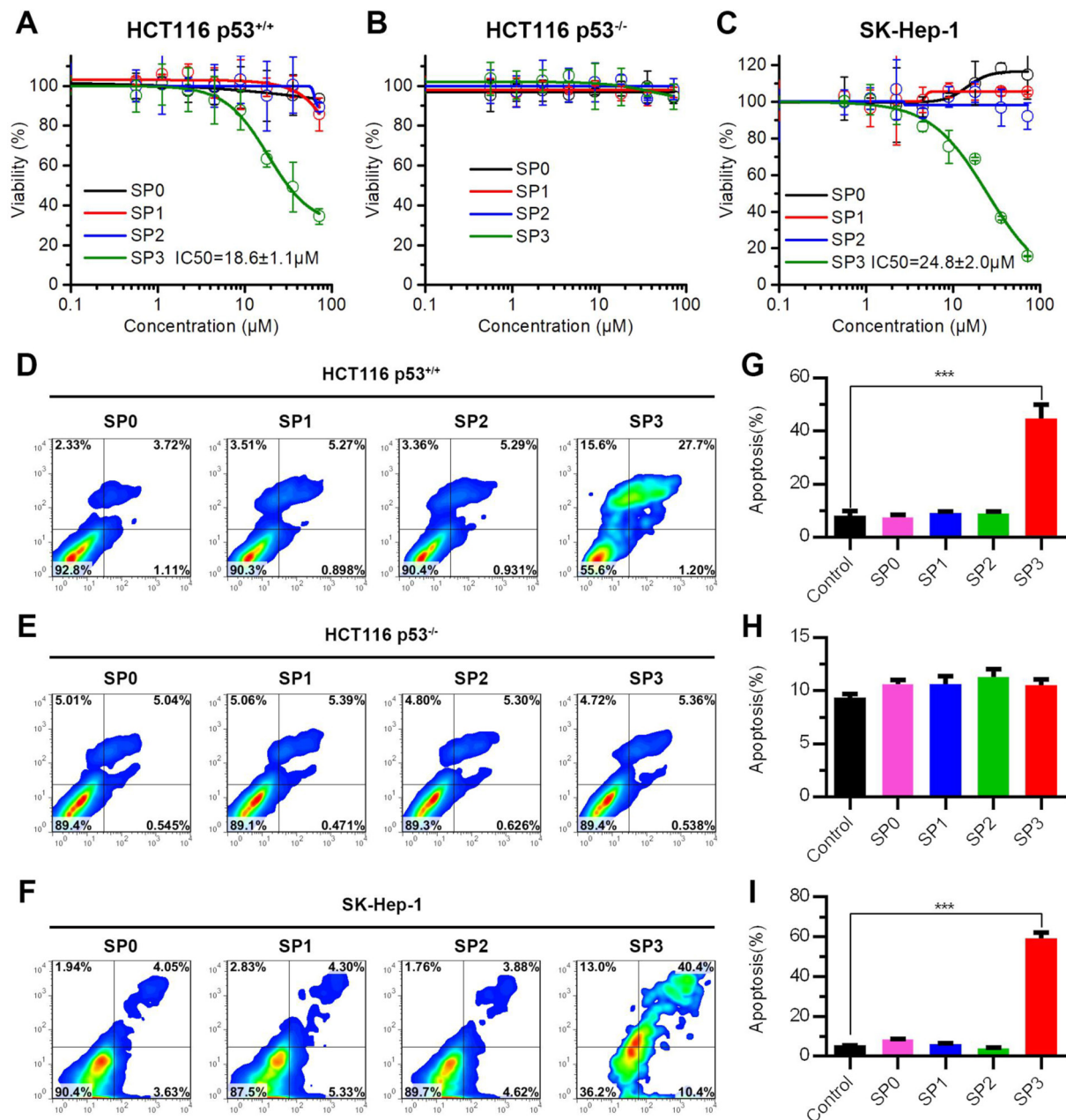


**Figure 3.** The stapled peptides specifically targets intracellular complexes of p53/MDM2 and p53/MDMX. A: Schematic diagram for that stapled peptides can compete with p53 for MDM2 or MDMX binding; B and C: fluorescence polarization-based competitive binding assay of p53-MDM2/MDMX inhibitor (PMI) or stapled peptides to MDM2/p53 complex (B) and MDMX/p53 complex. For fluorescence polarization measurements at room temperature on a Tecan Infinite M2000 plate reader, FITC was covalently conjugated to the N-terminal of 15-29 p53. Non-linear regression analyses were performed to give rise to IC<sub>50</sub> values (mean ± SEM, *n* = 3); D: table for the results from B and C. Kd of PMI was cited from our previous reports, and Kd of SP1, SP2 and SP3 were calculated by the IC<sub>50</sub> ratio between SPx to PMI

concentrations of stapled PMI were applied to pre-incubated MDM2/<sup>18-26</sup>p53-FITC or MDMX/<sup>18-26</sup>p53-FITC complexes, respectively, [Figure 3B and C] and the IC<sub>50</sub> and Kd values are tabulated in Figure 3D. Compared with the N-acetylated and C-amidated wild-type peptide PMI, SP1 and SP2 were bound more strongly to MDM2 and MDMX. Meanwhile, SP3 showed a moderately higher ability to block MDMX/p53 interaction than PMI, but was slightly inferior than PMI to MDM2/p53. Notably, the half-maximal inhibitory concentrations (IC<sub>50</sub> values) of SP1, SP2 and SP3 were as the same order of magnitude as PMI, demonstrating that all of the three stapled PMI were capable of blocking p53-MDM2/MDMX interaction, thereby reactivating p53 to suppress tumor growth.

### Functional characterization of dimethylbromobenzene-cysteine PMI

Previous reports have shown that structurally permissible stapling of peptide, while enhancing  $\alpha$ -helicity and improving targets binding, is not sufficient to endow the peptide with an ability to kill tumor cells<sup>[15]</sup>. In fact, the amino acid composition and topological structure of a stapled peptide are critical for its ability to traverse the cell membrane to exert biological activity<sup>[15]</sup>. To address it, we firstly measured the



**Figure 4.** Functional characterization of dimethylbromobenzene-cysteine p53-MDM2/MDMX inhibitor. A-C: dose-dependent growth inhibition of HCT116 p53<sup>+/+</sup> (A), HCT116 p53<sup>-/-</sup> (B) and SK-hep-1 (C) cells upon various treatments as determined by the MTT assay to monitor the pesticide effects. Three cell lines were plated in 96-well plates at a density of 2,500 cells/well (100  $\mu$ L). After 24 h, cells were treated with drug sample at the indicated concentrations and times in FBS-free mediums, respectively. The *in vitro* cytotoxicity was then measured by using a standard MTT (Thermo Fisher scientific) assay after 72 h drug treatment. (mean  $\pm$  SD,  $n = 4$ ); D-F: apoptosis levels measured by FACS in three cell lines treated with SP0, SP1, SP2 and SP3 for 48 h incubation at concentration of 50  $\mu$ mol/L; G-I: the average means of the apoptosis calculated three independent experiments like D-F.  $P$  values were calculated by  $t$ -test (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ )

cellular uptakes of FITC-labeled SP0, SP1, SP2 and SP3 in HCT116 cells after 6 h incubation in 37  $^{\circ}$ C at a concentration of 100  $\mu$ mol/L. As shown in Supplementary Figure 1, SP3 showed the strongest ability of cellular internalization (> 75%), whereas neither SP1 nor SP2 showed exceed 15% cellular internalization. Moreover, there exists no cellular uptakes for the three stapled peptides at 4  $^{\circ}$ C incubation [Supplementary Figure 1], suggesting that the cellular uptakes of the stapled peptide most likely result from the ATP-dependent endocytosis. Furthermore, it is necessary to verify the biological activity of SPx to induce the

cancer cells apoptosis in a p53-dependent manner. To functionally validate SPx, we subjected them and their unstapled control to a cell viability assay in a FBS-free medium using a pair of cell lines with the same genetic background carried wild-type p53 (HCT116 p53<sup>+/+</sup>) or deleted p53 (HCT116 p53<sup>-/-</sup>). While the control peptide exhibited no anti-proliferative activity against both cell lines at concentrations of up to 100  $\mu\text{mol/L}$ , SP3 displayed dose- and p53-dependent growth inhibitory activity against HCT116 p53<sup>+/+</sup>, but not HCT116 p53<sup>-/-</sup>, with an IC50 value of  $\sim 18.6 \mu\text{mol/L}$  at 72 h [Figure 4A and B]. Besides, SP3 also showed an obvious suppression for a hepatocellular carcinoma cell line carried wild-type p53, named Sk-Hep-1, with an IC50 value of  $\sim 24.8 \mu\text{mol/L}$  in the absence of serum [Figure 4C and Supplementary Figure 2]. Consistent with this result, the induction of apoptosis of HCT116 p53<sup>+/+</sup> and SK-Hep-1 cells by SP3 was verified by fluorescence-activated cell sorting [Figure 4D-I]. Taken together, these findings support that SP3 actively traversed the cell membrane and killed tumor cells by reactivating the p53 pathway. It is worth pointing out that as is often the case with other stapled peptide activators, although its efficient blocking the interaction between p53-MDM2/MDMX, are rather weak in killing HCT116 p53<sup>+/+</sup> and SK-Hep-1 cells. The weak *in vitro* activity implies that stapling alone is insufficient to achieve optimal therapeutic efficacy of helical peptides, dictated by cell internalization, endosomal escape, proteolytic stability, spatio-temporal distribution, etc.

## DISCUSSION

The tumor suppressor protein p53 induces powerful cancer cell antiproliferation and apoptotic responses to cellular stress, plays a pivotal role in preventing damaged cells from cancerous<sup>[4]</sup>. Not surprisingly, the impairment of p53 signaling pathway is a hallmark of almost all human cancers, where either the TP53 gene is mutated or wild-type p53 is functionally inactivated by the E3 ubiquitin ligase MDM2 and its homolog MDMX<sup>[37,38]</sup>. In many tumor cells harboring wild-type p53, the up-regulated MDM2 and/or MDMX often cooperate to inhibit p53 transactivation activity and urge p53 for degradation, conferring tumor development and progression<sup>[2]</sup>. A great number of studies have validated that MDM2 and/or MDMX antagonism as a viable therapeutic regimen for cancer therapy, and several small-molecule antagonists specific for MDM2 are in various phases of clinical trials<sup>[39,40]</sup>. As for HCC, our results in Figure 1 further illustrates that p53-MDM2/MDMX is an important target for therapy, thus, the development of potent antagonists specific for MDM2 and MDMX is meaningful for HCC therapy.

Growing evidence suggests that the interplay between MDM2 and MDMX confers robust p53 inactivation in tumorigenesis and that antagonizing both MDM2 and MDMX affords a powerful, synergistic and sustained inhibition of tumor growth<sup>[41,42]</sup>. However, traditional small-molecule drugs are always limited by the comparatively small interaction area, resulting in the failure as dual specific inhibitors to target both MDM2 and MDMX simultaneously. To this end, a peptide therapeutics termed PMI was developed to competes with p53 for MDM2 and MDMX binding at high affinity<sup>[14,43]</sup>. However, major pharmacological hurdles still impede the development of anticancer peptide therapeutics with optimal therapeutic efficacy, including: short circulation half-life due to proteolytic degradation and poor cellular uptake. To overcome these technical obstacles, we developed a novel peptide stapling method to link the side chains of Cys and Cys at (i, i+4) positions by two bromine methyl group in benzene para-, ortho- or meta- positions to form the dimethylbromobenzene-cysteine structure. Of note, this stapling method is appropriate for all  $\alpha$ -helix after mutating two nonfunctional residues into Cys at (i, i+4) positions. After a series of characterization and functional verification, SP3, a stapled PMI crosslinked the side chains by 1,4-dimethylbromobenzene, can potently inhibit the growth of cancer cell in a p53 dependent manner. Of note, the remaining position of the benzene in the dimethylbromobenzene can be further modification for more hydrophilic and more appropriate charge characteristics.

In this work, we found that the expression of p53, MDM2 and MDMX were closely related to the survival of  $\sim 70\%$  HCC patients carrying wild-type p53, and provided strong evidence that reactivating p53 from MDM2 and MDMX was a potentially feasible program for HCC therapy. After that, we have developed a

novel stapling strategy for peptide drug design using the reaction between mercapto group and bromine to crosslink the side chains of the two Cys at (i, i+4) positions. By this way, we successfully induced the formation of and stabilized a productive  $\alpha$ -helical conformation of PMI - a dual-specificity peptide antagonist of MDM2 and MDMX, enabling it to traverse the cell membrane and kill tumor cells by reactivating the p53 pathway. This stapling functionally rescued PMI that, on its own, failed to activate p53 because of its poor membrane permeability and susceptibility to proteolytic degradation. Taken together, this work not only illustrates that the restoration of p53 is a potentially feasible program for HCC therapy, but promises an important new tool for peptide drug discovery and development for a variety of human diseases.

## DECLARATIONS

### Authors' contributions

Did experiments: Jiang W, Jin L

Designed this work: Liu M, Hou P, He WX

Wrote this paper: Hou P, He WX

Revised the manuscripts: All authors

### Availability of data and materials

The patient mRNA data was from TCGA. All experimental data did by the authors listed in this paper.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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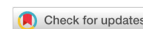


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Review

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# Circulating tumor DNAs and non-coding RNAs as potential biomarkers for hepatocellular carcinoma diagnosis, prognosis and response to therapy

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## Abstract

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide and despite improvement in therapeutic approaches, prognosis remains poor. This can be partly attributed to the fact that the majority of HCCs are diagnosed at intermediate or advanced stages. Availability of circulating biomarkers able to detect HCC at early stages could improve patients' prognosis. At present, however, alpha fetoprotein or des- $\gamma$ -carboxyprothrombin are unable to reliably detect HCC at early stages and better circulating biomarkers are needed. Circulating tumor DNA (ctDNA) and non-coding RNAs (ncRNAs) are emerging as promising biomarkers to achieve the goal. Genetic and epigenetic alterations in ctDNA allow to pinpoint tumor-specific biomarkers, reveal tumor heterogeneity, help monitor tumor evolution over time and assess therapy efficacy. It remains to be fully evaluated the possibility of detecting these biomarkers at early tumor stages. Circulating ncRNAs are quantitative biomarkers with potential use in diagnostic, prognostic and predictive clinical settings. They may help to reveal HCC at early stages. However, because of heterogeneous and sometimes conflicting reported results, they still require validation and standardization of pre-analytical and analytical approaches before clinical applications could be envisaged.

**Keywords:** Liquid biopsy, hepatocellular carcinoma, circulating tumor DNA, non-coding RNA, diagnosis, prognosis, therapy response



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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and second leading cause of cancer-related deaths worldwide. Despite new therapeutic approaches, prognosis remains poor. According to the Barcelona Clinic Liver Cancer (BCLC) system<sup>[1]</sup>, treatment options rely on cancer staging. Patients with an early stage HCC (BCLC stage A) can take advantage of curative treatments, such as tumor resection, ablation and liver transplantation. Although termed curative, risk of recurrence in post-surgical resection is about 70% at 5 years<sup>[2]</sup>. Patients who presents an intermediate (Stage B) or advanced (Stage C) HCC, about 70% of the total, can only benefit from palliative treatments, chemoembolization or sorafenib respectively, with survival in fewer than 10% of patients at 3 years<sup>[3-5]</sup>.

Poor prognosis of HCC can be partly attributed to the fact that the majority of neoplasms are diagnosed at intermediate or advanced stages. Availability of blood biomarkers would be extremely important to improve early diagnosis in individual at risk or for a better management of prognosis and response to therapy in HCC patients. Among biomarkers presently in use, alpha fetoprotein (AFP) and des- $\gamma$ -carboxy prothrombin (DCP) are the most commonly employed. AFP was the most widely used serum biomarker in HCC<sup>[6]</sup>, but due to suboptimal sensitivity (55%-65%)<sup>[7-9]</sup>, it is now employed to the monitoring of therapy effectiveness in HCC patients, together with ultrasound examination. DCP is a second biomarker utilized in HCC; although initially indicated as superior to AFP with a sensitivity of 92% and specificity of 93%<sup>[10]</sup>, other studies showed suboptimal sensitivity (48%-62%)<sup>[11,12]</sup>. These studies signify that more effective biomarkers are needed for better management of HCC patients at various clinical phases.

Since 80%-90% of HCCs develop in a cirrhotic liver, a distinction between regenerative nodules and early HCC can be a challenge<sup>[13]</sup>. Albeit DCP exhibits the potential capability of differentiating HCC from non-malignant liver diseases<sup>[14]</sup>, tissue biopsy remains the most dependable option for diagnostic purposes as well as for recognizing the molecular changes that characterize the tumor. However, tissue biopsy is invasive and associated with potential risks for the patients, it cannot be repeated and cannot be performed on patients with unresectable advanced HCC.

In recent years, liquid biopsy has become a valid alternative to overcome the above mentioned limitations. It is only modestly or not invasive at all and it offers the possibility of carrying out repeated tests over time. Moreover, it can be used for an early detection of tumors, for monitoring its growth dynamics, for evaluating the efficacy of treatments and for spotting tumor genetic heterogeneity and identifying mutations responsible for acquired resistance, becoming a highly promising approach for the clinical management of cancer patients. Liquid biopsy is the sampling and analysis of biological samples, such as blood, urine, saliva or stool, where nucleic acids originating from all or part of body districts can be found. In the presence of cancer, its derived materials, such as circulating tumor cells, cell-free tumor DNA (ctDNA) and microvesicles containing mRNAs, microRNAs (miRNAs) and proteins, are present in peripheral blood or other body fluids and can be measured through the use of specific tests [Figure 1]. This review is focused on ctDNA and circulating non coding RNAs, like miRNAs and long non-coding RNAs (lncRNAs), as potential biomarkers of HCC for early diagnosis, monitoring patients' follow-up and assessing response to treatments.

### ctDNA

The presence of free circulating DNA in serum/plasma has been used to reveal tumor-associated biomarkers, such as the increased abundance of cell-free DNA (cfDNA) in cancer patients or the presence of specific genetic or epigenetic alterations, which have been discovered in numerous types of cancer including HCC. Several studies have indeed proposed the cfDNA as a source of HCC biomarkers in diagnostic, prognostic or predictive clinical settings [Table 1].

### cfDNA as biomarkers in plasma or serum in HCC

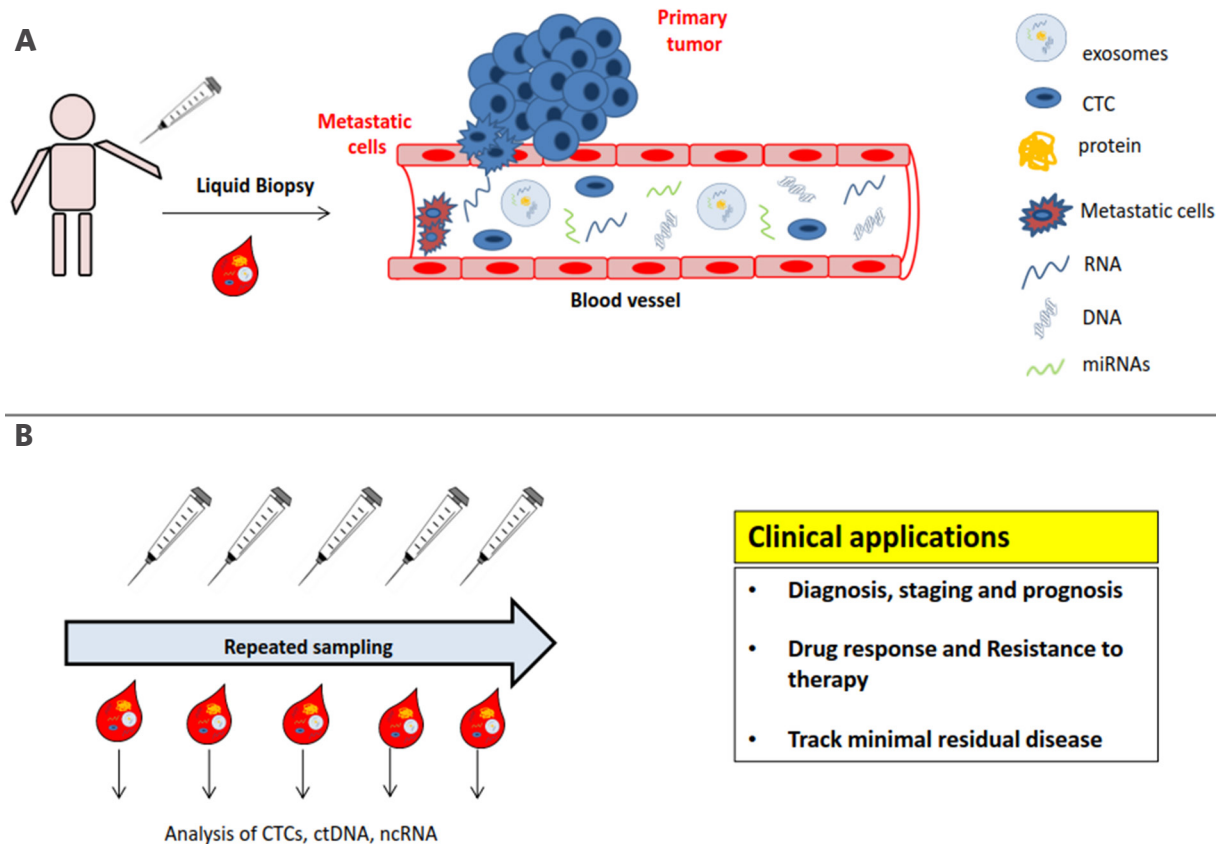
The level of plasma cfDNA was found significantly increased in patients affected by HCC, compared to individuals with liver fibrosis. A model that included this parameter together with patient age and AFP levels

**Table 1. Cell-free DNA as biomarker of hepatocellular carcinoma**

Target	Serum/ plasma	Technology	Experimental setting	Clinical setting	Cohort comparison	Ref.
DNA abundance	Plasma	Fluorimeter	cfDNA levels	Diagnosis	HCC vs. HBV-related LF	[15]
DNA abundance	Serum	Branched DNA	cfDNA levels	Diagnosis	HCC vs. HL	[114]
DNA abundance	Plasma	Spectrophotometer	cfDNA levels	Prediction of radiotherapy response	HCC	[19]
DNA abundance	Plasma	Fluorimeter	cfDNA levels	Prognosis	HCC	[115]
<i>GSTP1</i>	Serum	Real-time PCR	cfDNA levels	Diagnosis	HCC vs. HCV carriers	[16]
<i>GSTP1</i>	Serum	Real-time PCR	cfDNA levels	Prognosis	HCC	[17]
<i>hTERT</i>	Plasma	Real-time PCR	cfDNA levels	Diagnosis	HCC vs. CHC + LC	[18]
<i>hTERT</i>	Plasma	Real-time PCR	cfDNA levels	Prognosis	HCC	[18]
<i>APC, FHIT, p15, p16, E-cadherin</i>	Plasma	MSP	Methylation	Diagnosis	HCC <sup>1</sup>	[116]
<i>APC, GSTP1, RASSF1A, SFRP1</i>	Plasma	MSRE-qPCR	Methylation	Diagnosis	HCC vs. HL	[21]
<i>APC, GSTP1, RASSF1A, SFRP1</i>	Plasma	MSRE-qPCR	Methylation	Diagnosis	HCC vs. BLD	[21]
<i>APC, GSTP1, RASSF1A, SFRP1</i>	Plasma	MSRE-qPCR	Methylation	Prognosis	HCC	[21]
Gene panel (12 genes)	Plasma	NGS	Methylation	Diagnosis	HCC vs. CLD + HL	[25]
<i>p15, p16</i>	Plasma	MSP	Methylation	Diagnosis/ prognosis	HCC vs. CLD + HL	[23]
<i>SEPT9</i>	Plasma	MSP	Methylation	Diagnosis	HCC vs. LC	[117]
<i>SOC3</i>	Plasma	MSP	Methylation	Diagnosis/ prognosis	HCC vs. LC, BLD + HL	[24]
<i>UBE2Q1</i>	Serum	MSP	Methylation	Diagnosis	HCC vs. LC + CHB	[118]
Gene panel	Plasma	NGS	Mutations	Guiding therapy choice	HCC	[31]
Gene panel (574 genes)	Plasma	NGS	Mutations	Tumor heterogeneity and prognosis	HCC <sup>1</sup>	[32]
Gene panel (58 genes)	Plasma/serum	NGS	Mutations	Patient monitoring	HCC <sup>1</sup>	[27]
<i>KRAS, NRAS</i>	Plasma	BEAMing	Mutations	Guiding therapy choice	HCC	[31]
<i>TERT</i>	Plasma	ddPCR	Mutations	Diagnosis	HCC vs. LC	[119]
<i>TERT</i>	Plasma	ddPCR	Mutations	Prognosis	HCC	[119]
<i>TERT, CTNNB1, TP53</i>	Plasma	NGS	Mutations	Prognosis	HCC <sup>1</sup>	[120]
<i>TP53</i>	Plasma	ddPCR	Mutations	Diagnosis	HCC vs. CLD + HL	[26]
<i>TP53</i>	Plasma	COLD-PCR	Mutations	Patient monitoring	HCC	[121]
<i>TP53, CTNNB1, TERT</i>	Plasma	ddPCR	Mutations	Diagnosis	HCC <sup>1</sup>	[28]
Genome-wide	Plasma	NGS	CNV	Diagnosis	HCC vs. CH + LC	[122]
Genome-wide	Plasma	NGS	SNV, CNV	Diagnosis	HCC vs. HL	[123]
Genome-wide	Plasma	NGS	SNV, CNV	Patient monitoring	HCC	[123]
Gene panel (54-70 genes)	Plasma	NGS	SNV, CNV, fusions	Guiding therapy choice	HCC	[29]

<sup>1</sup>Tumor DNA vs. matched cfDNA. *GSTP1*: glutathione S-transferase p1; *hTERT*: telomerase reverse transcriptase; *APC*: adenomatous polyposis coli; *FHIT*: fragile histidine triad; *p15*: cyclin dependent kinase inhibitor 2B; *p16*: cyclin dependent kinase inhibitor 2A; *RASSF1A*: ras association domain family member 1; *SFRP1*: secreted frizzled related protein 1; *SEPT9*: septin 9; *SOC3*: suppressor of cytokine signaling 3; *UBE2Q1*: ubiquitin conjugating enzyme E2 Q1; *KRAS*: KRAS proto-oncogene; *NRAS*: NRAS proto-oncogene; *CTNNB1*: beta catenin 1; *TP53*: tumor protein p53; *MSP*: methylation-specific PCR; *MSRE-qPCR*: methylation-sensitive restriction enzymes qPCR; *NGS*: next generation sequencing; *BEAMing*: beads, emulsion, amplification, and magnetics PCR; *ddPCR*: droplet digital PCR; *CNV*: copy number variations; *SNV*: single nucleotide variations; *cfDNA*: cell-free DNA; *BLD*: benign liver disease; *CH*: chronic hepatitis; *LF*: liver fibrosis; *CHB*: chronic hepatitis B; *CHC*: chronic hepatitis C; *CLD*: chronic liver disease; *HBV*: hepatitis B virus; *HCC*: hepatocellular carcinoma; *HL*: healthy liver; *LC*: liver cirrhosis; *HCV*: hepatitis C virus

displayed 87.0% sensitivity and 100% specificity as diagnostic performance<sup>[15]</sup>. As a surrogate of abundance of circulating DNA, some studies evaluated the amount of cfDNA by quantifying specific circulating gene fragments. Iizuka *et al.*<sup>[16]</sup> found a significant increase in serum levels of the *GSTP1* gene in HCC patients and found correlations with tumor grade and size. An increased *GSTP1* gene in cfDNA was also associated with a shorter overall survival (OS) and metastasis occurrence<sup>[17]</sup>. Similarly, higher than normal levels of *hTERT* gene in plasma of HCC patients correlated with presence of advanced disease and shorter survival<sup>[18]</sup>. Quantification of cfDNA revealed its potential usefulness also for assessing therapy response. Reduction of plasma cfDNA after radiotherapy correlated with a better tumor response<sup>[19]</sup>.



**Figure 1.** Liquid biopsies and their clinical applications. A: Liquid biopsy is an approach for detecting and analyzing DNA and RNA in biological fluids, such as serum, plasma, urine and saliva. Being a minimally invasive procedure, it offers the possibility of performing repeated sampling over time, thus providing a practical method for patient surveillance. Blood plasma or serum from patients contain cancer derived material, such as CTCs, ctDNA, miRNAs and other RNAs; B: analysis of such DNA/RNA content can provide evidences on the presence of HCC at an early stage, assessment of prognosis and patients' monitoring during and after therapy, thus helping the clinical management of patients at different phases of disease. CTC: circulating tumor cell; ctDNA: cell-free tumor DNA; ncRNA: non-coding RNA

Quantification of circulating DNA is very easy to perform and inexpensive. However, this approach lacks specificity for type of cancer; in addition, levels of cfDNA can also increase in inflammatory conditions unrelated to cancer or in some physiological conditions, such as pregnancy. Furthermore, this analysis does not provide information about tumor genetic landscape and cannot reveal actionable targets. For these reasons, most investigations moved toward the detection of more specific genetic or epigenetic alterations in blood, as biomarkers for HCC.

#### Aberrant methylation of cfDNA in plasma or serum in HCC

Promoter methylation is a well-known mechanism for gene transcriptional repression. Aberrant methylation in promoters of cancer genes represents a tumor-specific event and its detection is potentially useful for the prediction or diagnosis of HCC. Concordance between aberrant methylations in tumor tissues and plasma is generally good, indicating that plasma could represent a tumor surrogate when tissue is not available. The field has been widely investigated and a meta-analysis of these studies has been published<sup>[20]</sup>. In the diagnostic setting, from the analysis of 150 plasma samples from patients with HCC, benign liver disease (including cirrhosis and chronic inactive hepatitis) and normal controls, Huang and co-workers found that the combined aberrant methylation of four genes (*APC*, *GSTP1*, *RASSF1A* and *SFRP1*) has a significant diagnostic value for HCC<sup>[21]</sup>, confirming the results obtained in tumor tissues<sup>[22]</sup>. In particular, the combination analysis of plasma methylation levels of these genes allowed to discriminate HCC from both benign or normal controls, with a sensitivity of 84.7% (in both cases) and a specificity of 81.1% and 87.8% respectively<sup>[21]</sup>.

For prognostic assessment, Wong *et al.*<sup>[23]</sup> evaluated the methylation status of p15 and p16 in tumor tissues, plasma, serum and buffy coat samples from HCC patients, non-HCC controls and healthy individuals and found promoter methylation in plasma or serum of 40%-50% HCC patients. Most of patients associated with gene methylation exhibited a poorer prognosis in comparison with patients negative for aberrant methylation<sup>[23]</sup>. The detection of methylation of *APC* and *RASSF1A* promoters was also associated with shorter OS in HCC patients and *RASSF1A* methylation was demonstrated to be an independent prognostic factor<sup>[21]</sup>. Very recently, another gene whose aberrant methylation detected in plasma was associated with patients' poorer prognosis was *SOCS3*<sup>[24]</sup>.

The analysis of panels of aberrantly methylated genes through the use of next generation sequencing (NGS) is expected to further improve sensitivity and specificity of aberrant methylation biomarkers. For example, targeted deep-sequencing of plasma DNA after bisulfite treatment could be used to simultaneously assess the methylation status of several targets. While Holmila *et al.*<sup>[25]</sup> identified two genes (*VIM* and *FBLN1*) whose promoters were differentially methylated in HCC using this approach, these areas of study have not been thoroughly investigated.

Compared to cfDNA abundance in plasma or serum, the detection of aberrant DNA methylations in cfDNA provides a more specific tumor biomarker, especially if a combination of multiple genes is employed. However, it still contains a limitation. Considering that aberrant methylation generally affects tumor suppressor genes, the analysis cannot reveal alterations in oncogenes potentially targets of specific therapies. With the development of more sophisticated approaches, the identification of tumor-specific genetic alterations has become feasible and has been applied to HCC.

### Cancer gene mutations in plasma or serum cfDNA

An analysis of cancer gene mutations in serum or plasma of HCC patients was investigated in the diagnostic, prognostic and predictive settings.

In the diagnostic setting, R249S mutation of the *TP53* gene is hallmark of aflatoxin B1 exposure, one of the major causes of HCC in certain geographic areas. Using droplet digital PCR the authors identified a higher prevalence of this mutation in plasma cfDNAs of HCC in Cameroonian and Central African patients in comparison with control subjects with and without liver disease (almost 25% of patients with HCC and 3%-9% of non-HCC subjects were R249S carriers), suggesting a potential use of this biomarker as an early risk factor for HCC in individuals exposed to aflatoxin B1<sup>[26]</sup>. Targeted deep sequencing was used to investigate several cancer genes involved in HCC. For example, the ultra-deep sequencing analysis of 58 cancer genes performed in 8 HCC tissues and paired plasma/serum samples revealed that 15 of the 21 somatic tumor mutations (71%) could also be detected in plasma/serum cfDNA<sup>[27]</sup>, thus indicating the translational potential of this approach for HCC diagnosis. In another recent study, cancer alterations in four hot-spot regions of *TP53*, *CTNNB1* and *TERT* genes were investigated in plasma cfDNA and corresponding tumor DNA from 48 HCC patients. Interestingly, the authors found that many gene alterations found in plasma DNA were different from those found in tumor tissues, an evidence of tumor heterogeneity<sup>[28]</sup>. Confirming tumor heterogeneity in HCC, a recent study analysed plasma cfDNA from 26 HCC patients for the presence of mutations in a large set of genes. Authors found tumor heterogeneity and evolution over time by tracking circulating mutation pattern in a patient who developed progression after capecitabine treatment<sup>[29]</sup>.

The identification of gene mutations offers the possibility of identifying potential actionable alterations, useful for guiding treatment choice. It has been demonstrated that HCC harbouring mutant *RAS* exhibited a better clinical response to refametinib plus sorafenib, compared to wild-type *RAS* tumors<sup>[30]</sup>. Notably, in the course of the study aimed at detecting *KRAS* or *NRAS* mutations in plasma cfDNA of a large cohort of HCC patients, authors found other actionable mutations in *EGFR*, *JAK2*, *BRAF*, *FLT3*, *PIK3CA*, and *cKIT*, suggesting that available target therapies could potentially be effective in defined, albeit small, subsets of



HCC patients<sup>[31]</sup>. These results highlight the usefulness of cfDNA analysis for identifying actionable targets and for stratifying patients according to the potentially most appropriate therapeutic approach.

The approach has also been employed to monitor therapy efficacy over time. The mutation analysis of 574 cancer genes applied to plasma cfDNA and matched HCC from four patients, demonstrated that 97% of tumor alterations were present in the blood and that it was possible to assess tumor progression, to track the possible sites of recurrence and understand tumor clonal dynamics in relation to sequential therapies. The possibility to track tumor dynamics from plasma analysis provides a valuable strategy for monitoring therapy efficacy and infer clinical outcomes<sup>[32]</sup>, helping clinician modulate therapeutic approaches in a more rational and proper direction.

## MIRNAS

miRNAs are 20-24 nucleotides long RNAs. By interacting with homologous target mRNAs, they act by fine-tuning gene expression through a post-transcriptional mechanism. Each tissue exhibits a unique profile of miRNAs, which is altered in pathological conditions. In tumor tissues, miRNAs are aberrantly regulated and it has been demonstrated that some deregulated miRNAs can act as oncogenes and others as tumor suppressors<sup>[33]</sup>. The interest in circulating miRNAs as non-invasive tumor biomarkers surfaced when their presence was reported as stable molecules in serum or plasma of healthy individuals and cancer patients<sup>[34,35]</sup>.

### Approaches for detection and quantification of circulating miRNAs

The most common technologies employed to measure miRNA expression in biological samples include microarray, NGS, quantitative real-time PCR (RT-qPCR) and droplet digital PCR (ddPCR)<sup>[36-38]</sup>. Microarray and NGS technologies are suitable for screening and discovery purposes, qPCR and ddPCR remain the choices for validation and clinical tests development. Both microarrays and NGS provide high throughput analysis of miRNA expression profiles. Microarrays can quantify all the known miRNAs. NGS can also identify new miRNA species and differentiate closely related sequences. NGS can also detect miRNA length variation (isoforms of miRNA)<sup>[38]</sup>. qPCR and ddPCR are not high throughput technologies, but technology is relatively inexpensive, available in most laboratories and can offer higher sensitivity by exploiting amplification steps.

Among quantitative PCR approaches, ddPCR was shown to be superior to conventional real time qPCR for quantifying circulating miRNAs, as it allowed an easier absolute quantification of circulating RNAs without requiring an internal standard for normalization. Furthermore, ddPCR proved to be more tolerant than real time qPCR to the presence of inhibitors<sup>[39]</sup>. Finally, ddPCR generally exhibits a higher precision and reproducibility than real time qPCR, thus allowing an easier discrimination between cases and controls<sup>[37,40,41]</sup>.

### Circulating miRNAs for HCC diagnosis

Circulating miRNAs have been tested for their ability of discriminating HCC patients from control individuals [Table 2]. As shown in Table 2, however, it is evident that published studies are heterogeneous as they often differ for technical characteristics and experimental design. This heterogeneity makes it difficult to compare results and limits their transferability into applications of clinical interest.

A first source of heterogeneity is given by the use of serum or plasma for measuring circulating miRNA levels. Albeit early studies reported that composition and levels of miRNAs in serum and plasma are similar<sup>[35]</sup>, there are several examples that subsequently contradicted this idea. Heegaard *et al.*<sup>[42]</sup> tested miRNA levels in paired serum and plasma samples of lung cancer patients and they concluded that these apparently similar sources of circulating miRNAs exhibit very different miRNA levels. Supporting this conclusion in liver cancer patients, miR-223-3p was found consistently low in plasma of HCC patients<sup>[43,44]</sup> but the same miRNA was high in the serum<sup>[45,46]</sup>; miR-21 was found low in serum of HCC patients in

**Table 2. Circulating miRNAs as diagnostic biomarkers of hepatocellular carcinoma**

miRNA	Expression	Body fluid	Experimental setting	Clinical setting	Cohorts comparison	Sample size	Sensitivity (%)	Specificity (%)	AUC	Ref.
miR-130b	Up	Serum	Single	Diagnosis	HCC vs. HL + CHB	57 vs. 30 + 29	87.7	81.4	0.91	[124]
miR-29a-3p	Up	Serum	Single	Diagnosis	HCC vs. HL + LC (T)	74 vs. 60 + 43	N/A	N/A	0.71	[125]
Let-7f, miR-16, miR-21	Down	Serum	Multiple	Diagnosis	HCC vs. HL	90 vs. 60	N/A	N/A	N/A	[47]
miR-101	Up	Serum	Single	Diagnosis	HCC vs. HL	25 vs. 20	N/A	N/A	N/A	[126]
miR-101	Down	Serum	Single	Diagnosis	HCC vs. HL	67 vs. 30	76.1	70.0	0.79	[53]
miR-101	Down	Serum	Single	Diagnosis	HCC vs. LC	67 vs. 61	95.5	90.2	0.98	[53]
miR-101	Down	Serum	Single	Diagnosis	HCC vs. HL + CH	52 vs. 43 + 42	54.9	76.9	0.62	[52]
miR-101	Down	Serum	Single + AFP	Diagnosis	HCC vs. HL + CH	52 vs. 43 + 42	N/A	N/A	0.85	[52]
miR-101, miR-106b, miR-122, miR-195	Down	Exosomes	Multiple	Diagnosis	HCC vs. CHB	20 vs. 20	N/A	N/A	N/A	[127]
miR-101-3p, miR-106b-3p		Serum	Multiple	Diagnosis	HCC vs. LC	24 vs. 14	84.6	94.1	0.96	[51]
miR-101-3p, miR-1246, miR-106b-3p	Up	Plasma	Multiple	Diagnosis	HCC vs. HL	22 vs. 11	100	100	1	[51]
miR-101-3p, miR-1246, miR-106b-3p	Up	Plasma	Multiple	Diagnosis	HCC vs. HL + LC	7 vs. 14 + 21	N/A	N/A	N/A	[51]
miR-101-3p, miR-1246, miR-106b-3p	Up	Plasma	Multiple	Diagnosis	HCC vs. LC	9 vs. 6	100	92.9	0.99	[51]
miR-122	Up	Serum	Single	Diagnosis	CH vs. HL	30 vs. 10	N/A	N/A	N/A	[56]
miR-122	Up	Serum	Single	Diagnosis	HCC vs. HL	30 vs. 10	N/A	N/A	N/A	[56]
miR-122	Down	Serum	Single	Diagnosis	LC vs. HL	30 vs. 10	N/A	N/A	N/A	[56]
miR-122	Up		Single	Diagnosis	HCC vs. HL + CHB	3423 vs. 1887 + 2403	68.0	73.3	0.77	[128]
miR-122	Up	Serum	Single	Diagnosis	HCC vs. CHB	71 vs. 45	77.6	57.8	0.63	[45]
miR-122	Up	Serum	Single	Diagnosis	HCC vs. HL	71 vs. 34	81.6	83.3	0.87	[45]
miR-122	Down	Serum	Single	Diagnosis	CH vs. HL	48 vs. 89	80.0	91.2	0.93	[46]
miR-122	Down	Serum	Single	Diagnosis	HCC vs. CHB	101 vs. 89	N/A	N/A	N/A	[46]
miR-122	Up	Serum	Single	Diagnosis	HCC vs. HL	101 vs. 89	70.7	69.1	0.79	[46]
miR-122, let-7b	Up	Serum	Multiple	Diagnosis	HCC vs. HBV-related DN	30 vs. 47	84.8	50.0	0.65	[129]
miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801		Plasma	Multiple	Diagnosis	HCC vs. HL (V)	196 vs. 66	83.2	93.9	0.94	[49]
miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-802		Plasma	Multiple	Diagnosis	HCC vs. CHB (V)	196 vs. 72	79.1	76.4	0.84	[49]
miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-803		Plasma	Multiple	Diagnosis	HCC vs. LC (V)	196 vs. 56	75.0	91.1	0.88	[49]
miR-122, miR-885-5p, miR-221, miR-181b		Serum	Multiple + AFP	Diagnosis	HCC vs. LC	192 vs. 96	N/A	N/A	0.98	[60]
miR-122, miR-885-5p, miR-221, miR-181b		Serum	Multiple	Diagnosis	HCC vs. LC	192 vs. 96	N/A	N/A	0.84	[60]
miR-122, miR-885-5p, miR-29b		Serum	Multiple + AFP	Diagnosis	HCC vs. HL	192 vs. 96	N/A	N/A	1	[60]
miR-122, miR-885-5p, miR-29b		Serum	Multiple	Diagnosis	HCC vs. HL	192 vs. 96	N/A	N/A	0.89	[60]
miR-122-5p	Up	Plasma	Single	Diagnosis	CHB vs. HL	20 vs. 24	N/A	N/A	N/A	[43]
miR-122-5p	Up	Plasma	Single	Diagnosis	HCC vs. HL	20 vs. 28	N/A	N/A	N/A	[43]

miR-122-5p	Up	Plasma	Single	Diagnosis	LC vs. HL	20 vs. 22	N/A	N/A	N/A	[43]
miR-122-5p	Up	Plasma	Single	Diagnosis	HCC vs. HL + LC	7 vs. 14 + 21	N/A	N/A	N/A	[51]
miR-122-5p	Up	Serum	Single	Diagnosis	HCC vs. LC	24 vs. 14	N/A	N/A	N/A	[51]
miR-122a	Down	Serum	Single	Diagnosis	HCC vs. HL	85 vs. 85	70.6	67.1	0.71	[130]
miR-122a	Down	Serum	Single + AFP	Diagnosis	HCC vs. HL	85 vs. 85	87.1	98.8	0.94	[130]
miR-1247-3p	Up	Serum	Single	Diagnosis	HCC vs. HL	110 vs. 25	N/A	N/A	N/A	[131]
miR-125a-5p	Down	Serum	Single	Diagnosis	HCC vs. HL + CHB	120 vs. 164 + 91	N/A	N/A	N/A	[76]
miR-125b	Down	Plasma	Single	Diagnosis	HCC vs. CHB	64 vs. 63	93.8	85.7	0.96	[58]
miR-125b	Down	Plasma	Single	Diagnosis	HCC vs. HL	64 vs. 56	85.9	78.6	0.89	[58]
miR-125b	Down	Plasma	Single	Diagnosis	HCC vs. LC	64 vs. 59	89.1	88.1	0.96	[58]
miR-125b, miR-223, miR-27a, and miR-26a	Down	Serum	Multiple + AFP	Diagnosis	HCC vs. HL	90 vs. 60	N/A	N/A	0.87	[50]
miR-125b, miR-27a	Down	Serum	Multiple + AFP	Diagnosis	HCC vs. HL	90 vs. 60	80.0	87.2	N/A	[50]
miR-125b-5p	Up	Plasma	Single	Diagnosis	CHB vs. HL	20 vs. 24	N/A	N/A	N/A	[43]
miR-125b-5p	Up	Plasma	Single	Diagnosis	HCC vs. HL	20 vs. 28	N/A	N/A	N/A	[43]
miR-125b-5p	Up	Plasma	Single	Diagnosis	LC vs. HL	20 vs. 22	N/A	N/A	N/A	[43]
miR-143	Down	Serum	Single	Diagnosis	HCC vs. HL	131 vs. 122	80.3	82.4	0.83	[132]
miR-145	Down	Serum	Single	Diagnosis	HCC vs. CHB	85 vs. 50	88.2	78.0	0.85	[133]
miR-148a	Down	Serum	Single	Diagnosis	HCC vs. HL + BLD	76 vs. 55 + 62	67.7	59.2	0.67	[71]
miR-148a, miR-148b, miR-152	Down	Serum	Multiple	Diagnosis	HCC vs. BLD	76 vs. 62	96.1	91.9	0.94	[71]
miR-15b	Up	Serum	Single	Diagnosis	HCC vs. HL + CHB	57 vs. 30 + 29	98.3	15.3	0.49	[124]
miR-15b, miR-130b	Up	Serum	Multiple	Diagnosis	HCC vs. HL + CHB	57 vs. 30 + 29	98.3	91.5	0.98	[124]
miR-16	Down	Serum	Single + AFP	Diagnosis	HCC vs. HL + CLD	105 vs. 71 + 107	92.4	78.5	N/A	[134]
miR-16, miR-195, miR-199a		Serum	Multiple	Diagnosis	HCC vs. HL + CLD	105 vs. 71 + 107	N/A	N/A	N/A	[134]
miR-18, miR-221, miR-222, miR-224	Up	Exosomes	Single	Diagnosis	HCC vs. LC	20 vs. 20	N/A	N/A	N/A	[127]
miR-18, miR-221, miR-222, miR-224	Up	Exosomes	Single	Diagnosis	HCC vs. CHB + LC	20 vs. 20 + 20	N/A	N/A	N/A	[127]
miR-182	Up	Serum	Single + AFP	Diagnosis	HCC vs. HL + BLD	103 vs. 40 + 95	82.5	94.7		[135]
miR-182	Up	Serum	Single	Diagnosis	HCC vs. HL + BLD	103 vs. 40 + 95	78.6	91.6	0.91	[135]
miR-182, miR-331-3p		Serum	Multiple + AFP	Diagnosis	HCC vs. HL + BLD	103 vs. 40 + 95	93.2	95.8	N/A	[135]
miR-192-5p	Up	Serum	Single	Diagnosis	HCC vs. HL + LC (T)	74 vs. 60 + 43	N/A	N/A	0.69	[125]
miR-192-5p and miR-29a-3p	Up	Serum	Single	Diagnosis	HCC vs. HL (T)	50 vs. 50	N/A	N/A	N/A	[125]
miR-192-5p and miR-29a-3p	Up	Serum	Single	Diagnosis	HCC vs. HL (V)	100 vs. 70	N/A	N/A	N/A	[125]
miR-199a	Down	Serum	Single	Diagnosis	HCC vs. CH	23 vs. 17	54.5	100	0.85	[136]
miR-21	Up	Serum	Single	Diagnosis	HCC vs. CH	23 vs. 17	100	82.1	0.94	[136]
miR-21	Up	Serum	Single	Diagnosis	CHC vs. HL	62 vs. 19	87.1	73.7	0.83	[137]
miR-21	Up	Serum	Single	Diagnosis	HCC vs. HL	29 vs. 19	N/A	N/A	N/A	[137]
miR-21	Up	Serum	Single	Diagnosis	HCC vs. LC (V)	175 vs. 78	80.8	72.9	0.81	[59]
miR-21	Up	Serum	Single	Diagnosis	HCC vs. CHB (V)	175 vs. 64	76.9	85.7	0.79	[59]
miR-21	Up	Serum	Single	Diagnosis	HCC vs. HL (T)	40 vs. 40				[59]
miR-21	Up	Serum	Single	Diagnosis	HCC vs. HL + CHB + LC (V)	175 vs. 136 + 64 + 78	82.1	83.9	0.85	[59]
miR-21	Up		Single	Diagnosis	HCC vs. HL + CHB	3423 vs. 1887 + 2403	86.6	79.5	0.88	[128]
miR-21	Down	Serum	Single	Diagnosis	HCC vs. HL	70 vs. 34	N/A	N/A	N/A	[45]
miR-21	Up	Plasma	Single + AFP	Diagnosis	HCC vs. HL + CH	127 vs. 50 + 30	90.0	92.9	0.82	[48]
miR-21	Up	Plasma	Single	Diagnosis	HCC vs. HL + CH	126 vs. 50 + 30	61.1	83.3	0.77	[48]

miR-21	Up	Exosomes	Single	Diagnosis	HCC vs. HL + CHB	30 vs. 30 + 30	N/A	N/A	N/A	[78]
miR-21	Up	Serum	Single	Diagnosis	HCC vs. HL	97 vs. 30	N/A	N/A	N/A	[79]
miR-21	Down	Serum	Single	Diagnosis	CH vs. HL	48 vs. 89	80.0	95.6	0.91	[46]
miR-21	Down	Serum	Single	Diagnosis	HCC vs. CHB	101 vs. 89				[46]
miR-21	Up	Serum	Single	Diagnosis	HCC vs. HL	101 vs. 89	84.0	73.5	0.87	[46]
miR-21	Up	Serum	Single	Diagnosis	HCC vs. HL	56 vs. 43	67.4	55.8	0.62	[52]
miR-21, miR-122, miR-192	Up	Serum	Multiple + AFP	Diagnosis	HCC vs. CHB	118 vs. 100	N/A	N/A	0.94	[61]
miR-21, miR-122, miR-192	Up	Serum	Multiple	Diagnosis	HCC vs. CHB	118 vs. 100	N/A	N/A	0.90	[61]
miR-21, miR-122, miR-192	Up	Serum	Multiple + AFP	Diagnosis	HCC vs. CHB + LC	118 vs. 100 + 69	N/A	N/A	0.88	[61]
miR-21, miR-122, miR-192	Up	Serum	Multiple	Diagnosis	HCC vs. CHB + LC	118 vs. 100 + 69	N/A	N/A	0.81	[61]
miR-21, miR-122, miR-192	Up	Serum	Multiple	Diagnosis	HCC vs. HL + CHB + LC	118 vs. 119 + 100 + 69	N/A	N/A	0.85	[61]
miR-21, miR-122, miR-192	Up	Serum	Multiple + AFP	Diagnosis	HCC vs. LC	118 vs. 69	N/A	N/A	0.88	[61]
miR-21, miR-26a, miR-101		Serum	Multiple + AFP	Diagnosis	HCC vs. HL + CH	52 vs. 43 + 42	87.0	81.0	0.91	[52]
miR-218	Down	Serum	Single + AFP	Diagnosis	HCC vs. HL + BLD	156 vs. 64 + 98	N/A	N/A	0.91	[73]
miR-218	Down	Serum	Single	Diagnosis	HCC vs. HL + BLD	156 vs. 64 + 98	66.7	69.1	0.73	[73]
miR-22, miR-199a-3p		Serum	Multiple + AFP	Diagnosis	HCC vs. CHC	192 vs. 96	N/A	N/A	0.98	[60]
miR-22, miR-199a-3p		Serum	Multiple	Diagnosis	HCC vs. CHC	192 vs. 96	N/A	N/A	0.66	[60]
miR-221	Up	Serum	Single	Diagnosis	CH vs. HL	30 vs. 10	N/A	N/A	N/A	[56]
miR-221	Down	Serum	Single	Diagnosis	HCC vs. HL	30 vs. 10	N/A	N/A	N/A	[56]
miR-221	Up	Serum	Single	Diagnosis	LC vs. HL	30 vs. 10	N/A	N/A	N/A	[56]
miR-221	Up	Serum	Single	Diagnosis	HCC vs. HL	46 vs. 50	N/A	N/A	N/A	[72]
miR-221	Up	Serum	Single	Diagnosis	HCC vs. HL	45 vs. 45	93.3	77.8	0.94	[138]
miR-221	Up	Serum	Single + AFP	Diagnosis	HCC vs. HL	45 vs. 45	96.5	88.0		[138]
miR-223	Down	Serum	Single	Diagnosis	HCC vs. HL + CLD	39 vs. 14 + 17	97.2	94.1	0.99	[139]
miR-223	Up	Serum	Single	Diagnosis	HCC vs. HL	73 vs. 34	N/A	N/A	N/A	[45]
miR-223	Up	Serum	Single	Diagnosis	CH vs. HL	48 vs. 89	80.0	75.0	0.88	[46]
miR-223	Up	Serum	Single	Diagnosis	HCC vs. HL	101 vs. 89	80.0	76.5	0.86	[46]
miR-223-3p	Down	Plasma	Single	Diagnosis	CHB vs. HL	20 vs. 24	N/A	N/A	N/A	[43]
miR-223-3p	Down	Plasma	Single	Diagnosis	HCC vs. HL	20 vs. 28	N/A	N/A	N/A	[43]
miR-223-3p	Down	Plasma	Single	Diagnosis	LC vs. HL	20 vs. 22	N/A	N/A	N/A	[43]
miR-223-3p	Down	Plasma	Single	Diagnosis	HCC vs. HL	8 vs. 28	N/A	N/A	N/A	[44]
miR-223-3p	Down	Plasma	Single	Diagnosis	LC vs. HL	30 vs. 28	N/A	N/A	N/A	[44]
miR-224	Up	Plasma	Single	Diagnosis	HCC vs. HL	20 vs. 20	N/A	N/A	0.91	[140]
miR-224	Up	Plasma	Single	Diagnosis	HCC vs. HL	87 vs. 55	93.1	80.0	0.91	[140]
miR-224	Up	Plasma	Single	Diagnosis	HCC vs. HL	33 vs. 22	87.7	86.3	0.91	[140]
miR-224	Up	Plasma	Single	Diagnosis	HCC vs. HL	54 vs. 33	87.7	86.3	0.91	[140]
miR-24-3p	Up	Serum	Single	Diagnosis	HCC vs. HL + CLD	84 vs. 46 + 31	N/A	N/A	0.63	[67]
miR-24-3p	Up	Serum	Single + AFP	Diagnosis	HCC vs. HL + CLD	84 vs. 46 + 31	N/A	N/A	0.83	[67]
miR-26a	Down	Serum	Single	Diagnosis	HCC vs. HL + CH	52 vs. 43 + 42	75.0	70.0	0.76	[52]
miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505		Serum	Multiple	Diagnosis	HCC vs. CHB + LC (T)	108 vs. 51 + 47	80.6	82.7	0.81	[141]
miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505		Serum	Multiple	Diagnosis	HCC vs. CHB + LC (V)	153 vs. 68 + 71	74.5	89.9	0.82	[141]
miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505		Serum	Multiple	Diagnosis	HCC vs. HL + CHB + LC (T)	108 vs. 51 + 51 + 47	80.6	85.5	0.82	[141]
miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505		Serum	Multiple	Diagnosis	HCC vs. HL + CHB + LC (V)	153 vs. 60 + 68 + 71	74.5	88.9	0.81	[141]

miR-30e	Down	Serum	Single	Diagnosis	HCC vs. HL + CLD	39 vs. 14 + 17	91.7	70.5	0.93	[139]
miR-331-3p	Up	Serum	Single	Diagnosis	HCC vs. HL + BLD	103 vs. 40 + 95	79.6	92.6	0.89	[135]
miR-331-3p	Up	Serum	Single + AFP	Diagnosis	HCC vs. HL + BLD	103 vs. 40 + 95	91.2	92.6	N/A	[135]
miR-335	Down	Serum	Single	Diagnosis	HCC vs. HL + CH	125 vs. 125 + 125	N/A	N/A	N/A	[86]
miR-519d	Up	Exosomes	Single	Diagnosis	HCC vs. LC	87 vs. 31	N/A	N/A	0.82	[57]
miR-595	Up	Exosomes	Single	Diagnosis	HCC vs. LC	87 vs. 31	N/A	N/A	0.92	[57]
miR-638	Down	Exosomes	Single	Diagnosis	HCC vs. HL	126 vs. 21	N/A	N/A	N/A	[75]
miR-665	Up	Exosomes	Single	Diagnosis	HCC vs. HL	30 vs. 10	N/A	N/A	N/A	[74]
miR-885-5p	Up	Serum	Single	Diagnosis	HCC + CHB + LC vs. HL	46 + 23 + 26 vs. 24	90.5	79.2	0.94	[142]
miR-939	Up	Exosomes	Single	Diagnosis	HCC vs. LC	87 vs. 31	N/A	N/A	0.84	[57]
miR-96	Up	Serum	Single	Diagnosis	HCC vs. CHB	104 vs. 100	77.9	75.3	0.83	[143]
miR-96	Up	Serum	Single + AFP	Diagnosis	HCC vs. CHB	104 vs. 100	83.6	82.4	0.88	[143]
miR-96	Up	Serum	Single	Diagnosis	HCC vs. CHB	104 vs. 100	77.9	75.3	0.80	[143]
miR-96	Up	Serum	Single	Diagnosis	HCC vs. HL	104 vs. 120	N/A	N/A	N/A	[143]
miR-96	Up	Serum	Single	Diagnosis	HCC vs. LC	104 vs. 90	N/A	N/A	N/A	[143]

AUC: area under the receiver-operating characteristic curve; AFP: alpha fetoprotein; BLD: benign liver disease; CH: chronic hepatitis; CHB: chronic hepatitis B; CHC: chronic hepatitis C; CLD: chronic liver disease; DN: dysplastic nodules; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HL: healthy liver; LC: liver cirrhosis; (T): training set; (V): validation set; N/A: not available data

two reports<sup>[45,47]</sup>, but others reported miR-21 high in plasma of HCC patients<sup>[48,49]</sup>. Analysis of miR-125-b and miR-101 levels also displayed variances between serum and plasma: an upregulation of plasma miR-125-b levels was reported in HCC patients in comparison with healthy controls<sup>[43]</sup>, while downregulation of the same miRNA was found in serum<sup>[50]</sup>; miR-101 levels were found high in plasma of HCC patients in comparison with healthy controls<sup>[51]</sup>, whereas this miRNA was found downregulated in serum of HCC patients in comparison with healthy controls at least by two reports<sup>[52,53]</sup>. These several examples support the concept that differences between plasma and serum are common and should be taken in consideration when comparing results from different studies.

The use of plasma or serum is not the only source of variability of achieved results. An analysis of published studies strongly suggests that both pre-analytical and analytical procedures can affect results. Any change in tissue collection steps (like type of blood tubes, centrifugation strength and sample conservation) can generate differences in miRNA levels<sup>[54,55]</sup>. Considering the different hard-to-control sources of variability, it is not difficult to understand how uneven and sometimes even opposite results can easily derive.

In addition to technical reasons, aspects linked to experimental design can also be added to factors responsible for heterogeneity of results. The existence of various types of control populations represents indeed a source of variability and, in some cases, a limitation to the practical value of such results. In fact, for identifying useful biomarkers for the early detection of HCC it is very important to compare HCC not only with healthy controls but vs. cirrhotic patients, considering that 80%-90% of HCCs arise in this group of high-risk patients. A paradigmatic example is miR-122, a liver-specific miRNA whose level was found increased in serum/plasma of HCC patients in comparison with healthy patients<sup>[45,51]</sup>, but studies found no significant differences when HCC patients were compared to cirrhotic or chronic hepatitis patients<sup>[51,56]</sup>. These findings indicate that increased circulating miR-122 levels likely reflect liver damage rather than the presence of an underlying HCC, indicating the importance of controls to draw conclusions. Among studies that produced results on differential circulating miRNAs between HCC vs. cirrhotic patients, Fornari *et al.*<sup>[57]</sup> found that serum miR-939, miR-595 and miR-494 could separate cirrhotic patients with and without HCC, performing better than AFP. Moshiri *et al.*<sup>[51]</sup> showed that the combination of three plasma miRNAs, miR-101-3p, miR-106b-3p and miR-1246, exhibited a high diagnostic accuracy in discriminating HCC from cirrhotic patients. Combination of two of the same miRNAs, miR-101-3p and miR-106b-3p, exhibited also an excellent diagnostic accuracy in serum of HCC vs. cirrhotic patients. Chen *et al.*<sup>[58]</sup> proved that plasma miR-



**Table 3. Circulating miRNAs as prognostic biomarkers of hepatocellular carcinoma**

miRNA	Expression <sup>1</sup>	Body fluid	Experimental setting	Clinical setting	Sample size	Kaplan-Meier analysis ( <i>P</i> value)	Ref.
miR-101	Down	Plasma	Single	Prognosis (DFS)	163	<i>P</i> < 0.001	[144]
miR-122	Down	Serum	Single	Prognosis (OS)	122	<i>P</i> < 0.01	[145]
miR-1247-3p	Up	Serum	Single	Prognosis (OS)	85	<i>P</i> < 0.05	[131]
miR-1247-3p	Up	Serum	Single	Prognosis (DFS)	85	<i>P</i> < 0.01	[131]
miR-125a-5p	Down	Serum	Single	Prognosis (OS)	120	<i>P</i> < 0.01	[76]
miR-125b	Down	Exosomes	Single	Prognosis (OS)	128	<i>P</i> < 0.01	[77]
miR-143	Down	Serum	Single	Prognosis (OS)	131	<i>P</i> < 0.05	[132]
miR-148a	Down	Serum	Single	Prognosis (OS)	76	<i>P</i> < 0.001	[71]
miR-152	Down	Serum	Single	Prognosis (OS)	76	<i>P</i> < 0.05	[71]
miR-192-5p	Up	Serum	Single	Prognosis (OS)	74	<i>P</i> < 0.01	[125]
miR-192-5p	Up	Serum	Single	Prognosis (PFS)	74	<i>P</i> < 0.01	[125]
miR-29a-3p	Up	Serum	Single	Prognosis (OS)	74	<i>P</i> < 0.01	[125]
miR-29a-3p	Up	Serum	Single	Prognosis (PFS)	74	<i>P</i> < 0.05	[125]
miR-21, lncRNA-ATB	Up	Exosomes	Single + lncRNA	Prognosis (OS)	79	<i>P</i> < 0.05	[80]
miR-21, lncRNA-ATB	Up	Exosomes	Single + lncRNA	Prognosis (PFS)	79	<i>P</i> < 0.05	[80]
miR-218	Down	Serum	Single	Prognosis (OS)	156	<i>P</i> < 0.05	[73]
miR-221	Up	Serum	Single	Prognosis (OS)	46	<i>P</i> < 0.05	[72]
miR-224	Down	Serum	Single	Prognosis (OS)	182	<i>P</i> < 0.05	[146]
miR-24-3p	Up	Serum	Single	Prognosis (OS)	84	<i>P</i> < 0.01	[67]
miR-24-3p	Up	Serum	Single	Prognosis (DFS)	84	<i>P</i> < 0.01	[67]
miR-638	Down	Exosomes	Single	Prognosis (OS)	126	<i>P</i> < 0.01	[75]
miR-96	Up	Serum	Single	Prognosis (OS)	49	<i>P</i> < 0.05	[143]

<sup>1</sup>Expression in the group with the poorest prognosis. lncRNA: long noncoding RNA; OS: overall survival; PFS: progression free survival; DFS: disease-free survival

125b could differentiate HCC from cirrhotic patients, and Guo *et al.*<sup>[59]</sup> found that serum miR-21 analysis had also a good diagnostic efficacy in discriminating HCC patients both from non-HCC populations or from cirrhotic patients. Analyses of miRNA panels have been used for discriminating cirrhotic patients with or without HCC. For example, two different miRNA panels have been employed to efficiently distinguish HCC patients from cirrhotic patients, one in serum (miR-122, miR-885-5p, miR-221, miR-181b<sup>[60]</sup>) and one in plasma (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-803<sup>[49]</sup>). More recently, Tat Trung *et al.*<sup>[61]</sup> showed that a miRNA panel including miR-21, miR-122 and miR-192 had a good diagnostic performance in discriminating HCC patients from other groups, in particular from cirrhotic and chronic hepatitis patients.

Another potential limitation of several published studies is the fact that they were based on the assumption that cell-free miRNA levels were altered as a consequence of their release by neoplastic cells, and tried to validate as circulating cancer biomarkers the same miRNAs deregulated in tumor tissues. This assumption may not be correct<sup>[62,63]</sup>. In fact, if we consider that ctDNA represents a small or very small fraction of cfDNA (approximately 0.1%-1% or less for non-metastatic tumors and 1%-10% for large metastatic tumors), it is difficult to conceive that cancer cells could instead release such a high amount of RNA to significantly change content and levels of specific circulating RNAs. While this consideration raises doubts about the source of circulating RNAs, a number of studies (see review by McAllister and Weinberg<sup>[64]</sup>) offer a possible explanation by indicating that cancer should be considered a systemic disease. In this view, tumor-associated systemic processes may unbalance the release of miRNAs from non-tumor cells and therefore change circulating RNA profiles. Thus, finding altered circulating miRNA profiles would represent evidence of a systemic pathophysiological process closely linked to the presence of a tumor, and such RNAs would then represent actual circulating tumor biomarkers, although largely not directly released by tumor cells. In this viewpoint, the numerous evidences that the altered levels of miRNAs in circulation do not necessarily reflect the miRNA deregulation found in cancer tissues would become plausible and results should be re-

**Table 4. Circulating miRNAs as predictive biomarkers of therapy response in hepatocellular carcinoma**

miRNA	Expression in circulation <sup>1</sup>	Body fluid	Experimental Setting	Clinical setting	Treatment	Sampling (pre/post therapy)	Ref.
miR-1246	Up	Plasma	Single	Monitoring	LT	Pre/post	[82]
miR-182	Up	Serum	Single	Monitoring	TACE	Pre/post	[135]
miR-331-3p	Up	Serum	Single	Monitoring	TACE	Pre/post	[135]
miR-122	Up	Plasma	Single	Response prediction	TACE	Pre	[84]
miR-122	Up	Plasma	Single	Response prediction	RFA	Pre	[147]
miR-181a-5p	Down	Serum	Single	Response prediction	Sorafenib	Pre	[89]
miR-200	Up	Serum	Single	Response prediction	TACE	Pre	[87]
miR-21	Up	Serum	Single	Response prediction	Resection	Pre	[79]
miR-21, miR-26a, miR-29a-3p		Plasma	Multiple	Response prediction	TACE	Pre	[148]
miR-221	Up	Serum	Single	Response prediction	Sorafenib	Pre	[88]
miR-26a	Down	Plasma	Single	Response prediction	Resection or RFA	Pre	[149]
miR-29a	Down	Plasma	Single	Response prediction	Resection or RFA	Pre	[149]
miR-339-5p	Down	Serum	Single	Response prediction	Sorafenib	Pre	[89]
miR-34a	Down	Serum	Single	Response prediction	Resection	Pre	[150]
miR-665	Up	Serum exosomes	Single	Response prediction	Resection	Pre	[74]
miR-718	Down	Serum exosomes	Single	Response prediction	LT	Pre	[151]
miR-1246	Up	Plasma	Single	Responsive vs. non responsive	LT	Post	[82]
miR-148, miR-1246	Up	Plasma	Multiple	Responsive vs. non responsive	LT	Post	[82]
miR-148a	Up	Plasma	Single	Responsive vs. non responsive	LT	Post	[82]
miR-148a, miR-148b, miR-152	Down	Serum	Multiple	Responsive vs. non responsive	Resection	Post	[71]
miR-182	Up	Serum	Single	Responsive vs. non responsive	TACE	Post	[135]
miR-221	Down	Serum	Single	Responsive vs. non responsive	Sorafenib	Post	[88]
miR-331-3p	Up	Serum	Single	Responsive vs. non responsive	TACE	Post	[135]
miR-335	Down	Serum	Single	Responsive vs. non responsive	TACE	Post	[86]
miR-423-5p	Down	Serum	Single	Responsive vs. non responsive	Sorafenib	Post	[152]
miR-122	Down	Exosomal	Single	Responsive vs. non responsive	TACE	Pre/Post	[85]

<sup>1</sup>Circulating miRNA levels in non-responsive patients. LT: liver transplantation; RFA: radiofrequency ablation; TACE: transcatheter arterial chemoembolisation

interpreted in accordance. In this context, an example is miR-101-3p, which was found downregulated in HCC tissues<sup>[65]</sup> but upregulated in plasma<sup>[51]</sup>. Other examples include miR-21, upregulated in HCC tissues<sup>[66]</sup> but in some cases they are reported to be downregulated in patients serum<sup>[45,47]</sup> or the previously mentioned miR-122, whose altered circulating level is predominantly a sign of hepatic injury<sup>[51,56]</sup>.

The combination of biomarkers can potentially overcome the individual limitations. For this reason, miRNAs have been tested in association with AFP for improving test performance. Some studies indicated that the combination of miR-21 and AFP improved the discrimination between HCC patients vs. chronic hepatitis patients<sup>[48]</sup>. Meng *et al.*<sup>[67]</sup> showed that the combination of miR-24-3p and AFP allowed to better separate HCC from chronic liver disease affected patients and also a combination of miRNA panels with AFP provided a very good discriminating power between HCC vs. cirrhotic or chronic hepatitis patients<sup>[52,60,61]</sup>.

### Circulating miRNAs for HCC prognosis

Differences in median level of plasma/serum miRNAs as a cut-off value provided information about tumor stage and prognosis in HCC patients [Table 3]. Some studies have shown correlations of miRNA levels with pathological characteristics associated with prognosis. Members of the miR-148/152 family (miR-148a, miR-148b and miR-152) are important modulators of cell growth and progression of HCC<sup>[68-70]</sup>. Wang *et al.*<sup>[71]</sup> showed that low levels of miR-148a and miR-148b were significantly associated with tumor size and TNM stage, whereas low levels of miR-152 correlated with TNM stage. Additionally, the combination of circulating

miR-148/152 family could discriminate HCC from non-malignant chronic liver diseases<sup>[71]</sup>. Other studies associated circulating miRNA levels with HCC patients' prognoses. For example, Li *et al.*<sup>[72]</sup> showed that high serum levels of miR-221 correlated with tumor size, cirrhosis, tumor stage and with a lower OS in comparison with patient with low miR-221 expression levels, suggesting serum miR-221 as an independent risk factor for poor prognosis. A study by Yang *et al.*<sup>[73]</sup> reported that low serum levels of miR-218 were associated with clinic-pathological features such as tumor size, vascular invasion and TNM stage, as well as OS of patients. All these reports supported the potential role of circulating miRNAs in the assessment of prognosis in HCC. Studies described also the correlation between exosomal serum miRNA levels with pathological features and survival in HCC patients. For example, HCC patients with low levels of miR-665 showed a strong association with large tumour size (> 5 cm), local tumour invasion and metastases<sup>[74]</sup>; Shi *et al.*<sup>[75]</sup> showed that decreased levels of exosomal miR-638 had poor OS. Zheng *et al.*<sup>[76]</sup> reported that low levels of serum miR-125a-5p were associated with a lower OS compared with those exhibiting higher expression levels, and more recently Liu *et al.*<sup>[77]</sup> confirmed by a Kaplan-Meier analysis that HCC patients with lower serum miR-125b levels showed reduced time to recurrence and OS. Many studies involved the analysis of circulating miR-21. In one study, high levels of serum miR-21 were found correlated with cirrhosis and tumor stage<sup>[78]</sup>, another revealed an association with metastasis<sup>[79]</sup>; Lee *et al.*<sup>[80]</sup> reported that exosomal miR-21 was an independent predictor of disease progression in HCC patients and high circulating levels of exosomal miRNA-21 were associated with lower OS and progression-free survival. These data support the role of miRNAs as potential prognostic biomarker in HCC.

### Circulating miRNAs for prediction of HCC recurrence and treatment response

Many studies focused their attention on miRNAs ability to predict treatment response and monitor disease relapse after surgery or drug therapy [Table 4].

Surgery is the treatment of choice for early HCC, however relapse is common<sup>[81]</sup>. Levels of circulating miRNAs were studied in patients who underwent surgical resection, revealing a correlation with the post-operative survival. For example, subjects with low serum miR-21 levels had a 29% 5-year survival rate, whereas those with high expression had a 14.3% 5-year survival rate<sup>[79]</sup>. Wang *et al.*<sup>[71]</sup> showed that levels of serum miR-148/152 family decreased in case of relapse after surgery. Ng *et al.*<sup>[82]</sup> showed that miR-1246 was an independent predictor of OS and disease-free survival of HCC patients after liver transplant.

In patients at intermediate HCC stage, recommended first-line therapy is trans-arterial chemoembolization (TACE)<sup>[83]</sup>, while sorafenib is the standard first line-systemic therapy for patients with advanced tumors (BCLC C)<sup>[81]</sup>. The association between circulating levels of miR-122 and treatment outcome after TACE was evaluated in two recent studies. Kim *et al.*<sup>[84]</sup> found that high plasma miR-122 expression levels could be predictive for early and overall TACE insufficient responses and refractoriness in HCC patients. Suehiro *et al.*<sup>[85]</sup> found that exosomal miR-122 expression levels were significantly decreased after TACE, especially in patients with cirrhosis, and suggest that the reduction in exosomal miR-122 levels may reflect a decrease in the liver function, rather than the anti-tumor effects of the procedure. Other miRNAs were studied in HCC patients treated with TACE. For example, lower serum miR-335 levels were associated with a shorter OS<sup>[86]</sup>, while lower expression of miR-200 in HCC patients predicted a better prognosis in HCC patients treated with TACE<sup>[87]</sup>. Considering the data reported, circulating miRNAs could be associated with clinical outcome of HCC patients treated with TACE.

There are no biomarkers to predict response to sorafenib. A small number of studies evaluated whether circulating miRNAs could predict or anticipate therapy responsiveness. From the analysis of miR-221 levels in sera from HCC patients who received sorafenib, Fornari *et al.*<sup>[88]</sup> found that the treatment determined an increase of miR-221 only in responders. Moreover, analyzing miR-221 levels in sera from HCC patients before sorafenib treatment, lower miR-221-circulating levels were associated with better response to the drug<sup>[88]</sup>. Analysing serum miRNA profiles during sorafenib therapy, Nishida *et al.*<sup>[89]</sup> found that miR-181a-

**Table 5. Circulating long noncoding RNAs as biomarkers of hepatocellular carcinoma**

lncRNA	Expression	Body fluid	Experimental setting	Clinical setting	Cohorts comparison	Ref.
AFO85935	Up	Serum	Single	Diagnosis (early)	HCC vs. HL	[95]
AFO85935	Up	Serum	Single	Diagnosis (early)	HCC vs. HBV carriers	[95]
CTBP + LAMP2 + miR-16-2 + miR-21-5p		Serum	Multiple + miRNA	Diagnosis	HCC vs. HL + CHC	[153]
CTBP + LAMP2 + miR-16-2 + miR-21-5p		Serum	Multiple + miRNA	Prognosis (PFS)	HCC	[153]
DANCR	Up	Plasma	Single	Diagnosis	HCC vs. HL + CHB + LC	[105]
DANCR	Up	Plasma	Single	Prognosis	HCC	[105]
ENSG00000258332.1 + LINC00635	Up	Serum	Multiple + AFP	Diagnosis	HCC vs. HL + LC + CHB	[96]
ENSG00000258332.1 + LINC00635	Up	Serum	Single	Prognosis (OS)	HCC	[96]
HULC	Up	Plasma	Single	Diagnosis	HCC vs. HL	[104]
HULC	Up	Plasma	Single	Diagnosis	HCC vs. HL	[154]
HULC + LINC00152	Up	Plasma	Multiple + AFP	Diagnosis	HCC vs. HL	[104]
JPX	Down	Plasma	Single + AFP	Diagnosis	HCC vs. HL	[101]
JPX	Down	Plasma	Single	Diagnosis	HCC vs. HL	[101]
JPX	Down	Plasma	Single	Prognosis	HCC	[101]
JUN + UCA1	Up	Serum	Multiple	Diagnosis (early)	HCC vs. HL + CHC	[155]
JUN + UCA1	Up	Serum	Multiple + AFP	Diagnosis (early)	HCC vs. HL + CHC	[155]
LRB1	Up	Serum	Single + AFP/DCP	Diagnosis	HCC vs. HL	[102]
LRB1	Up	Serum	Single	Prognosis (OS)	HCC	[102]
LINC00152	Up	Plasma	Single	Diagnosis	HCC vs. HL	[104]
LINC00974	Up	Plasma	Single	Diagnosis (early)	HCC vs. HL	[156]
LINC01225	Up	Serum	Single	Diagnosis	HCC vs. HL	[157]
lnc-PCDH9-13:1	Up	Saliva	Single	Diagnosis (early)	HCC vs. HL + HBV carrier + CHB + LC	[107]
LOC149086 + RP11-160H22.5 + XLOC_014172	Up	Plasma	Multiple	Diagnosis	HCC vs. cancer free (T)	[158]
LOC149086 + RP11-160H22.5 + XLOC_014172	Up	Plasma	Multiple	Diagnosis	HCC vs. cancer free (V)	[158]
PIVKAI1 + MALAT1	Up	Plasma	Multiple + AFP	Diagnosis	HCC vs. HL	[159]
PVT1 + uc002mbe.2	Up	Serum	Multiple	Diagnosis	HCC vs. HL	[100]
SNHG1	Up	Plasma	Single	Diagnosis	HCC vs. HL + CHB + LC	[97]
SNHG1	Up	Plasma	Single + AFP	Diagnosis	HCC vs. HL + CHB + LC	[97]
SPRY4-IT1	Up	Plasma	Single + AFP	Diagnosis	HCC vs. HL	[160]
SPRY4-IT1	Up	Plasma	Single	Diagnosis	HCC vs. HL	[160]
uc001ncr + AX800134		Serum	Multiple	Diagnosis (early)	HCC vs. HL + HBV carriers	[161]
uc003wbd	Up	Serum	Single	Diagnosis (early)	HCC vs. HL	[95]
uc003wbd	Up	Serum	Single	Diagnosis (early)	HCC vs. HBV carriers	[95]
UCA1	Up	Serum	Single	Diagnosis	HCC vs. HL + CHC	[99]
UCA1	Up	Serum	Single	Prognosis (PSF)	HCC	[103]
UCA1 + WRAP53	Up	Serum	Multiple + AFP	Diagnosis	HCC vs. HL + CHC	[99]
UCA1 + WRAP53	Up	Serum	Multiple	Diagnosis	HCC vs. HL + CHC	[99]
WRAP53	Up	Serum	Single	Diagnosis	HCC vs. HL + CHC	[99]
WRAP53	Up	Serum	Single	Prognosis (PSF)	HCC	[99]
XIST	Down	Plasma	Single	Prognosis	HCC	[101]
ZFAS1	Up	Plasma	Single + AFP	Diagnosis	HCC vs. HL (T)	[98]
ZFAS1	Up	Plasma	Single + AFP	Diagnosis	HCC vs. HL + CHB + LC (V)	[98]
ZFAS1	Up	Plasma	Single	Diagnosis	HCC vs. HL (T)	[98]
ZFAS1	Up	Plasma	Single	Diagnosis	HCC vs. HL + CHB + LC (V)	[98]

lncRNA: long noncoding RNA; AFP: alpha fetoprotein; DCP: des-carboxyprothrombin; CHC: chronic hepatitis C; CHB: chronic hepatitis B; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HL: healthy liver; LC: liver cirrhosis; (T): training set; (V): validation set; OS: overall survival; PFS: progression free survival

5p was decreased in non-responder patients compared to responders, suggesting this miRNA as a candidate serum biomarker for predicting response to sorafenib.

## LNCRNAS

lncRNAs are a class of > 200 nt RNA transcripts linked to the modulation of several pathways through various different molecular mechanisms<sup>[90,91]</sup>. Their expression differs between cancer and non-cancer tissues and their role in cancer biology is well recognized<sup>[92,93]</sup>. Expression deregulation of several lncRNAs has been reported in HCC<sup>[94]</sup> and abnormal levels of an increasing number of lncRNAs are being found in serum/plasma of HCC patients, suggesting their potential use as circulating tumor biomarkers. Table 5 summarizes the studies that have linked circulating lncRNAs to HCC. Technologies for detection and quantification of lncRNAs in biological samples are the same previously described for miRNAs.

A number of studies demonstrated that circulating lncRNAs could discriminate HCC from healthy controls or patients with non-malignant chronic liver diseases. In several cases, the studies involved detection of early HCCs, a crucial factor for the application of curative strategies. lncRNAs were evaluated either as single biomarkers or in combination. As single biomarkers, results indicated a sensitivity ranging from 51% to 92%. In this experimental setting, lncRNA - uc003wbd<sup>[95]</sup>, ENSG00000258332.1<sup>[96]</sup>, small nucleolar RNA host gene 1 (SNHG1)<sup>[97]</sup>, zinc finger antisense 1 (ZFAS1)<sup>[98]</sup> were able to differentiate HCC patients from chronic hepatitis B virus (HBV) patients or healthy controls, urothelial carcinoma associated-1 (UCA1) or WRAP53<sup>[99]</sup> were significantly higher in HCC patients' sera in comparison with chronic hepatitis C virus (HCV) patients or healthy volunteers. Diagnostic accuracy improved when lncRNAs were combined among them or with AFP and DCP. Combination of the lncRNAs plasmacytoma variant translocation 1 (PVT1) and uc002mbe.2 could discriminate early HCC patients from either HBV or HCV positive patients<sup>[100]</sup>. ZFAS1<sup>[98]</sup> or the expressed neighbor of XIST (Enox or JPX)<sup>[101]</sup> levels in combination with AFP could discriminate HCC from healthy individuals or chronic liver diseases patients with a better accuracy than each biomarker considered individually. The same was found for the combination of serum lncRNA uc007biz.1 (LRB1) with AFP and DCP biomarkers<sup>[102]</sup> or the combination of PVT1 and uc002mbe.2 with AFP<sup>[100]</sup>.

Many circulating lncRNAs were also found to correlate with unfavourable pathologic features<sup>[96,97,100-105]</sup> and their association with prognosis was also evaluated. The increased levels of UCA1<sup>[106]</sup> or differentiation antagonizing non-protein coding RNA (DANCR)<sup>[105]</sup> or LRB1<sup>[102]</sup> were all associated to a poorer OS. Some of the studies investigated the combination of lncRNAs. The combined up-regulation of ENSG00000258332.1 and LINC00635<sup>[96]</sup>, or of SNHG and UCA1<sup>[106]</sup> or low levels of JPX and X inactive-specific transcript (XIST)<sup>[101]</sup> significantly correlated with a poorer prognosis in HCC patients.

lncRNAs were also evaluated as biomarkers for monitoring tumor recurrence after surgery. In this clinical setting, it was found that patients with higher DANCR levels after surgery were prone to develop HCC recurrence<sup>[105]</sup>. It was also reported that the circulating levels of PCDH9-13:1 were significantly reduced after curative hepatectomy. However, it increased again in case of a relapse, suggesting that this lncRNA could be used to monitor patients after surgery<sup>[107]</sup>. Similarly, based on the decreased levels of SNHG1<sup>[97]</sup> or PVT1<sup>[100]</sup> after surgery, it was speculated that a subsequent increase of their expression might serve as biomarkers to monitor patients for HCC relapse.

## CONCLUSION

Analysis of cell free DNA and RNA in body fluids, the so-called liquid biopsy, represents a very promising strategy for the early detection of cancer at an early stage or during monitoring of patients for the early detection of cancer relapse. The approach has the potential to significantly improve the clinical management of HCC cancer patients. Studies on circulating DNA/RNA in HCC originate from the need to identify more effective biomarkers than those currently in use, AFP and DCP. This review presents the results obtained so far in HCC. Although the specific studies still require further validation, overall they demonstrate a good sensitivity and specificity, higher than the current biomarkers and, once present limitations are over, can successfully find a valuable clinical use.



Although there are small amounts of nucleic acids in the circulation and only a fraction originate from tumor cells, the current technologies allow to pinpoint the changes induced by the presence of a tumor both qualitatively and quantitatively. Deep sequencing-based approaches for high-throughput and quantitative PCR analyses for targeted investigations demonstrated the appropriate ability to highlight such decisive traces of disease. However, it is clear that there are differences deriving from the analysis of DNA or RNA in the circulation.

From this point of view, DNA analysis is simpler and provides a straightforward interpretation: the presence, even in traces, of genetic or epigenetic alterations typically associated with a neoplastic disease provides a tangible sign of the presence of tumor cells. However, ctDNA analysis can also provide additional information. In fact, the technologies either based on NGS or ddPCR are quantitative and therefore allow to monitor the quantitative evolution of genetic or epigenetic alterations over time, thereby providing an assessment of therapy effectiveness. Furthermore, it is possible that distinct tumor clones may differentially respond to therapy. The mutational analysis of ctDNA offers the possibility of highlighting tumor heterogeneity, that tissue biopsy does not allow, thus making possible the detection of tumor clones differentially responsive to therapy. In this regard, identification of mutations in specific “actionable” genes offers the clinician the possibility of using targeted therapies if a molecular target is spotted. At present, the situation in HCC is limited, but auspiciously in evolution. Although sorafenib and regorafenib were the only few available options in advanced HCC until now, two new kinase inhibitors, namely lenvatinib and cabozantinib, have been recently approved by FDA for first-line and second-line treatment<sup>[108,109]</sup>. Unfortunately, an important limitation is that none of these drugs is associated with specific molecular alterations. As seen in other tumor types, it is however reasonable to expect that molecular subgroups of HCCs might be recognized for their differential responsiveness to specific targeted therapies. From these considerations, it is therefore clear that ctDNA analysis, which widely surpasses AFP and DCP circulating biomarkers in many aspects, can find clinical application for the management of HCC patients. It possibly presents a limitation, namely the possibility of detecting the presence of gene mutations in the early stages of disease, which can be difficult due to the very little amount of DNA released by small tumors. Overcoming this current limit will certainly be a goal of future research. Another limitation in HCC is the availability of a limited number of effective drugs against the most frequently mutated genes in HCC, such as catenin, as currently there are no target drugs capable of acting against the encoded oncoproteins.

The results from circulating RNA studies have probably a different conceptual meaning and so far the produced outcomes are not yet ready for clinical use. From a practical point of view, unlike genetic or epigenetic DNA-based tests that display characteristics intrinsically distinctive of neoplastic cells, in the case of circulating RNAs, they are also present in circulation of unaffected individuals and differences with cancer patients are exclusively quantitative. This indicates that for clinical application it will be necessary to define significance thresholds and appropriate methods to obtain reliable data. Quantitative PCR methods can be easily applied to diagnostic applications and, at present, ddPCR emerged as a robust method to quantify circulating miRNAs<sup>[37,39,40,110,111]</sup>.

In summary, the analysis of circulating miRNA/lncRNA is potentially useful in different phases of the disease including early stages and the ddPCR method is optimally effective for performing the analysis. However, the approach is at present immature for clinical use both for methodological and scientific issues that need to be solved. Both pre-analytical and analytical procedures need to be standardized to guarantee solid results from independent laboratories<sup>[112]</sup>. On the scientific side, the levels of circulating RNAs show a normal variability among individuals. To diagnose HCC is essential to reaching an understanding of the variability in circulating miRNA/lncRNA levels not only in healthy individuals, but also and more importantly in patients affected by chronic liver diseases, such as chronic HBV/HCV infection, alcoholic and non-alcoholic fatty liver disease, steatohepatitis and cirrhosis. To this end, large study cohorts are needed<sup>[113]</sup>.

Albeit often very exciting, results from the scientific literature are so far inconsistent. To change this status and open the way to translational applications, prospective well-designed large multicenter trials are needed.

## DECLARATIONS

### Authors' contributions

Conceived and designed the study: Callegari E, Sabbioni S, Negrini M

Wrote the manuscript: Guerriero P, Moshiri F, Lupini L, Callegari E, Negrini M

Read and approved the manuscript: All authors

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Case Report

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# Hepatocellular carcinoma in non-alcoholic fatty liver disease with and without cirrhosis

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## Abstract

Non-alcoholic fatty liver disease has become the leading chronic liver disease in the developed world, with a prevalence of 6%-35%. Its pathological spectrum ranges from simple steatosis (non-alcoholic fatty liver) to different degrees of inflammation and liver cell damage [non-alcoholic steatohepatitis (NASH)]. NASH has gained attention in recent years because of its association with hepatocellular carcinoma (HCC). Although the occurrence of HCC is more frequent in the presence of cirrhosis, studies have shown that hepatic carcinogenesis may also develop in the context of NASH without association with advanced fibrosis, as well as from simple steatosis. Evidence of the onset of HCC in the absence of cirrhosis is of concern, since recent surveillance and screening guidelines for liver cancer do not include this population subgroup. Therefore, it is imperative that new effective screening and monitoring measures for HCC, or even the reformulation of these recommendations, be taken to handle these patients considered to be at high risk. The present paper aims to review the literature on the occurrence of HCC in patients with NASH with or without cirrhosis. In addition, we report a case showing the development of HCC in a patients with NASH without cirrhosis.

**Keywords:** Non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, cirrhosis, hepatocellular carcinoma

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the leading cause of liver disease in the world and its pathological spectrum ranges from simple steatosis [non-alcoholic fatty live (NAFL)] to various degrees of inflammation and liver cell damage, a condition known as non-alcoholic steatohepatitis (NASH)<sup>[1-4]</sup>. The



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diagnosis of NAFLD requires the exclusion of excessive alcohol consumption, defined separately for men and women, and other secondary causes of liver disease. For definitive diagnosis of NASH, currently, a liver biopsy is needed<sup>[4]</sup>.

A hypercaloric diet with excess saturated fats, refined carbohydrates and high fructose consumption has been related to weight gain and, more recently, to NAFLD<sup>[4,5]</sup>. Thus, NAFLD is associated with metabolic syndrome and is characterized by adipose tissue dysfunction and insulin resistance. These two factors generate deregulation in the production of adiponectin, an anti-inflammatory protein, and increase the release of several proinflammatory cytokines, such as tumor necrosis factor alpha, leptin and interleukin-6. The effects of this imbalance lead to the deposition of lipids in hepatocytes, causing lipotoxicity and the production of free radicals by the oxidation of fatty acids. The progression to NASH occurs in 25 percent of cases<sup>[1]</sup>. Also, gut microbiota plays a role in inflammation, through alterations in gut epithelial permeability, choline metabolism, endogenous alcohol production, release of inflammatory cytokines, regulation of hepatic toll-like receptor, and bile acid metabolism<sup>[6]</sup>.

Estimates of NAFLD prevalence range from 25%-45% in the US, while NASH currently affects 5 percent of the population<sup>[7]</sup>. As diabetes and obesity have become global epidemics, the WHO predicts an exponential increase in cases of NAFLD in the coming decades<sup>[2]</sup>. The objective of this study is to review the literature on the occurrence of hepatocellular carcinoma (HCC) in the context of NAFL/NASH with or without associated cirrhosis.

## CASE REPORT

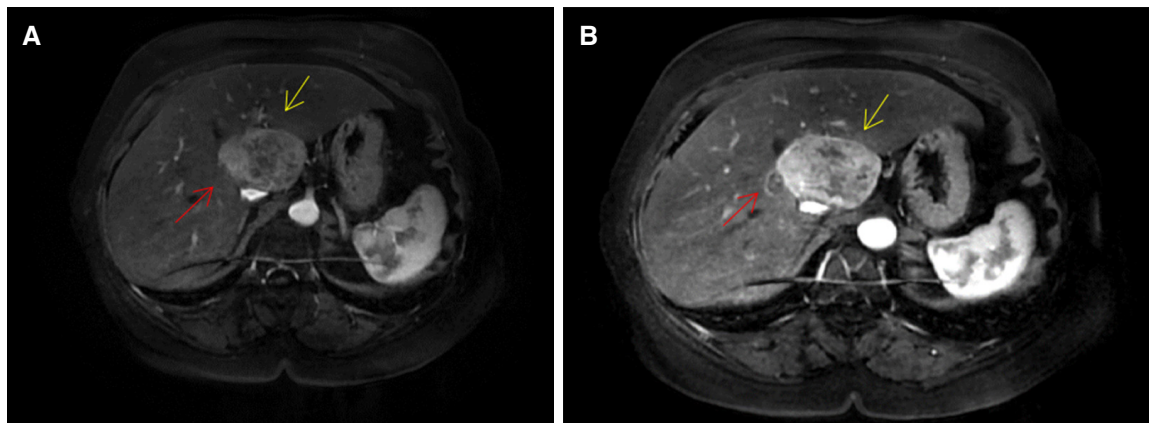
A 67-year-old female patient, from Caxias do Sul, sought care due to a complaint of asthenia, inappetence and a weight loss of 3 kg over the last month. The patient displayed metabolic syndrome, with a previous diagnosis of grade I hepatic steatosis, diabetes mellitus type 2, mild obesity and arterial hypertension. The patient was admitted in the hospital for investigation of hepatic nodules identified in an ultrasonography. Magnetic resonance imaging showed a heterogeneous nodule, in hepatic segment I, measuring 5.7 cm (largest measurement) and another nodular image in segment II measuring 1.9 cm (largest measurement), with homogeneous arterial impregnation (suspected for HCC - [Figure 1](#)). A biopsy of both nodules and of the liver was performed, guided by ultrasonography.

The patient remained in good general condition throughout the hospital stay. Hemoglobin 11.3 g/dL, hematocrit 32.9%, prothrombin time 11.1 s, total bilirubin 0.2 mg/dL, alkaline phosphatase 113 U/L, aspartate aminotransferase 25 U/L, alanine aminotransferase 28 U/L, gama glutil transferase 73 U/L, albumin 5.01 g/dL and alpha-fetoprotein of 6.7 ng/mL were performed. The biopsy revealed that liver presented: nodulo I with macrovacuolar steatosis in 10 percent of hepatocytes, portal lymphocytic infiltrate, no fibrosis and hepatocellular ballooning and nodule II with hepatic lesion with desmoplastic stroma and small cells with mild nuclear pleomorphism, an image corresponding to HCC.

The patient was diagnosed with NASH associated to HCC. After histological confirmation, she was referred to the clinical and surgical oncology service for tumor resection.

## DISCUSSION

NASH has gained attention in recent years because of its association with HCC<sup>[1,3]</sup>. It is known that there is a risk of progression to advanced fibrosis in up to 20 percent of patients with this pathology, thus increasing the chance of developing liver cancer<sup>[1,8-11]</sup>. Studies have found that 11.3% of patients with cirrhosis due to NASH developed HCC within 5 years, while in patients with cirrhosis due to alcohol, the rate is 12.5% over the same time frame<sup>[1]</sup>. Compared with the benign course of NAFL, NASH patients have an 8-fold increased chance of progressing to advanced fibrosis, in addition to an increased risk of liver-related death and of



**Figure 1.** Hepatocellular carcinoma demonstrated in magnetic resonance of the abdomen. Yellow arrow - larger nodule (5.7 cm); red arrow - smaller nodule (1.9 cm)

cardiovascular disease<sup>[2,12]</sup>. The main risk factors involved in the occurrence of HCC in cirrhotic patients due to NASH are male gender, age over 70 years-old, diabetes and hypertension<sup>[13]</sup>. It was estimated that the presence of NAFLD-associated HCC is 7.6-fold greater than in a same sex and age control group<sup>[14]</sup>. Nevertheless, the impact of hepatitis C virus (HCV) in HCC is still greater than that of NAFLD - the risk for HCC in cirrhotic patients with HCV is three times greater than that of patients with NAFLD<sup>[15]</sup>. Considering only studies strictly including patients with or without cirrhosis, the reported incidence of HCC in NAFLD patients with cirrhosis was between 6.7% and 15% at 5-10 years, whereas the incidence in NAFLD patients without cirrhosis was 2.7% at 10 years and 23 per 100,000 person-years<sup>[16]</sup>.

The prevalence of NAFLD has become similar in the West and the East<sup>[17]</sup>. Obesity, which has been mostly a health problem of the Western world, has emerged rapidly in Asia, due to globalization and rapid urbanization, which lead to a change of dietary patterns to those of the West<sup>[18]</sup>. In China, the number of obese people has increased from below 0.1 million in 1975 to over 43.2 million in 2014, accounting for 16.3% of obese people worldwide. In India, the number of obese people increased from 0.4 million to 9.8 million during the same period<sup>[19]</sup>. This will increase the prevalence of NAFLD in Asia, which will in turn increase the cases of HCC not only from the increasing prevalence of NAFLD but also from the anticipated decreasing burden of HBV and HCV infections. It is a fact that primary, secondary and tertiary preventive strategies for HCC due to NAFLD are lacking. NAFLD has been estimated to contribute to 10%-12% of HCC cases in Western populations and 1%-6% of HCC cases in Asian populations. The increasing burden of NAFLD-related HCC over time has been demonstrated in studies from both Western and Asian populations<sup>[20]</sup>. For example, in a Sri Lanka cohort, the most common cause of HCC was NAFLD-related cirrhosis<sup>[21]</sup>. Hence the global incidence of NAFLD is increasing rapidly, its impact on HCC incidence may be explosive<sup>[22,23]</sup>.

Although HCC is more frequent in the presence of cirrhosis, several studies have shown that hepatic carcinogenesis may also develop in the context of NASH or NAFL, without association with advanced fibrosis<sup>[1-3,7,24]</sup>. A 2.5-fold increased risk of developing HCC in patients with NAFLD without cirrhosis was observed when compared to other etiologies of chronic liver disease<sup>[1]</sup>. This is particularly concerning, since in a recent study 20% of NAFLD-related HCC occurred in the absence of cirrhosis<sup>[14]</sup>. The patient with non-cirrhotic NASH presenting HCC is older, male, and meets one or more criteria for metabolic syndrome<sup>[3,7]</sup>.

The pathogenesis of HCC related to NAFLD is different, once metabolic syndrome and obesity manifest several exclusive mechanisms that favor the occurrence of tumors: increased release of free fatty acids, of multiple proinflammatory cytokines, and the reduction of activity of anti-inflammatory agents such as



adiponectin<sup>[9]</sup>. The presence of these chemical mediators leads to apoptosis of hepatocytes, compensatory proliferation and, finally, carcinogenesis<sup>[2,7,13,25]</sup>. Accumulating evidence supports the importance of lipid metabolic reprogramming in various situations of hepatocarcinogenesis<sup>[26]</sup>. Given the increasing incidence of NAFLD and advances in curative options for hepatitis C viral infection, NAFLD is expected to become the leading cause of HCC in developed countries<sup>[2,3,27]</sup>. Absent washout and capsule appearance are associated with increasing hepatic steatosis in patients with non-cirrhotic, NAFLD-associated HCC<sup>[28]</sup>. Increased incidence of NASH-related cirrhosis is also influencing trends in liver transplantation: there has been a 4-fold increase in the number of liver transplants due to NASH compared to a 2-fold increase in those due to hepatitis C<sup>[2,5]</sup>. However, comparing to other etiologies of cirrhosis, it is not clear whether HCC-related NAFLD has similar outcomes<sup>[29,30]</sup>. In addition, NASH has already become the second leading cause of HCC-related liver transplantation in USA<sup>[2]</sup>.

HCC represents the fifth most common neoplasm and the second largest cause of cancer mortality worldwide<sup>[2,31,32]</sup>. Despite its increasing incidence and the development of new therapies, overall 5-year survival is still low, no more than 30%<sup>[33,34]</sup>. NAFLD is considered to be the third cause for HCC in the USA<sup>[35]</sup>. Early detection of HCC provides greater treatment options, significantly improving the prognosis of patients<sup>[31]</sup>. NAFLD increased substantially over the past 20 years among resectable HCCs and it is now the leading cause of HCC occurrence without or with minimal fibrosis<sup>[36]</sup>. In terms of clinicopathological findings, most studies agree that noncirrhotic NAFLD-related HCC patients were more likely to present with larger tumors<sup>[37-39]</sup>. Although there are guidelines for routine HCC surveillance that allow early diagnosis and improvement in curative outcomes, overall screening rates are below those considered ideal<sup>[31,32]</sup>. In addition, it has been found that patients with cirrhosis due to NASH are less likely to undergo adequate checks and monitoring of HCC compared to patients with other etiologies for chronic liver disease<sup>[1,5,31]</sup>. This may be due to the lack of HCC screening in noncirrhotic NAFLD patients<sup>[31]</sup>. It is widely reported that the deficiency in HCC surveillance among NAFLD patients, with only 13% of HCC discovered through surveillance, resulted in delayed detection in the majority of patients<sup>[40]</sup>. Several factors may contribute to this phenomenon: visceral adiposity, for example, is associated with a lower degree of ultrasonographic tumor identification, limiting its sensitivity for screening<sup>[1]</sup>. Other related variables are attributed to difficulties in access to adequate health care<sup>[31]</sup>. Patients with NAFLD-related HCC are older, have a shorter survival time, have more cardiovascular diseases and diabetes and are more likely to die from their HCC than other patients<sup>[41-43]</sup>. It has been demonstrated that curative treatment for HCC and serum albumin level > 3.7 g/dL suggest best prognostic profile for NAFLD-related HCC<sup>[44]</sup>.

Evidence of hepatocarcinogenesis arising in the absence of advanced fibrosis is of concern, as recent guidelines recommending ultrasonographic abdominal screening and surveillance for HCC, every 6-12 months, only for patients with cirrhosis or chronic hepatitis B infection, failed to address this growing patient population<sup>[1,2,7]</sup>. In addition, those with NAFLD-related HCC have a worse prognosis, since they have a shorter survival time, a more advanced tumor at diagnosis and a lower probability of liver transplantation<sup>[31,45]</sup>. This reinforces the idea that a rewording of current HCC screening recommendations is needed so that these high-risk patients can be diagnosed via routine assessment<sup>[3,5,46]</sup>. It is also understood that, because of the high prevalence of this type of liver disease, the extension of screening to this whole group would greatly increase health spending, making it less viable<sup>[47]</sup>. Stratification by fibrosis score may offer some additional benefit to the subgroup of patients with non-cirrhotic NASH; however, reports of HCC in patients with NAFLD and no fibrosis have been described, such as the reported case. Further research to elucidate the association between the degree of fibrosis and the risk of HCC would provide a useful tool in the screening for HCC in these patients<sup>[1]</sup>. *PNPLA3* gene polymorphism has been associated with an increased risk of HCC and may assist in assessing the patient's risk and personalizing surveillance. However, it has not yet been validated for routine use because there is no well-documented cost-benefit ratio<sup>[4]</sup>.

American Association for the Study of the Liver, European Association for the Study of the Liver and the Brazilian Society of Hepatology do not recommend routine HCC screening in non-cirrhotic NASH

patients justifying that the large population of NAFL/NASH patients makes systematic surveillance impracticable<sup>[4,12,35]</sup>.

## CONCLUSION

With increasing cure rates for chronic liver disease related to HBV and HCV, NASH may become the leading cause of HCC and liver transplantation in the coming decades. Recent evidence shows that a significant proportion of patients with NAFL and NASH progresses to HCC even in the absence of cirrhosis or fibrosis. However, new effective monitoring and screening measures should be established to address these high-risk patients, thereby reducing the future impact of HCC in this population.

## DECLARATIONS

### Authors' contributions

Conception and design of the study, data analysis and interpretation: Onzi G, Moretti F, Soldera J  
Data acquisition, provided administrative, technical, and material support: Onzi G, Moretti F, Soldera J, Balbinot RA, Balbinot SS

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

None.

### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Review

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# Telomerase reverse transcriptase promoter mutations in hepatocellular carcinogenesis

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## Abstract

Through several studies exploiting next-generation sequencing, we are obtaining a clearer picture of the complex genetic and molecular landscape of hepatocellular carcinoma (HCC). Consistent with the findings of other cancer types, telomerase reverse transcriptase (TERT) promoter mutations have been frequently reported in HCC. C228T and C250T are two major types of hot spot mutations in the TERT promoter region. Besides, in hepatitis B virus (HBV)-related HCC cases, the TERT promoter is recurrently interrupted by integration of HBV DNA. TERT promoter mutations are thought to be an early event in HCC carcinogenesis, and they are significantly associated with disease progression. In this review, we provide an updated overview of the somatic mutations in the TERT promoter region and discuss their possible roles in the development of HCC.

**Keywords:** Hepatocellular carcinoma, telomerase reverse transcriptase, mutation, hepatitis B virus

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and deadliest cancers worldwide, ranking fifth and ninth in incidence, and second and sixth in mortality for males and females, respectively<sup>[1,2]</sup>. So far, only three molecular targeted agents, including sorafenib, lenvatinib and regorafenib, have been approved by the Food and Drug Administration for the treatment of HCC<sup>[3,4]</sup>, and they only extend median survival by a few weeks to months<sup>[5]</sup>. Therefore, more research is needed to fill the gaps in knowledge of the genetic



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and molecular landscape of HCC in order to develop target therapies. The genetic landscape of HCC is complicated and involves a number of pathways as well as a considerable amount of somatic mutations in a wide range of genes<sup>[6]</sup>. Among all these genetic alterations, telomerase reverse transcriptase (TERT) promoter mutations occur most frequently, affecting ~60% of all HCC patients<sup>[6-8]</sup>. In this mini-review, we mainly summarize the frequency, mechanisms and clinical prospect of TERT promoter mutations in HCC. To provide more background information, this review also briefly touches upon the TERT promoter mutations in various cancers, although HCC remains the main focus of our discussion throughout the whole paper.

## THE STRUCTURE AND FUNCTION OF TERT

Human telomerase is a ribonucleoprotein polymerase that reverses the continuous telomere shortening in cell division by adding 5'-TTAGGG-3' repeats to the ends of chromosome<sup>[9]</sup>. It consists of two core subunits: the catalytic component TERT and the RNA component (TERC) that serves as a template for elongating telomeres<sup>[10,11]</sup>.

The TERT component is encoded by the *TERT* gene, located on chromosome 5 in humans. It spans a length of about 40,000 base pairs (bp) with 16 exons<sup>[12]</sup>. Of note, the *TERT* gene is suppressed in most normal somatic cells (excluding germ cells and stem cells), ensuring that these cells only divide a finite number of times and do not surpass the Hayflick limit<sup>[13,14]</sup>. Normal somatic cells stop dividing when their telomeres become critically short, whereupon they enter a stage called senescence<sup>[15]</sup>. Cancer cells, however, overcome replicative senescence and achieve immortality by reactivating the *TERT* gene and upregulating TERT expression<sup>[14]</sup>.

The regulation of TERT expression largely depends on the activity of the TERT promoter, especially the core functional fragment that consists of a 260 bp DNA sequence with several transcription factor binding sites, but distinctly lacking a TATA box or a similar sequence<sup>[16,17]</sup>. The binding motifs in the TERT promoter include two evolutionarily-conserved E-boxes (CACGTG), located at -242 bp and -34 bp to the translational start site, for c-Myc binding<sup>[18]</sup>. The binding of c-Myc to the E-box activates TERT transcription, suggesting a role of c-Myc in regulation of the expression of TERT<sup>[19,20]</sup>. GC-boxes (GGGCGG), the binding sites for zinc finger transcription factor Sp1, are the other characteristic sequences in the TERT promoter region<sup>[21]</sup>. There are at least five GC-boxes within the core promoter of TERT, and they function synergistically to maintain the promoter activity of TERT<sup>[22]</sup>. P53 has been shown to down-regulate TERT transcription in an SP1-dependent manner<sup>[23]</sup>.

## TERT PROMOTER MUTATIONS IN SEVERAL CANCERS

TERT promoter mutations are the most frequent somatic mutations in a variety of cancers. It has been widely reported that the two most common types of recurrent TERT promoter mutations are C228T and C250T, located at positions 1,295,228 and 1,295,250 on chromosome 5, or -124 bp and -146 bp of the ATG translational start site of the *TERT* gene<sup>[24-27]</sup>. In a systematic analysis involving 1,581 cancer cases of different types, 27.0% were found to have TERT promoter mutations<sup>[25]</sup>. Killela et al.<sup>[28]</sup> examined 1,230 tumor specimens of 60 different types and identified 231 TERT promoter mutations (18.8% of the total), among which C228T and C250T mutations accounted for 98%. Similarly, in a study where 1,515 tumors of the central nervous system were tested, 327 (21.6%) had TERT promoter mutations, and all except two contained either C228T or C250T<sup>[29]</sup>. Another study examined 150 cell lines of several cancer types from the Cancer Cell Line Encyclopedia and noted that 24 cell lines (16%) harbored either C228T or C250T mutations<sup>[26]</sup>. Statistics show that C228T is somewhat more prevalent than the C250T mutation [Table 1] in a wide range of cancer types, including various subtypes of CNS cancers, urogenital cancers, melanoma and thyroid cancer<sup>[25,26,28-37]</sup>.

**Table 1. Telomerase reverse transcriptase promoter mutations in multiple cancers**

Cancer type	Number of cancer cases	Number of TERT mutations*	Number of different types of TERT promoter mutations**			Methods	Ref.
			C228T	C250T	C228T or C250T		
Cancer tissue							
Glioma, medulloblastoma, hepatocellular carcinoma, etc.	1230	231 (18.8)	179 (77.5)	48 (20.8)	227 (98.3)	PCR/Sanger sequencing	[28]
Bladder cancer, liver cancer, glioma, etc.	1581	426 (26.9)	/	/	/	Whole-genome/low-pass whole-genome sequencing	[25]
CNS cancers	1515	327 (21.6)	257 (78.6)	68 (20.8)	325 (99.4)	PCR/bidirectional sequencing	[29]
CNS, bladder, thyroid cancers, etc.	741	142 (19.2)	99 (69.6)	43 (30.3)	140 (98.6)	PCR/Sanger sequencing	[36]
Urogenital cancers	302	130 (43.0)	100 (76.9)	24 (18.5)	124 (96.4)	PCR/Sanger sequencing	[37]
Medulloblastoma	466	98 (21.0)	/	/	/	PCR/Sanger sequencing	[35]
Melanoma	287	109 (38.0)	51 (46.8)	40 (36.7)	91 (83.5)	PCR/Sanger sequencing	[32]
Bladder cancer	262	218 (83.2)	165 (75.7)	32 (14.7)	197 (90.4)	SNaPshot assay and Sanger sequencing	[34]
Melanoma	77	24 (31.2)	7 (29.2)	5 (20.8)	12 (50.0)	High-throughput sequencing/Sanger sequencing	[33]
Cancer cell line							
Melanoma	168	125 (74.4)	46 (36.8)	64 (51.2)	110 (88)	High-throughput sequencing/Sanger sequencing	[33]
Melanoma, liver, bladder cancers, etc.	150	24 (36.0)	/	/	24 (100)	Whole-genome sequencing, Sanger sequencing,	[26]
Urothelial bladder cancer	23	20 (87.0)	16 (80.0)	2 (10.0)	18 (90.0)	PCR/Sanger sequencing	[31]
Urothelial bladder cancer	32	28 (87.5)	25 (89.3)	3 (10.7)	28 (100)	PCR/Sanger sequencing	[30]

\*Percentage in all cancer cases; \*\*percentage in telomerase reverse transcriptase (TERT) mutation cases

Overall, it is widely accepted that glioma, melanoma, bladder cancer and HCC are among those commonly-affected by TERT promoter mutations<sup>[25,28,38]</sup>.

## TERT PROMOTER MUTATIONS IN HCC

The genomic landscape of HCC involves a number of pathways as well as somatic mutations in a wide range of genes, including *TP53*, *CTNNB1*, *AXIN1*, *CDKN2A*, *ARID2*, *ARID1A*, *TSC1/TSC2*, *RPS6KA3*, *KEAP1*, *MLL2*, and several epigenetic modifications<sup>[6]</sup>. Despite the complexity of the genomic landscape of HCC, the single most significant factor is genomic changes on TERT promoter, which include point mutations, hepatitis B virus (HBV) DNA integrations, amplifications and epigenetic modifications. TERT promoter point mutations contribute more frequently (54%-60%) to the reactivation of telomerase in HCC than the exclusively-present HBV insertions in the TERT promoter (10%-15%) and TERT amplification (5%-6%)<sup>[6-8]</sup>. Therefore, we are going to thoroughly discuss TERT promoter mutations while briefly touching upon other genomic and epigenomic alterations on TERT promoter in HCC.

### TERT promoter point mutations

A few prominent studies on HCC demonstrated that TERT promoter mutations were found in about 30%-60% of the total cases<sup>[8,39-49]</sup>. Consistent with the findings in other cancer types, the two most common mutations were C228T and C250T, and the former was more prevalent than the latter in HCC [Table 2]<sup>[8,39-47]</sup>. As shown in Table 2, there are no cases with both C228T and C250T mutations, which implies that these two hot spot

**Table 2. Telomerase reverse transcriptase promoter mutations in hepatocellular carcinoma**

Number of HCC cases	Number of TERT mutations (%)	Number of different types of TERT promoter mutations (%)			Methods	Ref.
		C228T	C250T	C250T or C228T		
469	254 (54.2)	236 (92.9)	11 (4.3)	247 (97.2)	PCR/bidirectional sequencing	[8]
316	103 (32.6)	96 (93.2)	5 (4.9)	101 (98.1)	PCR/Sanger sequencing	[43]
305	179 (58.7)	166 (92.7)	11 (6.1)	177 (98.9)	PCR/Sanger sequencing	[42]
276	85 (30.8)	84 (98.8)	1 (1.2)	85 (100)	PCR/Sanger sequencing	[44]
196	87 (44.4)	/	/	/	Whole-genome sequencing	[48]
195	57 (29.5)	54 (94.7)	3 (5.3)	57 (100)	PCR/Sanger sequencing	[45]
160	46 (28.8)	32 (69.6)	14 (30.4)	46 (100)	PCR/Sanger sequencing	[39]
44	15 (34.1)	10 (66)	5 (34)	15 (100)	PCR/Sanger Sequencing	[40]
190	57 (30.0)	50 (87.7)	7 (12.3)	57 (100)	PCR/bidirectional sequencing	[46]
127	64 (50.4)	62 (96.9)	2 (3.2)	64 (100)	PCR/Sanger sequencing	[47]
123	45 (36.6)	43 (95.6)	2 (4.4)	45 (100)	PCR/Sanger sequencing	[41]
125	85 (68.0)	/	/	/	PCR/Sanger sequencing	[49]

\*Percentage in telomerase reverse transcriptase (TERT) mutation cases. HCC: hepatocellular carcinoma

mutations are mutually exclusive. Furthermore, a comprehensive review evaluating the distribution of TERT promoter mutations in 1,939 primary HCC from four continents also showed that TERT promoter mutations had almost the same level of prevalence in all continents, with slightly higher mutation rates in Europe (56.6%) and Africa (53.3%) than in America (40%) and Asia (42.5%), and that C228T mutation was universally more frequent than C250T<sup>[41]</sup>.

Apart from the high frequency of TERT promoter mutations in HCC, another piece of useful information indicated by several lines of evidence is that TERT promoter mutations are associated with a few factors, including virus status, gender, age and tumor size of the patients. TERT promoter mutations were more frequent in HCC patients infected with hepatitis C virus<sup>[7,8,39,41,42,47,48,50]</sup> than in those infected by HBV. One study suggested that this phenomenon could be explained by the high rate of HBV DNA insertions in the TERT promoter<sup>[42]</sup>. Furthermore, several studies reported higher TERT promoter mutations rate in men<sup>[7,39,42]</sup>, in older patients<sup>[7,50]</sup>, in patients with smoking<sup>[51]</sup>, in patients with smaller tumors<sup>[42]</sup>, in patients with low serum levels of alpha-fetoprotein<sup>[42]</sup>, and in patients with *CTNNB1* mutations<sup>[8,42,47]</sup>, while other papers either disagreed with or did not find these associations.

Further, TERT promoter mutations are early somatic genetic alterations in hepatocarcinogenesis, playing important roles in malignant transformation of preneoplastic cirrhotic lesions<sup>[42,52]</sup>. Nault *et al.*<sup>[52]</sup> found that the frequency of TERT promoter mutations increased as premalignant lesions transformed into HCC, from 6% in low-grade dysplastic nodules and 19% in high-grade dysplastic nodules to 61% in early HCC and 42% in small and progressed HCC; mutations in 10 other recurrent genes only emerged in small and progressed HCC. Similarly, Huang *et al.*<sup>[43]</sup> demonstrated that the mutation rates also increased in a stepwise manner during advanced HCC progression and reached a maximum of 45% in patients with stage C. Calderaro *et al.*<sup>[53]</sup> found that there were 64.6% (208/322) cases with TERT promoter mutations; HCC phenotypes were tightly associated with gene mutations, including TERT promoter mutations, and transcriptomic classification.

As the proportion of nonalcoholic fatty liver disease (NAFLD)-related HCC patients is increasing due to increased prevalence of metabolic syndrome, especially in Western countries<sup>[54-56]</sup>, there have been studies investigating TERT promoter mutations in NAFLD-related HCC. One research analyzed the genetic aberrations of 11 tumor samples from 10 NAFLD-HCC patients and found that TERT promoter mutation C228T occurred in 9/11 (82%) cases<sup>[56]</sup>. On the contrary, in another study, the prevalence of TERT promoter mutations C228T and C250T was very low (3.2%) in patients with NAFLD<sup>[57]</sup>. Obviously, the TERT promoter mutation state in NAFLD-related HCC is far from conclusive.

### TERT promoter insertional mutations by HBV DNA integration

HBV infection has been shown to be a causative factor of HCC, especially in Asians where chronic hepatitis B infection is prevalent. Integration of HBV DNA into the human genome of HCC cells is evident in HBV-related HCC<sup>[8,40,48,58-64]</sup>. Several lines of evidence demonstrate that the integration sites of HBV are not random. Integration of certain genomic sites, including near or within the genes of TERT<sup>[8,48,59-65]</sup>, MLL4<sup>[48,59,61-63,65]</sup> and CCNE1<sup>[48,61-63,65]</sup> are more frequently identified in HCC<sup>[48]</sup>.

To date, 13 independent studies have identified a total of 262 integrations of HBV DNA in the *TERT* gene, meaning that in more than 20% HBV-related HCC cases, *TERT* gene is interrupted by HBV integration<sup>[7,58,65-75]</sup>. *TERT* is the most susceptible gene for HBV integration, followed by MLL4 (79 integrations), CCNE1 (22 integrations) and CCNA2 (19 integrations)<sup>[76]</sup>. According to our pool analysis of the results from these articles<sup>[7,58,65-75]</sup>, among the 262 HBV integrations in *TERT*, 73.28% (192/262) occur in the *TERT* promoter region, including 26% in the core functional fragment (-223 bp to -14 bp from the ATG translational start site). As the regulation of *TERT* expression largely depends on the activity of the *TERT* promoter region, especially the core functional fragment, HBV integration in the *TERT* promoter may have an important functional role in HCC development.

A few studies suggested that HBV tended to integrate in common chromosomal fragile sites, where DNA replication was delayed and DNA sequences were more susceptible to breakage<sup>[63,64]</sup>. Nevertheless, the findings that *TERT* was a recurrent integration site but not a fragile site demand new explanation<sup>[64]</sup>. More recent studies have therefore presented new possibilities. One study proposed that HBV preferentially integrates into *TERT* gene because disruption at these loci lowers the threshold for malignant transformation and thus grants a selective advantage to carcinogenesis<sup>[59]</sup>. Another two studies, using a similar line of reasoning, suggested that the recurrence of HBV integrations into *TERT* promoter region in HCC could be due to the potential growth advantage that augmented *TERT* expression provides for the clonal expansion and carcinogenesis of hepatocytes<sup>[60,62]</sup>. In TCGA database, the HCC with HBV DNA insertion into the *TERT* promoter displays the highest level of *TERT* RNA expression among all HCCs, suggesting an HBV cis-activating event did exist<sup>[48]</sup>.

HBV integrations promote the development of HCC by inducing global genomic instability, elevating expression of adjacent genes, viral-host fusion transcripts and secondary mutations of host or viral genes, as well as by DNA copy number variations and proteins with oncogenic activity (*X* and *preS* gene products)<sup>[58,61,64,65]</sup>. Recently, based on the discovery that both HBV integration and somatic mutations in the *TERT* promoter were more frequent in male patients with HCC, Li et al.<sup>[69]</sup> proposed a novel mechanism in which sex hormones, along with GABPA play a role in regulating *TERT* expression. They analyzed 101 HBV-related HCC cases using a capture-next-generation sequencing platform and concluded with convincing evidence that the integration of HBV DNA, whose sequence contains both androgen- and estrogen-responsive elements, into the *TERT* promoter permits the androgen-receptor to up-regulate and the estrogen-receptor to down-regulate *TERT* transcription in a HNF4 $\alpha$ -dependent manner<sup>[62]</sup>.

### OTHER GENOMIC AND EPIGENOMIC ALTERATIONS ON TERT PROMOTER IN HCC

#### TERT amplification in HCC

Totoki et al.<sup>[8]</sup> showed that *TERT* focal amplification was detected in 6.7% of the total 608 cases. Schulze et al.<sup>[77]</sup> observed less than 5% of *TERT* focal amplification in the 243 liver tumors. However, while both studies described the occurrence of *TERT* focal amplification in HCC, none of them investigated its effect on *TERT* expression level. Thus, more research is needed to confirm the role of *TERT* amplification in liver carcinogenesis.

#### Epigenetic modification of TERT promoter in HCC

As for epigenetic regulation of *TERT* promoter in HCC, Iliopoulos et al.<sup>[78]</sup> observed a strong negative correlation between *TERT* promoter methylation and *TERT* expression in all liver tissues they studied,

proposing for the first time that the hypermethylation of TERT promoter and the methylation of histone H3-K9 resulted in the inhibition of c-Myc binding in E-box 1, which in turn inactivated TERT expression. However, this result contrasts with previous studies, which showed that TERT promoter epigenetic modification had either a positive correlation or no correlation with TERT expression and telomerase activity in other cancer types<sup>[79-83]</sup>. A more recent study examining 125 HCC cases in the Han Chinese population found that the promoter of the *TERT* gene is significantly hypermethylated, and it further showed that the hypermethylation is associated with higher expression of TERT, suggesting that TERT promoter hypermethylation contributes to the progression of liver carcinogenesis via elevating TERT expression level<sup>[84]</sup>. Overall, there is no definite conclusion regarding whether hypermethylation of TERT promoter has a positive or negative correlation with TERT expression and telomerase activity.

## MECHANISMS OF TERT PROMOTER MUTATIONS CONTRIBUTING TO THE DEVELOPMENT OF HCC AND OTHER CANCERS

Although TERT promoter mutations are strongly associated with several cancers, the mechanism by which TERT promoter mutations lead to cancer development is not fully understood. How TERT promoter mutations increase TERT expression and whether the up-regulation of TERT directly translates into active telomerase activity that eventually contributes to tumorigenesis are two important questions requiring answers.

### Mechanisms of TERT promoter in other cancers

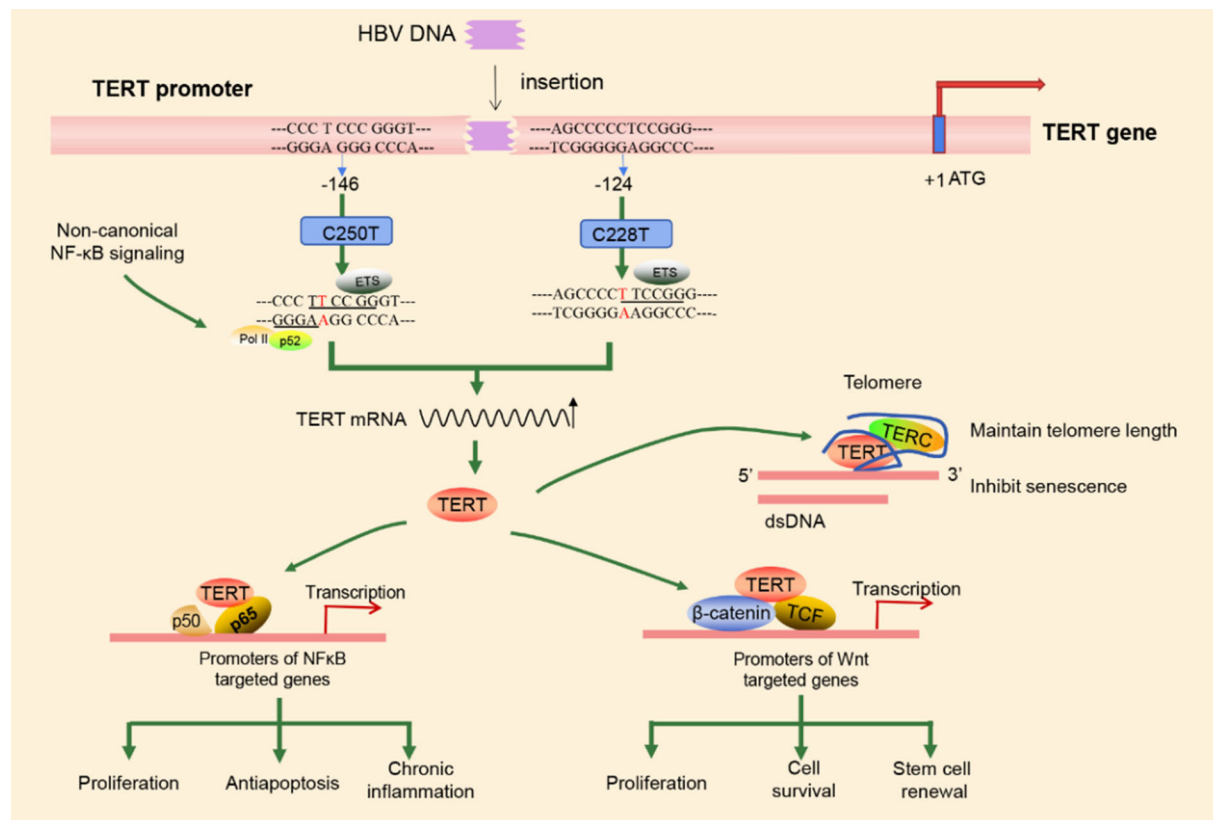
It is currently accepted that C228T and C250T, the two most common mutation types in TERT promoter region, both create an 11-bp binding motif (5'-CCCCTTCCGGG-3') for E-twenty-six (ETS) transcription factors<sup>[26,85,86]</sup>. In glioblastoma, a total of five ETS transcription factors were found (ELF1, ETS1, ETV3, ETV4 and GABPA) that modulate TERT expression. GABPA complexes with GABPB to form a fully functional heterodimer GABP transactivator, it was the only factor that reproducibly regulated TERT expression in a mutation-specific manner<sup>[86]</sup>. Akincilar *et al.*<sup>[24]</sup>, using cell lines from several cancer types, including melanoma, glioblastoma, colon, and prostate cancers, *etc.*, reported that TERT promoter mutations enhanced the binding of GABPA, mediating long-range chromatin interaction (at chr5: 1,556,087-1,558,758, a region 300 kb upstream of promoter), enrichment of active histone markers H3K4Me3 and H3K9Ac and subsequent POL2 recruitment, thus driving TERT transcription. Another study suggested a slightly different mechanism. According to work by Li *et al.*<sup>[85]</sup>, the TERT promoter with C250T mutation was driven by NF- $\kappa$ B signaling. On activation of this signaling pathway, p52 (NF- $\kappa$ B2) is recruited to the C250T region, but not the C228T region, and cooperates with ETS factors ETS1/2 to drive efficient TERT transcription<sup>[85]</sup>. TERT promoter mutations are widely found together with BRAF V600E alteration in human cancers, particularly in thyroid cancer and melanoma<sup>[87-92]</sup>. A recent study found that that TERT promoter mutations and BRAF V600E cooperatively upregulated TERT expression and promoted the oncogenic behaviors in the papillary thyroid cancer cells<sup>[93]</sup>.

### Mechanisms of TERT promoter mutations in HCC

TERT promoter mutation was a later oncogenic event. Pilati *et al.*<sup>[94]</sup> have screened TERT promoter in a large series of liver cancers including adenomas, borderline lesions hepatocellular adenomas (HCA)/HCC, HCC derived from adenomas and classical HCC, and found TERT promoter mutations did not exist in classical adenomas, but in borderline lesions HCA/HCC (17%) and HCC cases derived from adenomas (56%) which frequency was similar to that in classical HCC (54%).

There are only a few studies focusing on the mechanism of how TERT promoter mutations influence TERT expression and lead to malignant transformation of liver cells [Figure 1]. Telomerase activation is important to maintaining telomere length that confers cancer cells infinite ability to overcome the proliferation barrier. One study demonstrated that *TERT* mRNA expression and telomerase activity were higher in patients with





**Figure 1.** Proposed model for telomerase reactivation by telomerase reverse transcriptase (TERT) promoter mutations. The C228T and C250T TERT promoter mutation both create an E-twenty-six (ETS) binding motif (the mutational hotspots are in red) to modulate TERT mRNA expression. P52 (NF-κB2) is recruited to the C250T region, but not the C228T region, and cooperates with ETS factors to drive efficient TERT transcription. The elevated TERT expression enhances cell malignant behavior through a telomere lengthening-dependent manner (maintaining telomere length or inhibiting senescence), and/or a telomere lengthening-independent manner (TERT acting as a transcriptional modulator regulating genes related to Wnt and NF-κB signaling pathways thereby promoting cell proliferation, antiapoptosis, and stem cell renewal). Hepatitis B virus (HBV) DNA insertion into TERT promoter is another possible mechanism of hepatocarcinogenesis, which may cause HBV promoter/enhancer-driven transcription of TERT

HCC who had both single nucleotide polymorphism (SNP) rs2853669 and promoter mutations of *TERT* gene<sup>[95]</sup>. The rs2853669 variant and the TERT promoter mutation C228T combined to induce TERT promoter methylation and increase TERT expression, resulting in a longer telomere length compared to the wild-type rs2853669 and TERT promoter<sup>[95]</sup>.

In recent years, TERT has been considered to have some other direct effects on carcinogenesis in addition to its function on maintaining telomere length<sup>[96]</sup>. Studies revealed that TERT acts as a transcriptional activator that activates the transcription of genes targeted by Wnt and NF-κB signaling to play a role in cell proliferation, antiapoptosis, and stem cell renewal<sup>[96,97]</sup>. In HCC, TERT expression level was higher in almost all cases with TERT promoter mutations than that in those without the mutations, and elevated TERT expression is closely related to the development of HCC<sup>[42,94]</sup>. Based on the significant association between TERT promoter and CTNNB1 mutations as well as previous studies showing the interaction between TERT and Wnt/β-catenin pathway, it was proposed that TERT promoter mutations and activation of the Wnt/β-catenin pathway together lead to malignant transformation<sup>[42,97]</sup>. By contrast, another research revealed that, while TERT expression did increase in the HCC cohort overall, it was not significantly correlated with TERT promoter mutations<sup>[48]</sup>. They suggested that TERT promoter mutations might cooperate with CDKN2A silencing to promote *TERT* mRNA expression. CDKN2A gene encodes the tumor suppressor gene p16<sup>INK4A</sup>, whose down-regulation together with up-regulated TERT expression is critical for epithelia cell

immortalization<sup>[98]</sup>. Anyhow, there are only a few studies focusing on the mechanisms of TERT promoter mutations in HCC. Whether it shares the same mechanisms with other cancers requires further research in the future.

### TERT PROMOTER MUTATIONS IN DIAGNOSIS, PROGNOSIS AND THERAPY OF HCC

A study detected the TERT promoter mutations in plasma cell-free DNA (cfDNA) in 218 patients with HCC, and the prevalence of TERT mutations was 47.7%, which was similar to the prevalence (44.4%) of 196 HCCs derived from the TCGA database<sup>[57]</sup>. Meanwhile, they also measured the prevalence of TERT promoter mutations in cfDNA of 81 patients with cirrhosis, and the frequency was 8.6%<sup>[57]</sup>. Since the frequency of TERT promoter mutations gradually increases during the process of cirrhosis and liver cancer, the TERT promoter mutations in the cfDNA in the serum can be detected as an important index for evaluating the development of HCC. However, there still remains a problem with specificity since the TERT promoter mutation is very common in various tumors so that the mutations in cfDNA cannot accurately reveal the source of the lesion.

The prognostic value of TERT promoter mutations remains controversial. Kawai-Kitahata *et al.*<sup>[7]</sup> and Huang *et al.*<sup>[43]</sup> performed survival analyses and demonstrated that TERT promoter mutations were associated with poor overall survival and could be prognostic markers for HCC<sup>[7,43]</sup>. However, Ko *et al.*<sup>[95]</sup> found that the presence of TERT promoter mutations alone did not translate into poor prognosis, but that the SNP rs2853669 and the -124C>T mutation combined were associated with poor survival rates. Further, Lee *et al.*<sup>[39]</sup> reported that longer telomere length, but not TERT promoter mutations, was independently associated with poor overall survival. Besides showing TERT promoter mutations' correlation with poorer overall survival in HCC, Li *et al.*<sup>[99]</sup> also demonstrated that TERT amplifications were associated with shortened overall survival independent of other clinicopathological parameters such as age, gender and TNM staging. Thus, while we are sure that genetic changes at *TERT* gene have prognostic value, we are uncertain about exactly which factor(s) - TERT promoter mutations alone, the combination of the SNP rs2853669 and the -124C>T mutation, longer telomere length or TERT amplifications - directly indicate(s) poor prognosis.

It is believed that TERT is a promising but also challenging driver gene to target. There are no drugs specifically targeting *TERT* gene yet, although a few inhibitors have been used to target amplified genes in HCC: epidermal growth factor receptor inhibitors like Gefitinib targeting amplified EGFR, MET, MAPK1, MAPK3 and CRKL, Crizotinib and vemurafenib targeting BRAF and ERBB2, and alisertib targeting amplified AURKA<sup>[99]</sup>. According to Dhanasekaran *et al.*<sup>[100]</sup>, the somatic mutations associated with liver tumor development lie in genes whose products are not easily or safely targeted, and that mutant TERT, TP53, CTNNB1, and MYC are even believed to be undruggable. Nevertheless, the study also reveals that a synthetic TERT DNA vaccine, INO-1400, is being tested in a phase 1 trial of patients with solid tumors (NCT02960594) and that some trials are using TERT promoter mutation as a biomarker for study enrollment (NCT02766270)<sup>[100]</sup>. Since a traditional strategy to target TERT is challenging, it is suggested that new strategies, such as microRNA-based therapeutics, should be developed to target driver genes like *TERT* or their pathways<sup>[100]</sup>. In fact, one study explored the potential of a novel immunotherapy using TERT-derived peptide (TERT461) as a vaccine by investigating its safety and immunogenicity and characterizing the TERT-specific T cell responses induced<sup>[101]</sup>. Their results showed that the vaccination induced TERT-specific immunity in 10/14 (71.4%) of the patients, and that 57.1% of patients treated with TERT461 peptide-specific T cells could prevent HCC recurrence after vaccination<sup>[101]</sup>. Another study also concluded that CypB, SART2, SART3, p53, MRP3, AFP, and TERT are promising tumor-associated antigens (TAAs) in HCC immunotherapy<sup>[102]</sup>. Besides, not only do they suggest that the administration of the TAAs or peptides containing their epitopes as vaccines after HCC treatment is likely to be effective, but they also demonstrated that the concurrent use of anti-CTLA-4 antibodies may further improve antitumor immunity<sup>[102]</sup>. Therefore,

while it remains challenging to target *TERT* gene, new strategies are emerging to achieve this goal and make more effective therapy possible.

## CONCLUSION

Our knowledge regarding the role of *TERT* promoter mutations in HCC is expanding; nevertheless, there remain many puzzles to be solved. Although the pattern of *TERT* promoter mutations in HCC is well-established, little is known about the mechanism through which *TERT* promoter mutations reactivate telomerase and promote tumor development. We are not yet sure how either somatic mutations or HBV integrations in the *TERT* promoter lead to malignant transformation and whether they can be prognostic biomarkers in HCC; nevertheless, we are confident that untangling the mechanisms relevant to *TERT* promoter can be a key for developing target therapy for HCC.

## DECLARATIONS

### Authors' contributions

Writing the initial manuscript: Ma ZX, Yang CM

Revision of the manuscript: Yang CM, Ma ZX, Li MG

Drafting the outline of the manuscript, critical revision of the manuscript for intellectual content, finalizing the manuscript, and obtaining the funding: Tu H

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Review

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# Molecular diagnosis of hepatocellular carcinoma: trends in biomarkers combination to enhance early cancer detection

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## Abstract

Despite of the advances in clinical imaging and applied research in proteomic biomarkers, liver cancer, especially hepatocellular carcinoma remains detected at the very late and advanced stages when curable treatments are unavailable and ineffective. In this regard, there are still huge unmet medical needs in developing and clinically validating those high-potential protein biomarkers preferably in liquid biopsy samples. This review provides a glimpse of emerging biomarkers together with detection tools and techniques which are potentially commercially available to the markets. We also discuss several diagnostic biomarkers having therapeutic potential for developing first-in-class medicines.

**Keywords:** Hepatocellular carcinoma, biomarkers, targets,  $\alpha$ -fetoprotein, cadherin-17, Yes-associated protein, AXL, Trop2

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related deaths worldwide<sup>[1]</sup>. The prognosis of HCC is generally poor, especially for late-stage malignancies, but a cure is possible if it is diagnosed at the early stages. In fact, 5-year survival for early stage HCC after curative treatments is as high as 70%<sup>[2]</sup>. This highlights the uttermost importance of having a convenient, accurate and affordable diagnostic technique for early stage HCC.



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The current clinical diagnosis of early HCC is mainly based on medical imaging, including ultrasonography, computed tomography (CT) and magnetic resonance imaging. Early stage HCC is classified by having less than 4 tumour nodules with less than 3 cm in diameter<sup>[3]</sup>. The sensitivity and specificity for ultrasonography to pick up these tumour nodules are 60% and 97%, respectively, while CT has sensitivity and specificity of 68% and 93%, respectively<sup>[4]</sup>. There have been several attempts to increase the sensitivity by combining imaging with  $\alpha$ -fetoprotein (AFP) biomarker but with limited success so far, indicating the urgent need for new potential biomarkers<sup>[5,6]</sup>. Many patients have no access to diagnostic imaging due to the lack of necessary equipment and imaging specialists in local and regional hospitals.

In this review, clinically approved biomarker, such as AFP, will be discussed in detail followed by updates on other diagnostic biomarkers under development, including AXL, thioredoxin and golgi protein-73 (GP73). A few promising HCC targets that can be used as both a diagnostic and a therapeutic biomarkers, such as glypican 3 (GPC3), Yes-associated protein 1 (YAP1), trophoblast cell-surface antigen 2 (Trop2) and vasorin (VASN), will also be described. Nucleic acids-based biomarkers, such as non-coding RNA, are beyond the scope of this review and are covered elsewhere in this special issue.

## DIAGNOSTIC BIOMARKERS FOR HCC

### AFP

AFP is the most well studied biomarker for HCC and is also the first biomarker approved for HCC detection in liquid biopsy. It is a 591 amino acids glycoprotein encoded by the *AFP* gene on human chromosome 4 (4q13). AFP transports a variety of molecules, including fatty acids and bilirubins, across the body<sup>[7]</sup>. It is mainly produced by the visceral endoderm of the yolk sac and fetal liver during development<sup>[8]</sup>. The highest AFP plasma concentration is detected during week 12 to week 16 of a fetal life and subsequently declines to virtually undetectable after birth<sup>[9]</sup>. However, unusually high serum concentration of AFP is also detected in patients with HCC<sup>[10,11]</sup>.

Nonetheless, the use of AFP as a biomarker for HCC has been controversial ever since its discovery nearly half a century ago<sup>[12,13]</sup>. One study that evaluated AFP as a standalone HCC biomarker on 5,581 men in China exhibited sensitivity and specificity of 55.3% and 86.5%, respectively<sup>[14]</sup>. Although more early stage HCCs were reported in the test group than in the control group, there was no survival benefit in the test group. Another study that evaluated 18,816 patients with chronic hepatitis B demonstrated that combining ultrasonography with AFP test in a biannual screening scheme reduced the mortality of HCC in the test group by 37%<sup>[15]</sup>. However, given the high false positive rate and additional costs, some argued the practicality of recommending such a biannual screening scheme<sup>[5]</sup>. A systematic review of five trials conducted on patients with hepatitis C, a high risk group, between 1999 and 2002 concluded that AFP had limited ability to detect early HCC<sup>[13]</sup>. The high false positive rate is not only because only 61% of HCC expresses AFP<sup>[16]</sup>, but also the fact that AFP expression is detected in other liver abnormalities such as cirrhosis and acute hepatitis<sup>[17]</sup> and other tumours, including endodermal sinus tumour<sup>[18]</sup> and gastrointestinal malignancies<sup>[19]</sup>.

AFP exists in three different glycoforms, namely AFP-L1, AFP-L2 and AFP-L3. Interestingly, AFP-L3 expression only increases in HCC but not in hepatitis or cirrhosis, suggesting that it could be a better HCC biomarker<sup>[20]</sup>. However, a trial using AFP-L3 as the sole biomarker on 372 patients with hepatitis C virus demonstrated sensitivity of only 37%, despite a specificity of 92%<sup>[21]</sup>. In the same study, combining AFP-L3 with another biomarker, des-gamma-carboxy prothrombin increased the sensitivity to 61% but sacrificed the specificity down to 71%. Another phase II study using the combo for early stage HCC reported sensitivity and specificity of 78% and 62%, respectively<sup>[22]</sup>. The very low sensitivity is probably because AFP-L3 is minimally expressed and usually undetectable when the patients' AFP level is below 20 ng/mL. Kagebayashi *et al.*<sup>[23]</sup> utilized a microfluidic device in an attempt to detect low level of AFP-L3 in patient serum but reported

**Table 1. Performance of various HCC diagnostic biomarkers and tools**

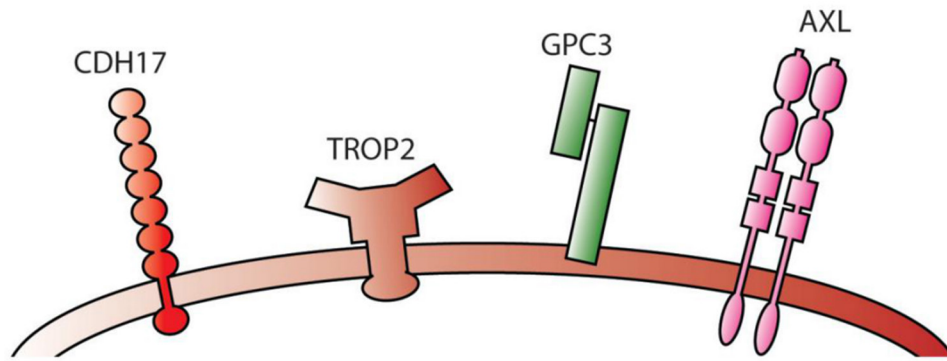
Biomarker	Sensitivity (%)	Specificity (%)	Note	Ref.
Ultrasonography	60	97	Meta-analysis on 14 studies	[4]
CT	68	93	Meta-analysis on 14 studies	[4]
AFP	55	87	$n = 5,581$	[14]
	66	82	Early stage HCC, $n = 836$	[22]
	59	89	Early stage HCC, $n = 1,100$	[47]
	35	88	Meta-analysis on 19 studies	[75]
	41-65	80-94	Meta-analysis on 5 studies, cirrhotic patients	[139]
	97	40	$n = 100$	[140]
	58	85	$n = 4,217$	[52]
AFP-L3	37	92	$n = 372$	[21]
	57	64	Detect using microfluidic device	[23]
DCP	61	70	Early stage HCC, $n = 836$	[22]
AFP-L3 + DCP	61	71	$n = 372$	[21]
	78	62	Early stage HCC, $n = 208$	[22]
AXL	71	73	$n = 584$	[43]
AXL + AFP	84	92	$n = 584$	[43]
Thioredoxin	75	89	Early stage HCC, $n = 1,100$	[47]
Thioredoxin + AFP	83	94	Early stage HCC, $n = 1,100$	[47]
GP73	75	97	$n = 4,217$	[52]
GP73 + AFP	89	85	$n = 4,217$	[52]
GPC3	55	84	Meta-analysis on 19 studies	[75]
	55	97	Early stage HCC, meta-analysis on 19 studies	[75]
OPN	75	62	Early stage HCC, $n = 312$	[126]
SCCA	84	49	$n = 961$	[141]
Annexin A2	83	68	Early stage HCC, $n = 224$	[142]
Annexin A2 + AFP	87	68	Early stage HCC, $n = 224$	[142]
suPAR	76	90	$n = 267$	[143]
MDK	93	83	$n = 100$	[140]

CT: computed tomography; AFP:  $\alpha$ -fetoprotein; GPC3: glypican 3; MDK: Midkine; OPN: osteopontin; SCCA: squamous cell carcinoma antigen; suPAR: soluble urokinase plasminogen activator receptor; DCP: des-gamma-carboxy prothrombin; HCC: hepatocellular carcinoma

sensitivity and specificity of only 57% and 64%, respectively. Comparing AFP-L3 with AFP, Marrero *et al.*<sup>[22]</sup> concluded that AFP was more sensitive than AFP-L3 for detecting early HCC. Taken together, these results suggest that AFP-L3, despite having higher specificity, is inferior than AFP as an HCC biomarker. The low sensitivity of AFP encourages combining AFP with other biomarkers that are significantly overexpressed in HCC [Table 1]. Three of these biomarkers that have performed extraordinarily when used in combination with AFP are AXL, thioredoxin and GP73.

## AXL

AXL is a receptor tyrosine kinase that is expressed in a number of malignancies, including HCC<sup>[24]</sup>, lung cancer<sup>[25]</sup>, ovarian cancer<sup>[26]</sup>, colon cancer<sup>[27]</sup>, breast cancer<sup>[28]</sup> and pancreatic ductal adenocarcinoma<sup>[29]</sup> [Figure 1]. AXL is stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. Stimulated AXL in turn activates the PI3K-AKT-mTOR, MEK-ERK, NF- $\kappa$ B and JAK/STAT signaling pathways that lead to tumour growth, immune escape and drug resistance<sup>[30-35]</sup>. AXL is also expressed in normal bone marrow stroma and myeloid cells to clear apoptotic material, suppress inflammatory responses and control natural killer cell activity<sup>[36,37]</sup>. Loss of AXL, therefore, leads to inflammation and autoimmunity<sup>[38,39]</sup>. AXL is a key downstream target that drives YAP-dependent oncogenic functions<sup>[40]</sup>. Knocking down AXL by RNAi decreased the ability of YAP-expressing MIHA and the primary HCC cell line to proliferate and invade. Furthermore, AXL also serves as a putative entry receptor for Zika Virus, Ebola Virus and West Nile Virus to infect the host cells<sup>[41]</sup>. Activated AXL undergoes proteolytic processing to yield a soluble protein that can be detected in the serum<sup>[42]</sup>. Detection of very early HCC (i.e., BCLC stage 0) by soluble AXL (sAXL)



**Figure 1.** Cartoon depicting structures of cadherin-17 (CDH17), Trop2, glypican 3 (GPC3) and AXL

showed an area under the curve (AUC) of 0.848 upon receiver operating characteristic curve analysis. The sensitivity and specificity of the detection was 76.9% and 69.2%, respectively. The accuracy of the detection was increased to 0.936 by the combined use of sAXL and AFP. Interestingly, sAXL combined with AFP could differentiate very early HCC from liver cirrhosis with an accuracy of 0.901, of which the sensitivity and specificity was 88.5% and 76.7%, respectively<sup>[43]</sup>. Nevertheless, multicenter clinical studies are needed to validate these findings.

### Thioredoxin

Thioredoxin, together with thioredoxin reductase, forms a ubiquitous oxidoreductase system that plays an important role in regulating intracellular redox environment, controlling cellular proliferation and providing defense mechanism against oxidative stress<sup>[44]</sup>. Thioredoxin expression is detected in HCC, non-small cell lung cancer and colorectal cancer<sup>[45,46]</sup>. It is generally associated with a more aggressive tumour phenotype, poor prognosis and a lower survival rate. As a sole early stage HCC biomarker, Li *et al.*<sup>[47]</sup> found that thioredoxin (sensitivity, 75%; specificity, 89%) surprisingly outperformed AFP (sensitivity, 70%; specificity, 79%) in their study. When used in combination, they could detect early HCC with an impressive sensitivity and specificity of 83% and 94%, respectively. This supports the idea that an ideal combination of biomarkers can outperform a single biomarker in giving both lower false positive and false negative rates.

### GP73

GP73 is a 400 amino acid, type II Golgi-specific membrane glycoprotein normally expresses on epithelial cells of liver and kidney<sup>[48]</sup>. GP73 resides within the cis-Golgi complex but it can be secreted into the extracellular space by cleavage at the proprotein convertase site<sup>[49]</sup>. In fact, soluble GP73 was detected in the medium cultured with HeLa, foreskin fibroblasts (HCA) and osteosarcoma (MG63) cell lines, suggesting that it may have some functions in the extracellular environment<sup>[50]</sup>. A number of studies noted elevated level of serum GP73 in HCC patients<sup>[49,51]</sup>. Mao *et al.*<sup>[52]</sup> compared serum GP73 and AFP biomarkers in 4,217 subjects with a mixture of healthy adults, HBV carriers and patients with cirrhosis, HCC or others cancers. They found GP73 to be a superior biomarker (sensitivity: 75%, specificity: 97%) than AFP (sensitivity 58%, specificity 85%). The combination of both biomarkers improved the sensitivity further to 89% but with a drop-in specificity down to 85%.

### Annexin A2

Annexin A2 (ANXA2), a member of the annexin family, is a 36-kDa calcium-dependent phospholipid-binding protein that plays a role in immune responses, phospholipase A2 regulation and anti-inflammation. The serum ANXA2 was found to be elevated in HCC patients ( $n = 50$ ) as compared with patients with chronic disease ( $n = 30$ ) or healthy subjects ( $n = 20$ ) by ELISA<sup>[53]</sup>. In the same study, follistatin, a potential serological HCC biomarker, was found elevated in both HCC patients and patients with chronic liver



disease. The authors highlighted the superiority of ANXA2 over follistatin but given the low number of patient samples, more comprehensive studies are required to draw a conclusion.

## BIOMARKER COMBINATIONS FOR HCC DIAGNOSIS

In addition to individual biomarkers and those combined with AFP, biomarker combinations for the diagnosis of early HCC have also been extensively studied. Cytokeratin-1 (CK-1) and nuclear matrix protein-52 (NMP-52) elevated in sera of patients with HCC. Combination of CK-1, NMP-52 and AFP showed an AUC of 0.9 for identifying HCC with 80% sensitivity and 92% specificity. More interestingly, this triple combination could differentiate HCC from liver fibrosis with an AUC of 0.94 with 80% sensitivity and 92% specificity<sup>[54]</sup>. Epithelial membrane antigen and fibronectin, of which the serum levels were increased in HCC, when in conjunction with total bilirubin and AFP, could identify HCC from cirrhosis with an AUC of 0.92 with 89% sensitivity and 85% specificity<sup>[55]</sup>. Combined use of plasma protein with immune cells in HCC diagnosis has been published as well. A combination of plasma Dickkopf-1, Tie2-expressing monocytes and AFP yielded an AUC of 0.833 for HCC diagnosis<sup>[56]</sup>. The clinical utility of these biomarker combinations undoubtedly requires further validation in independent cohorts.

## Diagnostic biomarkers with therapeutic potential

Although early diagnosis of HCC should translate into better overall survival, Chen *et al.*<sup>[14]</sup> did not find this link in their study, citing lack of effective treatment as the main reason. Biomarkers that can serve as both a diagnostic tool and a therapeutic target would, undoubtedly, be more beneficial to the patients, as the diagnostic results can immediately assist physicians in planning treatment regimen. A number of promising biomarkers of this type are discussed below.

## GPC3

GPC3 is a member of heparin sulfate proteoglycan family, which is bound to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor<sup>[57]</sup>. A total of 6 glypicans have been identified to date, namely GPC1 to GPC6, and they are predominantly expressed during development<sup>[58]</sup>. The amino acid sequence homologies amongst glypicans are low but the location of 14 cysteine residues are conserved, indicating that they may share similar high-dimension structures. The location of the heparin sulfate insertion sites of the glypicans appears to be restricted to the C terminus, putting the heparin sulfate chains near to the cell membrane<sup>[58]</sup>. GPC3 is a 580 amino acid protein encoded by the *GPC3* gene located on human chromosome X (Xq26). Despite being a cell membrane protein, GPC3 is cleaved by the Notum lipase at the GPI anchor and released into the serum<sup>[59]</sup>, making it easy for clinical detection. GPC3 is frequently upregulated in HCC and melanoma<sup>[60]</sup>. By fixed tissue staining, GPC3 expression was detected in up to 72% of samples from patients with HCC, but not in healthy subjects or patients with benign liver diseases<sup>[59]</sup>. The mRNA level of GPC3 was also upregulated in HCC<sup>[61,62]</sup>. Moreover, at least three independent groups reported significant elevation of serum GPC3 in HCC but not hepatitis<sup>[63-65]</sup>. Taken together, the data strongly suggest GPC3 to be an attractive serum and histochemical biomarker for HCC.

GPC3 can stimulate Wnt signaling through canonical and non-canonical pathways, which are initiated by Wnt ligands and Frizzled receptors<sup>[66]</sup>. Given that Wnt proteins bind to heparin sulfate, it was suggested that GPC3 acts as a facilitator of the interaction between Wnt ligands and Frizzled receptors<sup>[66,67]</sup>. GPC3 may promote tumorigenesis by facilitating canonical Wnt signal activation, which is frequently observed in HCC<sup>[65,68,69]</sup>. In contrast, GPC3 expression is downregulated in breast and ovarian cancers, suggesting that the functions of GPC3 may be tissue-specific<sup>[70-72]</sup>. Indeed, GPC3 is found to be a negative regulator of Hedgehog signaling pathway. Downregulation of GPC3 causes hyperactive Hedgehog signaling, which promotes ovarian and breast cancer progression<sup>[73]</sup>. Filmus and Capurro<sup>[67]</sup> proposed that GPC3 may exert different functions depending on cell types. In tissues that proliferate mainly via Hedgehog signaling, overexpression of GPC3 has an inhibitory effect on proliferation whereas in tissues where canonical Wnt signaling exerts a dominant influence, upregulation of GPC3 promotes cell proliferation.

As a HCC diagnostic biomarker, GPC3 outperformed AFP in a number of independent studies. Tangkijvanich *et al.*<sup>[74]</sup> reported sensitivity of 56% for GPC3 while AFP stood at 33%. Interestingly, GPC3 overexpression did not correlate with AFP level, tumour size or stage of HCC but was significantly associated with the presence of viral hepatitis markers. A meta-analysis of 19 studies reported the superior sensitivity for GPC3 (pooled sensitivity, 55%; pooled specificity, 84%) over AFP (pooled sensitivity, 35%; pooled specificity, 88%)<sup>[75]</sup>. Notably, the specificity for GPC3 was significantly higher if the analysis focused only on early HCC (pooled sensitivity 97%).

The potential of GPC3 goes well beyond being a diagnostic biomarker. GPC3 is an oncofetal antigen, a protein that is predominantly expressed in cancer and during fetal development. Murine model injected with GPC3 transgenic colon cell line showed that GPC3 was able to elicit T-cell mediated tumour rejection without autoimmunity<sup>[76]</sup>. Similar results were reported using highly metastatic mouse melanoma<sup>[77]</sup>. Importantly, the anti-tumour effects appeared to be mediated by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which are essential for optimal anti-tumour response. CD4<sup>+</sup> T cells have a broad role in orchestrating host anti-tumour responses, such as secreting cytokines to enhance cytotoxic T cell response, activating eosinophils and tumouricidal macrophages and secreting granulocyte/macrophage colony-stimulating factor. These results encourage development of immunotherapy targeting against the tumor-specific GPC3 isoform.

A phase I clinical study of a GPC3-derived peptide vaccine for 33 advanced HCC patients demonstrated that the vaccine was well-tolerated<sup>[78]</sup>. Most patients had only grade I and grade II side effects. Four patients developed grade III hematological adverse events but were likely due to disease progression rather than the vaccine. In terms of efficacy, 1 patient showed partial response while 19 patients had stable disease 2 months after initiation of treatment. Given the favorable safety profile, it is rational to target GPC3 with other therapies, such as adoptive cell transfer. Along this line of thinking, there have been several Phase 1 clinical studies of chimeric antigen receptor (CAR)-T trials directed against the GPC3 antigen in HCC patients.

Recently, a phase I clinical trial of anti-GPC CAR-T cells was conducted on Chinese patients ( $n = 13$ ) with refractory or relapsed GPC3<sup>+</sup> HCC. The 3rd generation CAR-T was engineered with CD28, 4-1BB and CD3 $\zeta$  downstream signaling domains<sup>[79]</sup>. No dose-limiting toxicities were identified and only one patient experienced grade 3 fever<sup>[80]</sup>. Without lymphodepletion, none of the five patients responded to the treatment but with lymphodepletion, 4/6 (67%) clinical response was reported. The authors concluded that the anti-GPC3 CAR-T treatment is safe and tolerable.

A bispecific T cell-redirecting antibody that binds both GPC3 and CD3 is also under active development by Chugai Pharmaceutical (a Roche subsidiary). Early studies on animal models showed that the GPC3/CD3 bispecific antibody showed anti-tumour efficacy against various GPC3-positive xenografts including liver tumours<sup>[81]</sup>. This bispecific antibody is now being investigated in a phase I clinical trial on patients with GPC3 positive advanced solid tumours (NCT02748837).

### Cadherin-17

Cadherin-17 (CDH17) is a calcium-dependent cell adhesion molecule that belongs to the 7D-cadherin superfamily, characterized by the presence of 7 cadherin-like ectodomains followed by a short cytoplasmic tail<sup>[82]</sup>. It is normally present in fetal liver and gastrointestinal tract during embryogenesis, hence the name liver-intestinal cadherin (LI cadherin). It is a peptide transporter and plays an important role during embryonic gastrointestinal development<sup>[83,84]</sup>. CDH17 expression was reported in normal human colon, intestine and pancreas but not normal liver and stomach<sup>[85-89]</sup>. However, the overexpression of CDH17 was observed in HCC as well as breast, ductal pancreatic, colorectal and gastric cancers<sup>[90-93]</sup>. The upregulation was associated with malignant transformation of these cancers. Knock-down of CDH17 by RNAi inhibited proliferation of primary and metastatic HCC cell lines *in vitro* and *in vivo*<sup>[94]</sup>. This anti-tumour effect was likely due to inactivation of Wnt signaling pathway because CDH17-knockdown HCC tumours

showed re-localization of  $\beta$ -catenin to cytoplasm, concomitant reduction in cyclin D1 and increase in tumour suppressor retinoblastoma. In addition, CDH17 was reported as an useful diagnostic marker for adenocarcinomas of the digestive system<sup>[95]</sup>. It was also associated with bone marrow metastasis of breast cancer<sup>[96]</sup> and liver metastasis of colorectal cancer<sup>[93]</sup>.

CDH17 expression was upregulated by 2.5 to 800 folds in over 80% HCC but not in healthy liver, making it an attractive diagnostic and therapeutic biomarker for HCC<sup>[88]</sup>. Half of the CDH17<sup>+</sup> HCC patients have gained genomic copy of this gene. Importantly, alternately spliced mRNA transcripts, characterized by loss of exon 7, were reported in roughly half of the HCC patient specimens. The splicing introduced a premature stop codon in the open-reading frame and resulted in a truncated CDH17 protein. It was speculated that overexpression of the truncated variant may act as a dominant inhibitor of wild-type CDH17, thereby enhancing tumour invasion. In consistent to this speculation, expression of this variant CDH17 was strongly associated with poorer overall survival, higher risk of relapse and venous infiltration after hepatectomy. The spliced transcripts were only detected in HCC samples but not normal liver samples, implying that the splicing is likely to be an aberrant cancerous event rather than a normal splicing phenomenon. Importantly, an antibody against the RGD motif of CDH17 has shown promising anti-tumour effects against metastatic colon cancer and melanoma, suggesting that it is likely to be effective against HCC<sup>[97]</sup>.

## YAP1

YAP1, also known as YAP or YAP65, is an oncogene encoded by the *YAP1* gene located on human chromosome 11 (11q22)<sup>[98]</sup>. It is a downstream nuclear effector of the Hippo signaling pathway, which is important for development, cell proliferation, repair and homeostasis<sup>[99]</sup>. Given its importance in cell proliferation, YAP1 knockout mice showed development arrest and died prematurely<sup>[100]</sup>. Studies on the *Drosophila* Yorkie (Yki) protein, an ortholog of YAP1, suggested that YAP1 is negatively regulated by the Hippo pathway<sup>[101]</sup>. Inactivation of Hippo pathways leads to accumulation of Yki proteins in the nucleus and upregulation of genes associated with cell survival and proliferation, including *cycE*, *diap1/thread* and *bantam*<sup>[102]</sup>. In mammalian cells, overexpression of YAP1 caused aberrant expression of genes associated with cell proliferation, anti-apoptosis, survival and migration, such as *CTGF*, *CCND1*, *ITGB2* and *BCL2L1*<sup>[103]</sup>.

Analysis on 177 HCC samples by immunohistochemistry, Western blot analysis and RT-PCR showed that approximately 62% of both YAP protein and mRNA were upregulated as compared to adjacent non-tumour tissues<sup>[104]</sup>. The YAP proteins were mainly accumulated in the tumour nucleus. In an independent study, Zhao *et al*<sup>[101]</sup> also reported YAP overexpression in 63 of the 115 HCC samples tested by tissue microarray. Similar results were reported in non-small lung cell cancer, suggesting that YAP may have broad implications in different solid cancers<sup>[105]</sup>. Importantly, YAP expression was associated with poorer tumour differentiation, high serum AFP level and lower overall survival rate, indicating that it may be used as an independent prognostic marker<sup>[104]</sup>.

Both YAP and transcriptional co-activator with PDZ-binding motif (TAZ) are downstream effectors of Hippo pathway. Hayashi *et al*<sup>[106]</sup> reported that knocking down TAZ, under normal condition, inhibited cell growth in HCC. However, treating the TAZ knockdown cells with 5-fluorouracil induced YAP expression that conferred chemoresistance. The drug resistance was not observed when both TAZ and YAP were knockdown, suggesting that a shift to predominantly YAP expression when TAZ was depleted led to chemoresistance and tumorigenicity. The authors concluded that targeting both YAP and ZAP is essential for a complete anti-tumour response. Given that YAP expression is an early event in HCC tumorigenesis and its expression is critical to chemoresistance and proliferation of malignant hepatocytes, YAP is a promising HCC target for therapeutic intervention.

The oncogenic activity of YAP depends on its interaction with transcriptional enhancer activation domain family member 1 (TEAD1) that resides in the nucleus and therefore, disrupting YAP-TEAD1 interaction is

believed to have anti-cancer efficacy in YAP positive tumours<sup>[107]</sup>. YAP-like peptides occupying the interface 3 on YAP/TEAD complex were shown able to block YAP-TEAD1 interaction<sup>[108]</sup>. Small-molecule inhibitors (SMIs) targeting the same interface were recently shown to suppress the expression of YAP target genes<sup>[109]</sup>. Whether these peptides or SMIs would inhibit tumour growth in vivo however remains to be investigated.

## Trop2

Trop2, also known as tumour-associated calcium signal transducer 2 (TACSTD2) or epithelial glycoprotein-1 antigen, is a calcium signal transducer encoded by the *TACSTD2* gene located on human chromosome 1 (1p32.1). Trop2 is a cell surface glycoprotein that is associated with regulation of cyclin D1 and protein kinase C levels. Trop2 stimulates the expression of cyclin D1 and cyclin E via the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway to promote cell proliferation<sup>[110]</sup>. Numerous reports have confirmed that Trop2 is an oncogene associated with tumour development, progression and metastasis in various cancers, including pancreatic cancer<sup>[111]</sup>, squamous cell carcinoma<sup>[112]</sup>, gastric carcinoma<sup>[113]</sup>, hilar cholangiocarcinoma<sup>[114]</sup>, colorectal cancer<sup>[115]</sup>, cervical cancer<sup>[116]</sup>, ovarian carcinoma<sup>[117]</sup>, gallbladder cancer<sup>[118]</sup> and breast cancer<sup>[119]</sup>. Unsurprisingly, Trop2 overexpression is often associated with poor cancer prognosis. A recent gene network analytic study found aberrant expression of Trop2 in HCC<sup>[120]</sup>. Given that Trop2 is an oncogene in many cancers, it is speculated that it may be a potential biomarker candidate and a therapeutic target for HCC.

## VASN

VASN is a cell surface and secreted protein that modulates the arterial response to injury by inhibiting the TFG- $\beta$  signaling pathway<sup>[121,122]</sup>. It was identified as a potential HCC biomarker using a subtractive EMSA-SELEX strategy from AFP negative serum of HCC patients with secondary metastasis<sup>[123]</sup>. VASN expression can be detected in aorta, kidney, placenta, brain, heart, liver, lung and skeletal muscle tissues. It was highly expressed in HCC samples ( $n = 100$ ) but not in normal liver ( $n = 97$ ) or hepatitis samples ( $n = 129$ ), as verified by both Western blotting and quantitative PCR. This high VASN expression appeared to be negatively regulated by microRNAs miR145 and miR146a. Downregulation of these microRNAs led to overexpression of VASN, which promoted cell proliferation and migration and inhibited apoptosis. As a membrane protein, VASN has the potential to be a therapeutic target.

## Osteopontin

Osteopontin (OPN), a matrix glycoprotein secreted by a wide variety of cell types, has also emerged as a biomarker with diagnostic potential<sup>[124]</sup>. Plasma level of OPN in patients with HCC was significantly higher than in or healthy subjects or patients with chronic liver diseases<sup>[125]</sup>. In a prospective study on 22 patients who developed HCC during follow-up, OPN was elevated in plasma one year before cancer diagnosis<sup>[126]</sup>. A meta-analysis on 12 published studies showed that the sensitivity in HCC diagnosis was higher than that of AFP (OPN, 0.813; AFP, 0.639)<sup>[127]</sup>. Plasma OPN could be used to differentiate HCC from other non-malignant liver diseases including chronic hepatitis C, cirrhosis, and nonalcoholic fatty liver disease<sup>[128]</sup>. Importantly, serum OPN was associated with dismal overall survivals of patients with HCC with a hazard ratio of 2.38<sup>[129]</sup>.

In addition to its diagnostic value, OPN can be a potential therapeutic target for HCC treatment. Antiviral therapy suppressed early progression of hepatitis B-related HCC by modulating the expression of OPN in patients<sup>[130]</sup>. Knockdown of OPN using RNA interference suppressed in vivo growth and lung metastasis of liver cancer xenograft in mice<sup>[131]</sup>. Monoclonal antibodies (mAbs) against OPN have been reported to demonstrate anti-cancer effects in animal models. An antibody named AOM1, which abrogated the integrin binding of OPN, was illustrated to suppress the in vivo of Kras-mutant non-small cell lung adenocarcinoma in mice<sup>[132]</sup>. Hu1A12, another OPN mAb that bound to the calcium binding domain of OPN, was demonstrated to inhibit primary tumor growth and spontaneous metastasis in a mouse lung metastasis

model of human breast cancer<sup>[133]</sup>. There studies strongly support OPN as a potential target for the antibody-based cancer therapy, although the anti-cancer efficacy of OPN mAbs in HCC has remained to be studied.

## CONCLUSION

HCC is an extremely difficult to treat cancer, which generally involves multiple pathologic complications including hepatitis, metabolic (NASH and diabetic), fibrotic and cirrhotic diseased conditions in addition to the notorious tumor burden. As a result, both the current diagnosis and treatments of HCC remain largely ineffective. Therefore, bringing new biomarkers and innovative treatments to the patients are in critical demand. With the recent advent of cancer immunotherapy, it is more than ever necessary to find tumour-specific biomarkers as therapeutic targets. One of the major challenges in immunotherapy on solid tumours is the extreme scarcity of highly specific targets<sup>[134]</sup>. Failure to find such a target has led to not only ineffective treatment but also high toxicities and even deaths in clinical trials<sup>[135-138]</sup>. Despite being extremely rare and highly challenging, fortunately, recent advances in next-generation sequencing and high-throughput technologies would, undoubtedly, accelerate discovery of such biomarkers and make progress for the diagnosis and treatment of HCC.

## DECLARATIONS

### Authors' contributions

Wrote the manuscript: Chia TS, Wong KF, Luk JM

Reviewed the manuscript: Luk JM

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All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

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Review

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# Novel insights of HBV RNA in hepatitis B virus pathogenesis and clinical application

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## Abstract

Hepatitis B virus (HBV) infection is still a severe health problem in the world, and chronic hepatitis B (CHB) is the major cause of serious HBV-related complications, including fibrosis, hepatic failure, and hepatocellular carcinoma. It is difficult for CHB patients to achieve complete cure as the currently available antiviral drugs can hardly eradicate covalently closed circular DNA (cccDNA) in the infected liver. Since detecting intrahepatic cccDNA needs invasive procedure, it is urgent to find a noninvasive indicator to reflect the activity of cccDNA. Recently, growing numbers of studies have indicated that serum HBV RNA could be regarded as a new biomarker for CHB activity. In order to illustrate the molecular biology and clinical characteristics of HBV RNA, we systematically reviewed the latest research to summarize the role of HBV RNA in HBV replication and pathogenicity, and to better estimate its potential function as a remarkable biomarker in clinical application. Meanwhile, we will also point out the deficiencies of current research, and discuss the future direction of HBV RNA study.

**Keywords:** Hepatitis B virus, pregenomic RNA, serum biomarker, chronic hepatitis B, hepatocellular carcinoma, cirrhosis

## INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most common communicable diseases, with over 240 million people chronically infected all over the world<sup>[1]</sup>. Chronic hepatitis B (CHB) is the major etiological cause



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of developing serious liver complications, such as cirrhosis, hepatic failure, and primary hepatocellular carcinoma. Although the application of anti-HBV drugs [i.e., nucleos(t)ide analogues and pegylated interferon (peg-IFN)] can effectively suppress HBV replication and decrease the occurrence of corresponding complications, still more than 680,000 patients die of the fatal consequences of CHB every year<sup>[2]</sup>. The proportion of cirrhosis and liver cancer caused by HBV infection is about 30% and 45% worldwide. In China, the proportion is 60% and 80%, which is much higher<sup>[3]</sup>. Therefore, it is urgent to acquire a better understanding of virus-host interaction to develop new therapeutics that increases the HBV cure rate.

HBV is an enveloped DNA virus which contains a 3.2 kb circular, partially double-stranded DNA genome. HBV establishes its genome as a covalently closed circular DNA (cccDNA) in the nucleus of the infected hepatocytes<sup>[4]</sup>. In the life cycle of HBV, the transcription of cccDNA to generate HBV mRNA is the beginning of HBV replication and the key factor for the continuous infection. Therefore, cccDNA is the most direct evidence of HBV infection and replication *in vivo*. Since the detection of cccDNA counts on biopsy, which is an invasive procedure, it is warrant to find some serum biomarkers that reflect the activity of intrahepatic cccDNA. The cccDNA is transcribed to 5 mRNAs during the viral replication. One of these transcripts, the pregenomic RNA (pgRNA) is not only the template for reversing transcription of viral DNA but also the coding mRNA for core protein and polymerase of HBV (pol)<sup>[5]</sup>. Recently, an increasing number of studies suggest that the HBV RNA can also be detected in serum. Circulating HBV RNA is HBV pgRNA and it may be used as a new serum biomarker for HBV infection, treatment and prognosis<sup>[6-8]</sup>. Thus, further comprehension of HBV pgRNA may provide new horizon for the better understanding the virus-host interaction and the development of new HBV therapeutics. In this paper, we will review the current knowledge on the clinical significance of HBV RNA and its biological impact on host liver cells.

## THE ORIGIN AND NATURE OF HBV RNA

The life cycle of HBV begins with the invasion of viral particles containing a 3.2-kb long partially double-stranded genome called relaxed circular DNA (rcDNA) into the hepatocytes through the sodium taurocholate co-transporting polypeptide (NTCP) receptor. The rcDNA is converted into cccDNA when coming into the nucleus<sup>[9]</sup>. The cccDNA plays a role as the transcription template for all the viral transcripts, involving the 3.5 kb pgRNA and precore mRNA (pre-C RNA), the 2.4 kb and 2.1 kb surface mRNAs, and a 0.7 kb X mRNA<sup>[10]</sup>. Among the 5 HBV mRNA, pgRNA not only serves as the template for reverse transcription of HBV, but also is the template for translation of pol and core proteins<sup>[11]</sup>. The 5'-ε region of pgRNA has the ability to combine with the pol. Once combined, they are packaged into viral capsid<sup>[12]</sup>. And inside the capsid, with the assist of the pol, pgRNA produces rcDNA through reverse transcription. Since newly created viral capsids can re-infect the nucleus, a small part of the newly formed rcDNAs re-enter into the nucleus to replenish the cccDNA pool. The remaining capsids are enveloped by the viral surface protein and released as Dane particles to infect new cells<sup>[13]</sup>. A recent research shows that HBV RNA can be detected in the serum of CHB patients, especially in those who have been taking antiviral drugs for a long time with their serum HBV DNA low or even undetectable. The research also revealed that serum HBV RNA is pgRNA which was presented in the virion-like particles, and serum pgRNA is produced by the transcription of cccDNA inside the hepatocytes<sup>[8]</sup>. The discovery of HBV RNA virion-like particle may complete the traditional life cycle of HBV infection. In theory, after the encapsidated pgRNAs get into hepatocytes, the reverse transcription to form rcDNA and cccDNA might be restarted, and this process eventually leads to HBV re-infection. However, more and stronger evidences are needed to prove the infection potential of HBV RNA virion-like particles.

## ROLES OF HBV RNA IN HBV-ASSOCIATED DISEASES

HBV RNA not only is the template for both viral DNA reverse transcription and viral protein synthesis, but also plays a crucial role in the pathogenesis of HBV-associated diseases. Recently, many studies reveal

that HBV RNA itself contributes to the progression of HBV-associated diseases through direct and indirect ways<sup>[14-23]</sup>. Nucleos(t)ide analogues (NAs) exerts its antiviral function through inhibiting the reverse transcription of HBV. As cccDNA is unaffected and its transcriptional activity remains, the formation of HBV RNAs continues. Therefore, in some CHB patients, although HBV DNA was maintained at extremely low level by anti-HBV drugs, serious HBV-related complications still occurred.

### HBV RNA and CHB

Persistent infection of HBV is the main cause of CHB, and continuous virus replication will eventually results in the inflammation and fibrosis of liver, which is the key characteristic of CHB. The effect of HBV RNA on HBV replication other than serving as reverse transcription template is poorly understood by now. However, HBV RNA may facilitate viral replication through deregulating the functions of host microRNAs. For example, one of the micro RNAs which is highly and specifically expressed in hepatocytes, miR-122, inhibits the replication of HBV in the liver. It has been reported that HBV RNA could act as sponges to bind and sequester endogenous miR-122, then the down-regulated expression or decreased function of miR-122 would increase level of cyclin G1, which further represses the expression of p53, leading to upregulation of HBV transcription via blocking specific combination of p53 with HBV enhancer elements<sup>[14,16]</sup>. It also has been reported that the miR-15 family might regulate HBV replication. The overexpression of the miR-15 family members, miR-15a and miR-16-1, inhibits HBV replication. As HBV RNA can sequester these miRNAs, cyclin D1, the target of miR-15a and miR-16-1, is up-regulation, which makes a significant contribution HBV replication<sup>[17]</sup>. Furthermore, the viral-derived miRNA, miR-3, suppressed the transcription of pgRNA and HBc protein translation by targeting the 3.5-kb transcript of HBV<sup>[18]</sup>. Yang *et al.*<sup>[18]</sup> thought that the inhibition of HBV replication might contribute to the development of persistent infection in CHB patients. However, more and further studies are need to verify his hypothesis.

### HBV RNA and hepatic fibrosis

The cycle of continuous inflammation caused by viral replication and self-repairing of hepatocytes leads to the accumulation of extracellular matrix proteins, and eventually results in the development of fibrosis. The transforming growth factor- $\beta$  (TGF- $\beta$ ) pathway and nuclear factor- $\kappa$ B pathway together play an important role in the process of liver fibrosis. As mentioned above, miR-122 is down-regulated through sequestration caused by the role of HBV RNA as miRNA sponge in HBV-infected liver<sup>[14]</sup>. The altered expression of miR-122 activates the synthesis of collagen via the TGF- $\beta$  pathway, which participates in the liver fibrosis process<sup>[19]</sup>. Furthermore, the study of Sato *et al.*<sup>[20]</sup> indicated that the inflammatory factors induced by the 5'- $\epsilon$  region of HBV pgRNA may aggravate the degree of inflammation and exacerbate the fibrosis of liver.

### HBV RNA and hepatocellular carcinoma

Although the direct relationship between HBV RNA and the occurrence of hepatocellular carcinoma (HCC) has not been reported so far, the study of Wang *et al.*<sup>[6]</sup> showed that serum HBVRNA level correlates with the intrahepatic HBVRNA level, and serum HBVRNA level reflects the severity of histological changes and is associated with liver disease progression during NAs therapy, even in the CHB patients whose viral replication is suppressed. This means that serum HBV RNA is associated with the degree of intrahepatic inflammation and may be used as a new biomarker for reflecting hepatocarcinogenesis, especially in the CHB patients whose serum HBV DNA is suppressed by NAs therapy. It has also been reported that the 5'- $\epsilon$  region of HBV pgRNA induces the production of interferons and inflammatory cytokines in hepatocytes, which may lead to histological changes and the aggravation of fibrosis<sup>[20]</sup>. As we all know, the cirrhosis caused by liver fibrosis is a precancerous lesion of HCC.

These results together show that HBV RNA itself might promote the occurrence of HCC, at least to some extent. Moreover, the study of Halgand *et al.*<sup>[7]</sup> showed that HBV pgRNA is detectable more frequently in HCC non-tumor tissues (90%) than in HCC tumor tissues (67%). When detectable in both compartments, the level of pgRNA was higher in non-tumor tissues than in tumor tissues. And the detection of pgRNA in

tumour was correlated to the absence of tumorous microvascular invasion and better survival of patients. The results of microarrays and analysis of gene expression profiles showed that pgRNA positive HCCs were characterized by lower expression of cell cycle and DNA repair markers, and higher expression of the HBV receptor NTCP, which indicates a well-differentiated tumor. The replication of HBV in HCC may represent a sub-type of weakly invasive and hyper-differentiated HCC<sup>[7]</sup>. The possible mechanism of this phenomenon might be because the high metabolic status in poorly differentiated HCC is not suitable for the survival of HBV. Furthermore, it has also been reported that the circulating HBV RNA may be used as a biomarker for predicting the occurrence of HCC<sup>[21,22]</sup>.

HBV RNA, as miRNA sponge, can also promote the carcinogenesis of HCC by sequestration of host miRNAs. For example, HBV RNA could bind and sequester endogenous miR-122 through sponge adsorption, which upregulates PTTG1-binding protein and promotes the growth and invasion of HCC<sup>[14]</sup>. miRNA let-7 family is considered as tumor suppressors miRNAs. The expression of miRNA let-7 family is decreased in HCC, and it inhibits the progression of HCC via suppressing oncogenic targets, such as LIN28B, HMGA2 and c-Myc. Studies have demonstrated that let-7 family miRNAs (e.g., let-7a and let-7g) could be adsorbed and sequestered by HBV RNA, resulting in the promotion of tumorigenesis of HCC<sup>[15,23]</sup>.

### CLINICAL SIGNIFICANCE OF HBV RNA

In most CHB patients, the application of NAs potentially decreases HBV DNA and is associated with HBV induced complications. However, as the function of NAs is blocking HBV reverse transcription, the cccDNA is unaffected. The formation of pgRNA and the produce of HBV proteins would still continue in a long period of time. Hence cccDNA is the ultimate root of HBV replication. Since detecting cccDNA depends on liver biopsy, serum biomarkers reflecting the intrahepatic cccDNA activity are warranted. Giersch *et al.*<sup>[24]</sup> found that levels of serum pgRNA significantly correlated with hepatocyte pgRNA levels in humanized uPA/SCID/beige (USB) mouse model of HBV infection treated with NAs and peg-IFN- $\alpha$ , while in untreated HBV-infected mice, serum pgRNA levels not only apparently correlated with hepatocyte pgRNA levels, but also clearly correlated with intrahepatic cccDNA levels, indicating that serum pgRNA might serve as a useful clinical indicator to estimate the intrahepatic activity of cccDNA in HBV-infected patients<sup>[24]</sup>. Recently, more and more studies suggested that the pgRNA can be detected in serum and it may serve as a potent serum biomarker for reflecting the dynamic change of HBV replication.

### Evaluating the efficacy of CHB patients receiving NAs therapy

During NAs therapy, it is important to monitor the dynamics of serum HBV DNA for assessing the virological response (VR) of CHB patients. According to clinical practice guidelines, VR is defined as serum HBV DNA being under the lowest limit of detection during NAs treatment, and that has been regarded as withdrawal indication of NAs therapy<sup>[3,25-27]</sup>. However, the virological rebound and hepatitis relapse often occurred when CHB patients discontinued the application of NAs. As mentioned above, the existence and transcription of cccDNA cannot be affected by NA. So merely detecting serum HBV DNA may not completely reflect the activity of cccDNA in CHB patients under NAs therapy. As HBV pgRNA can be detected in serum and its level reflects the transcriptional activity of intrahepatic cccDNA<sup>[6]</sup>, it is more valuable to detect both serum HBV pgRNA and serum HBV DNA than detecting serum HBV DNA alone when it comes to the better prediction of VR during NAs therapy. A growing number of studies have inferred that serum HBV RNA can be a useful marker for evaluating the efficacy of antiviral therapy<sup>[28-31]</sup>. Huang *et al.*<sup>[31]</sup> demonstrated that in CHB patients receiving NAs therapy, the low serum HBV RNA levels at week 12 of treatment could predict the initial VR. Another related study revealed that in HBeAg-positive CHB patients treated with NAs, baseline serum 3' full-length polyadenylated HBV RNA (flRNA) expression level could predict HBeAg seroconversion, and the decline of serum HBV RNA also showed a higher possibility of HBeAg seroconversion compared with HBV DNA and HBsAg during antiviral treatment<sup>[29]</sup>.

Although these studies mentioned above showed that serum HBV RNA may have great potential to act as a supplementary biomarker for judging the effect of antiviral therapy, it remains unclear whether serum HBV RNA is superior to existing biomarkers, or whether it can replace other biomarkers for the same clinical applications.

### Monitoring safe discontinuation of NA-therapy in CHB patients

As HBV cccDNA cannot be completely cleared during NAs therapy, most CHB patients have been suffering from virological rebound and HBV relapse, making it difficult to decide the timing of NAs therapy withdrawal. So, the majority of CHB patients have to receive NAs therapy for a long time, even their entire lifetime, which aggravates the financial burden for both the patients and the society<sup>[26,27]</sup>. As mentioned above, serum HBV RNA could be regarded as a potential indicator for cccDNA activity, so the vanishment of serum HBV RNA may represent the transcription silence of cccDNA. Therefore, serum HBV RNA could serve as a potential predictable marker for safe withdrawal of NAs therapy<sup>[24,32]</sup>. A study on 36 CHB patients treated with NAs for at least 6 months revealed that after discontinuation of NA therapy for 24 weeks, their HBV DNA and HBV RNA titer on the third month of treatment was significantly associated with HBV DNA rebound and alanine aminotransferase rebound<sup>[33]</sup>. Another study of 33 CHB patients who had received NAs treatment for at least 3 years and whose serum HBV DNA was undetectable afterwards showed that all patients with HBV RNA positive experienced virological rebound at the end of treatment after withdrawal of NAs for 24 weeks, while virological rebound occurred in only 25% of patients with negative serum HBV RNA<sup>[8]</sup>. However, as the sample size of these studies and the follow-up time are insufficient, additional studies with larger sample size and longer follow-up time are needed to further verify whether HBV RNA can be used as a predictive biomarker to reflect the rebound of HBV after discontinuation of antiviral treatment.

### To assess the prognosis of HBV-associated HCC

Few studies have reported the relationship between HBV RNA and the prognosis of HBV-associated HCC. A recent study on 99 HBsAg-positive, virologically suppressed patients treated by tumour resection or liver transplantation indicated that HBV pgRNA was detectable more frequently in non-tumor (55/61; 90%) than in tumor samples (40/60 (67%);  $P < 0.01$ ). When detectable in both compartments, the levels of pgRNA were slightly higher in non-tumor than in tumor samples. Moreover, the detection of pgRNA in HCC is significantly associated with lower incidence of vascular invasion and better survival rate. HCC expressing higher HBV pgRNA may represent a kind of well differentiated, less-proliferative and low-invasive HCC subtype<sup>[7]</sup>. Therefore, HBV pgRNA might be used as a new biomarker for assessing the prognosis of HCC. However, in consideration of the insufficient sample size of this study, further research is still needed. Moreover, serum circulating HBV RNA may act as a biomarker for predicting the occurrence of HCC, which needs further study<sup>[21,22]</sup>.

### THE MEASUREMENT OF HBV RNA

For the first time, Kock *et al.*<sup>[34]</sup> successfully detected the HBV RNA in the serum of CHB patients through the method of rapid amplification of complementary DNA (cDNA)-ends (RACE) in 1996. The specific primer with a special anchored sequence was used to form cDNA after the extraction of HBV RNA from CHB patient serum. To ensure the high specificity for HBV RNA amplification, cDNA was amplified by PCR with HBV-specific forward primer and the reverse primer which is identical to the special anchored sequence. Since then, similar methods have been used to detect intrahepatic and serum HBV RNA in CHB patients<sup>[35,36]</sup>. Using unique primers designed for reverse transcription, Kairat *et al.*<sup>[37]</sup> developed RACE-based real-time quantitative PCR to specifically quantify serum 3' flRNA and 3' internally truncated polyadenylated HBV RNA later. Conventional RT-qPCR method with HBV-specific primers was also used to quantify intrahepatic and serum HBV RNA. However, DNase I pretreatment of the nucleic acids extracted is necessary to avoid DNA contamination before RT-qPCR<sup>[7,8]</sup>. Recently, super-sensitive droplet digital PCR was used to quantify serum HBV RNA by Wang *et al.*<sup>[6]</sup> with HBV-specific primers. Collectively, many



methods can be used to detect and quantify HBV RNA. As the widely accepted standardized method for HBV RNA detection is not available, further studies are needed to develop a more accurate and reliable technique of HBV RNA detection and quantification.

## **FUTURE STRATEGY ON HBV RNA**

Before HBV RNA can be applied as a biomarker in clinic for CHB patients on a large scale, there are still many questions that need to be solved. Firstly, the methodology for detecting and quantifying serum HBV RNA should be standardized to make it possible that the results of different studies are comparable. Secondly, more clinical and molecular biology research is needed to further clarify details dynamics of HBV RNA under different conditions in CHB patients, for example, different HBV replication states, different stages of CHB, and receiving what kind of antiviral drugs, NAs therapy or interferon therapy. Thirdly, more studies should focus on detecting HBV RNA among different ethnic groups and genotypes of CHB patients. Moreover, further and more exploration should be made to illuminate the correlation between HBV RNA and hepatocarcinogenesis.

## **CONCLUSION**

In this review, we summarized the current progress and knowledge on the role of HBV RNA in HBV replication and pathogenicity. As mentioned above, HBV RNA may reflect the activity of intrahepatic cccDNA, even in CHB patients whose HBV DNA is maintained at low or undetectable levels through long-term antiviral therapy. And HBV RNA might play an important role in viral replication, promoting cirrhosis, and hepatocarcinogenesis. Moreover, serum HBV RNA has the potential of evaluating the efficacy of anti-viral drugs and predicting safe discontinuation of NA-therapy. And the intrahepatic HBV pgRNA could be used for assessing the prognosis of HCC. Therefore, HBV RNA possesses great potentials to be a new surrogate or complementary biomarker for HBV DNA in CHB patients. However, more research concerning the molecular biology of HBV RNA and more multi-centered and large-scale cohort studies should be conducted to assess and testify the feasibility and safety of HBV RNA as a novel biomarker for CHB in the future. Moreover, better understanding of HBV RNA will also provide new methods and strategies for anti-HBV therapy.

## **DECLARATIONS**

### **Authors' contributions**

Contributed the central idea and wrote the initial draft of this paper: Ding WB

Refining the ideas, carrying out the editing, revision, and finalizing of the manuscript: Zhou WP, Yang F

Discussed the ideas and did the literature research: Wang MC, Zhang JN, Sun DP, Dong JP

Writing and revisions of the manuscript: All authors

### **Availability of data and materials**

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### **Conflicts of interest**

All authors declared that there are no conflicts of interest.

**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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Editorial

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# HCC incidence and recurrence after DAAs: new insights

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Hepatit C virus (HCV) infection is a global health and economic problem in the world. It is the cause of chronic active hepatitis, liver cirrhosis, hepatic decompensation, hepatocellular carcinoma (HCC) and extra-hepatic manifestations. Before 2013, standard care of HCV infection was the combination of Pegylated Interferon (PEG IFN) and Ribavirin. Sustained Virologic Response (SVR) rate of this combination was approximately 50% after 48 weeks of therapy. One fifth of patients who had this combination had to stop treatment because of severe side effects. Adding of Telaprevir or Boceprevir (first generation of protease inhibitors) to PEG IFN and Ribavirin therapy for 24 or 48 weeks result in a SVR rate around 70%. However, because of severe adverse events, these combinations are not recommended.

HCV infection is the second most common cause of death in man, and incidence of HCC varies between 2%-8% in cirrhotic patients in a year. In a number of studies, it was shown that IFN-based treatments reduce the complication of advanced liver diseases such as decompensation, or HCC, liver related or all causes of mortality<sup>[1-6]</sup>. Although occurrence of SVR after the treatment of IFN-based or IFN free therapies is associated with a decrease of HCC occurrence, it does not eliminate the disease entirely, and HCC occurs annually in a rate of 0.4%-2% in advanced liver disease<sup>[5]</sup>.

Ninety HCV related cirrhotic patients were 1:1 randomized to receive IFN alfa thrice in a week for 12-24 weeks and as controls. SVR rate was 16% and HCC occurred in 19 patients who were followed up for 2-7 years. Two out of 19 patients had SVR while the remaining 17 did not have SVR ( $P = 0.002$ )<sup>[6]</sup>.

Direct-Acting Antivirals (DAAs) is a revolution for the treatment of HCV infection with a more than 95% of SVR rates. Long-term results of treatment with DAAs on liver parenchymal disorders, hepatic



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decompensation and occurrence or recurrence rates after radical treatment of HCC are not fully understood.

Recently several studies reported unexpectedly high occurrence or recurrence rates of HCC after DAAs treatment for HCV and curative treatment for HCC<sup>[1,7,8]</sup>.

In a retrospective study, 344 patients with liver cirrhosis were treated with DAAs. 285 of those patients did not have a history of HCC, but 59 patients did. SVR rate was 91%. Six months after follow-up, 26 patients were shown to have HCC (7.6%). HCC occurred in 9 out of 285 (3.2%) patients without a history of HCC, while recurrence of HCC developed in 17 (28.8%) patients with a previous history of HCC. Besides, disease free survival was short as a median of 376 days in this group. They postulated that SVR after the treatment with DAAs was not associated with the reduced risk of HCC occurrence. However this study had several gaps such as historical control was used instead of normal control group, absence of information in detail about surgical resection of HCC for 59 patients with a history of HCC preceding the treatment of DAAs, and radiological control was performed in 6 months rather than 3 months. There were different HCC treatment modalities such as ablation, resection, and percutaneous ethanol injection. In addition, there were differences in terms of confounding factors between the treatment groups, and DAAs were started as a median of 376 days after the curative treatment of HCC. Normally HCC recurrence occurs at a rate of 20% after curative treatment of HCC. Unexpected increased rate of HCC recurrence (28.8%) may be a missed diagnosis of remnant HCC in several cases<sup>[1]</sup>.

In another similar study, 58 patients with HCV-related early HCC who achieved complete response after curative HCC treatment [resection *n*: 20; ablation *n*: 32; Trans Arterial Chemo Embolization (TACE) *n*: 6] were treated with DAAs after 11.2 months. SVR rate was 97.5%. Median follow-up time was 5.7 (0.4-14.6) months. Sixteen patients (27.6%) had unexpected recurrence of HCC in a median of 3.5 (1.1-8) months. It is difficult to definitely estimate early recurrence of HCC due to small sample size of study, high clinical, biological, and epidemiological heterogeneity of early HCC<sup>[7]</sup>.

In a meta-analysis which included 30 observational studies, 31,538 patients were treated for IFN-based regimen. SVR occurred in 10,853 patients (34.4%). Overall HCC occurrence was 5.5%. A hundred forty-five out of 9,185 patients (1.5%) with SVR developed HCC while 990 out of 16,312 patients (6.2%) had HCC. No matter if the patient had varying degrees of fibrosis or advanced liver disease, SVR was associated with 54% reduction in all causes of mortality, histologic improvement, risk for progression of liver disease, liver related mortality, and HCC<sup>[8]</sup>.

Ravi *et al.*<sup>[9]</sup> reported 6 out of 66 HCV patients with HCC (9.1%) after the treatment of DAAs during 6 months follow-up. Cardoso *et al.*<sup>[10]</sup> reported that HCC occurrence rate was 7.4% in 54 patients with cirrhotic HCV treated with DAAs after 12 months of follow-up. Yang *et al.*<sup>[11]</sup> compared the 81 patients who had liver transplantation in terms of HCC occurrence. The patients receiving DAAs in pre-liver transplantation showed 27.8% of HCC while those who did not take DAAs demonstrated 9.5% (6/63) of HCC. Among 17,487 patients who were treated with DAAs, 624 of them had HCC. 142 patients with HCC received liver transplantation. 482 patients still had HCC. Overall SVR was found 91% in non-HCC, 74% in HCC, 94% in transplanted due to HCC. It was concluded that the patients who had transplantation due to HCV and HCC can successfully be treated with DAAs with high SVR rates<sup>[12]</sup>.

In a large retrospective cohort study comparing 2,400 chronic hepatitis C patients treated with IFN and 490 untreated patients, all patients had a liver biopsy, and they were followed up for a mean of 4.3 years. HCC occurred in 89 treated and 59 untreated patients. It was found that both F2 and F3 patients had less HCC in treated group compared to untreated group ( $P = 0.0128$  for F2;  $P = 0.0011$  for F3 patients). Predictive factors for less HCC occurrence were SVR, normal Alanine Aminotransferase (ALT) levels, and less than two times



of the upper normal limits for aspartate aminotransferase (AST) levels<sup>[13]</sup>. In another retrospective study, 463 patients with HCV cirrhosis were treated with the combination of PEG interferon and Ribavirin. Three hundred of 463 patients had SVR (64.8%). Development of HCC was seen in 3 and 9 patients with SVR and non-SVR, respectively. It was found that SVR group had less HCC (1%) compared to non-SVR group (5.5%) ( $P = 0.005$ ) after 36.1 months follow-up<sup>[14]</sup>.

Innes *et al.*<sup>[15]</sup> reported that 857 patients with HCV infection were treated with IFN-based or IFN free regimens to compare the occurrence of HCC in both groups. Patients were followed up for 2.4 years, and 46 patients out of 857 had HCC. Incidence of HCC occurrence was two-fold high [(2.53 vs. 1.26 per 100 person years)  $P = 0.21$ ] in patients who took IFN free regimen. However, those patients were more thrombocytopenic, were at the higher Child Pugh stages, had more treatment experiences and were older than the patients who took IFN-based regimens. When confounding factors were corrected, there was no difference between two treatment regimens in terms of HCC occurrence. Adjusted (HR: 1.15, 95%CI: 0.49-2.71;  $P = 0.744$ ).

Similarly, Telep *et al.*<sup>[16]</sup> presented US administrative claims data which contained 4,887 patients who were curatively treated for HCC and HCV infection. Those patients were treated with IFN-based or IFN free treatment regimens. The latter patients were followed up for 182 days while the former were followed up 349 days. The patients in the IFN free treatment regimen had cirrhosis more frequently (95.7% vs. 88.2%), more liver necrosis (34.8% vs. 9.8%), more portal hypertension (58% vs. 35.3%) and were older than the patients who took IFN-based treatment regimen. Statistical difference could not be shown after adjusting the confounding factors (HR: 0.97; 95%CI: 0.49-1.92) between two treatment regimen groups in terms of HCC occurrence after following three, six and twelve months periods.

In a large retrospective cohort study, Group A ( $n = 3534$ , PEG IFN treated group) and Group B ( $n = 834$ , Sofosbuvir and Simeprevir or Sofosbuvir and Ledipasvir treatment group), Group C ( $n = 8468$ ) untreated group were compared for occurrence of HCC in patients with liver cirrhosis. There were not statistical differences for basic characteristics among the groups. Mean follow-up time was 2,719.2 days for IFN-treated persons and 396.4 days for DAAs-treated persons. It was found that there was no association between HCC occurrence rates and DAAs treatment compared to IFN treatment<sup>[17]</sup>.

In an Italian multi-centric study, 328 patients with HCV related early HCC followed up for the recurrence of HCC. Median time for the recurrence of HCC was 31 months (26-38) in the group of active hepatitis, 72 months in the group of SVR by Interferon free therapies, 82.3 months in group of SVR by Interferon based therapies. There were statistical differences between active hepatitis and entire SVR groups. However there was not a difference between Interferon free or Interferon based treatment groups. In the multivariate analysis, serum bilirubin, creatinine and alpha-feto protein (AFP) levels were found to be an independent predictor for recurrence of HCC<sup>[18]</sup>.

In a well-designed prospective large cohort study, 143 consecutive HCV infected patients who had complete response after curative treatment of HCC with stage Barcelona Clinic Liver Cancer (BCLC) 0/A were treated with DAAs. Those patients were followed up for a mean of 9.1 (3-19) months. SVR rate was 96%. The 6-, 12- and 18-month HCC recurrence rates in the whole cohort were 12%, 26.6% and 29.1%, respectively. The 6-, 12- and 18-month HCC recurrence rates in patients without prior history of HCC recurrences and in those with prior history of HCC recurrences were 9.2%, 20.9%, 24.2% and 18.5%, 39.7%, 39.7% respectively. Predictive factors for the recurrence of HCC were prior history of HCC and tumor size bigger than 2.5 cm in diameter<sup>[19]</sup>.

Sixty-eight consecutive cirrhotic patients with HCV and HCC under remission were treated with DAAs ( $n = 23$ ) or not treated ( $n = 45$ ). SVR rate reached 96%. Median time between HCC remission and initiation of

DAAs was 7.2 months, while between the time of starting of DAAs and HCC recurrence was 13.0 months. Recurrence rate was 1.7/100 person-months among treated patients *vs.* 4.2/100 person-months in not treated patients. HCC recurrence rate was significantly lower in patients who were treated with DAAs compared to untreated group<sup>[20]</sup>.

Mettke *et al.*<sup>[21]</sup> compared DAAs treated ( $n = 158$ ), and untreated control patients ( $n = 184$ ) in terms of HCC occurrence. Treated and untreated control patients were followed up for a mean of 440 (91-408) days and a mean of 592 (90-1000) days, respectively. HCC occurred in treated and untreated patients at rates of 2.9% ( $n = 6$ ) and 4.48% ( $n = 14$ ). They concluded that DAAs therapies do not change the short time occurrence of *de novo* HCC; however, it reduces HCC developing risk after 1.5 years.

In a prospective study of Calvaruso *et al.*<sup>[22]</sup>, in 2,249 patients with HCV related cirrhosis (Child Pugh A 90.5%, Child Pugh B 9.5%) were treated with DAAs. SVR occurred in 95.2% (2140/2249; Child Pugh A 95.9%, Child Pugh B 88.3%;  $P < 0.001$ ). Patients were followed-up for a median of 14 (6-24) months. In Child Pugh A patients who maintained SVR, HCC developed in 2.1% of the cases while HCC was seen in 6.6% of those cases without SVR. Accordingly, in Child Pugh B patients who maintained SVR, HCC developed in 7.8% of the cases while HCC was observed in 12.4% of those cases without SVR ( $P < 0.001$ ). The predictive factors for occurrence of HCC were the absence of SVR, serum albumin levels less than 3.5 g/dL, platelet level  $< 120 \times 10^9/L$ .

Patients with 218 Stage-1 and 226 Stage-2 were treated with PEG-IFN and ribavirin. Patients with SVR had less esophageal varices compared to non-SVR patients (HR 0.23; 95%CI: 0.11-0.48;  $P < 0.001$ ). However, there was no difference in terms of the progression of esophageal varices between the groups (HR 458; 95%CI: 0.33-1.03;  $P = 0.7$ ). SVR was found to be associated with reduced risk of HCC<sup>[23]</sup>.

In a multicenter retrospective study, 22,500 patients (39% cirrhotic) were treated with DAAs based regimens. The patients were followed up for 20 months; 19,500 of them had SVR (group A) and 2,982 did not have SVR. A hundred eighty-three patients (0.9%) with HCC were detected in Group A, while HCC occurred in 88 patients (3.4%) in Group B. (HR 0.28; 95%CI: 0.22-0.36;  $P < 0.0001$ ). Even if SVR occurred, the patients over 65 years of age and patients with advanced fibrosis or cirrhosis were associated to increased rates of HCC development. In this study, there were several comorbid conditions like alcohol use (61.4%), drug addiction (54.2%), and diabetes mellitus (43.6%) which all may facilitate HCC development. SVR was associated with 76% reduced risk of HCC occurrence<sup>[24]</sup>.

In a prospective study, 3,917 patients who included stage F3 fibrosis and CP-A cirrhosis were treated with DAAs based regimen. They were followed up with a mean of  $536.2 \pm 197.6$  days after the start of DAAs. Overall incidence of HCC was found to be 0.97% patients/year, 95%CI: 0.73-1.26. HCC incidence of cirrhotic patients was found to be 1.18% patients/year. When patients were stratified according to the stage of liver disease at baseline, HCC incidence rates during the first year of follow-up were 0.46x100 patients/year (95%CI: 0.12-1.17) in patients with fibrosis F3, 1.49'100 patients/year (95%CI: 1.03-2.08) in CTP-A cirrhosis and 3.61 100 patients/year (95%CI: 1.86-6.31) in CTP-B cirrhosis. HCC incidence rates in the second year of follow-up declined to 0% in F3, to 0.20'100 patients/year (95%CI: 0.05-0.51) in CTP-A cirrhosis and to 0.69'100 patients/year (95%CI: 0.08-2.49) in CTP-B cirrhosis and these differences were statistically significant (Mantel-Cox test,  $P = 0.00008$ )<sup>[25]</sup>.

In a retrospective study 421 patients who had HCV infection with or without cirrhosis were treated with DAAs therapy. Thirty-three per cent of patients had active or a history of HCC. Twenty-nine out of 421 patients resulted in failed SVR. Twenty-one per cent of patients who had HCC did not have SVR while, SVR failed in 12% of patients without HCC. Twenty-seven out of 29 patients who failed SVR resulted in active period of HCC. If DAAs treatments were given in an inactive period of HCC or after transplantation, SVR was excellent similar to those without HCC ( $P < 0.0001$ )<sup>[26]</sup>.

In a retrospective study 1,170 patients with HCV were treated with DAAs for 12-24 weeks. The patients were followed up for 1.3 years. Twenty-two patients had HCC during the follow-up. Cumulative incidences of HCC were 1.8% and 2.3% at 1 year and two years respectively. However, SVR was associated with reduced risk of HCC occurrence in 1.4% and 1.8% at 1 year and two years respectively. Non-SVR, hypoalbuminemia, thrombocytopenia, high AFP levels are risk factors for the development of HCC<sup>[27]</sup>.

The occurrence and recurrence rate of HCC is lower among patients who receive DAAs treatment compared to those who are not treated. HCV infection should be treated as early as possible in order to reduce the progression of parenchymal damage. Occurrence of SVR after treatment with both DAAs and Interferon is strongly associated with reduced developing or recurrence of HCC in patients with all fibrotic stages and advanced liver diseases compared to those patients who had no SVR. Occurrence of SVR does not exclude the development of HCC. However, reduced recurrence (0.4%-2%) of HCC may take a longer time than the patients who had active HCV infection. The patients who had SVR should be followed up periodically in every 3 or 6 months. Presence of active HCC reduces of SVR with the treatment of DAAs. The patients with HCV infection and active stage of HCC should be treated for HCV infection after curative treatment of HCC and/or after liver transplantation.

In our recently published multi-centric study, 200 patients with chronic hepatitis C were treated with the fixed dose combination of Sofosbuvir and Ledipasvir for 12 weeks. Thirty-five out of 200 patients had a history of HCC. Nineteen of those 35 patients had curative treatment at the beginning of anti-viral therapy. Median follow-up time was 22.1 months (15.7-30.3 months). Overall HCC occurrence was detected in 18 (9.0%) out of 200 patients. Recurrence of HCC was detected in 12 out of 16 (75%) patients who had non-curative treatments, while it was detected in 5 out of 19 (26.3%) patients who had curative treatments. This study also has several limitations. There is no control group, treatment modalities are different, time period between the beginning of anti-viral therapy and the time for recurrence of HCC varies between 3-14 months<sup>[28]</sup>.

Seventy-one million people are still infected with HCV infection in the world. DAAs are very effective with more than 95% of SVR rates. World Health Organization and some countries like Japan, Egypt, Mongolia, and Turkey have an elimination program for HCV infection. It may be an important problem to have a claim without evidence that DAAs treatment for HCV infection is associated with the occurrence and recurrence of HCC.

In conclusion, unexpected results concerning high occurrence and recurrence rates of HCC after the treatment of HCV infection with DAAs and complete curative treatment are heterogenic and incompatible in retrospective studies due to clinic, biologic, epidemiologic differences and methodological biases. In most of the studies which are HCV-related, occurrence and recurrence of HCC are retrospective and small case groups. Confounding factors such as age, sex, fibrotic stages, genotypes, Plt, AFP, serum albumine and bilirubine levels, number of liver cirrhosis are different between comparative groups. Curative treatment modalities such as radical surgery and Radio Frequency Ablation, TACE, Trans Arterial Radio Embolization of HCC are different. Prognosis of these modalities are different. Most of the articles did not describe pathological examinations, lymphatic and vascular infiltration of the tumor. Follow-up times are also different in similar studies. In order to make more precise decisions for occurrence or recurrence of HCC after DAAs treatment, we need to do large prospective randomized studies.

## DECLARATIONS

### Authors' contributions

Prof. Necati Örmeci contributed solely to the article.

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Author declared that there are no conflicts of interest.

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Not applicable.

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Review

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# Liver cancer screening in China: practices and its extended questions

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## Abstract

Screening for liver cancer (hepatocellular carcinoma) in China started in early 1970s with the application of alpha-fetoprotein (AFP) in high-incidence regions. It has been extended to nationwide areas, emerging from the concepts of conducting screening in populations at-risk with positive hepatitis B surface antigen to the practice programs in rural and urban areas, and finally to the development of recommendations to guide medical practice for health care providers. The implementation of screening for liver cancer has resulted in earlier detection and hence the early curable treatment for patients who have gained short- or long-term survival, and even reduction in mortality rates, although these outcomes are more anecdotal than rigorously evidence-based. AFP or ultrasound examination has been considered as sensitive and specific methods for early detection but are with limitations. The combined use of these two modalities for screening populations at-risk every six months seems to have been reached consensus. The feasibility of screening for liver cancer is still debated because of differing opinions and even opposition to the choice of targeted sub-populations, the intrinsic necessity, and the contributions of the main risk factors among Western countries and China/Asian areas. Yet, the over 51% of global burden of liver cancer is in China, the solution to the early detection and treatment of liver cancer should fully consider the actual situation in China. The effectiveness of screening for liver cancer is worthy of anticipation.

**Keywords:** Hepatocellular carcinoma, screening, alpha-fetoprotein, ultrasound, early detection, high risk population

## INTRODUCTION

Liver cancer [hepatocellular carcinoma (HCC)] is currently the second leading cause of cancer deaths worldwide, accounting for about 8.2% of the global burden of cancer<sup>[1]</sup>. China has the most patients with



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this disease. Based upon the Chinese Cancer Registry Annual Report 2016, the incidence and mortality rates of liver cancer were 28.17 and 24.70 per 100,000, respectively, thereby contributing to total incident cases of 394 thousand, and total death cases of 346 thousand a year in mainland China<sup>[2]</sup>. Liver cancer has long been a public health challenge in China. While improvements in therapies for this cancer have been developed widely, and have achieved some progress in the past decades<sup>[3-6]</sup>, the overall survival from liver cancer remains unsatisfactory<sup>[5-7]</sup>. This outcome is because the choice of treatment is driven by the cancer stage, the resources available, and the level of practitioner expertise<sup>[5]</sup>. Liver cancer often has no obvious clinical symptoms and signs in its early stages, and the tumor lumps grow quietly and rapidly. Most patients have been detected only in an advanced stage, resulting in limited treatment options and a very poor prognosis. A United States population-based study, for instance, reported that, in 2963 HCC patients diagnosed between 1992 and 1999, only 13% of the patients received a potentially curative therapy<sup>[8]</sup>.

Recent survival rate data show that the 5-year survival rates of liver cancer from population-based cancer registries in China were around 9.8%-12.1%<sup>[9]</sup>, and that the 5-year survival rate of liver cancer from a hospital-based cancer registry was 11.69%<sup>[7]</sup>. Furthermore, the 5-year survival rate from clinical series of data was 4.8% in 1958-1970, 11.2% in 1971-1982, and 45.4% in 1983-1994 for patients who received surgical resection<sup>[10]</sup>; and 63.8% for patients who had resection of small liver cancer<sup>[11]</sup>. The 5-year relative survival rates for liver cancer during 2002-2012 in Taiwan were 52.0% for stage I, 2.9% for stage IV and 28.9% for all stages<sup>[12]</sup>. A recent Australia report based on cancer registration shows that the 5-year survival was 5% during the years 1984-1993, and 16% during 2004-2013<sup>[13]</sup>. A current multicenter retrospective investigation shows that the overall survival is 19.6%, and is derived from 18,275 liver resection patients with HCC in China<sup>[14]</sup>.

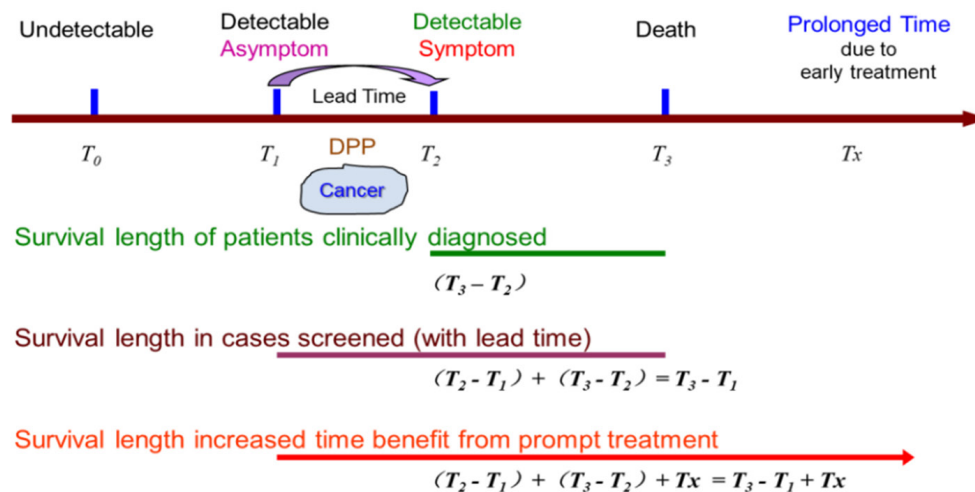
The fact that small or early stage liver cancer had better outcomes for survival has long been recognized<sup>[11]</sup>, and attracted great efforts for the early detection of liver cancer by mass screening in the general population since the 1970s. In the Qidong area, for instance, population-based mass screening programs were first applied to field practice<sup>[15,16]</sup> when alpha-fetoprotein (AFP) was established to be synthesized in cancer of the liver, and had been proven to be a serological test for this cancer<sup>[17,18]</sup>.

In the past 4 to 5 decades, the application value of AFP and the screening benefit for early detection has demonstrated the mixed results<sup>[15-23]</sup>. So far, there is no internationally recognized program of screening for the cancer of the liver, nor has a scientific consensus been formed in the academic world. Yet, case reports and research reports have provided evidence that screening is an effective way to achieve early detection, early diagnosis and opportunity for early treatment for liver cancer. Screening may have positive and important significance to improving prognosis and reducing mortality, especially in epidemic areas of hepatitis B/liver cancer. Here we describe and review the practice of the screening for liver cancer and discuss the problems arising from this approach.

## DISCOVERY OF AFP AND ITS CLINICAL APPLICATION

After Bergstrandh and Czan discovered alpha-fetoprotein (AFP) in human fetal serum in the 1950s, former Soviet scholar Abelev discovered that AFP was mainly synthesized from placenta and yolk sac, and correspondingly AFP could be detected in both human and mammalian embryo serum<sup>[17,18]</sup>. AFP begins to be synthesized at 6 weeks of gestation and peaks at 12-15 weeks (AFP in fetal plasma can reach 3 µg/L). After birth, AFP synthesis is inhibited (content reduced to 50 µg/L); at the end of 12 months, the concentration is close to the adult level. Plasma AFP concentration in healthy adults is lower than 20 µg/L<sup>[24]</sup>. However, AFP would be re-expressed when hepatocytes become cancerous or have severe injury or other forms of diseases<sup>[17,25]</sup>. Human hepatic stem cells (*hHpSCs*) are currently considered to express AFP<sup>[26]</sup>.

The probable association of AFP with liver diseases and liver cancer was noted in the 1960s. Tatarinov *et al.*<sup>[27]</sup> and O'Connor *et al.*<sup>[28]</sup> found that AFP was detected in the serum of patients with liver cancer; and a human



**Figure 1.** Schema for cancer detection and survival time

AFP variant was isolated in 1970<sup>[29]</sup>. However, since the relationship between AFP and liver diseases was not very clear, and the incidence of liver cancer in some developed countries was not high, the value of applying measures of AFP in the clinical diagnosis of liver cancer was totally ignored for a long time.

Fortunately, AFP detection technology was quickly introduced to China, and applied in high-risk areas of liver cancer, such as in Jiangsu (Qidong), Guangxi (Fusui), and Shanghai. From 1971-1976, 1,967,511 people were screened for serum AFP in the Shanghai general population: 300 patients with liver cancer were detected, of them, 134 (44.7%) cases were diagnosed as subclinical HCC<sup>[30]</sup>. During the period of 1974-1980 in Qidong, 1,310,871 people were screened for serum AFP, 499 patients with liver cancer were found, 177 (35.5%) patients being at early stage<sup>[15]</sup>. These results and subsequent studies have shown that AFP is a useful marker for the early detection of liver cancer<sup>[16]</sup>.

## RATIONALE FOR SCREENING

The overarching principles for screening are prevention and early detection. A detectable early cancer stage must exist for reasonable duration of time to allow the screening test to pick it up<sup>[31]</sup>. Hence screening trials must be based on an understanding of the natural history of the disease. From the view of clinical observation, any disease will have two states: an undetectable disease (normal) state, and a detectable disease state with symptoms or asymptom. Asymptomatically detectable, also known as the “detectable preclinical phase” (DPP)<sup>[32]</sup>, detectable and curable preclinical phase (DCPP)<sup>[33]</sup>, or “pre-clinical detectable phase (PCDP)”<sup>[34,35]</sup>, is the ideal stage for early diagnosis. At this stage, it is possible to detect the disease and even reverse the disease through active and curable treatments. When the cancer was detected in the symptomatic stage, the disease was often developed fully, and showed the medium- or late-stage clinical manifestations. As to screening, a disease that can be detected asymptotically is a disease suitable for screening. Liver cancer could have abnormal changes of AFP at early phase, vary from several months to several years before the appearance of subjective symptoms, thus providing a possibility for early detection of liver cancer.

In the debate about whether screening can prolong the survival rate of liver cancer, lead time is a key word that cannot be circumvented. Lead time is the time by which diagnosis is anticipated by screening with respect to the symptomatic detection of a disease, or is the time by which the diagnosis has been made in advance by screening<sup>[36]</sup>. Any screening program, including surveillance for liver cancer, is subject to lead time bias<sup>[23,31,35,37-40]</sup>. The relation of the lead time, natural survival length, and the prolonged time due to prompt treatment after screening may be expressed as Figure 1.

Assuming that any disease or the carcinogenic process started at  $T_0$ , and developed before the time  $T_1$ , this evolution process ( $T_1 - T_0$ ) is undetectable. After the point  $T_1$ , the disease (cancer) is asymptomatic but can be detected, until the point  $T_2$ , when symptoms occur. This length of time ( $T_2 - T_1$ ) is called lead time, or “detectable preclinical phase” (DPP), and also “sojourn time”. In the absence of any screening test, a patient may be diagnosed as an outpatient due to symptoms and/or signs, and then experiences a natural disease course accompanying the process of healing and rehabilitation until the end of life ( $T_3 - T_2$ ). Obviously, screening cases will survive with “cancer” for an additional time (lead time:  $T_2 - T_1$ ); therefore, their full survival time will be  $(T_3 - T_2) + (T_2 - T_1) = T_3 - T_1$ , which is “longer” than the survival time ( $T_2 - T_1$ ) of the outpatient cases. This “increase” in time essentially has no clinical significance for the patient, but it is artificially diagnosed earlier as a cancer. However, the real purpose of screening is to enable timely treatment of cases that are diagnosed early, and hence to prolong the time ( $T_3 - T_2$ ) benefit from the prompt treatment, whose increment is assumed to be  $T_x$ . Thus, the survival time of the cases after actual screening is  $(T_3 - T_2) + (T_2 - T_1) + T_x$ , or  $T_3 - T_1 + T_x$ , where  $T_x$  is the patient’s real extension of survival length due to early detection and treatment.

## MEANS OF SCREENING IMPLEMENTATION

It is possible to achieve the intended purpose of screening by mastering the principles of screening, identifying the target population for screening, using appropriate tests/examination or diagnostic tools, and relying on reliable and effective treatment. In terms of liver cancer screening, the evidence supporting feasible means of screening in populations at-risk or of specific individuals are summarized as follows.

### Major risk factors should be point of focus

Epidemiological studies have confirmed the main risk factors for liver cancer in China, and high-risk areas as well as high-risk groups (populations) of liver cancer can be defined scientifically<sup>[5,16,19,41-44]</sup>. In accord with recent understanding, the risk factors for primary liver cancer may be categorized into 3 groups<sup>[41,45]</sup>: (1) established factors: chronic HBV infection, chronic HCV infection, alcoholic cirrhosis, dietary aflatoxins, tobacco smoking; (2) likely factors: diabetes mellitus, inherited metabolic disorders- $\alpha$ -antitrypsin deficiency, hemochromatosis, porphyria cutanea tarda, cirrhosis of any etiology, obesity; and (3) possible factors: decreased consumption of vegetables, oral contraceptives, high parity, ionizing radiation, organic trichloroethylene solvent, clonorchis sinensis infection. However, as a population screening strategy, it is impossible to consider all of these etiological factors or risk factors. Moreover, some characteristics of disease or risk factors need to be confirmed in advance by specific test methods; in addition, factors that are considered “established” and “likely” in some areas may only be “possible”, or even “not possible” in other regions. For instance, liver fluke infection may be a “very likely” important risk factor for cholangiocarcinoma (CC) in Southeast Asia and Guangdong-Guangxi regions<sup>[46,47]</sup>; Hepatitis C virus (HCV) is considered to be the established factors in many countries<sup>[48-50]</sup>, but these factors seem “impossible” in the etiology of liver cancer in some regions such in Qidong, Jiangsu<sup>[41,51]</sup>. The other example is the nonalcoholic fatty liver disease (NAFLD), which is becoming an increasingly important health issue nowadays in China, with an overall pooled prevalence of 20.09% (17.95%-22.31%)<sup>[52]</sup>. It is considered as an alarming important risk factor of HCC development<sup>[53]</sup>. Therefore, population screening in high-incidence areas should be implemented in conjunction with the major local risk factors that can be easily identified within the general population.

### AFP could be used as a useful marker

AFP has been shown to be a sensitive and specific marker for screening<sup>[17,18,54-57]</sup>. In the early stage of liver cancer, abnormal levels ( $> 20 \mu\text{g/L}$ ) can be detected in serum, which can ensure that most patients with liver cancer have a long enough DPP characterized with positive AFP even 2 years before the clinical presentation of liver cancer<sup>[58]</sup>. When an AFP cut-off of  $20 \mu\text{g/L}$  is used, the sensitivity and specificity of AFP for HCC were in the range of 41%-65% and 80%-95%, respectively<sup>[56,59-61]</sup>; and the sensitivity and specificity of AFP would be changed if the cut-off value is modified<sup>[56,62]</sup>. In Qidong’s screening program the sensitivity and

specificity were 55.3% and 86.5%, respectively<sup>[19]</sup>. A systematic review evaluating AFP in cirrhotic patients with HCV infection showed sensitivities and specificities of 41%-65% and 80%-94%, respectively, for HCC diagnosis<sup>[63]</sup>. In a Taiwan study, screening with AFP was reported to be feasible screening marker of risk identification, and could result in good prognosis in an aged population<sup>[64]</sup>. A recent report in a South East Scotland HCC Surveillance Study (January 2009 and December 2014)<sup>[65]</sup> showed that AFP as an HCC surveillance tool detects a significant number of treatable HCC in patients with satisfactory outcomes. They also found that the use of serum AFP in HCC surveillance has facilitated the early diagnosis of HCC in a large proportion of the patients undergoing HCC surveillance in whom the HCC was otherwise not detected by ultrasound (US) alone, and that AFP should be included in the liver cancer surveillance<sup>[66]</sup>.

### US could be used for early detection

The application of US and other imaging modalities facilitate localized diagnosis for liver cancer. In the 1980s, US examination began to be used widely in the clinical detection of liver diseases in China. The advantages of US are manifold. It is non-invasive, produces no radioactive damage, is easy to repeat, has high sensitivity and at a relatively low cost. US is considered as the preferred method for liver cancer localization in screening<sup>[6,67,68]</sup>. US has a sensitivity of 60%-80% and a specificity of over 90% when it is done expertly<sup>[69]</sup>. An early prospective study reported in the United States in 1985<sup>[70]</sup> showed that in the initial screening for 528 patients, 17 liver cancer patients were found after an average follow-up of 1.4 years. In tumors < 5 cm, AFP levels were normal in 46.2%, 20-400 µg/L in another 46.2%, and only 7.6% were over 400 µg/L. Another 7 patients were found by further follow-up to have cancer varying from 1.6 to 4.7 cm, with normal serum AFP levels in 3 cases. Hence the authors concluded that real-time ultrasonography is more sensitive than AFP assay for the early detection of HCC, and that high-risk subjects should receive this procedure at regular intervals. A randomized trial<sup>[71]</sup> compared two US periodicities: 3 months vs 6 months, in a surveillance of HCC in cirrhotic patients. The results showed that 3-month US detection may find more small focal lesions than 6-months US detection, but does not improve detection rate of small HCC, nor improve the 5-year survival. The efficacy of US screening every 6 months for HCC or CC in a selective high risk group in endemic areas of hepatitis B such as in Thailand, Taiwan have been reported<sup>[72-74]</sup>.

### The combined application of AFP and US

AFP or US detection have their limitations. It is a common practice to combine these two methods for HCC surveillance. Many studies using a combined AFP and US surveillance/screening have proven survival benefit to patients by detecting smaller and curable liver cancers<sup>[20,55,61,75-78]</sup>, US combined with AFP for screening for liver cancer is believed to be superior to AFP alone, but periodic US examination would be expensive, while AFP testing is relatively inexpensive<sup>[79,80]</sup>. At present, computed tomography (CT) and dynamic magnetic resonance imaging (MRI) as robust imaging location techniques for the diagnosis of liver cancer are used widely in clinical practice<sup>[81,82]</sup>. A prospective randomized study comparing two different HCC screening procedures (biannual ultrasonography vs. annual triphasic CT) with biannual AFP has suggested that biannual US is comparable to annual CT in detecting early-stage HCC, with lower costs<sup>[83]</sup>. So there is no evidence to support the use of CT or MRI for routine liver cancer surveillance/screening; while its disadvantages are obvious: significant cost and radiation exposure<sup>[81,82,84]</sup>. Furthermore, findings are frequently discordant even on both CT and MRI<sup>[85]</sup>. In an Alaskan Native screening cohort study during 1983-2012, the cost-effectiveness of two HCC screening methods (by US-alone, or screening by AFP initially and switching to US) was evaluated<sup>[86]</sup>. The sensitivity analysis demonstrated that AFP→US was more cost-effective than US-alone over a broad range of differences in sensitivity between the two HCC screening methods. It was also pointed out that for many of the patients in rural Alaska, AFP is the only locally available option for HCC screening, and it could potentially identify patients at high risk for HCC who could benefit from referral for a liver US or CT. Thus, public health officials should evaluate the cost-effectiveness of AFP→US to increase access to HCC screening for persons living in remote communities



without access to US. A balance between the application of AFP test with or without US in screening should be considered. General speaking, the combination of AFP and US can ensure early detection and improve detection rates, thus enabling early diagnosis for liver cancer<sup>[20]</sup>. As such, the combined use of US and AFP is recommended<sup>[5]</sup>.

### **Early detection could lead to curable treatment**

Early detection of liver cancer has led to effective early treatment, especially by means of surgical resection, and has led to long-term survival for those treatable patients<sup>[11,21,30,76,87]</sup>, although the lead time due to screening may range from 2 to 6 months (70 to 200 days)<sup>[23,37,39]</sup>. Based upon a study of surveillance in cirrhotic patients, semiannual surveillance maintained a survival benefit over symptomatic diagnosis after lead time adjustment, and this benefit became durable in a long-term perspective<sup>[39]</sup>. In a community-based surveillance program<sup>[88]</sup>, significantly improved survival rates were noted in HCC patients detected by surveillance, and in those who received surgical and loco-regional therapies, indicating that HCC patients identified by surveillance were more suitable for surgical and local regional therapies, and would improve survival and should be included as standard of care for patients with hepatitis B. A recent prospective population-based study in Australia<sup>[89]</sup> showed that increased survival was associated with participation in surveillance programs and curative treatment. The 1-, and 2-year survival rates for surveillance participants were 79% and 66%, compared with 49% and 33%, respectively, for non-participants.

## **HISTORY OF LIVER CANCER SCREENING IN CHINA**

### **Pioneering start of screening in high-incidence area**

The most representative region for liver cancer screening was in Qidong<sup>[16,19,90]</sup>: from the early 1970s to the early 1980s, a sensitive AFP test was used to detect more than 2 million person-times in the general population from Qidong, including about 1.8 million persons who joined the screening program. More than 1000 cases of liver cancer confirmed by screening, of which early (stage I) cases represented 35%<sup>[15]</sup>. The practice of screening in this period helped to answer a question of primary importance: could liver cancer be detected at an early stage? - it could be. The application of AFP in population-based screening in the field has demonstrated that it is a simple, easy, sensitive and specific way of detection for liver cancer. A large number of patients with liver cancer at early stage in that period has resulted in the improvement of the overall survival rate<sup>[15,16,30]</sup>.

### **Formation of concepts of screening for high-risk populations**

The large requirements in human resources and financial resources for the mass screening of liver cancer impeded further implementation of screening. Screening in the general population was halted in the 1980s in Qidong and other areas in China. Based on strategic considerations for early detection and early treatment in the high-incidence area, the role of AFP screening was reevaluated, recognizing that the economic benefits of screening through AFP detection are determined by the preferred choice of the target population at high-risk. The specific age (with high incidence rate), gender (males) and risk factors (such as infection with HBV) of liver cancer should be given prioritized consideration for screening<sup>[42]</sup>. Hence, men aged 30-59 who were positive for hepatitis B surface antigen (HBsAg) were identified as high-risk population of liver cancer in Qidong<sup>[90,91]</sup>. In the same time, a Shanghai report suggested that screening should be focused on those aged over 35 or 40 with hepatic diseases for more than 5 years and who are positive HBsAg<sup>[92]</sup>.

### **Practice of screening in high-risk populations**

From late 1980s to early 1990s, a selected population of 36,381 males at the ages of 30-59 were screened<sup>[19,90,93]</sup>. 5581 HBsAg carriers were identified, enrolled and then randomly assigned to a periodical screening group (once every six months) or a control group to investigate the effectiveness of screening for liver cancer. This research program and practice has helped to confirm and optimize a scheme of screening in populations at high risk that includes such indicators for periodic screening as the subclinical mean sojourn time, sensitivity and predicted values, the lead time (DPP) and the best interval of screening for liver cancer<sup>[19,93,94]</sup>.

**Table 1. Status of national screening for liver cancer in rural areas (2011-2018)**

	No. of screened (areas)	No. of cases detected	Detection rate (%)	No. of early cases	Early detection* rate (%)	No. of treated	Treatment rate (%)
2011.7-2012.6	7,732 (6)	65	0.84	44	67.69	58	89.23
2012.7-2013.6	14,972 (11)	119	0.79	64	53.78	110	92.44
2013.7-2014.6	19,441 (13)	100	0.51	59	59.00	96	96.00
2014.7-2015.6	21,603 (13)	123	0.57	75	60.98	115	93.50
2015.7-2016.6	22,460 (13)	119	0.53	78	65.55	108	90.76
2016.7-2017.6	21,024 (13)	115	0.55	66	57.39	113	98.26
2017.7-2018.6	20,194 (13)	127	0.63	92	72.44	122	96.06
Total	127,426	768	0.60	478	62.24	722	94.01

Data from Ref.<sup>[98]</sup>. \*The diameter of the tumor is less than 5 cm<sup>[96]</sup>

In the Qidong screening program, the lead time for screened patients with liver cancer was estimated to be 12 months<sup>[94]</sup>. In an Italian study, after 10-year follow-up, they found that the median lead-time calculated for all surveilled patients was 6.5 months (7.2 for semiannual and 4.1 for annual surveillance). Lead time bias accounted for most of the surveillance benefit until the third year of follow-up after HCC diagnosis<sup>[39]</sup>.

### Implementation of the national project on cancer early diagnosis and treatment

In 2004, the China Cancer Foundation launched a project for early diagnosis and treatment of cancer, subsidized by central financial transfer payment program<sup>[95]</sup>, and in 2006, the demonstration project of early detection and early treatment of liver cancer was officially launched in Qidong, Jiangsu Province and in Fusui, Guangxi Zhuang Autonomous Region where screening was carried out in high risk populations, i.e., male residents aged 35-64 and female residents aged 45-64 with positive HBsAg, who should be followed up every 6 months by using repeat monitoring examinations of combined AFP and US. This project has been described in the “Chinese Technical Scheme for Early Diagnosis and Early Treatment of Cancer”<sup>[96,97]</sup>.

## CURRENT STATUS AND PROGRESS IN CHINA

### Extensions of the screening program

After 2010, in order to meet the requirements for expanding the scale of liver cancer screening, two areas of Haimen, Jiangsu Province, and Tong'an, Fujian Province were included into the National screening project. Later on, Gong'an, Yidu, Yingshan, Dangyang, Honghu, Huangzhou, Jiayu of Hubei Province, Zherong of Fujian Province, Chongzuo, Guigang, Cenxi, Wuming of Guangxi Zhuang Autonomous Region, Zhongshan of Guangdong Province, and Huanchi, Shangdan of Gansu Province, were included into the program as well. At that time there were 19 areas included in the program, but some of them withdrew after one or more years, with 13 counties (cities) remaining nowadays. From 2007 to 2018, individuals with positive HBsAg have been repeatedly screened 146,637 times; 965 liver cancer patients were found/detected. The annual detection rate was 0.66%, the early detection rate was 62.38%, and the treatment rate was 91.09%. Among them, 127,426 high-risk individual-times were screened during the period of 2011-2018, and 768 liver cancer patients were found/detected. The detection rate was 0.60%, the early detection rate was 62.24%, and the treatment rate was 94.01% [Table 1]<sup>[98]</sup>.

### Cancer Screening Project in Huaihe River Region

A cancer screening program (include liver cancer) was issued by the Bureau of Disease Control of the National Health Commission of the PR China in 2008 which has included Sheyang of Jiangsu Province, Fuyang, Suzhou of Anhui Province, Wenshang of Shandong Province, and Xiping, ShenQiu of Henan Province<sup>[99]</sup>. Now this program has been increased to 32 counties (cities) in four Provinces, and has screened more than 53,400 person-times. The results on the screening of liver cancer have not been reported.

### Cancer Screening Program in Urban China

In 2012, the National Cancer Center of China proposed a Cancer Screening Program in Urban China (CanSPUC), which is also a National Major Medical Reform Project that includes screening for cancers of

the lung, colon-rectum, upper digestive tract (esophagus and stomach), and liver. Residents at ages of 40-69 were enrolled into the screening groups. About one to two medium-sized or more cities in each of 14 Provinces/Municipalities across the country joined the project. During the first 5-year round (2012-2016) of screening, it aimed to cover areas of some 3,500,000 of the population, and to screen about 700,000 individuals at high risk<sup>[100]</sup>. So far, 42 cities of 20 provinces were included into CanSPUC. However, there have not been any reports to show the findings of the detection rate or the effectiveness of screening for liver cancer, except on medical expenditures for liver cancer in urban China. The CanSPUC program analyzed the medical expenditure for liver cancer during 2002-2011 in urban areas of China<sup>[101]</sup> and found that the medical expenditure per case for liver cancer diagnosis and treatment was ¥31,020 (\$4,528) from the year 2002 to 2011 and ¥35,248 (\$5,146) from the year 2009 to 2011, indicating that the economic burden of liver cancer is high in China and the related medical expenditures are increasing.

### Recent advances in screening from 2 rural areas

As one of the bases for demonstration of early detection and early treatment of liver cancer, Qidong launched its Special Fiscal Transfer Payment Project of the Central Government in 2006<sup>[97]</sup>. The screening scheme followed the recommendations of the Expert Committee of Early Detection and Early Treatment by China Cancer Foundation<sup>[96,98]</sup>. The high risk population screened was defined as those with positive HBsAg at ages of 35-64 for men and of 40-64 for women. Periodically diagnostic screening by using combined methods of AFP and US monitoring were recommended. Since 2007, a target population of 38,016 has been screened in the Qidong area: 3,703 (9.74%) individuals with positive HBsAg were found. Excluding for 29 patients with liver cancer at the initial screening, 3,674 persons in the cohort were followed up until the 31st of March, 2016. The 268 patients with liver cancer were detected from the 33,199 person-times screened, with an annual detection rate of 1.12%. Of them, 186 patients were found via repeated periodic screening (Group A), in which 149 patients were the early cases, with an early detection rate of 80.11%. Some participants with positive HBsAg were not followed by the suggested periodical screening schedule, but they (82 cases) were diagnosed as outpatients within the intervals of screening points (Group B). Calculated by the life-table method, the 1-, 3-, 5-, and 8-year survival of all patients with liver cancer in Group A were 77.16%, 49.04%, 38.53%, and 24.25%, and in Group B were 36.25%, 21.21%, 21.21%, and 0%, respectively, with significant differences between two groups ( $P < 0.01$ ). This finding shows that the screening of individuals at high-risk with semiannual AFP and US detection is effective not only in increasing detection rate of early stage liver cancer but also in improving patients' survival. Ji *et al.*<sup>[102]</sup> reported another example from Zhongshan, Guangdong Province that started in 2012. The biannual screening also used serum AFP and US examination for subjects positive for HBsAg. Of the 68,510 eligible residents, 17,966 were screened for HBsAg. Within the first 4 years of follow-up, 57 incident cases of liver cancer (43 from 2,848 HBsAg-positive participants, 14 from 15,118 HBsAg-negative participants) were found. Compared with cases (104) identified from non-participants (50,544), the cases detected among screening participants were more likely to be at early stage and had better survival than those among non-participants. The 1-, 3-year overall survival rates for liver cancer cases in the screened group were 48.7% and 29.1%; and in non-screened group were 36.9% and 15.5%, respectively, showing better prognosis in screened group (HR = 0.64, 95%CI: 0.42-0.98, after adjustment for gender and age). However, this screening study did not show a reduction in liver cancer mortality within the first 4 years of follow-up by comparison of the two groups (RR = 1.04, 95%CI: 0.68-1.58).

## GLOBAL DISPUTES AND CONSENSUS ON LIVER CANCER SCREENING

### Notable randomized trials of screening from China

Whether liver cancer is suitable for screening, or whether screening has a significant effect, has caused much controversy globally. As one of the methods of cancer control, the values of population screening are often disputed because of differences in understanding of goals, benefits, disadvantage, costs, and potential adverse effects of screening, and of disagreements in assessing the effectiveness of screening<sup>[103]</sup>. Two randomized trials of screening for liver cancer were published in early this century: one from Qidong

in 2003<sup>[19]</sup>, one from Shanghai in 2004<sup>[20]</sup>, in which both screened carriers of HBsAg every 6 months. In the Qidong study, the percentage of cases in stage I were significantly higher in the screening group (29.6%) than in control group (6.0%), showing short survival benefit from screening, but no difference in 5-year survival between the groups. The mortality rate in the screened group (1,138 per 100,000 person-years) was not significantly different from that in the controls (1,114 per 100,000). This trial concluded that screening with AFP resulted in earlier diagnosis of liver cancer, but the gain in lead time did not result in overall reduction in mortality in this reported period. In the Shanghai study, the authors reported that the HCC mortality rate was significantly lower in the screened group (83.2 per 100,000) than in controls (31.5 per 100,000), with a mortality rate ratio of 0.63 (95%CI: 0.41-0.98). It concluded that the biannual screening with combined AFP and US in individuals aged 35-59 years reduced HCC mortality after 5-year follow-up. These two trials have been noticed and/or cited by over a hundred reports or guidelines, irregardless of whether they were in support or opposition to screening<sup>[5,8,21,57,68,103-108]</sup>.

### Screening recommendation in Western countries

After China's randomized trials were published, the benefit from screening in people at high risk was noted by professional societies, such as AASLD<sup>[21,105,108]</sup>, simply because of the surveillance/screening for liver cancer had become widely applied, but, there was no evidence of benefit from it worldwide. In these guidelines on management of HCC, the two randomized trials performed in China mentioned above were evaluated. The guideline authors were interesting in the result of HCC related mortality that was reduced by 37% throughout the screening for 18,816 individuals with HBV infection in Shanghai, and added positive comments that these results probably represent the minimum benefit that can be expected from surveillance, because of poor compliance of less than 60%<sup>[20]</sup>. They also cited the earlier study conducted in Qidong<sup>[19]</sup> that failed to show long term survival/mortality-reduction benefit due to patients who were diagnosed with liver cancer did not undergo appropriate treatment, and suggested that these results should be validated in other geographical areas, and that assessing the benefits of surveillance by RCT are still considered necessary<sup>[21]</sup>. Since the recommendation was issued, other guidelines or suggestions have been published<sup>[106-110]</sup>, and various studies have examined physicians' knowledge of or adherence to the guidelines and reported deficiencies and need for improvement<sup>[81]</sup>. Most gastroenterologists correctly identified the common high-risk scenarios, methods, and interval of HCC screening as recommended by AASLD<sup>[111]</sup>. A recent systematic review on surveillance detection demonstrated improved survival and increased detection rate of early stage HCC<sup>[68]</sup>. Forty-seven studies from January 1990 through January 2014 with 15,158 patients were identified, of whom 6,284 (41.4%) had HCC detected by surveillance, being associated with improved early stage detection (OR: 2.08, 95%CI: 1.80-2.37) and curative treatment rates (OR:2.24, 95%CI:1.99-2.52). HCC surveillance was associated with significantly prolonged survival (OR: 1.90, 95%CI: 1.67-2.17), even after adjusting for lead-time bias. It is believed that HCC surveillance is associated with significant improvements in early tumor detection, receipt of curative therapy, and overall survival in patients with cirrhosis<sup>[75]</sup>, and may also reduce the mortality of HCC<sup>[20,74]</sup>.

### Debates on screening effectiveness

Although the effectiveness of liver cancer screening has been recognized in the literature and is also included in the AASLD surveillance guidelines for liver cancer<sup>[55,21]</sup>, there have been different opinions and even opposition to the choice of at-risk populations, the necessity, and the effectiveness of screening. Lederle and Pocha<sup>[112]</sup> were opposed to the existing screening programs by criticizing the 2005 AASLD recommendations for HCC screening<sup>[21]</sup>, arguing that the recommendations were based upon trials from China<sup>[19,20]</sup>, which failed to account for clustering in the analysis (a cluster randomized trial cannot be analyzed at the patient level), hence they state "Ignoring the clustering results in confidence intervals which are too narrow and *P* values which are too small; hence it is likely to produce spuriously significant differences"<sup>[57,113]</sup>. Furthermore, they questioned the evidence obtained from the study that is not a level I evidence to support the liver cancer screening, and is not necessarily applicable to Western populations because it was conducted in a hepatitis B population in China, and most HCC in West countries and North America is caused by hepatitis

C<sup>[21,114]</sup>. In an editorial comment in the BMJ<sup>[115]</sup>, Law points out that screening of unproved value should not be advocated, and that before any screening for cancer is introduced, large randomized trials with mortality end points should be conducted to establish and quantify any benefit. Evaluation of mortality of liver cancer in a screening population is a point of concern. A recent matched case-control study within the American Veterans Affairs (VA) health care system found that screening patients with cirrhosis for HCC by US or AFP alone, or both tests was not associated with decreased HCC-related mortality<sup>[116]</sup>. Some authors thought that randomized screening trials are bothersome, but there is no second-best option<sup>[103,112]</sup>; others illustrated that RCTs of screening for HCC is difficult and ethically questionable<sup>[40]</sup>, is now not ethically feasible in clinical practice because screening for liver cancer in cirrhotic patients is routine practice for the majority of clinicians<sup>[117]</sup>, even if patients show no interest in such a program<sup>[118]</sup>. In addition, the AFP use in screening has long been criticized because of its lower sensitivity and specificity than imaging modalities<sup>[60,119]</sup>. In the European clinical practice guidelines for HCC, US was seen as the most appropriate test to perform surveillance, but the combination with AFP is not recommended<sup>[108]</sup>. A meta analyses showed that AFP provided no additional benefit to US<sup>[69]</sup>, while others concluded that there is not enough evidence to support or refute the value of AFP or US screening, or both, of HBsAg positive patients for HCC<sup>[120]</sup>. More emphatically, early in this century, it has been stated that “the time has come to bid a fond adieu to AFP”<sup>[121,122]</sup>, or it is “the demise of a brilliant star”<sup>[123]</sup>, as a test for HCC diagnosis and particularly for HCC surveillance.

### Consensus on liver cancer screening

Despite the large debate over liver cancer screening, there is still much consensus on many of the relevant aspects of screening. For example, it is emphasized that the cancer screened must have DPP, or the cancer should be detected early by better sensitive and specific methods; moreover, the appropriate effects of the screening results can be evaluated, and could prolong the survival and may reduce mortality<sup>[20,124,125]</sup>. Many guidelines for the management and monitoring of liver cancer have been issued around the world; for example, they are available in the United States, Europe, and Asia<sup>[105-110]</sup>. However, evaluation of current liver cancer screening has not been carried out in a large scale because there is no consensus on the best strategy for liver cancer screening. On the other hand, it also believed that there is an urgent need to improve the strategies of screening and monitoring for liver cancer, in order to detect early stage liver cancer and improve the survival rate of patients<sup>[37,57]</sup>. The current problem is that, compared to other cancers, the development of globally accepted guidelines seems to be less relevant due to the existence of regional differences in etiologies underlying the resultant tumor biology as well as the resources available for management of liver cancer<sup>[126]</sup>. However, in recent years, research and practice of targeted liver cancer screening, screening methods and time intervals have become consistent and reached a point of consensus. For example, screening should be performed in high-risk populations<sup>[19,20,22,43,44,72,87,93,108,127]</sup>; chronic hepatitis B is a high-risk population of liver cancer<sup>[128]</sup>. The cost effectiveness of screening will be principally related to the sensitivity and specificity of the surveillance tools, as well as the efficacy of treatment<sup>[123]</sup>, and surveillance is deemed cost-effective if the expected HCC risk exceeds 1.5% per year in patients with hepatitis C and 0.2% per year in patients with hepatitis B<sup>[105]</sup>; The screening methods used included AFP and US, with a recommended interval of 6 months<sup>[5,19,20,54,55,59,66,75,76,86,108,129-131]</sup>. In a two-stage screening intervention in Taiwan, potential cost-effectiveness compared with opportunistic screening in the target population of an HCC endemic area is reported<sup>[132]</sup>.

### PROSPECTS FOR LIVER CANCER SCREENING

Although there is currently no internationally recognized program for the screening for liver cancer, except for some aspects of the consensus, in the past decades China has experienced many screening trials<sup>[15,19,20,90,93,97,100,102]</sup>, which have fully demonstrated the Chinese characteristics (most patients are HBV-related liver cancer) and the need for the management and control for the one of its most common malignancies. Professional societies in Western countries had proposed recommendations and guidelines



on this special issue<sup>[108]</sup>, although in the recent American Cancer Society Guidelines, the screening for liver cancer is not mentioned<sup>[133]</sup>. Even in China, there are several clinical practice guidelines for liver cancer<sup>[134]</sup>. Therefore, any users of these guidelines should be aware that the recommendations are intended to guide clinical practice in circumstances where all possible resources and therapies are available; hence, they should adopt the recommendations in the context of their local regulations and/or team capacities, infrastructure and cost-benefit strategies<sup>[108,129]</sup>. Liver cancer nowadays is the second leading cause of cancer deaths worldwide, accounting for about 8.1% of the global burden of cancer, in which China represents its 51% of this burden<sup>[1]</sup>. The global solution to the early diagnosis and treatment of liver cancer should fully consider the actual situation in China. We present some suggestions in summary for liver cancer screening/surveillance.

### **Combined use of US and AFP are recommended**

So far, AFP remains an effective screening tool or marker for liver cancer detection, especially in undeveloped countries/areas, on Asia, and even in some areas in developed countries<sup>[55-57,65,80,86]</sup>, because there is no a single “all-in-one” biomarker that fits all-surveillance, diagnosis, or prediction of prognosis<sup>[62]</sup>. In order to improve the sensitivity and specificity of screening and prevent missed diagnosis, the combination use of US and AFP test are strongly recommended. Since about 30% of liver cancers are negative for serum AFP, novel diagnostic markers need to be established<sup>[56]</sup>. There are no data to support the use of multidetector CT or dynamic MRI for surveillance<sup>[108]</sup>, but one report<sup>[133]</sup> showed that the sensitivity estimates of CT and MRI for liver cancer detection were 0.70 and 0.86, respectively, and the combined use was 0.94. CT or MRI could be used for patients with cirrhosis and those suspected cases (such as with AFP positivity) requiring further clinical ascertainment<sup>[57,130,135]</sup>.

### **Novel diagnostic markers are urgently needed**

In addition to AFP (AFP-L3), DCP, GPC3, GP73, AFU, GGT and others are still recommended as markers for monitoring and diagnosis of liver cancer; DKK1, MDK, and microRNA are also being used as new markers<sup>[55,56,62,61,136-141]</sup>. For instance, a European study found that osteopontin (OPN) is a promising marker for early detection of HCC<sup>[142]</sup>. In this study, each of 100 HCC cases was matched with 2 controls. Conditional logistic regression model was used to calculate the multivariate OR and 95%CI for OPN levels in relation to HCC. The results showed that OPN levels were positively correlated with HCC risk: the multivariate OR was 1.30 (1.14-1.48) for every 10% increase. For cases diagnosed within 2 years, the combination of OPN and AFP was best able to predict the risk of HCC, indicating that the measurement of OPN and AFP could independently identify high-risk groups in liver diseases. In order to make up for the deficiency of sensitivity and specificity of diagnostic markers such as AFP, novel early diagnosis and early precursory (predictive) markers are urgently needed for research-development and verification.

### **Translating early detection to effective curable treatment**

According to the current economic conditions and medical conditions (especially in undeveloped countries/areas), screening in high-risk groups of liver cancer every 6 months is particularly appropriate and acceptable. The key for a successful screening program should be a focus on individuals at high risk, conducting repeated or periodical screening and follow-up. Some authors may suggest patients with HCV, NAFLD or with cirrhosis should be screened, but so far there are no data from randomized trials of surveillance to evaluate effectiveness<sup>[5]</sup>. Liver cancer patients found in screening who fail to receive timely treatment will not improve survival and mortality. Any guidelines for screening on liver cancer should emphasize not only the early detection of liver cancer but also access and uptake of early curable or life-extending treatment.

### **Effectiveness of screening is in anticipation**

For evaluating the efficacy of population-based cancer screening modalities, the reduction of mortality rate within the screened population is the gold-standard indicator<sup>[20,72]</sup>, but it should not be a mandatory

requirement, since these outcomes will not be observable for many years<sup>[143]</sup>; survival rate change is indeed a necessary indicator. Any benefits and risks should be compared and reviewed before adopting a certain method of screening<sup>[57,117,144]</sup>. If the risks outweigh the benefits, it cannot be regarded as effective and is therefore not recommended. The surveillance adherence rates should be increased and improved<sup>[40,145]</sup>, and should be supported by patients, providers, and health care systems/governments<sup>[37]</sup>. From the perspective of public health, cost-effective evaluation should be considered, and the benefits and risks of screening should be compared, as well<sup>[31,86,144,146]</sup>. Obviously, benefits of liver cancer screening, at least in terms of greater benefits than harms from the surveillance, have been evident so far.

## CONCLUSION

The success of screening depends on having sufficient numbers of personnel to perform the screening tests by using the technology appropriately or to achieve adequate coverage of the population, and on the availability of facilities that can undertake subsequent diagnosis, treatment, and follow-up, as has been addressed by the WHO<sup>[147]</sup>. The bulk of available evidence suggests that screening for liver cancer is beneficial, certainly benefits outweigh harms. Inasmuch as symptomatic presentation of liver cancer has an almost universally fatal outcome, screening for liver cancer is an appropriate method that could be used to detect early stage liver cancer in China and other endemic countries/areas where liver cancer burden is substantial. The combined use of AFP and US for liver cancer screening, in the view of its relative cost-effective or applicability in community/population-based screening, are recommended while other novel markers or techniques remain to be developed. High risk individuals with established risk factors (etiological) and or characteristics (clinically identified) are the target populations; and opportunities for screening at-risk persons is to be encouraged even in regions with financial and medical limitations. Only in this way will it be possible to find more early and curable liver cancers.

## DECLARATIONS

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### Authors' contributions

Design of the work, data analysis and interpretation: Chen JG

Data acquisition, material support: Chen JG, Zhang YH, Lu LL, Chen HZ

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Wrote the manuscript: Chen JG, Shen AG

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Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Original Article

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# Transarterial chemoembolization combined with radiofrequency ablation in the treatment of hepatocellular carcinomas larger than 5 cm

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## Abstract

**Aim:** This meta-analysis was designed to compare the effectiveness of the combination of transarterial chemoembolization (TACE) and radiofrequency ablation (RFA) vs. that of TACE alone in hepatocellular carcinoma (HCC) tumors larger than 5 cm.

**Methods:** PUBMED, CNKI, and CBM were searched for all related randomized controlled trials (RCTs) up until October 22, 2018. Eleven studies were identified that compared TACE with RFA vs. TACE alone for HCC treatment. Tumor response rate, the proportion of patients with either complete or partial shrinkage of tumors, and survival rate were the major evaluation indices.

**Results:** Meta-analysis data revealed that TACE with RFA showed significantly better tumor response rate (risk ratio (RR) = 1.452, 95% confidence interval (CI): 1.308-1.610,  $P < 0.001$ ) and 1-year overall survival rate (RR = 1.412, 95% CI: 1.249-1.596,  $P < 0.001$ ) than that of TACE alone treatment.

**Conclusion:** The data of our study indicates that TACE combined with RFA in the treatment of HCC larger than 5 cm is an effective comprehensive interventional therapy.



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**Keywords:** Transarterial chemoembolization; radiofrequency ablation; hepatocellular carcinoma; meta-analysis

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and malignant tumor in the world, with an annual incidence of over 700,000 patients worldwide<sup>[1]</sup>. As the symptoms of HCC often do not present in the early stages, most patients are in the middle and late stage at the time of diagnosis, among which only 20%-30% of patients have the chance to receive surgical resection or liver transplantation<sup>[2]</sup>. Patients with large tumors that cannot undergo surgical resection or liver transplantation are usually offered comprehensive treatment based on transarterial chemoembolization (TACE)<sup>[3,4]</sup>. However, the long-term outcome of treating HCC with TACE alone is not ideal, due to incomplete tumor necrosis<sup>[5,6]</sup>. Studies have shown that TACE combined with RFA in the treatment of HCC is more efficacious than either TACE or RFA alone<sup>[7,8]</sup>. Nevertheless, some studies have reported contradictory results<sup>[9,10]</sup>. Of note, the sample sizes of these studies are small and the observations need further validation. Additionally, it is unknown whether this combined treatment is more effective than single modality treatment for HCC tumors larger than 5 cm.

Therefore, in order to determine whether TACE plus RFA is more effective in patients with HCC than TACE alone, this current meta-analysis was performed to compare the efficacy of TACE plus RFA with TACE monotherapy. This comparison is expected to provide more convincing evidence for HCC patients having to choose between two methods. In this study, the clinical efficacy of TACE combined with RFA was compared with that of TACE alone in the treatment of HCC larger than 5 cm, to provide evidence to guide clinical practice.

## METHODS

### Search methods and quality assessment

As of October 22, 2018, randomized controlled trials (RCT) comparing the clinical efficacy of TACE with RFA *vs.* TACE alone in the treatment of HCC was performed using a computerized search on PUBMED, Chinese Journal Full-text Database (CKNI), and CBM. Search terms include “Liver Neoplasms/therapy” [Mesh], “Chemoembolization, Therapeutic” [Mesh], “TACE”, “Radiofrequency ablation”. The literature language is limited to Chinese and English.

Evaluation of literature quality (including literature data extraction and quality scoring) was carried out by the authors. According to the Jadad quality standard, the scoring method is as follows. Whether it is randomly assigned: 2 points is awarded for detailed random allocation, 1 point when it was not specifically described, and 0 point if it was not mentioned. Whether analysis was blinded, 2 points for double-blind, 1 point for blinding without detailed description, 0 point for open trial. Whether there was a detailed reason for loss of follow-up: 1 point for yes, 0 point for no. High quality research literatures are those that received 3 to 5 points; and low quality literatures are those that received 0 to 2 points.

### Inclusion criteria

Literature reports were eligible for inclusion if: (1) they are domestic or international publications, that compared the clinical efficacy of TACE combined with RFA *vs.* TACE alone in the treatment of intermediate and advanced staged HCC; (2) they report complete case data; (3) the results of the study include tumor response rate; (4) the maximum diameter of tumor lesions is greater than 5 cm; (5) the clinical study design is consistent with that of a RCT.

### Exclusion criteria

Literature reports were excluded if: (1) they are review articles or case reports, are of poor literature quality as evaluated by the above method, or have no proper controls; (2) they are animal studies; (3) there are

**Table 1. Main characteristics of studies concerning tumor response rate between TACE with RFA vs. TACE alone**

Ref.	Year study was conducted	Gender	Sample size	TACE		Tumor response rate	TACE + RFA		Tumor response rate
				Total	Events		Total	Events	
Dong et al. <sup>[11]</sup>	2011-2012	Both	44	22	6	0.272727273	22	11	0.5
Du et al. <sup>[12]</sup>	2015-2016	Both	80	40	14	0.35	40	23	0.575
Ge and Zhang <sup>[13]</sup>	2008-2009	Both	43	24	12	0.5	19	14	0.736842105
Kuang et al. <sup>[14]</sup>	2015-2017	Both	87	40	21	0.525	47	35	0.744680851
Li et al. <sup>[15]</sup>	2012-2013	Both	80	42	21	0.5	38	27	0.710526316
Liang <sup>[16]</sup>	2006-2008	Both	55	24	9	0.375	31	25	0.806451613
Liu et al. <sup>[17]</sup>	2011-2013	Both	128	64	10	0.15625	64	22	0.34375
Shen et al. <sup>[18]</sup>	2004-2005	Both	40	19	9	0.473684211	21	17	0.80952381
Song et al. <sup>[19]</sup>	2006-2008	Both	29	15	4	0.266666667	14	11	0.785714286
Zhang et al. <sup>[20]</sup>	2012-2014	Both	70	33	6	0.181818182	37	17	0.459459459
Yang et al. <sup>[21]</sup>	2006-2008	Both	35	11	6	0.545454545	24	16	0.666666667

duplicate reports of similar content by the same author, or if there are too few patients and unclear data; (4) the maximum diameter of tumor lesions is less than 5 cm.

### Data acquisition

The literature and extracted the data were screened independently by authors. After articles were screened by their titles and abstracts, they were filtered by reading the full text. During the screening process, the literature was selected in strict accordance with the set inclusion and exclusion criteria. After the screening was completed, the articles were read again to verify that they meet the requirements.

### Statistical methods

Statistical analysis was performed using Comprehensive Meta Analysis V2. Before the meta-analysis, the heterogeneity  $I^2$  test of each test result was performed. If the homogeneity of each test included in the study was good ( $P > 0.05$ ), the fixed effect model was used. If heterogeneity existed, the random effect model was used. A funnel chart was used to evaluate the bias risk of the inclusion test, and asymmetric funnel charts suggest that there may be publication bias.

## RESULTS

### Literature search results

Manual search of electronic databases identified a total of 1,487 studies. After checking for duplicates, there were 1,304 remaining. A large number of these studies were excluded based upon our inclusion and exclusion criteria, leaving only 11 articles to be included in the meta-analysis [Figure 1 and Table 1].

### Tumor response rate

There were 11 reports with tumor response rate data comparing TACE with RFA vs. TACE alone. Tumor response rate was measured by the proportion of patients with either complete or partial shrinkage of tumors. Since the heterogeneity test had a  $P = 0.983$ , the fixed-effects model was used. The results showed that the tumor response rate of TACE with RFA in the treatment of HCC was significantly superior to TACE alone [risk ratio (RR) = 1.452, 95%CI: 1.308-1.610,  $P < 0.001$ ,  $I^2 = 0\%$ ] [Figure 2].

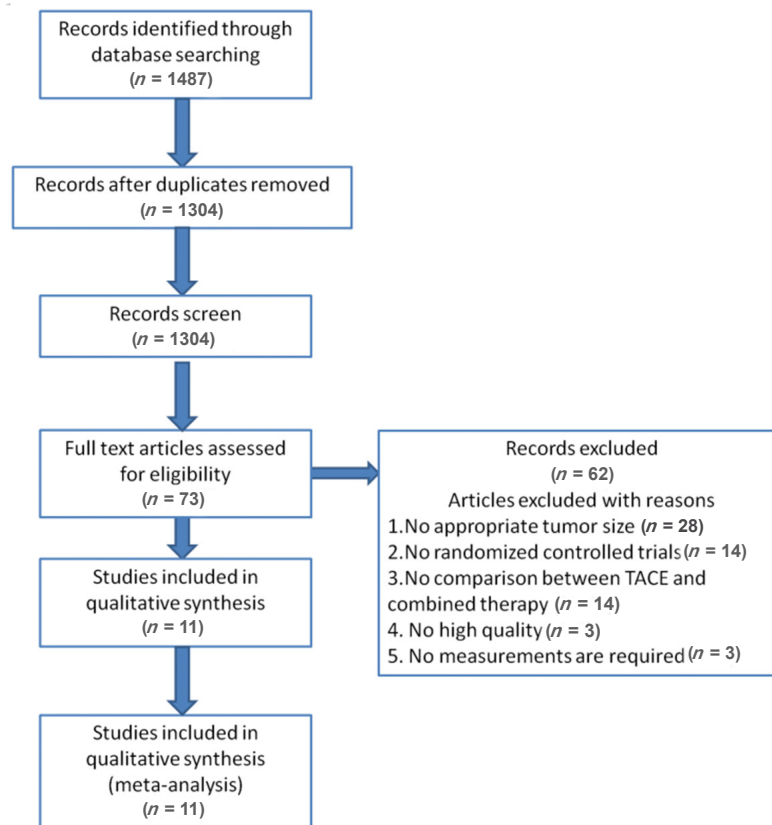
### Six-month survival rate

Six studies<sup>[15,16,18-21]</sup> (involving 309 participants) compared the half-year survival of the TACE with RFA group vs. the TACE alone group. The results showed that half-year survival rate was higher in the TACE with RFA group than in the TACE alone group [RR = 1.257, 95%CI = 1.128-1.401,  $P < 0.001$ ,  $I^2 = 0\%$ ] [Figure 3].

### One-year survival rate

Eight studies<sup>[14-21]</sup> (involving 524 participants) compared the 1-year survival of the TACE with RFA group vs. the TACE alone group. The results showed that 1-year survival rate was higher in the TACE with RFA group compared to the TACE alone group [RR = 1.412, 95%CI = 1.249-1.596,  $P < 0.001$ ,  $I^2 = 0\%$ ] [Figure 4].





**Figure 1.** Flow diagram of the detailed selection process of this meta-analysis. A total of 11 RCTs<sup>[11-21]</sup> [Table 1] were included in the study. There were 691 eligible patients, of whom 357 received TACE with RFA, and 334 received TACE alone. The baseline characteristics of the trials included in the meta-analysis are shown in Table 1. The quality of the included studies was assessed using the Cochrane Collaboration Tool

### Eighteen-month survival rate

Six studies<sup>[15,16,18-21]</sup> (involving 309 participants) compared eighteen-month survival of the TACE with RFA group vs. the TACE alone group. The results showed that eighteen-month survival rate was higher in the TACE with RFA group than in the TACE alone group [RR = 1.792, 95%CI: 1.423-2.256,  $P < 0.001$ ,  $I^2 = 0\%$ ] [Figure 5].

### Two-year survival rate

Three studies<sup>[14,17,20]</sup> (involving 285 participants) compared the 2-year survival rate of the TACE with RFA group vs. the TACE alone group. The results showed that 2-year survival rate was higher in the TACE with RFA group than in the TACE alone group [RR = 1.675, 95%CI: 1.233-2.275,  $P = 0.001$ ,  $I^2 = 0\%$ ] [Figure 6].

### Incidence of fever

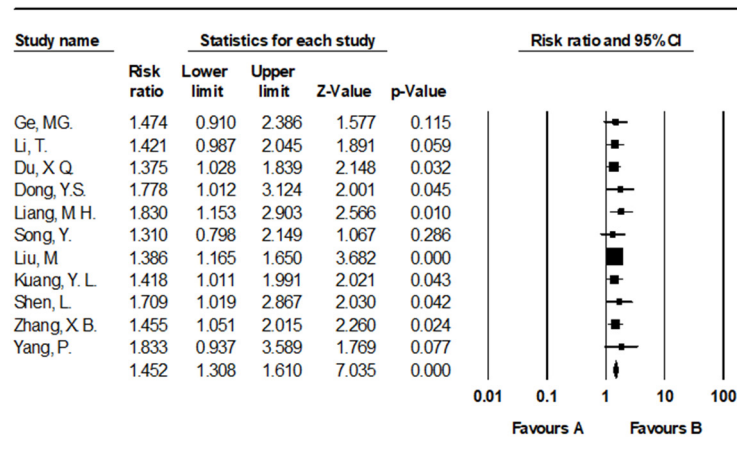
Three studies<sup>[11,12,20]</sup> (involving 194 participants) compared the incidence of fever of the TACE with RFA group vs. the TACE alone group, and showed that there was no significant difference between the two groups [RR = 1.177, 95%CI: 0.904-1.532,  $P = 0.227$ ,  $I^2 = 0\%$ ] [Figure 7].

### Publication bias assessment

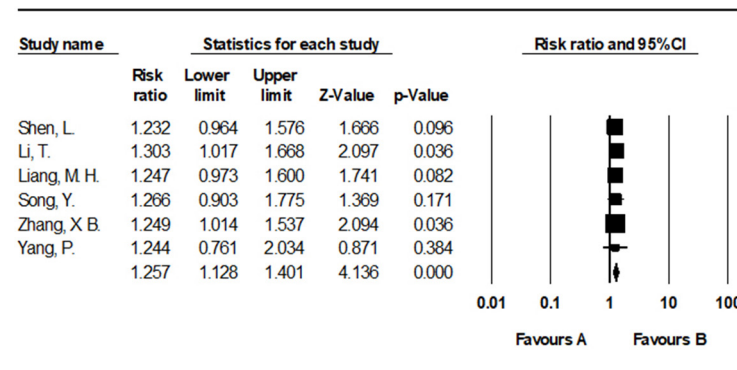
Based on statistical analysis, the meta-analysis of TACE with RFA vs. TACE alone obtained better symmetry of the funnel plot<sup>[22]</sup> and can be assessed without significant publication bias in the study literature [Figure 8].

## DISCUSSION

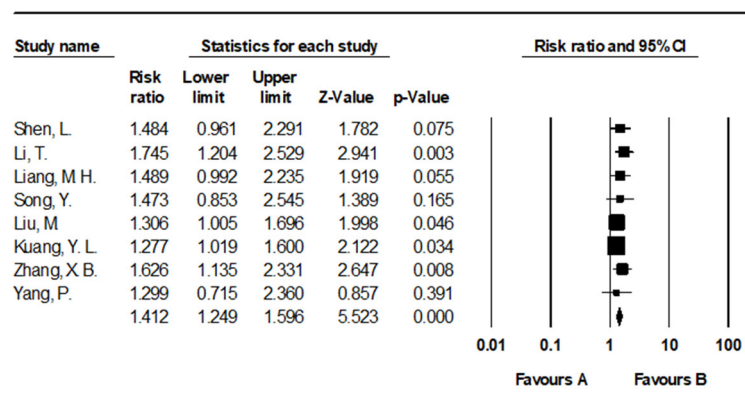
Compared to treatment with TACE alone, this study showed that TACE combined with RFA showed significantly better outcomes on tumor response rate [RR = 1.452, 95%CI: 1.308-1.610,  $P < 0.001$ ], six-month



**Figure 2.** Tumor response rate of comparison TACE with RFA vs. TACE alone

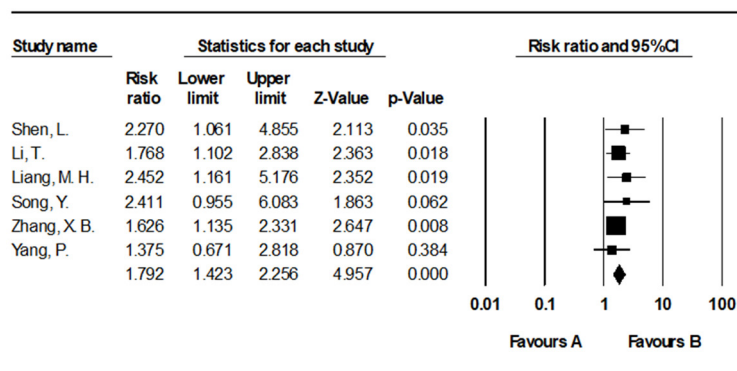


**Figure 3.** Six-month survival rate of TACE with RFA vs. TACE alone

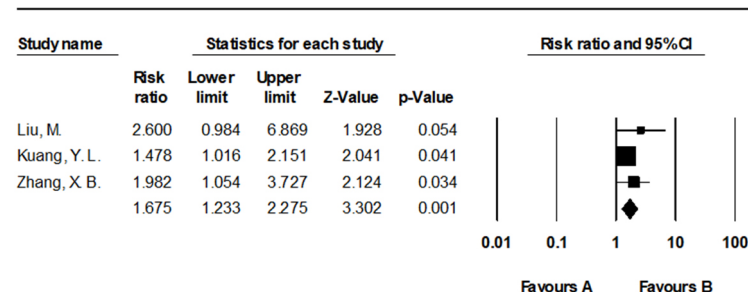


**Figure 4.** One-year survival rate of TACE with RFA vs. TACE alone

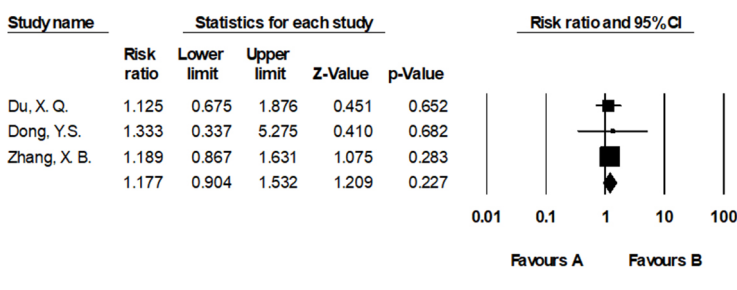
survival rate [RR = 1.257, 95%CI: 1.128-1.401,  $P < 0.001$ ], 1-year overall survival rate [RR = 1.412, 95%CI: 1.249-1.596,  $P < 0.001$ ], eighteen-month survival rate [RR = 1.792, 95%CI: 1.423-2.256,  $P < 0.001$ ], and 2-year overall survival rate [RR = 1.675, 95%CI: 1.233-2.275,  $P = 0.001$ ]. To our knowledge this study is the first meta-analysis to disclose the efficacy of TACE combined with RFA for HCC tumors larger than 5 cm, compared with TACE alone. The publication bias of this study was evaluated using the symmetry level of the funnel plot<sup>[22]</sup>. In the analysis of the tumor response rate and survival rate, the symmetry of the shape of the



**Figure 5.** Eighteen-month survival rate of TACE with RFA vs. TACE alone



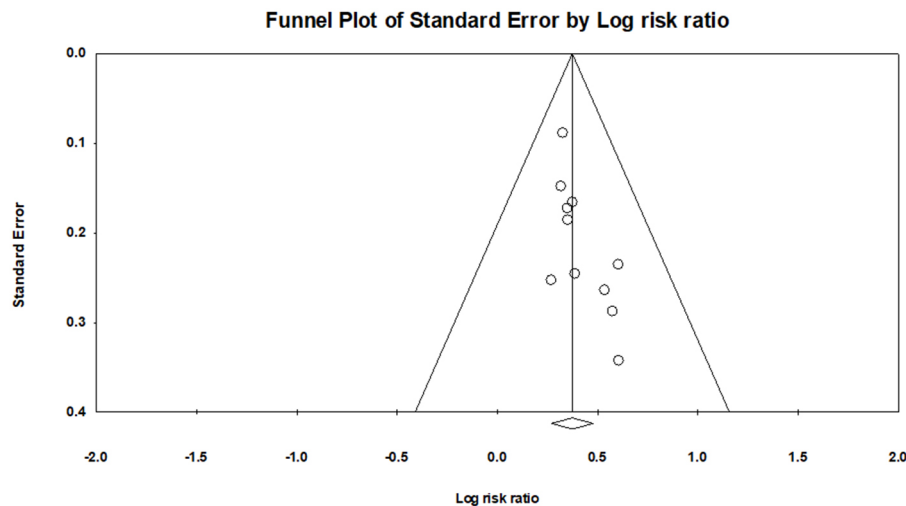
**Figure 6.** Two-year survival rate of TACE with RFA vs. TACE alone



**Figure 7.** Incidence of fever in the TACE with RFA group vs. TACE alone group

funnel plots indicates that there is no significant bias in this meta-analysis. The overall quality of the studies included in this meta-analysis was evaluated to be of high quality, which gives confidence to our results.

HCC is a serious global health problem and the third most common cause of cancer death. Most patients with HCC are diagnosed with intermediate or advanced stage, with baseline liver dysfunction, intrahepatic metastasis or excessive load, and are not suitable for surgical resection. The established local treatment options include TACE, RFA, ethanol injection, and microwave coagulation; however, it is still unclear which method is the most efficacious<sup>[23-25]</sup>. In the 2018 NCCN Clinical Practice Guidelines for Malignancies, TACE is recommended as a first-line palliative treatment for unresectable HCC. However, the tumor response rate and survival rate of patients treated with TACE alone are not ideal. Therefore, the treatment of TACE combined with other local treatment options such as RFA for comprehensive treatment is gradually being adopted.



**Figure 8.** Meta-analysis of funnel plots of tumor response rate

Based on our meta-analysis, combination therapy of TACE with RFA is an effective method for HCC treatment. HCC is mainly supplied by the hepatic artery. Even when the hepatic artery blood flow is blocked by TACE, the thermal coagulation effect of RFA is not affected. Thus, it increases the area of necrosis induced by RFA. Additionally, the effects of expanded ablation zones and anticancer agents on liver cancer cells during treatment may reduce the chance of tumor recurrence<sup>[26]</sup>.

This meta-analysis has some limitations. Firstly, the complications and adverse reactions of combination therapy cannot be assessed fully due to the lack of original research data. Therefore, future studies can further evaluate these indicators. Secondly, the sample size of this current meta-analysis is limited; large-scale randomized controlled trials of long-term follow-up are needed to validate this result.

In conclusion, our study suggests that TACE combined with RFA is superior to TACE alone in the treatment of HCC larger than 5 cm. Patients in the combined treatment group showed significantly increased tumor response rate and survival rates compared with those treated with TACE alone. This article provided clinical and systematic evidence for the improved treatment of HCC larger than 5 cm.

## DECLARATIONS

### Authors' contributions

Design of the work: Yang Y, Long Y, Zhang WL

Acquisition, analysis of data: Yang Y, Lv ZM, Yan M

Wrote this paper: Yang Y, Zhang HX, Long Y, Zhang WL

Revised the manuscripts: All authors

### Availability of data and materials

All data did by the authors listed in this paper.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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Review

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# Molecular diagnosis and therapy of hepatocellular carcinoma: achievements and challenges

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## Abstract

Hepatocellular carcinoma (HCC) is often associated with pre-existing chronic liver pathologies of different origin infections of hepatitis B virus (HBV) and hepatitis C virus. Clinically, the diagnosis and therapy for HCC are very important for the prognosis of patients. However, current methods for HCC diagnosis and therapy have no an optimal accuracy due to the tumor heterogeneity and the frequent late diagnosis. This review summarizes the new advances in molecular diagnosis and therapy of HCC, based on the recent novel biomarkers and new therapeutic strategies for HCC, including alpha-fetoprotein-L3, glypican-3, heat shock protein 90, dickkopf WNT signaling pathway inhibitor 1, paraoxonase 1, highly up-regulated in liver cancer. Moreover, epigenetic regulation, signal pathway, cellular and molecular targets for the immunotherapy, tumor microenvironment and genome sequencing analysis may serve as the molecular expression signatures in clinical practice. For promising new treatment strategy of HCC, targeting molecular therapy based on the restoration of tumor suppressor genes lost and inhibition of oncogenic genes is attractive. The new clinical trials for other molecular-targeted agents, including pembrolizumab, nivolumab, tivantinib, lenvatinib, cabozantinib, and ramucirumab, are ongoing in clinic. Interestingly, anti-HBV drugs display an amazing therapy for HBV-related HCC. In future, the global determination of more biomarkers may provide new insights into the diagnosis of HCC. More importantly, the diagnostic markers should be used to trace patient's follow-up disease progression, guiding doctors to judge and prescribe drugs for status of an illness, prognosis and other processes.

**Keywords:** Molecular diagnosis, therapy, hepatocellular carcinoma, hepatitis B virus, hepatitis C virus

## INTRODUCTION

Hepatocellular carcinoma (HCC) is a serious health issue globally. The increased trends of HCC will remain until 2030<sup>[1]</sup>. According to the World Health Organization, HCC is the fifth most common cancer



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worldwide and the second most common cause of cancer-related death in 2015. Hepatitis B virus (HBV) or hepatitis C virus (HCV) infection accounting for 80%-90% of all HCC cases are well-known major risk factors for the development of HCC. The other risk factors, such as aflatoxin B1 exposure, alcoholic and non-alcoholic liver cirrhosis, obesity, diabetes, vitamin D deficiency, are involved in HCC occurrence<sup>[2,3]</sup>. Although treatment with HBV and HCV infection by some recent antiviral therapies is available, virally mediated hepatocarcinogenesis is still the etiology for the majority of HCC cases worldwide<sup>[4]</sup>. In patients with advanced HCC, there is low response to chemotherapy, and sorafenib is the only standard treatment recommended in international guidelines<sup>[5]</sup>. Thus, it is very important to identify novel diagnosis biomarkers and therapeutic targets for prognosis of HCC.

Molecular mechanisms of malignant cells will lead to the development of successful HCC therapies. NcRNAs and epigenetic regulation have been considered as a potential non-invasive biomarkers due to their experimental and clinical versatility. Whole-genome sequencing analysis has promoted molecular profiles transduced in expression “signatures” which will help in the comprehension of liver physiopathology. HCC etiology seems to be a factor that should be included in several clinic association studies.

The development of novel and useful biomarkers can be employed as a screening strategy for early diagnosis and prognosis in these high-risk populations, since HCC presents a high mortality rate<sup>[6]</sup>. Because late diagnosis, resistance to treatment, tumor recurrence, and metastasis cause to low survival, it is essential for developing novel diagnostics and therapeutics of HCC<sup>[7]</sup>. However, current methods for HCC diagnosis and therapy have no an optimal accuracy due to the tumor heterogeneity and the frequent late diagnosis. Therefore, the most urgent needs for early diagnosis and novel therapies of HCC should be developed.

## DIAGNOSIS FOR HCC

The present methods for diagnosis of HCC can be divided into the following major aspects: magnetic resonance imaging, abdominal ultrasonography, and contrast-enhanced computed tomography, liver biopsy, and serological test. However, the diagnostic effectiveness of above technologies is not very satisfied, particularly for the diagnosis of small lesions and early diagnosis of HCC. The abdominal ultrasound is an operator-dependent test. Liver biopsy is an invasive method not exempt of mortality risk<sup>[8]</sup>. Therefore, the serological test should be developed. In this review, we focused on the advances of gene diagnosis for HCC.

### MiRNAs serve as HCC diagnostic markers

As we know, cellular miRNAs released into the extracellular circulation system can be detected by serological test. Owing to circulating miRNAs relevant to HCC, miRNAs may serve as potential biomarkers. Thus, some circulating miRNAs can be considered as representative of certain pathological conditions. Moreover, circulating miRNAs possess accessibility well and high stability in the detection system, particularly for supervision of early stage, pre-symptomatic diseases in at-risk patients<sup>[9]</sup>. It has been reported that a serum diagnostic test, based on a 34-miRNAs signature, can recognize the early stage lung cancer with 80% accuracy<sup>[10]</sup>.

MiRNAs may be used as biomarkers for prognosis or diagnosis in HCC. Down-regulation of miR-let-7g, miR-22, miR-26, miR-29, miR-99a, miR-124, miR-139, miR-145 and miR-199b is involved in the cell's life activities, including proliferation, apoptosis, angiogenesis, disease recurrence, disease-free survival (DFS) and poor prognosis<sup>[11-19]</sup>. On the contrary, the increase of miR-10b, miR-17-5p, miR-21, miR-135a, miR-155, miR-182, miR-221 and miR-222 is taken part in the metastasis, angiogenesis and poor prognosis<sup>[20-26]</sup>. Additionally, miRNA profiling categorizes HCC into three main parts<sup>[27]</sup>. The above discoveries display the important value of miRNA detection in prediction of HCC survival. Some microRNAs may be used for HCC diagnosis. MiR-101 in serum sample was 95.5% for sensitivity and 90.2% for specificity, respectively<sup>[28]</sup>. MiR-18a in serum sample was 86.1% for sensitivity and 75% for specificity, respectively<sup>[29]</sup>. The expression of miR-25 was

**Table 1. Serum diagnostic markers in hepatocellular carcinoma**

Biomarkers	Sensitivity (%)	Specificity (%)
AFP <sup>[6]</sup>	60.0	85.0
AFP-L3 <sup>[6]</sup>	84.9	86.4
DCP <sup>[6]</sup>	80.0	81.0
AFP + AFP-L3 + DCP <sup>[6]</sup>	94.3	86.4
PON <sup>[6]</sup>	41.6	85.7
Fuc-PON1 <sup>[6]</sup>	79.1	53.5
Hsp90α <sup>[53]</sup>	93.32	90.27
Hsp90α + AFP <sup>[53]</sup>	93.70	94.40
MiR-101 <sup>[28]</sup>	95.5	90.2
MiR-18a <sup>[29]</sup>	86.1	75

AFP: alpha-fetoprotein; AFP-L3: lens culinaris agglutinin (LCA)-reactive AFP; DCP: C-carboxy prothrombin; PON1: paraoxonase 1; Fuc-PON1: PON1-fucosylated level protein; HSP90α: heat shock protein 90 alpha

significantly up-regulated in HCC tissues, which may be used in HCC prognosis<sup>[30]</sup>. And miR-155 reflected tumor recurrence, micro-vascular invasion and recurrence-free survival<sup>[31]</sup> [Table 1].

Furthermore, it has been reported that miR-500 is highly expressed in the sera of HCC patients. While, after surgical treatment, the expression level is reduced<sup>[32]</sup>. What's more, other miRNAs including miR-25, miR-375 and let-7f, can also be used for distinguishing HCC from normal tissue<sup>[33]</sup>. Thus, the levels of extracellular miRNA expression are steady in the body circulation. It suggests that miRNAs may be used as biomarkers for HCC diagnosis.

### LncRNAs function as HCC diagnostic markers

Long non-coding RNAs (lncRNAs) are a subgroup of non-coding RNA transcripts greater than 200 nucleotides in length with little or no protein-coding potential. Emerging evidence indicates that lncRNAs may play important regulatory roles in the pathogenesis and progression of human cancers, including HCC. Certain lncRNAs may be used as diagnostic or prognostic markers for HCC, a serious malignancy with increasing morbidity and high mortality rates worldwide. LncRNA HOX transcript antisense intergenic RNA (HOTAIR) which can bind to lysine-specific demethylase 1 (LSD1) is a 2.2 kilobase ncRNA residing in the HOXC locus. HOTAIR serves as a scaffold of histone modification complexes including LSD1 and polycomb-repressive complex 2, leading to the development of various tumors<sup>[34,35]</sup>. For example, owing to HOTAIR serving as a scaffold, we found that HBXIP/HOTAIR/LSD1 complex function as a critical effector of c-Myc in transcriptional activation of downstream target genes<sup>[36]</sup>. Silencing HOTAIR increased response of HepG2 to apoptosis stimulation from TNF-α and chemo-drug Cisplatin and Doxorubicin on a dose-manner<sup>[37]</sup>. Highly upregulated in liver cancer (HULC) was detected in 63% (19/30) of the HCC patient's serum, which was much higher than in the healthy control group (10%, 2/20)<sup>[38]</sup>. Among the HCC patients, HULC detection frequencies increase with Edmondson grades. The detection rates are 14%, 62%, and 100% for Edmondson grades I-II, II-III, and III-IV, respectively<sup>[38]</sup>. HULC was detected more frequently in the plasma of HBV<sup>+</sup> HCC patients (90%) than in HBV-HCC patients (25%)<sup>[38]</sup>. These observations indicate that the presence of HULC is an indication of HCC and its progression. Interestingly, HULC contributes to the abnormal lipid metabolism in HCC cells<sup>[39]</sup>. Hepatitis B virus X protein (HBx) is able to raise the expression of HULC in both normal liver L-O2 cells and liver cancer HepG2 cells<sup>[40]</sup>. In addition, HULC significantly enhances the hepatocellular proliferation by promoting the HMGA2 expression by sequestration of the microRNA-186 in HCC<sup>[41]</sup>. Therefore, the data support the clinical usage of HULC lncRNA as a potential biomarker for HCC diagnosis and prognosis.

Taken together, the development of lncRNA expression profiling using high-throughput technology for specific HCC biomarkers will no doubt lead to more accurate and precise clinical decision-making having the consequence of better patient care in the future. While the first miRNA (lin-4) was identified two

decades ago, other ncRNAs including lncRNAs, snoRNAs, siRNAs and piRNAs have been surfaced and proved to be essential players in cancer pathogenesis<sup>[42]</sup>. Therefore, the combination of lncRNAs with other ncRNAs should not be underestimated in the onset and progression of HCC.

### Epigenetic markers

DNA methylation affects the phenotype mainly through expression of the corresponding genes, methylation profiles could reflect the biological characteristics of HCC if “passive methylation” could be eliminated appropriately. A number of results have shown the predictive value of selected methylation events on survival<sup>[43]</sup>. After hepatectomy, the methylation map of liver may reflect the recurrence-free survival of HCC patients<sup>[44]</sup>. It has been reported that the determination of DNA methylation as a potential tumor marker is able to monitor the circulating tumor DNA in plasma samples<sup>[45]</sup>. High levels of trimethylated histone H3 lysine 4 (H3K4me3) were usually accompanied by the decreased overall survival and poor prognosis in HCC<sup>[46]</sup>. Another research also indicated that high levels of H3K27me3 forecasted poor prognosis and aggressive tumor characteristics, such as large tumor size, vascular invasion, multiplicity of tumors and poor differentiation<sup>[47]</sup>. To better understand the roles in HCC, more studies using accurate detection methods, such as ChIP-sequencing, may be developed to evaluate these specific DNA-protein modifications.

About the traditional biomarkers, because the false negative rate of alpha-fetoprotein (AFP) is about 40% for early-stage HCC patients, 15%-30% of all the patients, even patients with advanced HCC, AFP levels remain normal<sup>[48]</sup>. Lens culinaris agglutinin (LCA)-reactive AFP (AFP-L3) as an isoform of AFP may improve the detective rate for small lesion of liver cancer<sup>[49]</sup>. It has been reported that C-carboxy prothrombin (DCP) has a higher specificity for HCC than AFP but is less sensitive. Thus, the combination of AFP, AFP-L3, and DCP seems to significantly improve HCC diagnostic accuracy<sup>[50]</sup>. The combination of Glypican-3 (GPC3) as a novel tumor marker of HCC with AFP proves the sensitivity but not the specificity in HCC diagnosis<sup>[51]</sup>. Moreover, the combination of the NH<sub>2</sub>-terminal portion of GPC3 which is also called soluble GPC3 with AFP can improve overall sensitivity from 50% to 72%<sup>[52]</sup>. Interestingly, the dynamic changes of plasma Hsp90 $\alpha$  in liver cancer patients can detect the condition of treatment, such as surgery and interventional therapy<sup>[53]</sup>. Serum Dickkopf WNT signaling pathway inhibitor 1 (DKK1) was reported to be a useful biomarker for diagnosis of HCC by a large-scale and multicenter study<sup>[54]</sup>. Paraoxonase 1 (PON1) has been proposed as a circulating protein biomarker since high serum levels in HCC patients concomitantly infected with HCV infection has been observed<sup>[55]</sup>. PON1-fucosylated level protein has been helpful in distinguishing early HCC from liver cirrhosis patients even with low AFP levels<sup>[56]</sup>.

## THERAPY FOR HCC

### Prevention and management of HBV infection

Based on hepatocarcinogenesis, prevention of HBV infection is a key step to reduce the incidence of liver cancer. There is a renewed interest regarding the understanding of various steps of the HBV replication cycle, as well as specific virus-host cell interactions, to define new targets and develop new antiviral drugs. Basically, the HBV covalently closed circular DNA (HBV cccDNA) is pivotal for persistent HBV infection and recurrence by the end of treatment. As far as we know, the cccDNA usually organizes into a minichromosome with histone 3 and H4 proteins and other nonhistone proteins, such as HBx, HBV core protein, and host transcription factors<sup>[57]</sup>. Even though recent therapies can successfully control the viral replication, but they fail to eliminate cccDNA completely. Demonstrating the molecular mechanisms and screening critical factors involved in cccDNA may make it come true to develop more precisely targeted therapeutic strategies and cure HBV-related HCC patients<sup>[58]</sup>. It covers a series of inhibition of viral replication processes such as entry inhibitors, capsid assembly modulators, approaches aiming at the secretion of viral envelope proteins, drugs targeting HBV cccDNA, and siRNAs targeting viral transcripts. Restoration of immune responses is a complementary approach. HBV chronic infection and high viral load have been associated with higher levels of soluble programmed cell death protein 1, and this results



in cytotoxic T-cell inhibition and 6.3-fold increase in risk for HCC development<sup>[59]</sup>. Using HBV infection models *in vitro* and *in vivo*, new targets and compounds will be available<sup>[60]</sup>.

HBx plays a crucial role in the various signal transduction pathways and HBV-induced hepatocarcinogenesis<sup>[61]</sup>. HBx accelerates the development of hepatoma<sup>[62]</sup>. HBx-elevated male-specific lethal 2 can strengthen HBV replication by regulating cccDNA in liver cancer cells, resulting in the development of HCC<sup>[63]</sup>. Moreover, we report that anti-HBx in sera may serve as one of the markers involving HBV-related liver cirrhosis and liver cancer<sup>[64]</sup>. Developing drugs targeting HBx is crucial for HBV-related HCC therapy. Two types of drugs, conventional interferon, and nucleoside analogs, have become available for the treatment of chronic hepatitis B infection. We also report that anti-HBV drugs such as entecavir, telbivudine and IFN- $\alpha$ 2b inhibit the tumor growth of HBV-related HCC through depressing HBx<sup>[65]</sup>. The finding gives innovative insights into the mechanisms of anti-HBV drugs in HCC therapy.

### Molecular targets for the immunotherapy

The research about a cohort of 956 HCC patients, 25% had high expression of programmed death-ligand-1 (PD-L1) and programmed cell death protein-1 (PD-1) in HCC tissues. Moreover, the study found that infiltrating CD8<sup>+</sup> TILs (tumor-infiltrating lymphocyte) could induce PD-L1 expression *via* IFN- $\gamma$ <sup>[66]</sup>. Icotinib decreases the growth of hepatoma cells *in vitro* and *in vivo*, relying on EGFR activation and PD-L1 expression<sup>[67]</sup>. Thus, the PD-1/PD-L1 pathway is available for prognosis and therapy in HCC. Patients with positive PD-L1 expression had significantly poorer DFS and overall survival (OS) than PD-L1 negative patients. The median DFS and OS were 14.9 and 29.6 months for PD-L1 positive patients compared with not reached and 59.4 months for PD-L1 negative patients, thus confirming the findings of the prognostic value of PD-1/PDL-1 in HCC<sup>[68]</sup>. Currently, nivolumab, a monoclonal antibody targeting PD-1, obtained an accelerated FDA approval in view of tumor response and durability for the therapy of HCC patients already treated with sorafenib in the phase 1/2 single-arm CheckMate 040 study<sup>[69]</sup>. Nivolumab and pembrolizumab targeted PD-1 are ongoing in clinic<sup>[70]</sup>.

### Clinical trial status of molecular-targeted agents

Tivantinib as the first drug was used to a phase III trial grounded in receptor overexpression analyses after disease progression on sorafenib in HCC<sup>[71]</sup>. Lenvatinib as an oral multikinase inhibitor for differentiated thyroid cancer and renal cell cancer treatment initially was approved. In a phase 2 trial of HCC patients in Japan and South Korea, lenvatinib treatment was obtained with a 37% response rate (by mRECIST), a median TTP of 7.4 months, and an available toxicity profile<sup>[72]</sup>. In addition, Regorafenib as the first agent showed a good survival benefit over placebo in patients progressing on sorafenib<sup>[73]</sup>. Regorafenib acting as an oral multikinase inhibitor, largely interdicted the activity of multiple protein kinases including tumor proliferation, metastasis, angiogenesis, microenvironment, and tumor immunity. Regorafenib exhibited a favorable survival regardless of the last dose of prior sorafenib (HR 0.67 for 800 mg/day; 0.68 for < 800 mg/day)<sup>[74]</sup>. Further approvals are coming, with good results from phase 3 trials evaluating cabozantinib and ramucirumab in the second-line setting. Cabozantinib, a small-molecule multikinase inhibitor was better than the placebo in the randomized phase 3 CELESTIAL trial<sup>[75]</sup>. Based on the trial analyses 707 advanced HCC patients previously received sorafenib treatment, cabozantinib distinctly increased OS over placebo (10.2 months vs. 8.0 months, respectively,  $P = 0.0049$ )<sup>[76]</sup>, as shown in Table 2.

### Sorafenib

Sorafenib as a molecular-targeted agent can attenuate HCC proliferation and angiogenesis by inhibiting RAF serine threonine kinase and VEGF, PDGF, Flt-3, c-Kit receptor tyrosine kinase, getting approved in Europe and North America in 2007 and in Japan on May 20, 2009. To our delight, a subanalysis of the SHARP study, such as sorafenib in combination with resection, ablation, transcatheter arterial chemoembolization or hepatic arterial infusion chemotherapy, will overtly extend the overall survival in early-, intermediate- or

**Table 2. Overview of clinical trial agents for hepatocellular carcinoma therapy**

Agents	Developmental status	Targets	Outcomes
Nivolumab	Phase III	PD-1 <sup>[69]</sup>	Ongoing <sup>[70]</sup>
Pembrolizumab	Phase III	PD-1 <sup>[76]</sup>	Ongoing <sup>[70]</sup>
Ramucirumab	Phase III	VEGFR2 <sup>[77]</sup>	Survival benefit for the subgroup with AFP $\geq$ 400 ng/mL <sup>[79]</sup>
Lenvatinib	Phase III	VEGFR1-3, FGFR1-4, PDGFR $\alpha$ , RET and KIT <sup>[70]</sup>	Non-inferior OS, improved PFS, TTP, and ORR <sup>[70]</sup>
Cabozantinib	Phase III	TIE-1, TIE-2, FLT3, c-MET, KIT, RET and VEGFR <sup>[78]</sup>	Significant improvement in OS, PFS, and ORR <sup>[75]</sup>
Tivantinib	Phase III	c-MET <sup>[71]</sup>	Improved OS and PFS <sup>[71]</sup>

HCC: hepatocellular carcinoma; OS: overall survival; ORR: objective response rate; PFS: progression-free survival; TKI: tyrosine kinase inhibitor; TTP: time to progression

advanced-stage HCCs<sup>[80]</sup>. However, it increases the potential risk of invasion and metastasis of HCC although it significantly delays tumor progression time<sup>[81]</sup>.

## FUTURE CHALLENGES

Although the effective diagnosis and therapies have been developed in HCC at present, it is unsatisfied to improve the patients' survival. The challenges in the field of diagnosis and therapy for HCC are still ongoing. Therefore, developing new diagnostic approach and drugs are urgent. About gene diagnosis of HCC, high-throughput means combined with bioinformatics methods will be used to find out the root cause of HCC in large-scale sample research. Clinically, it is necessary to monitor the biomarkers in the development of HCC involving treatment and prognosis, but not only in the early stage. It is vital to develop kits for determining the replication activity of HBV (or HCV) and HBV cccDNA to evaluate the risk of HCC incidence. For HCC therapy, identifying innovative targets and combination with multiple drugs are still needed in the treatment strategy. Effective combination of antiviral therapies with anti-inflammation drugs involving inflammation factors is available to treat chronic HBV-related HCC. It is necessary to examine the sensitivity, specificity, predictive value positive, predictive value negative, and validity of any candidate biomarker in a large pool of HCC patients with or without HBV infection, furthermore, it is also important to follow up large patients with HBV or other risk factor exposure for the prediction of occurrence and postoperative recurrence of HCC using representative markers. If biomarkers are valid, it is necessary to develop kits for molecular diagnosis, monitoring the efficacy, prognosis and treatments of HCC patients.

## CONCLUSION

In summary, this review lists the recent progresses in gene diagnosis and therapy for HCC. The achievements include the recent novel biomarkers and novel therapeutic strategies for HCC, such as AFP, AFP-L3, GPC3, HSP90, DKK1, PON1, *etc.* Moreover, epigenetic regulation, signal pathway, cellular and molecular targets for the immunotherapy, tumor microenvironment and genome sequencing analysis may also serve as the molecular expression signatures in clinical practice. More studies are necessary to find new biomarkers for prognosis and treatment response in patients under standard treatment of sorafenib. The new clinical trials for other molecular-targeted agents, including pembrolizumab, nivolumab, tivantinib, lenvatinib, cabozantinib, and ramucirumab, are ongoing in clinic. Anti-HBV drugs are available in the therapy of HBV-related HCC.

## DECLARATIONS

### Authors' contributions

Drafted the outline of this review: Zhang XD

Drafted the manuscript: Zhang XD, Zhao M

Finalized the manuscript: Zhang XD, Zhao M

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Not applicable.

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**Conflicts of interest**

All authors declare that there are no conflicts of interest.

**Ethical approval and consent to participate**

Not applicable.

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Review

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# Liver carcinogenesis: diagnostic and clinical aspects of preneoplastic nodules

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## Abstract

In multistep hepatocarcinogenesis, sizable lesions can precede the development of hepatocellular carcinoma (HCC). These lesions are currently classified as low grade (LG)- and high grade (HG)-dysplastic nodules. Following international guidelines recommending the surveillance of cirrhotic patients, a growing number of 1-2 cm hepatocellular nodules are recognized including early hepatocellular carcinoma (eHCC) and DN the latter accounting for as many as 70% of nodules < 1 cm. HG-DN are currently considered the most advanced HCC precursors. The histological diagnosis of low-grade dysplastic nodule (LG-DN), high- grade dysplastic nodule (HG-DN) and eHCC in small liver biopsies requires a comprehensive stepwise morphological and immunocytochemical approach. By imaging the differential diagnosis among these lesions is a challenge. According to vascular enhancement at dynamic computed tomography (CT) or magnetic resonance imaging (MRI) these precursors are classified as hypo-vascular/ indeterminate nodules even though distinction between LG-DN and HG-DN is almost impossible. The introduction of gadoexetic acid-enhanced MRI has represented an extremely important advance in this field allowing a better differentiation of dysplastic lesions from eHCC and progressed HCC. Additional MRI features as diffusion-weighted imaging further improved diagnostic accuracy of imaging. According to Liver Imaging Reporting and Data System (LI-RADS), either CT/MRI or Contrast-Enhanced Ultrasound LI-RADS, the dysplastic lesions should be categorized as LR-3 or LR-4. Natural history of these lesions confirmed that HCC can develop from HG-DN but which nodule and when it will undergo malignant transformation is not predictable. The search and validation of radiological and tissue markers able to select lesions more prone to HCC development, is currently underway. Whether and how HG-DN should be ablated or closely followed up is currently debated.

**Keywords:** Low-grade dysplastic nodule, high-grade dysplastic nodule, early hepatocellular carcinoma, progressed hepatocellular carcinoma, dynamic imaging, gadoexetic acid-enhanced resonance imaging, hepatobiliary phase



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## INTRODUCTION

It has long been known that, like in others human carcinogenetic models as colo-rectal cancer, in hepatocellular multistep carcinogenesis precancerous lesions both micro- and macroscopic precede the development of hepatocellular carcinoma (HCC)<sup>[1,2]</sup>. Chronic hepatitis and cirrhosis represent the natural background in which precancerous lesions grow through stepwise sequence. These lesions have been described many years ago and include microscopic dysplastic foci (DF) and sizable dysplastic nodules (DN). The latter are further categorized as low-grade (LG-DN) and high-grade dysplastic nodules (HG-DN) with specific and distinctive morphologic characteristics and different carcinogenetic propensity<sup>[3]</sup>. Representation of multistep hepatic carcinogenesis is schematized in [Figure 1]. Given the low attitude to perform liver biopsy in cirrhotic patients, microscopic preneoplastic lesions have been losing interest in clinical practice. Hence, the present review will focus specifically on preneoplastic nodules which can be routinely detected by radiologic techniques and monitored over time for their potential neoplastic transition. Histologic classification, histopatologic features, radiologic diagnostic criteria, natural history and treatment of these lesion will be discussed.

In cirrhosis, application of regular ultrasound surveillance resulted in the discovery of an increasing number of small nodules [ $< 2$  cm according to the definition of small lesion by International Working Party (IWP)]<sup>[4]</sup>. Small nodules usually include definite hepatocellular carcinoma (HCC), either early hepatocellular carcinoma (eHCC) and very-early hepatocellular carcinoma (veHCC) or progressed hepatocellular carcinoma (pHCC) and the large majority of pre-neoplastic nodules<sup>[5-9]</sup>. Other small lesions as metastases or mesenchymal neoplasms are much less frequently detected during surveillance.

Distinction of pre-neoplastic nodules from well-established HCC is mandatory for decision making process, particularly in transplant or surgical setting. To decide whether a given nodule is malignant, premalignant or benign is therefore of paramount importance demanding a great radiologic, morphologic and clinical expertise. The following key points should help clinicians to get oriented in the management of such small nodules:

1. Precancerous nodules have a maximum diameter seldom exceeding 2 cm and are typically detected in cirrhosis/chronic hepatitis.
2. Their prevalence is relevant ranging from 60% to 70% among nodules  $< 1$  cm and from 20% to 30% among nodules  $> 1 < 2$  cm.
3. They can be intercepted as single or multiple lesions and often concomitant with already established HCC.
4. HG-DN account for 30 % of all precancerous nodules and are considered true precancerous lesions whereas LG-DN have a trivial neoplastic risk comparable to that of large regenerative nodules.
5. Transforming risk of HG-DN is largely unpredictable at baseline.

## HISTOPATOLOGICAL CLASSIFICATION AND FEATURES OF DYSPLASTIC NODULES

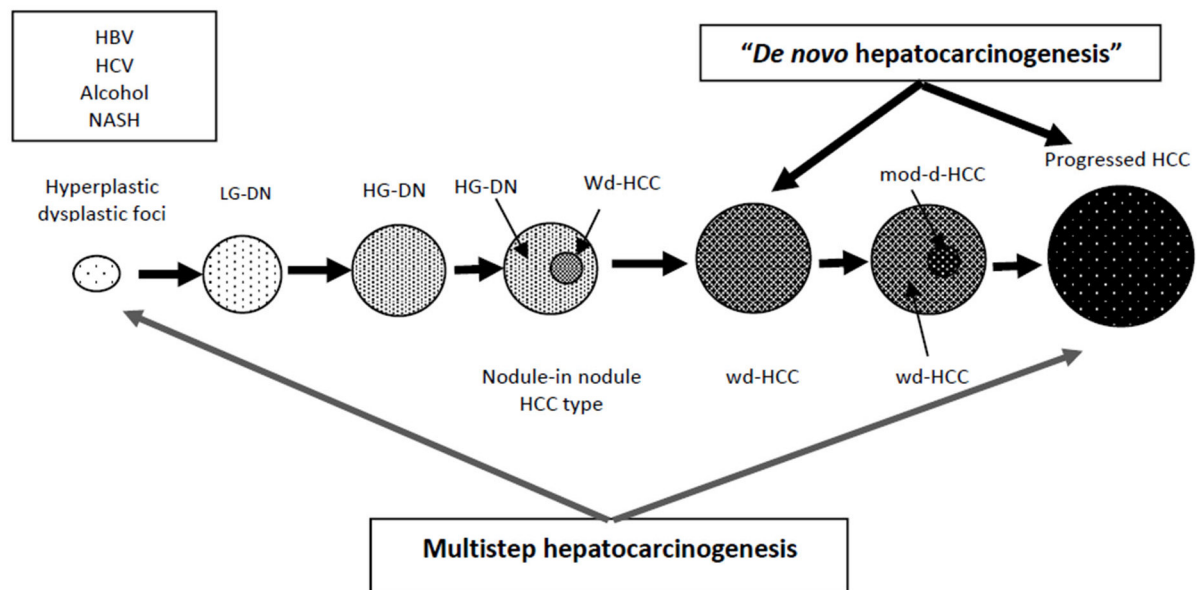
### Nomenclature

Definition and nomenclature of precancerous nodules was firstly proposed by the International Working Party in 1995<sup>[4]</sup> and further updated in an east-western consensus of pathologists in 2008<sup>[10]</sup>.

Non-malignant hepatocellular nodules include large regenerative nodules (LRN) and DN. The latter are subclassified as LG-DN and HG-DN. However, LG-DN share a number of features to non-neoplastic LRN and therefore, the distinction between these two lesions is particularly difficult and unpractical. Hence, the panel of experts recommended not to separate LG-DN from LRN and classify as LG-DN any nodule that cannot be classified as HG-DN<sup>[4]</sup>. Their size ranges from few millimetres to 2 cm rarely exceeding 3 cm.

### Histologic differential diagnosis

Histologic differentiation of LG-DN, HG-DN and eHCC is challenging, particularly when pathologists have to deal with small samples obtained by fine needle biopsy. It has been repeatedly stressed that histology is



**Figure 1.** Schematic illustration of lesions occurring in hepatocarcinogenesis. Two models of human hepatocarcinogenesis. HCC: hepatocellular carcinoma; DN: dysplastic nodules; HG-DN: high-grade dysplastic nodules; LG-DN: low-grade dysplastic nodules

mandatory, and that comparison between intra nodular and extra nodular tissue is of great importance. Cytology should be avoided since it does not allow to appreciate architectural changes useful for differential diagnosis. First diagnostic step is to evaluate whether or not the sample is adequate. Schematically, adequacy can be checked by evaluating the overall nodularity at low magnification [Figure 2] by comparing intra- and extranodular samples. In brief, when both intra- and extra-lesional samples show overlapping cirrhosis-like features, without a major nodule in the background, the sample should not be considered adequate and biopsy should be repeated shortly after. This is another reason why cytology is inadequate to approach this issue.

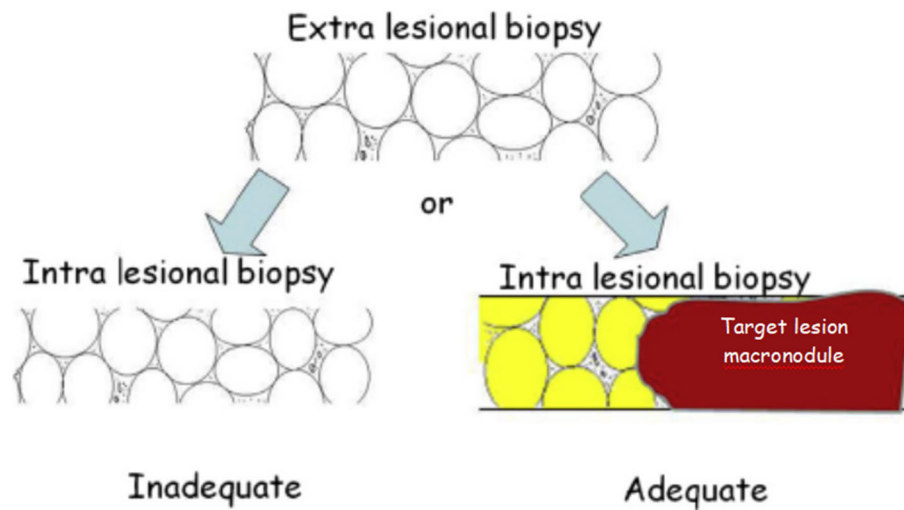
*Small liver cell dysplasia* (SLCD) is a key feature of HG-DN. It refers to areas of increased cell density (more than twice that of the surrounding tissue) and plate thickening, simulating a well-differentiated HCC. Formation of pseudoglands/acini along with SLCD are features helping to distinguish HG-DN from LG-DN. These features are also present in eHCC even though much more pronounced.

*Unpaired arteries*, so called because they are not coupled with bile ducts, is another distinctive feature of liver carcinogenesis and represent neoangiogenesis which is the morphologic counterpart of arterial contrast enhancement on imaging. Similarly, sinusoidal capillarization which can be highlighted by CD34 immunostaining is minimal in cirrhosis and increases from LG- to HG-DN with the highest levels in HCC.

*Reticulin framework* is another feature useful to distinguish benign from malignant nodules since it is usually well preserved in both regenerative and dysplastic nodules whereas it is decreased or lost in advanced HCC; however, in early well-differentiated HCC, the reticulin framework may be retained or only slightly decreased.

*Portal tracts* are retained in HG-DN and can be retained in eHCC as well and their presence does not help differential diagnosis.

*Stromal invasion* is the key feature of definitive malignant transition separating eHCC from HG-DN. Stromal invasion has to be looked for in portal tracts but, unfortunately, it is not always detectable, especially on samples obtained by fine needle biopsy. For iconography please refer to recent review by Roncalli et al.<sup>[11]</sup>.



**Figure 2.** Schematic illustration of adequacy judgement of liver biopsy for small nodules in cirrhosis. Intra- and extra-lesion samples are mandatory. Schematically, adequacy can be checked by evaluating the overall nodularity at low magnification by comparing intra- and extranodular biopsy samples. When both intra- and extra-lesional samples show overlapping cirrhosis-like features, without a major nodule in the background, the sample should not be considered adequate and biopsy should be repeated shortly after

The immunostaining of tissue biomarkers is a promising tool in the armamentarium of pathologists for improving the accuracy of the differential diagnosis between benign and premalignant/malignant nodules [Table 1]. In the last two decades, translation of data from gene expression profiles to clinical practice has been focused on the search of serum and tissue markers involved in hepatocarcinogenesis and useful for diagnostic purposes. Most of these markers can be tested on paraffin embedded, hematoxylin-eosin stained tissue samples making this diagnostic technique suitable for employment in clinical practice even on fine needle liver biopsies. Among them alpha-fetoprotein and des- $\gamma$ -carboxy prothrombin are serum markers of HCC but are not sensitive enough for eHCC<sup>[12]</sup>. Other tissue markers as heat shock protein 70 (HSP70), glutamine synthetase (GS), glypican 3 (GPC3), CD34, p53, proliferating cell nuclear antigen (PCNA) and Ki67 have been tested in lesions at different neoplastic evolution in the multistep process leading to mature HCC<sup>[12]</sup>. A panel of three biomarkers: HSP70, GS and GPC3 has been applied to differentiate non-malignant nodules (either LG or HG-DN) from HCC and a positivity for any two or three markers detected malignancy with a sensitivity of 72% and specificity of 100 %. Sensitivity dropped to 50% when the panel was applied to tissue obtained by fine needle biopsy samples whereas specificity remained absolute<sup>[13]</sup>. These findings were validated by other authors<sup>[14]</sup>. More recently it has been demonstrated that the addition of clathrin heavy chain (CHC) to the above-mentioned panel of biomarkers increased the diagnostic accuracy for small HCCs from 76.9% to 84.3%, with an important gain in sensitivity (from 46.8% to 63.8%)<sup>[15]</sup>. Currently, the use of three biomarkers panel with CD34 is endorsed by AASLD-EASL guidelines as a diagnostic instrument for diagnosis of HCC<sup>[16,17]</sup>. An algorithmic workflow based on sample adequacy and histopathological features including biomarkers immunostaining useful for characterization of small nodules in cirrhosis has been recently proposed by Roncalli *et al.*<sup>[11]</sup>.

### The role of biopsy

International guidelines recommend performing a biopsy whenever a nodule results atypical or indeterminate at conventional dynamic imaging (see below)<sup>[16-20]</sup>. If a biopsy should be done, a 18-20 gauge cutting needle should be employed and intra- and extra-lesion sample should be obtained. In the setting of small nodules in cirrhosis however, the role of biopsy is hampered by the risk of false negative results due to sampling error. Smaller the nodule higher the risk of sampling error. The rate of false negative sample is quite high and a successful result achievable in no more than two-third of patients<sup>[8]</sup>. In the remaining one-third of cases a second biopsy within a short interval is recommended. It is important to note that, contrarily to

**Table 1. Morphologic features and diagnostic tools for differential diagnosis between dysplastic nodules and early HCC**

	LG-DN	HG-DN	eHCC	Diagnostic value between HG-DN and eHCC
Elementary morphologic feature				
Parenchymal changes, cytologic alterations				
SCC	-	+	+	Low
LCC	±	±	-	Low
Clone like	±	+	+	Low
Architectural changes				
Cell density	±	+	+	Low
Pseudoglands/acini	-	±	+	Medium
Non-parenchymal changes				
Portal tracts	+	+	±	Low
Reticulin framework	+	+	±	Medium*
Unpaired arteries or sinusoidal capillarization (CD34)	±	±	+	Low
Diagnostic tools				
Stromal invasion/loss of ductular reaction (K7/19)	-	-	±	High
HCC biomarkers expression (at least 2 markers among HSP70, GPC3, GS, CHC)	-	-	++	High
Nodule-in-nodule	-	-	±	High

\*If frankly decreased or lost, the discriminatory value of reticulin framework is high. -: absent; ±: may be present but not necessarily detectable in biopsy; ++: present and usually detectable in biopsy. CHC: clathrin heavy chain; GPC3: glypican 3; HSP70: heat shock protein 70; LCC: large cell change; SCC: small cell change; LG-DN: low-grade dysplastic nodule; HG-DN: high-grade dysplastic nodule; eHCC: early hepatocellular carcinoma

what can be expected, a second biopsy has the same probability of success as the first. Thus, repeated biopsy would finally lead to a successful diagnostic yield in more than 90% of cases<sup>[8,21]</sup>. However, this invasive strategy is seldom adopted in clinical practice mainly for the awareness that *a priori* probability that such indeterminate 1-2 cm nodules are malignant is low<sup>[8,9,22,23]</sup> and that 2 cm is the size threshold to achieve the best result by percutaneous ablation. Based on these assumptions, a *wait and see* strategy with the adoption of a strict monitoring of the nodule by dynamic imaging is often preferred (see below). Nodule location and coagulative disorders are additional features making biopsy difficult or impossible. Lastly, the potential and theoretical risk of tumour seeding, should be considered even though, this risk, ranging between 1% and 2.7%, seems to balance favourably with the risk of inappropriate or delayed treatment<sup>[24-26]</sup>.

## RADIOLOGIC CRITERIA FOR DIAGNOSIS AND CHARACTERIZATION OF PRENEOPLASTIC NODULES IN CIRRHOSIS DYNAMIC IMAGING CT AND MRI

To date, the differential diagnosis between eHCC and dysplastic nodules still remains a radiologic challenge. Premalignant nodules are usually detected by standard US as hypo- or, less frequently, as hyper-echoic nodules completely indistinguishable from well-established HCC. Thus, standard US is inadequate for differential diagnosis and other imaging tools are needed. The physiopathologic basis for the diagnosis and characterization of small hepatic nodules by imaging rests on characteristics of their vascular supply. It is well established that during cirrhosis-related oncogenesis, progressive loss of normal portal vascular supply in favour of increase arterial one, the so called neoangiogenesis, occurs. This nodular arterialization becomes progressively more evident by transition from regenerative to dysplastic and neoplastic nodules reaching the full expression in high-differentiated HCCs. Dynamic CT, magnetic resonance imaging (MRI) and Contrast-Enhanced Ultrasound (CEUS) are the contrast-enhanced imaging to investigate the vascular pattern of nodules detected under surveillance in cirrhosis. A recent meta-analysis including several comparative studies confirmed that MRI is more accurate than dynamic CT for detection and characterization of small lesions and therefore, it should be preferred as a first line panoramic imaging<sup>[27]</sup>. In addition, a comparative 13-years meta-analysis<sup>[28]</sup> showed that CEUS had a sensitivity and PPV close to that of MRI with gadoexetic contrast media.



At imaging using the so-called extracellular contrast media, mature HCC shows a typical vascular pattern characterized by homogeneous and intense contrast uptake in the arterial phase known as “wash in” followed by a progressive washout of contrast in venous or late phases. Opposite, regenerative nodules, being a simple hypertrophic inflammatory growth of normal hepatocytes, maintain a normal vascular supply and share the same contrast uptake as the surrounding cirrhotic parenchyma during arterial and venous phases. Dysplastic nodules, either LG-DN or HG-DN lack a well-developed neoangiogenesis and maintain normal or partially normal portal inflow appearing as non-enhancing or partially enhancing nodules. Conventional dynamic imaging “per se” cannot differentiate LG-DN from HG-DN. Diagnostic difficulties are also represented by those incipient eHCCs with still poorly developed neoangiogenesis which usually appear as non-enhancing lesions (the so-called hypovascular HCC) and, therefore, hardly distinguishable from HG-DNs.

According to all the international guidelines<sup>[16-20]</sup> arterial wash-in and late washout, when present, are sufficient for the diagnosis of HCC and histologic confirmation is not necessary even for small lesions. This paradigm served to design the non-invasive diagnostic algorithm for nodules detected in cirrhotic patients under surveillance proposed by AASL-EASL-ERTO guidelines<sup>[16-17]</sup>. According to this algorithm, new lesions < 1 cm should be strictly monitored without biopsy. Nodules > 1 cm showing definite hallmarks of malignancy by a single enhancing imaging do not require confirmation by a second one and diagnosis of HCC is accepted. In case of equivocal/inconclusive results by the first imaging, a second one should be sequentially performed. If uncertainty still persists after two dynamic techniques, lesion should be categorized as indeterminate or atypical and biopsy is recommended<sup>[16,29]</sup>.

This sequential strategy demonstrated high specificity and almost absolute PPV for diagnosis of HCC and, in clinical practice, proved to be cost-effective and useful for minimizing the number of futile biopsies<sup>[9,22]</sup>.

Although specificity and positive predictive value of radiologic hallmarks of malignancy (wash in and late washout) are close to 100%, their sensitivity in small lesions is suboptimal (71%)<sup>[30,31]</sup>. According to vascular-based imaging modalities one-third of small nodules discovered under surveillance remain indeed indeterminate or atypical even by dynamic MRI. Indeterminate/atypical lesions include approximately 30% of hypovascular eHCCs<sup>[7,22,32]</sup> and the large majority of pre-neoplastic lesions (the one-third rule: one-third of small lesions are indeterminate and one-third of them are HCCs).

Premalignant nodules are usually detected by standard US as hypo- or, less frequently, as hyper-echoic small nodules. Hence, standard US cannot be used for differential diagnosis being dysplastic nodules completely indistinguishable from well-established HCC. On dynamic imaging these nodules appear as non- or weakly enhancing lesions on arterial phase and hypo/iso-enhancing on venous/delayed phases (hypovascular nodules). Therefore, they fall into the group of indeterminate/atypical lesions and, as recommended by AASLD/EASL guidelines, they should require histologic diagnosis. In this setting however, the role of biopsy is still debated (see above). The differentiation between DN and eHCC is challenging on conventional dynamic imaging. The recent introduction of MRI hepato-specific post-vascular contrast media has represented a relevant diagnostic advance in the characterization of small nodules in cirrhosis<sup>[33,34]</sup>. Gadolinium-chelate agents as gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd EOB DTPA, Primovist®) or gadobenate dimeglumine (Gd-BOPTA, MultiHance®) enter the hepatocyte by the organic anion transporter polypeptide 1 (OATP1) and are excreted in the bile canaliculi by MRP2,3,4 protein transporters. These agents make possible to achieve information not only on the vascular profile of a given lesion (dynamic phase) but also on specific hepatocyte functions in the so-called hepatobiliary phase (HE phase). During carcinogenetic process, proteins responsible of uptake and excretion of gadolinium-chelates are progressively lost and this derangement may precede changes on vascular profile<sup>[35]</sup>. Most eHCCs and veHCCs, are in fact no longer able to incorporate gadolinium-chelates and appear hypo-intense on HE phase. Opposite, LG-DN and most HG-DN maintain still preserved uptake function and consequently

appear as iso- or even hyper-intense nodules. These contrast agents are therefore potentially able to distinguish differently progressed lesions<sup>[35]</sup>. Although the full potential of liver-specific-contrast media needs to be fully assessed in prospective studies, the use of these agents is now considered mandatory in this setting since they provide better diagnostic accuracy than the vascular contrast media<sup>[36]</sup>. Recent data on histologically well characterized small atypical nodules in cirrhosis, confirmed the high diagnostic accuracy of Gd EOB DTPA MRI to correctly distinguish eHCC from premalignant nodules. Ninety-seven percent of iso/hypovascular nodules were correctly classified as premalignant or malignant in HE-phase with a specificity of 100% for malignancy when hypointensity in HP phase was coupled with hypervascularity in arterial phase<sup>[32]</sup>. These data are well in keeping with those of Choi *et al.*<sup>[37]</sup>. In a recent prospective study<sup>[38]</sup> carried out on 111 small nodules defined as atypical at CEUS or dynamic CT and histologically classified as eHCCs or dysplastic, either LG-DN and HG-DN, emerged that HE-phase hypointensity by itself using Gd-EOB-DTPA was the strongest predictor of malignancy superior to vascular-based hallmarks and T2w behaviour. All hypointense nodules at HE phase were malignant/premalignant (overt HCC or eHCC/HG-DN) and hypointensity captured 45/51 malignant nodules either hyper- or hypovascular, with a sensitivity of 88% and specificity of 97% reaching 100% when associated with arterial enhancement. In this study, all benign nodules (large regenerative or LG-DN) displayed iso-hyperintensity at HE-phase. However, it could be outlined that a very low rate (roughly 3%) of well-differentiated hepatocellular carcinomas (wdHCCs), are hyper vascularized at arterial phase but are iso-hyperintense on HE-phase mainly because they retain still functioning hepatocytes<sup>[39-43]</sup>. It is wise to consider nodules with arterial enhancement but hyperintense in HE as highly suspicious of incipient malignant transition.

The differential diagnosis between HG-DN and eHCC still remains difficult even by Gd-EOB-DTPA MRI since HG-DN show variable behaviour on HE-phase being frequently hypointense like eHCC<sup>[38,44-48]</sup>. Indeed, the specificity of hypointensity on HE-phase as a unique hallmark of malignancy, is suboptimal ranging from 33% to 97%<sup>[38,49]</sup>. It has been recently outlined that coupling Gd-EOB-DTPA MRI with diffusion-weighted imaging (DWI) may result in increased accuracy for diagnosis of overt HCC and also for the differential diagnosis of eHCC and HG-DN<sup>[44,45]</sup>. DWI is based on the simple assumption that water diffusion in the extracellular compartment is influenced by cellular density which, in turn, reduces the width of interstitial spaces and water diffusion<sup>[50,51]</sup>. Water diffusion can be quantified by a mathematical index called apparent diffusion coefficient (ADC); low ADC values mean low diffusivity, namely hypercellularity and, as previously stated, hypercellularity progressively increases from HG-DN, to eHCC reaching maximum expression in progressed HCC. Unfortunately, DWI as a unique tool for assessing hepatic lesions is inaccurate due to the considerable overlap between benign and malignant lesions and normal liver tissue<sup>[52,53]</sup>. In addition, DWI images are highly influenced by artefacts of liver motion due to respiration and artefacts in the left lobe derived from the heart beating<sup>[54,55]</sup>. Therefore, DWI should be used in conjunction to the other conventional imaging features. Renzulli *et al.*<sup>[56]</sup> reported data from a prospective study evaluating the imaging criteria of HCC, early HCC and HG-DNs using gadoxetic acid-enhanced liver MRI in 228 patients prospectively enrolled with 480 small nodules detected under surveillance for cirrhosis. Using three MRI findings: HE-phase hypointensity, arterial hyperintensity and diffusion-weighted imaging (DWI) the authors designed an algorithm which yielded an overall sensitivity of 96.6% and a specificity of 92.7% and a very high sensitivity (94.7%) and specificity (99.3%) in classifying HG-DN. Although some technical aspects need to be fully standardized (type of MRI machine, breath old method, *b* value for DWI), gadoxetic acid-enhanced MRI including DWI analysis as part of a diagnostic algorithm, may represent a promising approach to better defining those small lesions still atypical at vascular and HE phases being eHCC hyperintense and HG-DN isointense at DWI. DWI behaviour is currently part of ancillary features for liver nodules characterization in Liver Imaging Reporting and Data System (LI-RADS) v17 system (see above)<sup>[57]</sup>.

These advances on MRI prompted JHS and APASL guidelines to incorporate gadoxetic acid-enhanced MRI for definition of atypical nodules before performing biopsy<sup>[18,19]</sup>. Conversely, the late version of guidelines from the Western world<sup>[58,59]</sup>, still endorse a diagnostic algorithm for HCC that have remained the same for

at least 16 years not giving due relevance to the above discussed imaging innovation. Furthermore, western guidelines erroneously consider CT as equivalent to MRI in diagnostic accuracy even though recent meta-analyses<sup>[60-61]</sup>, one of which conducted by the same authors who contributed to the last version of AASLD guidelines, confirmed, the superiority of the latter. Other hepato-specific contrast media as Sonazoid for CEUS (s-CEUS) or Super paramagnetic iron oxide agents for MRI (SPIO-MRI) showed promising results but they are not available worldwide and the experience with these compounds is limited to Eastern countries<sup>[18,19]</sup>. Sonazoid (GE Healthcare, Waukesha, WI, USA), is a second-generation contrast agent available in Japan, South Korea, and Norway (August 2015). The microbubbles of this agent are captured in the liver parenchyma by reticuloendothelial (Kupffer) cells and, therefore, s-CEUS provides information in both haemodynamic-phase and accumulated-phase images. Because malignant tumours contain few or no Kupffer cells, they appear as perfusion defects in late phase of s-CEUS. Conversely, non-neoplastic nodules with preserved or only minimally reduced reticuloendothelial system lack wash-out in the late-phase. Sonazoid proved to be particularly accurate for characterization of small lesions in cirrhosis. In a literature review covering 10 years experience with Sonazoid<sup>[62]</sup>, s-CEUS emerged as accurate as gadoxetic acid-enhanced MRI in differentiating benign nodules from wdHCC. Furthermore, s-CEUS provides a better characterization of some small lesions at contrast-enhanced CT or MRI. Takahashi *et al.*<sup>[63]</sup> in a study comparing s-CEUS and gadoxetic acid-enhanced MRI on characterization of small < 3 cm lesions in cirrhosis reported that 27% of indeterminate hypo-vascular lesions at MRI turned out to be hyper-enhancing at s-CEUS and were histologically diagnosed as HCC. For these reasons s-CEUS is currently included by Japanese and APASL guidelines in the diagnostic algorithm for indeterminate lesions discovered in cirrhosis during surveillance.

## LI-RADS CRITERIA

In 2011 the American College of Radiology issued a new system of liver imaging reporting (LI-RADS) aimed at providing a precisely defined terminology for interpreting and reporting contrast-enhancing CT and MRI examination of nodules detected in cirrhosis<sup>[64]</sup>. This system also included guidance for the interpretation of data obtained by MRI with hepato-specific agents. According to LI-RADS, lesions detected at dynamic CT or MRI in patients at high risk of having HCC (cirrhotic patients under surveillance), are categorized as definitively benign (LR-1), probably benign (LR-2), intermediate probability of being HCC (LR-3), probably HCC (LR-4) and definitively HCC (LR-5). This system would provide more guidance to clinicians to adopt proper clinical decisions such as accelerated follow-up, biopsy or even treatment of small lesions in cirrhosis. According to criteria proposed by LI-RADS the great majority of dysplastic nodules are categorized as LR-3 and LR-4. LR-3 lesions should be strictly monitored and LR-4 lesions require biopsy<sup>[64]</sup>.

LI-RADS system was strongly encouraged by the US regulatory agency for liver transplantation (UNOS) to avoid or minimize futile transplants for false positive cases. Indeed, LI-RADS criteria proposed by LI-RADS v2014 and by the updated 2017 version (v2017)<sup>[57]</sup> for diagnosis of HCC are more restrictive than those proposed by AASLD-EASL and do not accept the diagnosis of definitive-HCC (LR-5) for lesions sized 1-2 cm even in presence of wash in and washout. In these cases a 50% growth in at least 6 months is needed for definite classification as LR-5. These stringent criteria would limit *de facto* the early diagnosis of HCC in the group of 1-2 cm nodules. These limits were emphasized by a prospective study comparing LI-RADS classification with AASLD criteria for HCC on 133 small (< 2 cm) newly detected nodules in cirrhosis studied by MRI<sup>[65]</sup>. In this study 21 histologically proven eHCCs were erroneously subcategorized as LR-4 according to LI-RADS criteria even though they were hyper-enhancing at arterial phase with late wash-out. In addition, 29 small HCCs fell into LR-3 category making the overall sensitivity of LI-RADS for HCC 43%, relevantly lower than the sensitivity of AASLD criteria (58.6%). Similar results were obtained by Ronot *et al.*<sup>[66]</sup> in a prospective study on 595 nodules < 3 cm in cirrhosis, where specificity of LI-RADS criteria for diagnosis of HCC relevantly increased and became better than AASLD criteria when LR-4 and LR-5 categories were considered in combination. On the other hands and more important, in the study by Darnell *et al.*<sup>[65]</sup>

none of the non-malignant hepatocellular nodules fell into LR5 category making the LI-RADS specificity for HCC diagnosis close to 100%. The high specificity of LI-RADS criteria was further emphasized in a recent systematic review in which data from 2,760 patients were analysed<sup>[67]</sup>. The authors found that only 3% of observations categorized as LR5 were non-malignant giving a 97% overall specificity of LI-RADS for malignancy. To overcome the low sensitivity of LI-RADS, the most recent version (v2018)<sup>[68]</sup> provides updated criteria for small (10-19 mm) LR-5 observations and a simplified definition for threshold growth. In this version, in order to align LI-RADS criteria with AASLD/EASL criteria of HCC and increase simplicity, a 10-19 mm nodule with arterial phase hyperenhancement and non-peripheral “washout” is definitely categorized as LR-5.

According to LI-RADS v2018, hypointensity in the HE-phase and DWI hyperintensity are considered as ancillary features but they should not allow upgrading a suspected malignant lesion (LR-4) to LR-5<sup>[66]</sup>. In order to significantly increase the diagnostic efficacy of the MRI LI-RADS criteria it has been suggested that they should be modified (mLI-RADS) by incorporating hypointensity in the HE-phase and hyperintensity at diffusion restriction among the major MRI features of malignancy<sup>[68]</sup>.

A further progress in the diagnostic accuracy small HCCs comes from the recently released Contrast-Enhanced Ultrasound LI-RADS (CEUS LI-RADS v2017) which provides a refined definition of the typical CEUS pattern of HCC<sup>[69]</sup>. According to CEUS LI-RADS criteria, a nodule showing a rapid arterial enhancement and a delayed wash out (> 60 s) can be classified as definite HCC (LR-5) regardless its size. These criteria were recently validated in a series of 1086 well-defined lesions detected in cirrhosis and studied by CEUS. Applying CEUS LI-RADS criteria 58.5 % of HCCs were correctly classified as LR-5 with a PPV of 98.5% and only 3% of non-malignant nodules were erroneously classified as HCC<sup>[70]</sup>. Thus, LR-5 CEUS is an extremely reliable criteria for HCC, given its excellent PPV, without misdiagnosis for other malignancies. Using CEUS LI-RADS criteria most of LG and HG-DN were classified as LR-3 and LR-4<sup>[70]</sup>. These results are extremely important and support the use of CEUS in clinical practice not only for the definite diagnosis of HCC when other imaging is inconclusive but also for monitoring indeterminate lesions at risk of neoplastic transformation considering that it is cheaper and more accessible than MRI.

## NATURAL HISTORY OF PRENEOPLASTIC LESIONS

Although preneoplastic lesions have been described several years ago, their natural history has been clarified only recently by prospective studies carried out on cirrhotic patients undergoing US surveillance for early detection of HCC. Early small series from Japan<sup>[71-73]</sup> collected in a pre-dynamic imaging era, and including ultrasonically detected non malignant lesions classified according to different histologic criteria, provided preliminary evidence of the preneoplastic role of adenomatous/dysplastic nodules. More robust and convincing data derived from two successive prospective studies<sup>[5,74]</sup> comparing the natural history of different non-malignant lesions detected by ultrasounds in cirrhosis and categorized according to IWP classification. These studies confirmed that HG-DN were the true precursors of HCC with a risk of neoplastic transformation significantly higher as compared to LG-DN and LRN. Similar conclusions emerged from a single centre study on 66 LRNs and 20 DN with a 28-years follow-up by Sato *et al.*<sup>[75]</sup>. However, in these studies, the overall rate of malignant evolution of HG-DN nodules was relatively low ranging from 9% to 31% and neoplastic evolution could be documented in large regenerative/LG-DN as well albeit at a lower rate<sup>[5,74]</sup> [Table 2]. In addition, transforming progression of HG-DNs was hardly predictable in terms of elapsing time from US detection to HCC transformation. In fact, some HG-DNs remained stable over a long time (20% to 50%), often exceeding the follow-up time suggested by AASLD/EASL guidelines (18 months), and some disappeared during follow-up. These data rise concern on the low PPV of morphology as a unique tool for the correct identification of nodules at risk of transformation when applied to small samples supporting a “watchful waiting” policy based on a strict radiologic surveillance. Conversely, Iavarone *et al.*<sup>[76]</sup> in a retrospective-prospective study carried out on 36 non-malignant nodules histologically classified as



**Table 2. Natural history of regenerative/dysplastic nodules in cirrhosis**

Author (years)	Type	No	Malignant changes	Unchanged	Disappeared	Time (months) to HCC progression Mean (range)
Kondo <i>et al.</i> <sup>[71]</sup> , 2011	LRN	17	-	13 (76%)	4 (24%)	NA
Terasaky <i>et al.</i> <sup>[72]</sup> , 1998	LRN/DN	34	5 (15%)	4 (12%)	25 (73%)	NA
Seki <i>et al.</i> <sup>[73]</sup> , 2000	DN	33	4 (9%)	14 (42%)	15 (49%)	18 (16-21)
Borzio <i>et al.</i> <sup>[5]</sup> , 2003	LRN/DN	90	28 (31%)	44 (49%)	18 (20%)	22 (8-48)
Kobayashi <i>et al.</i> <sup>[74]</sup> , 2006	LRN/DN	154	29 (19%)	81 (53%)	44 (28%)	NS
Iavarone <i>et al.</i> <sup>[76]</sup> , 2013	LRN/DN	36	11 (31%)	21 (53%)	4 (16%)	13 (7-27)
Sato <i>et al.</i> <sup>[75]</sup> , 2015	LRN/DN	92	19 (21%)	30 (32%)	43 (47%)	NS

NA: not available; NS: not specified. DN: dysplastic nodules; LRN: large regenerative nodules

LG- or HG-DNs found that neither size increase nor changing on enhancing pattern emerged as accurate predictors of neoplastic transformation since some DNs transformed into HCC without enlarging and others without acquiring arterial hypervascularity.

In the dynamic imaging era, the natural history of pre-malignant nodules can be extrapolated from mainly retrospective studies including nodules classified as non-enhancing/indeterminate at both CT and MRI to differentiate them from progressed HCC. Studies before 2010 and mainly based on dynamic CT, confirmed that some of these hypo-enhancing nodules carry a risk to acquire true radiologic hallmarks of malignancy over time even though this risk at an individual level still remains unpredictable. From these studies, size at baseline (larger the nodule, higher the risk) and changes in size and/or in the vascular pattern during follow-up (shift from hypo- to hyper-enhancing pattern on arterial phase), were reliable predictors of malignant transformation<sup>[77,78]</sup>. In the retrospective study by Chuo *et al.*<sup>[79]</sup> including a large series of indeterminate (hypo-vascular) small nodules observed during surveillance of HBV-related cirrhosis, emerged that old age, initial size > 1 cm and arterial enhancement were risk factors for neoplastic progression. The authors developed a useful and accurate risk score model for predicting HCC progression of indeterminate nodules. Unfortunately, this risk score model was not further validated in series with different etiologic liver diseases.

From 2010 onwards, most data on natural history of small hypovascular lesions observed in cirrhosis derived from studies carried out by gadoxeti-acid enhanced MRI<sup>[80-86]</sup>. In these studies neoplastic evolution was assumed to occur by acquisition of hypervascularity. Unfortunately, all these studies were retrospective and hypovascular/indeterminate nodules lacked histological classification. Furthermore, in most of these studies, one of the inclusion criteria was hypointensity at HE-phase. As previously stated, most of small nodules hypovascular, hypo-intense at He-phase, are eHCC/HG-DN. Thus, in the strict sense, these studies focused on the natural history of early hypovascular HCCs or progressed HG-DN rather than on precursor lesions as a whole<sup>[80-86]</sup>. This may partially explain why the transition rate to hyper-vascularized pHCC found in these studies was extremely high ranging from 12% to 35% at one year [Table 3].

At the best of our knowledge, only two studies by gadoexetic-acid enhanced MRI provided reliable information on the natural history of premalignant lesions with exclusion of hypovascular HCC.

The first by Kim *et al.*<sup>[85]</sup> focused on hypovascular, HE hypo-intense lesions without T2W hyperintensity. Authors assumed that having excluded T2 hyperintense nodules, the risk of inclusion of eHCC among hypovascular hypointense nodules on HE phase was marginal.

In this study the rate of hypervascular transformation of precursor nodules, such as LG-DN and HG-DN was 23 % at 3 years. In the second study, Sano *et al.*<sup>[86]</sup> in a large series of small hypo-vascular nodules, iso/hyperintense at HE found that acquisition of hypervascularity was extremely rare (0.6%) over 3 years follow-up and no nodules evolved into mature HCC after the fourth year. In this study the only independent risk factor for progression was the initial size of the nodule (> 10 mm). In addition, the nodule growth rate showed 85% PPV in predicting of hypervascularization.



**Table 3. Natural history of small hypovascular nodules at MRI and evaluated by gadoxetic acid uptake at hepatic phase**

Author (years)	Type	HE intensity at baseline	Nodules (n)	Acquired hypervascularity	Mean follow-up (months)	Risk factors
Kumada <i>et al.</i> <sup>[81]</sup> , 2011	Retrospective	Hypo-	49	6 (27%)	12	Size ≥ 15 mm
Motosugi <i>et al.</i> <sup>[80]</sup> , 2011	Retrospective	Hypo-	135	16 (12%)		Size ≥ 10 mm Fat content enlargement
Kim <i>et al.</i> <sup>[83]</sup> , 2012	Retrospective	Hypo-	214	75 (35%)	11	Hyperintensity at DWI
Hyodo <i>et al.</i> <sup>[82]</sup> , 2013	Retrospective	Hypo	160	50 (31%)	12	Rapid growth (tumor volume doubling time = 542 days) T2W hyper-intensity
Higaki <i>et al.</i> <sup>[84]</sup> , 2014	Retrospective	Hypo	60	10 (17%)	12	Higher growth rate
Kim <i>et al.</i> <sup>[85]</sup> , 2016	Retrospective	Hypo No T2W hyperintensity	114	26 (23%)	42	T1w hyperintensity Size > 10.5 mm Previous HCC Rapid growth rate
Sano <i>et al.</i> <sup>[86]</sup> , 2017	Retrospective	Iso-hyper	663	6 (0.9)	36	Size > 10 mm

HCC: hepatocellular carcinoma

In summary, results from imaging-based studies provide evidence showing that indeterminate hypovascular nodules may evolve into mature HCC but this transition is hardly predictable based on initial clinical characteristics and MR imaging features. The risk and speed of neoplastic evolution of hypovascular nodules seems to depend mostly on their behaviour at HE phase on gadoxetic-acid enhanced MRI. Hypo-intense nodule at HE phase, have an elevated risk to become hypervascular pHCC in a short interval, whereas nodules iso/hyper-intense at HE phase, showed a minimal oncogenic risk<sup>[80-86]</sup>. Among the numerous risk factors found, the growth rate per se seems to have the highest positive predictive value and should be regarded as the most reliable alarm ring for radiologic evolution to progressed HCC.

## SURVEILLANCE AND TREATMENT

The proper follow-up of non-malignant lesions is still debated and, theoretically, it should be dictated by the intrinsic risk of neoplastic evolution [Table 4]. Guidelines do not specifically address this issue even though a strict follow up is recommended. Once a nodule is histologically classified as dysplastic, either LG-DN or HG-DN, it should enter an enhanced follow-up the goal of which is to promptly capture its neoplastic transformation. However, indications on such an enhanced surveillance are not uniform among different guidelines. An interval of 3-4 months seems to be reasonable since it would ensure that, in case of malignant transformation, nodule would not grow beyond curability. For nodule diagnosed only by imaging, the interval should be dictated by radiologic characteristics. Hypovascularity coupled with hypo-intensity at HE by gadoxetic-acid MRI call for a strict follow up. Conversely, hypovascular nodules showing iso/hyperintensity at HE-phase should be monitored by standard six months interval and with a follow-up no longer than 3 years. Eighteen months observation period as that recommended by AASLD/EASL guidelines seems to be inadequate since neoplastic transformation of some DN may take longer interval. As to which imaging technique should be employed, ideally it should be able to detect changes either in size and/or in vascular profile. Therefore, a dynamic imaging is preferable to standard ultrasound. CEUS, being cheaper, safer and more accessible than CT or MRI seems to be preferable in clinical practice since it can catch changes either in size or vascularity. Gadoxetate-enhancing MRI with DWI evaluation remains the recall imaging of reference to confirm neoplastic transition of nodules.

Early treatment of dysplastic nodules, which may theoretically improve survival is controversial. Unlike in others human models of gastrointestinal carcinogenesis (colo-rectal and gastroesophageal cancer) where treatment of precancerous lesions is recommended, in hepatocarcinogenetic model data supporting this policy are still lacking and evidences from the natural history of preneoplastic lesions discourage their systematic treatment. Recommendations on this issue by international guidelines are discordant. American

**Table 4. Clinical, radiologic and morphologic features useful to predict neoplastic evolution of dysplastic nodules**

Features	Risk of neoplastic evolution
Size	
< 1 cm	Low
> 1.5 cm	High
Arterial enhancement at CT, MRI, CEUS	
Hypo-vascularity	Low
Hyper-vascularity	High
HE phase at gadoxetate-enhanced MRI	
Iso/hyper-intense	Low
Hypo-intensity	High
DWI	
Hypo-intensity	Low
Hyper-intensity	High
Imaging features at follow-up	
Stable size	Low
Increasing size	High
Stable vascular pattern	Low
Acquired hyper-vascularity	High
Synchronous HCC	
No	Low
Yes	High
Histologic diagnosis	
LG-DN	Low
HG-DN	high

LG-DN: low-grade dysplastic nodule; HG-DN: high-grade dysplastic nodule; HCC: hepatocellular carcinoma

and European guidelines do not recommend systematic treatment of these lesions while Asian and Japanese guidelines are in favour of treatment of HG-DN. These discrepancies can be explained by the confusion in the pathological interpretation of early HCC and DNs among Japanese and Western pathologists. In particular, many of the vaguely nodular well-differentiated HCCs diagnosed by Japanese pathologists tend to be interpreted as high-grade DNs rather than HCC by Western pathologists while, many of the high-grade DNs diagnosed by Western pathologists are interpreted as well-differentiated HCC by Japanese pathologists<sup>[87]</sup>. This grey zone is particularly worrisome considering that the pursued goal is to treat any lesion arising in cirrhosis within an optimal curable stage (within 2 cm as the maximum diameter). However, according to western point of view, concerns are raised on the indiscriminate treatment of DNs that might be regarded as futile due to their longer and unpredictable natural history. In addition, the accurate selection of lesions with true neoplastic potential is still difficult in particular when multiple lesions are encountered. To date, only few studies addressed this issue with questionable conclusions. In 2008, Kim *et al.*<sup>[88]</sup> reported in a retrospective study the results of radiofrequency ablation (RFA) of 21 HG-DNs as compared to 41 small HCCs. Although complete necrosis was successfully obtained in 100 % of DN, this result did not translate into either long-term overall and disease-free survival benefit owing to the occurrence of “*de novo*” HCCs aside the initial DNs (48%) as the natural course of multicentric hepatocarcinogenesis. Owing to the lack and the difficulties to organize and conduct well-designed prospective controlled trials, Korean authors addressed this issue by a simulation model comparing two treatment strategies: RFA versus follow-up and timely resection. This model could not provide any evidence supporting that nodular ablation was superior to follow-up and timely resection for overall survival. Furthermore, in patients with multiple HG-DNs, RF ablation of all nodule is not clinically feasible, as it can compromise liver function<sup>[89]</sup>. In conclusion, the rationale for systematic treatment of DN in cirrhosis at present is weak and carries the risk of falling into an overtreatment, i.e., treatment of lesions which may not cause significant disease in the patient.

## CONCLUSION

Like in other gastrointestinal oncogenetic models, in multistep cirrhosis-related hepatocarcinogenesis the development of HCC is preceded by sizable dysplastic lesions. The IWP classification distinguishes DN into

LG- and HG-DN, being the latter the true HCC precursors. Although relevant advances have been obtained in the last 2 decades as to morphology, and radiologic behaviour of these precursors, their management still represents a challenge for clinicians, radiologists and pathologists. In particular, major difficulties arise in surgical and transplant setting where experts have to face with these small lesions and make decisions about early treatment or simple observation without impacting negatively on the single patient outcome<sup>[90]</sup>. This remain an open and hot issue and efforts of scientific community are targeted to search and validate radiological and tissue markers helping to select lesions more prone to evolve into well-established HCC and deserving early treatment. Due to the limitations of biopsy and considering the complexity of radiologic work up, strict follow-up remains the most reliable alternative and the nodule growth the most confident and easy-to-use parameter driving decision-making process.

## DECLARATIONS

### Authors' contributions

Planned the review: Borzio M

Performed data research and data extraction: Borzio M, Francica G

Drafted the manuscript and approved the final version of the manuscript: Borzio M, Paladino F, Francica G

Controlled for data extraction: Borzio M, Paladino F, Francica G

### Availability of data and materials

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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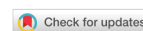


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Original Article

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# Hepatocellular carcinoma recurrence in HCV patients treated with direct-acting antivirals after curative treatment

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## Abstract

**Aim:** The increased risk of hepatocellular carcinoma (HCC) recurrence in hepatitis C virus (HCV)-infected patients treated with direct-acting antivirals (DAAs) after curative treatment for HCC is controversial. The purpose of this study was to examine the risk of HCC recurrence after DAA therapy.

**Methods:** We conducted a retrospective cohort study of 312 consecutive patients with HCV-related HCC who received DAA therapy in participating institutions between September 2014 and July 2016. All patients received curative



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hepatectomy or radio-frequency ablation. We calculated the annual incidence of HCC recurrence after DAA therapy and identified the risk factors for HCC recurrence using Cox regression models.

**Results:** The median age was 74 years old, and a sustained virological response was achieved by 288 patients. The 3-year-overall survival rate was 95.4% in a median follow-up period of 855 days. HCC recurred in 135 patients. The 1-, 2- and 3-year recurrence rates were 18.3%, 38.8% and 55.4%, respectively. A multivariate analysis revealed that the following factors were associated with HCC recurrence: multiple tumors at the first HCC treatment [hazard ratio (HR) = 2.21; 95%CI: 1.41-3.49], a history of multiple treatments for HCC (HR = 1.97; 95%CI: 1.28-3.02), and  $\alpha$ -fetoprotein (AFP-L3)  $\geq 10\%$  at the initiation of DAA therapy (HR = 4.74; 95%CI: 2.10-10.7).

**Conclusion:** Among patients treated with DAAs after the curative treatment of HCC, multiple tumors at the first HCC treatment, multiple prior HCC treatments and a high AFP-L3 level before DAA therapy were associated with recurrence, and the rate of recurrence was comparable to that before the DAA era.

**Keywords:** Hepatocellular carcinoma, hepatitis C virus, direct-acting antiviral, recurrence

## INTRODUCTION

Worldwide, primary liver cancer is the second and sixth leading cause of cancer mortality in men and women, respectively<sup>[1,2]</sup>. The most frequent cause of HCC is liver cirrhosis due to HCV infection<sup>[3]</sup>.

DAA therapy have made it possible for most patients with HCV infection to achieve a sustained virological response, even if they cannot tolerate interferon-based therapy. The introduction of DAA therapy is expected to improve the prognosis of patients with liver cirrhosis due to HCV infection, and it is also expected that the recurrence rate will decrease in patients after HCC treatment. However, recent studies have suggested that DAA therapy might increase the risk of HCC recurrence<sup>[4,5]</sup>. For example, a Spanish multicenter study reported by Reig *et al.*<sup>[4]</sup> warned that DAA therapy may increase the risk of HCC recurrence. In their paper, 16/58 (27.6%) patients who received DAA therapy after HCC treatment experienced tumor recurrence after a median follow-up period of 5.7 months. Subsequently, several studies reporting contradictory findings have been published<sup>[6-15]</sup>.

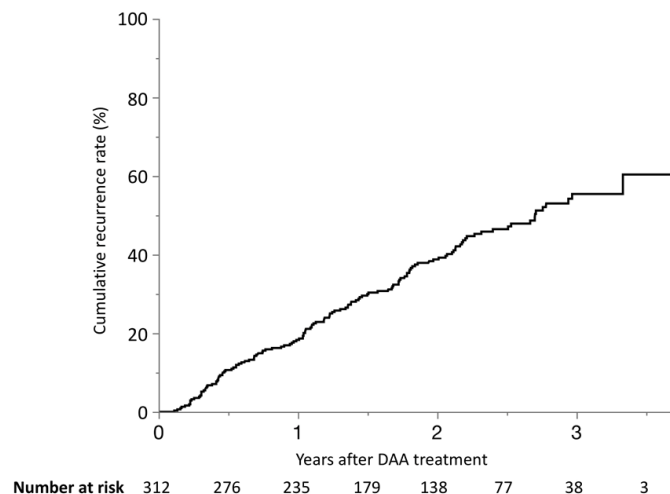
Although DAAs have been demonstrated to lower carcinogenicity in patients without a history of HCC treatment<sup>[8,16]</sup>, the effect of DAAs for preventing recurrence after HCC treatment has not been proven. In this study, we investigated the outcomes of HCC patients who received DAA therapy after curative treatment with hepatectomy or radio-frequency ablation (RFA) in a multicenter collaborative study and attempted to elucidate the effect of DAAs on recurrence.

## METHODS

### Patients

We performed a multicenter, retrospective cohort analysis of HCC patients who had previously been treated with hepatectomy or RFA and who received anti-HCV treatment with DAAs between September 2014 and July 2016. In this study, 312 consecutive patients were enrolled: 224 (71.8%) and 88 (28.2%) patients received RFA and hepatectomy before DAA treatment, respectively. A flowchart of the patient selection is shown in the [Supplementary Figure 1](#).

All patients were diagnosed as cancer-free prior to DAA treatment based on triple-phase multidetector computed tomography (CT), dynamic contrast-enhanced magnetic resonance imaging (MRI) or ultrasonography (US). We confirmed the cancer-free status at least with two imaging modalities. The study protocol conformed to the ethical



**Figure 1.** The cumulative recurrence rate in patients treated with DAAs after curative treatment for HCC. HCC recurred in 135 patients. The 1-, 2- and 3-year cumulative recurrence rates after DAA therapy were 18%, 39% and 55%, respectively. HCC: hepatocellular carcinoma; DAA: direct-acting antiviral

guidelines of the World Medical Association and the Declaration of Helsinki and was approved by the ethics committees of each institute.

### DAA treatment

The DAA regimens were daclatasvir/asunaprevir ( $n = 130$ , 41.7%), sofosbuvir/ledipasvir ( $n = 116$ , 37.2%), sofosbuvir/ribavirin ( $n = 61$ , 19.6%) and ombitasvir/paritaprevir/ritonavir ( $n = 5$ , 1.6%). We assessed the response to DAA treatment based on the presence of HCV-RNA at 12 weeks after the end of the treatment. Patients negative for HCV-RNA at this time were considered to have achieved a sustained virological response (SVR12). The presence of HCV-RNA was examined using real-time polymerase chain reaction.

### Follow-up

After DAA treatment, the patients were assessed every three months by US, triple-phase CT, or MRI. The diagnosis of HCC recurrence was confirmed via typical HCC imaging patterns obtained by angiography, CT, MRI and US. The criteria for the imaging-based diagnosis of HCC have been described in previous reports: hyper-attenuation at the hepatic arterial phase, hypo-attenuation at the portal venous phase in triple-phase CT or MRI and tumor staining on angiography, or hyper-enhancement in the arterial phase and hypo-enhancement in the portal venous and late phases on contrast-enhanced US<sup>[17,18]</sup>.

### Statistical analyses

The clinical characteristics of the patients were obtained at the initial treatment for HCC and before and after DAA treatment. The data collected included the age, gender, platelet count, albumin (ALB), total bilirubin (T.Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGTP), prothrombin time-international normalized ratio, Child-Pugh grade, AFP, des- $\gamma$ -carboxy prothrombin (DCP), lens culinaris agglutinin-reactive AFP-L3, fibrosis (FIB-4) index, HCV genotype, amount of HCV-RNA, history of HCC treatment, number of tumors, maximum diameter of the tumor, date of HCC recurrence, treatment methods and survival.

Patients without recurrence were censored at the last visit or on the date of death. We calculated the annual HCC recurrence rate after DAA therapy and examined the risk factors for recurrence using Cox proportional hazard models. Variables with a  $P$  value of  $\leq 0.1$  in a univariate analysis were incorporated into a multivariate analysis by stepwise selection. The recurrence rate and overall survival rate after DAA therapy were calculated using the Kaplan-Meier method and evaluated with a log-rank test.  $P$  values of  $< 0.05$  were considered to indicate statistical significance. All analyses were performed using the JMP Pro 14.1.0 software package (SAS Institute, Inc., Cary, NC, USA).

**Table 1. The baseline characteristics of 312 patients at the time of direct-acting antiviral treatment**

Characteristics	All patients (n = 312)	Final HCC Tx before DAA	
		Hepatectomy (n = 89)	RFA (n = 223)
Age (years)	74 (68-79)	74 (68-78)	74 (68-79)
Gender (male)	182 (58.3%)	50 (56.2%)	132 (59.1%)
Platelet ( $\times 10^3/\mu\text{L}$ )*	105 (76-140)	113 (98-137)	99 (73-142)
ALB (g/dL)*	3.8 (3.4-4.0)	3.9 (3.6-4.2)	3.7 (3.4-4.0)
T.Bil (mg/dL)**	0.8 (0.6-1.1)	0.73 (0.6-0.9)	0.89 (0.6-1.2)
AST (U/L)	52 (37-67)	51 (39-70)	52 (36-66)
ALT (U/L)	41 (28-60)	42 (31-65)	39 (27-60)
GGTP (U/L)	37 (25-55)	40 (26-62)	36 (24-52)
PT-INR	1.07 (1.0-1.14)	1.07 (0.99-1.11)	1.06 (1.00-1.15)
Child-Pugh grade A**	280 (89.7%)	83 (93.3%)	197 (88.3%)
Fib-4 index	5.64 (3.75-8.04)	5.06 (3.65-6.85)	5.88 (3.75-8.53)
HCV genotype*			
Genotype 1	251 (80.5%)	65 (73.0%)	186 (83.4%)
Genotype 2	60 (19.2%)	24 (27.0%)	36 (16.2%)
Genotype 1 + 2	1 (0.3%)	0 (0%)	1 (0.4%)
HCV-RNA (log IU/mL)	6.0 (5.4-6.4)	6.0 (5.4-6.5)	6.0 (5.4-6.4)
SVR at 12 weeks	288 (92.3%)	83 (93.3%)	205 (91.9%)

\* $P < 0.001$ ; \*\* $P < 0.05$ ; The values indicate the median (interquartile range) unless otherwise noted. ALB: albumin; T.Bil: total bilirubin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGTP: gamma-glutamyl transpeptidase; PT-INR: prothrombin time-international normalized ratio; FIB-4 index =  $\text{Age (years)} \times \text{AST (U/L)} / [\text{PLT}(10^9/\text{L}) \times \text{ALT}^{1/2} (\text{U/L})]$ ; HCV: hepatitis C virus; HCC: hepatocellular carcinoma; Tx: treatment; SVR: sustained virological response

## RESULTS

### Patient characteristics

The clinical characteristics of the patients and the characteristics of HCC before DAA treatment are shown in [Tables 1 and 2], respectively. The median age was 74 years old, and 182 (58.3%) patients were male. Two hundred and fifty-one (80.4%) and 60 patients (19.2%) had HCV genotypes 1 and 2, respectively, and 1 patient had both. An SVR12 was achieved by 288 patients (92.3%). The median tumor size at the initial treatment was 18 mm, and 244 patients (78.2%) had a single tumor. The interval from the final HCC treatment and DAA therapy was 297 days (median). The median follow-up time from the end of DAA treatment was 855 days.

### The recurrence and survival rates

HCC recurred in 135 patients (43.2%), and the 1-, 2- and 3-year cumulative recurrence rates after DAA therapy were 18.3%, 38.8% and 55.4%, respectively [Figure 1]. The 1-, 2- and 3-year-overall survival rates were 99.4%, 98.6% and 95.4%, respectively [Figure 2].

### Risk factors for recurrence

In the univariate analysis, the factors associated with HCC recurrence included AFP  $\geq 10$  ng/mL and multiple tumors at the initial HCC treatment, T.Bil  $> 0.8$  mg/dL, AFP-L3  $\geq 10\%$  at the initiation of DAA therapy, a history of multiple treatments before DAA therapy, RFA as the final HCC treatments before DAA, period between the final HCC treatment and DAA therapy  $< 1$  year, AFP  $\geq 10$  ng/mL, DCP  $\geq 28$  mAU/mL, AFP-L3  $\geq 10\%$  and non-SVR12 [Table 3].

In the multivariate analysis, the factors associated with HCC recurrence were multiple tumors at the first HCC treatment [HR = 2.21; 95% confidence interval (CI): 1.41-3.49], a history of multiple treatments for HCC before DAA therapy (HR = 1.97; 95%CI: 1.28-3.02) and AFP-L3  $\geq 10\%$  at the initiation of DAA therapy (HR = 4.74; 95%CI: 2.10-10.7) [Table 4].

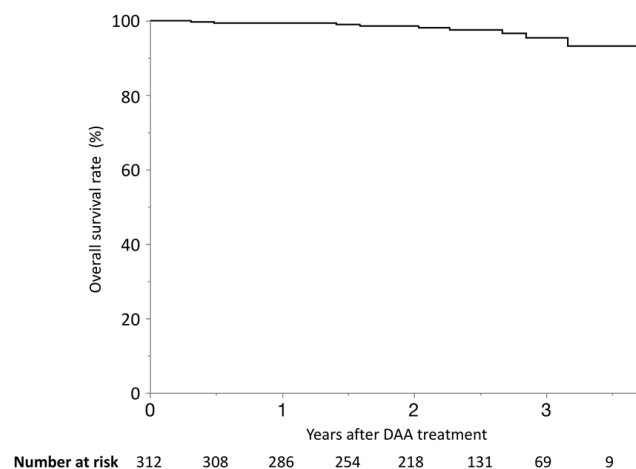
The relationship between the number of times the patient had received treatment for HCC and recurrence is shown in [Figure 3]. The recurrence rates increased as the number of previous treatments for HCC increased: the



**Table 2. Characteristics of hepatocellular carcinoma before direct-acting antiviral treatment**

Characteristics	All patients (n = 312)	Final HCC Tx before DAA	
		Hepatectomy (n = 89)	RFA (n = 223)
At the first HCC Tx			
Max tumor diameter (mm)*	18 (14-25)	22 (16-30)	16 (13-22)
Tumor number (1/2/≥ 3)**	244 (78.2%)/42 (13.5%)/26 (8.3%)	73 (82.0%)/14 (15.7%)/2 (2.3%)	171 (76.7%)/28 (12.6%)/24 (10.7%)
AFP (ng/mL)	14.8 (7.2-57.4)	19.4 (8.1-99.3)	13.7 (6.8-52.1)
DCP (mAU/mL)	24.5 (17-56.8)	25 (16-142)	24 (17-48)
AFP-L3 (%)	4.3 (0-7.3)	6.4 (0-15.8)	4.3 (0-6.6)
At the final HCC Tx before DAA			
Max tumor diameter (mm)*	15 (12-21.3)	22 (15.5-30)	15 (11-20)
Tumor number (1/2/≥ 3)**	246 (78.8%)/43 (13.8%)/23 (7.4%)	74 (83.1%)/13 (14.6%)/2(2.3%)	172 (77.1%)/30 (13.5%)/21 (9.4%)
At the initiation of DAA			
History of HCC Tx (1/2/≥ 3)*	190 (60.9%)/62 (19.9%)/60 (19.2%)	82 (92.1%)/6 (6.7%)/1 (1.1%)	108 (48.4%)/56 (25.1%)/59 (26.5%)
The period from the final HCC Tx (days)*	297 (117-96)	495 (209-1101)	233 (104-544)
AFP (ng/mL)*	8.4 (4.9-16.9)	7.5 (4.1-11.4)	9.1 (5.1-23.6)
DCP (mAU/mL)**	16.5 (12-23)	15 (11-19)	18 (12-25)
AFP-L3 (%)**	3.5 (0-5.6)	0 (0-4.6)	3.8 (0-5.8)
After DAA Therapy			
AFP (ng/mL)**	5.3 (3.3-7.7)	4.8 (3.1-6.7)	5.5 (3.4-8.5)
DCP (mAU/mL)*	17 (13-23)	15 (12-20)	18 (14-24.8)
AFP-L3 (%)**	0 (0-2)	0 (0-0)	0 (0-3.2)

\* $P < 0.001$ ; \*\* $P < 0.05$ ; The values indicate the median (interquartile range) unless otherwise noted; AFP: alpha-fetoprotein; DCP: des-gamma-carboxy prothrombin; AFP-L3: lens culinaris agglutinin-reactive alpha-fetoprotein; HCC: hepatocellular carcinoma; Tx: treatment; DAA: direct-acting antiviral



**Figure 2.** The overall survival in patients treated with DAAs after curative treatment for HCC. The survival rates at 1, 2 and 3 years were 99%, 98% and 95%, respectively. HCC: hepatocellular carcinoma; DAA: direct-acting antiviral

1-, 2- and 3-year cumulative recurrence rates after DAA therapy were 11.4%, 31.6% and 49.4% in patients with 1 previous treatment; 25.1%, 45.3% and 57.5% in patients with 2-4 previous treatments; and 47.6%, 70.9% and 100% in patients with more than 4 previous treatments, respectively.

### Patterns of recurrence

The patterns of HCC recurrence after DAA therapy are shown in [Table 5]. The median diameter of the tumors was 13 mm, and 44 patients (32.6%) had multiple tumors, including 3 patients (2.2%) with extrahepatic metastasis. Two patients (1.5%) had rapidly progressing tumors: 1 had massive infiltrative-growing tumors with invasion to the

**Table 3. Risk factors associated with hepatocellular carcinoma recurrence, as determined by a univariate Cox regression analysis**

Risk factors	Category	HR	95%CI	P value
At the initiation of DAA therapy				
Age (years)	≥ 75 (vs. < 75)	0.99	0.70-1.38	0.94
Gender	Male (vs. female)	1.16	0.82-1.63	0.41
Platelet ( $\times 10^3/\mu\text{L}$ )	< 100 (vs. ≥ 100)	0.93	0.66-1.31	0.69
ALB (g/dL)	< 3.8 (vs. ≥ 3.8)	1.05	0.75-1.48	0.76
T.Bil (mg/dL)	> 0.8 (vs. ≤ 0.8)	1.65	1.17-2.33	< 0.01
AST (U/L)	> 52 (vs. ≤ 52)	0.95	0.68-1.33	0.75
ALT (U/L)	> 41 (vs. ≤ 41)	0.87	0.62-1.22	0.41
GGTP (U/L)	> 37 (vs. ≤ 37)	0.84	0.60-1.17	0.30
PT-INR	> 1.0 (vs. ≤ 1.0)	0.99	0.67-1.46	0.95
Child-Pugh grade	A (vs. B)	0.93	0.60-1.43	0.73
AFP (ng/mL)	≥ 10 (vs. < 10)	1.14	0.81-1.60	0.46
DCP (mAU/mL)	≥ 28 (vs. < 28)	1.05	0.67-1.64	0.83
AFP-L3 (%)	≥ 10 (vs. < 10)	5.22	2.39-11.4	< 0.01
Fib-4 index	≥ 3.25 (vs. < 3.25)	1.26	0.77-2.04	0.36
HCV genotype	Genotype 1 (vs. others)	1.42	0.17-11.7	0.75
HCV-RNA (log IU/mL)	> 6.0 (vs. ≤ 6.0)	1.22	0.87-1.71	0.25
History of HCC Tx	Multiple (vs. single)	1.89	1.35-2.65	< 0.01
The period from the final HCC Tx (year)	≤ 1 (vs. > 1)	1.47	1.04-2.09	0.03
At the first HCC Tx				
Maximum tumor diameter (mm)	> 20 (vs. ≤ 20)	1.19	0.84-1.69	0.33
Tumor number	Multiple (vs. single)	2.01	1.39-2.90	< 0.01
AFP (ng/mL)	≥ 10 (vs. < 10)	1.50	1.04-2.16	0.03
DCP (mAU/mL)	≥ 28 (vs. < 28)	1.24	0.88-1.76	0.22
AFP-L3 (%)	≥ 10 (vs. < 10)	1.23	0.71-2.14	0.46
At the final HCC Tx before DAA				
Maximum tumor diameter (mm)	> 20 (vs. ≤ 20)	1.05	0.71-1.55	0.80
Tumor number	Multiple (vs. single)	1.72	1.18-2.50	< 0.01
The final HCC Tx before DAA	RFA (vs. hepatectomy)	1.49	1.00-2.22	0.049
After DAA Therapy				
AFP (ng/mL)	≥ 10 (vs. < 10)	1.45	0.93-2.25	0.10
DCP (mAU/mL)	≥ 28 (vs. < 28)	1.62	1.05-2.49	0.03
AFP-L3 (%)	≥ 10 (vs. < 10)	4.19	1.94-9.04	< 0.01
SVR (12 weeks)	No (vs. yes)	1.68	0.93-3.05	0.09

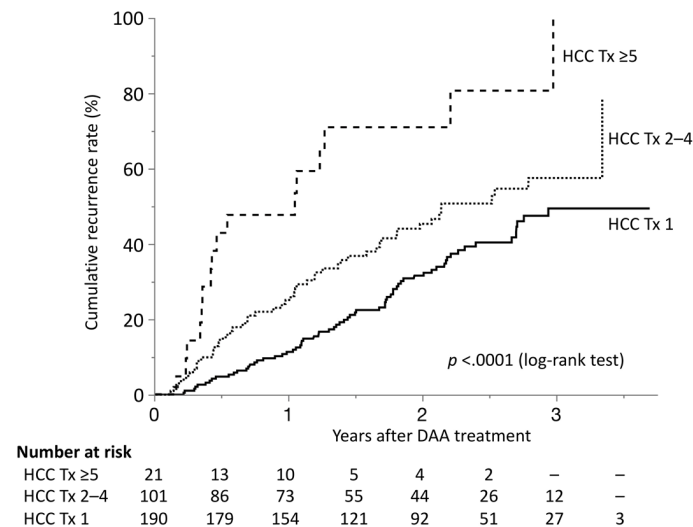
CI: confidence interval; ALB: albumin; T.Bil: total bilirubin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGTP: gamma-glutamyl transpeptidase; PT-INR: prothrombin time-international normalized ratio; AFP: alpha-fetoprotein; DCP: des-gamma-carboxy prothrombin; AFP-L3: lens culinaris agglutinin-reactive alpha-fetoprotein; FIB-4 index =  $\text{Age (years)} \times \text{AST (U/L)} / [\text{PLT}(10^9/\text{L}) \times \text{ALT}^{1/2} (\text{U/L})]$ ; HCV: hepatitis C virus; HCC: hepatocellular carcinoma; Tx: treatment; DAA: direct-acting antiviral; SVR: sustained virological response

**Table 4. Risk factors associated with hepatocellular carcinoma recurrence, as determined by a multivariate Cox regression analysis**

Risk factors	HR	95%CI	P value
Multiple tumors at the first HCC Tx	2.21	1.41-3.49	< 0.01
History of multiple Tx for HCC before DAAs	1.97	1.28-3.02	< 0.01
AFP-L3 ≥ 10 % at the initiation of DAAs	4.74	2.10-10.7	< 0.01

CI: confidence interval; AFP-L3: lens culinaris agglutinin-reactive alpha-fetoprotein; HCC: hepatocellular carcinoma; Tx: treatment; DAA: direct-acting antiviral

umbilical portion of the portal vein and lung metastasis at 1 month after DAA therapy (3 months after RFA), and the other had 2 infiltrative-growing tumors of > 40-mm diameter at 8 months after DAA therapy (15 months after RFA); they died 56 days and 15 months after recurrence, respectively.



**Figure 3.** Recurrence in patients treated with DAAs after curative treatment for HCC according to the number of previous HCC treatments. The recurrence rates increased as the number of previous HCC treatments increased ( $P < 0.0001$ , log-rank test). HCC: hepatocellular carcinoma; DAA: direct-acting antiviral; Tx: treatment

**Table 5. Hepatocellular carcinoma status at the time of recurrence after direct-acting antiviral therapy**

	The final HCC Tx before DAA	
	Hepatectomy	RFA
Number of patients with recurrence	32	103
Non-SVR	4 (12.5%)	8 (7.8%)
Maximum tumor diameter (mm)	13 (10-17)	13 (10-17)
Tumor number (1/2/3/≥ 4)	21 (65.6%)/8 (25.0%)/1 (3.1%)/2 (6.3%)	70 (68.0%)/19 (18.4%)/6 (5.8%)/8 (7.8%)
Extrahepatic metastasis (positive)	0 (0%)	3 (2.9%)
Vascular invasion (positive)	1 (3.1%)	1 (1.0%)
AFP (ng/mL)	4.9 (3.1-7.1)	6.4 (4.3-11.2)
DCP (mAU/mL)	20 (13-27)	24 (18-41)
AFP-L3 (%)	0 (0-4.9)	0.5 (0-6.7)

The values indicate the median (interquartile range) unless otherwise noted; AFP: alpha-fetoprotein; AFP-L3: lens culinaris agglutinin-reactive alpha-fetoprotein; DCP: des-gamma-carboxy prothrombin; HCC: hepatocellular carcinoma; Tx: treatment; DAA: direct-acting antiviral

The treatment methods for HCC after DAA therapy were as follows: RFA ( $n = 83$ , 61.4%), hepatectomy ( $n = 14$ , 10.4%), TACE ( $n = 22$ , 16.3%), radiation therapy ( $n = 3$ , 2.2%) and particle beam, percutaneous ethanol injection, hepatic arterial infusion, systemic chemotherapy and best supportive care ( $n = 1$  each).

## DISCUSSION

In the present study, we found that the 1- and 3-year recurrence rates of curatively treated HCC patients who received DAA therapy were 18.3% and 55.4%, respectively. In contrast, the 3-year overall survival rate was extremely high (95.4%). In addition, we revealed that the factors associated with recurrence were multiple tumors at the first HCC treatment, a history of multiple treatments for HCC and AFP-L3  $\geq 10\%$  at the initiation of DAA therapy.

Table 6 summarizes the published data on HCC recurrence after DAA administration in patients with an HCC treatment history. The unexpectedly high rate of HCC recurrence reported by the Spanish group raised a number of concerns and prompted a great deal of discussion<sup>[4]</sup>. According to the original paper, 3 patients died, and 16 of 55 patients (27.6%) developed HCC recurrence after a median follow-up period of 5.7 months. However, that group

**Table 6. Summary of hepatocellular carcinoma recurrence in patients administered direct-acting antivirals after hepatocellular carcinoma treatment**

Authors	Number of patients	HCC treatment before DAA	HCC recurrence	Follow-up period
Reig <i>et al.</i> <sup>[4]</sup>	58	Hepatectomy 20 Ablation 32 TACE 6	16/58 (27.6%)	5.7 months
Conti <i>et al.</i> <sup>[5]</sup>	59	Hepatectomy 23 Ablation 28 TACE 5	17/59 (28.8%)	24 weeks
Nagata <i>et al.</i> <sup>[7]</sup>	83	N/A	SVR: 22.9%/3 years Non-SVR: 40.0%/3 years	2.3 years
Ikeda <i>et al.</i> <sup>[9]</sup>	89	Hepatectomy 43 RFA 38 TACE 4 PRT 4	21.8%/2years	20.7 months
ANRS <sup>[10]</sup>	189	N/A	8.8%/year	20.2 months
Minami <i>et al.</i> <sup>[13]</sup>	27	RFA 27	29.8%/2 year	1.3 years

HCC: hepatocellular carcinoma; DAA: direct-acting antiviral; TACE: transcatheter arterial chemoembolization; PRT: particle radiation therapy; RFA: radio-frequency ablation; SVR: sustained virological response

included patients treated with TACE, which was not a curative treatment; recurrence occurred only in cases treated with hepatectomy or RFA before DAA, and no recurrence was reported in cases treated with TACE, possibly because of the small number of TACE cases (only 6). Another Italian group also reported that HCC recurrence was observed in 17 of 59 (28.8%) cirrhotic patients with a history of previous liver cancer during a 24-week follow-up period<sup>[5]</sup>.

In contrast to these studies, a French prospective cohort study did not observe an increased risk of HCC recurrence after DAA therapy in patients who underwent curative HCC treatment: the rates of recurrence were similar when comparing 189 patients who received DAAs (recurrence,  $n = 24$ ; incidence, 8.8%/year) to 78 patients who did not receive DAAs (recurrence,  $n = 16$ ; incidence, 7.9%/year)<sup>[10]</sup>. A Canadian group examined the effect of HCV eradication pre- and post-liver transplantation (LT) and reported that the treatment of HCV with DAAs prior to LT ( $n = 13$ ) enabled an SVR to be achieved in 92.3% of patients with no influence on the HCC progression or mean waiting time<sup>[14]</sup>. In 2017, Zanetto *et al.*<sup>[15]</sup> examined the dropout rate from an LT waiting list because of HCC progression in HCV-infected patients treated with DAAs. They reported that 2 of 23 DAA-treated patients (8.7%) and 1 of 23 controls (4.3%) were registered as dropout events due to HCC progression ( $P = 0.90$ ) and concluded that HCV eradication did not seem to be associated with an increased risk of dropout from the waiting list.

Several such studies have been reported from Japan<sup>[7,9,13]</sup>. Minami *et al.*<sup>[13]</sup> examined the recurrence of HCC after RFA treatment and noted no difference in the rate or aggressiveness of recurrence between cases treated with DAA ( $n = 27$ ) and those treated with interferon ( $n = 38$ ) within 2 years after ablation. Ikeda *et al.* showed that the 1- and 2-year recurrence rates after curative treatment for HCC were 18.1% and 25.0%, respectively, in patients with DAA therapy and 21.8% and 46.5%, respectively, in those without DAA therapy<sup>[9]</sup>. Nagata *et al.*<sup>[7]</sup> showed that the rate of cumulative HCC recurrence in patients with an SVR after interferon-free therapy was 28.9% (22/76) during a median follow-up period of 2.3 years and concluded that the risks of early HCC recurrence after viral eradication were similar between interferon-based and interferon-free therapies. However, the populations of the studies were small, and the follow-up periods were short. We therefore tried to eliminate these problems as much as possible by increasing the number of cases and prolonging the observation period.

In the period before the DAA era at our institution, the 1-, 3- and 5-year recurrence rates of HCC patients after RFA were 23.8%, 56.2% and 68.0%, respectively<sup>[19]</sup>. A study from Korea<sup>[20]</sup> reported that the 1-, 3- and 5-year cumulative intrahepatic distant recurrence rates were 24.4%, 59.5% and 73.1%, respectively. These recurrence rates were quite similar to those of our study of DAA-treated patients. Considering that the subjects in these studies were limited

to patients with HCC at the initial treatment and that 39% of the patients in our study had recurrent HCC, we concluded that DAA treatment did not induce recurrence more frequently than interferon treatment, as we found that a history of multiple treatments for HCC before DAA therapy was an important risk factor for recurrence after DAA therapy.

We previously reported that AFP > 10 ng/mL after RFA for HCC is a significant risk factor for recurrence<sup>[21]</sup>. In the present study, however, AFP elevation before DAA treatment was not extracted as a significant factor for HCC recurrence. The discrepancy may be due to the small number of high-AFP cases in the present study due to curative treatment and the suppression of carcinogenic potential by DAA therapy. The median AFP values before and after DAA treatment were 8.4 ng/mL and 5.3 ng/mL, respectively, in the present study. Of note, however: the recurrence rate was higher in patients with high AFP-L3 levels at the initiation of DAA therapy than in patients with low AFP-L3 levels. High AFP-L3 levels are known to indicate that the HCC has a high malignant carcinogenic potential and is associated with a poor prognosis<sup>[22]</sup>. Although most published studies did not report the AFP-L3 levels of their patients, it is possible that case studies showing high recurrence rates after DAA included patients with high AFP-L3 levels.

We experienced two HCC patients who showed recurrence with portal vein invasion. Considering that both patients had a history of multiple treatments and the frequency of such a recurrence pattern was not high, it is difficult to conclude whether or not DAA treatment was responsible for this type of recurrence. Careful observation will be necessary in order to confirm whether or not the prevalence of such cases will increase in the future.

Our study was associated with some limitations. First, although the observation period was relatively long among studies in which DAA therapy was administered after curative treatment of HCC, the duration was still not sufficient to estimate the long-term survival. Second, the number of deaths was extremely small; thus, the effect of DAA therapy on prolonging the survival could not be evaluated. In addition, some factors affecting the recurrence of HCC, such as alcohol consumption and coexisting diabetes mellitus, have not been well studied.

In conclusion, the recurrence rate in patients treated with DAAs after curative treatment of HCC was comparable to that before the DAA era. We also found that multiple tumors at the first HCC treatment, a history of multiple treatments for HCC, and high AFP-L3 at the time of DAA treatment were risk factors for HCC recurrence. Given that the incidence of recurrence after DAA therapy was non-negligible, a long-term follow-up is necessary to ensure a long survival, especially for patients who have risk factors for recurrence.

## DECLARATIONS

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### Authors' contributions

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Analysis of data: Nakamura S, Nouse K

Drafting of the manuscript: Nakamura S, Nouse K

Statistical analysis: Nakamura S

Study supervision: Nouse K, Okada H, Tanaka M



### Availability of data and materials

The original raw data used to support the findings of this study have not been made available because of the risk that will come into conflict with Personal Information Protection Law in Japan.

### Financial support and sponsorship

Not applicable.

### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Copyright

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Original Article

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# Efficacy and safety of stereotactic body radiation therapy combined with transarterial chemoembolization for Chinese intermediate-to advanced-stage hepatocellular carcinoma patients: a systematic review and meta-analysis

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## Abstract

**Aim:** According to the current guidelines, transarterial chemoembolization (TACE) remains the first-line therapies for hepatocellular carcinoma (HCC) patients at Barcelona Clinic Liver Cancer (BCLC) B-stage and sorafenib is a small molecule target drug for BCLC C-stage. In clinical practice, clinicians have attempted to use stereotactic body radiation therapy (SBRT) plus TACE for treating intermediate- to advanced-stage HCC. However, the therapeutic effects are still inconsistent. This meta-analysis was conducted to elucidate the validity and safety of the combination therapy of SBRT plus TACE in the patients with intermediate-to advanced-stage HCC.

**Methods:** PubMed, MEDLINE, Web of Science, China Biology Medicine, Chinese Knowledge resources integrated and Chinese Scientific Journal Full-Text Database was searched from their inception date to November 2018. The survival rates (half-year, one-year and two-year) were analyzed and compared between the observation groups and the control groups. The negative conversion rate of AFP and the total effective rate were also assessed. Risk ratios (RR) and 95%CI were calculated to express therapeutic effects.



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**Results:** A total of 1,210 patients from 13 eligible studies were included. The cooperation of TACE and SBRT notably ameliorated the whole survival rates of half-year, one-year, two-year, the negative conversion rate of AFP, and the total effective rate, compared with TACE or SBRT monotherapy [RR (the total effective rate), 1.412, 95%CI: 1.309-1.523,  $P < 0.001$ ], [RR (half-year survival rate), 1.196, 95%CI: 1.121-1.276,  $P < 0.001$ ], [RR (one-year survival rate), 1.327, 95%CI: 1.236-1.424,  $P < 0.001$ ], [RR (two-year survival rate), 1.479, 95%CI: 1.284-1.703,  $P < 0.001$ ] and [RR (negative conversion rate of AFP), 1.756, 95%CI: 1.502-2.059,  $P < 0.001$ ]. Sensitivity analysis supported the above results.

**Conclusion:** Combination therapy of SBRT and TACE provides survival benefits in intermediate-to advanced-stage HCC patients compared to monotherapy of SBRT or TACE.

**Keywords:** Transcatheter arterial chemoembolization, hepatocellular carcinoma, stereotactic body radiation therapy, meta-analysis

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death and the fifth most common malignancy worldwide<sup>[1,2]</sup>. The incidence and mortality rates of HCC shows an increasing trend year by year<sup>[3]</sup>. Although the application of new HCC biomarkers and advanced imaging methods may improve the sensitivity and specificity of HCC in an early stage, a large proportion of HCC patients have been already at the intermediate-to advanced-stage at the time of diagnosis.

TACE, radiofrequency ablation, microwave ablation are widely used in clinically, and each of them has been proved to produce a great healing effect on patients in clinical practice<sup>[4-6]</sup>. However, the limited indications and contraindications restrict the clinical use of the monotherapy which may lead to a high recurrence rate. In recent years, researchers have tried relevant clinical trials to seek the treatment effect of combined treatment on patients with intermediate-and advanced-stage HCC<sup>[7-10]</sup>.

With the development of liver radiobiology and the significant progress of radiotherapy technology, SBRT has been gradually applied to HCC in the intermediate and advanced stage<sup>[11]</sup>. However, due to the relatively small sample size of related clinical trials and the lack of multi-center and large-sample randomized controlled studies, the efficacy of SBRT combined with TACE in the treatment of intermediate-and advanced-stage HCC is difficult to draw definite conclusions. Therefore, the current meta-analysis was carried out to evaluate the efficacy and safety of combination therapy and provide evidence for clinical decision making.

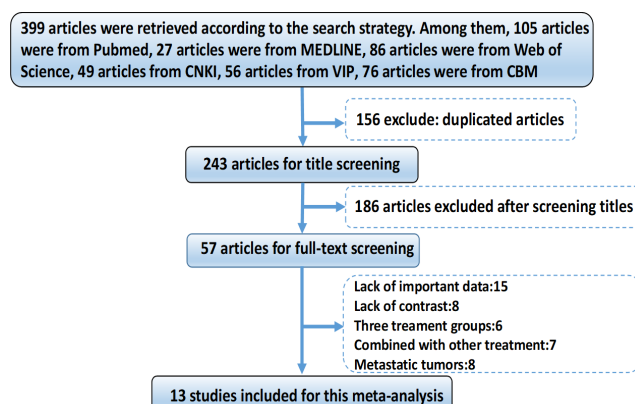
## METHODS

### Search strategy

Studies were acquired via searching English databases, covering PubMed, MEDLINE, and the web of science (SCI). Chinese databases were also examined, including Chinese Knowledge resources integrated, Chinese Scientific Journal Full-Text Database and China Biology Medicine. The closing date for documents search was November, 2018, “transcatheter arterial chemoembolization” or TACE) or “transarterial chemoembolization” and “hepatocellular carcinoma” or “liver carcinoma” or “liver cancer” or “HCC” and (“stereotactic body radiation therapy” or “Gamma Knife”) were used as search terms. Additionally, the references of relevant articles were also retrieved until no new potential material could be found.

### Study selection

Including criteria for this meta-analysis were as follows: (1) randomized controlled trials and language limited to Chinese or English; (2) the studies that included an observation group adopted SBRT combined



**Figure 1.** The search and selection of eligible clinical studies. CNKI: Chinese Knowledge resources integrated; VIP: Chinese Scientific Journal Full-Text Database; CBM: China Biology Medicine

with TACE, while a control group passed TACE or SBRT merely; (3) HCC should be diagnosed by computed tomography (CT), magnetic resonance imaging or pathology; (4) the research results should include the total effective rate at least. The overall effective rate =  $(CR + PR) / \text{the total participants} \times 100\%$ , CR: tumor completely subsided and no re-occurrence of new tumors for at least four weeks; PR: tumor size shrunk more than 50% and no re-occurrence of new tumors for at least four weeks.

Publications complied with the following criteria were excluded: (1) the repetitive studies and unsuitable publication types, narrative reviews, systematic reviews, letters, comments, case reports or studies unrelated to our topics; (2) the studies including patients with metastatic liver cancer, under other therapies or three intervention procedures; (3) the studies including patients with severe cirrhosis, massive ascites or severe hepatic insufficiency; (4) the data were unable to be extracted from the reviews; (5) no control group was established in the reviews; (6) the studies including patients who had metastatic or recurrent liver carcinoma.

### Identification of eligible studies

After searching the literature within several databases, 399 potentially relevant studies were identified initially. After the examination of titles and abstracts, 156 surveys were excluded and 57 articles were selected for full-text screening. Finally, 13 studies were included for this meta-analysis. The study recruitment flowchart was shown in [Figure 1].

### Data extraction of the studies

All included studies were published from 2008 to 2017. A total of 1,209 patients were enrolled, including 625 patients from the observation group and 584 patients from the control group. Among the patients, the male was 862 and female was 347. All patients were followed up for at least one year. Also, KPS score, Child-pugh score and TNM stage of the patients were also described. The data of the baseline characteristics of the patients in the included studies are presented in Table 1.

### Study quality assessment

Two researchers (Shoujie Zhao and Baishu Dai) independently evaluated the included studies. The authors' name and institution were blinded to researchers. The risk of bias in RCTs was assessed according to the Cochrane Risk of Bias tool<sup>[12]</sup> which is based on the following aspects: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, and selective reporting. All the disagreements were discussed with the third researchers (Yong Long) to reach consensus.



**Table 1. Baseline characteristics of studies included in the meta-analysis**

Authors	Published year	Type of study	Total number	Number of participants		KPS score	Child-pugh score	TNM stage	Follow-up time
				Observation	Control				
Ji <i>et al.</i> <sup>[29]</sup>	2010	RCT	120	62	58	≥ 70	A or B	ND	3
Liu <i>et al.</i> <sup>[30]</sup>	2016	RCT	86	43	43	ND	ND	ND	2
Luo <i>et al.</i> <sup>[31]</sup>	2015	RCT	74	38	36	ND	A or B	ND	2
Pan <i>et al.</i> <sup>[32]</sup>	2015	RCT	84	47	37	≥ 70	A or B	≥ II	1
Sha <i>et al.</i> <sup>[33]</sup>	2013	RCT	105	52	53	≥ 60	≥ B	ND	1
Song <i>et al.</i> <sup>[34]</sup>	2016	RCT	78	39	39	ND	A or B	ND	2
Sun <i>et al.</i> <sup>[35]</sup>	2014	RCT	62	32	30	ND	A or B	ND	1
Wei <i>et al.</i> <sup>[36]</sup>	2009	RCT	104	52	52	≥ 60	≥ B	≥ III	1
Xiu <i>et al.</i> <sup>[37]</sup>	2011	RCT	48	25	23	≥ 60	A or B	≥ III	1
Yang <i>et al.</i> <sup>[38]</sup>	2012	RCT	259	135	124	ND	≥ B	≥ II	2
Ye <i>et al.</i> <sup>[39]</sup>	2011	RCT	62	30	32	ND	A or B	ND	2
Zhang <i>et al.</i> <sup>[40]</sup>	2010	RCT	72	36	36	ND	ND	ND	1
Zhou <i>et al.</i> <sup>[41]</sup>	2011	RCT	56	34	22	≥ 70	A or B	ND	5

KPS: Karnofsky scores; ND: Not described; RCT: randomized controlled trial; TACE: transcatheter arterial chemoembolization; SBRT: stereotactic body radiation therapy

## Statistical analysis

Comprehensive Meta-Analysis V2 software was used in the data analysis. Pooled risk ratios (RRs) and 95%CI were calculated to express therapeutic effects which were identified to be statistically significant if  $P$  value < 0.05. The heterogeneity was assessed using the  $I^2$  statistic and associated  $P$  values. Statistically, heterogeneity was deemed to have existed among the studies if  $P$  value < 0.1 or  $I^2$  > 50.00%. A random-effect model was used to analyze the results if the heterogeneity existed. On the contrary, the fixed-effect model was used. Publication bias was assessed by the outcomes of the Egger test and the Begg test. If the number of included studies was less than 5, publication bias was not assessed.

## RESULTS

### Total effective rate

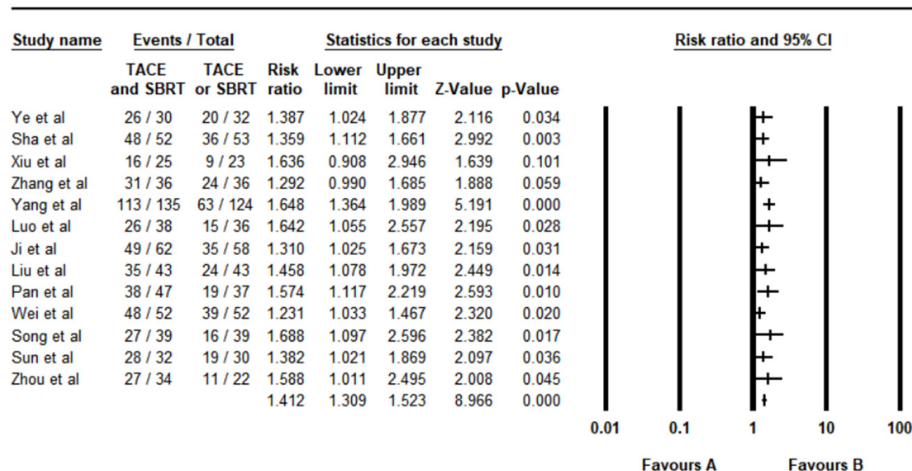
The results of the total effective rates were reported in 13 studies. No statistical heterogeneity was found among the studies, and a fixed-effect model was used ( $P = 0.791$ ,  $I^2 = 0.00\%$ ). The results showed that the tumor response in the combined therapy group (TACE + SBRT) was significantly higher than that of the monotherapy group (RR = 1.412, 95%CI: 1.309-1.523,  $P < 0.001$ ). The Egger test ( $P = 0.124$ ) and the Begg test ( $P = 0.0769$ ) revealed no publication bias. The result of the total effective rates was shown in [Figure 2].

### Half-year survival

There were only 4 out of 13 studies included in the half-year follow up the group, and the rest were not included. No statistical heterogeneity was found among the studies, and a fixed-effect model was used for meta-analysis ( $P = 0.917 > 0.1$ ,  $I^2 = 0.00\%$ ). The results showed that the half-year survival of the combined therapy group (TACE + SBRT) was significantly higher than that of the monotherapy group (RR = 1.196, 95%CI: 1.121-1.276,  $P < 0.001$ ). The result of the half-year survival was shown in [Figure 3].

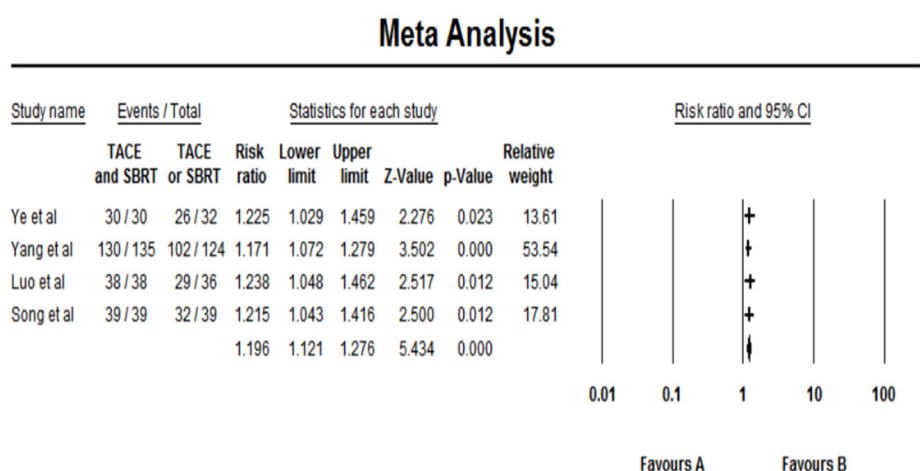
### One-year survival rate

The results of the one-year survival rate were reported in 13 studies. No statistical heterogeneity was found among the studies, and a fixed-effect model was selected ( $P = 0.793 > 0.10$ ,  $I^2 = 0.00\%$ ). The results showed that the 1-year survival rate of the combined therapy group (TACE + SBRT) was higher than that of the TACE monotherapy group (RR = 1.326, 95%CI: 1.234-1.424,  $P < 0.001$ ). The Egger test ( $P = 0.10092$ ) and the Begg test ( $P = 0.0509$ ) revealed no publication bias. The result of one-year survival rate was shown in [Figure 4].



## Meta Analysis

**Figure 2.** Tumor response comparing transarterial chemoembolization (TACE) plus stereotactic body radiation therapy (SBRT) with TACE or SBRT monotherapy in intermediate-to advanced-stage hepatocellular carcinoma patients



## Meta Analysis

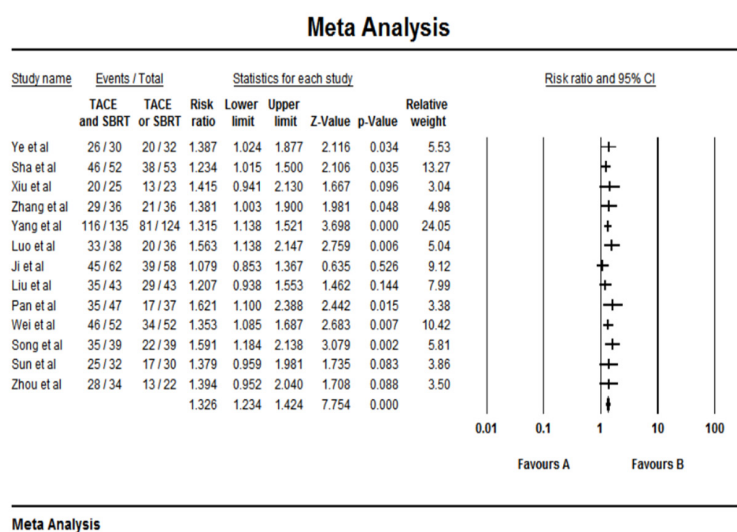
**Figure 3.** Meta-analysis of the half-year survival in 4 studies comparing transarterial chemoembolization (TACE) plus stereotactic body radiation therapy (SBRT) with TACE or SBRT monotherapy in intermediate-to advanced-stage hepatocellular carcinoma patients

## Two-year survival rate

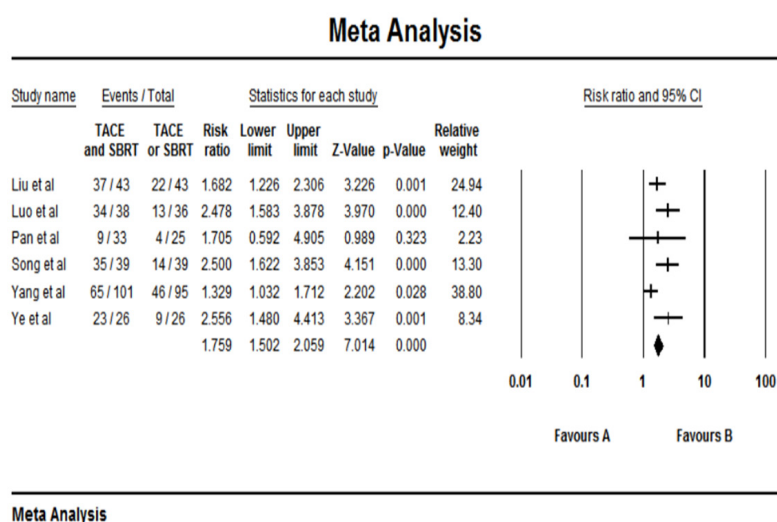
There were only 6 out of 13 studies included in the two-year survival follow up the group and the rest were not added. No statistical heterogeneity was found among the studies, and a fixed-effect model was used ( $P = 0.930 > 0.1$ ,  $I^2 = 0.00\%$ ). The results showed that the 2-year survival of the combined therapy group (TACE + SBRT) was significantly higher than that of the monotherapy group (RR = 1.153, 95%CI: 1.282-1.783,  $P < 0.001$ ). The Egger test ( $P = 0.36738$ ) and the Begg test ( $P = 0.57303$ ) revealed no publication bias. The result of the two-year survival rate was shown in [Figure 5].

## The negative conversion rate of AFP

The negative conversion rate of AFP was reported in 6 studies. A random-effect model was used to analyse the result on account of the statistical heterogeneity which was found among the studies ( $P = 0.045$ ,  $I^2 = 56.00\%$ ).



**Figure 4.** Meta-analysis of the one-year survival rate in 13 studies comparing transarterial chemoembolization (TACE) plus stereotactic body radiation therapy (SBRT) with TACE or SBRT monotherapy for intermediate-to advanced-stage hepatocellular carcinoma patients

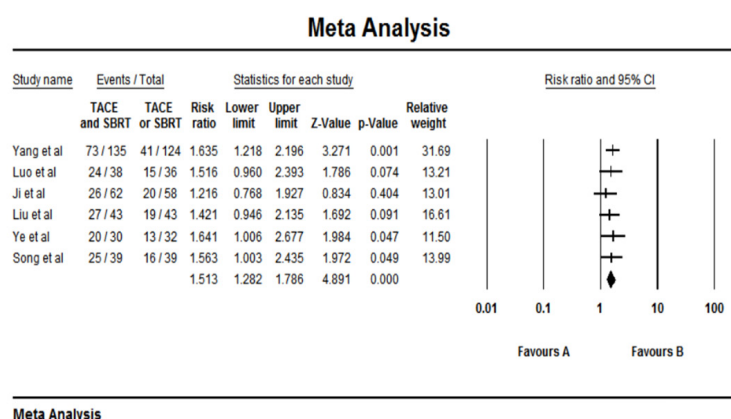


**Figure 5.** Meta-analysis of the two-year survival in 6 studies comparing transarterial chemoembolization (TACE) plus stereotactic body radiation therapy (SBRT) with TACE or SBRT monotherapy for intermediate-to advanced-stage hepatocellular carcinoma patients

The results showed that negative conversion rate of AFP of the combined therapy group (TACE + SBRT) was significantly higher than that of the monotherapy group (RR = 1.756, 95%CI: 1.502-2.059,  $P < 0.001$ ). The Egger test ( $P = 0.175$ ) and the Begg test ( $P = 0.707$ ) revealed no publication bias. The result of the negative conversion rate of AFP was shown in [Figure 6].

## DISCUSSION

This current meta-analysis aimed to assess the validity and safety of SBRT combined with TACE for patients in intermediate-to advanced-stage HCC. The pooled results showed that TACE plus SBRT notably ameliorated the total survival rates of half-year, one-year and two-year ( $P < 0.05$ ). Combination treatment of SBRT and TACE were also benefited to the negative conversion rate of AFP and the total effective rate ( $P < 0.05$ ). The results revealed that SBRT combined with TACE had superior efficacy than that of SBRT or TACE alone for HCC patients in intermediate-to advanced-stage.



**Figure 6.** Meta-analysis of the negative conversion rate of AFP in 6 studies comparing transarterial chemoembolization (TACE) plus stereotactic body radiation therapy (SBRT) with TACE or SBRT monotherapy for intermediate-to advanced-stage hepatocellular carcinoma patients

HCC is a malignant tumor which seriously endangers human health. Due to the difficulty in early diagnosis and its hidden character, most patients are diagnosed at the intermediate- to advanced-stage. Sorafenib or lenvatinib is currently used as a first-line standard therapeutic agent for advanced HCC according to the BCLC criteria<sup>[13,14]</sup>. Besides, apatinib may be a substitute for HCC patients with sorafenib resistance in the future, especially for those with high expression of VEGF<sup>[15]</sup>. With the rapid development of clinical medicine, the appearance of more and more treatment methods which lead to no uniform suggestion for the treatment of intermediate to advanced HCC patients.

TACE is recognized as an alternative treatment option for intermediate-to advanced- HCC patients<sup>[16]</sup>. It is to inject chemotherapy drugs directly into tumor blood supply artery through a catheter which can improve local drug concentration of tumor to increase the ability to kill cancer cells, achieve embolization of tumor blood vessels and block the blood supply of a tumor, tumor tissue necrosis, shrinkage, and disappearance. However, tumor tissues could not be eliminated through TACE<sup>[17]</sup>. There are mainly three reasons. Firstly, after TACE, some infiltrating cells and metastatic liver cells are still alive, and repeated treatment by TACE may produce a specific resistance to chemotherapy drugs. Secondly, the liver tissue is damaged due to hypoxia and ischemia, embolization agents and chemotherapy drugs, which influences the clinical efficacy of transcatheter arterial chemoembolization. Thirdly, after TACE, part of the tumor tissue will recover blood supply. Therefore, although the short-term effectiveness of TACE is justifiable, it still has limitations, and the long-term effectiveness remains unsatisfactory.

The liver is a radiosensitive organ which ranks only behind bone marrow, lymphoid tissue, and kidney<sup>[18]</sup>. As a result, in spite of the rapid development of radiotherapy for HCC, the efficacy was not significantly improved. In recent years, with the growth of stereotactic radiotherapy, SBRT is gradually appropriate for intermediate-to advanced-HCC<sup>[19-22]</sup>. SBRT delivers a high dose of radiation to HCC within a short period time and is effective and less invasive for the delivery of high radiation doses to the tumor with hypofractionation. Employing the high-dose irradiation to the tumor area which can reduce irradiation dose of the healthy liver tissue at the same time, SBRT can make the tumor vascular degrade and mortify, lower the blood supply of the cancer to achieve the goal of killing tumor cells. Besides, it is efficient that multiple lesions can be operated at the same time by the use of SBRT. The features of SBRT above, to a large extent, make up the defect of TACE.

Recently, the therapeutic role of SBRT combined with TACE for intermediate-to advanced-stage HCC has been emphasized more than before<sup>[23,24]</sup>. Jun and Kim<sup>[25]</sup> showed that SBRT combined with TACE is a feasible

option for patients with HCC ( $\leq 5$  cm) without increased liver toxicity compared with TACE. Chung and Hwang<sup>[26]</sup> suggested that SBRT combined with TACE can be a therapeutic option for HCC at the caudate lobe with marginal resectability. In the study by Kang *et al.*<sup>[27]</sup> stereotactic body radiation therapy combined with TACE in the treatment of primary HCC with portal vein cancer thrombus can significantly improve the local control rate, survival rate, the effective rate of portal vein cancer thrombus, and AFP improvement rate. Besides, SBRT before TACE may have superiority in protecting liver function. Furthermore, SBRT combined with TACE may be a useful complementary treatment approach for HCC  $> 5$  cm in diameter<sup>[28]</sup>.

The application of SBRT combined with TACE in the treatment of intermediate-to advanced-HCC patients produced a synergistic therapeutic effect which may be related to the following factors: (1) TACE can shrink tumor volume and reduce normal liver tissue damage; (2) Chemotherapeutics have the effect of enhanced sensitivity for radiotherapy; (3) SBRT can denature vascular endothelial cells and block blood capillaries, prolong the storage time of iodide oil and drugs in the body, and avoid repeated TACE treatment; (4) TACE and SBRT have different therapeutic effects on cancer cells at various growth stages; (5) TACE can promote the transformation of the remaining cells from non-proliferative stage cells to the proliferative phase which can improve the sensitivity and the therapeutic effect of SBRT.

Six studies revealed that the side effects in the combined therapy group were slightly more substantial than those in the monotherapy group, such as decrease of hemoglobin, leukocyte, thrombocytopenia, gastrointestinal reactions, and liver function damage, but there was no significant difference between the experimental group and control group ( $P > 0.05$ ). The prognosis of the patients generally did not be affected through the active symptomatic treatment<sup>[29,30,32,33,36,39]</sup>.

The results of our meta-analysis are subject to several limitations. Firstly, although a total of 13 studies including 1210 patients were enrolled and the whole studies selected were high-quality RCTs, the sample sizes of most studies were relatively small<sup>[29-41]</sup>. As a result, the studies selected maybe not capable of finding out the details of all aspects and performing more subgroup analyses to evaluate the effect of the patients treated by SBRT plus TACE compared with SBRT or TACE monotherapy. To verify and extend the observations, a more randomized controlled, multi-center, large sample of trials are necessary. Secondly, for the sake of clinical practice guidelines and ethical issues, there might be produced potential selection bias which may derive from the characteristics of the patients such as the age, the liver function, tumor size. The above limitations may influence the final results. Thirdly, due to lack of sufficient data, the sequence of the two therapies and the interval of them, the frequency of TACE and the dose of radiotherapy were not performed in this meta-analysis which was expected to be answered by further clinical studies. Fourthly, the included studies were all conducted in China, which may bring the regional bias.

In conclusion, compared to the treatment of TACE or SBRT alone, SBRT combined with TACE is a mild, safe and effective treatment which can extend the survival time and be beneficial to the prognosis of intermediate-to advanced-stage HCC patients without any significant increase in severe untoward effects. Further studies should be performed to confirm the impact of the combined therapy.

## DECLARATIONS

### Authors' contributions

Discussed, agreed upon the content, contributed to the development and revision of the draft manuscripts, read and approved the final manuscript: all authors

Drafted the initial version: Zhao SJ, Dai BS

Contributed to constructive suggestions and modification: Shao ZJ

Developed the idea for the study and contributed useful criticism and suggestions: Du XL, Zhang WL, Long Y



### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Copyright

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Editorial

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# Controversies of hepatectomy and adjuvant therapy for hepatocellular carcinoma: moving forward

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Due to the prevalence of hepatitis B virus (HBV) and hepatitis C virus (HCV), occurrence of hepatocellular carcinoma (HCC) is increasing in many countries/regions, including China<sup>[1]</sup>. HBV and HCV infections, alcohol consumption, non-alcoholic steatohepatitis cirrhosis, or obesity pandemic are risk factors of HCC development. A recent survey study found about 70% patients with HCC are diagnosed as intermediate or advanced disease because of the lack of significant syndrome in their early stage<sup>[2]</sup>. Main treatments of HCC include hepatectomy, liver transplantation, ablation (radiofrequency, microwave, cryoablation), transarterial chemoembolization, radiotherapy, chemotherapy, target therapy, and so on. Among these treatments, only hepatectomy, liver transplantation, and ablation are curative treatments, with a 70% 5-year overall survival (OS) for early stage HCC. Hepatectomy is not recommended by Western official guidelines for intermediate and advanced stage HCC. However, Eastern official guidelines and many liver centres recommend hepatectomy for such patients who are with preserved liver function. Tumor recurrence, which occurs in 70% within 5 years after hepatectomy, is a major cause of death after hepatectomy<sup>[3]</sup>. This recurrence can be true recurrence relating to primary tumor (intrahepatic metastases), which occurs less than two years, or it can be due to the development of *de novo* tumors relating to liver disease (such as HBV/HCV and cirrhosis), which occurs at least two years later. Even so, none Western official guidelines recommend any effective adjuvant therapy to prevent HCC recurrence.

Therefore, there are at least three controversies in the field of HCC treatment between literature evidence and official guidelines. Namely:

1. Which HCC stage system is the best? Do we need more stage systems?
2. Should patients with intermediate or advanced stage HCC receive hepatectomy?
3. Should postoperative HCC patients receive adjuvant treatments?



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In recent two decades, nine systems have been proposed for staging HCC from Western to Eastern, including Cancer of the Liver Italian Program (CLIP)<sup>[4]</sup>, French Score<sup>[5]</sup>, Barcelona Clinic Liver Cancer (BCLC) staging<sup>[6]</sup>, the Model to Estimate Survival for HCC patients<sup>[7]</sup>, China liver cancer (2017 Edition)<sup>[8]</sup>, Chinese University Prognostic Index<sup>[9]</sup>, Hong Kong Liver Cancer (HKLC) system<sup>[10]</sup>, Japan Integrated Staging Score<sup>[11]</sup>, and Italian Liver Cancer (ITA.LI.CA) system<sup>[8,12]</sup>. Among these stage systems, only the BCLC<sup>[6]</sup>, HKLC<sup>[10]</sup>, and China liver cancer (2017 Edition)<sup>[8]</sup> staging systems propose stage-appropriate treatment modalities. Even so, BCLC stage system is the only one endorsed by each version of the EASL<sup>[13]</sup> and AASLD<sup>[14]</sup>.

However, each stage system has its own limitations. They leave large treatment gaps. For example, not each individual with HCC fall completely into his/her prespecified treatment modalities, and even those within the same HKLC or BCLC stage system may differ completely because of their different liver disease background. Many studies compared performance of different stage systems. Studies based on Western population found the BCLC system can predict overall survival and/or disease-free survival more accurately for Western patients with HCC than Eastern ones. However, studies based on Eastern population found HKLC or China liver cancer (2017 Edition) staging system is better than Western ones<sup>[15,16]</sup>. Therefore, selection of stage system should be based on population characteristics.

Intermediate stage disease of BCLC system includes HCC involving asymptomatic multinodular tumors with a maximum diameter > 3 cm or > 3 tumors without vascular invasion or extrahepatic spread. Earlier version of the BCLC system classified large solitary HCC beyond 5 cm with an expansive growth as intermediate disease. Namely, intermediate disease definition includes a wide range of patients according to liver function and tumour burden, which triggered a major controversy to further stratify intermediate stage HCC according to tumor burden and liver function<sup>[17-19]</sup>. Nowadays, guideline from European Association for the Study of the Liver<sup>[13]</sup> and several reviews written by BCLC proponents seems trying to recalibrate their position stating that if technically feasible patients with large solitary HCC beyond 5 cm should be classified as BCLC stage A. Anyhow for patients with solitary HCC, hepatectomy is first-line treatment with good long-term OS.

Western official guidelines only recommend palliative treatments for intermediate disease, but not hepatectomy. Their recommendations did not completely reflect newest evidence by continuing to recommend transarterial chemoembolization, particularly in comparison with hepatectomy. The efficacy of transarterial chemoembolization is far from clear. Our systematic review involving large sample size with large solitary or multinodular HCC found median 1-, 3-, and 5-year OS after hepatectomy were 81%, 56%, and 42%<sup>[20]</sup>. For 4,945 patients with multinodular HCC, the corresponding OS were 75%, 48%, and 30%<sup>[21]</sup>. A recent large meta-analysis found significant OS benefits for hepatectomy over transarterial chemoembolization in BCLC stage B patients (hazard ratio, 0.59; 95% confidence interval, 0.51-0.67;  $P < 0.001$ )<sup>[22]</sup>. Nowadays, substantial evidence supported that hepatectomy would provide better OS than other palliative therapies, implying the possibility that some Western HCC guidelines are restricting many populations with intermediate stage HCC to palliative treatment. But actually, these populations could obtain more benefit from more aggressive hepatectomy.

In China, about half of HCC patients are diagnosed as HCC in an advanced stage<sup>[2]</sup>. Many studies compared the safety and efficacy of hepatectomy to transarterial chemoembolization<sup>[3,23,24]</sup>. Patients receiving either treatment modality showed similar safety. However, hepatectomy provided significantly longer median survival than transarterial chemoembolization, even after using propensity score analysis. Two recent large retrospective studies from Japan also found hepatectomy was associated with better OS for patients with portal vein tumor thrombus (PVTT) or hepatic vein thrombus<sup>[25,26]</sup>. The first study compared OS of 2,093 HCC patients with PVTT who underwent hepatectomy and 4,381 patients who received palliative

therapies<sup>[25]</sup>. Patients in the hepatectomy group had significantly longer median OS than those received other treatments (2.87 years vs. 1.10 years). However, hepatectomy provided no OS benefit for those with PVTT affected the main trunk or contralateral branch (Vp3 or 4). Our systematic review involving 4,389 HCC patients with macrovascular invasion showed that hepatectomy provided median OS of 50% at 1 year and 18% at 5 years<sup>[20]</sup>. However, median OS after sorafenib therapy was less than 1 year in presence of PVTT<sup>[27]</sup>. Moreover, radioembolization is associated with similar median OS with sorafenib in HCC patients with PVTT<sup>[27]</sup>. These median survival time is not much higher than that of about 5 months after the best supportive care<sup>[13,28]</sup>. The OS benefit of palliative treatment is not obvious. Moreover, we should also consider that these treatments are always associated with risk of adverse events and high costs<sup>[29]</sup>.

These findings argue for expanding the Western official liver guidelines<sup>[13,14]</sup> to recognize hepatectomy as a therapeutic option for selected HCC patients with intermediate or advanced disease with good liver function (mainly Child-Pugh A). It may be true that some situations could decrease the efficacy and/or safety of hepatectomy. For instance, hepatectomy may be less effective and associated with more morbidity in HCC patients with multinodular tumors due to the possibility of microvascular invasion and liver/lung metastasis. In addition, liver cirrhosis and hepatitis activity may increase the risk of mortality, perioperative morbidity, and long-term tumor recurrence. However, continuous improvements in perioperative care and surgical technique support expanding the indications of hepatectomy. Surgeons and oncologists should not shy away from hepatectomy selection when it is feasible. At the same time, doctors should be fully conscious of the fact that the procedure is technically demanding<sup>[30]</sup>. This highlights the need to expand indications for hepatectomy.

But in fact, expanding indications of hepatectomy will translate into higher rate of tumor recurrence. Therefore, effective adjuvant therapy to prevent the recurrence of HCC is important to improve patients' long-term OS after hepatectomy. In recent decades, lots of studies have explored such therapies to prevent the recurrence of HCC, but until now, none has been officially recommended<sup>[13,14]</sup>.

Nowadays, many types of postoperative therapies to prevent HCC recurrence were reported, such as transarterial chemoembolization, nucleos(t)ide analogues (NAs), interferon- $\alpha$ , adoptive immunotherapy, vitamin K2 analog, autologous tumor vaccination, sorafenib, capecitabine, and so on. Meta-analysis found a significant improvement in recurrence-free survival and OS when adjuvant transarterial chemoembolization is used for patients with high risk of early-phase recurrence, such as large tumor, vascular invasion, and multinodular tumors<sup>[31]</sup>. The other postoperative therapies with positive efficacy is NAs for patients with HBV-related HCC<sup>[32,33]</sup>, interferon- $\alpha$  for patients with HCV-infected HCC<sup>[34]</sup>. All these three therapies are with acceptable safety. However, the safety and efficacy of the following adjuvant therapies have not been definitively established, and need further clinical investigation: interferon- $\beta$  for patients with HCV-related HCC; interferon- $\alpha$  for patients with HBV-related HCC; vitamin K2 analog, autologous tumor vaccination, adoptive immunotherapy, heparanase inhibitor PI-88, iodine-131-labeled lipiodol, or capecitabine for patients with HCC<sup>[35,36]</sup>. In contrast, the following adjuvant therapies are not recommended for clinical use: tamoxifen, sorafenib, intravenous chemotherapy and systemic chemotherapy, octreotide, and branched-chain amino acid supplementation<sup>[37-39]</sup>. Though most of these reports can create a base for clinical use and further studies, their findings should be interpreted with caution due to their clinical heterogeneity among the trials (patients, liver disease, drugs, dosages, treatment duration, *etc.*) and their small sample size.

Early- and late-phase recurrence of HCC are associated with different risk factors, and patients will have different prognoses. Macrovascular invasion, tumor rupture, multinodular tumors, large tumor size, absence of a tumor capsule, poorly differentiated tumor, and narrow resection margin are associated with early-phase recurrence. Liver cirrhosis, which is the risk factor of liver carcinogenesis, is associated with late-phase tumor recurrence. Moreover, HBV infection may contribute to both early- and late-phase recurrence. In



China, nearly 90% patients with HCC are infected with HBV. In addition, many of them have liver cirrhosis, show microvascular invasion or micrometastases before hepatectomy<sup>[38]</sup>. Therefore, almost each patient with HCC presents risk factors for early- and/or late-phase tumor recurrence. Adjuvant therapy for patients should be take into account the risk factors that they possess. Individuals presenting several such risk factors may benefit most from combination treatment modality dedicated to against both early- and late-phase tumor recurrence. However, few trials have investigated the safety and efficacy of combined therapies. This content is urgently needed for further trials. They should think over the full profile of prognostic risk factors in included individuals so that ensure that individuals with similar risk factors are assigned the appropriate combination therapy.

In summary, official guidelines have been shown to be clinically useful for guiding research and treatment of HCC<sup>[13,14]</sup>. Nevertheless, despite the sometimes substantial evidence indicating the safety and efficacy of adjuvant therapies for specific patients, official guidelines do not recommend them as treatment options<sup>[13,14]</sup>. More and more worldwide studies suggest that (1) hepatectomy could be a suitable treatment for selected patients with intermediate or advanced stage HCC, as long as preserved liver function is adequate; and (2) adjuvant transarterial chemoembolization for individuals with high risk of early-phase recurrence, NAs for individuals with HBV-related HCC, and interferon- $\alpha$  for individuals with HCV-infected HCC, are associated with better OS. There is now room, rather than debating whether or not hepatectomy and adjuvant therapies should have a room for these individuals, for focusing better on selection criteria to further enhance the long-term benefits of hepatectomy and adjuvant therapies.

## DECLARATIONS

### Authors' contributions

Conceived the study: Xiang BD

Wrote and reviewed the manuscript: Zhong JH, Xiang BD

### Availability of data and materials

Not applicable.

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### Conflicts of interest

Both authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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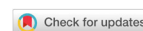
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Meta-Analysis

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# Efficacy and safety of immune checkpoint therapy in hepatocellular carcinoma: meta-analysis

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## Abstract

**Aim:** Immune checkpoint inhibitors (ICIs) are proven to be an effective way to treat the disease of hematologic malignancies. But there is still plenty of uncertainty about the effectiveness of ICIs on hepatocellular carcinoma. The Meta-analysis was conducted to evaluate the efficacy and safety of ICIs treatment in patients with HCC.

**Methods:** Four electronic databases, including PubMed, Embase, Cochrane database, and ClinicalTrials.gov, were systematically retrieved for relevant observational studies published before November 1, 2018. The objective response rate (ORR) and adverse events were analyzed. Meta and Metafor Packages in R were utilized to accomplish meta proportion analysis.

**Results:** A total of 462 patients from 7 studies were included in this meta-analysis. The pooled estimated ORR of ICIs was 19.8% (95% CI 16.4% to 23.7%). No substantial heterogeneity was observed among studies ( $Q = 2.0427$ ,  $P = 0.92$ ,  $I^2 = 0.0\%$ ). The common adverse events on any grade were saw in increased AST (22.7%, 95%CI 13.8% to 35.2%), fatigue (20.9%, 95%CI 10.9% to 36.3%), rash (18.5%, 95%CI 8.9% to 34.4%) and pruritus (17.3%, 95%CI 13.5% to 21.8%). Increased AST (9.9%, 95%CI 4.4% to 21.0%) and increased ALT (5.8%, 95%CI 3.7% to 8.9%) were the most common adverse events on grade greater than 3.

**Conclusion:** Although ICIs treatment has a certain efficacy on liver cancer, it also causes some adverse events which should be noticed by clinicians.

**Keywords:** Hepatocellular, immune-checkpoint inhibitor, CLAT-4, PD1/PD-L1



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## INTRODUCTION

Liver cancer is the fourth most common cause of cancer-related death worldwide. Among all liver cancer type, hepatocellular carcinoma (HCC) is the most common neoplasm, accounting for approximately 90% cases<sup>[1,2]</sup>. The common risk factors of HCC are cirrhosis, hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, alcohol abuse and metabolic syndrome<sup>[3]</sup>. The median overall survival of untreated HCC was 7 months, suggesting that its poor prognosis is attributable to advanced stages of diagnosis<sup>[4]</sup>. First line usage of multi-kinase inhibitor such as sorafenib was able to increase survival in advanced HCC from 7.9 months to 10.7 months (hazard ratio, 0.69). Unfortunately, this benefit was usually restricted by high resistance<sup>[5,6]</sup>. It's obviously that other approaches are still needed in treatment with advanced HCC. Immune checkpoints inhibitors (ICIs) therapy aiming to restore anticancer immunity has emerged as a promising therapy in liver cancer. Both clinical and preclinical studies revealed that there was a highly immunosuppressive tumor microenvironment and defective T cell recruitment in advanced HCC<sup>[7]</sup>. Exhaustion of CD4<sup>+</sup> T cells has also been reported as a mechanism of immune evasion in HCC<sup>[8]</sup>. ICIs are monoclonal autoantibodies (mAbs) specifically targeting the inhibitory receptors on T cells (the so-called immune checkpoints). The most common types of ICIs are the cytotoxic T lymphocyte antigen 4 (CTLA4) and the programmed death 1 (PD-1) and its ligand PD-L1. Those all act as negative co-regulators to limit further T cell activation, which are normally responsible for limiting the escalated and chronic immune responses with deleterious autoimmune effects<sup>[9,10]</sup>. ICIs have been evaluated in a series of clinical trials for melanoma, non-small cell lung cancer (NSCLC) and renal cell carcinoma, and they have yielded favorable outcomes<sup>[11-13]</sup>. Some of the clinical trials with ICIs in liver cancers have been conducting in recent years, and more studies are still in the stage of recruiting. During the 2nd phase of clinical trials, ORR is an important outcome to evaluate the efficacy of anticancer drugs, which is also an essential factor to determine the carrying out of the 3rd phase of clinical trials. In this system review, we will retrieve studies about ICIs on liver cancer with outcome of ORR and analysis of the efficacy and safety.

## METHODS

We carried out a comprehensive systematic search to identify studies about immune checkpoint inhibitor conducted on patients with HCC. The study was performed with adherence to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) guidelines<sup>[14]</sup>.

### Literature search strategy

We mainly searched four databases (PubMed, Embase, Cochrane database, and ClinicalTrials.gov) for articles published before November 1st, 2018. Controlled vocabulary and text word for synonymous terminology were both used in the search strategies. The following keywords were combined with Boolean logistical strategy for search: nivolumab, pembrolizumab, atezolizumab, avelumab, durvalumab, ipilimumab, tremelimumab, checkpoint inhibitors, PD1, programmed death 1, PD-L1, programmed cell death ligand 1, CTLA-4, cytotoxic T lymphocyte associate protein 4, and hepatocellular carcinoma, liver cancer, liver neoplasm, hepatic cancer, hepatic tumor. The search strategy in pubmed was as follow: (nivolumab OR pembrolizumab OR atezolizumab OR avelumab OR durvalumab OR ipilimumab OR tremelimumab OR "checkpoint inhibitors" OR "PD1" OR "programmed death 1" OR "PD-L1" OR "programmed cell death ligand1" OR "CTLA-4" OR "cytotoxic T lymphocyte associate protein 4") and ("hepatocellular carcinoma" OR "liver cancer" OR "liver neoplasm" OR "hepatic cancer" OR "hepatic tumor").

### Selection criteria

Inclusion criteria was as follow: (1) randomized controlled clinical trials (RCTs) or non-randomized controlled clinical trials (n-RCTs); (2) patients pathologically diagnosed with hepatocellular carcinoma; (3) patients treated with PD1/PD-L1 or CTLA-4 monoclonal antibody; (4) studies with an outcome of objective response rate. Studies were excluded if they met the following criteria: (1) reviewer or case-report; (2) duplications with early publications from same authors or institutions; (3) unable to obtain full test.



### Study selection

Two investigators (Tang WN, Deng Y) independently screened the titles and abstracts of retrieved articles to choose potential relevant articles. Disagreement about particular studies were discussed and resolved by consensus.

### Data extraction

Data extraction was carried out independently by the two reviewers (Tang WN, Deng Y). The following information was extracted from the eligible studies: information of the articles (author, published year, and study design), patient characteristics (number, age, area or nationality, race, and gender), liver disease condition (hepatitis virus infection, Eastern Cooperative Oncology Group (ECOG) performance scale, Child-Pugh stage, prior therapy), intervention in patients (agent, target, dosage, duration of dosing), outcome of efficacy (ORR).

### Statistical analysis

The data analysis process was initially conducted by the third author (Ma LT). The pooled estimated ORRs and their 95%CI were derived. Meta and Metafor Packages in R were utilized to accomplish meta proportion analysis. Logit transformation of raw proportion was performed before further analyses to increase validity. The ratio of between-study heterogeneity to total heterogeneity was quantified by  $I^2$  and  $P$  value. The assumption of homogeneity was considered invalid for  $I^2 > 25\%$  and  $P < 0.10$ . A chi-square test ( $Q$ -test) was performed to test whether the heterogeneity between studies existed or not. If between-study heterogeneity were not significant, a fixed model would be applied to get a summarized proportion; otherwise a DerSimonian and Laird random effects model would be adopted. Two side  $P < 0.05$  was considered statistically significant.

## RESULTS

### Eligible studies

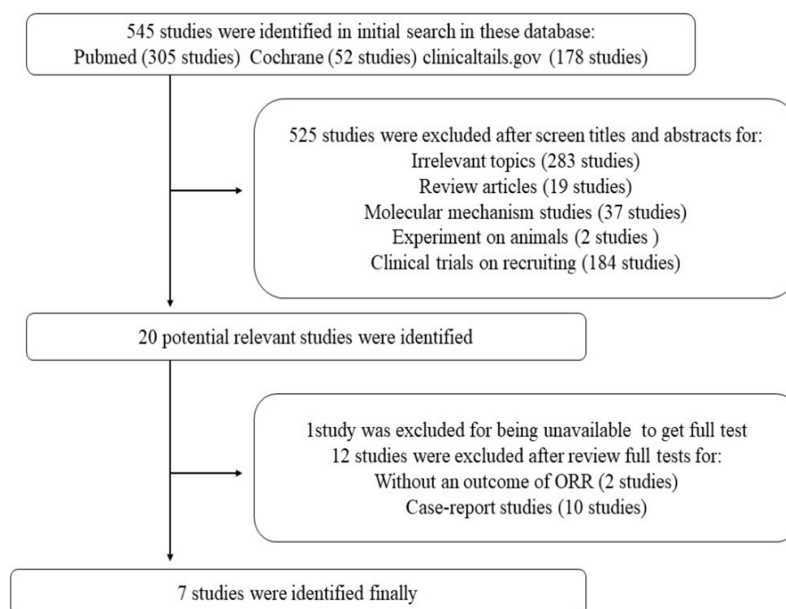
A total of 545 related articles were identified by the initial search strategy. After screening titles and abstracts, 525 studies were excluded because of irrelevant topics, review articles, molecular mechanism studies, experiments on animals, and clinical trials on recruiting. We then carefully reviewed the full texts of the remaining 20 potentially eligible papers. And then, 13 articles were excluded, because one of them was not able to obtain full test, two of them did not have the outcome of ORR, and ten papers were case-reports. Finally, seven studies were chosen for the following analysis. [Figure 1](#) shows the study selection flowchart. Data from all eligible studies were obtained from published manuscripts.

### Study characteristics

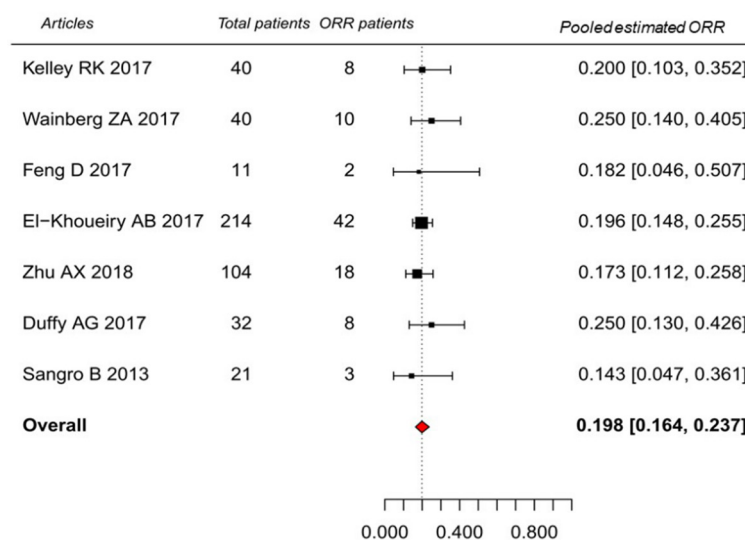
A total of 462 populations from seven studies were included in this meta-analysis. There was one paper published in 2013, five papers in 2017 and one paper in 2018. Six studies were carried out with multi-center clinical trials and one study was carried out with mono-center in China. Most of studies were in phase 1 or phase 2 of clinical trials. There were two studies conducting with CTLA-4 inhibitor (Tremelimumab), four studies with PD1/PD-L1 inhibitor (Pembrolizumab, Nivolumab, Durvalumab) and one study with combination of PD1 inhibitor and CTLA inhibitor (durvalumab and tremelimumab). [Table 1](#) shows the main characteristics of the eligible trials.

### Efficacy of immune checkpoint inhibitors in HCC

Overall, the pooled estimated ORR of patients treated with ICIs was 19.8% (95%CI: 16.4%-23.7%). No substantial heterogeneity was observed among single-study ( $Q = 2.0427$ ,  $P = 0.92$ ,  $I^2 = 0.0\%$ ). Study of El-Khoueiry *et al.*<sup>[18]</sup> weighed most with estimated proportion of 19.6% (95%CI: 14.8%-25.5%). And the second weighted article was Zhu *et al.*<sup>[17]</sup> with estimated proportion of 17.3% (95%CI: 11.2%-25.8%). See [Figure 2](#).



**Figure 1.** Flowchart program of selected studies



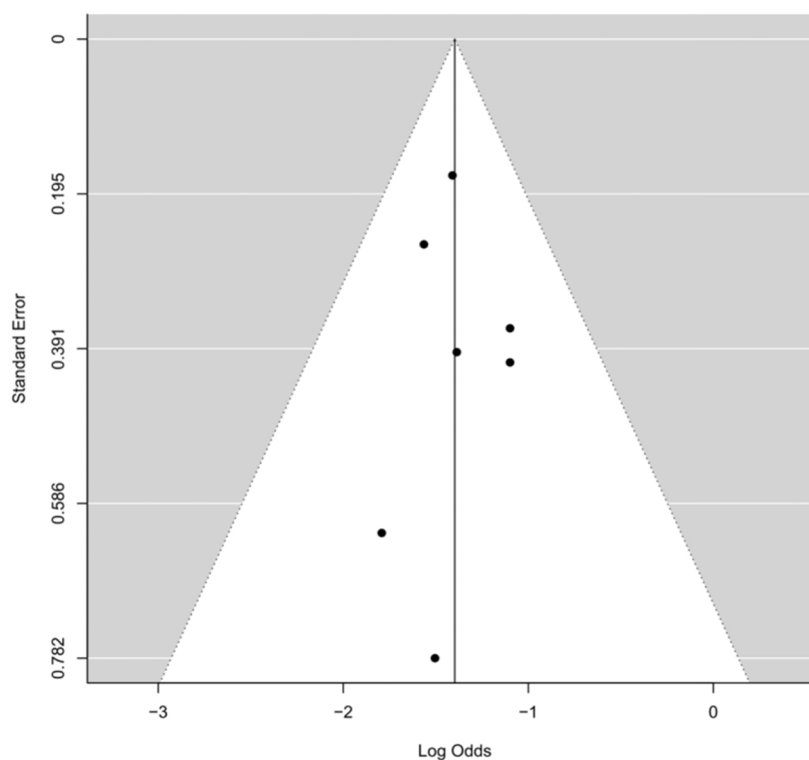
**Figure 2.** Objective response rate (ORR) of immune checkpoint inhibitor

### Publication bias

A funnel plot was made to investigate potential publication bias. Unweighted Egger regression test was performed in each analysis to test whether the funnel plot is symmetrical or not [Figure 3].

### Adverse events of ICIs

If an adverse event was mentioned in more than 4 published papers, the incident rate of adverse event was estimated by R software. The pooled estimated incident rate and its 95% confident interval were calculated on all grades and on grade greater than 3 respectively. The adverse events included in our study were fatigue, pruritus, rash, diarrhoea, nausea, asthenia, pulmonary toxicity, increased AST and increased ALT. The increased ALT was the most common adverse event whose pooled estimated incident rate was 22.7% (95%CI: 13.8%-35.2%), which was followed by fatigue 20.9% (95%CI: 10.9%-36.3%), rash 18.5% (95%CI:



**Figure 3.** Publication biases of included references

8.9%-34.4%), and pruritus 17.3% (95%CI: 13.5%-21.8%). The most common adverse event on grade greater than 3 was increased AST, whose pooled estimated incident rate was 22.7% (95%CI: 13.8%-35.2%). The second was increased ALT 13.9% (95%CI: 8.8%-21.3%). The remaining adverse events of grade greater than 3, such as fatigue, pruritus, rash, diarrhoea, nausea, pulmonary toxicity, showed a small difference among pooled estimated incident rates, which were around 1%-2%. The incident rate of Asthenia was only 0.9%, as shown in Table 2.

## DISCUSSION

This meta-analysis was performed to investigate the ORR published by papers which aimed to analyze the effectiveness of ICIs in patients with HCC. The derived overall estimated ORR reported on these non-heterogeneity papers is 19.8% (95%CI: 16.4%-23.7%,  $P < 0.001$ ). The result of this study is different from the investigation of other tumors. The difference maybe mainly caused by the heterogeneity among tumors. High response rate (50%-90%) of ICIs can be obtained with classical Hodgkin lymphoma, desmoplastic melanoma, Merkel cell carcinoma and microsatellite instability carcinoma. But the response rate of ICIs is reduced to 15%-25% when treating solid tumors such as non-small cell lung cancer, head and neck cancer, gastroesophageal cancer, bladder and urothelial cancer<sup>[22]</sup>. Compared to hematological malignancies, HCC as a solid tumor, shows not only a more complicated tumor microenvironment but the unique immune escape. All of these reasons may cause the low ORR in HCC patients treated with ICIs. One of the effective ways to enhance the drug response to ICIs is to obtain specific markers of liver cancer<sup>[23]</sup>. A high rate of ORR (> 30%) can be regarded as a proper goal in the single arm clinical trial aiming at groundbreaking treatment<sup>[24]</sup>. There are currently five anti- PD-1/PD-L1 antibodies and two CTLA-4 blocking antibodies approved by the United States Food and Drug Administration (FDA). But only Nivolumab and Tremelimumab were approved to treat with HCC, with clinical trials of ICI agents currently ongoing<sup>[25]</sup>. The overall estimated ORR is only 19.8% based on the current study, which needs to be verified with multicenter randomized controlled studies

**Table 1. Characteristics of patients in included studies**

No	Author	Year	Clinical trial phase	Area	Race	Target	Agent	Male, n (%)	Dosage	Duration of dosing	Patient number	Age in year (range)	Viral status	ECOG	Child-Pugh score	Child-Pugh stage	Alpha-fetoprotein ng/mL	BCLC stage	Prior antitumor therapy
1	Sangro <i>et al.</i> <sup>[15]</sup>	2013	Phase 2	Multi-center	NG	CTLA-4	Tremelimumab	15 (71.4%)	15 mg/kg	360 days	21	65.2 (48-79)	HCV (100%)	0 (71.4%) 1 (28.6%)	6.5 (100%) B (43%)	A (57%) B (29%)	AFP U 400 ng/mL (29%)	A (14%) B (29%) C (57%)	Any (57%) Surgical resection (5%) RFA (19%) TACE (33%) Radioembolization (29%)
2	Duffy <i>et al.</i> <sup>[16]</sup>	2017	Phase 1	Multi-center	NG	CTLA-4	Tremelimumab	28 (87.5%)	3.5 mg/kg; 10 mg/kg	6 months	32	61 (36-76)	HBV (16%) HCV (59%)	0 (25%) 1 (75%)	5 (44%) 6 (16%) 7 (9%)	NG	NG	B (25%) C (75%)	Sorafenib (24%) Sorafenib (22%) Other systemic therapies (32%) TACE (39%) Resection (28%) Ablation (28%)
3	Zhu <i>et al.</i> <sup>[17]</sup>	2018	Phase2	Multi-center	White (81%) Asian (13%) Black (3%) Other (2%) Unknown (1%)	PD-1	Pembrolizumab	86 (3%)	200 mg intravenous injection	2 years	104	68 (62-73)	HBV (21%) HCV (25%)	0 (61%) 1 (39%)	NG	A (94%) B (6%)	AFP >200 ng/mL (41%)	B (24%) C (76%)	NG
4	El-Khoueiry <i>et al.</i> <sup>[18]</sup>	2017	Phase1/2	Multi-center	white (49%) Asian (47%) Black (3%) Other 2 (1%)	PD-1	Nivolumab	171 (80%)	3 mg/kg	Depend on disease progression	214	64 (56-70)	HBV (83%) HCV (23%) uninfected (53%)	NG 6 (29%) 7-9 (2%)	5 (70%) 6 (29%)	NG	AFP U 400 ng/mL (37%)	NG	Surgical resection (60%) Radiotherapy (19%) TACE (55%) Systemic therapy (74%) Sorafenib (68%)
5	Feng <i>et al.</i> <sup>[19]</sup>	2017	NG	China	Asian (100%)	PD-1	Nivolumab	8 (72.7%)	4 mg/kg	6 cycles	11	54.8 (42-70)	HBV (100%)	0 (81.8%) 1 (18.2%)	NG	NG	AFP U 400 ng/mL (46%)	B (36%) C (64%)	Surgical resection (27.3%) Radiotherapy (9.1%) TACE (100%) sorafenib (54.5%)
6	Wainberg <i>et al.</i> <sup>[20]</sup>	2017	Phase1/2	Multi-center	White (55.9%) Asian (26.5%) Black or African (8.8%) American (8.8%) Native Hawaiian or other Pacific Islander (5.9%) Other (2.9%)	PD-L1	Durvalumab	32 (80.0%)	10 mg/kg Q2W	12 months	40	61.5 (20-77)	HBV (25%) HCV (20%) uninfected (55%)	0 (42.5%) 1 (57.5%)	NG	NG	NG	NG	Any (95%) Biologic (38%) Chemotherapy (83%) Radiation (30%) Surgery (45%) Other (40%) Prior treatment with sorafenib (93%)
7	Kelley <i>et al.</i> <sup>[21]</sup>	2017	Phase 1	Multi-center	NG	PD-1 and CTLA4	Durvalumab/ tremelimumab	NG	NG	8 months	40	NG	HBV (28%) HCV (23%) uninfected (50%)	NG	NG	A (93%)	NG	NG	Systemic therapy (70%)

AFP: alpha fetoprotein; BCLC: barcelona clinic liver cancer; CTLA-4: cytotoxic T-lymphocyte associated antigen 4; ECOG: the eastern cooperative oncology group; PD1: programmed death 1; PD-L1: programmed death ligand 1; HCV: hepatitis C virus; HBV: hepatitis B virus; NG: not given; RFA: radiofrequency ablation; TACE: transcatheter arterial embolization

**Table 2. The rate of major adverse events and their 95% confidence interval**

Adverse Events	Event rate on all grade (95%CI)	Ref.	Event rate on grade > 3 (95%CI)	Ref.
Fatigue	20.8% (10.9%-36.3%)	[15,17-21]	1.9% (0.9%-3.9%)	[15,17-21]
Pruritus	17.3% (13.5%-21.8%)	[16-21]	1.3% (0.4%-3.6%)	[16-21]
Rash	18.5% (8.9%-34.4%)	[15-20]	1.6% (0.5%-5.1%)	[15-20]
Diarrhoea	12.5% (7.9%-19.1%)	[15,17-20]	1.5% (0.3%-6.6%)	[15,17-20]
Nausea	7.3% (5.0%-10.6%)	[15,17-19]	1.8% (0.4%-8.3%)	[15,17-19]
Asthenia	6.2% (4.1%-9.1%)	[17-20]	0.9% (0.2%-3.4%)	[17-20]
Pulmonary toxicity*	3.3% (1.5%-6.8%)	[15-17,19-21]	1.9% (0.7%-5.1%)	[15-17,19-21]
Increased AST	22.7% (13.8%-35.2%)	[16-21]	9.9% (4.4%-21.0%)	[16-21]
Increased ALT	13.9% (8.8%-21.3%)	[16-20]	5.8% (3.7%-8.9%)	[16-20]

and clinical trials with other endpoints (such as overall survival, OS).

ICIs targeting CTLA-4 and PD-1/PDL-1 have dramatically changed the outcomes of patients with advanced-stage malignancies. However, ICIs may cause unique side effects, known as immune-related adverse events (irAEs). These side effects are mostly transient and mild, but can occasionally be fatal. Our analysis indicated that the most common AEs associated with ICIs treatment in HCC patients was increased AST (22.73%, 95%CI: 13.8%-35.2%), which was also the most common AEs of grade greater than 3 (9.94%, 95%CI: 4.4%-21.0%). This result is inconsistent with previous studies on other cancers. Respectively, the most common AEs and severe AEs (grade 3-4) were fatigue in NSCLC<sup>[26,27]</sup>, fatigue and lipase elevation or fatigue and rash in melanoma<sup>[28,29]</sup>, low appetite and asthenia in urothelial carcinoma<sup>[30]</sup>, rash and lipase elevation in Hodgkin's lymphoma<sup>[31]</sup>, and neutropenia in lymphoma<sup>[32]</sup>. Patients with HCC treated with ICIs presented adverse event of fatigue in the second common place followed by rash and fatigue. Rash and fatigue are high incidence skin AEs. Skin AEs are the most original irAE, taking place every 3.6 weeks after treatment<sup>[33]</sup>. The pooled estimated incident rate of diarrhea is 12.46% (95%CI: 7.9%-19.1%), which was the most common reported gastrointestinal toxicity. Other gastrointestinal toxicities such as abdominal pain, constipation, vomiting, were rarely reported and not taken into consideration here. Result from recent research showed that the incidence rate of diarrhea was higher with CTLA-4 blocked than the PD-1/PD-L1 blocked<sup>[34]</sup>. Adverse events of instance nausea, asthenia and pulmonary toxicity were less commonly reported in this study.

There are still some limitations in this study. Firstly, the final 7 studies included were all non-randomized controlled clinical trials; it might produce bias and downgrade the level of evidence. Secondly, some factors such as the origin of the HCC and patients' race might produce bias on outcomes, which can not be controlled in this meta analysis. However, the ORR is a straightforward index in evaluating the effectiveness of immunotherapy, and result from this meta analysis can be referred in clinical application.

## DECLARATIONS

### Authors' contributions

Conception and design: Zhang HW, Tang WN

Date analysis and interpretation: Tang WN, Ma LT, Deng Y, Wang W

Manuscript preparation: Tang WN, Wang W

Critical revision and finalizing of the manuscript: Zhang HW

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.



**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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Review

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# Contrast-enhanced ultrasound with sulphur-hexafluoride in diagnosis of early HCC in cirrhosis

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## Abstract

Contrast-enhanced ultrasound (CEUS) with pure blood stream contrast agents allow the study of blood supply of focal liver lesions and especially of hepatocellular carcinoma (HCC) in cirrhosis. Its sensitivity and specificity in diagnosis of small tumors is very high. This review summarizes the recent results on CEUS with SonoVue, which is one of the second generation contrast agents, in the diagnosis of early HCC in cirrhosis emphasizing its increasing role in routine clinical practice.

**Keywords:** Hepatocellular carcinoma, contrast-enhanced ultrasound, cirrhosis, early hepatocellular carcinoma

## INTRODUCTION

Today the use of dynamic imaging modalities allows the study of liver vasculature and in particular the study of the blood supply of the focal liver lesions. Focal liver lesions can be non-invasively differentiated and characterized in benign or malignant on the basis of their vascular support, so to avoid the need of biopsy. This revolution was provided non-invasively by the dynamic study of liver vasculature using contrast-enhanced CT (CECT), contrast-enhanced MRI (CEMRI) and, lastly, Contrast-enhanced ultrasound (CEUS). These new dynamic imaging techniques have had a strong impact especially in the field of diagnosis of hepatocellular carcinoma (HCC) in cirrhosis, and have achieved considerable importance in the management



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of this kind of tumor. HCC is the most frequent primary epithelial malignant tumor of the liver<sup>[1]</sup>. The main feature of this tumor is that it arises mainly in patients with cirrhosis through the transformation of normal regenerating nodules in dysplastic nodules and finally overt HCC<sup>[1-5]</sup>. For non cirrhosis HBV patients, it is via HBV-DNA integration into the host genome, which occurs at early steps of clonal tumor expansion and induces both genomic instability and direct insertional mutagenesis. Therefore, patients with cirrhosis represent a high risk population for developing HCC and should undergo a 6 months surveillance with ultrasound (US) to allow the detection of the tumor at an early stage<sup>[6-13]</sup>. In clinical practice, CECT and CEMRI are not recommended in the surveillance programs. Viceversa, when US examination shows a new nodule, CECT and/or CEMRI are recommended for the staging of the disease.

## VASCULAR CHANGES IN HEPATOCARCINOGENESIS AND CEUS

It is well known that in the case of HCC arising on cirrhosis the normal vascular support is reversed: while in normal subjects the vascular support of the liver is provided by the portal venous system up to 75%, in HCC the blood supply of the nodule is only arterial.

The process of hepatocarcinogenesis in cirrhosis includes the progression from Low Grade Dysplastic nodule to High Grade Dysplastic nodule (HGDN) and overt HCC. During this process, unpaired arteries progressively substitute tumoral portal tracts so that overt HCC blood supply is only arterial. This pathological phenomenon explains the arterial hyperenhancement of typical HCC nodules on dynamic imaging modalities such as CECT, CEMRI and CEUS<sup>[14,15]</sup>.

In recent years, CEUS has gained significant popularity in the characterization of focal liver lesions. CEUS has shown to have a great capability in distinguishing between benign or malignant hepatic nodules on the basis of characteristic patterns of blood supply of the lesions<sup>[16]</sup>. The 2nd generation contrast agent SonoVue is a pure blood stream agent formed by micro bubbles with inert gas sulphur- hexafluoride and a palmitic acid shell. After Intra venous injection of 2.4 mL of SonoVue, in real time and second by second, the arterial phase appearance of contrast agent distribution within the nodule's vessels (duration 10-30 s after contrast injection) can be studied and recorded, followed by the portal phase (30-60 s after injection) and the late or sinusoidal phase (60-240 s)<sup>[16,17]</sup>. The typical CEUS pattern of HCC in cirrhosis is reported in [Figures 1-5](#)<sup>[18,19]</sup>.

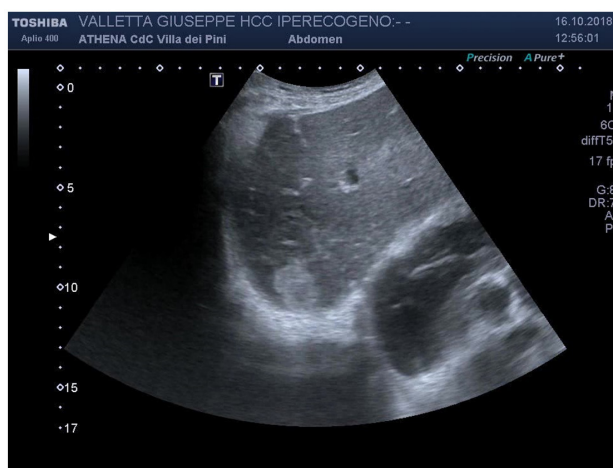
## HCC DIAGNOSIS IN CIRRHOSIS AND ROLE OF CEUS

### US surveillance of HCC in cirrhosis and CEUS

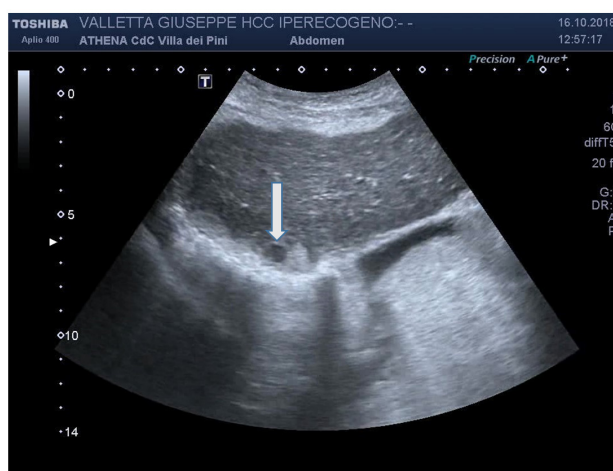
It is well known that conventional US, although a unique tool for surveillance, has a great sensitivity but a very low specificity in the characterization of HCC in cirrhosis<sup>[16]</sup>. CEUS has shown to significantly improve US accuracy<sup>[16,20]</sup>. CEUS using SonoVue easily shows the characteristics of liver nodules blood supply and therefore allows the characterization of malignant nodules<sup>[17]</sup>.

CEUS has determined a real revolution by eliminating the low specificity of conventional US in diagnosing and managing HCC after recognition of a new nodule in a cirrhotic liver: this is due to the immediate and real time visualization of its vascular supply<sup>[16]</sup>. Other advantages of CEUS are the absence of ionizing radiation, the low cost, repeatability, safety and, more important, the possibility to be performed in patients with renal insufficiency<sup>[16,21]</sup>. Moreover, it has been reported that CEUS adds significant diagnostic information in the characterization of atypical or indistinctive lesions on conventional US<sup>[21,22]</sup>.

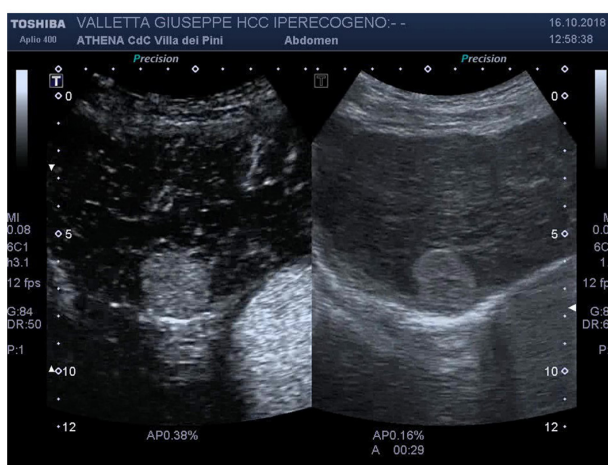
The main limitation of CEUS using pure blood stream contrast agents is based on the fact that only one lesion at a time can be studied and characterized, due to the very short duration of the arterial phase (see later). Consequently, CECT and/or CEMRI are the only dynamic imaging modalities to be used for the staging of the tumor. For the same reasons, CEUS with pure blood stream agents such as sulphur hexafluoride cannot be used for surveillance, unlike Sonazoid which is a new US contrast agent using perflubutane (see later).



**Figure 1.** A 75-year old man with HCV related cirrhosis, successfully treated one year before with DAAs and presenting with a small 2 cm angioma-like hyperchoic nodule in the 7th segment of the liver, not present during the surveillance in the previous ultrasound (US) exam 6 months before

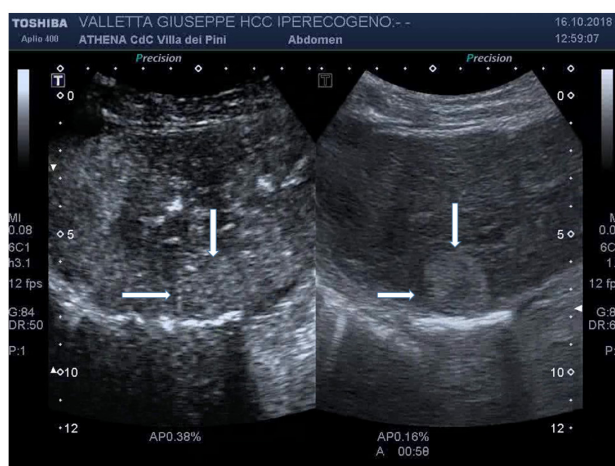


**Figure 2.** A very small hypoechoic round portion (white arrow) is seen within the hyperechoic nodule ( nodule in nodule)

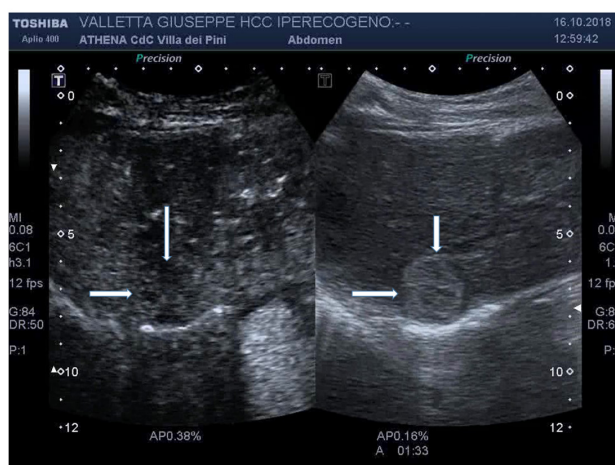


**Figure 3.** CEUS appearance: in the arterial phase, the nodule becomes homogeneously hyperechoic (hyperehanced) (left of the figure)





**Figure 4.** At the end of the portal phase (59 s) the nodule (white arrows) appears iso-enhanced



**Figure 5.** Only at 1.33 min in the late phase, the nodule (white arrows) becomes slightly hypoechoic (hypo-enhanced)

### CEUS patterns of small HCC in cirrhosis and international guidelines

Nowadays, all practice guidelines on the management of HCC in cirrhosis have endorsed CEUS as a dynamic imaging modality capable of diagnosing HCC in cirrhosis per lesion<sup>[8-13]</sup>. The Italian Society for the Study of the Liver, in cooperation with the Italian Societies of Oncology, Radiology, Surgery, Hepatobiliary Surgery and Organs Transplant, published a position paper stating that CEUS can diagnose non-invasively as HCC a hepatic nodule when the characteristics of arterial hyperenhancement and wash out are present, like CECT and CEMRI<sup>[12]</sup>. The guidelines of CEUS hepatic applications of EFSUMB and WFUMB date back to 2012<sup>[13]</sup>.

The recent EASL guidelines for the management of HCC recognized that “CEUS can be effectively utilized to characterise lesions in cirrhosis” although CT and MRI are panoramic technique useful for the staging of the tumor, since the rapid CEUS arterial enhancement does not allow the detection of eventual multiple nodules scattered in the liver<sup>[10]</sup>. In fact, CEUS “can be utilised to characterise one or few nodules detected on conventional US surveillance”. Nonetheless, EASL does not recommend CEUS as first line tool, but only in cases where CT/MRI are contraindicated or inconclusive<sup>[10]</sup>.

2017 Chinese guidelines stated that in HBV/HCV chronic hepatitis or cirrhosis patients diagnosis of HCC > 2 cm can be made with only one dynamic imaging tool (CECT, CEMRI or CEUS) when the typical findings are present. If the nodule has a diameter < 2 cm, two dynamic imaging techniques are needed<sup>[23]</sup>.

The new 2017 Japanese guidelines on management of HCC stated that CEUS sensitivity is similar to dynamic CT or dynamic MRI in diagnosis of HCC and therefore CEUS is able to characterize nodules detected on sonography. The US contrast agent indicated in these guidelines is Sonazoid, which is a US contrast agent phagocytized by Kupffer cells<sup>[24]</sup>. In the past, controversies arose over the possibility of misdiagnosis between small (< 3 cm) Intrahepatic Cholangiocarcinoma (ICC) arising in cirrhosis and HCC. In such cases, CEUS was considered unable to distinguish between these two entities in cirrhotic livers because, in the experience of Spanish authors, CEUS washout patterns of ICC can mimic those of HCC<sup>[25]</sup>. As of today, it is well established that the differential diagnosis between small < 3 cm ICC in cirrhosis and small HCC is no longer a problem. It is well known that, at CEUS, small ICC can present an intense (as HCC) arterial phase hyperenhancement, but a more rapid and marked washout in the portal phase (always < 42 s), differently from the mild and very late wash-out (> 60 s) of HCC in the sinusoidal phase, avoiding any pitfalls<sup>[26,27]</sup>. Therefore, the old diatribe that for several years has labelled CEUS not able to distinguish between small HCC and ICC nodules arisen in a cirrhotic liver is now to be considered surpassed<sup>[25-31]</sup>. Nevertheless, we should consider that small HCC nodules (< 2 cm) can present with hyperenhancement in the arterial phase followed by isovascularity in the portal and sinusoidal phases in more than 50% of cases (as is shown in [Figure 4](#) and Giorgio's 2011 results as reported below.

### CEUS LI-RADS and HCC

A so-called CEUS LI-RADS was proposed by the American College of Radiology based on the Liver Imaging Reporting and Data System (LI-RADS) using CECT and CEMRI patterns for HCC in cirrhotic livers. LI-RADS was originally developed for CECT and CEMRI, but expanded to include CEUS. Based on CEUS features, focal liver lesions ("observations" in radiologic terminology) detected in a cirrhotic liver can be classified in 5 major classes ranging from "definitely benign" (LR-1) to "definitely HCC" (LR-5)<sup>[32]</sup>. Sonovue is included in the CEUS LI-RADS version 2017<sup>[32]</sup>. The 5 major categories (LR-1-LR-5) are classified according to the diameter of the lesions and their contrast enhancement patterns.

The CEUS pattern characterized by the presence of rapid, intense and homogeneous hyperenhancement in the arterial phase (APHE) followed by mild and late (> 60 s) wash-out is termed as CEUS LI-R 5. When a hepatic nodule discovered in a cirrhotic liver presents with the CEUS LR-5 pattern, the nodule can be managed as HCC and there is no need for biopsy. This classification is applied to nodules > 10 mm<sup>[32-35]</sup>.

Very recently, Terzi *et al.*<sup>[36]</sup> reported very interesting data that strongly influenced the last 2018 EASL guidelines in diagnosis of HCC. In a multicentre retrospective study, these authors evaluated CEUS patterns of 1,006 nodules in 848 patients with chronic liver disease at risk for HCC. Median size of nodules was small: 2 cm. Five hundred twenty one (52%) out of all nodules showed APHE and a mild, late wash-out. The 17% of nodules showed APHE and isoechogenicity in the portal and late phase, while 16% of nodules were iso-enhancing in the arterial and portal-late phases. The most important data was that 512 (98.5%) of all nodules classified as CEUS LR-5 were HCC. When authors included in their analysis 3 other CEUS LR-5 cases that were judged underdiagnosed and that resulted HGDN at biopsy, the rate of HCC diagnosis became 99%. In their study, Terzi *et al.*<sup>[36]</sup> did not report any case of misdiagnosis with ICC.

Moreover, studies on inter-observer agreement suggest that the classification of small hepatic nodules (< 2 cm) with LI-RADS-CEUS is reproducible with good consistency in patients with chronic liver disease<sup>[37-39]</sup>.

### CEUS arterial hyper enhancement and early HCC

Some authors studied the interobserver agreement for CEUS-based standardized algorithms in diagnosis of HCC in high-risk patients. The interobserver agreement was good for arterial phase hyper enhancement, which is the key diagnostic feature for HCC nodules in a cirrhotic liver<sup>[39]</sup>. For what has been said so far, although it is evident that HCC diagnosis on CEUS relies also on the washout findings (type and time),

arterial hyper-enhancement remains the main element for the visualization of the HCC nodule when pure blood stream contrast agents are used.

This feature was also studied by Giorgio *et al.*<sup>[40]</sup> who reported a considerable effectiveness of CEUS in detection of arterial hyperenhancement in small nodules (7-20 mm) discovered in cirrhotic patients during surveillance, so to shorten the diagnostic work-up for the management of HCC.

In Giorgio's experience, CEUS showed arterial hyperenhancement in 95.5% of HCC nodules, with a sensitivity of 94.48%, a specificity of 100% and 100% PPV. In this study, CEMRI showed 97% sensitivity, 80% specificity and 97% PPV. The authors concluded that CEUS has a great capability in detection of arterial hypervascularity in < 2 cm HCC. In Giorgio *et al.*<sup>[40]</sup>'s experience, only 4.5% of new nodules escaped the demonstration of arterial hypervascularity. Therefore authors concluded that "CEUS must be performed immediately after conventional US to contrast the malignant fate of small lesions arising in a cirrhotic liver". Moreover, "CEUS should be included in the diagnostic management of HCC in order to avoid a late diagnosis, enable an early treatment and improve survival".

It was shown that CEUS vascular patterns of HCC lesions are related to size and histologic differentiation of the tumor. Ling *et al.*<sup>[41]</sup> reported that < 3 cm HCC nodules show more homogeneous hyperenhancement compared to > 3 cm lesions<sup>[41]</sup>. Moreover, heterogeneous arterial enhancement of HCC nodules > 3 cm were followed by faster washout compared to < 3 cm nodules. The portal and late phase washout was faster in poorly differentiated HCC compared to well-differentiated lesions.

Italian authors also reported that CEUS has high capability in the differential diagnosis of dysplastic nodules (DN), early hepatocellular carcinoma and progressed HCC<sup>[42]</sup>. According to this study, DN, early HCC and progressed HCC have different and characteristic CEUS patterns. Progressed HCC is characterized by rapid, intense and homogeneous arterial hyperenhancement, while early HCC displays the so called "reticular pattern". This pattern is characterized by inhomogeneous enhancement during arterial phase and complete enhancement in the late phase. In the experience of the authors, the "reticular pattern" identified early HCC nodules with a sensitivity of 85.7% and a specificity of 96.1%<sup>[42]</sup>.

### **Comparison among CEUS, enhanced CT and enhanced MRI in diagnosis of small (2-3 cm) HCC nodules in cirrhosis**

Many authors studied the diagnostic capability of CECT, CEMRI and CEUS alone or in combination for the diagnosis of small HCC on cirrhosis. Aubé *et al.*<sup>[43]</sup> carried out a large multicentre study in a large number of cirrhotic patients (544 nodules in 381 patients). Authors aimed at evaluating the accuracy of CECT, CEMRI and CEUS alone and in combination, in diagnosing small (10-30 mm) HCC nodules. The best combination for the 10-20 mm nodules was CEMRI -CECT. They found that, when a first imaging tool was inconclusive and CEUS was used as second dynamic technique, this combination allowed the highest specificity with only a slight drop of sensitivity for 10-20 mm nodules and the highest sensitivity and specificity for 20-30 mm nodules. The authors concluded that in diagnosis of small HCC nodules the best combination is CEMRI followed by CEUS<sup>[43]</sup>.

Moudgil *et al.*<sup>[44]</sup> compared the role of CEUS and CECT in diagnosis of HCC. In their experience, CEUS and CECT were similar in demonstrating the arterial hypervascularity of HCC nodules. Vice versa, they found a better capability of CEUS in the demonstration of washout pattern and the presence of the capsule of the nodules, when present.

Finally, Intraoperative Contrast-Enhanced Ultrasound (CEUS/IOCEUS) is routinely performed during surgical resection of HCC in cirrhosis. It has been shown that such technique allows the detection of

additional liver lesions. This advantage was demonstrated when IOCEUS was compared to preoperative MRI, as well as to preoperative CEUS. According to results of Huf *et al.*<sup>[45]</sup>, in 27% of their cases IOCEUS allowed the detection of further liver lesions not detected preoperatively. Such detection of further lesions modified the treatment planning and resection was extended if necessary.

## CONCLUSION

Today, CEUS plays an essential role in the clinical recognition of small nodules arising *de novo* or recurrent in cirrhotic livers at risk for HCC. The advantages of CEUS over CT/MRI are unique and are represented by: the high sensitivity in depiction of arterial hypervascularity of HCC; the better demonstration of rapid washout for non-HCC malignant nodules; the very late washout of HCC.

In 2016, SonoVue was approved for the first time in the United States for the diagnostic imaging of liver tumors in adults and children. It is undisputable that this approval represents a milestone for CEUS<sup>[46]</sup>. In clinical practice, CEUS demands are constantly increasing in Europe, Asia and Canada (and we are hoping also in USA after the FDA approval) in the Hepatology Units and not only (see Gastroenterology Units, Infectious disease Units, Internal Medicine Units and Surgical Units).

It is undoubtable that the most important benefit of CEUS is based on the fact that physicians can perform CEUS soon after the detection on conventional US of a new nodule during surveillance of cirrhotic patients. Thanks to this technique, physicians can immediately exclude typical benignancy, non-HCC malignant nodules such as ICC and, mainly, in case of CEUS recognition of early HCC, physicians can define a rapid therapeutic work-up choosing among liver transplantation, resection or ablation.

## DECLARATIONS

### Authors' contributions

Design of the work, data analysis and interpretation: Giorgio A

Data acquisition, material support: Giorgio A, Gatti P, Matteucci P, Giorgio V

Wrote the manuscript: Giorgio A, Giorgio V

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

None.

### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Copyright

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Case report

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# Three primary malignancies in 17 years in a man with chronic hepatitis B

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## Abstract

The occurrence of three primary malignancies in a single patient is an infrequent phenomenon with an estimated occurrence at 0.1%. Notably, patients with hepatocellular carcinoma (HCC) are particularly unlikely to develop extrahepatic primary malignancies. In this light, we present a case of patient with chronic hepatitis B who developed HCC, as well as two other primary malignancies. This case exhibits an exceedingly rare combination of cancers, underlining the importance of continued cancer surveillance in those with a history of primary malignancy.

**Keywords:** Hepatitis B virus, hepatocellular carcinoma, antiviral therapy, lung cancer, multiple malignancy

## INTRODUCTION

Multiple primary malignancies (MPMs) is a phenomenon that has been described in the literature for nearly a century<sup>[1]</sup>. The frequency of MPMs has increased over time, which has been partly attributed to improved surveillance and treatment options for cancer. Notably, a similar uptick in cases of second primary



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malignancies occurring in those with HCC has also been observed<sup>[2]</sup>. This is particularly enlightening that HCC has typically carried a grim prognosis as the third leading cause of cancer mortality worldwide<sup>[3]</sup>.

The present case report describes a patient with chronic hepatitis B, who sequentially developed bladder cancer, HCC, and lung adenocarcinoma. His complex sequence of diagnostic evaluations and therapeutic interventions are first discussed, providing context for a review of the literature on MPMs involving HCC.

## CASE REPORT

A 40-year-old Asian man initially presented to an outpatient office in 1987 with a chief complaint of chronic fatigue for several months. There was no family history of malignancy or hepatitis B. He was found to be positive for the hepatitis B surface antigen [HBsAg (+)]. He initially had an elevated alanine aminotransferase, but this returned within normal limits on repeat bloodwork. He demonstrated hepatitis B envelope antigen (HBeAg) seroconversion on follow-up labs in 1991. At the time of his diagnosis, no antiviral therapy existed, therefore he was not started on any treatment.

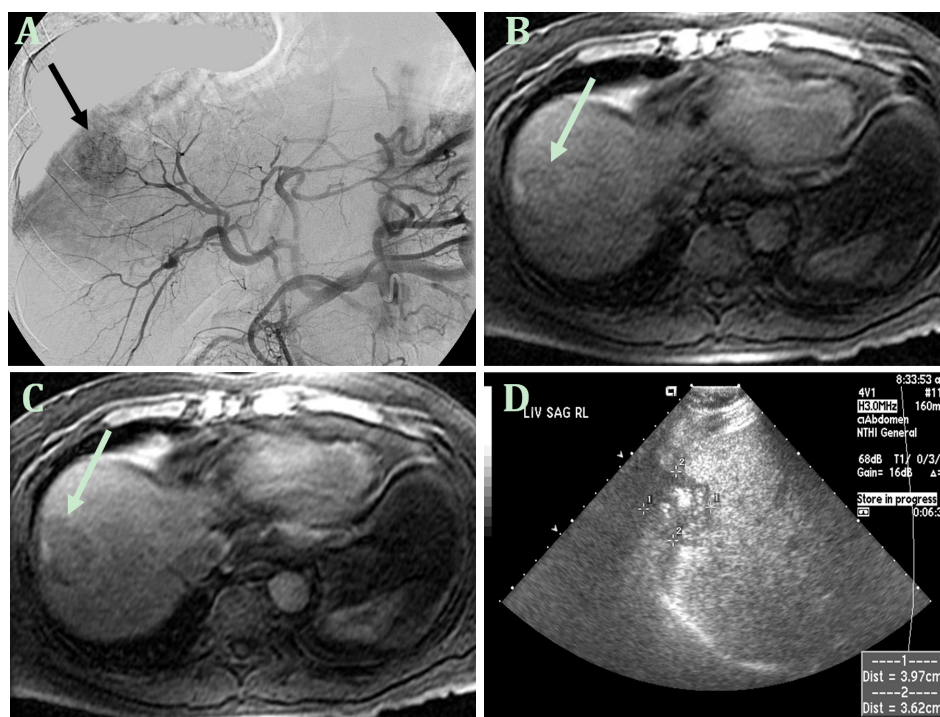
Nine years later, in 2000 at age 53, he developed proteinuria and hematuria and was diagnosed with bladder cancer. He underwent cystectomy with ureterostomy without complication. Three years later, he underwent cardiac angioplasty and stopped smoking cigarettes shortly thereafter. In September 2004 at age 57, abdominal imaging identified a liver mass consistent with HCC (3.4 cm, right lobe, segment 8) with mild medial segment atrophy suggestive of underlying cirrhosis. He was started on lamivudine 150 mg daily and then underwent transarterial chemoembolization (TACE) [Figure 1] followed by radiofrequency tumor ablation. Tenofovir 300 mg daily was then added in May 2010. Hepatitis B virus (HBV) serology at that time demonstrated HBsAg (+), HBeAg (-), anti-HBe (+) with undetectable HBV DNA.

Despite continued antiviral therapy and undetectable HBV DNA, an abdominal MRI ten years after initial HCC [Figure 2] in 2014 revealed a new 1.0 cm LI-RADS 5 lesion in segment 7 consistent with recurrent HCC. At that time, liver function tests were normal, alpha-fetoprotein 2.1 and HBV DNA were still undetectable. He underwent TACE on 4/14/2014 and remained on lamivudine and tenofovir.

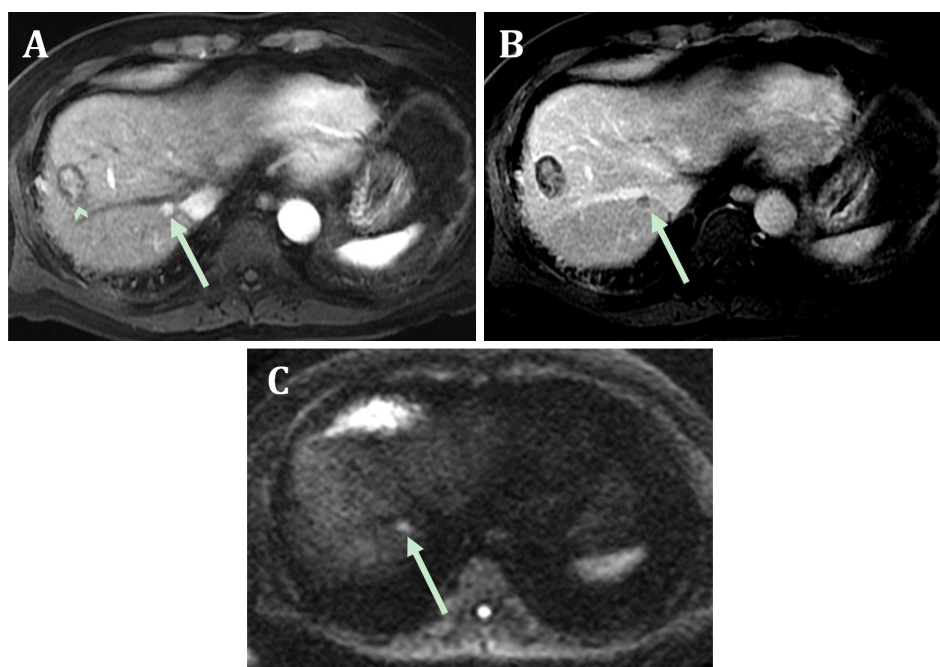
However, three years later, abdominal MR imaging in March 2017 revealed a 0.9 cm liver mass located next to the prior treatment site in segment 7. Follow-up imaging in June 2017 showed that the mass nearly doubled in size to 1.9 cm [Figure 3]. This prompted an evaluation for orthotopic liver transplantation (OLT). During the workup, a chest CT in August 2017 at age 70 showed a lung mass (2.8 cm × 2.4 cm) in the right upper lobe [Figure 4]. A PET scan characterized the lung mass as hypermetabolic and subsequent biopsy showed mucinous adenocarcinoma of the lung. Of note, the patient had a 60 pack years smoking, stopping in 2016.

Given this new diagnosis of lung cancer, the patient was no longer eligible for OLT and therapy for both his recurrent HCC and new lung adenocarcinoma was initiated. For his HCC, he underwent TACE on early October 2017 followed by two sessions of CT-guided microwave tumor ablations. He also received five fractions of stereotactic body radiation therapy (SBRT) for his lung adenocarcinoma during this same time period. Unfortunately, surveillance PET scan in April 2018 [Figure 5] revealed a new left-sided paratracheal lymph node that was biopsy-proven mucinous adenocarcinoma representing a recurrence which was wild type for activating genetic aberrations and had a PD-L1 (by SP 263) tumor proportion score of 25%. Concurrent carboplatin and paclitaxel with radiation to the mediastinum was planned. It was delayed for a month per patient request.

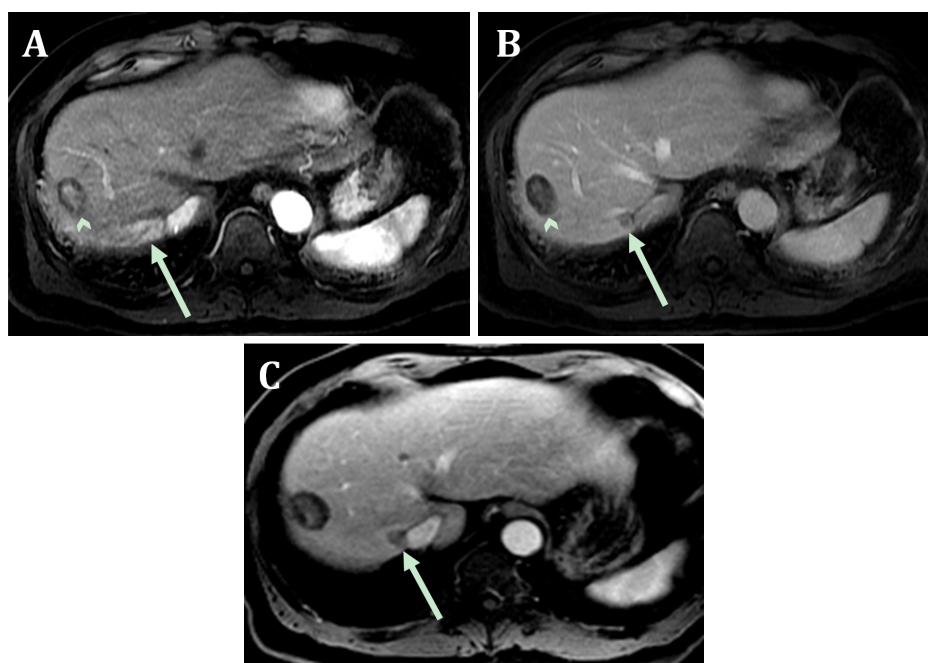
Concurrent chemotherapy radiation was not done as a repeat CT chest in May 2018 to establish a new baseline, which showed a new right-sided lung nodule (1.2 cm) [Figure 6] when biopsied was HCC. This was



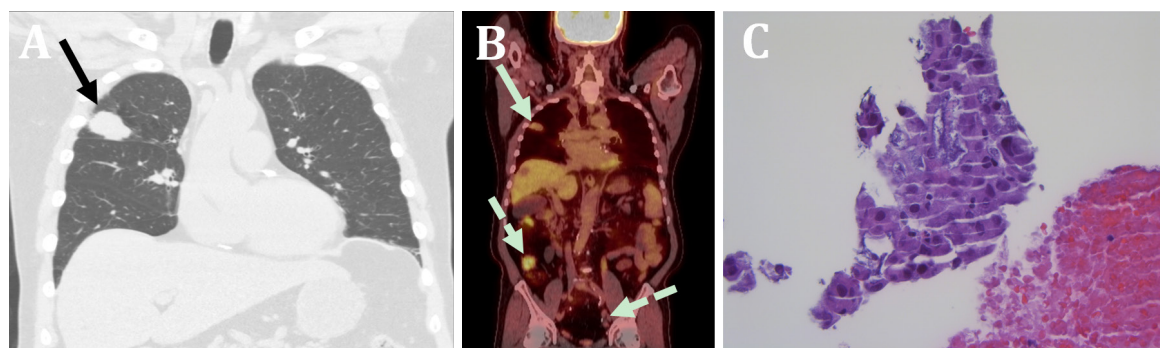
**Figure 1.** First diagnosis and treatment of hepatocellular carcinoma in 2004. The image from the celiac axis arteriogram in 2004 (A) shows a hyperenhancing lesion (arrow) corresponding to the hepatocellular carcinoma in segment 8; The axial T1-weighted fat-suppressed precontrast image (B) following chemoembolization shows the lesion (arrow) which fails to enhance on the subsequent postcontrast image (C); The longitudinal ultrasound image (D) shows the hypoechoic treated lesion (marked by calipers) with intralesional echogenic foci, likely reflecting posttreatment changes



**Figure 2.** Recurrence of hepatocellular carcinoma on MRI in 2014. The arterial phase T1-weighted fat-suppressed postcontrast image (A) shows a small hyperenhancing lesion (arrow) in segment 7 and the previously treated lesion in segment 8 (arrowhead); The corresponding portal phase image (B) shows washout and capsule appearance and the findings conform to a LI-RADS 5 observation; the treated lesion fails to enhance (arrowhead). The diffusion-weighted image (C) reveals hyperintensity (arrow), or diffusion restriction, supporting the diagnosis of malignancy



**Figure 3.** A second recurrence of hepatocellular carcinoma in March 2017. The arterial phase postcontrast T1-weighted, fat-suppressed image (A) shows hyperenhancement in segment 7 (arrow) adjacent to the right hepatic vein (0.9 cm); the previously treated segment 8 lesion is again noted (arrowhead). The portal phase postcontrast T1-weighted, fat-suppressed image (B) shows washout in the segment 7 lesion (arrow); the previously treated lesion (arrowhead) does not enhance. A follow-up post-contrast T1-weighted fat-suppressed three months (C) later shows an increase in lesion size to 1.9 cm (arrow)

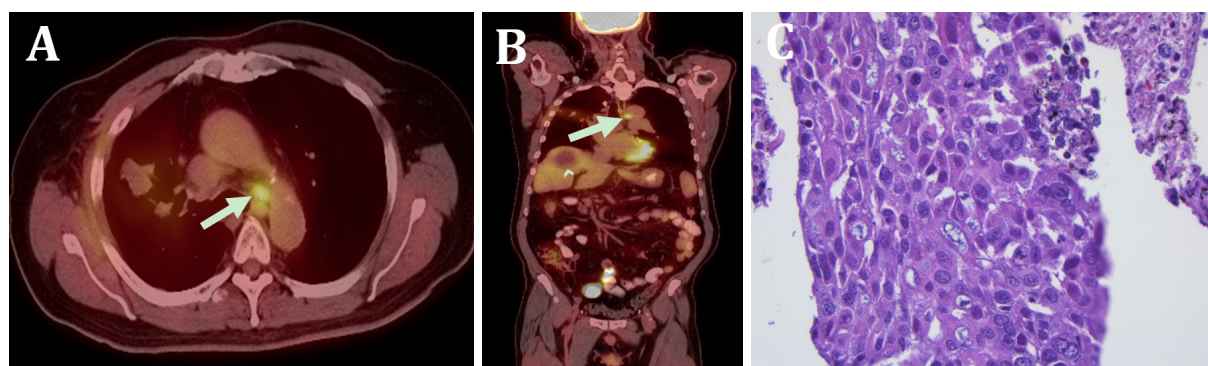


**Figure 4.** Diagnosis of lung adenocarcinoma in August 2017. The coronally formatted CT image (A) shows a well-circumscribed, mass-like lesion in the right upper lobe (arrow) that measures 2.8 cm × 2.4 cm; The image from a subsequent PET/CT (B) shows: hypermetabolism in the right upper lobe lesion (arrow) confirming malignancy; lack of metabolic activity in the previously treated segment 8 liver lesion (arrowhead) and; changes of cystectomy and right lower quadrant ileal conduit with excreted radiotracer (dashed arrows); H&E stain (400x) of tissue from the lung mass obtained by core needle biopsy (C) show lung mucinous adenocarcinoma displaying marked cellular pleomorphism with abundant mucin production

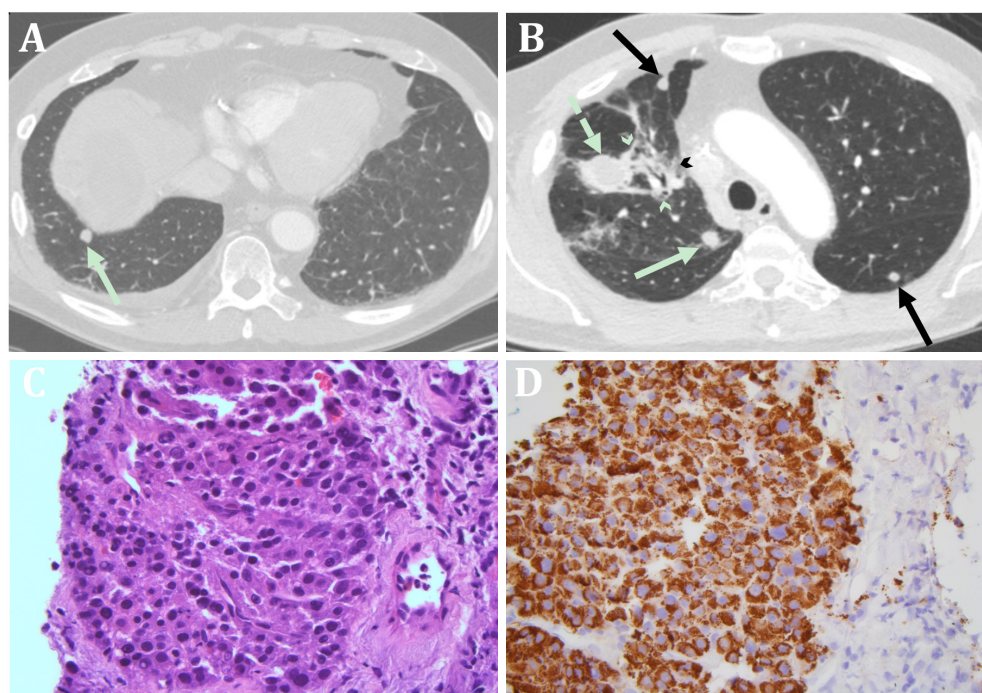
treated with SBRT. A repeat CT chest July 2018 showed new bilateral lung nodules. A biopsy of a left lung nodule showed HCC metastasis to the lung based on positive immunohistochemical staining for hepatocyte specific antigen, Hep Par-1.

He was started on nivolumab which is active in both hepatocellular and non-small cell lung cancer manifested by the lymph nodes as above. However, after one infusion treatment, follow-up imaging [Figure 7] showed a new right hilar mass compressing the right upper bronchus. Biopsy in August 2018 showed mucinous adenocarcinoma consistent with his known lung cancer. His therapy was then switched to carboplatin, pemetrexed, and pembroluzimab of which he received four cycles with stable disease of the lung mass and





**Figure 5.** Metastasis of lung adenocarcinoma to paratracheal lymph node in April 2018. The axial PET/CT image (A) shows a hypermetabolic left paratracheal lymph node (arrow); The corresponding coronal PET/CT image (B) shows the hypermetabolic lymph node (arrow), as well as the treated segment 8 lesion (arrowhead); H&E stain (400x) of the lymph tissue obtained by fine-needle aspiration (C) reveal sheets of pleomorphic cells with mucin production. This is the same morphology of lung adenocarcinoma seen on prior lung biopsy, thereby consistent with a metastasis from the lung primary tumor

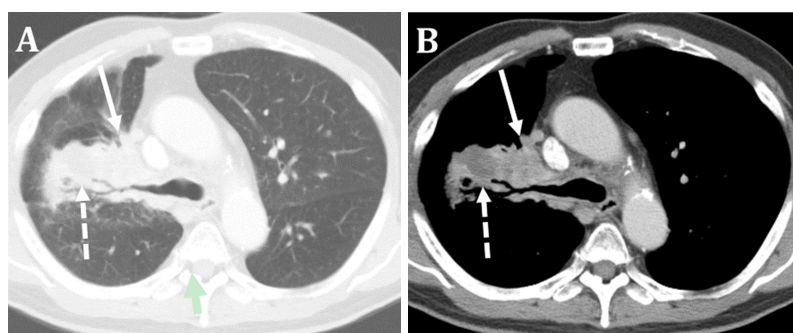


**Figure 6.** Metastasis of hepatocellular carcinoma to the lung in June 2018. The axial postcontrast chest CT image (A) shows the 1.2 cm right lower lobe nodular lesion (arrow). The axial image more superiorly shows additional nodular lesions (arrows), patchy airspace opacity (arrowheads) representing radiation treatment changes targeted to the previously noted left upper lobe mass (dashed arrow); H&E stain (400x) of tissue from the right lower lobe lesion obtained by CT-guided core needle biopsy (C), which reveals metastatic hepatocellular carcinoma displaying a dissimilar morphology to the primary lung adenocarcinoma with relatively small and monotonous nuclei; Mucin production is also absent. Immunohistochemical stain for hepatocyte specific antigen (Hep Par-1) of the same tissue from the right lower lobe (D) shows strong positivity in tumor cells, further supporting hepatocellular origin

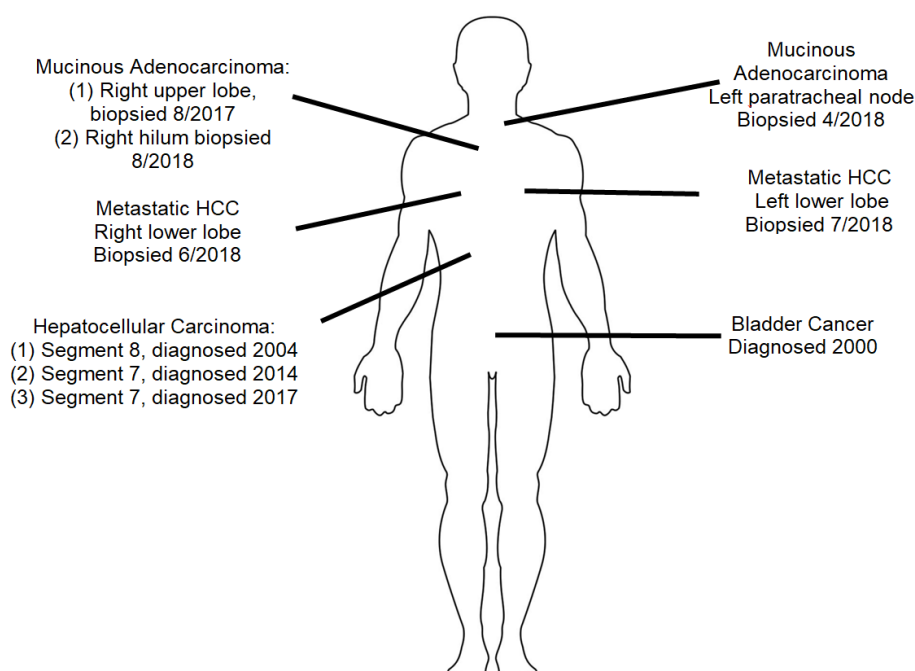
the bilateral lung nodules. The patient unfortunately passed away due to complications from aspiration pneumonia.

Sites and years of cancer development in the bladder, liver, lung and metastatic HCC in the lungs are shown in the diagram below. [Figure 8]





**Figure 7.** Progression of lung adenocarcinoma to parahilar region in August 2018. The axial postcontrast chest CT image (A) reveals the radiation scar (broken arrow) continuous with the new large right parahilar mass (solid arrow) encasing the right mainstem bronchus; The corresponding soft tissue window image (B) shows the heterogeneous enhancement of the radiation scar (broken arrow) and the right parahilar mass (solid arrow). CT-guided biopsy of this lesion (not shown) demonstrated histology compatible with metastasis of the primary lung tumor



**Figure 8.** Site and time of cancer development in the bladder, liver, lung and HCC metastasis to the lung.

## DISCUSSION

In 1932, Warren and Gates first described and defined MPMs as two or more malignant tumors of different histopathological origin in a single patient<sup>[1]</sup>. MPMs specifically in patients with HCC were initially characterized as an infrequent occurrence. This was attributed to the fact that the prognosis of HCC was historically poor. However, due to advances in screening and therapeutic options for HCC, there has been an increase in patients with HCC developing extra-hepatic primary malignancies (EHPMs)<sup>[2-4]</sup>.

This case report describes a patient who developed three primary malignancies over the span of 17 years from bladder cancer in 2000 at age 53, HCC in 2004 at age 57, and lung adenocarcinoma in 2017 at age 70. Importantly, the documented frequency of HCC occurring with two other primary malignancies in a single patient is exceedingly rare<sup>[2]</sup>. A large retrospective review of MPMs found only 57 (0.1%) of the 52,398

patients in their database had three or more primary malignancies in which none of these patients had HCC<sup>[5]</sup>. Other retrospective studies reported similar results<sup>[6,7]</sup>.

The most common sites of EHPMs in patients with HCC tend to correlate with the typical distributions of cancers found in different geographic regions. For example, studies with patient cohorts of Asian descent identified gastric and nasopharyngeal cancers as common EHPMs<sup>[2-4]</sup>, whereas studies from Western nations revealed genitourinary and colorectal cancers as the most common EHPMs<sup>[8]</sup>.

In regards to risk factors for EHPMs, studies have found similar clinical characteristics in patients with HCC who developed EHPMs. A retrospective analysis of 1506 Taiwanese patients found that in comparison to HCC patients without EHPMs, patients with HCC who developed EHPMs were more likely to be older, have earlier stage HCC, and exhibit better liver functional reserve<sup>[3]</sup>. They also found that patients with HCC and EHPMs were less likely to be chronically infected with Hepatitis B virus (HBV) or Hepatitis C virus (HCV)<sup>[3]</sup>. Retrospective studies of American, Japanese, and South Korean cohorts found similar correlations<sup>[4,9,10]</sup>.

To date, studies have been inconclusive in identifying iatrogenic or hereditary factors that may contribute to second primary malignancies in HCC patients. No clear treatment differences between HCC patients with and without EHPMs have been identified<sup>[2]</sup>. Similarly, genetic factors that may contribute to second malignancies in HCC patients have not been determined. Indeed, HCC patients with and without EHPMs have similar frequencies of family members with cancer<sup>[2]</sup>, which suggests that patterns of inheritance may be involved but these have not yet been elucidated.

In conclusion, improvements in surveillance, diagnosis, and treatment of HCC has led to an increased occurrence in the diagnosis of second primary malignancies. The case discussed here presents a rare presentation of HCC with two additional EHPMs, especially given the patient's infection with chronic HBV. Notably, these three malignancies occurred over a period of nearly two decades. This highlights the importance of continued regular cancer screening in patients with HCC, particularly those with early stage HCC with favorable prognoses. It is therefore important for physicians to be aware of EHPMs and to provide appropriate cancer screening for early detection and treatment. With this increased awareness, we hope to see an increase in overall survival in the patients with HCC and EHPMs in the future.

## **DECLARATIONS**

### **Authors' contributions**

Provided care for the patient with HBV and HCC, the conception and the design of the manuscript: Hann HW

Searched literature and wrote the text: Block PD, Shinn BJ

Radiological images and legends: Roth CG

Pathological examination and images: Baliff JP

Provided the lung cancer treatment: Zinner RG

### **Availability of data and materials**

Not applicable.

### **Financial support and sponsorship**

None.

### **Conflicts of interest**

All authors declared that there are no conflicts of interest.

**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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Correction

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## Correction: Three primary malignancies in 17 years in a man with chronic hepatitis B

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The [Case Report](#) was published on 21 Jun 2019.

The author's statement of conflict of interest was confused with the original and published incorrectly due to the negligence of the Editorial Office. Here is the correction:

### Conflict of Interest:

Hie-Won Hann: Clinical Research Grant from Gilead, Assembly Biosciences, Arbutus and serves at the National Advisory Board of Gilead Sciences.

Ralph Zinner: Bristol Myers Squibb, Merck

Peter Block, Brianna Shinn, Christopher Roth, Jeffrey Baliff: No conflict of Interest.

We, Editorial Office of Hepatoma Research apologize for any inconvenience we may cause.



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Case Report

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# Third time re-irradiation of liver metastasis with robotic radiosurgery: a case series

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## Abstract

Re-irradiation (Re-RT) in liver tumours is rarely reported owing to poor tolerance of liver and high incidence of radiation induced liver disease incidence. Fiducial based robotic radiosurgery allows to deliver high dose radiation to the liver tumour and restricts the dose to healthy uninvolved liver, thereby increasing the potential for Re-RT. Tolerance to radiation is low for entire liver and hence re-radiation is a challenge. On the other hand, as regenerative potential of hepatocytes is rapid, replacement of necrotic liver tissue occurs with regenerated hepatocytes. These regenerated hepatocytes are radiation naïve, do not have “memory” of radiation therapy treatment and hence have potential of Re-RT. We are reporting a series of two breast cancer patients presented with liver oligometastasis treated with fiducial based CyberKnife system (CK). Both the patients were treated multiple times with CK and had long-term survival (> 2 years) without any clinical features of radiation induced liver injury. Appropriately selected patients are suitable for multiple sessions of CK for liver lesions with long-term outcome.

**Keywords:** Liver lesions, re-radiation, robotic radiosurgery

## INTRODUCTION

Liver tissue has poor tolerance to radiation therapy (RT), hence RT was rarely considered for liver tumours. Mean liver dose as low as 15 Gy to the whole liver can cause injury to the liver [radiation induced liver disease (RILD)], presenting with anicteric hepatitis, ascites and progressive deterioration of liver



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**Table 1. Case report of breast cancer with liver metastasis at diagnosis and re-treated with radiosurgery**

Date	Event	Treatment	Investigations
Sept 2014	Left Breast carcinoma pT2N2M1 ER/PR +ve; Her2neu +ve	Lumpectomy à Left MRM	PETCT - Hypermetabolic liver metastases in Seg VII (size - 2 cm × 1.6 cm)
Oct 2014	SBRT (1st CK)	SBRT to Segment VII liver lesions - 45 Gy in 3 fractions, ( prescription to 88% isodose )	
Nov 2014	Adjuvant chemotherapy	TCH -Taxol, carbolatin, Herceptin × 6 cycles ; Herceptin continued × 1 yr	
Mar 2015	Adjuvant radiation therapy	EBRT to left chest wall and region nodes -50 Gy/25 fr/ 5 wk	
	Hormonal therapy	Inj Goserelin and Tab Tamoxifen	
April 2016	Restaging	PETCT - No significant metabolically active disease	
Sept 2016	Restaging	PETCT - Interval new hepatic metastases in segment VIII (3 cm × 2.4 cm)	
Sept 2016	SBRT (2nd CK)	SBRT to Seg VIII liver lesion - 45 Gy/3 fr ( prescription at 88% iso-dose)	
Oct 2016	Change hormonal therapy	Letrozole , Herceptin restarted, Inj Goserlin continuing	
March 2018	Restaging	PETCT - Abnormal increased uptake in subtle hypodensity in segment VI of liver (SUVMax 6.5)	
April 2018	SBRT (3rd CK)	SBRT to seg VI liver lesion - 50 Gy/5 fr, prescription at 88% Continuing Herceptin, Inj Goserlin	
Sep 2018	Restaging	PETCT - liver lesion completely resolved. No other abnormality detected	

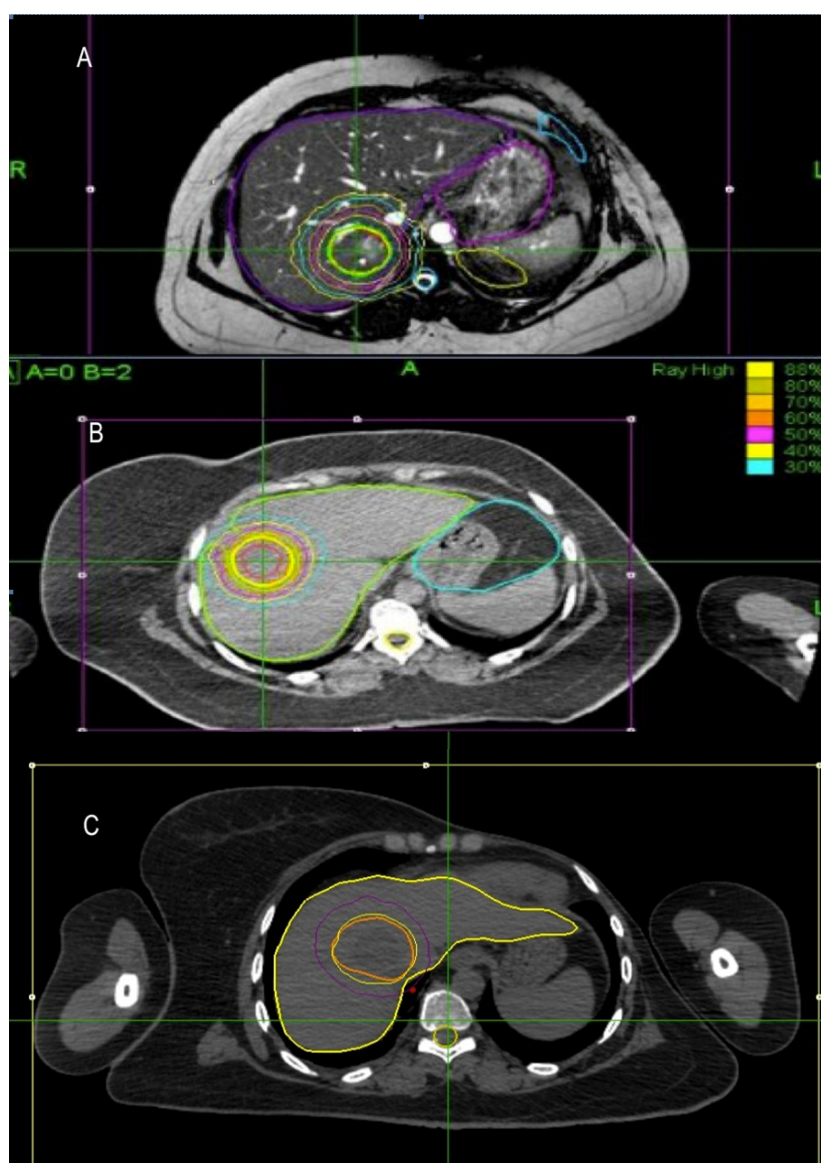
SBRT: stereotactic body radiotherapy; PETCT: positron emission tomography-computed tomography; EBRT: external beam radiation therapy

function<sup>[1,2]</sup>. Only after high precision RT was introduced and clinicians had knowledge regarding partial liver radiation, RT gained momentum in the treatment of liver tumours. Stereotactic radiation therapy with real time liver tracking have helped in delivering safely high dose precise short course RT (radiosurgery) in liver tumours. However, the risk of liver injury is always a concern in treatment of liver tumours. In such a situation it is likely that re-irradiation (Re-RT) in liver tumours will be rarely reported.

Fiducial based robotic radiosurgery (CyberKnife, CK, *Accuray*®; Sunnyvale, CA) gives liberty to deliver high dose radiation to liver tumours and restrict dose to surrounding healthy liver cells, thereby increasing the potential for Re-RT<sup>[3,4]</sup>. Re-RT in liver tumours is a challenge, and needs active evaluation of possible toxicities before initiating the treatment. There are higher risks of liver decompensation and incidence of RILD. Toxicity and response to treatment after stereotactic body radiotherapy (SBRT) depends upon mean liver dose, amount of spared normal liver volume, previous treatment and modality of treatment<sup>[5]</sup>. On the other hand, regenerative potential of hepatocytes is rapid and rapidly proliferating hepatocytes replacement of necrotic liver tissue, those expected to have no “memory” of RT as they are naïve to radiation<sup>[2]</sup>. Hence, there is a potential for Re-RT in liver with rapid regeneration of hepatocytes<sup>[2]</sup>. We are reporting a series of two breast cancer patients presented with liver oligometastasis and are treated with fiducial based CyberKnife system (CK).

## CASE REPORT

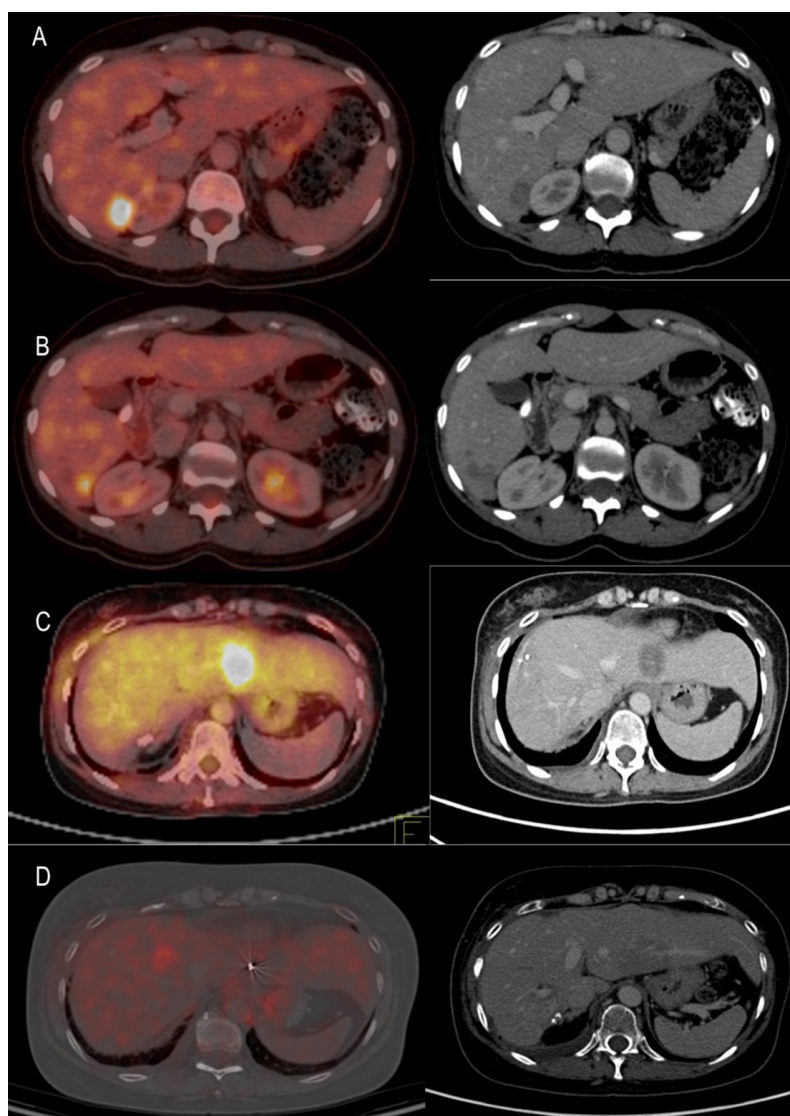
A forty-five year-old female was diagnosed in Sept 2014 with left breast cancer (2 cm × 2 cm, Upper Outer Quadrant, mobile axillary nodes) [Table 1]. Metastatic workup with PET-CT revealed solitary metastasis in segment VII of liver (2 cm × 1.6 cm, SUVmax -7). She had normal liver function (Child Pugh A) and viral markers were negative. She underwent Left Modified radical mastectomy and was subsequently treated with CK (45 Gy in 3 fractions, prescribed to 88%, mean liver dose 681 cGy) in Oct 2014 [Figure 1]. Histopathology - Infiltrating ductal carcinoma (IDC), Gr III, 5/11 nodes positive, ER/PR +ve, Her2neu +ve. She received chemotherapy (Taxane based chemotherapy) followed by adjuvant RT to chest wall (45 Gy/25 fr/5 wk) and then received Trastuzumab for 1 year along with Tamoxifen (HT). Follow up PET scan in 2015 was complete response (CR) and no focal lesion seen in liver. Repeated PET scan (Sept 2016) showed a new solitary liver lesion (3 cm × 2.5 cm, SUVmax -8) in segment VIII. She was re-irradiated with CK (45 Gy/3 fr, 88% isodose, mean liver dose 771 cGy). Follow-up PET scan (Mar 2017) showed no evidence of disease and repeat scan in Nov 2017 showed complete regeneration of both segment VII and VIII region without any



**Figure 1.** CyberKnife treatment plans. A: First CyberKnife plan (45 Gy/3 fr); B: Second CyberKnife plan (45 Gy/3 fr); C: Third CyberKnife plan (50 Gy/5 fr)

evidence of disease. Follow-up scan in March 2018 showed a new lesion in segment II (2.5 cm × 2.3 cm). She was treated the third time with fiducial based SBRT 50 Gy/5 fr [(prescription 88% isodose) three Accuray defined fiducials, distributed by *Alphamed*®]. Response evaluation scan (Sep 2018) showed significant regression of SUV uptake and mass in liver. She had no sign of radiation induced liver injury [Figure 2]. Three fiducials were placed close to the tumour under USG guidance by radiologist. Same fiducials were used for tracking during Re-RT. In situations where new lesion is in another lobe of liver, larger PTV margin (5 mm) was given to the GTV.

A thirty-six year-old female was diagnosed with carcinoma right breast (cT1N1M0, IDC Gr III, ER/PR +ve, Her2neu -ve) in Nov 2010, was treated with breast conservative surgery (BCS). She received adjuvant systemic therapy (FEC × 4 and Docetaxel × 4 cycle), followed by adjuvant loco-regional RT (45 Gy/25 fr/5 wk) [Table 2]. She was on periodic follow-up and had controlled disease until Feb 2014 when routine PET scan showed multiple liver lesions in both lobes, largest measuring 7 cm × 8 cm in Seg VI/



**Figure 2.** Patient 1: PET-CT scan. A: Sep 2014: 2 cm × 1.6 cm mass in seg VII of liver; B: Sep 2016: 3 cm × 2.4 cm mass in seg VIII of liver; C: March 2018: 2 cm × 1.5 cm mass in seg VI of liver; D: June 2018: PET scan showing complete resolution of mass lesions

VII suggestive of metastasis. There was no other focus of distant metastasis in any other organs. Biopsy from liver lesion was IDC Gr III, ER/PR +ve, Her2neu -ve. She received multiple lines of systemic therapy (Abraxane, Gemcitabine/Carboplatin, capecitabine, HT), but had partial response to treatment. PET-CT (Feb 2016) revealed multiple small lesions in liver with significant metabolic activity in two residual lesions in segment VI (2 × 1.5 SUVmax -5) and VII (2 cm × 2 cm, SUVmax -8). In March 2016, she was treated with CK (45 Gy/3 fr to both lesions, 86% isodose). Then she was on immunotherapy with Ipilimumab (PDL1 antagonist). Follow-up PET scan (June 2016) revealed complete metabolic and anatomic resolution of previous liver lesions as well. PET scan in Oct 2016 showed a new solitary lesion in segment II of liver (2.3 cm × 2.5 cm, SUVmax -8.0) and re-treated with CK (45 Gy/3 fr, 87% isodose) [Figure 3]. PET scan (March 2017) showed resolution of liver lesions with signs of regeneration/ hypertrophy of the irradiated liver segments. Patient was evaluated with USG abdomen, liver function test for any signs of RILD and no signs of RILD was found on evaluation. Target (GTV) was contoured on contrast CT scan and MRI scan (T1 contrast and T2 flair) images. Usual PTV margin of 3 mm given. In re-radiation, where the new lesion is away (> 5 cm) from the fiducials, 5mm PTV margin was given for setup uncertainties. Overlapping of

**Table 2. Case report of breast cancer with liver metastasis at follow up evaluation and re-treated with radiosurgery**

Date	Event	Treatment	Investigations
Nov 2010	Right breast carcinoma ER/PR +ve, Her2neu -ve	Right BCS (pT1N1MO) – Lumpectomy + SNLB and axillary dissection. 4 × CEF + 4 × Docetaxel – RT- 50 Gy/25 fr + 10 Gy, then Tamoxifen	
Feb 2014	Metastatic disease detected	USG Abdomen – Multiple lesions in Liver. PET CT – Multiple metastatic deposits in both lobes of liver, largest 7.6 cm × 9 cm –Seg VI, VII. No other focus of distant metastases. Needle biopsy Liver lesion – Metastatic high grade ductal carcinoma (ER/PR +ve, Her2neu negative); CA 15.3: 1291 Started on Abraxane × 4 Cycle	
June 2014	Restaging	Partial response PETCT – Significant reduction in no of lesions, metabolic activity and size. CA 15.3 = 60. Started on LHRHA + Anastrozole	
Oct 2014	Chemotherapy	Abraxane × 3 cycles, then Gem/Carbo × 4 Cycle	Rising CA 15.3 = 39 à 66 PET CT - Disease progression - Increase in size and metabolic activity of liver lesion Segment VII ( 4.5 cm × 4 cm; SUVmax 6.1 ) . New metabolic active liver lesion segment VI (1.4 cm × 1.4 cm; SUVmax 5.4 )
March 2015	Restaging	Complete metabolic response	PETCT - Complete resolution of metabolic activity and reduction in size of lesions in segment VI, VII. On Tamoxifen
Aug 2015	Progression	Inj Goserlin monthly + Exemestane + Everolimus Then on Capecitabine	CA 15.3 = 25 PETCT - Disease progression – New small liver lesions in segment V, VI, VII . No other site of distant metastases.
Feb 2016	Restaging	PETCT – Significant reduction in size of all lesions. 2 lesions appear metabolically active – Segment VI – 1.8 cm × 1.5 cm , SUVmax 4.7 ; Seg VI/VII – 2.2 cm × 2.2 cm , SUVmax 7.6	
March 2016	1st CK Liver	SBRT – 45 Gy in 3 fractions (prescribed to 86% isodose line) delivered to the two FDG avid liver lesions Then on Ipilimumab + Nivolumab × 7 cycles	
July 2016	Restaging	CECT scan – resolution of all lesions , except lesion in Seg VI/VII which has substantially reduced in size.(2 cm × 1.2 cm)	
Nov 2016	3rd CK Liver CK – Brain	MRI Brain –2.3 cm × 2.8 cm × 3.2 cm in left premotor region . CK 27 Gy/3 fr PET-CT - Disease Progression - New FDG avid lesion in left lobe of liver - Segment II (2.3 cm × 2.5 cm × 2 cm, SUVmax 8.0) SBRT to Segment II liver lesion – 45 Gy in 3 fractions, (prescribed to 87% isodose line).	
March 2017	Restaging	PET-CT - Disease Progression – Segment II lesion - CR. Three new FDG avid poorly enhancing ill defined lesions appeared Seg IVa (1.1 cm × 1.3 cm , SUVmax 4.2), Seg III/IV (1.8 cm × 1.3 cm, SUVmax 8.0), Seg VI (1.2 cm × 1.2 cm, SUVmax 5.8) FDG avid small lesion on left side of sacrum near neural foramina.	
April 2017	4th CK	Dose of 25 Gy in 5 fractions to sacral lesion	
June 2018	Expired due to disease progression		

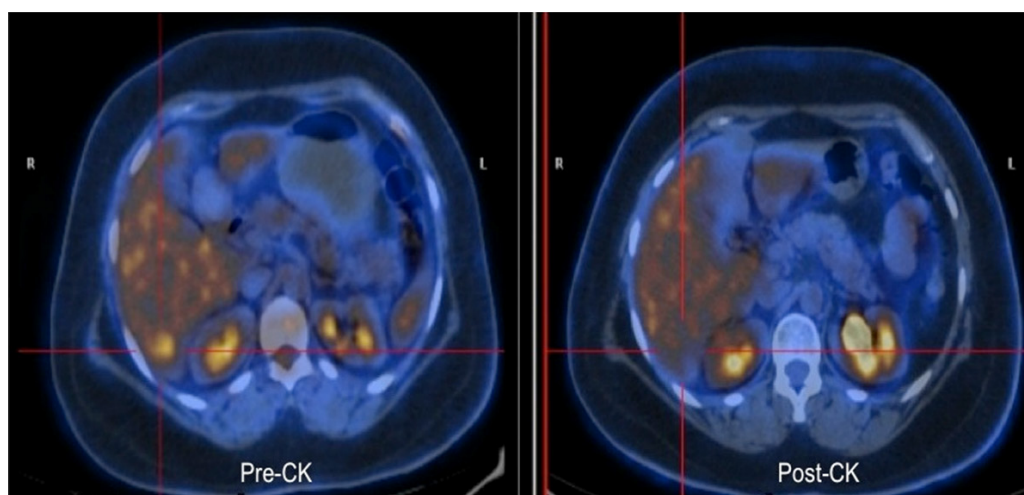
FDG: fluorodeoxyglucose; SNLB: sentinel lymph node biopsy; BCS: breast conservative surgery; SUV: ; CECT: ; CEF:

previous dose distribution was evaluated. No “hot spot” (more than prescribed dose) outside the target volume was seen. Follow-up PET scan (Oct 2017) showed extensive metastasis in other organs. She was on metronomic chemotherapy and expired with progressive disease on June 2018.

## DISCUSSION

Re-radiation in liver tumours are not common in clinical practice. There are only few published literatures in this aspect and no standard consensus regarding dosage schedule<sup>[6-8]</sup>. In most of the subsites, such as in head and neck cancer or cervical cancer, in re-irradiation setting there is usually reduction of total dose (BED)<sup>[9]</sup>. Treatment volume is limited and fractionation schedule modified depending upon “time to re-treat”. Irradiated volume is also important in selection of fractionation schedule<sup>[8]</sup>. Usually, in head and neck cancer “seven” year time is considered “safe” to re-challenge with full dose of RT. In case of re-radiation before that period, there is a reduction of dose depending upon the “time to re-treat”. Usually 15% dose “decay” considered in 1st year after RT and then every year 10% “decay” in dose. As the time gap between primary RT and re-irradiation is increasing, it’s safer to deliver higher (adequate) dose of RT





**Figure 3.** Patient 2: PET scan uptake showing response to treatment. Pre-CK: pre-cyberknife; Post-CK: post-cyberknife

to the target<sup>[9]</sup>. This standard practice is not applied in re-radiation of liver tumours. In fact, in few studies there are better results (OS) in patients treated with higher dose in re-radiation setting. Child Pugh Score and “time to re-treat” are considered significant prognostic factors<sup>[6]</sup>. There is no compromise in irradiated volume as well<sup>[6]</sup>. Tolerance of liver is low, but fortunately in re-radiation setting, liver tolerates radiation comparatively better than other subsites<sup>[2,5]</sup>. High dose RT work like thrombo-embolism, embolizing blood supply to a portion of liver and stimulating proliferating of hepatocytes from adjacent normal liver<sup>[7]</sup>. Proliferating hepatocytes cause hypertrophy of the liver portion which is naïve to RT<sup>[7]</sup>. This proliferating hepatocytes replace the post-CK necrotic liver. Hence, the “new” regenerated portion of liver tolerate better than previously treated liver. Different cytokines liberated from the necrosed liver tissue may also stimulate hypertrophy of liver. It is assumed that the new hepatocytes are naïve to RT and will tolerate radiation better. However, there is no prospective study neither any laboratory model to establish this notion.

After RT, there is fibrosis as well, and this fibrosis may lead to shrinkage of liver volume. Post-CK, there is 50% regression of the involved liver due to radiation injury, on the other hand there is 320% compensatory hypertrophy of the contralateral liver lobe<sup>[2]</sup>. This phenomenon negates the implications of fibrosis, and hypertrophy has more predominant impact. Shrinkage of liver volume is expected to be more with higher integral dose of RT. In few studies, there is transient reduction of liver volume of about 20% at 3 months post-CK. However, at one year follow up there is only 10% shrinkage compared to pre-treatment volume. Even after repeating CK, liver volume is mostly maintained due to compensatory hypertrophy.

Most severe complication after re-radiation is RILD<sup>[7,8]</sup>. It is a syndrome of ascites, elevated transaminase level, and anicteric hepatomegaly. Usually occurs in a proportion of patient after receiving whole liver doses of > 30-35 Gy<sup>[8]</sup>.

However, retrospective series of partial liver radiation have demonstrated that liver tolerance not only depends upon the total dose of RT, but also on pre-treatment Child-Pugh score, viral load and volume of tumour as well<sup>[8]</sup>. Partial liver may be safely treated with radiation if adequate liver volume is preserved<sup>[3]</sup>. In re-radiation, as the hypertrophied liver is mostly radiation naïve, re-radiation is possible with adequate dose in small volume recurrences.

In this present case series, breast cancer patients with liver metastasis were treated with radiosurgery for multiple times in recurrent setting. There was a time gap of more than six months between two treatments.



There was no clinical sign of “RILD” after re-CK. Radiological evaluation, clinical examination and liver function test done to exclude RILD at all post-CK follow up evaluation. In both the cases, liver volumes were maintained after post-CK long-term follow up (> 2 years). There was regeneration of the treated portion of liver. In the present series, patient was treated three times with CK for liver metastasis. There are few small series of liver metastasis patients treated with SBRT for twice in two different lobes. There is limited or no published literature on SBRT for three times in liver metastasis. In present study is novel in terms of thrice CK treatment for liver metastasis and had complete response to treatment. Modern systemic therapy improves the probability of control of distant metastasis as well as survival<sup>[9]</sup>. Hence, the probability of repeating focal treatment with RT has increased significantly with usage of modern systemic therapy. Focused RT with Robotic Radiosurgery (CK) has minimal internal target volume and spares maximum liver volume, hence enables to re-treat with radiosurgery in small volume recurrent or new lesions in liver. Toxicity was assessed by liver function test parameters, ascitis and clinical symptoms<sup>[8]</sup>. There was no gross derangement of secretory or excretory functions (serum bilirubin, alkaline phosphatase, SGOT, SGPT) of the liver. There was no ascitis after treatment or at follow up evaluation. Patients were asymptomatic with liver metastasis and were on routine close follow up. Usually, after liver metastasis survival outcome is poor, mean overall survival is six to eight months after diagnosis. In this case series, both the patients survived more than 2 years and after CK there is acceptable survival outcome in these patient cohort.

In recent years, with advent of modern more potent systemic therapies as second and third line treatment, possibility of re-radiation of liver metastasis has increased<sup>[9,10]</sup>. RT for liver metastasis at diagnosis and at follow up evaluation is more common and needs to be addressed. Long-term survival (> 2 years) is seen in breast cancer patients with liver metastasis and also in known patients with liver metastasis on routine follow up evaluation.

In summary, re-radiation for liver lesions is feasible but uncommon in clinical practice. In the present series, two patients with liver metastasis were treated three times with radiosurgery for metastasis at different segments of liver without any clinical signs of liver decompensation. There were signs of early regeneration in the irradiated regions of the liver in USG scan. High regeneration capacity and hypertrophy of the irradiated region of liver suggest potential for Re-RT. Re-radiation of liver with CK will be an exciting option in the era of highly potent systemic therapies.

## DECLARATIONS

### Authors' contributions

Concept and design: Dutta D, Krishnamoorthy S

Data analysis and interpretation: Dutta D, Krishnamoorthy S, Nair H

Manuscript preparation: Dutta D, Das R, Madhavan R, Holla R

Critical review and finalization of the manuscript: Dutta D

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Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

**Consent for publication**

Not applicable.

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Review

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# Advances in early diagnosis of hepatocellular carcinoma

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## Abstract

Hepatocellular carcinoma (HCC) is the most recurrent hepatic malignancy and the third in the cancer-related casualties in the west. The frequently-documented causes of HCC are chronic liver infections by hepatitis B virus or hepatitis C virus, nonalcoholic fatty liver disease, cirrhosis, exposure to aflatoxins and tobacco smoking, *etc.* Clinical presentation of this fatal disease ranges from asymptomatic to upper abdominal pain or common health conditions like weight loss or lethargy. Among current surveillance strategy for suspected patients, liver imaging and serum alpha fetoprotein estimation has been regularly recommended. However, sensitivity of this diagnostic methodology especially in early detections, often suffers from compromised sensitivity and selectivity. Various image based and serological biomarkers for HCC has been introduced in recent decades with varied sensitivity as stand-alone or combined diagnostic protocol. The current article will review the status of HCC diagnosis with respect to common diagnostic protocol, and upcoming novel biomarkers.

**Keywords:** Alpha fetoprotein, extracellular vehicles, hepatocellular carcinoma, indocyanine green, magnetic resonance imaging, non-coding RNA

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary liver malignancy and the third cause of cancer-related death in the western countries that trended the highest increase in occurrence throughout the last decade<sup>[1]</sup>. Patients with chronic HCV, hepatitis B (HBV), alcoholic liver disease and non-alcoholic fatty liver



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disease (NAFLD) often develop cirrhosis and are at high threat of developing HCC, with a significantly high annual incidence rates nearing 2% per year<sup>[2]</sup>. However, cirrhosis may or may not be present in HCC. Studies have revealed that NAFLD patients would be at a high risk of developing HCC, even in absence of cirrhotic transformation. The development of HCC is a complex multi-step process named as “hepatocarcinogenesis” and characterized by progressive genetic aberrations. Studies of these HCC associated molecular aberrations have also exposed that this malignancy is orchestrated by accumulation of some key genetic as well as epigenetic events which in turn lead to anomalous activation or inhibition of diverse cellular signaling cascades in crucial cellular processes like proliferation, cellular survival, differentiation, and angiogenesis. Further, the emergent views coming out of different studies suggest that the key cell biological events including regulation of p53/ARF, RB/INK4A and Wnt/ $\beta$ -catenin pathways are also found to be affected in most cases of HCCs irrespective of etiology of the disease, indicating the presence of a shared oncogenic pathway in HCC development that regulates the mentioned biochemical events.

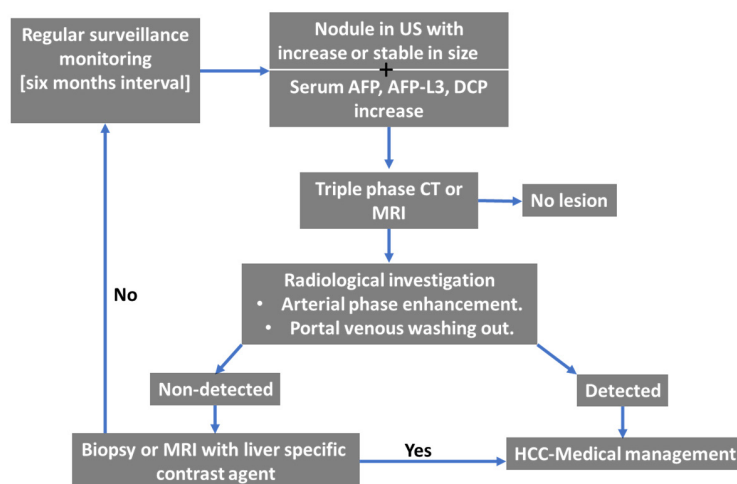
Clinical manifestation of HCC is diverse and significantly heterogeneous; asymptomatic cases are of suggestively high in incidence while symptoms often encompass from pain in upper right-abdominal quadrant, weight loss to obstructive jaundice and fatigue that are not strikingly exclusive compared to other hepatic ailments, viral infections, alcohol abuse associated functional loss of liver, *etc.* These make the differential detection, screening and monitoring of this fatal disease highly challenging.

Imaging is one of the gold standard non-invasive methods along with assessment of the biomarker alpha-fetoprotein (AFP) for diagnosis, staging and follow-up monitoring of HCC patients. Further, in standard clinical practice, AFP > 10 ng/mL or a composite AFP index ensures satisfactory sensitivity for early HCC detection<sup>[3]</sup>. However, there have been limitations often reported in sensitivity and specificity of AFP assessment and ultrasound based imaging with regard to clinical correlations with HCC. AFP-L3 and des- $\gamma$  carboxy prothrombin (DCP) are also used as important biomarkers for HCC determination<sup>[4]</sup>. HCC have a strong sex dominance in male (2 to 8 times more common) than in female in low - and high incidence areas. It has also been reported that the incidence of HCC holds a significant correlation with age, but a tendency of early onset of HCC in high-incidence areas has been observed too. With the continuous increase in incidence and mortality of HCC, along with the limitations of the current diagnostic strategy, the development of improved strategy for early detection is of greatest importance. The current review will discuss the latest clinical practice along with some recently introduced techniques in the early and sensitive diagnosis of HCC.

## METHODOLOGY

### Clinical manifestation and image based analysis

Often HCC progresses without prominent clinical symptoms and with no detectable aberration in liver function escaping early diagnosis. This makes HCC diagnosis usually delayed in developing countries with limited surveillance resources. On the other hand, clinical symptoms are emphasized in cases with compromised liver function. In advanced stages, symptoms include right upper quadrant abdominal pain, hepatomegaly, obstructive jaundice, hemobilia, and persistent fever. Some associated non-specific symptoms of malignant disease including anorexia, nausea, lethargy and weight loss are often reported. In many cases patients with unrecognized cirrhosis or known compensated cirrhosis may also present. Regular clinical practice employs various imaging studies such as ultrasonography (US), contrast enhanced computed tomography (CT) and magnetic resonance imaging (MRI) *etc.* for diagnosis, treatment management and follow up of HCC [Figure 1]<sup>[5]</sup>. According to the European Association for the Study of the Liver (EASL), liver lesions of length 2 cm or more observed on MRI or computed tomograph angiography (CTA), with AFP > 400 ng/mL or rising within sequential measurements do not require biopsy confirmation<sup>[6]</sup>. According to American Association for the Study of Liver Disease (AASLD) guideline however, liver lesions smaller



**Figure 1.** Current clinical practice for hepatocellular carcinoma (HCC) surveillance and diagnosis. AFP: alpha-fetoprotein; DCP: des-γ carboxy prothrombin; MRI: magnetic resonance imaging

than even 1 cm has been recommended for re-examinations twice in a year and in case of no detectable radiographical changes over a period of two years, routine surveillance guideline should be followed<sup>[7]</sup>. In the case of non-cirrhotic patients vascular profiling of the liver tumor on imaging studies reveals no consistent clue for HCC, a biopsy of the lesion is recommended to rule out HCC. According to the recommendations of the Asia-Pacific Association for the Study of the Liver 2010, every nodular lesion with uncharacteristic vascular profiles should undergo further imaging investigation such as endoscopic ultrasonography (EUS) as the confirmation for HCC<sup>[8]</sup>. Contrast enhanced CT and MRI scans are most efficient in differentiate and analyze diverse liver nodules and can characterize late stage HCC by its exclusive appearance of arterial-phase hypervascularity, however, this feature often lacks in early HCC<sup>[9,10]</sup>.

Diffusion weighted imaging (DWI) is one of the upcoming techniques that map the free diffusion of water molecules in three dimensions, which reflects the restricted diffusion in different stages of HCC and other liver diseases depending on highly congested cellularity, distortion of the extracellular interface, and density of the hydrophobic cell membrane within the tissue. This methodology has ability to detect liver lesions with quantitative investigation using no contrast media. Therefore, DWI can be used safely for the patients allergic to contrast media and the risk of nephrogenic complications associated with it can be avoided. However, in real life DWI is sensitive in detecting liver nodules, but it cannot precisely differentiate between HCC and dysplastic nodules or other malignant and benign lesions. Therefore, DWI results still require validations from contrast enhanced MRI for sensitive and specific determination of HCC cases<sup>[11]</sup>.

Indocyanine green (ICG) retention assay is another crucial fluorescence-based imaging technique that is being used for preoperative and perioperative dynamic diagnosis of HCC affected tissues. Both HCC affected tissue and normal tissue can take up ICG at same rate but follow up discharge of ICG from HCC tissues to the bile is impaired. There are few parallel phenomena that explain the reduced discharge rate of ICG from HCC tissues. Expression of glutathione S-transferase, an ICG binding protein, decreases significantly in HCC affected tissues than the healthy hepatocytes thus the excretion of ICG becomes impaired in cancer affected tissue of liver. Further, portal up-taking proteins like Na<sup>+</sup>/taurocholate co-transporting polypeptide (NTCP) and organic anion transporting polypeptide 8 (OATP8) are found to be overexpressed in case of HCC than the normal hepatocytes. Therefore, sustained portal intake of ICG by HCC by over expressed NTCP and OATP8 and impaired biliary excretion mechanism enhance the accumulation of ICG in HCC affected tissues that allows highly sensitive visualization of HCC affected part of liver following intravenous administration of ICG<sup>[12]</sup>.



## Serological tests

Serological biomarkers may provide crucial diagnostic hint in support of the results of ultrasound and may provide a crucial breakthrough in detecting biochemical changes related to liver malignancy prior to the image-based identification of hepatic nodules.

Assessment of the serum biomarker AFP has been one of the most extensively used clinical tests routinely performed for the determination of HCC. However, sensitivity of serum AFP test in determination of HCC ranges from 25% for nodules smaller than 3 cm to 50% for lesions larger than 3 cm in diameter<sup>[13]</sup>. Further, for the patients with cirrhosis of different stages AFP levels are found to be varying within a broad range and same trend has been found in the cases with underlying liver diseases where elevated serum AFP levels must be supported with high resolution image-based analysis to avoid false positive HCC detection<sup>[14]</sup>.

Other informative serological tests for HCC diagnosis are protein-induced by vitamin K absence or antagonist-II (PIVKA-II), also known as DCP, and the percentage of *Lens culinaris* agglutinin-reactive alpha-fetoprotein (AFP-L3). Technically, AFP-L3 is a glycoform of AFP that exclusively originates from cancer cells and demonstrates higher specificity for HCC in combination with AFP<sup>[14]</sup>. However, AFP-L3 is not typically detected when AFP levels are < 20 ng/mL<sup>[8]</sup> and it has a low sensitivity for early stage HCC diagnosis.

DCP or abnormal prothrombin cross-reacts with prothrombin antibodies in blood but fails to generate functional activity because it lacks  $\gamma$ -carboxy glutamate (GLA) unit which is crucial for binding  $\text{Ca}^{2+}$ . Structural studies of HCC associated DCP revealed that it has only 5 GLA unit as compared to 10 GLA units in native prothrombin structure. The mean level of DCP in HCC patients were often found to be as high as 900 ng/mL when it was determined for the first time in a cohort of 76 patients; a significantly 67% of those patients had DCP levels above 300 ng/mL<sup>[15]</sup>. However, there are evidence that DCP sometimes may be present cases of hepatitis and metastatic carcinoma, with a lower level of less than 300 ng/mL. Notably, plasma levels of abnormal prothrombin (DCP) in HCC could not be normalized by supplementing vitamin K, whereas, native prothrombin levels were recorded as normal. This rule out any correlation between HCC associated DCP production and vitamin K deficiency, but its biosynthesis in malignant hepatocyte is linked with an acquired defect in the vitamin K-dependent carboxylase system<sup>[16]</sup>. However, often AFP and DCP assay has found to be poorly correlated as it is expected from the very different origin of these biomarkers; the re-expression of a fetal antigen in the tumor tissues and an independently acquired posttranslational aberration respectively. DCP measured in biopsy homogenates of HCCs showed a high upward trend compared to the plasma concentration of the corresponding patients. Notably, in patients having normal plasma levels of DCP showed no changes in DCP concentrations within biopsy homogenates and surrounding healthy hepatic tissues. Currently, plasma DCP levels greater than 100 ng/mL on ELISA are taken as suggestive of HCC<sup>[17]</sup>. However, DCP levels and tumor size do not correlate well, studies found that DCP levels increased in only 20% of the HCC cases with nodules less than 3 cm<sup>[18]</sup>. A recent French study adopting a lower cut-off of DCP (42 ng/mL) recorded a sensitivity and specificity of 77% and 82%, respectively for early diagnosis of HCC; however, less sensitivity and specificity (61% and 50% respectively) were registered with a lower AFP cut-off of 5.5 ng/mL<sup>[19]</sup>. A recent study demonstrated that DCP was superior to AFP or its variant biomarker, AFP-L3 in detecting HCC and a combination of DCP with the other two mentioned tests provided in better accuracy than DCP alone<sup>[20]</sup>.

In current clinical practice, diagnostic methodology and surveillance program for HCC follow a sequence of image based and serological tests as described in [Figure 1](#). Under clinical guideline of AASLD, the European Association for the Study of the Liver and the European Organization for Research and Treatment of Cancer (EASL-EORTC), and the Japan Society of Hepatology (JSH), those with cirrhosis and those with chronic HBV infection regardless of cirrhosis has been considered as the high-risk population for HCC surveillance. However, EASL-EORTC also includes patients with chronic HCV and advanced liver fibrosis in this high-

risk group. In a real clinical surveillance protocol a combination of ultrasound based analysis and serological determination of biomarkers: AFP, AFP-L3 and DCP increases the diagnostic accuracy to a significant level compared to only image based study. However, ultrasound is reported to be precision compromised in visualizing the liver in patients with morbid obesity, therefore, in next level of confirmation triple phase CT or MRI is recommended. However, ultrasound positive, MRI negative cases are further cross-checked and confirmed either by biopsy or MRI with liver specific contrast agent under this HCC surveillance guidance. Based on 85-171 days of median doubling time in HCC volume, a 6-month interval in the surveillance protocol is currently recommended. However, the JSH guidelines propose a 3-4 month interval for HBV and HCV associated liver cirrhosis patients.

The widely approved strategy for the surveillance of HCC as mentioned above is a combination of image based and serological analysis with varied stand alone and combined sensitivity and diagnostic accuracy which provide impetus for the clinical assessment of new biomarker for HCC.

A recent study on the diagnostic performance of serum aldo-keto reductase family 1 member B10 (AKR1B10) in hepatitis B virus/hepatitis C virus (HBV/HCV)-related liver diseases demonstrated a crucial correlation in detection of HCC. Significant and stage dependent elevation of serum levels of AKR1B10 were recorded in patients with HCC compared to liver disorder cases. Importantly, comparison of advanced and terminal of HCC cases, a crucial increase in AKR1B10 levels was reported in early and intermediate HCC stages. The reported sensitivity (81.0%) and specificity (60.9%) for AKR1B10 based HCC diagnosis were significantly high at a cutoff value of 1.51 ng/mL. Further, conjoint measurement of serum AKR1B10 and AFP significantly increased sensitivity and specificity of the combined diagnostic parameters<sup>[21,22]</sup>.

Cell secreted small membrane-enclosed spheres, present in biological fluids are known as extracellular vehicles (EVs). EVs contain diverse types of biomolecules, including proteins, RNA, DNA, various metabolites and lipids, *etc.* that often carries the signature of the ailing conditions of the tissues of their origin. Thus, EVs are often potential source of biomarkers for different human pathobiology. Several lines of different diagnostic reports suggested that HCC cell-derived EVs carries the key effectors for autocrine and/or paracrine cellular communications, chemoresistance, angiogenesis, and tumor dissemination. A study has been successful in identifying EVs secreted by sorafenib-treated HCC cells rich in long intergenic non-coding RNA regulator for reprogramming (linc-ROR) that enables them to escape chemotherapy-induced apoptosis by the mechanism of p53 repression, upregulating the expression of CD133 marker and the stimulation of the signaling pathway for hepatocyte growth factor (HGF)/c-Met/Akt in liver cancer cells<sup>[23]</sup>. The notable abundance of EVs in biological fluids and their varied molecular payload has recently upgraded EVs as a key source of non-invasive biomarkers in liver diseases. Diagnostic studies have demonstrated the significantly elevated levels of different micro-RNAs in serum EVs of HCC patients (miRs 18a, 221, 222, and 224) as compared to cirrhosis or HCB patients, whereas a prominent drop in the concentration of miR-21 was consistently observed in the serum EVs of HCC patients as compared to HBV or healthy controls<sup>[24]</sup>. Another important molecular cargo of EVs isolated from HCC has been miR-665, whose concentration has found to be positively correlated with tumor size, and clinical stages along with local invasion<sup>[25]</sup>. Reduced expression of another EV cargo, miR-718 is highly correlated with increasing tumor size, recurrence and poor histological differentiation of HCC cells<sup>[26]</sup>. Furthermore, low concentrations of serum EV packed miR-125b have found to be associated to advanced TNM staging parameters, which is a suggestive of miR-125b as a potential prognostic biomarker for recurrence and overall survival of HCC<sup>[27]</sup>. Further, recent studies suggest that apart from miRNAs, serum EVs has been the delivery mode of diverse set of HCC associated proteins such as LG3BP, polymeric immunoglobulin receptor (PIGR) and alpha-2-macroglobulin (A2MG). These proteins were found to be over expressed in HCC patients compared to healthy individuals and serum EV concentrations of these proteins provided even better diagnostic value than AFP in early diagnosis of HCC<sup>[28]</sup>. Serum EV concentration itself is an implicative of HCC progression; it has been demonstrated that stage I and II HCC patients recorded higher serum EV abundance compared to liver cirrhosis patients<sup>[29]</sup>.

**Table 1. Aberrant concentration of miRNAs observed in hepatocellular carcinoma patients (with hepatitis B virus infection)**

miRNA	Change in concentration	Isolated sample
miR-221	Upregulation	Tissue and Serum
miR-21	Upregulation	Tissue and Serum
miR-222	Upregulation	Tissue and Serum
miR-222a	Upregulation	Serum
miR-224	Upregulation	Tissue and Serum
miR-101	Downregulation	Tissue
miR-18a	Upregulation	Tissue and Serum
miR-223	Upregulation	Serum

**Table 2. Aberrant concentration of miRNAs observed in hepatocellular carcinoma patients (with hepatitis C virus infection)**

miRNA	Change in concentration	Isolated sample
miR-765	Upregulation	Urine
miR-200a	Upregulation	Urine
miR-610	Upregulation	Urine
miR-323	Downregulation	Urine
miR-449	Downregulation	Urine
miR-502d	Downregulation	Urine
miR-92b	Downregulation	Urine
miR-122 and miR-221	Upregulation	Serum
miR-181a	Upregulation	Tissue and PBMC
miR-9, -10a, -15a, -16	Upregulation	Tissue
miR-198, -302b, -145, -368, -218, -330, -137, -147	Downregulation	Tissue from primary liver tumor
miR-155	Upregulation	Tissue

Recent study suggests that a significant number of non-coding RNAs (miRNA and long non-coded RNA; lncRNA) have been associated with HCC, more precisely that caused by HCV infection. These noncoding RNAs are found to be differentially expressed to promote pathogenesis of HBV and HCV-induced HCC. Apart from miRNAs packed in EVs (as discussed above); serum, urine, tissue concentration of various miRNAs also afford promise to be potential future biomarkers for both HBV or HCV-induced HCC as they are correlated consistently with progression, staging, survival rate and recurrence [Tables 1 and 2]<sup>[30]</sup>.

Many of the lncRNAs (long noncoding RNAs) are found to be dysregulated significantly in HCC and most are associated with the maintenance of the pathophysiological ambience of HCC tissue [Table 3]. The recent trend is also suggestive of their potential use as future biomarker candidates for HCC diagnosis. A decisive upregulation is observed in a lncRNA, HULC (highly upregulated in liver cancer) with a significantly consistent correlation with HCC progression, hepatic colorectal metastasis in HCC *etc.* Further, a key single nucleotide polymorphism in HULC (SNP) has been identified in the serum sample that can be a susceptibility marker for the risk of HBV infection<sup>[31,32]</sup>. H19 is another crucial lncRNA highly expressed in fetal liver, faintly expressed in normal adult liver. However, during tumorigenesis, it is highly upregulated and expressed, and might play a crucial part in tumorigenesis. Further, H19 was found to be over expressed and associated with cell proliferation, invasion, chemoresistance in HCC and thus it holds the promise of being a potential future serum biomarker for the same<sup>[33]</sup>. Among other lncRNAs, HOTAIR, MALAT-1, MEG3, GAS5, UCA1, HOTTIP, XIST are found to be consistently dysregulated in HCC and have the potential to act as crucial HCC biomarkers in future if validated by different cohorts of clinical data<sup>[34-40]</sup>.

A recent study demonstrated that a secretory protein, Trefoil factor 3 (TFF3), was highly expressed in HCC tissues, suggesting it to be a potential serum biomarker for HCC. Further, two circulating microRNAs (miR-7-5p and miR-203a-3p) reported to target TFF3 have also been proposed as future biomarkers for HCC. However exhaustive clinical data are awaited<sup>[41]</sup>.

**Table 3. Dysregulated lncRNAs observed in hepatocellular carcinoma**

lncRNA	Change in concentration	Isolated sample
HULC	Upregulation	Hepatic colorectal metastasis samples
H19	Upregulation	Malignant liver tissues
UCA1	Upregulation	Liver tissues and serum
HOTAIR	Upregulation	Malignant liver tissues
MVIH	Upregulation	Malignant liver tissues
ATB	Upregulation	Malignant liver tissues
HOTTIP	Upregulation	Malignant liver tissues
MALAT-1	Upregulation	Malignant liver tissues
VLDLR	Upregulation	Malignant liver tissues and EVs
TUC339	Upregulation	EV
MEG3	Downregulation	Malignant liver tissues
PTENP1	Downregulation	Malignant liver tissues
DREH	Downregulation	Malignant liver tissues
WT1-AS	Downregulation	Malignant liver tissues
Uc002mbe.2	Downregulation	Malignant liver tissues
XIST and FTX	Downregulation	Expressed more in liver malignant tissues of female than male
CPS1-IT1	Downregulation	Malignant liver tissues
AOC-4P	Downregulation	Malignant liver tissues
HEIH	Upregulation	Malignant liver tissues

Recently, among various non-protein biomarkers, aberrant methylation, like hypomethylation in DNA and/or hypermethylation in the CpG promoter gene, are found to be associated in the pathogenesis of different tumors, including liver HCC<sup>[42,43]</sup>. A recent study identified a set of aberrantly methylated DNA markers showing significant association with the HCC progression and interestingly, most of these hypermethylation occur within the CpG domain<sup>[44]</sup>. The identification of these circulating tumor DNAs carrying the hypermethylated aberration within in the large cohort of HCC patients provides a promise of development of a highly sensitive, noninvasive and accurate early detection platform for HCC.

## CONCLUSION

HCC remains to be the most fatal malignant liver cancer worldwide even after the advances that has been achieved in diagnostic and invasive medicine since last decades. The contemporary HCC treatment has been intensive to early diagnosis and hepatic transplantation as medical management of this fatal disease. Combination therapies performs well to downgrade the tumor and make it removable, that significantly improve basic liver function and improve the timeline of survival, however, the early diagnosis has been the most crucial deciding factor. In summary, informative feedback on the preclinical performance of image-based techniques, AFP, AFP-L 3%, and DCP in the detection of HCC has created a vast and varied data set since last few decades that serves as a fulcrum in the early diagnosis and medical management of HCC. Recent observations demonstrate that many miRNAs and lncRNAs are differentially expressed in malignant liver tissues, and their dysregulation as reflected in aberrant concentrations in various clinical samples (tissues, serum, EVs *etc.*) were found to be correlated well with HCC progression, recurrence after liver transplantation, chemoresistance *etc.* Thus, these miRNAs or lncRNAs may serve as potential biomarkers for the diagnosis, prognosis, prediction of recurrence and therapeutic response of HCC<sup>[45]</sup>.

However, a substantial heterogeneity among various cohorts of patients in terms of diagnostic criteria leaves the space for evaluating the clinical performance of novel biomarkers, comprehensive studying different variables associated with the malignant transformation in HCC and inclusion of some of the newer biomarkers in surveillance strategy may prove to be crucial in future clinical management of this fatal disease.

## DECLARATIONS

### Authors' contributions

Literature survey; Data analysis and comparison of published data; Draft of the manuscript: Bose PP  
Draft of the Manuscript and comparison of published data: Chatterjee U

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

Not applicable.

### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Copyright

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Original Article

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# Hepatocellular carcinoma occurred in a Hepatitis B carrier clinic cohort during a mean follow up of 10 years

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## Abstract

**Aim:** Chronic persistent hepatitis B virus carriers are generally asymptomatic until the advanced stage of the disease. The “Hepatitis B-Carrier Clinics” of Chang Gung Memorial Hospital has been using alpha-fetoprotein (AFP) and liver ultrasound for early detection of hepatocellular carcinoma (HCC) in hepatitis B surface antigen (HBsAg) carriers since 1980.

**Methods:** We evaluated the results of surveillance between 1980 and 2012 by collecting clinic data, matched cancer registry status, and national mortality database status.

**Results:** Of 15,235 HBsAg carriers, 238 instances of HCC (1.5% or 156.2/100,000 person-years) were detected over a mean follow-up period of  $10.0 \pm 7.6$  years. There were more men (89.1%) and patients with liver cirrhosis (70.2%) in the HCC group ( $P < 0.001$ ), and both the initial and maximal alanine aminotransferase (ALT) levels were higher in this group ( $P < 0.001$ ). One hundred and thirty cases of HCC (54.6%) were identified during regular follow-up sessions, 55 (23.1%) were detected after the regular schedule had lapsed (“out-of-schedule”), and 53 (22.3%) were lost to follow-up completely. The mean tumor size was smaller in the regular group than in the out-of-schedule group (2.72 cm vs. 4.59 cm,  $P < 0.001$ ), and the survival rate was higher (43.8% vs. 30.9%,  $P < 0.001$ ).

**Conclusion:** The incidence of HCC was relatively low in the HBsAg-Carrier Clinics cohort. Surveillance for early



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diagnosis of HCC improved the survival of high-risk HBsAg carriers. To ensure cost-effectiveness, we suggest using different screening strategies according to the individual risk of hepatocarcinogenesis.

**Keywords:** Hepatitis B surface antigen, retrospective cohort, hepatocellular carcinoma, surveillance

## INTRODUCTION

Chronic persistent hepatitis B virus (HBV) infection is a global disease, and its prevalence is highest in Africa and East Asia<sup>[1-4]</sup>. The chronic relapsing inflammation caused by HBV leads to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC)<sup>[4]</sup>. Patients with HBV are generally asymptomatic and unaware of their illness until they reach the advanced stage. Early diagnosis of HCC through periodic surveillance has become one of the strategies for management of chronic hepatitis B surface antigen (HBsAg) carriers<sup>[5-7]</sup>. Diagnosis of HCC in the early, treatable stages leads to improved survival<sup>[7,8]</sup>. This is especially important for areas with endemic HBV infection<sup>[1-3,9]</sup>. The methods for early detection of HCC mainly rely on alpha-fetoprotein (AFP), AFP-lectin 3 fraction (AFP-L3), des- $\gamma$ -carboxy prothrombin, and liver ultrasound (US)<sup>[5-8,10,11]</sup>. Many new biomarkers are under investigation<sup>[12-14]</sup>, but they appear to be more expensive and were not found to be significantly superior to AFP and US in a large population survey<sup>[15]</sup>.

In addition to detection tools, a number of other variables, including duration of surveillance, individual risk, and patient attitude, may also contribute to the effectiveness of surveillance. We have been performing surveillance for early detection of HCC in a hepatitis B carrier clinic for more than 30 years<sup>[5,6]</sup>. This analysis was conducted to assess our results and to improve our surveillance strategy.

## METHODS

The HBsAg-Carrier Clinics of Chang Gung Memorial Hospital in Taipei and Linkou Medical Centers have been in operation since 1980, and they provide an easily accessible service for chronic HBsAg carriers in Taiwan. Most patients who visit the HBsAg-Carrier Clinics are asymptomatic upon entry. They visit the clinics because the presence of HBsAg has been incidentally detected upon blood donation, in a general check-up, or in a work-up for a non-liver-related disease, or they are referred from our outpatient department as stable HBsAg carriers with normal alanine aminotransferase (ALT).

A total of 15,235 HBsAg carriers with persistent HBsAg for more than six months had been registered in the HBsAg-Carrier Clinics by 2012. Patients with dual infections were excluded. The subjects underwent 275,324 visits and had a mean follow-up duration of  $10.0 \pm 7.65$  years. Upon registration with the clinic, the subjects underwent liver biochemical tests and testing for serologic markers of hepatitis viruses, AFP, and real-time liver US. After this initial visit, the subjects were followed-up every 3-12 months, with ALT, AFP, and US as the basic measures.

This HBsAg-Carrier Clinics has three full-time clinic staffs and a private line telephone to arrange registration and visits. The clinic staffs recorded basic information, delivered patient education, collected data from each visit, and entered these data into the hospital's main computer. One of the main aims of this clinic is to detect HCC at the early stage. For patients unable to keep up with the follow-up schedule, reminder letters are sent.

In this study, we examined data from the HBsAg-Carrier Clinic from 1980-2012. This long-term follow-up analysis of chronic HBsAg carriers was approved by the human research committee of Chang Gung Memorial Hospital (IRB No: 201600523B0).

### Identification of hepatocellular carcinoma

One of the main aims of this study was to determine the incidence of HCC in this cohort. Diagnosis of HCC was ascertained based on cytology or histology reports. We also included subjects with at least two findings from the AFP, liver US, computer tomography (CT), magnetic resonance imaging (MRI), and angiography tests indicating they were positive for HCC<sup>[16]</sup>. In addition, disease progression based on one imaging-modality study was accepted as a diagnosis of HCC.

In addition to the medical records maintained in this clinic, the study subjects had linked records in the Cancer Registration Database of Chang Gung Memorial Hospital. This database contains all information recorded about all cancers diagnosed or treated at this hospital since 1987. We linked our cohort with this database to retrieve all HCC cases identified between 1987 and 2007 in other departments.

The subjects' national identification numbers were also linked with our national mortality database, which has been maintained by the Statistics Office of Taiwan's Department of Health since 1985. Cause of death was classified using the ninth revision of the International Classification of Diseases, Injuries and Causes of Death (ICD-9, World Health Organization, 1977). We matched our cohort with this database between 1985 and 2007. For patients lost to follow-up in our hospital, the national mortality database was the only available source of information regarding diagnosis of HCC.

### Classification of HCC according to follow-up schedule

The patients in this study differed with respect to the point at which HCC was detected during their follow-up schedule, and they were therefore classified into three groups. The first group comprised patients with HCC identified within one year of last follow-up (regular follow-up group). The second group comprised patients who were lost to follow-up for more than one year but returned with HCC, and those for whom HCC was identified upon initial presentation (out-of-schedule group). The third group comprised patients who were completely lost to follow-up, but who were identified as having died from HCC based on the national mortality database (lost group).

### Statistical analysis

To compare the characteristics of the three groups, the  $\chi^2$  test, Fisher's exact test, Student's *t*-test, linear-by-linear association, Pearson's correlation, and ANOVA were used as appropriate. Survival analysis was done using the Kaplan-Meier actuarial curve method with the log-rank test. Univariate and multivariate analyses were performed to identify factors associated with mortality and HCC or development of cirrhosis. Variables found to be significant in the univariate models were tested in a multivariate setting using the Cox proportional hazards regression model. All statistical analyses were performed using SPSS software (version 22; SPSS Inc., Chicago, IL) and  $P < 0.05$  was considered significant.

## RESULTS

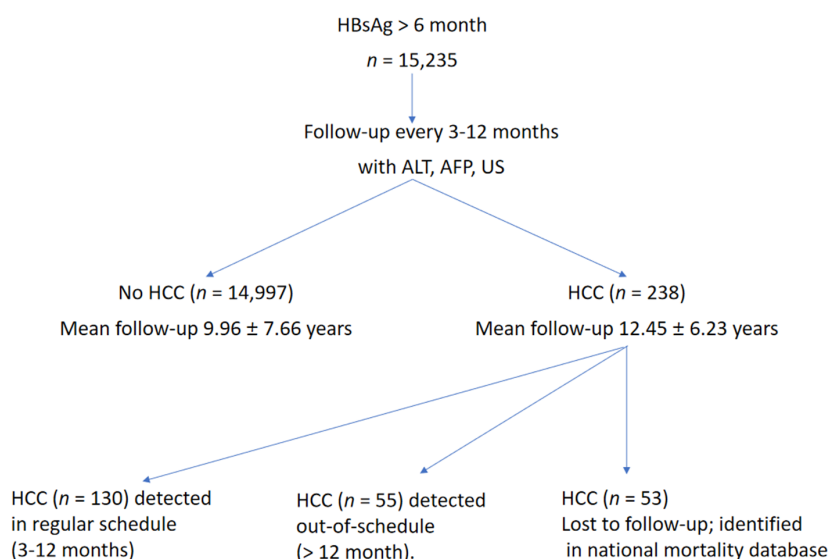
During the study period, 238 HCC cases were identified (1.5% or 156.2/100,000 person-years, [Table 1](#)). Male sex (89.1% in the HCC group *vs.* 58.6% in the non-HCC HBsAg carriers,  $P < 0.001$ ), age at enrollment (43.32 *vs.* 34.96 years,  $P < 0.001$ ), prevalence of liver cirrhosis (70.2% *vs.* 2.7%,  $P < 0.001$ ), and initial ALT (123.72 *vs.* 63.22 U/L,  $P < 0.001$ ) and maximal ALT levels (310.73 *vs.* 130.63 U/L,  $P < 0.001$ ) were significantly associated with HCC [[Table 1](#) and [Figure 1](#)]. In the HBsAg carriers without HCC, 72.6% had initial ALT levels lower than  $2 \times$  the upper limit of normal (ULN; [Figure 2A](#)) and 69.3% had a maximal ALT level lower than  $5 \times$  ULN [[Figure 2B](#)]. In contrast, 63.5% HCC patients had an initial ALT level greater than or equal to  $2 \times$  ULN and 71.5% had a maximal ALT level greater than or equal to  $5 \times$  ULN.

Of the patients with HCC, 130 (54.6%) cases were identified during regular follow-up, 55 (23.1%) were in the out-of-schedule group, and 53 (22.3%) cases were lost to follow-up [[Table 2](#)]. The diagnosis was established

**Table 1. Demographics of chronic HBsAg carriers with and without HCC**

	HBsAg carriers		P value	
	Without HCC	HCC	Univariate	Multivariate
Total	14,997	238		
Male	8,793 (58.6%)	212 (89.1%)	< 0.001	< 0.001
Age at enrollment (years)	34.96 ± 10.08	43.32 ± 11.18	< 0.001	< 0.001
Liver cirrhosis	407 (2.7%)	167 (70.2%)	< 0.001	< 0.001
Initial ALT (U/L)	63.22 ± 206.16	123.72 ± 264.12	< 0.001	0.434
Maximal ALT (U/L)	130.63 ± 310.75	310.73 ± 487.70	< 0.001	< 0.001
Anti-HBV therapy	876 (5.8%)	55 (23.1)	< 0.001	< 0.001
Thymosin	13 (0.08%)	1 (0.4%)		
IFN/pegIFN	130 (0.8%)	6 (2.5%)		
LAM/ADV/TBV	342 (2.3%)	31 (13.0%)		
ETV/TDF	386 (2.6%)	17 (7.1%)		
Follow-up (years)	9.96 ± 7.66	12.45 ± 6.23	< 0.001	0.001

ALT: alanine aminotransferase; ADV: adefovir; ETV: entecavir; IFN: interferon; pegIFN: pegylated interferon; LAM: lamivudine; TBV: telbivudine; TDF: tenofovir

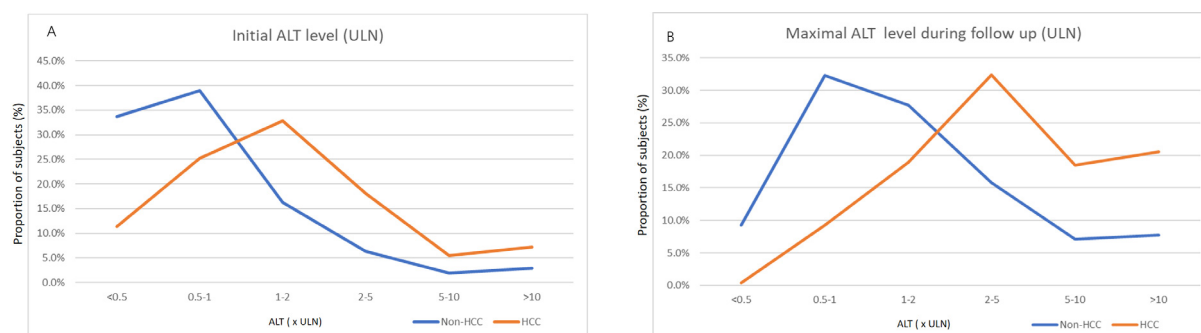
**Figure 1.** Patient flowchart. AFP: alpha-fetoprotein; ALT: alanine aminotransferase; HCC: hepatocellular carcinoma; US: ultrasound

via histology or cytology in 119 (50%) patients. The mean tumor size was smaller (2.72 vs. 4.59 cm,  $P < 0.001$ ) in the regular follow-up group than in the out-of-schedule group. The regular follow-up group had the highest survival rate (43.8%) of the three groups ( $P < 0.001$ ) at end of the study period. There was no difference in age at diagnosis or age at death between the groups.

Of the 130 HCC cases detected during regular follow-up, 47 (36.2%) patients had at least one liver nodule identified via US during the three months before the diagnosis. Sixteen (12.3%) of these patients had three or more nodules.

During the study period, 931 patients received anti-HBV therapy. The medications administered were: thymosin (14 patients); interferon or pegylated interferon (136 patients); lamivudine, adefovir, or telbivudine (373 patients); and entecavir or tenofovir (403 patients). More patients in the HCC group received anti-HBV therapy than in the non-HCC HBsAg-carrier group (Table 1;  $P < 0.001$ ).





**Figure 2.** A: the hepatocellular carcinoma (HCC) group had higher initial alanine aminotransferase (ALT) levels than HBsAg carriers without HCC ( $P < 0.001$ ). The majority of HCC cases were patients who had presented with an initial ALT level around 1-2  $\times$  the upper limit of normal (ULN), while most of HBsAg carriers without HCC had presented with normal ALT levels; B: the maximal ALT level was higher in HCC cases than in HBsAg carriers without HCC ( $P < 0.001$ ). Most of the HBsAg carriers without HCC had maximal ALT levels below 2  $\times$  ULN

**Table 2. HCC classification according to follow-up schedule**

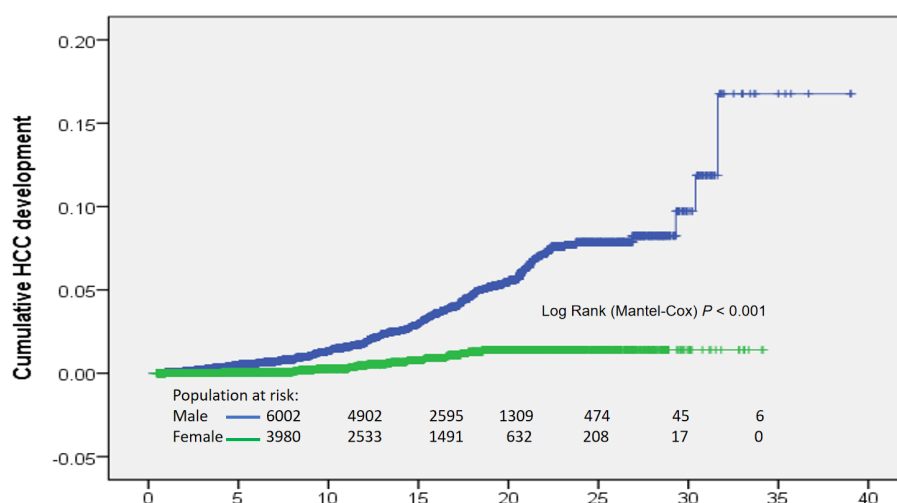
	Follow-up schedule			P value
	Regular	Out-of-schedule	Lost	
Total	130	55	53	
Male	115 (88.5%)	51 (92.7%)	44 (83.0%)	NS
Cirrhosis	93 (71.5%)	37 (67.3%)	?	NS
Diagnosis				
Histology	56 (43.1%)	28 (50.9%)	?	NS
Cytology	27 (20.8%)	8 (14.5%)	?	
Age at diagnosis (years)	55.8 $\pm$ 10.3	55.2 $\pm$ 11.9	?	NS
Tumor size (cm)	2.72 $\pm$ 1.58	4.59 $\pm$ 3.64	?	< 0.001
Survivors	57 (43.8%)	17 (30.9%)	0 (0%)	< 0.001
Age at death (years)	61.8 $\pm$ 10.6*	59.7 $\pm$ 12.6	57.1 $\pm$ 10.3*	0.035*

\*Groups for comparison

## DISCUSSION

Only 238 (1.5% or 156.2 person-years) HCC cases were identified among 15,235 HBsAg carriers during a mean follow-up of 10 years. The annual incidence of HCC was 0.53% in men and 0.04% in women [Figure 3]. The incidence of HCC in this cohort was relatively low compared with the 826 person-years reported in our previous study of 432 patients with chronic hepatitis B<sup>[5]</sup>. In general, the incidence in our study is lower than in studies of patients with chronic hepatitis and higher than in studies of inactive carriers<sup>[17]</sup>. In a study examining HCC-risk stratification<sup>[18]</sup>, the 10-year cumulative risk score for developing HCC was 1.2%-2% when only male sex and subjects aged 50-59 years were considered<sup>[18]</sup>. The HCC diagnostic age in this cohort was around 55 years [Table 2]. Therefore, our HCC incidence (1.5%/10 years) is within the range of that prediction. HCC screening is cost-effective and is recommended for groups in which annual HCC incidence exceeds 1.5%<sup>[19]</sup>. Therefore, lifelong screening of all HBsAg carriers for liver cancer would not be cost-effective in this cohort. We do have other reasons for maintaining this clinic, such as screening for chronic hepatitis and liver cirrhosis that require therapy, understanding the history of chronic HBV infection, and others. However, we should focus surveillance efforts on high-risk HBsAg carriers<sup>[20]</sup>. Without complicated parameters, this study has identified male gender (89.1%), maximal ALT level greater than or equal to 5  $\times$  ULN (71.5%), liver cirrhosis (70.2%), and age over 40 at enrollment (95%) as risk factors for HCC (Table 1; logistic regression,  $P < 0.001$ ). Patients who have these clinical and demographic characteristics warrant active surveillance.

A large proportion of the patients were unable to adhere to the follow-up schedule in this cohort. Only 54.6% (130/238) of the HCC cases were identified during the regular follow-up schedule, while 55 (23.1%)



**Figure 3.** Cumulative hepatocellular carcinoma (HCC) development in men and women. The annual HCC incidence was 0.53% in men and 0.04% in women

were patients who returned with HCC and 53 (22.3%) were patients who had been completely lost to follow-up. This suggests that ongoing surveillance at 3-12-month intervals was difficult to maintain for nearly half of the patients. The results from our single hospital with 15,235 cases are similar to a meta-analysis of 22 reports covering 19,511 cases<sup>[21]</sup>. In that study, Wang *et al.*<sup>[21]</sup> found that the adherence rate to HCC surveillance was only 52%.

The regular follow-up group had a higher survival (43.8% vs. 30.9%) and smaller mean tumor size (2.72 vs. 4.59 cm) than the out-of-schedule group (Table 2;  $P < 0.001$ ). These results are in agreement with a randomized control study that included 18,816 participants<sup>[22]</sup>. Zhang *et al.*<sup>[22]</sup> found that regular surveillance leads to early detection of HCC, resulting in better survival than in those without surveillance. A recent extensive review confirms surveillance improved survival<sup>[23]</sup>. There is therefore no doubt that surveillance should be carried out in high-risk HBsAg carriers. The current Asian Pacific Association for Study of the Liver (APASL) guideline specifies that male HBsAg carriers aged greater than 40 years and females aged greater than 50 years are high-risk groups<sup>[7]</sup>. In our previous analysis, which used a subset of patients from this cohort, we found that the incidence of HCC was relatively low if the initial ALT level was lower than  $2 \times \text{ULN}$  or if the patients maintained persistent normal ALT<sup>[23,24]</sup>. In this study, 72.6% of chronic HBsAg carriers without HCC had initial ALT levels lower than  $2 \times \text{ULN}$ , and 69.3% had maximal ALT levels lower than  $5 \times \text{ULN}$ . In contrast, 63.5% HCC patients had initial ALT levels greater than or equal to  $2 \times \text{ULN}$  and 71.5% had maximal ALT levels greater than or equal to  $5 \times \text{ULN}$ . Therefore, we might not encourage patients with persistent ALT levels lower than  $2 \times \text{ULN}$ , no cirrhosis, female gender, or age under 40 years to receive early full surveillance. Repeated negative findings during regular follow-up visits may cause the patient to feel it is less necessary to continue surveillance. For such patients, we may continue with simple ALT and AFP surveys. Full surveillance, including HBV viral load, US, elastography, or new markers may then be started once a risk factor is identified. Active call-back mechanisms focusing on these high-risk patients will be mandatory.

The prevalence of liver cirrhosis was relatively high (70.2%) in patients with HCC. In addition, lesions resembling liver regeneration nodules were found in 47 (36.2%) patients preceding HCC diagnosis. Of these, 16 (12.3%) had three or more nodules. These preexisting findings decreased the likelihood of early diagnosis of HCC. Indeed, cirrhotic nodules have been reported as a problem in the diagnosis of small HCC<sup>[25]</sup>. New parameters to allow the discrimination of HCC from regeneration nodules will therefore be needed<sup>[26-28]</sup>.

More patients with HCC received treatment than those without HCC (Table 1;  $P < 0.001$ ). This could be because active viral replication is typically associated with HCC<sup>[29,30]</sup>. Since the treatment regimens were complicated, whether anti-HBV therapy decreased the incidence of HCC could not be evaluated in this study.

We conclude that surveillance for early diagnosis of HCC improved the survival of high-risk HBsAg carriers. Most HBsAg carriers are low-risk and can be screened using simple parameters, such as periodic AFP and liver biochemistry tests at 6-12-month intervals. When risk factors appear during this follow-up schedule, we may then add US, elastography, or other new markers for early diagnosis of HCC in high-risk patients.

## DECLARATIONS

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### Authors' contributions

Designed the study and wrote the manuscript: Tai DI

Update treatment data and wrote the manuscript: Chen CJ, Tai DI

Collected and organized data: Tai J

### Availability of data and materials

The data source is from Carrier Clinics of Chang Gung Memorial Hospital. Please contact the author through E-mail for information if necessary.

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### Conflicts of interest

The author declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

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Not applicable.

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Editorial

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# Hepatocellular carcinoma: a new hope?

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In this special issue of *Hepatoma Research*, we highlight certain Novel Approaches to Hepatocellular Carcinoma. In the article “Stereotactic ablative radiotherapy for hepatocellular carcinoma” Dr Spieler and Dr Portelance discussed the development of improved toxicity models and highly conformal radiation delivery systems which allows for stereotactic radiosurgery to ablate liver tumors in few fractions and spare noncancerous liver tissue<sup>[1]</sup>. Stereotactic body radiation therapy or SABR is an advanced form of external body radiation therapy. SABR combines both tumor/organ motion management with multiple beams of high energy so that very high doses of radiation can be administered precisely in one to five fractions. Advantages to SABR is that this treatment is minimally invasive, can treat large tumor volume, or tumors close to liver capsule, major blood vessels or diaphragm and when disease is associated with portal vein thrombosis. This enables treatment for patients whose liver function tests may preclude radioembolization or chemoembolization, or when the portal vein is occluded which may preclude chemoembolization. In addition, as mentioned in the article, there is a rationale for this treatment to be considered to combine with immunotherapeutic agents to enhance response. Preclinical and clinical studies demonstrate that radiation therapy can upregulate PD-L1 expression in tumors so checkpoint inhibitors may be more effective. Currently there are multiple clinical trials combining radiation therapy with checkpoint inhibitors such as anti-PD-1 or anti-PD-L1 inhibitors or with cytotoxic T-lymphocyte associate protein inhibitor (anti-CTLA-4) to treat hepatocellular carcinoma<sup>[2]</sup>.

In the case report of “Congenital absence of the portal vein complicated by hepatocellular carcinoma in the liver of an adult”, Dr Mehta highlights the rarity of this condition<sup>[3]</sup>. There are only 101 previously reported cases of congenital absence of the portal vein and 40% of which were associated with hepatic tumors, including hepatocellular carcinoma. They discussed different approaches to treatment for patients



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with this unusual anatomy and vasculature. This congenital abnormality represents a challenge for clinical management and should involve a multidisciplinary team experienced in the treatment of liver cancer.

Dr Ayoub and Dr Jones review the “Impact of nucleos(t)ide analog therapy in hepatitis B on the incidence of hepatocellular carcinoma”<sup>[4]</sup>. Hepatitis B is a major cause of hepatocellular carcinoma worldwide, particularly in the Far East. This article discusses the role of treatment of hepatitis B, including the new antiviral agents and how they may reduce but not eliminate the risk of hepatocellular carcinoma. The third generation nucleos(t)ide analogs, tenofovir and entecavir, which both have a high genetic barrier to resistance, has led to further decreases in HCC incidence.

Finally, in the article “Immunotherapy for hepatocellular carcinoma: the force awakens in HCC?”, we discuss newer therapeutic approaches with immunotherapeutic drugs<sup>[5]</sup>. It is known that hepatocellular carcinoma is an inflammation-associated malignancy and so can be immunogenic. Reasons for immune tolerance are included such as the presence in the liver of myeloid-derived suppressor cells, regulatory dendritic cells, T regulatory cells, invariant natural killer T- cells, and tumor-associated macrophages. Furthermore, there is evidence of T-cell exhaustion and apoptosis associated with chronic hepatitis C infection. Increased expression of TRAIL, indoleamine 2,3-dioxygenase (IDO), and LAG-3 have been found which may contribute to immunosuppression. In terms of therapy, recently, two checkpoint inhibitors, nivolumab and pembrolizumab, have been granted conditional approval for the treatment of hepatocellular carcinoma.

In the 2019 American Society of Clinical Oncology meeting, over 70 abstracts can be found under search for hepatocellular carcinoma and immunotherapy. Promising early results from clinical trials have been reported with combination of immunotherapy agents, or other modalities of treatment such as surgery or radiation therapy<sup>[6]</sup>. Thus, immunotherapy can now be considered, along with surgery, radiation therapy and chemotherapy, as a viable option and offers a new hope for our patients with hepatocellular carcinoma.

## **DECLARATIONS**

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Feun LG Contributed solely to the article.

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There is no conflict of interest.

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Review

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# Advances in surface markers of liver cancer stem cell

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## Abstract

Liver cancer stem cells (LCSCs), a small subpopulation that constitutes liver cancer heterogeneity, play a vital role in cancer initiation, invasion, recurrence, metastasis, and resistance to chemo-radiotherapy. It is believed that therapies targeting LCSCs can improve the efficacy of conventional chemotherapy and radiotherapy by completely eliminating tumors while preventing recurrence. Therefore, during last decades, numerous surface markers for LCSCs have been identified and characterized in many subtypes of liver cancer, especially in hepatocellular carcinoma (HCC). These well-recognized surface markers significantly promote the therapeutic efficacy that identifies, targets and destroys LCSCs. Meanwhile, there have been intensive studies that aim to investigate the molecular mechanism of how stemness contributes to liver cancer relapse, recurrence and resistance. However, liver cancer stemness seems to be regulated by a hierarchical organization and crosstalk of a wide variety of signaling pathways. Using individual or few LCSC surface markers may not be able to completely reveal the intrinsic stemness hierarchy. From an integrated perspective, understanding of recent advances in LCSC surface markers remains important and urgent. In this review, we concentrate on demonstrating the indispensable roles of LCSC surface markers in identification and characterization of multiple cancer stages including initiation, invasion, metastasis, resistance and highlighting the cutting-edge therapeutic strategies against cancer stem cells in HCC.

**Keywords:** Liver cancer, hepatocellular carcinoma, cancer stem cell, surface marker



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## INTRODUCTION

Liver cancer is the seventh most frequently diagnosed cancer and the third death causing of cancer around the world, which has 841,080 newly diagnosed cases and caused 781,631 deaths in 2018<sup>[1]</sup>. Hepatocellular carcinoma (HCC) comprises 75%-85% of the primary liver cancer cases and intrahepatic cholangiocarcinoma and other rare types comprise 15%-25%<sup>[1]</sup>. Chronic hepatitis B virus infection, hepatitis C virus infection, steatohepatitis and cirrhosis are the most prevalent precursors to HCC. Despite of the recent advances in liver cancer therapies, the current treatment cannot effectively prevent tumor recurrence and metastasis due to the existence of (liver cancer stem cells) LCSCs. The concept of cancer stem cells (CSCs) is raised from clinical and experimental observations that there exists a subpopulation of cancer cells that possess stem cell-like characteristics including self-renewal and differentiation that eventually lead to cancer relapse and resistance. LCSCs have been reported in varied types of HCC and are deemed to be one of the major causes of HCC recurrence, metastasis, chemoresistance and radioresistance.

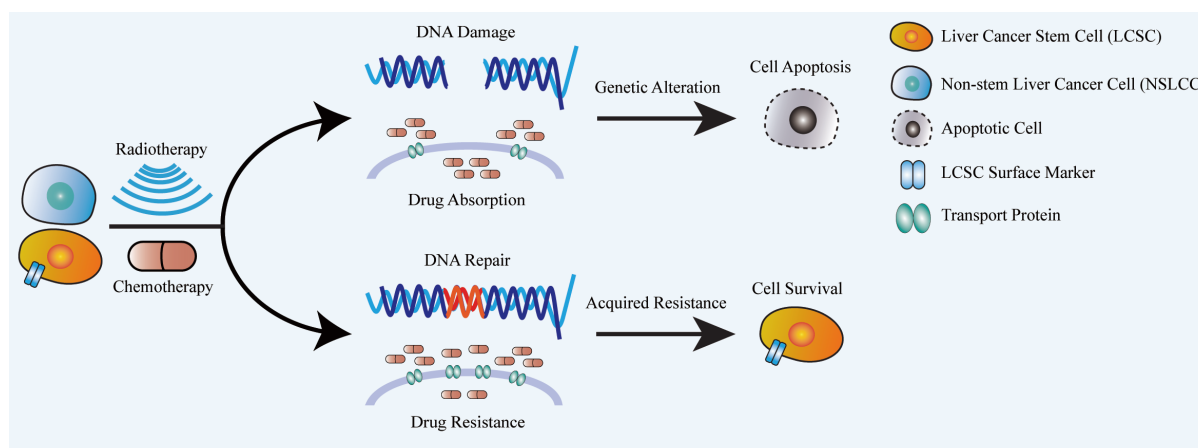
Conventional therapies against non-stem liver cancer cells such as chemotherapy and radiotherapy, have multiple limitations that result in cancer recurrence and metastasis due to acquired resistance. The survival LCSCs can re-initiate tumor development and invasion [Figure 1]. Hence, in order to develop feasible therapies that can prevent tumor recurrence and metastasis, it is important to specifically identify, target and eliminate LCSCs. Recent advances in LCSC surface markers and understanding of cellular features related to LCSC phenotypes greatly improve the efficacy of treatments that target LCSCs. Targeting the LCSCs with high expression of certain stemness surface markers, can manipulate the abilities of LCSCs in proliferation, growth, maintenance, differentiation, resistance and apoptosis via cellular signaling pathways so that tumor regeneration can be impeded.

In order to develop patient-specific therapies that target LCSCs, multiple stemness surface markers have been identified consisting CD133<sup>[2]</sup>, CD44<sup>[3]</sup>, CD90<sup>[4]</sup>, epithelial cell adhesion molecule (EpCAM)<sup>[5]</sup>, CD47<sup>[6]</sup>, CD34<sup>[7]</sup>, C-kit<sup>[8]</sup>, CD13<sup>[9]</sup>, CD24<sup>[10]</sup>, calcium channel  $\alpha 2\delta 1$  isoform5<sup>[11]</sup>, oval cell marker OV6<sup>[12]</sup>, DLK1<sup>[13]</sup>, K19<sup>[14]</sup>, and Lgr5+<sup>[15]</sup> [Table 1]. The integrated therapy using conventional anti-carcinogenic inhibitors such as sorafenib with LCSCs-targeting drugs, may provide an effective therapeutic strategy for complete elimination of liver cancer.

## CD133

CD133, also referred to as PROM1, is a member of prominin family that has a structure of five transmembrane single-chain glycoprotein with a molecular weight of 115 ~ 120 kDa, including an extracellular N-terminus, two large extracellular loops, two small intracellular loops and an intracellular C-terminus<sup>[16-19]</sup>. CD133 was originally identified as a surface marker of hematopoietic stem cells<sup>[16]</sup>. In solids tumor, CD133 was firstly identified and further isolated in brain tumors<sup>[20]</sup>. Later, the role of CD133 as a surface marker of CSC is been reported in a wide variety of tumor tissues such as lung cancer<sup>[21]</sup>, stomach carcinoma<sup>[22]</sup>, pancreatic cancer<sup>[23]</sup>, colon cancer<sup>[24]</sup>, and liver cancer that was identified by our team<sup>[25-27]</sup>.

In 2006, Suetsugu *et al.*<sup>[28]</sup> reported that CD133+ liver cancer cells, sorted from the Huh7 cell line, exhibited a more potent capability of proliferation and metastasis compared to the CD133- counterparts. Our previous study indicated that CD133+ cells also processed a stronger colony-forming characteristic, greater tumorigenicity and potential to differentiate into angiomyogenic-like lineages<sup>[2]</sup>. We also further characterized the liver CD133+ CSCs, revealing that CD133+ cells were endowed with high *in vivo* tumorigenicity and the capability to form spheroids with an upregulated expression of stemness-associated genes *in vitro*<sup>[29-31]</sup>. Liu *et al.*<sup>[32]</sup> reported that CD133 was crucial to monitor the migratory capability of LCSCs, tumor-initiating properties, and the epithelial-mesenchymal transition (EMT) process. Tang *et al.*<sup>[33]</sup> demonstrated that CD133+ liver tumor-initiating cells (TICs) had angiogenesis ability. In addition, Li *et al.*<sup>[34]</sup> and other researchers<sup>[35]</sup> also found that CD133+ HCC cells could exploit autophagy to maintain their survival. Liver CD133+ CSCs are



**Figure 1.** Acquired chemo- and radioresistance in liver cancer stem cells. Traditional chemo-/radiotherapy can induce genetic alteration in non-stem liver cancer cells (NSLCCs) via DNA damage and cytotoxic agent intake, in order to activate cellular apoptosis. However, upon treated with traditional chemo-/radiotherapeutic agents, liver cancer stem cells (LCSCs), can acquire chemo-/radioresistance including an increased level of drug intake and an enhanced DNA repairing mechanism, which eventually lead to a higher survival rate of LCSC subpopulation.

**Table 1. Summary of liver cancer stem cell biomarkers and related pathways**

Surface markers of LCSCs	Related pathways
CD133	AKT/PKB <sup>[30]</sup> , EGFR-AKT <sup>[38]</sup> , IL-8/CXCL1 <sup>[33]</sup> , Aldehyde dehydrogenases <sup>[25]</sup> , JNK <sup>[39]</sup> , mTOR <sup>[40]</sup> , TGF-β <sup>[41,42]</sup> , Aurora kinase/RalA <sup>[43]</sup> , Notch1 <sup>[44]</sup> , PTEN <sup>[45]</sup> , NF-κB <sup>[45]</sup> , ZFP42/REX1 <sup>[46]</sup> , miR-150/c-Myb <sup>[47]</sup> , miR-142-3p <sup>[48]</sup> , miR-152/KIT <sup>[49]</sup> , miR-130b/TP53INP1 <sup>[29]</sup> , miR-1246/Wnt/β-catenin <sup>[50]</sup> , LncSox4/Stat3 <sup>[58]</sup>
CD44	AKT <sup>[81]</sup> , YAP1/TEAD <sup>[82]</sup> , anti-miR-27a/QD-HA-PEI <sup>[84]</sup> , TGFβ1/ALK5 <sup>[85]</sup> , mTOR <sup>[86]</sup> , FoxM1/ROS <sup>[83]</sup>
CD90	SHH/Gli and IL6/JAK2/STAT3 <sup>[91]</sup> , ABCG2 and Oct5 <sup>[93]</sup> , miR-125a/b <sup>[96]</sup> , has 0067531 <sup>[97]</sup>
EpCAM	Wnt-β-catenin <sup>[5,104]</sup> , CHD4 <sup>[105]</sup> , OSM <sup>[106]</sup> , ATRA <sup>[107]</sup> , EZH2 <sup>[108]</sup> , miR-155 <sup>[109]</sup> , miR-181 <sup>[111]</sup> , miR-216a/217/PTEN/SMAD7 <sup>[110]</sup>
CD47	CTSS/PAR2 <sup>[6,120]</sup> , NF-κB <sup>[122]</sup> , SIRPα <sup>[119]</sup>
CD34	OCT4, SOX2, NAONG, Klf4, c-Myc, and Lin28 <sup>[7]</sup>
C-kit	TGF-β/SMAD2 and c-KIT/JAK1/STAT3 <sup>[132]</sup>
CD13	TGF-β-/EMT <sup>[139]</sup>
CD24	STAT3/NANOG <sup>[10,144]</sup> , Twist2 <sup>[144]</sup>
α2δ1	OCT4, SOX2, NANOG, and BMI1 <sup>[11]</sup> , miR-31/ISL1 <sup>[148]</sup>
OV6	Wnt/β-catenin <sup>[12]</sup>
DLK1	Nanog, SMO, SOX2, Oct3/4 <sup>[153]</sup>
K19	EMT and TGFβ/Smad <sup>[14,155]</sup> , PDGFRα-laminin <sup>[156]</sup> , MET-ERK1/2-API and SP1 <sup>[157]</sup>
LGR5	HGF/ Rspo1 <sup>[173]</sup> , LSD1/Prickle1/APC/β-catenin <sup>[175]</sup>

shown to be more resistant to radiotherapy<sup>[36]</sup> and chemotherapy<sup>[37]</sup>. Our previous study found that CD133+ cancer stem cells conferred chemoresistance caused by abnormal activation of the Akt/PKB pathway<sup>[30]</sup>. Other Aberrant signaling pathways related to CD133+ LCSCs have also been reported and characterized including EGFR-AKT<sup>[38]</sup>, IL-8/CXCL1<sup>[33]</sup>, aldehyde dehydrogenases<sup>[25]</sup>, JNK<sup>[39]</sup>, mTOR<sup>[40]</sup>, TGF-β<sup>[41,42]</sup>, aurora kinase/RalA pathway<sup>[43]</sup>, Notch1 signaling pathway<sup>[44]</sup>, PTEN signaling pathway<sup>[45]</sup>, NF-κB signaling pathway<sup>[45]</sup>. Recently, our team identified ZFP42/REX1 as a key regulator of cancer stemness in CD133+ LCSCs by genome-wide DNA methylation analysis<sup>[46]</sup>. A panel of miRNAs that include miR-150, miR-142-3p, miR-152, miR-130b and miR-1246 have also been found to regulate proliferation, tumorigenicity, invasion, migration and angiogenesis in CD133+ HCC cells<sup>[29,47-50]</sup>.

In summary, aforementioned studies demonstrate that the maintenance of CD133+ LCSCs is modulated by an intricate network of signaling pathways. Cells with varied morphological structures primarily constitute HCC and express distinct hepatic lineage genes. Thereby, there might also be functionally different cancer cell subpopulations that express distinct stemness-associated markers. Wilson *et al.*<sup>[51]</sup> have shown that the



most widely used CSC markers including CD133, CD44, CK19, CD90, EpCAM, and ALDH are not specific to LCSCs. CD133+/ALDH+ cells showed to possess stronger tumorigenicity than their CD133-/ALDH- or CD133-/ALDH+ counterparts<sup>[25]</sup>. We also found and confirmed<sup>[30]</sup> that CD133+/ALDH+ cells possess stronger tumorigenicity than their CD133-/ALDH- and CD133-/ALDH+ counterparts both *in vivo* and *in vitro*. Furthermore, we established a hierarchical organization in HCC to demonstrate HCC tumorigenicity from the highest to the lowest: CD133+/ALDH+ > CD133+/ALDH- > CD133-/ALDH-. Zhao *et al.*<sup>[11]</sup> reported that some subpopulations of liver cancer cells, including CD133+/1B50-1+, CD133+/1B50-1- and EpCAM+/1B50-1+ cells, exhibited high tumorigenicity. CD133+/EpCAM+ cells displayed the highest tumor-initiating activity, compared to CD133+/EpCAM- and CD133-/EpCAM+ cells<sup>[52]</sup>. Elevated CD133 expression is associated with tumor differentiation grades, disease stages and alpha-fetoprotein (AFP) levels. Furthermore, a higher CD133 expression level indicates higher recurrence rates as well as poorer overall survival<sup>[36,53-57]</sup>. Recently, Chen *et al.*<sup>[58]</sup> reported that a long noncoding RNA termed LncSox4, is upregulated in CD133 and EPCAM high-expressed HCC tissues, modulating the self-renewal of liver tumor-initiating cells via Stat3-mediated Sox4 expression.

When CD133 as a target was concerned, Sasaki *et al.*<sup>[54]</sup> developed a DC-based vaccine inhibited the tumorigenicity of CD133+ HCC cells subcutaneously injected into nude mice. Our previous study demonstrated that AKT1 inhibitor can significantly reduce the expression of the survival proteins that was primarily expressed endogenously in CD133+ HCC cells<sup>[30]</sup>. Smith *et al.*<sup>[59]</sup> developed an anti-CD133 antibody-drug conjugate that could inhibit growth of CD133+ HCC cells. Lang *et al.*<sup>[60]</sup> prepared a 131I-CD133 monoclonal antibody (mAb) with specific selectivity that could lead to clinical significance in liver cancer treatment. Huang *et al.*<sup>[61]</sup> developed a bispecific antibody (BsAb) of anti-CD3/anti-CD133 and coagulate it to the cytokine-induced killer (CIK) cells to effectively target and kill CD133+ cells.

## CD44

CD44, firstly was recognized as a lymphocyte homing receptor<sup>[62]</sup>, can be broadly detected in multiple tissues including embryonic<sup>[63]</sup>, hematopoietic<sup>[64]</sup>, mesenchymal<sup>[65]</sup>, and cancer stem cells<sup>[66-69]</sup>. In humans, CD44 gene comprises 20 exons and 19 introns and undergoes complicated alternative splicing to generate CD44 standard form (CD44s)<sup>[70-72]</sup> and CD44 variant splice isoforms<sup>[73]</sup>. CD44 is involved in the interaction between cells and extracellular matrix<sup>[74]</sup>.

Williams *et al.*<sup>[75]</sup> emphasized on the behavior of CD44-regulating stem cell, including cell differentiation and self-renewal and cell-matrix interactions during tumor progression and migration. Isolated CD44s+ cells can effectively form colonies and possess hepatic markers<sup>[76]</sup>. In HCC, CD44s expression is involved to modulation of the mesenchymal phenotype mediated by TGF-beta and its expression level is an unfavorable prognosis factor<sup>[77]</sup>. Proliferation of CD44+ cells and its tumorigenesis can be stimulated by IL6 produced by tumor-associated macrophages (TAMs)<sup>[78]</sup>. CD44 expression is known to be related to invasive and metastatic behavior of liver cancer<sup>[79]</sup>. For instance, FAM83D promotes HCC recurrence by increasing CD44 expression and modulating CD44+ CSCs malignancy<sup>[80]</sup>. Coexpression of CD44 with other markers such as CD133 and CD90 help well identify LCSC phenotypes. CD133+/CD44+ subpopulation is associated with the metastatic capability in the xenotransplantation assay in nude mice<sup>[36]</sup>. CD133+/CD44+ HCC cells exhibits elevated expression of many CSC-related genes and are more chemotherapy-resistant owing to the increased expression of transporters that belong to ATP-binding cassette superfamily<sup>[79]</sup>. Most of CD90+ cells coexpress CD44 and these CD90+/CD44+ cells exhibit an aggressive behavior than the CD90+/CD44- counterpart and easily develop metastases in the nude mice lung<sup>[4]</sup>. Yang *et al.*<sup>[4]</sup> found that administration of anti-CD44 antibody was able to induce apoptosis of the CD90+ and CD90- cells in a dose-dependent manner, and prevented CD90+/CD44+ CSC-derived tumor both locally generated and distantly metastasized<sup>[4]</sup>.

The mechanism of the conversion from terminally differentiated cells that expose to oncogenic factors into CSCs remains largely uninvestigated. Dhar *et al.*<sup>[81]</sup> explained this phenomenon that CD44 could activate AKT to induce Mdm2 phosphorylation nuclear translocation, which terminated the p53 DNA-damage surveillance. This process enables DNA- sequestered hepatocytes to avoid p53-induced apoptosis and to respond to proliferation-related signals that promotes daughter cells transfer to HCC progenitors. CD44s, regulated by the YAP1/TEAD axis, can positively modulate the YAP1 expression along with its target genes through the PI3K/Akt pathway in HCC. This processes composed a feedback loop consisting of CD44s and YAP1, promoting HCC tumorigenesis by regulating cell proliferation and invasion during<sup>[82]</sup>. Kopanja *et al.*<sup>[83]</sup> find that FoxM1 expression level is associated with CD44 expression, suggesting that FoxM1 is required for the expression of CD44 in HCC cells. In liver cancer, anti-miR-27a/QD-HA-PEI exhibit effective anti-cancer effects *in vitro* and *in vivo* via down-regulation of FOXO1 and PPAR- $\gamma$ <sup>[84]</sup>. Galunisertib (LY2157299), a selective ATP-mimetic TGF- $\beta$  inhibitor, can effectively reduce tumor cell vitality via alleviating expression of CD44 and THY1<sup>[85]</sup>. INK128, an ATP-competitive mTOR inhibitor, can suppress CD44+ and sorafenib insensitive HCC *in vitro* and *in vivo*<sup>[86]</sup>.

## CD90

In 1964, CD90 was initially named as  $\theta$  antigen because it had identified in a process to develop an antileukemia xeno-antibody in CH3 AKR strain mice<sup>[87]</sup>. Later in 1969,  $\theta$  antigen was renamed as Thy-1 since the thymus was found to the location where precursors of T cells got mature<sup>[88]</sup>. In the 1980s, Ades *et al.*<sup>[89]</sup> isolated CD90 from MOLT-3, a human T-cell leukemia cell line, demonstrated the presence of CD90 in human. CD90 is a 25-37 kDa glycosylphosphatidylinositol-anchored glycoprotein, and a crucial modulator of multiple cellular events, including immunologic function of promoting T cell activation and nonimmunologic functions such as nerve regeneration, tumorigenesis, metastasis, inflammation, and fibrosis<sup>[90]</sup>. The CD90+ LCSCs isolated from liver cancer tissue specimens shows a strong tumorigenic potential after being implanted into nude mice<sup>[4]</sup>. Zhang *et al.*<sup>[91]</sup> illustrated that by activating the IL6/JAK2 pathway, SHH/Gli could regulated the stem-cell like characteristics of CD90+ LCSC. Cytotoxic drugs 5-FU or epirubicin treatment result in the generation of CD90+ and CD105+ cells *in vitro* in Huh1 and Huh7 cells, which primarily have no CD90+ nor CD105+ cells<sup>[92]</sup>. It was shown by Jia *et al.*<sup>[93]</sup> that being as a closely related cause to chemoresistance, the overexpression of ABCG2 and Oct5 was frequently enriched in CD90+/CD133+ LCSCs. Subcutaneous transplantation of CD90+/CXCR4+ HCC cells to NOD/SCID mice are easily detected in the peripheral blood and able to develop distal metastatic tumors<sup>[94]</sup>. The expression of CD90+ does not overlap with the expression of EpCAM+. Gene expression analysis shows that EpCAM+ cells display epithelial characteristics, while CD90+ cells exhibit a vascular endothelial type of gene profile<sup>[95]</sup>. Exosomes containing miR-125a and miR-125b derived from TAMs mediate stem cell properties in HCC by targeting CD90<sup>[96]</sup>. Zhang *et al.*<sup>[97]</sup> demonstrated that has 0067531 affected the biological functions of CD90+ HCC cells by regulating P13K-AKT signaling pathway. Moreover, CD90 overexpression is shown to be associated with unfavorable prognosis<sup>[98]</sup>. Overall, the results of present studies have suggested that CD90 is a potential biomarker for HCC diagnosis and targeting therapy.

## EpCAM

EpCAM is the first human tumour-associated antigen identified with monoclonal antibodies (mAb)<sup>[99]</sup>, and also the first monoclonal antibody manufactured against for human cancer is murine mAb 17-1A targeting EpCAM<sup>[100,101]</sup>. According to an early elaborate review about EpCAM in cancer<sup>[102]</sup>, it is a type I membrane protein of 314 amino acids, containing two epidermal growth factor-like domains at the extracellular domain and 26 amino acids at intracellular domain. EpCAM is a cell surface marker expressed in almost all the epithelial tumors<sup>[103]</sup>. The EpCAM+ HCC cells possess CSC-like characteristics including an enhanced self-renewal ability and differentiation potential, and are able to initiate the development of highly tumorigenic cancer in NOD/SCID mice. EpCAM is a target gene in Wnt- $\beta$ -catenin signaling pathway<sup>[5,104]</sup>. Chemoresistance as well as stemness of EpCAM+ LCSCs are modulated by abnormal expression of CHD4<sup>[105]</sup>,

OSM<sup>[106]</sup>, ATRA<sup>[107]</sup>, EZH2<sup>[108]</sup>. A group of microRNAs including miR-181, miR-155, miR-181, miR-216a/217 have been found involved in regulating stemness of EpCAM+ HCC cells<sup>[109-111]</sup>. Patients with EpCAM+/AFP+ HCC have higher frequency of portal vein invasion and significantly shorter survival than EpCAM-/AFP- HCC patients<sup>[112]</sup>. Chen *et al.*<sup>[113]</sup> proposed a novel EpCAM-antibody-labeled polymer in nano-vesicles for cancer stem cells-targeted drug and siRNA and displayed higher tumor selectivity and killing efficacy. A recent study revealed that metformin decreased both the EpCAM+ HCC cells abundance and self-renewal capability<sup>[114]</sup>. Babaei *et al.*<sup>[115]</sup> reported that EpCAM targeted nanoparticles of PEG-Au@Si-5-FU exhibited higher cytotoxicity than nontargeted PEG-Au@Si-5-FU in 2D and 3D HepG2 cell cultures. Many EpCAM antibodies are currently available to treat patients with EpCAM+ malignant ascites in preclinical and clinical trials including edrecolomab, adecatumumab, MT110 and catumaxomab<sup>[116]</sup>.

### CD47

CD47 is firstly discovered in 1992 as a surface protein that is frequently expressed in ovarian carcinoma<sup>[117]</sup>. Later studies have exhibited that CD47 is a highly expressed transmembrane protein with various functions<sup>[118,119]</sup>. Lee *et al.*<sup>[6]</sup> identified that CD47 was preferentially expressed in liver TICs, which result in cancer development, self-renewal, metastasis and chemoresistance and significantly influence the clinical prognosis of patients. CD47+ HCC cells preferentially secrete cathepsin S (CTSS), which manipulates liver TICs through the CTSS/protease-activated receptor 2 (PAR2) loop. Suppression of CD47 by morpholino decreases HCC viability and exerts a chemo-sensitization effect through blockade of CTSS/PAR2 signaling pathway<sup>[6,120]</sup>. Increased CD47 expression level has been considered as a negative prognostic factor for a wide variety of cancer<sup>[118,121]</sup>. Lee *et al.*<sup>[6]</sup> unraveled that CD47 expression was enriched on CD133+/CD24+ TICs isolated from a HCC cell line and was increased by serial passage in the presence of doxorubicin and cisplatin, and high CD47 expression conferred chemoresistance and increased the stemness characteristics of TICs. CD47 blockade or down-regulation suppresses HCC development and elevated sensitivity to chemotherapeutic drugs such as sorafenib<sup>[6,122-124]</sup>, while NF- $\kappa$ B-mediated CD47 up-regulation enhances sorafenib resistance<sup>[122]</sup>. Importantly, not only being considered as a LCSC surface marker, expression of CD47 is also involved in innate immune response<sup>[123]</sup>. CD47 is a ligand for signal regulatory protein- $\alpha$  (SIRP $\alpha$ ), which expressed on macrophages and dendritic cells<sup>[125]</sup>. After binding CD47, SIRP $\alpha$  activates a signaling cascade that leads to the inhibition of phagocytosis<sup>[119]</sup>. Macrophage phagocytosis of HCC cells is enhanced after treatment with CD47 antibodies (CD47mAbs) that impede CD47 binding to SIRP $\alpha$ <sup>[118,126]</sup>. Treatment to mice with tumor burden with antibodies that blockade CD47 signaling can produce intensive tumor regression when used solely or integrated with existing therapeutic strategies<sup>[118,121,127,128]</sup> and humanized CD47 antibodies have recently entered human clinical trials (NCT02678338, NCT03717103, NCT03763149, NCT02216409, NCT02367196).

### CD34

Park *et al.*<sup>[7]</sup> identified CD34+ as a newfound LCSC surface marker. SOX2 is one of the vital factors maintaining CD34+ LCSC stemness before colonization, and OCT4, SOX2, NAON, Klf4, c-Myc, and Lin28 are supposed to be associated with stemness maintenance of CD34+ LCSC on feeder cells<sup>[7]</sup>. Park *et al.*<sup>[129]</sup> found that CD34+ LCSCs possessed stemness characteristics and three types of liver carcinomas were directly produced from CD34+ PLC/PRF/5 hepatoma cells (PLC): hepatocellular carcinoma (HCC), cholangiocarcinoma (CC), as well as combined hepatocellular cholangiocarcinoma (CHCs). CD34+ PLCs that express OV6 and their progeny OV6+ cells primarily produce CHC and CC, suggesting that the OV6+ antigen is correlated with human CHC and CC<sup>[129]</sup>. Crosby *et al.*<sup>[130]</sup> addressed that c-kit+ or CD34+ liver cancer cells had the potential to transfer to biliary epithelial cell lineage and might represent biliary epithelial stem cells. Zeng *et al.*<sup>[131]</sup> demonstrated that CD34+ LCSCs and xenografts generated by CD34+ LCSCs exhibited a blended phenotypes, coexpressed stemness and myelomonocytic cell markers. CD34+ LCSCs are often coexpressed with CD45, suggests that the origin appears to be from a hematopoietic precursor, which illuminate a comprehensive understanding of the molecular mechanism of how LCSCs are originated and developed<sup>[131]</sup>.

### C-kit

C-kit, also named as stem cell factor receptor, is a receptor protein of transmembrane type III with intrinsic tyrosine kinase activities to generate human embryonic stem cells. Besides having been used to identify human hematopoietic progenitor cells or hepatic stem cells, c-kit also is capable to sustain the stem cells in an undifferentiated state. The presence of c-kit on HCC cell lines suggests that stem cell factor (SCF) have been considered to play an indispensable role in the manipulation of the proliferative capability of liver cancer cells<sup>[8]</sup>. Fujio *et al.*<sup>[132]</sup> demonstrated that C-kit could be an important factor in the receptor systems, a growth factor related to the biological functions of liver stem cells and the development of bile ducts. It has been reported<sup>[133]</sup> that TGF- $\beta$ /SMAD2 signaling pathway mediates the expression of the c-KIT receptor ligand in a transcriptional level by activating c-KIT/JAK1/STAT3 signaling pathway. SCF activates TGF- $\beta$ 1 ligand expression through STAT3, thereby result in a positive feedback loop between TGF- $\beta$ /SMAD and SCF/c-KIT signaling pathway. The signaling network attenuates TGF- $\beta$ -mediated cell cycle arrest and activates tumor cell to into proliferation, epithelial-to-mesenchymal-transition, migration, and invasion<sup>[133]</sup>. Blockade of C-kit in late cirrhosis might restore TGF- $\beta$  inhibitory effect on normal liver stem cells and prevent initiation and progression of HCC<sup>[134]</sup>. The expression of C-kit is significantly higher in liver cancer patients with advanced clinical stage and is an independent poor prognostic factor of DFS in HCC patients<sup>[135]</sup>.

### CD13

CD13, also referred as aminopeptidase N, is a membranous glycoprotein that has been used to identify leukemia or lymphoma cells<sup>[136]</sup>. CD13 plays vital roles in cancer progression including cell proliferation, invasion, and angiogenesis<sup>[137-139]</sup>. Nagano *et al.*<sup>[140]</sup> demonstrated that CD13 is a surface marker of CSCs in human liver cancer and may have promising therapeutic potentials. It was found by Haraguchi *et al.*<sup>[9]</sup> that CD13 attenuated ROS-induced DNA injury after chemo/radiation treatment and protected cells from apoptosis. They also found that ubenimex, a CD13 inhibitor, alleviated oncogenic and self-renewal ability of CSCs and suppressed CD13+ tumor growth with co-treatment of 5-FU. Kim *et al.*<sup>[141]</sup> reported that upregulated CD13 expression was associated with TGF- $\beta$ -induced EMT-like process, which prevents further increasing of ROS level as well as the induction of apoptosis, supporting the survival of CD13+ CSCs in liver cancer cells. It was also shown by Yamashita *et al.*<sup>[142]</sup> that ubenimex synergistically enhanced the antitumor effects of a chemotherapy regimen composed of 5-FU, CDDP and DXR on HCC cells, and the functions of ubenimex were associated with enhanced intracellular ROS levels.

### CD24

CD24 is a glycosylated and mucin-like cell surface glycoprotein with relatively high expression in stem/progenitor cells and related to formation and development of CSCs isolated from breast, colon, ovary, pancreas<sup>[143,144]</sup>. Huang *et al.*<sup>[145]</sup> firstly cloned the full-length CD24 cDNA sequence from human HCC cells and identified that CD24 mRNA overexpression was associated with p53 mutation and tumor differentiation. Lee *et al.*<sup>[10]</sup> reported CD24 as a surface marker of LCSCs. They<sup>[10]</sup> also documented that CD24 was upregulated in chemoresistant tumors after cisplatin treatment in immunodeficient mice model. Significantly, CD24 expression largely overlaps with expression of CD133 and EpCAM in HCC<sup>[10]</sup>. CD24+ HCC cells have a great impact on clinical prognosis of patients, and play a vital role in self-renewal, differentiation, maintenance, and metastasis of tumors<sup>[10]</sup>. Self-renewal and tumor initiating behavior of CD24+ LCSCs is regulated by STAT3-mediated NANOG regulation<sup>[10]</sup>. It was demonstrated by Liu *et al.*<sup>[146]</sup> that the pathway of Twist2-CD24-STAT3-NANOG was crucial to the regulation of self-renewal of CD24+ LCSCs.

### $\alpha 2\delta 1$

In 2010, García *et al.*<sup>[147]</sup> reported that when the expression of calcium channel  $\alpha 2\delta 1$  subunit was inhibited, migration, adhesion as well as spreading of myoblasts were impaired, whereas the L-type calcium maintained unaffected, suggesting a newfound function of the  $\alpha 2/\delta 1$  subunit in extracellular signaling. Later studies

have confirmed that calcium channel  $\alpha_2/\delta_1$  subunit is a potential marker for CSCs in laryngeal squamous cell carcinoma<sup>[148]</sup> and in non-small cell lung cancer<sup>[149]</sup>. Zhao *et al.*<sup>[11]</sup> identified  $\alpha_2/\delta_1$  subunit as a LCSCs marker and developed its monoclonal antibody named 1B50-1, which had positive therapeutic effects on HCC xenograft by eradicating LCSCs.  $\alpha_2/\delta_1$  + liver cancer cells have stemness characteristics, including the expression of stemness-related genes such as SOX2, OCT4, BMI1, and NANOG, the capability of self-renewal, invasiveness, and to produce both  $\alpha_2/\delta_1$  + and  $\alpha_2/\delta_1$  - cells<sup>[11]</sup>. Recently, Zhang *et al.*<sup>[150]</sup> discovered that miR-31 could negatively manipulate the self-renewal capability of  $\alpha_2/\delta_1$  + LCSCs via sequestering ISL1, implying a potential therapeutic strategy for directly targeting liver TICs.

## OV6

1998, Roskams *et al.*<sup>[151]</sup> identified that reactive ductules and intermediate hepatocyte-like cells originated partially from differentiation and activation of progenitor cells. It has been put forward that OV6 in human liver can help identify cells owing a progenitor stem cell-like characteristics, which has the ability to differentiate into OV6+ ductular cells or lobular hepatocytes. OV6+ is a specific phenotype of oval cells that has been originally identified in the livers of tumor transplanting rats, and is identified as a surface marker of human liver progenitor cells in 2008<sup>[152]</sup>. In 2012, Yang *et al.*<sup>[12]</sup> further demonstrated that OV6+ HCC cells not only possessed a stronger capacity to form spheroids, but also showed stronger tumorigenic and metastatic characteristics. These results suggest that OV6+ HCC cells are highly capable of self-renewal and forming tumors. Wnt/ $\beta$ -catenin signaling plays an indispensable role in the induction and expansion of OV6+ subpopulation within tumor tissues. Thereby, OV6 is considered as an effective LCSCs surface marker. Additionally, Yang *et al.*<sup>[12]</sup> also demonstrated that overexpression of OV6 enhanced the invasive and metastatic characteristics of HCC CSCs so that the number of OV6+ CSCs increased in patients diagnosed with liver cancer indicated poorer clinical outcomes and prognosis.

## DLK1

DLK1 has shown to be expressed in fetal liver, but scarcely expressed in neonatal and adult liver in mice and rats<sup>[153]</sup>. Huang *et al.*<sup>[154]</sup> demonstrated that proliferation of SMMC-7721 cells was significantly enhanced by exogenous DLK1, whereas colony-forming ability, cell growth, and tumorigenicity of Huh-7, Hep3B, and HepG2 cells were significantly impeded by the suppression of endogenous DLK1 via RNA interference. It was identified by Li *et al.*<sup>[13]</sup> that the enhancing effect of DLK1 in tumorigenicity and cancer stemness could potentially be used as a molecule target for therapies against LCSCs. DLK1+ cells have been discovered in all 17 HCC cell lines and showed a more potent capability of clonogenicity *in vitro* and tumorigenicity in animal models. In addition, some stemness markers have been identified upregulated in DLK1+ Huh-7 and Hep3B cells including NANOG, SMO, SOX2, Oct3/4, CD133, CD90, and EpCAM. The isolated DLK1+ HCC cells are possess strong therapeutic resistance to conventional cytotoxic agents such as doxorubicin, cisplatin, epirubicin, and 5-FU<sup>[155]</sup>.

## K19

Cytokeratin 19(K19) is a newfound CSC surface marker associated with EMT and TGF $\beta$ /Smad signaling pathway<sup>[14]</sup>. K19 disappears from liver cells but remains in bile duct cells at the 10th differentiation week, which is an important step in the organogenesis of liver<sup>[156]</sup>. It has been reported that using 18F-FDGPET and CYFRA 21-1 can identify K19+ LCSCs in HCC. In patients with HCC, K19 expression is significantly correlated with GLUT1 expression and FDG accumulation, and K19 regulated 18F-FDG uptake via TGF $\beta$ /Smad signaling pathway<sup>[157]</sup>. Besides the TGF $\beta$ /Smad signaling pathway, many other signaling pathways have been documented as well. The PDGFR $\alpha$ -laminin B1-K19 cascade drives tumor development at the invasive front of HCC<sup>[158]</sup>. Rhee *et al.*<sup>[159]</sup> reported that expression of K19 in HCC is modulated by fibroblast-derived HGF via a MET-ERK1/2-AP1 and SP1 axis. K19+ cells have high proliferation potential and doxorubicin, 5-fluorouracil and sorafenib resistance<sup>[14,160]</sup>. K19 expression exhibits strong correlation with increased



tumorigenicity, decreased tumor differentiation potential, metastasis and invasion and poor prognosis in HCC<sup>[160-163]</sup>, with profiling study shows that K19+ HCCs highly express invasion or metastasis-related genes (TACSTD2, VASP, LAMC2, LAMB1, PDGFRA), biliary/HPC markers (NOTCH2, GSTP1, CD133, JAG1) and members of the miRNA-200 family (miR-200c, miR-141)<sup>[160]</sup>. A recent study showed that K19+ cells were not involved in the early clonal expansion of rat hepatocarcinogenesis, and K19 expression arose in preneoplastic hepatocyte lesions undergoing malignant transformation. In addition, they also indicated that K19 positivity in HCCs did not necessarily reflect the cell of origin of the tumor, but rather the plasticity of preneoplastic cells during the tumorigenic process<sup>[164]</sup>.

## LGR5

In 2007, LGR5, a G protein-coupled receptor with a seven transmembrane domains<sup>[165]</sup>, was firstly identified as a surface marker of intestinal stem cells<sup>[166]</sup>. Later, it has been applied to identify homeostatic stem cells in various organs such as ovaries, hair follicles, mammary gland, and stomach<sup>[166-169]</sup>. LGR5 has been reported to involved in regeneration of damaged tissues in the small intestine and colon, liver, pancreas, and stomach<sup>[15,170-172]</sup> and in CSCs that regulates tumor proliferation<sup>[173,174]</sup>. Carbon tetrachloride treatment enhances both fibrosis and LGR5+ liver stem cell growth, whereas LGR5 downregulation aggravates fibrosis. HGF together with Rsp01 increases the number of LGR5+ liver stem cells and enhances hepatic function by inhibiting fibrosis<sup>[175]</sup>. Both Carbon tetrachloride-induced acute damage and oval cell response to damage can induce LGR5+ stem cells/progenitors actively engaged in hepatic reconstitution via *de novo* generation of hepatocytes<sup>[15]</sup>. Effendi *et al.*<sup>[176]</sup> addressed that LGR5 upregulated HCC cells showed more potent colony-forming capability and possessed higher therapeutic resistant to a cytotoxic drug and weakened migration ability than the controls. Further, LGR5 overexpressed HCC cells produces nodule-type metastases in the livers of immunodeficient mice, whereas vector-transfected HCC cells generates more invasive tumors<sup>[176]</sup>. Lei *et al.*<sup>[177]</sup> unraveled that the LSD1/Prickle1/APC/ $\beta$ -catenin signaling axis is engaged in regulating the stem characteristics and chemoresistance of hepatic LGR5+ LCSCs.

## TREATMENT TARGETING LCSCs

Using surface markers to identify and isolate LCSCs remains an initial and important step of CSC-targeting therapy. Immunotherapy uses specific antibodies to target LCSC surface makers can be integrated with conventional chemotherapy, radiotherapy and surgery to promote therapeutic effects. The most frequently-used LCSC-associated surface markers along with clinical strategies that target them are demonstrated as follows.

One of the current therapeutic approaches to target directly LCSCs is nanomedicine-based therapy, in which medication delivery and intake are effectively controlled in nanoscale<sup>[178]</sup>. Epirubicin-adsorbed nanodiamonds displayed high efficacy in inducing the elimination of chemoresistant LCSCs<sup>[179]</sup>. Poly lactic-co-glycolic acid-encapsulated disulfiram strongly inhibits LCSCs and has a synergistic cytotoxicity with 5-FU or sorafenib<sup>[180]</sup>. Gao *et al.*<sup>[181]</sup> developed a GPC3-targeted CAR and found that it obviously suppressed HCC growth. Overexpression of ANXA3 increased the number of CD133+ cells and positively associated with tumorigenicity of CD133+ cells. The underlying mechanism of ANXA3-mediated maintenance of LCSCs stemness involved the HIF1A/Notch pathway. ANXA3 upregulated dendritic cells could induce more active T cells, which could preferentially kill CD133+ LCSCs<sup>[182]</sup>. Xu *et al.*<sup>[183]</sup> addressed that Hep-12 cells owing stemness properties, are susceptible to autologous-activated tumor-infiltrating lymphocytes-mediated recognition and cytotoxicity. What's more remarkable, the authors put forward that it may be the first evidence to demonstrate the hypothesis that immunotherapy can be used to target recurrent HCC cells with stem cell-like properties. Bone morphogenetic protein-9 is a potent growth inhibitor of hepatocellular carcinoma and reduces the liver cancer stem cells population by suppressing the expression of five prominent LCSC markers, including CD44, CD90, AFP, GPC3 and ANPEP<sup>[184]</sup>. In current clinical practice (according to

NCCN guidelines for hepatobiliary cancers, version 4.2018), several oral targeted drugs have been approved, including sorafenib, lenvatinib, regorafenib, showed a median overall survival of 10.7 to 13.6 months<sup>[185-187]</sup>. Immunotherapy has also been considered as one of the promising treatments and is being actively studied and optimized in liver cancer progression and metastasis<sup>[188]</sup>. The most *ex vivo* investigated and clinically relevant check-point proteins are CTLA-4, PD-1, and PD-L1. Nivolumab and pembrolizumab, both as PD-1 antibodies with similar efficacy, are now approved to treat liver cancer clinically. Nivolumab showed an objective response of 20%, contained 1% complete response and 18% partial response, and stable disease is 45%<sup>[189]</sup>, when pembrolizumab is concerned, the objective response is 17%, and the complete responses, partial response, and stable disease were 1%, 16% and 44% respectively<sup>[190]</sup>. The development of new drugs enable the improvement of object responses and survival of advanced liver cancer, what's deserved notification is that drugs such as sorafenib, lenvatinib, regorafenib, nivolumab and pembrolizumab now available in clinic can obtained about 1% clinical complete response in small number of patients, revealing the pathways these drugs targeted may have the potential to diminished almost the whole tumor including the LCSC, and further exploration of the underlying mechanism of cancer development and progression is promising.

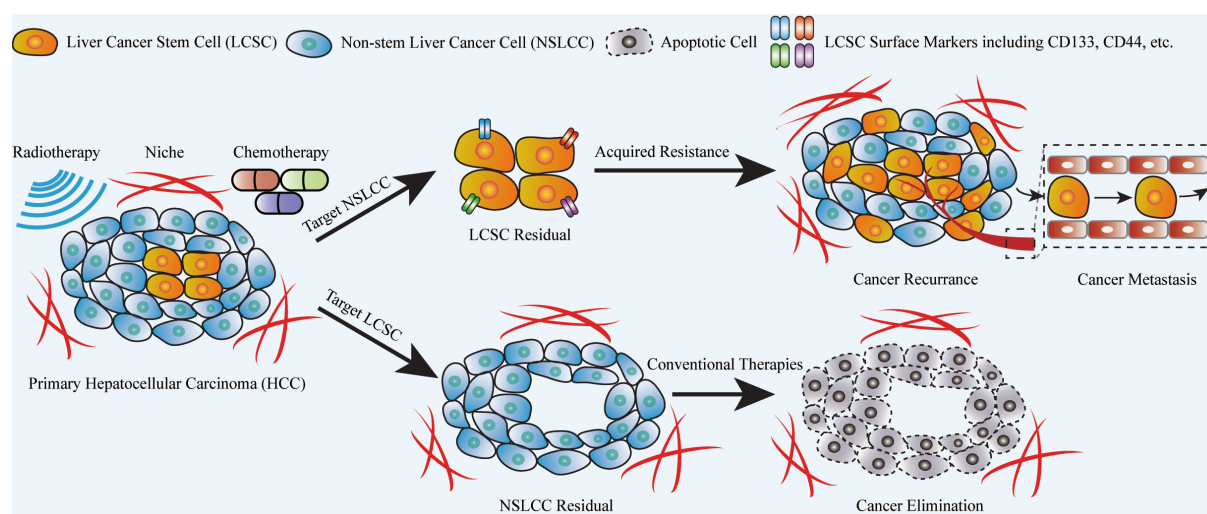
Alternative therapies including induction of LCSC differentiation and apoptosis are also promising. Conventional chemotherapy and radiotherapy have been proven to successfully eradicate terminally differentiated cancer cells but fail to influence CSCs<sup>[191,192]</sup>. Therapies that induce LCSC differentiation can be combined with those conventional therapies to efficiently diminish LCSC subpopulation and impede cancer development since the differentiation process obtains higher priority than cancer self-renewal process. There are intensive studies developing and optimizing the differentiation-inducing agents including retinoic acid, histone deacetylase inhibitors, tyrosine-kinase, Hippo/YAP signaling pathway inhibitors<sup>[192-195]</sup>. Apoptosis is also a vital cellular mechanism that regulates cell death through a complicated signaling network. LCSCs can escape from apoptosis process, therefore they possess unlimited and uncontrollable self-renewal ability to initiate cancer development and invasion. Induction of apoptotic mechanism in LCSCs by using microRNA hold great promise in cancer treatment so that many studies have been focused on developing therapies to activate apoptotic pathways in LCSCs<sup>[196]</sup>.

These inducing therapies would be feasible and efficient if LCSCs could be specifically identified according to the expression profile of varied LCSC surface markers. However, there is no LCSC surface marker has been identified and proven to have the ability to represent the entire subpopulation of LCSCs, thereby the inducing therapies remain challenging so far. It leads us to consider that whether or not the potential combination of varied LCSC surface markers can improve the specificity in identification of LCSCs.

## SUMMARY

The above-mentioned discussion offers a promising insight of how LCSCs can be employed in clinical diagnosis and treatment for liver cancer development, progression, metastasis and resistance [Figure 2]. During the last decades, the compelling knowledge about CSCs has enabled rapid advances of drugs targeting CSCs and gradually emerged as an indispensable class of therapies. Numerous agents with the capabilities to inhibit CSC-associated signaling pathways, including Notch pathway, Hedgehog pathway and WNT pathway, have been approved for clinical use. The recent development of culture condition allows CSCs to undergo long-term proliferation in spheroids and organoids, thus offer researchers with an innovative platform for identifying new CSC markers with high specificity and efficacy. Moreover, because organoids are directly derived from primary tumor tissues, the organoid technique provides a unique perspective to researchers so that we can comprehensively investigate the heterogeneous functions of CSCs in recurrence, metastasis, chemoresistance and radioresistance.

From a broader perspective, there is no doubt that drugs targeting CSC should be considered as a promising clinical strategy for therapeutic intervention, although the rate of treatment failure that aims to effectively



**Figure 2.** Clinical implication of conventional cancer therapy and LCSC-targeting therapy. Conventional chemotherapy and radiotherapy are frequently used to treat liver cancer, effectively targeting the non-stem liver cancer cells (NSLCCs) but not liver cancer stem cells (LCSCs). The LCSC residual can be re-activated to enrich the LCSC subpopulation and eventually trigger cancer recurrence and metastasis with a more aggressive phenotype. With the help of varied LCSC surface markers that can specifically and effectively identify LCSC heterogeneity, LCSC-targeting therapy is believed to be capable of eradicating the CSC subpopulation in liver cancer. Integration of LCSC-targeting therapy and conventional chemo-/radiotherapy might lead to complete cancer elimination without further development and invasion. LCSC-targeting therapy has generated many promising results in pre-clinical trials and there are intensive efforts from researchers and clinicians for further research.

eliminate CSCs remains relatively high so far. It is worth considering that such treatment failure might be resulted from inefficiency of drug delivery instead of inefficiency of the drugs. Promoting the efficacy of drug delivery and developing alternative approaches to target CSCs represent one of the most foremost fields to be explored. In addition, early diagnosis of cancer by using CSC markers remains as an important ramification that can prevent development, metastasis and resistance. Hence, there is an emergency for greater concentration on identification of CSC markers that can specifically and effectively represent tumor grades and disease stages. Integrating the development of early diagnosis techniques with a comprehensive understanding of CSC surface markers that drive a benign stage to a malignant stage can enable patient-specific and efficient early intervention and offer a balanced approach to regulating cancer development and invasion.

## DECLARATIONS

### Authors' contributions

Designed the structure and outline of the manuscript, instructed writing the intellectual part, and approved final publication: Guan XY

Reviewed the literatures and wrote the draft: Zhang JL

Revised the manuscript and drew the pictures: Gong LQ

Review and minorly revised the manuscript: Yan Q, Zhou NN, Lee VHF

### Availability of data and materials

Not applicable.

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## Conflicts of interest

All authors declared that there are no conflicts of interest.

## Ethical approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

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Review

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# Management of concomitant hepatocellular carcinoma and chronic hepatitis C: a review

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## Abstract

Our comprehensive review focuses on the treatment of hepatitis C virus in the context of hepatocellular carcinoma and vice versa, highlighting the ongoing complexity of this clinical scenario. There remain multiple unanswered questions when considering the management of these complex patients and, with a rapidly-changing treatment landscape for both chronic hepatitis C and hepatocellular carcinoma, these questions are only going to grow. Treatment timing, interactions and the impact of one disease condition on the other are vitally important, though guidance generally remains non-specific, suggesting that we make these decisions on a case-by-case basis. We focus on the current evidence for managing these cases, depending on disease stage and treatment type.

**Keywords:** Hepatocellular carcinoma, liver cancer, hepatitis C virus, direct-acting antiviral agents

## BACKGROUND

Hepatitis C virus (HCV) accounts for a third of all hepatocellular carcinoma (HCC) cases worldwide, with a 1%-8% annual risk of HCC development in cirrhotic HCV-infected patients<sup>[1-4]</sup>. The presence of cirrhosis greatly increases the risk of HCC development in HCV-positive patients, with the prominent pro-



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fibrotic effect of the virus undoubtedly playing a role<sup>[5]</sup>. Involvement of a direct mutagenic mechanism in addition to this is purely speculative at this stage, though animal and human models have demonstrated a potentially increased risk of HCC development in the non-cirrhotic HCV-positive patient<sup>[6-8]</sup>. Though HCV is a RNA-virus that cannot integrate into the host genome, it produces gene products that have been shown to have mutagenic effects in *ex vivo* human models, with further work in human models required to establish how this translates to *in vivo* processes<sup>[9,10]</sup>.

Following HCV clearance, patients see a reduction in liver-related morbidity and death<sup>[11,12]</sup>. This has also been shown in HCV-cirrhotic patients with successfully treated HCC, in which hepatic decompensation has been found to be the major driver of death, highlighting the importance of preserving liver function in this group<sup>[13]</sup>. Understanding how HCV clearance might impact upon the pro-carcinogenic environment remains uncertain, though this will likely become clearer as our understanding of the post-SVR liver progresses.

The timing and duration of HCV treatment in patients with HCC is becoming increasingly important, though guidance generally remains that we should make these decisions on a case-by-case basis. Another consideration is the interaction between HCC and HCV treatments, particularly with the swelling tide of HCC treatments waiting to break.

## HCV TREATMENT REGIMENS

HCV evokes a strong T cell-mediated reaction in the acute phase that successfully clears the virus in 30% of patients. In the remaining 70% of patients, multiple viral escape mechanisms - including inactivation of pathways that induce interferon - overwhelm the immune system, resulting in chronic infection<sup>[14]</sup>. Endogenous interferons are part of our natural arsenal against viruses, which explains the previous successes of exogenous interferon (IFN) in the treatment of HCV. Prior to 2011, prolonged courses of IFN were the mainstay of treatment outside of clinical trials for those infected with HCV, with or without concomitant ribavirin, with success rates ranging between 5%-50% depending on duration of therapy, stage of liver disease and genotype<sup>[15-17]</sup>. The exact mechanism by which ribavirin targets HCV is not completely understood, but is thought to have an effect on viral replication<sup>[18]</sup>. The addition of ribavirin improved outcomes but these regimens were poorly tolerated by many and improved alternatives were desperately sought.

The management of HCV has transformed over the past decade, with sustained virologic response (SVR) rates in excess of 95% following treatment with newer directly-acting antiviral agents (DAAs)<sup>[18]</sup>. Mechanistically, DAAs inhibit viral replication by inhibiting certain non-structural viral proteins, ultimately resulting in viral clearance<sup>[19]</sup>. DAA use has become more widespread and, with that, our understanding of their interaction with other treatments will improve.

Prior to the use of DAAs, IFN-based regimens were used in certain subgroups of patients, with significant histopathological improvements seen following successful treatment. It is more difficult to assess post-SVR histopathological changes as we are no longer required to perform pre-treatment biopsies as we were in the IFN-era. However, when assessing histopathology within 2 years of treatment, though there is suggestion of fibrosis regression, persistent inflammatory activity has been observed despite the absence of the virus<sup>[20]</sup>.

## Interferon-based therapies and HCC

Historical treatment with IFN-based therapies targeted patients with little or no fibrosis; a low-risk group in terms of HCC development<sup>[21]</sup>. In patients with advanced fibrosis or cirrhosis that were treated with maintenance pegylated interferon (PEG-IFN) in the HALT-C trial, it was noted that maintenance

therapy did not reduce the risk of HCC development<sup>[22,23]</sup>, though this and other studies have shown that reduction of HCV RNA correlated to a reduction in HCC risk, which reduced further still in cases of HCV eradication<sup>[23-26]</sup>. Further to this, IFN-based treatment may decrease the HCC recurrence rate in successfully treated HCC patients following curative therapy<sup>[17]</sup>. A speculative link has been drawn between the inhibitory effect of IFN on HCC proliferation, which may have an additional impact on HCC outcomes to the antiviral effect of IFN<sup>[17,27]</sup>.

Current HCC treatment guidance is widely dictated by the Barcelona Clinic Liver Cancer (BCLC) criteria, which stratifies liver cancer cases into stages based on tumour burden, liver disease and performance status, allocating treatments accordingly<sup>[28]</sup>. Curative treatments include resection, locoregional therapy (LRT) or liver transplantation for those that fall within Milan criteria. Outside of this, palliative LRTs, targeted systemic therapies or immunotherapy are the recommendation in advanced HCC.

Some work has been done to assess the role of IFN as an adjuvant agent in post-resection cases, with promising early results in terms of mortality<sup>[29-32]</sup>. In the DAA era, the use of IFN-based regimens has declined drastically and so, even in the absence of evidence for a similar role for DAAs, using IFN in this context is unlikely to be recommended. Some debate continues over timing of HCV treatment in this subset of patients; these medications are in their infancy and many questions remain that may take time to address.

### **DAAs and HCC**

DAAs have revolutionised HCV treatment with SVR rates exceeding 95%. In the presence of HCC, SVR rates are lower at 60%-90%<sup>[33]</sup>, the reasons for which are current sources of speculation [Figure 1]<sup>[33-35]</sup>. Certainly, the tumour microenvironment expertly creates multiple mechanisms of immune escape in order to survive and so it stands to reason that HCV-infected cells within the tumour may evade antiviral treatment in the same vein. In addition to this, it has been proposed that penetration of DAAs to the HCV-infected HCC tissue is suboptimal, not only due to altered architecture but also as tumour blood supply is from the hepatic arterial branches as opposed to the portal venous system<sup>[35]</sup> [Figure 1]. As original trials for newer DAAs often exclude patients with HCC from their eligibility criteria, data on this cohort is limited. Subsequent data has shown a decrease in SVR rates, though how this might shape our treatment regimens - be it duration or drug combination - is not yet clear.

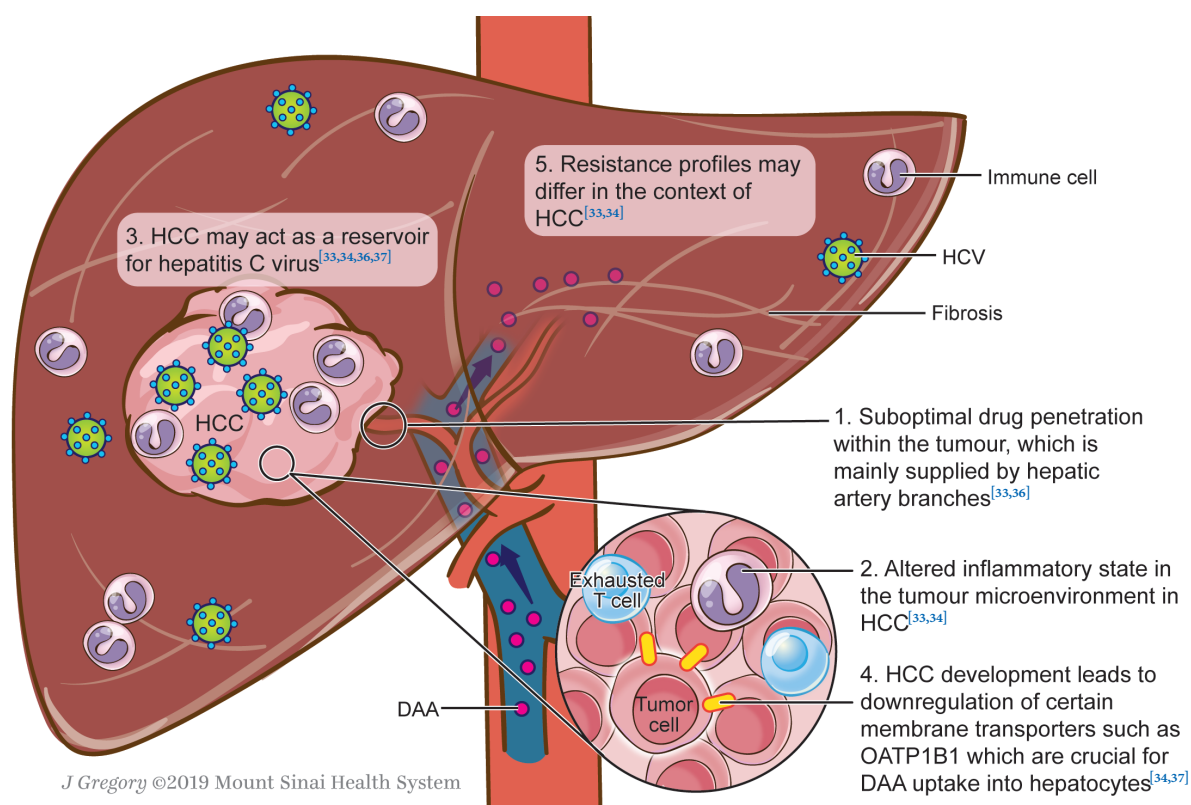
Some of the discordance between studies may be due to the fact that some of the studies that have shown association between active HCC and lower SVRs included DAA regimens with sub-optimal combinations (e.g., SOF/RBV). It will be important to assess SVR rates in this population with the new generation of DAAs.

### **DAA regimen adaptations for HCV in the context of HCC**

Recent data on efficacy of DAAs in patients that have concurrent HCC suggests that SVR rates are lower than those in the absence of HCC<sup>[35-40]</sup>. This includes both patients that respond and then relapse, as well as primary non-responders. It is, therefore, unclear whether these lower SVR rates are due to inadequate duration of therapy, treatment resistance or a combination thereof [Figure 1]. With this in mind, further trials are required to guide our treatment approach in this cohort of patients, be it prolonged courses of DAA therapy or treatment combinations.

### **HCC post-DAA treatment**

Some initial concerns regarding allegedly high rates of HCC after DAA-induced SVR compared with IFN-induced SVR have caused controversy<sup>[41]</sup>. This could be explained by altered immune surveillance in the post-DAA liver environment, which may alter T cell responses and therefore have an impact on



**Figure 1.** Proposed mechanisms for lower SVR rates in HCV in the presence of HCC, compared with non-HCC HCV<sup>[34-37]</sup>

cancer evasion from the host immune system<sup>[41,42]</sup>. On reassessment of the data, the apparent increase in HCC seen in the post-DAA population is at least in part thought attributable to bias within the patient cohorts<sup>[33,35,43]</sup>. A recent systematic review by Waziry *et al.*<sup>[44]</sup> was unable to find evidence that DAA therapy is associated with subsequent HCC development when compared with IFN therapy, though the reviewed studies were small, observational and sometimes lacking in useful clinical detail with significant inter-trial heterogeneity also noted. Furthermore, when assessing overall incidence of HCC rather than recurrence alone, the risk of developing HCC reduces by 71% in DAA-induced SVR compared with treatment failure<sup>[45]</sup>.

We eagerly await the outcome of ongoing clinical trials that are studying this potential association, which aim to assess recurrence rate of HCC as well as mapping the behaviour of HCC during and after DAA treatment of HCV<sup>[46-50]</sup>. Further research and debate are ongoing and in depth discussion on this topic is beyond the scope of this review.

## HEPATITIS C DAA TREATMENT CONSIDERATIONS BASED ON HCC THERAPY

### DAAs and locoregional therapies

LRT is used with curative intention in the early stages of HCC (for example microwave ablation, radiofrequency ablation, ethanol injection) and as palliative interventions in the intermediate/advanced stages [for example chemoembolization, selective internal radiation therapy (SIRT), stereotactic body radiotherapy (SBRT)]<sup>[51]</sup>. Multiple factors should be considered when deciding whether or not to prescribe DAAs in patients with HCC amenable to LRTs, and when.

Firstly, because LRTs are recommended only in patients with well-compensated liver disease<sup>[51]</sup>, achieving

SVR may significantly improve a given patient's clinical liver function, making them eligible for a therapeutic procedure. A recent multicentre study showed that 24% of the 122 patients with decompensated cirrhosis could be delisted due to improvement after HCV eradication<sup>[52]</sup>. Three patients with HCC that were originally listed for liver transplantation improved such that they were able to undergo resection or SIRT after achieving SVR.

Secondly, with some studies showing decreased SVR rates in the presence of active HCC, consideration should be given to treating the HCC with LRTs prior to DAA initiation. One retrospective study demonstrated that failure to achieve SVR rates was higher in patients with active HCC when compared to patients with inactive or resected HCCs or in patients with no HCC<sup>[37]</sup>. Similarly, a large prospective national multicentre study showed that successfully treated HCCs (resection, ablation, or chemoembolization) do not influence subsequent SVR rates with DAA therapy. DAA therapy was given at least 6 months after successful treatment (i.e., complete response) of the HCC<sup>[53]</sup>. As radiological response following LRT does not always accurately predict pathologic necrosis - and in some cases this may be overestimated - this underscores the importance of this time window before pursuing HCV treatment. Conversely, preliminary data from the HCV-TARGET study comparing SVR rates of cirrhotic patients with HCC with those of patients without HCC, again showed significantly inferior SVR rates in the former, but also showed no difference in SVR rates between those patients with active HCC versus those with complete response to LRTs<sup>[38]</sup>. In another study of 62 patients, who were started on DAAs just after radiological documentation of complete response to treatment (mainly radiofrequency ablation, TACE, microwave ablation, and percutaneous ethanol injection), the SVR rate was only 64.5%<sup>[54]</sup>. Importantly, 42% had HCC recurrence, and in most cases within the following 6 months after initiation of DAAs, suggesting the presence of residual HCC despite documentation of radiological response. Hence, in this case the presence of viable HCC could have contributed to the low SVRs.

Finally, in cases where LRTs fail to achieve complete necrosis of the tumour, DAA metabolite distribution to viable HCC areas may be compromised through multiple mechanisms. Impaired blood supply will impair penetration into the HCV-infected tumour tissue, particularly with procedures that include vascular embolization such as TACE. Altered tissue architecture may also have an impact on tissue penetration, as LRTs induce fibrosis, which seems to be particularly accentuated with SIRT<sup>[37,55]</sup>. There may, therefore, be a role for re-treatment of HCCs in an attempt to achieve a complete response before initiating DAAs. In reality, however, many physicians commence HCV treatment prior to HCC treatment, with an unmet need in research into this area.

In summary, the evidence is variable and further trials in this area may help to confirm the best approach where an HCC in chronic HCV cases is amenable to LRT. Until more evidence is available, it may be prudent to treat an active HCC with LRTs and achieve a complete and sustained response before initiating DAAs, in order to improve SVR rate. Where a patient is anatomically a candidate for LRT but is not suitable due to poor liver function, one might consider treating the HCV in order to improve the patient's clinical condition.

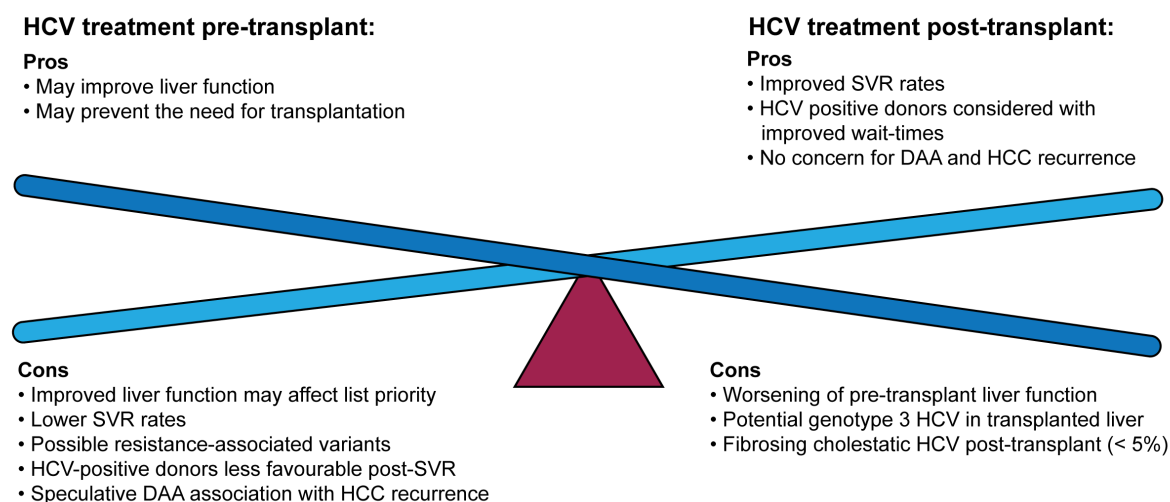
### **DAAs and liver transplantation**

There is also much speculation regarding timing of HCV treatment in patients with HCC, particularly in those for which liver transplantation is being considered<sup>[35]</sup> [Table 1]. Where no guidelines exist that prevent the transplantation of HCV-viraemic organs into HCV-negative recipients, limited data is available into this practice and so it is not generally accepted. In liver transplantation specifically, outcomes of HCV-viraemic organs into HCV-positive recipients do not appear to negatively impact patient or graft survival, therefore many centres have adopted this practice<sup>[35]</sup>. Treatment of HCV prior to transplantation may



**Table 1. Studies assessing DAAs in HCC patients** [35-38,52,69,78,79]

First author	Study characteristics	Patient characteristics		DAA therapy		Treatment Regimen	Treatment Duration	Outcome
		Genotype	Cirrhosis	HCC vs HCV	Timing			
Saberi <i>et al.</i> [35], 2017	Retrospective study assessing pre transplant HCV HCC, $n = 21$ with both HCC & HCV	71% genotype 1	All cirrhotic, 52% CPA	Pre-transplant patients, some had received HCC treatment prior to HCV treatment though exact timings unclear		Multiple regimens used	12-32 weeks	33.3% HCV relapse prior to transplant
Pascasio <i>et al.</i> [52], 2017	Retrospective study assessing delisting of patients post DAA therapy, $n = 116$ with HCC & HCV (also $n = 122$ HCV without HCC)	73% genotype 1	All cirrhotic, 72% decompensated with or without HCC, 28% compensated with HCC	Pre-transplant patients at the start of HCV therapy, no bridging HCC therapy given prior to HCV treatment		Variable	Not specified	86% SVR across all groups 92% SVR in compensated cirrhosis with HCC vs 83% in decompensated cirrhosis with or without HCC
Curry <i>et al.</i> [78], 2015	Prospective study assessing efficacy of Sof/Riba in pre-transplant HCV HCC, $n = 61$ with HCC & HCV (also $n = 122$ HCV without HCC)	73% genotype 1	All CPA cirrhotic	Pre-transplant patients, no comment on prior or subsequent bridging HCC therapy		Sofosbuvir + Ribavirin	Up to 24 weeks or liver transplant, whichever came first. Latterly protocol changed from 24 to 48 weeks due to observed relapses.	70% SVR12 achieved Of all 61 patients, 49% maintained post-transplant SVR 93% of the 46 patients that underwent liver transplant had HCV RNA < 25IU/mL at the time of transplant, but 30% of these relapsed.
Prenner <i>et al.</i> [37], 2017	Retrospective study comparing SVR in HCV with vs without HCC, $n = 135$ with HCC & HCV (also $n = 284$ HCV without HCC)	85% genotype 1	All cirrhotic, 81% CPA	Mixture of treated and untreated HCC, with 43% of patients with active tumour at the time of DAA treatment, exact timings unclear		Multiple regimens used	Not specified; the authors classified regimens as adequate or inadequate	15% treatment failure, with 93% of these cases having active tumour 54% SVR in cases with active tumour, 97% SVR in treated HCC
Chang <i>et al.</i> [79], 2017	Retrospective study assessing SVR and AEs in SOF-based DAA treatment of HCV in Asian Americans, $n = 17$ with HCC & HCV (also $n = 93$ HCV without HCC)	64.5% genotype 1	50% cirrhotic	HCC treatment given either prior or subsequent to HCV treatment - not specified how many in each category		Sofosbuvir-based	8-24 weeks	SVR12 93% overall, but 82% in patients with concomitant HCC - this did not reach statistical significance.
Beste <i>et al.</i> [36], 2017	Retrospective study assessing SVR in HCV HCC patients, $n = 624$ with HCC & HCV (also $n = 16,863$ HCV without HCC)	73% genotype 1	85% cirrhotic	Post-transplant ( $n = 142$ ) or post HCC therapy ( $n = 482$ ), exact timings unclear		Multiple regimens used	8-24 weeks	94% SVR post-transplant, 74% SVR pre-transplant 91.9% SVR in non-HCC patient Highest SVR in genotype 1 patients, lowest in genotype 3 patients
Radhakrishnan <i>et al.</i> [38], 2017	Retrospective study comparing SVR in cirrhotic HCV with vs without HCC, $n = 133$ with HCC & HCV (also $n = 89$ HCV without HCC)	78% genotype 1	All cirrhotic	Assessed complete response vs partial or non-response to HCC treatment, up to 6 months prior to DAAs		Not specified	Variable	Non-significant trend toward improved SVR rates in treated HCC vs active HCC 83.1% SVR in HCC (vs 90.3% non-HCC)
Revuelta-Herrero <i>et al.</i> [69], 2018	Prospective study assessing patients on a specific DAA regimen in combination with sorafenib, $n = 3$ with HCC & HCV	100% genotype 1b	Unclear, at least 1 non-cirrhotic patient	Patients on sorafenib, all had previous HCC treatments in the years preceding DAA treatment including LRT and resection		Ombitasvir/paritaprevir/ritonavir and dasabuvir	12 weeks	100% SVR24 Adverse effects of sorafenib reported after DAA Patients all discontinued sorafenib with no recurrence at average 16.6m follow up



**Figure 2.** Advantages and disadvantages of treating HCV prior to or post-liver transplantation<sup>[33,35]</sup>

therefore pose a disadvantage in terms of wait-list time, thus allowing potential for tumour progression<sup>[35]</sup>. This is particularly relevant in locations with high volumes of HCV-positive liver donors<sup>[35]</sup> [Figure 2].

In addition to the potential impact on HCC outcomes, an impact on HCV outcomes has been demonstrated in patients receiving DAA therapy pre- vs. post-transplant [Figure 2]. One recent large retrospective study demonstrated a difference in SVR rates between pre- and post-transplant treated patients, with the latter seeing improved clearance<sup>[34]</sup>. In terms of liver transplantation, there are certainly advantages and disadvantages of treating HCV prior to HCC [Figure 2], which should be considered on an individual basis.

The evidence to date offers a compelling argument for considering treatment of the HCC before treatment of HCV. These findings require further data in order to make concrete recommendations in terms of HCV treatment timing, and each case should still be reviewed on an individual basis.

### DAAs and systemic therapies

There is a paucity of data regarding concomitant use of DAAs and the systemic agents used in advanced HCC. Sorafenib was the breakthrough targeted therapy first used in the treatment of advanced HCC and although its effect on median overall survival does not extend life expectancy beyond one year, it is yet to be superseded a decade after the seminal SHARP trial<sup>[56-58]</sup>. Sorafenib is a multi-kinase inhibitor with a potent inhibitory effect on c-Raf<sup>[59]</sup>. NS5a - a non-structural protein produced by HCV that is integral in viral replication - has been shown to bind to cRaf<sup>[60]</sup> and, studied in vitro, inhibition of cRaf by sorafenib effectively blocks HCV replication<sup>[59]</sup>. Multiple other mechanisms of sorafenib inhibition of HCV replication, such as alteration of the viral entry step, the production of viral particles and Claudin-1 downregulation, have been demonstrated<sup>[61-63]</sup>. Though the antiviral effect of sorafenib in human studies to date have been disappointing, this association has not yet been excluded<sup>[56,64,65]</sup>. Interestingly, Sorafenib has been shown to provide a greater benefit in overall survival in HCV patients when compared to other aetiologies of liver disease<sup>[66]</sup>. Newer drugs including lenvatinib, a multi-kinase inhibitor, in the front-line and regorafenib, cabozantinib (both multi-kinase inhibitors) and ramucirumab (an antiVEGFR mAb) in the second-line have been incorporated into new guidelines and are now increasing in use<sup>[67]</sup>, but their potential interactions with DAA regimens have been explored still less.

As many trials for the new DAA regimens excluded patients with HCC, there is a little data on the interaction between targeted therapies and DAAs<sup>[68]</sup>. One small case series noted that there were no

deleterious effects in combining ombitasvir/paritaprevir/ritonavir and dasabuvir, either in terms of anti-neoplastic effect or SVR rate<sup>[69]</sup>, but more studies are required to assess these interactions.

### **DAA and immunotherapy**

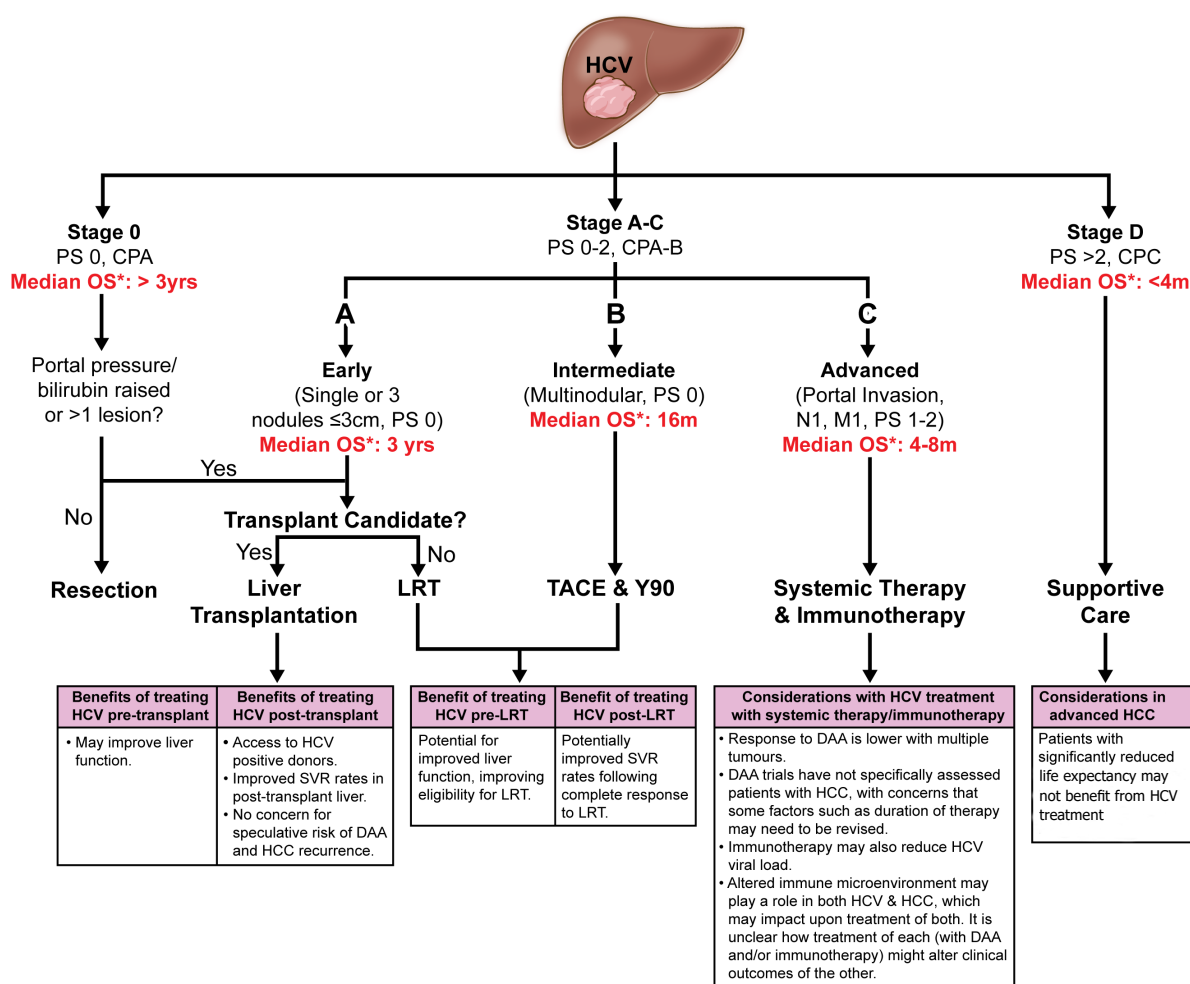
Though markedly different pathological processes are involved in chronic hepatitis C and the development of HCC, there are some similarities between the two when considering the role of the immune system, with T cell exhaustion implicated in both<sup>[70]</sup>. CD8+ T cells are integral in targeting and destroying both tumour cells and cells infected with HCV. T cell exhaustion exists to protect from tissue damage due to persistent and overzealous immunological response to antigens and is driven by upregulation of negative co-stimulatory pathways. With these pathways in action, key T cell effector processes are disrupted, they become tolerant of antigenic stimuli and ultimately apoptose<sup>[70]</sup>. T cell exhaustion is particularly efficient in T cells that are activated in the liver, causing the immunotolerant state required in an organ that encounters many antigenic threats. Chronic inflammation and development of cancer have both been shown to be associated with T cell exhaustion. These negative co-stimulatory pathways are multiple, including PD1, CTLA4, Tim3 and LAG3 - targets that are under scrutiny for potential new pharmacological options in the treatment of HCC. Inhibition of these targets aims to unlock the potential of these T cells to reinvigorate the immune cells. Though PD1 inhibitors in particular are now commonly associated with the treatment of HCC, they have also been trialled in the treatment of HCV in the past, with some success<sup>[71]</sup>. More studies assessing the impact of anti-PD1 immunotherapy on active HCV infection are required to fully understand its role in viral response. Multiple ongoing clinical trials using anti-PD1 antibodies are currently allowing patients with untreated HCV to enrol, which may go some way to answering this question. Nivolumab, a human monoclonal IgG4 antibody against PD1, is showing promise as a treatment in advanced HCC<sup>[72]</sup>. It has also been trialled in chronic HCV infection, showing a persistent suppression of HCV RNA in a subgroup of patients<sup>[71]</sup>. Pembrolizumab, another anti-PD1 monoclonal antibody, has also recently been granted accelerated FDA approval for the treatment of advanced HCC, with promising results in the Keynote-224 study<sup>[73]</sup>.

In addition to the impact of immunotherapy treatment of HCC on DAA treatment of HCV, we must also consider the opposite, as there is growing research showing the impact of DAAs on immune cells both within the liver and peripherally<sup>[74,75]</sup>. From this, we might extrapolate that this in turn may impact on the immune surveillance in this population, thus may affect HCC treatment outcomes. Currently the clinical impact of the immune environment and altered immune surveillance is not clear, but insight into these processes in the post-DAA liver is improving, which may be crucial in how we shape our treatment<sup>[74]</sup>.

Increased research into the immune environment in the post-DAA treated liver is vital to understand the potential impact viral clearance may have on HCC treatment response and vice versa. The effect of HCC-targeted immunotherapy on DAA treatment of HCV is not well-studied, but it would be interesting to see the impact on HCV treatment, and vice versa, be it synergistic, deleterious or non-existent.

### **Timing of HCV treatment in advanced HCC**

As previously discussed, timing of HCV treatment when considering curative options has been the source of some controversy, as the decreased efficacy of DAAs seen in the context of HCC offers a compelling argument for treating HCV after treatment of the tumour. In advanced HCC, the chance of cure is marginal and so delaying treatment of HCV for this reason is not practical. In patients where life-expectancy is significantly limited, the risk vs benefit of treating HCV at all must be considered. AASLD guidance recommends that patients with limited life expectancy within 12 months are unlikely to benefit from HCV eradication and therefore palliative measures should take precedence in this setting<sup>[76]</sup>. This will include patients with decompensated liver disease and advanced hepatocellular carcinoma. For those with a better prognosis, HCV eradication prior to sorafenib treatment of HCC may prolong post-progression



**Figure 3.** Modified from BCLC criteria for treatment of HCC<sup>[28]</sup>, with considerations for each treatment option outlined beneath

survival and improve overall survival<sup>[77]</sup>. Decisions regarding treatment timings should be considered on an individual basis, taking into consideration the advantages and disadvantages of treatment order [Figure 3].

## CONCLUSION

In summary, there is a paucity of clinical data surrounding the co-management of patients with both active HCV infection and HCC. The guidance for this challenging clinical scenario is to treat patients on a case-by-case basis, with conflicting evidence as to which condition to treat first. In cases where liver transplantation may be an option, there are advantages and disadvantages for treating one condition before the other, which should be considered on a case-by-case basis to enhance patient outcomes depending on individual clinical factors. Treatment of HCC through LRTs prior to HCV treatment may confer individual benefit in terms of SVR rates, but viral clearance conversely may improve liver function to allow more advanced treatment options. Again, assessment on an individual patient basis may be the most appropriate advice in the absence of robust clinical trials exploring this. For more advanced cases that are only eligible for systemic therapies, there are interesting parallels in the underlying immune processes that may have a significant impact on our management, though further trials into this are required before robust recommendations can be made. With newer treatments rapidly emerging for both conditions, this is an exciting area of hepatology that no doubt will be at the forefront of research in the coming decade.

**Key points**

There is a paucity of clinical data surrounding the co-management of patients with both active HCV infection and HCC.

As many trials for the new DAA regimens excluded patients with HCC, there is a little data on the interaction between targeted HCC therapies and DAAs, though there are interesting parallels in the underlying immune processes for HCV and HCC.

For patients with potentially curable HCC, deciding which pathology to treat first is complex and the data is conflicting. Improving liver function following SVR could enable the patient to undergo more favourable therapeutic HCC procedures. However SVR rates are significantly lower in patients with active HCC. In the absence of formal guidance and with conflicting evidence, we suggest this should be managed on an individual patient basis.

For patients awaiting liver transplantation, the ability to transplant an HCV-viraemic organ may improve waitlist times and thus guide decisions, but concrete data is lacking and so in the absence of formal guidance, we suggest this should be managed on a case-by-case basis.

**DECLARATIONS****Authors' contributions**

Study concept and design, literature search, drafting of the manuscript: Harrod E, Moctezuma-Velazquez C, Gurakar A, Ala A, Dieterich D, Saberi B

Critical revision of the manuscript for important intellectual content: Gurakar A, Ala A, Dieterich D, Saberi B

Study supervision: Saberi B

**Availability of data and materials**

Not applicable.

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**Conflicts of Interest**

Dieterich D: Gilead, Merck, AbbVie.

Harrod E, Moctezuma-Velazquez C, Gurakar A, Ala A, Saberi B: no conflicts of interest

**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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Editorial

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# The secondary prevention of hepatitis B virus-associated hepatocellular carcinoma

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Primary liver cancer can be classified into three categories according to different pathological types: hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC), and combined HCC-ICC. Among them, HCC accounts for more than 85%-90%. Therefore, the term “liver cancer” in this article refers specifically to HCC. Due to an insidious onset and no symptoms in the early stage of HCC, as well as lacking of awareness of disease screening of patients with HCC, they go to hospital only when the symptoms are vivid and diagnose with advanced HCC with a survival period of three to six months. For this reason, HCC was once known as “the king of cancer”. Clinically, we have noticed that most patients with HCC have a natural history of acute hepatitis B virus (HBV) infection - chronic hepatitis B (CHB) - liver cirrhosis (LC) - HCC, which shows that HBV infection is closely related to LC and HCC. According to the statistics, more than two billion people worldwide have been infected with HBV, and 240 million of them are CHB. Over 650 thousand people die every year from liver failure, LC and HCC caused by HBV, and 60% LC and 80% HCC are HBV-related<sup>[1]</sup>. The major hazard of HBV infection is considered to be chronic infection, which plays an important role in hepatocellular carcinogenesis. Hepatic fibrosis and LC are susceptible in CHB, which may eventually lead to the occurrence of HCC.

Since the implementation of planned immunization in China, the number of CHB patients has dropped steadily, while the stock of that is still large. For the carrying rate of hepatitis B surface antigen (HBsAg) in general population is estimated to be 7.18%, it is calculated that about 93 million people are chronically infected with HBV, about 20 million of whom are CHB patients. According to the natural history mentioned above, if these people do not receive whole course management and standard treatment, nearly 700 thousand people will develop into HCC in 8-10 years later.



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There may be two possible modes of HBV carcinogenesis: one is viral oncogene mode, that is, after the body is infected with HBV, HBV will integrate into the host genome by incorporating its own genes into the nucleus of hepatic cells, thus transforming normal hepatic cells into cancer cells<sup>[2-4]</sup>; the other is the cellular oncogene mode, that is, viral DNA will integrate into or rearranges with DNA of its host to produce HBV-activated genes or proto-oncogenes, leading to the transformation of normal cells into cancer cells and ultimately the development of HCC<sup>[5,6]</sup>.

It is not impossible to prevent HCC. If we can take intervention measures at the stage of CHB to prevent the disease progression to a great extent, it is impossible to prevent a considerable number of patients with CHB from developing into LC or even HCC. This article will focus on the secondary prevention of HBV-related HCC-early detection, early diagnosis, standard antiviral treatment, and whole-course management - to show its importance and clinical significance.

Screening of high-risk groups of HBV-associated HCC is helpful for early detection and diagnosis. Currently, it is known that the high risk factors closely lead to the progression of HCC in patients with CHB mainly include: hepatitis B e antigen (HBeAg) positive, genotype C HBV, high HBV DNA load, long-term intake of a lot of alcohol, people with LC basis and a family history of HCC, especially men over 40 years old and so on. Regular physical examinations should be carried out in high risk population. Regular examination of liver function, HBV serological tests, HBV-DNA load, AFP, abdominal ultrasound, and non-invasive liver fibrosis detection and the like every three to six months, enhanced CT test or nuclear magnetic resonance (MRI) should be further examined in suspected patients, in order to detect small HCC early. Early surgical resection and other radical treatment can improve the cure rate of HCC and prolong the life cycle of patients as far as possible.

Standard antiviral treatment plays a crucial role in secondary prevention of HBV-associated HCC. In patients with CHB, antiviral treatment has been shown to prevent disease progression to LC and HCC<sup>[7-11]</sup>. A retrospective cohort study showed<sup>[12]</sup> that after 5 years of follow-up, the cumulative incidence of HCC in nucleotide analogue (NUCs) antiviral treatment group and control group (without treatment) was 3.7%-13.7%, while the cumulative incidence of HCC was 7%-38.9% in these two groups of HBV-related LC (HBLC) patients, indicating a long-term of antiviral treatment can reduce the risk of HCC significantly. Similar studies also suggested<sup>[13]</sup> that long-term NUCs treatment was associated with 77% reduction of HCC risk in LC patients. Our team has been working on a cohort study of long-term standardized antiviral treatment and whole-course management for patients with CHB. Our previous studies showed<sup>[14]</sup> that compared with the control groups, the cumulative incidence of LC in CHB patients treated with NUCs for 3-5 years was 1.4% vs. 10.2% and 2.7% vs. 22.4%, respectively ( $P < 0.001$ ), the cumulative incidence of HCC in 3-5 years was 0.2% vs. 2.3% and 0.9% vs. 3.4%, respectively ( $P = 0.017$ ) [Figure 1]. All the results mentioned above illustrated that long-term and standard antiviral treatment can significantly reduce the risk of LC and HCC in patients with CHB.

Thus, a whole course management should be conducted in patients with high risk of HCC starting from the discovery of HBsAg positive, including chronic HBV carriers, non-active HBsAg carriers, HBeAg-positive and negative CHB patients, and HBLC patients in compensated and decompensated periods. As for the chronic HBV carriers and non-active HBsAg carriers, blood routine, biochemistry, HBV serological tests, AFP and the like should be monitored every 3 months, abdominal ultrasound, computed tomography (CT) or non-invasive liver fibrosis detection should be carried out every 6 months, and liver biopsy should be conducted if it is necessary. If antiviral treatment indications are met, treatment should be started in time. For HBeAg-positive CHB patients, after 3-6 months of observation, antiviral treatment could be started if alanine aminotransferase level continued to rise and there was no spontaneous HBeAg serological conversion. On the contrary, for HBeAg-negative CHB patients, antiviral treatment should be started as soon as possible if they meet the indication. For patients with HBLC, antiviral treatment should be initiated immediately [Figure 2]. After starting antiviral treatment, patients should be followed up regularly by telephone, text



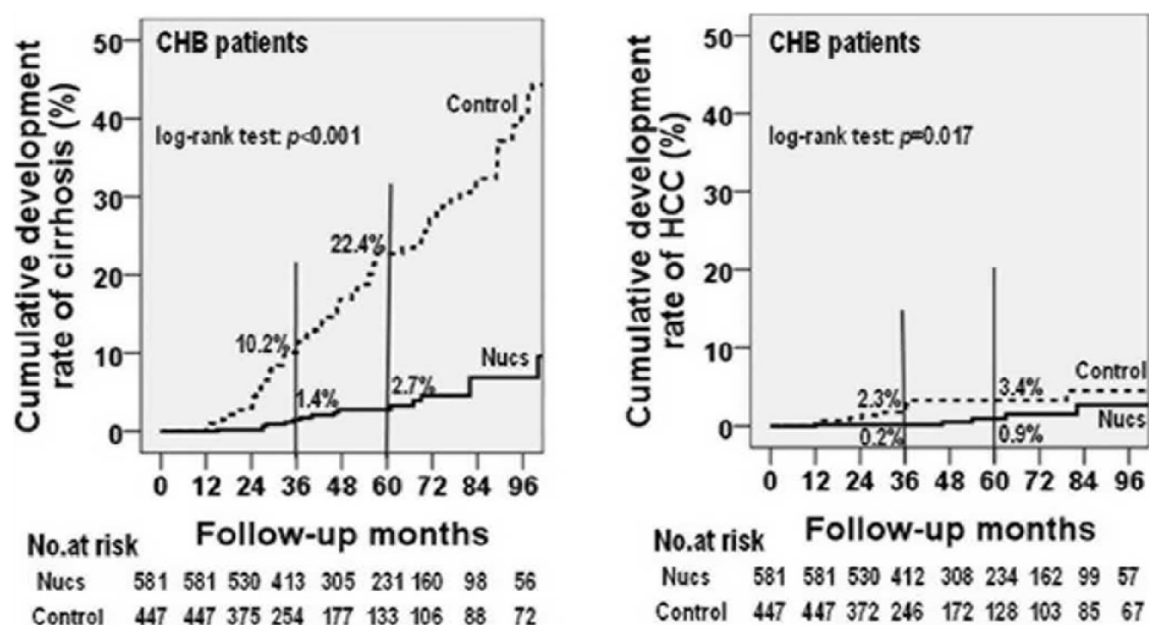
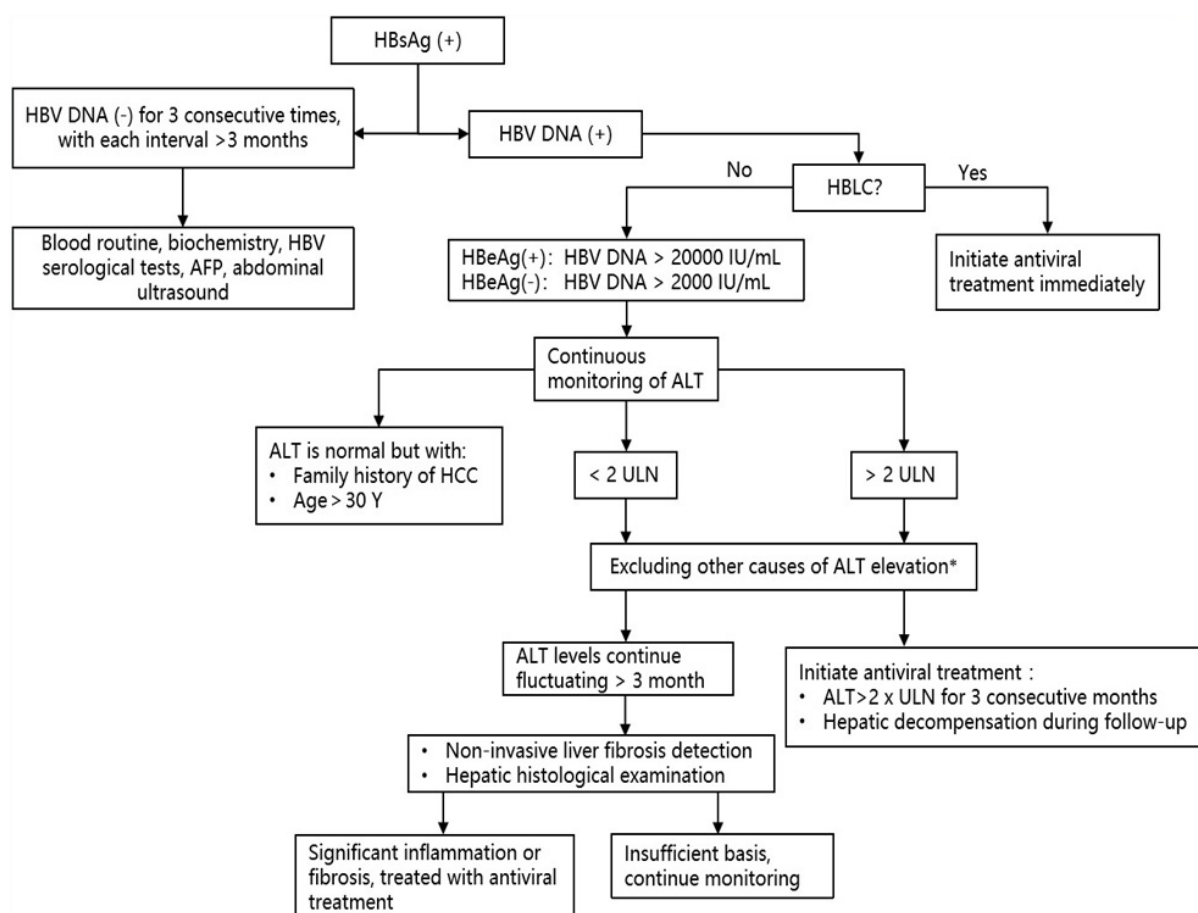
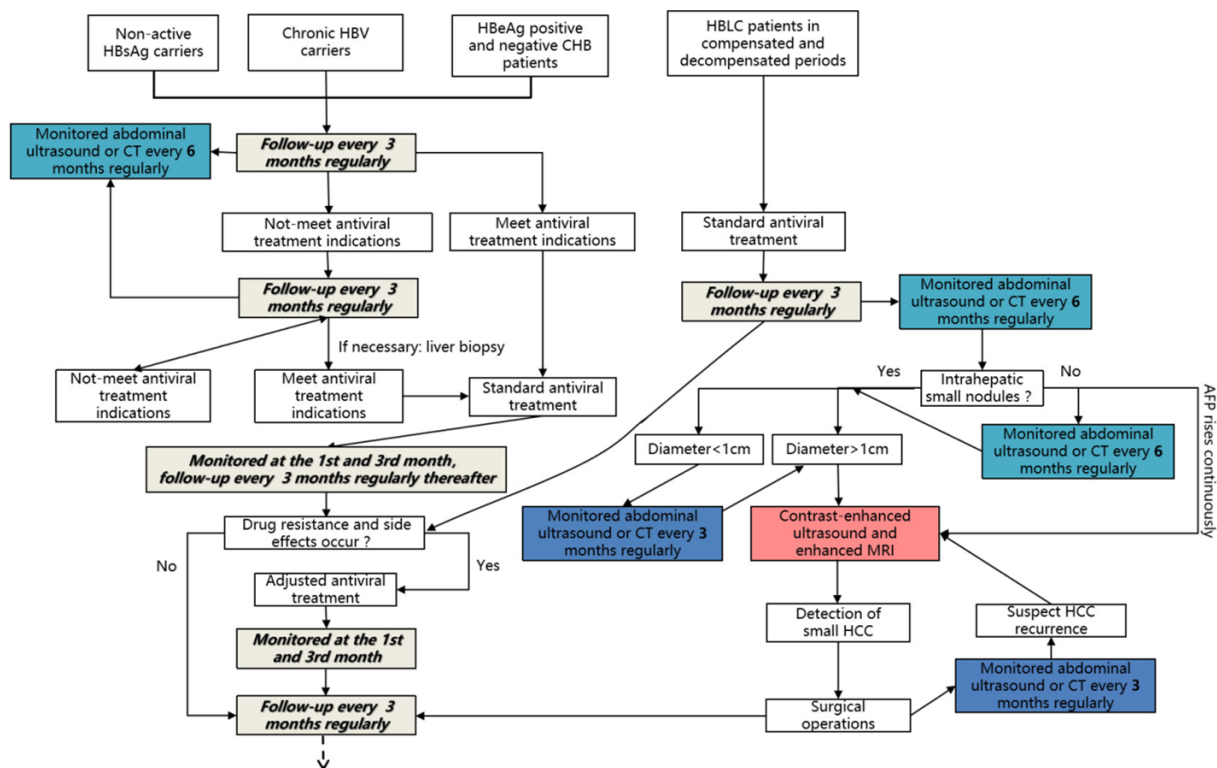


Figure 1. Clinical outcomes of long-term NUCs treatment



\* Other common causes of elevated ALT: other pathogenic infections, drugs, alcohol, immunity, fatty liver, etc.

Figure 2. Indications of antiviral treatment (2015 China CHB prevention and treatment guidelines)



**Figure 3.** Whole-course management-no end in sight

message, email, WeChat and other methods. Patients with hepatitis B cirrhosis should be reminded to examine HBV-DNA load, HBV serological tests, liver function, AFP and the like every 3 months. Abdominal ultrasound or CT test should be carried out every 6 months. Once the intrahepatic small nodules are found, and if the diameter of the nodules is larger than 1 cm, contrast-enhanced ultrasound and enhanced MRI should be performed to determine whether it is a small liver cancer. Once the diagnosis of small liver cancer is confirmed, surgical operation should be performed immediately. However, if the diameter of the nodules is smaller than 1 cm and unable to confirm diagnosis by imaging examination, closely follow-up should be performed instead, including monitoring abdominal ultrasound or CT test every 3 months. Once the intrahepatic small nodules are found to become bigger progressively, take intervene according to the above procedure. If no small intrahepatic nodules are found by imaging examination, abdominal ultrasound or CT test should be continued monitoring every 6 months. Once intrahepatic small nodules are found, interventions should be actively taken according to the above procedure. Patients with small liver cancers which are found early, after surgical operation, should be reminded monitoring abdominal ultrasound or CT test every 3 months. Once there is a possibility of recurrence of liver cancer, contrast-enhanced ultrasound and enhanced MRI should be performed, and surgical intervention should be taken immediately after diagnosis. Regular follow-up should be continued after surgery, repeatedly. Treatment plans should be adjusted in time once drug resistance and side effects occur during the follow-up period, and the biochemical and virological examinations should be performed in the 1st and 3rd months after the adjustment of the therapy, and continue to maintain regular follow-up every 3 months thereafter. Doctor-patient interaction should be advocated to supervise patients to continue taking antiviral drugs if they were found to stop taking medicines. At the same time, patients should be educated on the knowledge of hepatitis B to improve their understanding of the disease [Figure 3]. Through all the managements mentioned above, in our follow-up cohort, about 70% of HCC patients were found only with small HCC, and all of them received timely surgical resection and treatment, and continued to follow up postoperatively, so as to achieve the goal of early detection, early diagnosis, and early treatment.

Secondary prevention of HBV-associated HCC plays a key role in reducing the incidence of HCC, finding the occurrence of small HCC, carrying out early surgical treatments, and prolonging the survival cycle of patients, which deserved to be widely popularized.

## DECLARATIONS

### Authors' contributions

Collected the data and drew figures: Hu BB

Drafted the manuscript: Wang RM

Obtained the funding and revised the paper: Jiang JN

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

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### Copyright

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Editorial

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# The role of APOBEC3B in the development of hepatocellular carcinoma should be investigated with the consideration of hepatitis B virus evolution

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The chronic infection of hepatitis B virus (HBV) is the major cause of hepatocellular carcinoma (HCC) globally<sup>[1]</sup>. In Eastern China, chronic HBV infection contributes to 87.5% of HCC whereas chronic hepatitis C virus (HCV) infection contributes to 1.7%<sup>[2]</sup>. The mortality of HCC has increased in Europe and America over recent decades<sup>[3]</sup>. Although the infection of HCV is the leading cause of HCC in most European and American countries, the contribution of HBV is increasing possibly due to immigration<sup>[3]</sup>.

HCC represents a typical paradigm of inflammation-cancer transformation. Based on the advances in HBV-induced hepatocarcinogenesis, a scientific theory of Cancer Evolution-Development (*Cancer Evo-Dev*) was proposed<sup>[4-6]</sup>. The central aspects of this theory include: the interaction of HBV infection and immunogenetic predispositions maintains non-resolving inflammation. Immune imbalance promotes the generation of somatic and viral mutations via disbalancing Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3B (APOBEC3B) and mutation-repairing forces. Most mutant cells are eliminated by inflammatory microenvironment while only a small percentage of cells adapt the environment and survive. These survived mutant clones evolve to tumor-initiating cells (TICs) by altering the signal patterns mainly caused by de-differentiation mechanisms. TICs acquire the stemness and the ability of immune escape through recruiting tumor-associated macrophages (TAM) and myeloid-derived suppressor cells (MDSCs). Under the pressure of selection, TICs further obtain metastatic and drug-resistant potentials to adapt to distinct microenvironments. The evolution of HBV occurs along with this process. The mutant virus that selected by inflammatory environment can survive the immune elimination and facilitate the malignant



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transformation of normal cells. Thus, the HCC development is characterized by an evolutionary process of mutation-selection-adaptation.

APOBEC3B can generate cytosine-to-uracil (C>U) transversions through deamination. APOBEC3B related mutation pattern is proved to be widespread in the genome of tumors in many different organs including breast, lung, cervix, ovary, bladder, and head and neck<sup>[7]</sup>. Therefore, APOBEC3B is commonly believed to be a major force of generating somatic mutations.

In a recent study, Wang *et al.*<sup>[8]</sup> revealed another important role of APOBEC3B in the cancer evolutionary process of mutation-selection-adaptation. Their study suggests that APOBEC3B can also contribute to the “selection” and “adaption” of malignant cells via facilitating the immune escape in a deaminase-independent way. They reported that the elevated abundance of APOBEC3B in HCC predicts a poor prognosis. Authors demonstrated a high affinity  $\kappa$ B site in APOBEC3B promoter and non-canonical NF- $\kappa$ B signaling pathway up-regulates the expression of APOBEC3B via activating its transcription. With the animal models of HCC, it was demonstrated that elevated APOBEC3B facilitated the development of HCC only in the immunocompetent mouse rather than in the immune-deficient mouse. APOBEC3B was proved to recruit TAM, MDSC, and CD8<sup>+</sup> T cells positive for Programmed cell death ligand 1 through increasing the secretion of C-C motif chemokine ligand 2 (CCL2). The immunosuppressive effect of APOBEC3B depends on epigenetic modification. APOBEC3B can inhibit the activity of polycomb repressor complex 2, which is essential for maintaining methylation of H3K27. Therefore, the elevated APOBEC3B in HCC depresses global H3K27me3 abundance and reduces the occupancy of H3K27me3 on the promoter of CCL2. Thus, APOBEC3B promotes the immune escape and growth of HCC.

This remarkable study highlights the role of APOBEC3B in regulation of immune microenvironment as a factor of epigenetic modification. The understanding of APOBEC3B function and the theory of *Cancer Evo-Dev* are improved due to their solid evidences. In the meantime, two questions are proposed in this article. First, in addition to regulating epigenetic modification, APOBEC3B can promote HCC development by inducing mutation. Second, the role of interaction between HBV and APOBEC3B-mediated inflammatory microenvironment in HCC evolution should be further investigated. In this study, HBV infection was not taken into consideration. The immunosuppressive function of APOBEC3B was mainly demonstrated with the diethylnitrosamine-induced HCC animal model, which can hardly reflect the HBV-induced carcinogenesis in human. As described in the article, what authors investigated is the “hepatoma-intrinsic APOBEC3B” rather than APOBEC3B of hepatocytes with chronic inflammation. Therefore, results of this study cannot represent all the effects of APOBEC3B during HBV-induced hepatocarcinogenesis, especially its mutagenic function. APOBEC3B contributes the innate immune responses to HBV infection through inhibiting the replication of HBV via hyper-editing viral genome<sup>[9]</sup>. Although APOBEC3B induced HBV mutations are highly deleterious, a small percentage of viral mutations can facilitate the immune escape or the regeneration of hepatocytes<sup>[5]</sup>.

Interestingly, another recent study revealed the role of APOBEC3B in HCC development from another aspect, which answers the above questions. It discovered the associations among genetic predispositions, inflammation, APOBEC3B, and HBV mutations during the process of HCC development<sup>[10]</sup>. Interleukin-6 (IL-6) was proved to increase the expression of APOBEC3B and decrease the expression of uracil DNA glycosylase (UNG), an enzyme essential for DNA repair, thus leading to imbalance of mutagenic forces and mutation-repairing forces. Two genetic polymorphisms, rs2267401 (G) and rs3890995 (C), were proved to intensify the IL-6 induced APOBEC3B-UNG imbalance through affecting the activities of APOBEC3B promoter and UNG enhancer, respectively. These two genetic polymorphisms were also proved to be significantly associated with increased HCC risk by using a large case-control study involving 5221 participants. Besides, variant genotypes at rs2267401 were also demonstrated to improve the accumulation

of APOBEC3-signature HBV mutations and A1762T/G1764A in HBV-infected subjects, both of which were confirmed to associated with increased risk of HCC. The data of cohort studies demonstrated that APOBEC3B rs2267401-GG genotype, higher APOBEC3B expression, and higher APOBEC3B/UNG expression ratio in HCCs can predict a poor prognosis. Interestingly, APOBEC-signature somatic mutation predicts poor prognosis only in HBV-free HCC rather than in HBV-positive ones. These evidences strongly suggest that APOBEC3B facilitates HBV-induced HCC evolution via its mutagenic effect preferentially on the HBV genome. This result also explains why the APOBEC3-signature somatic mutation was not dominant in HCC genome<sup>[11]</sup>. APOBEC3B prefers to edit HBV genome possibly because the number of HBV genomic DNA is overwhelmingly more than that of human genome. Besides, during the replication of HBV, the partially double-stranded HBV DNA is generated from an intermediate RNA that is vulnerable to APOBEC3B.

To conclude, the work by Wang and related studies demonstrated the important role of APOBEC3B in HCC evolution from different aspects. APOBEC3B promotes HBV-induced carcinogenesis through its mutagenic activity and facilitating immune escape of HCC through regulating epigenetic modification. The investigation for APOBEC3B can be transformed not only into specific prophylaxis but also into target therapy.

## DECLARATIONS

### Authors' contributions

Study concept and design: Cao GW

Drafting of the manuscript: Liu WB

Discussion and revision of the manuscript text: Cao GW

### Availability of Data and Materials

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### Conflicts of interest

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### Ethical Approval and Consent to Participate

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### Consent for publication

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Meta-Analysis

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# No evidence for higher rates of hepatocellular carcinoma after direct-acting antiviral treatment: a meta-analysis

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## Abstract

**Aim:** Hepatitis C virus (HCV) is the leading cause of hepatocellular carcinoma (HCC) in the United States. Achieving sustained viral response with interferon (IFN) treatment reduces the risk from 3%-5% to 0.5%-1% annually. Several studies reported unexpectedly high rates of HCC after treatment with direct-acting antivirals (DAAs). The aim of our study was to compare HCC rates in DAA-, IFN-treated and untreated populations.

**Methods:** A literature search was conducted using ScienceDirect, Ovid®, Web of Science and MEDLINE through January 2019. Studies were included if they measured rates of *de novo* or recurrent HCC (following curative treatment) in HCV-infected persons. We included 138 studies ( $n = 177,512$ ). Simple pooling of data and meta-analysis were performed, using the random effects method.

**Results:** Mean age was higher in the DAA-treated *vs.* IFN-treated group (58.4 years *vs.* 52.6 years;  $P = 0.0073$ ), as were diabetes prevalence (34.5% *vs.* 11.7%;  $P \leq 0.001$ ) and incident cirrhosis (47.8% *vs.* 34.2%,  $P = 0.0017$ ). The incidence rate of *de novo* HCC was 2.01/100 person-years (py) (95%CI: 1.38, 2.67) in the DAA group and 1.45/100py (95%CI: 0.98, 1.94) in the IFN-treated group. HCC recurred at 16.76/100py (95%CI: 10.75, 22.91) in the DAA-treated group *vs.* 20.04/100py (95%CI: 2.58, 45.21) after IFN. After adjusting for factors such as age and cirrhosis, the hazard ratio was 0.58 (95%CI: 0.20, 1.07) for HCC occurrence and 0.59 (95%CI: 0.24, 1.03) for HCC recurrence after DAA treatment compared to IFN-based treatment.



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**Conclusion:** We did not find evidence for increased rates of HCC in DAA-treated compared with IFN-treated patients. Compared to those treated with IFN, older patients with additional risk factors for HCC were treated with DAAs. This imbalance appears to explain the higher numerical incidence of HCC among DAA-treated patients.

**Keywords:** Humans, hepatitis C virus, liver cirrhosis, liver neoplasms, interferons

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fastest rising cause of cancer-related death in the United States<sup>[1]</sup>. In most developed countries, chronic hepatitis C virus (HCV) infection is the leading risk factor for HCC. Approximately half of the increase in HCC cases in the United States may be accounted for by the aging cohort with chronic HCV infection<sup>[1]</sup>. Though the presence of cirrhosis is an important risk factor for the development of HCC in HCV-infected individuals, HCV itself may have pro-carcinogenic properties<sup>[2]</sup>. Specifically, the virus induces tumor development indirectly via inflammatory and pro-fibrotic host responses and may also exert direct oncogenic effects upon the infected cell, via deregulation of host cell checkpoints, oxidative stress and DNA damage<sup>[2]</sup>.

Patients with HCV-related cirrhosis have a risk of developing HCC, estimated at 3%-5% per year<sup>[3]</sup>. The risk is further enhanced by alcohol misuse, diabetes mellitus, obesity and coinfection with hepatitis B or HIV<sup>[4,5]</sup>. Studies have shown that achieving sustained viral response (SVR) after interferon (IFN) treatment reduces the risk of HCC to 0.5%-1% per year<sup>[6-8]</sup>. It was originally believed that IFN reduced the risk via antiviral as well as direct anti-tumor effects but non-sustained responders to IFN do not achieve the same reduction in HCC risk<sup>[9]</sup>. This may be related to the fact that IFN delays the development of HCC but does not prevent it in the presence of persistent viremia and cirrhosis.

With the FDA approval of IFN-free regimens in 2014, it was anticipated that the risk of HCC would be further reduced due to their high SVR achievement rates (upwards of 95% compared to approximately 56% with pegylated-IFN and ribavirin regimens)<sup>[10,11]</sup>. Thus it was surprising when several sentinel European reports published in 2016 raised concern for increased rates of HCC in patients treated with IFN-free direct-acting antiviral (DAA) regimens. Most of the studies reported increased rates of HCC recurrence but a few reported increased rates of *de novo* HCC. Some of these tumors were diagnosed within weeks to months of DAA treatment and several studies observed that tumors were unusually aggressive and locally invasive on imaging<sup>[12-15]</sup>. Later, conflicting studies were published that did not show evidence of increased risk of HCC, with follow-up periods of up to 15 months<sup>[16-18]</sup>.

However, all the studies had significant limitations. They were observational in nature, each with small numbers of patients. They evaluated heterogeneous populations with varying numbers of patients with cirrhosis. The question was raised as to whether the perceived increased risk was an artifact of selection bias, whereby older patients with more advanced liver disease and additional risk factors for HCC are being treated with DAA than would historically been treated in the era of IFNs.

The development of a safe, efficacious and well-tolerated treatment has revolutionized the landscape of HCV treatment. Patients treated with DAAs have been shown to have lower rates of decompensation and model for end-stage liver disease (MELD) score progression than those not treated with DAA agents<sup>[16,19]</sup>. Finding a correlation between DAAs and HCC development or recurrence would have major implications. Thus, to find a more definitive answer to this question, we compared rates of HCC occurrence and recurrence in DAA-treated persons with IFN-treated and untreated patients in a meta-analysis of all published studies.



## METHODS

### Data sources

A comprehensive literature search was performed using ScienceDirect, Ovid®, Web of Science, MEDLINE, Google Scholar and the Cochrane Library. Abstract books from the major international hepatology meetings including European Association for the Study of the Liver and American Association for the Study of Liver Diseases were also examined thoroughly for additional studies. We searched databases from inception through January 2019 and included studies with human subjects which measured rates of HCC occurrence or recurrence in persons infected with HCV.

### Study selection

We considered retrospective or prospective observational cohort studies and randomized controlled trials as eligible studies for analysis. We included studies if they assessed (1): *de novo* HCC development in patients with chronic HCV; or (2): HCC recurrence in patients with chronic HCV who had received successful HCC curative treatment and were believed to be cancer-free at the time of HCV treatment. HCC treatments which were categorized as being potentially curative included liver resection, microwave coagulation therapy, percutaneous ethanol injection therapy, radiofrequency ablation, and liver transplantation. We included HCV-infected patients regardless of the presence or absence of cirrhosis and regardless of HCV treatment status (DAA-treated, IFN-treated or untreated). Subjects with and without SVR were included. Where we found multiple studies from the same population, the most recent studies were included. All full text manuscripts and conference abstracts were considered for inclusion. Studies with missing essential data or with unclear or less rigorous methodology were excluded. We excluded studies with a follow-up period of less than 1 year, to avoid including cases where sub-clinical HCC was likely present at the time of treatment initiation. The quality of evidence in each included study was assessed using the Cochrane tool for risk of bias [Supplementary Tables 1 and 2].

### Data extraction

We manually pulled data from studies into a pre-formatted standardized spreadsheet containing clinical, demographic and epidemiological headings. In the spreadsheet, studies were categorized by treatment type (DAA-treated, IFN-treated or untreated) and primary endpoint of HCC occurrence or recurrence. They were then further sub-divided into SVR and non-SVR groups where this information was available from studies.

### Data analysis

The outcomes evaluated were HCC occurrence and HCC recurrence. Studies with zero events were excluded from the analysis. The incidence rates of HCC occurrence or recurrence were calculated per 100py. Meta-analyses, stratified by type of HCV treatment received (DAA, IFN and never treated), were undertaken to determine incidence rates for each group using a random-effects model. Several studies had performed multivariate analysis adjusting for a variety of factors including age, gender, baseline cirrhosis status, baseline alpha fetoprotein (AFP), ethnicity and Child-Pugh score. We used a mixed effects meta-analysis to calculate the overall adjusted and unadjusted hazard ratio of DAA treatment, using studies with multivariate analyses. Several sub-analyses were performed, including exclusion of any HCC event diagnosed within the first six months after the completion of treatment for HCV, and the calculation of the annualized HCC rate for the second year after HCV treatment. In order to obtain this data, we manually extracted information from studies where the time-to-event for each HCC event was recorded.

For baseline characteristics within individual studies, data were weighted, then pooled and *P*-value generated using GraphPad Prism software.

**Table 1. Baseline characteristics in the DAA-treated and IFN-treated groups**

Characteristic	DAA group	IFN group	P value
Sex, % male	91.0	90.3	0.1992
Age in years, mean $\pm$ SD	58.4 $\pm$ 6.05	52.6 $\pm$ 7.21	0.0073
Follow-up in years, mean $\pm$ SD	1.46 $\pm$ 0.54	7.75 $\pm$ 3.23	< 0.001
Cirrhosis, %	47.8	34.2	0.0017
Child-Pugh B/C, %	19.4	3.0	< 0.001
AFP level, ng/mL, mean $\pm$ SD	6.2 $\pm$ 5.4	5.6 $\pm$ 4.3	0.4456
Platelet count, mean $\pm$ SD	155 $\times 10^9$ /L $\pm$ 30	197 $\times 10^9$ /L $\pm$ 28	< 0.001
Albumin in g/dL, mean $\pm$ SD	3.8 $\pm$ 0.3	4.1 $\pm$ 0.3	< 0.001
Prevalence of genotype 1, %	85.0	60.8	0.0016
Prevalence of genotype 3, %	5.1	12.4	0.8381

AFP: alpha fetoprotein; DAA: direct-acting antiviral; IFN: interferon; SD: standard deviation

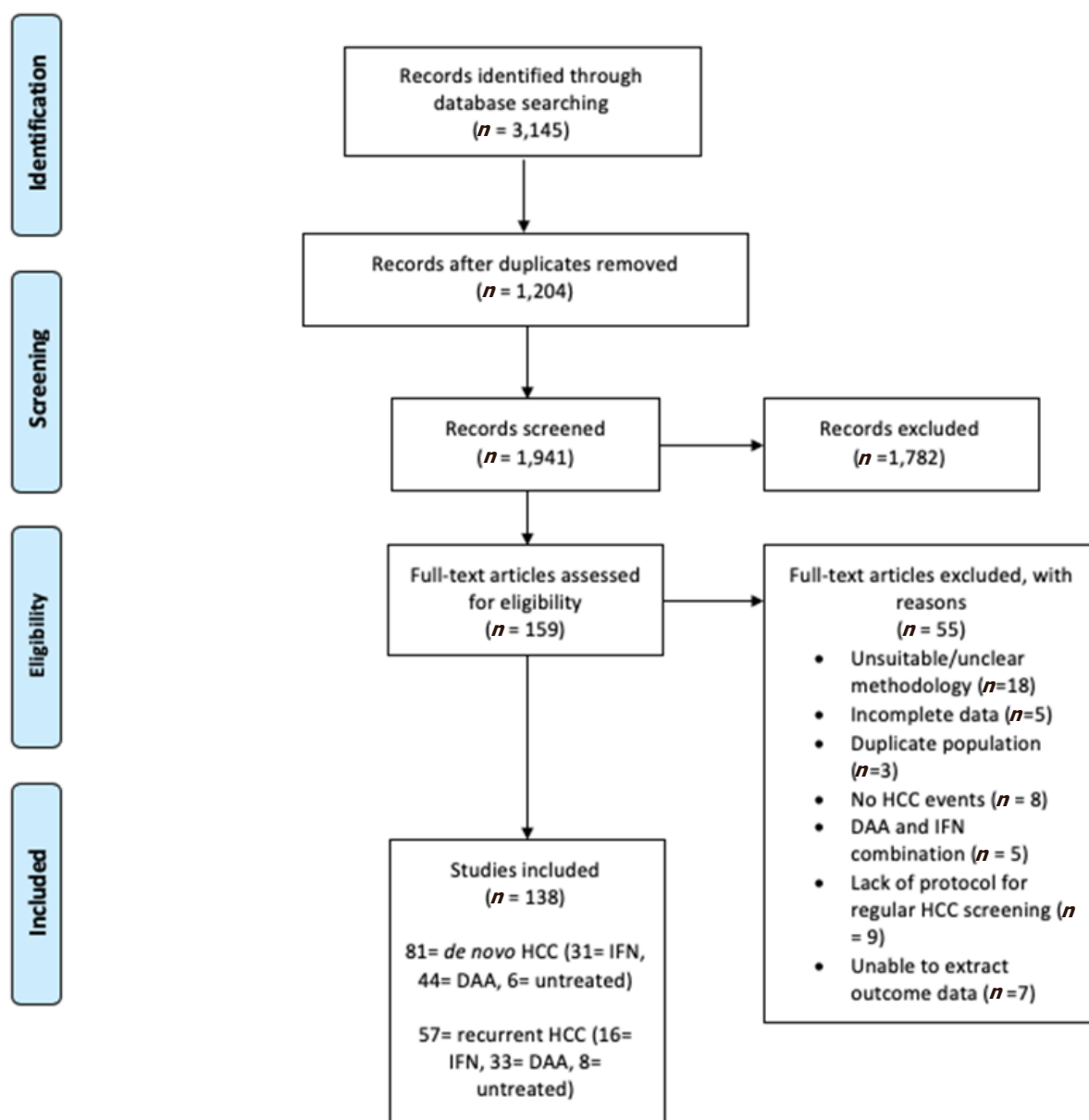
## RESULTS

We retrieved 3145 citations after the electronic database search. After excluding duplicates and studies which did not pertain to the patient population or outcomes in question, we included a total of 138 studies ( $n = 177,512$ ). We found 81 studies looking at *de novo* HCC occurrence ( $n = 172,636$ ): 31 studies of IFN-treated persons ( $n = 71,443$ ), 44 studies of DAA-treated patients ( $n = 91,249$ ) and 6 relating to untreated subjects ( $n = 9944$ ). We included 57 studies which evaluated HCC recurrence ( $n = 4876$ ): 16 studies of IFN-treated populations ( $n = 1043$ ), 33 relating to DAA-treated ( $n = 2186$ ) and 8 studies of untreated patients ( $n = 1647$ ). There were 16 DAA studies which examined both *de novo* and recurrence rates of HCC. Figure 1 contains study flow chart. Supplementary Tables 3 and 4 contain individual study details.

Both groups had similarly high rates of male patients, due to the inclusion of large studies of Veterans Affairs (VA) hospitals: 91% in the DAA group and 90.3% in the IFN group ( $P = 0.1992$ ). The rate of SVR in the DAA-treated group was 88.9%, compared to 45.9% in the IFN-treated group ( $P \leq 0.001$ ). Overall, mean age was higher in the DAA-treated vs. IFN-treated group (58.4 vs. 52.6 years;  $P = 0.0073$ ), as was the prevalence of diabetes (34.5% vs. 11.7%;  $P \leq 0.001$ ). As expected, mean follow-up was longer in the IFN group: 7.75 vs. 1.46 years ( $P \leq 0.001$ ). DAA-treated patients had higher prevalent cirrhosis compared to IFN-treated patients (47.8% vs. 34.2%,  $P = 0.0017$ ), and among persons with cirrhosis, Child-Pugh stage B/C disease was more frequent in the DAA-treated group (19.4% vs. 3.0%,  $P \leq 0.001$ ). The AFP levels at the time of initiation of HCV treatment were similar in both groups (6.2 ng/mL in DAA vs. 5.6 ng/mL in IFN-treated, ( $P = 0.4456$ )). Mean platelet count was lower in the DAA group ( $155 \times 10^9$ /L vs.  $197 \times 10^9$ /L), ( $P \leq 0.001$ ), as was mean albumin (3.8 g/dL vs. 4.1 g/dL in IFN ( $P \leq 0.001$ )). The prevalence of genotype 1 (GT-1) was 60.8% in the IFN and 85.0% in DAA group ( $P = 0.0016$ ); the prevalence of GT-3 was not significantly different between groups: 5.1% in DAA vs. 12.4% in IFN ( $P = 0.8381$ ) [see Table 1].

### Rates of *de novo* HCC

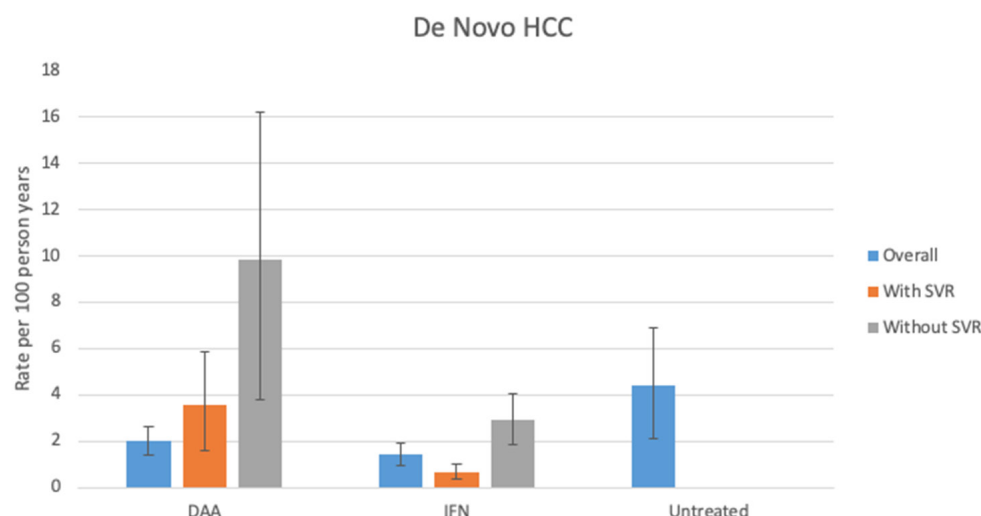
The estimated incidence of *de novo* HCC occurrence after DAA treatment was calculated to be 2.01/100py (95%CI: 1.38, 2.67) compared to 1.45/100py (95%CI: 0.98, 1.94) in IFN-treated subjects. In patients who had never been treated with HCV therapy, the rate of *de novo* HCC was significantly higher than IFN-treated groups at 4.41/100py (95%CI: 2.10, 6.90). We performed a sub-group analysis by SVR status: patients treated with DAAs who achieved SVR developed HCC at a rate of 3.57 per 100py (95%CI: 1.63, 5.88) while the IFN-treated SVR group had a lower estimated incidence rate of 0.70/100py, (95%CI: 0.41, 1.04). The DAA SVR sub-group had an unexpectedly higher rate of HCC occurrence (although not statistically significant) than the overall DAA group. This may be explained by the fact that not all studies reported SVR status, so marginally smaller numbers were available for this sub-group analysis, which may have arbitrarily included the studies with higher rates ( $n = 87,952$  in the sub-group analysis compared to 91,249 in the entire DAA group). In the non-SVR sub-groups, DAA-treated patients developed HCC at a rate of 9.83/100py



**Figure 1.** PRISMA study flow chart. HCC: hepatocellular carcinoma.

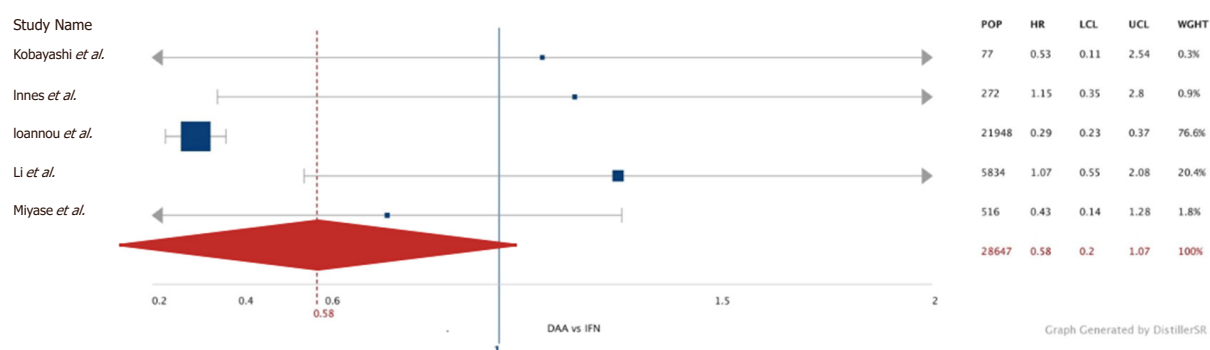
(95%CI: 3.77,16.22). Their IFN-treated counterparts who did not clear HCV had a *de novo* HCC incidence rate of 2.94/100py (95%CI: 1.88, 4.04) [Figure 2].

We performed an additional smaller meta-analysis of the five studies that performed multivariate-adjusted hazard ratios (adjusting for gender, baseline cirrhosis, and patient age), and found no increased risk of *de novo* HCC in patients treated with DAA compared to the IFN-treated population: unadjusted HR of 1.76 (95%CI: 0.001, 4.70) and adjusted HR of 0.58 (95%CI: 0.20, 1.07) [Figure 3]. When we looked at *de novo* rates in the second year after treatment with DAAs, we found a similar incidence rate of 0.88 per 100py, (95%CI: 0.0001, 1.94) compared to a second-year annualized incidence rate of 0.55 per 100py, (95%CI: 0.03, 1.29) in IFN-treated patients. We then excluded any cases of *de novo* HCC occurring within six months after end-of-treatment and obtained an incidence rate of 1.12 per 100py (95%CI: 0.43,1.98) in the DAA group and a higher incidence rate of 3.01 per 100py (95%CI: 0.033, 9.02) in the IFN group, which did not reach statistical significance.



**Figure 2.** Rates of *de novo* HCC by treatment group and SVR status. Colored bars represent rates of *de novo* HCC by SVR status, with 95% CIs depicted by the capped 95% CI vertical black lines. Rates of *de novo* HCC were as follows: DAA group, overall: 2.01/100py (95%CI: 1.38, 2.67), SVR: 3.57/100py (95%CI: 1.63, 5.88) and non-SVR: 9.83/100py (: 3.77,16.22). IFN group, overall: 1.45/100py (95%CI: 0.98, 1.94), SVR: 0.70/100py, (95%CI: 0.41, 1.04), non-SVR: 2.94/100py (95%CI: 1.88, 4.04). Untreated group: 4.41/100py (95%CI: 2.10, 6.90). SVR: sustained viral response; IFN: interferon; DAA: direct-acting antiviral; CI: confidence interval; HCC: hepatocellular carcinoma

#### Adjusted HR of *de novo* HCC

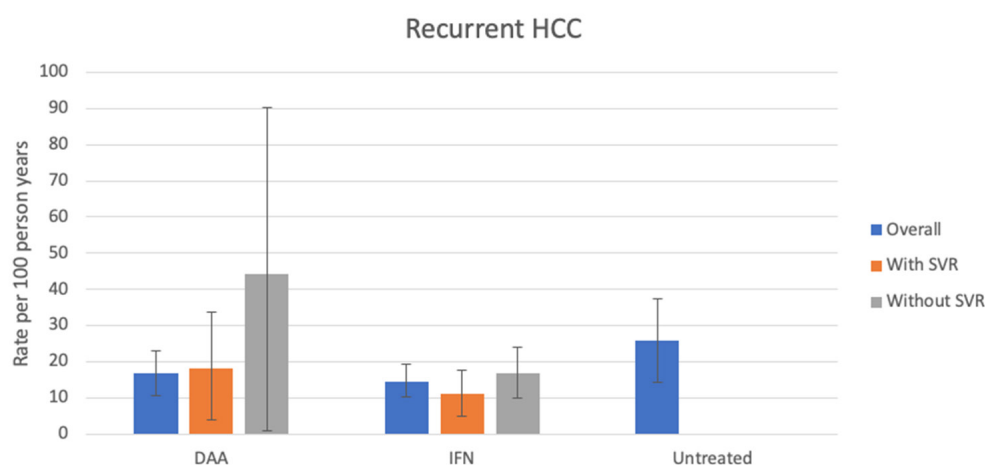


**Figure 3.** Adjusted hazards ratio of risk of *de novo* HCC in DAA- vs. IFN-treated populations. The multivariate-adjusted hazard ratio of each individual study is represented by the blue square with the size of the square being proportional to the n of the study. The thin horizontal grey bars represent the 95%CI of each study and the thick vertical blue line marks where the HR is equal to 1. The red diamond with the dashed vertical red line is the overall adjusted HR, which was 0.58 (95%CI: 0.20, 1.07) for *de novo* HCC in the DAA population compared to the IFN-treated population. HCC: hepatocellular carcinoma; IFN: interferon; CI: confidence interval

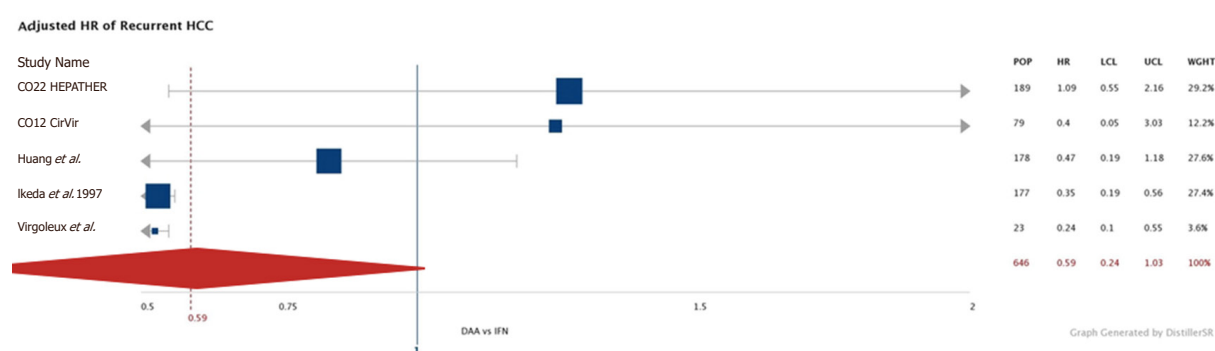
#### Rates of recurrent HCC

The recurrence rate of HCC was relatively high across all groups: 16.76/100py (95%CI: 10.75, 22.91) and 14.31/100py (95%CI: 10.17, 19.16) in DAA- and IFN-treated populations respectively. It was similar between untreated patients and IFN-treated patients without SVR [25.69/100py (95%CI: 14.44,37.27) compared to 16.89/100py (95%CI: 10.05, 24.02)], suggesting that IFN does not have significant anti-tumor effect in the absence of viral clearance. Patients treated with DAA who did not achieve SVR appeared to have a high rate of recurrent HCC; however, the numbers of patients with DAA failure were small, thus the CI is wide: 44.16/100py, (95%CI = 0.006, 90.35). DAA-treated patients who achieved SVR had a recurrence rate of 18.17/100py (95%CI: 3.84, 33.58) and in IFN-treated populations with SVR the recurrence rate was not significantly different at 11.01/100py (95%CI: 4.85, 17.63) [Figure 4].

When HCC recurrence that occurred during the first six months post-completion of treatment was excluded, the rates were similar between DAA and IFN groups: 10.75/100py, (95%CI: 5.50, 16.30) vs.



**Figure 4.** Rates of recurrent HCC by treatment group and SVR status. Colored bars represent rates of recurrent HCC by SVR status, with 95%CI's depicted by the capped vertical black lines. Rates of recurrent HCC were as follows: DAA group, overall: 16.76/100py (95%CI: 10.75, 22.91), SVR: 18.17/100py (95%CI: 3.84, 33.58) and non-SVR: 44.16/100py, (95%CI: 0.006, 90.35). IFN group, overall: 14.31/100py (95%CI: 10.17, 19.16), SVR: 11.01/100py (95%CI: 4.85, 17.63), non-SVR: 16.89/100py (95%CI: 10.05, 24.02). Untreated group: 25.69/100py (95% CI: 14.44,37.27). "Overall" group included some patients not included in SVR or non-SVR groups. SVR: sustained viral response; HCC: HCC: hepatocellular carcinoma; CI: confidence interval; IFN: interferon; DAA: direct-acting antiviral



**Figure 5.** Adjusted hazards ratio of risk of recurrent HCC in DAA- vs. IFN-treated populations. The multivariate-adjusted hazard ratio of each individual study is represented by the blue square with the size of the square being proportional to the n of the study. The thin horizontal grey bars represent the 95%CI of each study and the thick vertical blue line marks where the HR is equal to 1. The red diamond with the dashed vertical red line is the overall adjusted HR, which was 0.59 (95%CI: 0.24, 1.03) for recurrent HCC in the DAA population compared to the IFN-treated population. IFN: interferon; DAA: direct-acting antiviral; HCC: hepatocellular carcinoma

14.62/100py, (95%CI: 8.94, 20.52). Again, when we take the second-year rates post-treatment, we saw similar recurrence rates [DAA group: 6.66/100py (95%CI: 1.96, 12.11) and IFN group: 5.35/100py, (95%CI: 0.54, 11.06)]. Our additional meta-analysis of the five studies with multivariate analyses found that the unadjusted HR of recurrence in DAA vs. IFN-treated groups was 0.59 (95%CI: 0.11, 1.14) and after adjusting for age, gender, baseline AFP, ethnicity and Child-Pugh score, the HR was 0.59 (95%CI: 0.24, 1.03) [Figure 5].

## DISCUSSION

Our study is the largest meta-analysis to evaluate the risk of HCC after treatment with DAA therapy published to date. The results demonstrate that the risk of *de novo* HCC is similar between IFN- and DAA-treated cohorts. In the sub-group analysis by SVR, non-SVR IFN and non-SVR DAA groups had similar rates of *de novo* HCC, although the confidence interval for the DAA cohort was wide because of the very small numbers who did not achieve SVR. Those who achieved SVR with IFN had a significantly lower rate of HCC occurrence than the DAA-treated SVR group. We postulate that this is because patients who could



tolerate and achieve viral clearance with IFN therapy were a very well-compensated group with minimal liver disease. Indeed, we observed that the entire IFN-treated group was significantly younger and had less cirrhosis, less diabetes and lower Child-Pugh scores than DAA-treated patients. This echoes many of the other previously published studies<sup>[20-23]</sup>. In our meta-analysis of hazard ratios, after adjusting for a number of risk factors for HCC, we found that the rates of *de novo* HCC were lower in the DAA-treatment group compared to IFN-treated, although this did not reach statistical significance. This finding reinforces the hypothesis that “higher-risk” patients receive treatment with DAA agents than were treated with IFN in the past, thus leading to selection bias. There has been particular concern about the rates of HCC recurrence following DAA treatment, which were felt to be even more pronounced than the risk of *de novo* HCC<sup>[12,14,24]</sup>. However, we found no increased risk of HCC in DAA-treated patients compared with IFN-treated patients, and after adjusting for risk factors such as age and cirrhosis, the DAA-treated group trended towards a lower rate of recurrent HCC, although this did not reach statistical significance.

Our meta-analysis excluded any studies with less than one year of follow-up after end-of-treatment; this rigorous exclusion was not performed in another recent meta-analysis<sup>[25]</sup>. We believe that helped to mitigate any increase in rates of “early” HCC post-treatment due to sub-clinical HCC which may have been present prior to the initiation of antiviral therapy. We also performed a sub-group analysis, whereby HCC events occurring within six months of end-of-treatment were excluded, and we measured HCC rates in the second-year post-treatment. Our strict exclusion criteria and subgroup analyses were designed to mitigate surveillance bias, where patients who undergo treatment for HCV may be monitored more closely in the months following treatment due to more frequent visits to a hepatologist and may be more likely to undergo HCC screening with abdominal imaging. Limitations of our study include its retrospective observational nature thus allowing for confounding variables since many of these studies were not initially designed to compare rates of HCC, and the heterogeneous nature of the studies which had variable lengths of follow-up, differing percentages of patients with cirrhosis and different individual DAA treatment regimens. Furthermore, none of the studies which included both DAA-treated and IFN-treated persons adjusted for the exact same baseline risk factors for HCC, so this limited the validity of comparing the studies directly and deriving hazard ratios. There was minimal accounting for indication bias, which is one of the criticisms of these studies. Finally, there was a disproportionate number of male patients included in the meta-analysis, due to the high number of subjects in the VA studies: 64,306/93,435 DAA-treated and 50,143/72,486 IFN-treated subjects included in the meta-analysis were acquired from VA-based studies. Given that the veteran patient population has higher rates of smoking and alcohol use than the general population, the risk of HCC in this subgroup was likely higher which may have skewed the results (although approximately equal proportions of DAA and IFN-treated patients were obtained from VA data).

The debate on whether or not DAAs increase the risk of HCC has been ongoing for several years now. Initial reports from Europe first raised concern, and multiple studies confirming and refuting this theory have since been published<sup>[12-18,26]</sup>. The immune theory and liver regeneration theory are some of the most commonly cited theories for the perceived increase in HCC after treatment with DAA therapy. After treatment with DAAs, the HCV virus becomes undetectable within days to weeks, far more quickly than with IFN-based therapy. It has been suggested that clearing the hepatitis virus rapidly with fall in antigenic load removes the immune surveillance (with CD8+ T cells for example) which protected against the development of neoplasia<sup>[27]</sup>. It is also thought that as the liver regenerates rapidly after viral clearance, small sub-clinical tumors or areas of metaplasia may grow and become clinically evident<sup>[28]</sup>.

Small case series have found that tumors are more likely to be multi-focal and tend to have a more aggressive biology in DAA-treated individuals compared to IFN-treated or untreated subjects. Romano *et al.*<sup>[29]</sup> describe a particularly aggressive HCC pattern at diagnosis after DAA treatment. In 39% of the 27 patients treated with DAA therapy who developed HCC, there was an infiltrative pattern or more than three nodules present

(25% of these cases had vascular invasion or extrahepatic spread). Renzulli *et al.*<sup>[30]</sup> found imaging features of microvascular invasion in 70.7% of HCC nodules after DAA treatment; microvascular invasion was present in only 33.3% of HCC nodules that occurred before DAA treatment. However, conflicting studies have also been published: the large CIR-VIR study found infiltrative HCC in 10.8%<sup>[26]</sup>; typically up to 13% of all HCC cases are found to be infiltrating and are often associated with background hepatitis B infection<sup>[31]</sup>. The study by Zanetto *et al.*<sup>[32]</sup> evaluated 9 DAA-treated explanted livers with 14 control (untreated) liver explants and found no difference in median number and total tumor volume of HCC nodules, tumor differentiation, or microvascular invasion. Clearly, more studies evaluating the biology of tumors after DAA treatment are required before a definitive conclusion can be drawn.

The high efficacy and tolerability of DAAs has resulted in their use in patients who have more intrinsic risk factors for HCC, including advanced age, diabetes and cirrhosis. Several studies have shown the patients treated with DAAs have more risk factors than historical IFN-treated cohorts. The US Veterans Administration study by Li *et al.*<sup>[20]</sup> demonstrated the “warehousing” of HCV-infected patients which took place in the years leading up to the release of DAAs. They showed that patients who received the first available DAA agents had the most advanced liver disease and higher rates of HCC as a result, because HCV treatment had been deferred in anticipation of an efficacious tolerable regimen.

Fangazio *et al.*<sup>[33]</sup> showed that patients who developed *de novo* or recurrent HCC after DAA treatment were less likely to achieve SVR (SVR12 rate of 64% in patients with HCC compared to 95% in their counterparts without HCC, which is much more typical of the DAA viral clearance rates). This finding suggests that in patients who do not achieve SVR with DAA, clinicians should have heightened levels of suspicion for underlying undetected HCC. A study by Beste *et al.*<sup>[34]</sup> also found that HCC patients had lower rates of viral clearance than patients without HCC, even after adjusting for cirrhosis and genotype. It has been postulated that the virus within tumor cells could be inaccessible to DAAs because of differential blood supply, which prevents the clearance of virus. Furthermore, HCC arises in the setting of chronic inflammation with alterations in the hepatic architecture and micro-environment, including cytokine and chemokine populations<sup>[35]</sup>. This altered immune environment may predispose to treatment failure and to the development of liver cancer. A study by Tachi *et al.*<sup>[36]</sup> revealed that higher total bilirubin levels and higher liver stiffness measurements (as measured by ARFI elastography) prior to DAA treatment were positively associated with occurrence of HCC after achievement of SVR with DAA therapy. Clearly a risk of HCC still exists even after SVR with DAA treatment, so surveillance imaging should not be ceased even after treatment success.

Even in view of the mixed data, it is evident that the achievement of SVR is the ultimate arbiter of risk of HCC. While many studies have shown no increased risk of HCC after DAA treatment, multiple studies have demonstrated a lower risk of HCC in DAA-treated patients who achieve SVR compared to untreated patients<sup>[22,23,37]</sup>. Treatment with DAAs also portends other benefits such as a decrease in MELD and Child-Pugh score (which sometimes results in delisting for liver transplant and the so-called MELD “purgatory”), and a reduction in the risk of death as demonstrated by the French Hepather cohort<sup>[23,38-46]</sup>. Munoz *et al.*<sup>[39]</sup> have estimated that the DAA-induced reduction in MELD score to below the threshold for liver transplantation listing may occur in 592-993 listed patients/year during the first year after treatment, and that 213-515 donated livers/year could be redistributed as a result. As more time passes since their development and additional studies with longer follow-up are published, the benefit of treatment with DAAs and the lack of a causative effect on carcinogenesis becomes clearer. It is now evident that withholding DAA treatment denies patients the possibility of a significant improvement in liver disease and consigns patients to a higher risk of HCC development.

In conclusion, we did not find evidence of increased rates of *de novo* HCC or recurrence in DAA-treated compared with IFN-treated patients. Compared to those treated with IFN, older patients with additional

pre-existing risk factors for HCC development were treated with DAA. This imbalance would appear to explain the higher numerical incidence of *de novo* HCC among DAA-treated patients. Given the success and cost-effectiveness of DAA therapy for the treatment of HCV infection<sup>[47-49]</sup>, clinicians should not be dissuaded by prior studies that suggest an increased risk of precipitating HCC development, as this seems to largely be a product of the presence of more advanced liver disease and increased risk factors among DAA-treated patients. Rather, the practice of continued surveillance for HCC for those persons with baseline risk factors, should continue to be reinforced.

## DECLARATIONS

### Authors' contributions

Contributed to the final version of the manuscript: Rutledge SM, Li DK, Chung RT

Performed the statistical analysis: Zheng H

Supervised the project: Chung RT

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

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Not applicable.

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Review

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# Immune checkpoint blockade therapies for HCC: current status and future implications

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## Abstract

Hepatocellular carcinoma (HCC) is the most lethal and common type of liver cancer with limited treatment options at the advanced stage. The use of immune checkpoint inhibitor (ICI) based immunotherapy is exponentially increasing in the treatment of patients with advanced solid tumors. The expression of immune checkpoints on tumor cells leading to lower activity of T-cells is one of the major mechanisms of immune escape. Checkpoint blockade immunotherapies with antibodies against PD-1, PD-L1 or CTLA-4 are being investigated in clinical trials in HCC patients. ICIs have improved survival in patients with inoperable advanced stage HCC where other curative treatments are not applicable. However, the response rates remain low with only a small subset of patients responding to this therapy. There is an unmet need to identify predictive markers to select those HCC patients who would benefit from ICI therapies. Importantly, epithelial-to-mesenchymal transition (EMT), a major process driving HCC invasion and metastasis by regulating the phenotypic cellular switching from epithelial to mesenchymal state, has been implicated as a resistance mechanism associated with ICI therapies. The role of EMT as a regulator of immune checkpoint molecule in HCC is just emerging. However, the consequence of EMT as a resistance mechanism in HCC patients undergoing ICI treatments remains unexplored. In this review, we summarize the recent clinical studies with ICIs in HCC and highlight the trials underway featuring novel monotherapies and combinatorial approaches based on immune and non-immune therapies. We will discuss the ongoing efforts to discover new immune checkpoint molecules in HCC as potential drug targets. We also highlight the role of EMT in facilitating therapy resistance in HCC treated with ICIs and discuss potential strategies to circumvent resistance in ICI treated HCC patients.



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**Keywords:** Hepatocellular carcinoma, immunotherapy, immune checkpoint inhibitors, epithelial-to-mesenchymal transition, programmed cell death protein-1, programmed death-ligand 1, resistance

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent type of primary liver cancer and is associated with a high mortality rate<sup>[1]</sup>. The incidence of HCC is increasing annually by 3%-9% worldwide and the number of new cases and the number of deaths are almost in equal proportions<sup>[2]</sup>. Patients diagnosed with early stage HCC, have a better prognosis than advanced stage HCC patients with unresectable tumors<sup>[3]</sup>. Surgical resection and liver transplantation, the curative treatment approaches for early stage HCC provides 5-year survival rate of greater than 70%<sup>[4,5]</sup>. Loco-regional therapies such as radiofrequency ablation (RFA), thermal and non-thermal ablation and transarterial chemoembolization (TACE) are also available as alternative treatment options for unresectable early stage HCCs<sup>[6-8]</sup>. However, the multi-targeted tyrosine kinase inhibitor (TKI) Sorafenib and Lenvatinib are the only first-line treatment available for the inoperable advanced stages of HCC<sup>[9]</sup>.

As the survival benefit with Sorafenib is limited to only 3 months<sup>[10]</sup>, several clinical trials have examined the suitability of new drugs for the treatment of patients with advanced stage HCC<sup>[11]</sup>. TKIs such as Regorafenib, Ramucirab, and Cabozantinib have been recently approved by the Food and Drug Administration (FDA) as second-line treatment alternatives for HCC patients previously treated with Sorafenib<sup>[12-15]</sup>. In addition, a combination therapy of TACE plus Sorafenib from the TCTICS trial also reported improved progression-free survival<sup>[11]</sup>. However, the limited survival benefit and associated toxicity with TKIs suggests an urgent need for better and efficacious treatment approaches for advanced stage HCC.

Immunotherapy has emerged as a potential alternative in the treatment of cancers following the clinical success of immune checkpoint inhibitors (ICIs). ICIs target the negative immune regulatory pathways such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and the programmed cell death protein-1/programmed cell death ligand 1 (PD-1/PD-L1) which inhibit T-cell immune response. ICI treatments have demonstrated dramatic anti-tumor clinical effects in several malignancies including melanoma, lung cancer and renal cell carcinoma<sup>[16-19]</sup>. Immunotherapeutic approaches based on ICIs have substantially enhanced disease-free survival in HCC patients resulting in the approval of anti PD-1 monoclonal antibodies, Nivolumab and Pembrolizumab, as second-line treatment options for advanced HCC<sup>[20-22]</sup>. Notably, Nivolumab increases survival in HCC patients to 17 months, far exceeding the 3 months extension in survival offered by Sorafenib<sup>[20]</sup>.

In this review, we will highlight the clinical trials that address the utility of ICIs as therapeutic tools in the management of HCC. We will focus on ICIs as monotherapies and combination therapy regimen for HCC patients. Although ICIs have proven to be effective, therapeutic resistance occurs in the majority of patients, leading to tumor progression. We explore EMT process as a main resistance mechanism to immune checkpoint blockade therapy and review studies that link EMT to immune checkpoint regulation.

## IMMUNOTHERAPY BASED ON IMMUNE CHECKPOINT BLOCKADE

Immune equilibrium is vital for preventing uncontrolled immune responses leading to severe inflammatory conditions or autoimmune disorders<sup>[23,24]</sup>. The immune equilibrium is maintained by balance between co-inhibitory and co-stimulatory signals that regulate T-cell activation<sup>[23-25]</sup>. T-cells are activated when specific antigens are recognized by T-cell receptors, whereas, the immune checkpoints provide an inhibitory effect on the activation of T-cells<sup>[23,24]</sup>. Immune checkpoint molecules are thus responsible for self-tolerance and

prevent immune overstimulation in normal conditions<sup>[23,24]</sup>. However, the cancer cells hijack these immune checkpoint molecules to bypass T-cell-mediated cytotoxicity resulting in tumor immune evasion<sup>[26]</sup>.

ICIs are the class of immunotherapeutic drugs including monoclonal antibodies against immune checkpoint molecules that stops the inhibitory effects of immune checkpoint molecules on T-cells resulting in the restoration of immune-mediated antitumor activity<sup>[16,27]</sup>. The first ICI drug approved by FDA for cancer immunotherapy was Ipilimumab (anti-CTLA-4) for treatment of advanced melanoma<sup>[26]</sup>. The PD-1/PD-L1 pathway along with CTLA-4 are the most studied and targeted molecules in cancer immunotherapeutic research and clinical trials<sup>[28]</sup>. Several other immune checkpoint molecules have also been assessed as potential targets such as TIM-3, BTLA, VISTA, LAG-3, VTCN1, CD73, B7-H3 and OX40<sup>[23,25]</sup>. ICIs have shown clinical benefits in several other cancers such as lung cancer and renal cell carcinoma following its approval in melanoma<sup>[16-19]</sup>.

## FEASIBILITY OF IMMUNE CHECKPOINT BLOCKADE IN HCC

Immune checkpoint blockade therapy can be exploited as an alternative treatment approach in HCC similar to other cancers, as liver possess a unique immunobiology<sup>[29]</sup>. The tumor microenvironment (TME) in HCC is known to play a vital role in immune activation or suppression contributing to either tumor eradication or tumor progression<sup>[6,30]</sup>. The strong intrinsic immune suppressive microenvironment of the liver results in intrahepatic tolerogenicity<sup>[6,31]</sup>. Some of the key players contributing to immunological tolerance in liver are liver sinusoidal endothelial cells, Kupffer cells and hepatic dendritic cells<sup>[6]</sup>. This immune suppressive microenvironment is more evident during formation and progression of HCC depending on several mechanisms including expression of immune checkpoint molecules leading to the development of an anti-tumor immunity<sup>[6,32]</sup>. These immune evasive abilities of HCC make immunotherapy a plausible therapeutic option in HCC. Several clinical studies have already reported efficacy of ICI drugs in HCC. However, only two ICI drugs, Nivolumab and Pembrolizumab, have been approved for HCC patients previously treated with Sorafenib based on the CheckMate 040 trial and Keynote-224 trial respectively<sup>[20,22]</sup>.

## ICIS IN THE CLINICAL MANAGEMENT OF HCC

Several clinical trials have been conducted and many others are ongoing in HCC including ICIs alone or in combination with other therapeutic agents. The clinical studies of ICIs in HCC constitute targeting PD-1, PD-L1 and CTLA-4. The key findings from some major earlier clinical studies of ICIs in HCC are summarized in Table 1. The clinical immune checkpoint blockade studies have either been as a monotherapy or combination therapy.

## ICI AS MONOTHERAPY IN HCC

ICIs have been used as monotherapy in several clinical studies for HCC as summarized in Table 2.

## ICIS BLOCKING CTLA-4

CTLA-4 is a protein receptor expressed on activated T-cells and Tregs which binds to CD80 and CD86 upon stimulation such that it blocks the binding of CD28 to CD80 and CD86 and inhibits T-cell activation<sup>[23,33]</sup>. A study has shown that treatment with anti-CTLA-4 antibody resulted in increased frequency of tumor-associated antigens such as interleukin (IL)-1, IL-6 and macrophage inflammatory protein-1 in 60% of HCC patients<sup>[34]</sup>.

### Tremelimumab

In HCC, the first clinical trial using ICI was Tremelimumab, anti-CTLA-4, reported by Sangro *et al.*<sup>[35]</sup>. In this trial, HCC patients with chronic Hepatitis C viral infection were treated with Tremelimumab

**Table 1. Findings of initial clinical studies of immune checkpoint inhibitors in hepatocellular carcinoma**

Target	Immune checkpoint inhibitor	Phase	Overall survival	Clinical trial number	Approval	Reference
PD-1	Nivolumab	I/II	15 months dose escalation	NCT01658878	Approved	[20]
	Pembrolizumab	II	12.9 months	NCT02702414	Approved	[22]
CTLA-4	Tremelimumab	II	8.2 months	NCT01008358	Not approved	[35]
PD-L1	Durvalumab	I/II	13.2 months	NCT01693562	Not approved	[51]
PD-L1 and CTLA-4	Durvalumab + Tremelimumab	I/II	Not reported	NCT02519348	Not approved	[55]
CTLA-4 and ablation	Tremelimumab + ablation		12.3 months	NCT01853618	Not approved	[64]

PD-1: programmed death protein-1; CTLA-4: cytotoxic T lymphocyte-associated protein-4; PD-L1: programmed death protein ligand -1

**Table 2. Current clinical trials of immune checkpoint inhibitors as monotherapy in hepatocellular carcinoma**

Target	Immune checkpoint inhibitor	Phase	Clinical trial number	Design	Lines of therapy	End point
PD-1	Nivolumab	III	NCT02576509	Nivolumab vs. Sorafenib	First-line therapy	OS
	Nivolumab	III	NCT03383458	Nivolumab vs. placebo	Adjuvant therapy	PFS
	Pembrolizumab	III	NCT03062358	Pembrolizumab vs. placebo	Second-line therapy	OS
	Pembrolizumab	II	NCT03337841	Pembrolizumab	Neoadjuvant therapy	RFS
	Tislelizumab	II	NCT03419897	Tislelizumab	Second-line therapy	ORR
	Tislelizumab	III	NCT03412773	Tislelizumab vs. Sorafenib	First-line therapy	OS
	Camrelizumab	II/III	NCT02989922	Camrelizumab	Second-line therapy	ORR/OS
PD-L1	Avelumab	II	NCT03389126	Avelumab	Second-line therapy	ORR

PD-1: programmed death protein-1; PD-L1: programmed death protein ligand -1; OS: overall survival; PFS: progression free survival; RFS: recurrence free survival; ORR: overall response rate

and 3 out of 17 assessable patients showed partial responses (17.6%) and an additional 10 patients (58.8%) had stable disease resulting in time-to-progression of 6.48 months and overall survival of 8.2 months (NCT01008358)<sup>[35,36]</sup>. Tremelimumab is the only anti-CTLA-4 ICI which is undergoing a phase III trial as monotherapy in HCC as of September 2018<sup>[28]</sup>.

## ICIS BLOCKING PD-1

PD-1, a key regulator of T-cell mediated immune response, is expressed by activated T cells, B-cells, natural killer cells, Tregs, myeloid-derived suppressor cells (MDSCs), monocytes and dendritic cells<sup>[37]</sup>.

### Nivolumab

Nivolumab is the first recombinant monoclonal human IgG4 antibody specific for PD-1<sup>[11]</sup>. Nivolumab is also the first FDA approved ICI for HCC based on the CheckMate 040 trial (NCT01658878)<sup>[20]</sup>. The phase I/II study of CheckMate 040 trial with 262 treated patients and 202 patients with complete treatment reported a response rate of 20% with three complete responses and 39 partial responses in patients with advanced HCC and Child-Pugh A cirrhosis who progressed on or were intolerant to Sorafenib<sup>[20]</sup>. There are several ongoing clinical trials for Nivolumab in HCC either as monotherapy or in combination. The success of earlier clinical studies of Nivolumab led to a phase III clinical trial CheckMate 459 (NCT02576509) examining Nivolumab as a first-line therapy in HCC and comparing the effects with Sorafenib in 726 HCC patients<sup>[38]</sup>. However, a press release from Bristol-Myers Squibb recently announced that the topline results from the phase III clinical trial CheckMate 459 failed to meet its primary endpoint of overall survival. Nivolumab is also being studied as an adjuvant therapy after surgical resection or ablation therapy in a second phase III trial CheckMate 9Dx (NCT03383458)<sup>[28]</sup>. There are several ongoing clinical trials for Nivolumab in HCC either as monotherapy or in combination with other therapies.

### Pembrolizumab

Pembrolizumab is another recombinant monoclonal human IgG4 antibody specific for human PD-1. Pembrolizumab gained approval for HCC patients previously treated with Sorafenib in November 2018

based on a phase II clinical study of HCC patients, Keynote-224 (NCT02702414) that reported an overall response rate of 17% among 104 patients with 1 complete response and 16 partial responses<sup>[22]</sup>. A clinical study with 450 Asian HCC patients to evaluate efficacy and safety of Pembrolizumab or placebo with best supportive care (NCT03062358) is ongoing<sup>[16]</sup>. Another study is examining Pembrolizumab before and after surgery or ablation to evaluate HCC recurrence (NCT03337841)<sup>[16]</sup>. Recently, a phase III clinical study Keynote-240 investigating Pembrolizumab plus best supportive care compared to placebo plus best supportive care failed to meet its co-primary endpoints of overall survival and progression free survival in 413 patients with advanced HCC previously treated with systemic therapy<sup>[39]</sup>. Similar to Nivolumab, there are several ongoing trials of Pembrolizumab in HCC either as monotherapy or in combination with other treatments.

### **Tislelizumab**

Tislelizumab is also another human IgG4 against PD-1<sup>[40]</sup>. A phase I trial of Tislelizumab in 61 patients with solid cancers including HCC confirmed the safety of this drug<sup>[28]</sup>. In HCC, Tislelizumab is undergoing two clinical studies, one is a phase II clinical study assessing safety, efficacy and pharmacokinetics of the drug in 228 previously treated unresectable HCC patients (NCT03419897) and another is a phase III clinical study that compares safety and efficacy of Tislelizumab with Sorafenib as first line systemic treatment in 660 patients with unresectable HCC (NCT03412773)<sup>[28,41]</sup>.

### **Camrelizumab**

Camrelizumab is a human IgG4 mAb against PD-1 which was reported to exhibit an anti-tumor response in 58 patients with solid cancers including HCC in a phase I trial<sup>[42,43]</sup>. Currently, several clinical studies are ongoing with Camrelizumab in HCC either alone or in combination with other treatments<sup>[40]</sup>. A phase II/III trial of Camrelizumab reported a response rate of 13.8% and 6 month overall survival rate of 74.7% in HCC patients previously treated with systemic treatment (NCT02989922)<sup>[44]</sup>.

### **ICIS BLOCKING PD-L1**

PD-L1 is the main ligand for PD-1 that is responsible for suppression of T-cell migration, proliferation and secretion of cytotoxic mediators<sup>[45,46]</sup>. Studies have shown that higher expression of PD-L1 is associated with poor prognosis in HCC patients<sup>[25,47-50]</sup>. A study reported that PD-L1 expression by neoplastic and intra-tumoral inflammatory cells was associated with tumor aggressiveness<sup>[47]</sup>.

### **Durvalumab**

Durvalumab is an anti-PD-L1 antibody which has been approved for treatment of advanced or metastatic urothelial carcinoma and non-small cell lung cancer<sup>[40]</sup>. Durvalumab was reported with a 10% response rate and median survival of 13.2 months in a cohort of 40 HCC patients in a phase I/II clinical study of Durvalumab monotherapy for solid cancers including HCC (NCT01693562)<sup>[51]</sup>.

### **Avelumab**

Avelumab is a human IgG1 mAb targeting PD-L1 with ongoing trials for both monotherapy and combination therapy in HCC<sup>[40]</sup>. A phase II study of Avelumab is ongoing with 30 HCC patients previously treated with Sorafenib (NCT03389126)<sup>[40]</sup>.

### **ICI AS COMBINATION THERAPY IN HCC**

Despite promising results from clinical studies of ICIs as monotherapy in HCC, only a small patient population benefit from specific immune checkpoint blockade therapy<sup>[52]</sup>. Thus, several combination approaches have been utilized to improve the efficacy of ICI therapy. In HCC, the combination of anti-CTLA-4 and anti- PD-1/PD-L1 along with combinations of ICIs with other immune and non-

**Table 3. Current clinical trials of immune checkpoint inhibitors as combination therapy in hepatocellular carcinoma**

Target	Study design	Clinical trial number	Phase	End point
Combination with other immune-based therapies				
PD-1 and CTLA-4	Nivolumab + Ipilimumab	NCT03682276	I/II	ORR
	Nivolumab + Ipilimumab	NCT03510871	II	
	Nivolumab +/- Ipilimumab	NCT03222076	II	Safety
	Nivolumab +/- Ipilimumab	NCT03203304	I	Safety
	Tremelimumab <i>vs.</i> Tremelimumab + Durvalumab <i>vs.</i> Sorafenib	NCT03298451	III	OS
	Tremelimumab <i>vs.</i> Durvalumab <i>vs.</i> Tremelimumab + Durvalumab	NCT02519348	II	Safety
PD-L1 and TIM-3	LY3300054 +/- LY3321367	NCT03099109	I	Safety
PD-1 and LAG-3	REGN2810 +/- REGN3767	NCT03005782	I	Safety/ORR
Combination with molecular targeted agents				
PD-L1 and anti-VEGF	Atezolizumab + Bevacizumab	NCT02715531	I	Safety/ORR
PD-L1 and anti-VEGF	Atezolizumab + Bevacizumab <i>vs.</i> Sorafenib	NCT03434379	III	OS/ORR
PD-1 and TKI	Pembrolizumab + Lenvatinib <i>vs.</i> Lenvatinib	NCT03713593	III	PFS/OS
PD-1 and TKI	Pembrolizumab + Lenvatinib	NCT03006926	I	Safety/OR/DOR
PD-1 and TKI	Camrelizumab (SHR-1210) + Apatinib	NCT02942329	I/II	OS
PD-1 and TKI	Spartalizumab (PDR001) + Sorafenib	NCT02988440	I	Safety
PD-1 and c-MET inhibitor	Spartalizumab (PDR001) +/- Capmatinib (INC280)	NCT02795429	I/II	Safety/ORR
PD-1 and anti-TGF- $\beta$	Spartalizumab (PDR001) +/- NIS793	NCT02947165	I	Safety
PD-1 and FGFR4 inhibitor	Spartalizumab (PDR001) +/- FGF401	NCT02325739	I/II	Safety/TTP/ORR
PD-1 and TKI	Nivolumab +/- Lenvatinib	NCT03418922	I	Safety
PD-1 and TKI	Nivolumab + Cabozatinib	NCT03299946	I	Safety/Completion
PD-1 and anti-VEGF	Nivolumab + Bevacizumab	NCT03382886	I	Safety
PD-1 and TKI	Pembrolizumab + Regorafenib	NCT03347292	I	Safety
PD-1 and TKI	Pembrolizumab + Sorafenib	NCT03211416	I/II	ORR
PD-L1 and TKI	Avelumab + Axitinib	NCT03289533	I	Safety
PD-L1 and DNMT inhibitor	Durvalumab + Guadecitabine	NCT03257761	I	Safety/ORR
CTLA-4, PD-1 and anti-OX40	Nivolumab + INCAGN01949 <i>vs.</i> Ipilimumab + INCAGN01949 <i>vs.</i> Nivolumab + Ipilimumab + INCAGN01949	NCT03241173	I/II	Safety/ORR
PD-1 and anti-phosphatidyl-serine	Pembrolizumab + Bavixumab	NCT03519997	II	ORR
Combination with local therapies				
PD-1 and ischemia	Nivolumab + TACE	NCT03143270	I	Safety
PD-1 and radiation	Pembrolizumab + TACE	NCT03397654	I/II	Safety
PD-1 and radiation	Nivolumab + Y90	NCT03033446	II	ORR
CTLA-4, PD-L1 and ischemia	Tremelimumab + Durvalumab + Radiation	NCT03482102	II	ORR
PD-1 and HSV oncolytic virus	Pembrolizumab +/- Talimogene Laherparepvec (T-VEC)	NCT2509507	I	Safety/ORR

PD-1: programmed death protein-1; CTLA-4: cytotoxic T lymphocyte-associated protein-4; PD-L1: programmed death protein ligand -1; TKI: tyrosine kinase inhibitor; OS: overall survival; PFS: progression free survival; RFS: recurrence free survival; ORR: overall response rate; TTP: time to progression

immune based treatment approaches are being studied. The combination therapies with ICI for HCC are summarized in [Table 3](#).

## IMMUNE-BASED COMBINATION THERAPIES FOR HCC

The blockade of CTLA-4 and PD-1/PD-L1 is the most promising ICI combination therapy that could enhance the anti-tumor effects in HCC. This combination blockade therapy has been very effective as an immune dampener as CTLA-4 signaling prevents the initiation of a T-cell response, while the PD-1/PD-L1 axis limits T-cell activity in the TME<sup>[28]</sup>.



**Ipilimumab (anti-CTLA4) + Nivolumab (anti-PD-1)**

Since its FDA approval in 2011 for advanced melanoma, Ipilimumab (anti-CTLA4) has also been approved for renal cell carcinoma in combination with another ICI, Nivolumab (anti-PD-1), based on CheckMate 214<sup>[53,54]</sup>. In HCC, there are four ongoing trials combining Ipilimumab with other ICIs<sup>[40]</sup>. The first study is the combination therapy of Ipilimumab and Nivolumab for HCC patients before liver resection (NCT03682276)<sup>[40]</sup>. The second study is also a combination therapy with Nivolumab as neoadjuvant therapy for HCC (NCT03510871)<sup>[40]</sup>. A third study compares the combination of Ipilimumab and Nivolumab versus Nivolumab alone in resectable HCC (NCT03222076)<sup>[40]</sup>. The fourth study also compares combination of Ipilimumab and Nivolumab with Nivolumab alone in terms of safety and tolerability, after external beam photon stereotactic body radiotherapy in patients with unresectable HCC (NCT03203304)<sup>[40]</sup>.

**Tremelimumab (anti-CTLA4) + Durvalumab (anti-PD-L1)**

A phase I/II clinical study including combination of Tremelimumab (anti-CTLA4) and Durvalumab (anti-PD-L1) in 40 HCC patients reported a response rate of 25% and manageable toxicity profile<sup>[55]</sup>. Currently, a phase III study of combination therapy including various dosage regimens of Durvalumab and Tremelimumab versus Sorafenib is ongoing to compare the efficacy of these therapeutic approaches (NCT03298451)<sup>[56]</sup>. Similar combination therapy of Tremelimumab and Durvalumab is being studied in a phase II trial in HCC patients previously treated with Sorafenib (NCT02519348)<sup>[52]</sup>.

**Other ICI combinations**

Besides CTLA-4, other immune checkpoint molecules such as TIM-3 and LAG-3 are also being examined in combination with PD-1/PD-L1 blockade therapy<sup>[52]</sup>. There are ongoing clinical studies with combination of anti-TIM3 antibody LY3321367 with anti-PD-L1 antibody LY3300054 (NCT03099109), anti-LAG-3 antibody REGN3767 with or without the anti-PD-1 antibody REGN2810 (NCT03005782)<sup>[16]</sup>.

**NON-IMMUNE-BASED COMBINATION TREATMENTS WITH ICIS**

The effects of ICI therapy in HCC could be enhanced when combined with non-immune-based therapies such as chemotherapy with the aim to improve anti-tumor efficacy and survival in HCC.

**Atezolizumab (anti-PD-L1) + Bevacizumab (anti-VEGF)**

Atezolizumab is a human IgG1 mAb against PD-L1 which is being studied in combination with Bevacizumab (anti-VEGF antibody) in several clinical studies<sup>[57,58]</sup>. A phase I study of Atezolizumab and Bevacizumab as combination therapy reported a tolerable safety profile and promising response rates in patients (NCT02715531)<sup>[58]</sup>. Another phase III trial is ongoing for combination of Atezolizumab and Bevacizumab with 480 patients with advanced or metastatic HCC (NCT03434379)<sup>[57]</sup>.

**Pembrolizumab (anti-PD-1) + Lenvatinib (multikinase inhibitor)**

Pembrolizumab (anti-PD-1) in combination with Lenvatinib (a multikinase inhibitor) is currently being compared with Lenvatinib plus placebo as first-line treatment option in 750 HCC patients (NCT03713593)<sup>[59]</sup>. The combination therapy of Pembrolizumab and Lenvatinib reported a 42% response rate and median progression free survival of 9.69 months in HCC patients as per results presented at the ASCO 2018<sup>[28,57]</sup>. Another study is also ongoing with combination therapy of Pembrolizumab and Lenvatinib (NCT03006926)<sup>[16]</sup>.

**Camrelizumab (anti-PD-1) + Apatinib (TKI)**

Camrelizumab (SHR-1210) is an anti-PD-1 antibody, which in combination with Apatinib, a TKI, has been reported at the ASCO 2018 meeting in a phase I trial with 18 HCC patients to demonstrate a response rate of 38.9% and a median progression free survival of 7.2 months (NCT02942329)<sup>[60]</sup>.

### **Spartalizumab (anti-PD-1) + other agents**

Spartalizumab is a human IgG4 mAb against PD-1 that is currently being studied in combination with other drugs such as Sorafenib (NCT02988440), Capmatinib (c-Met inhibitor) (NCT02795429), NIS793 (anti-TGF- $\beta$ ) (NCT02947165) and FGF401 (fibroblast growth factor receptor 4 inhibitor) (NCT02325739)<sup>[40]</sup>.

### **Other combinations**

Several studies are ongoing for other combination of ICIs with molecular targeted agents such as Nivolumab + Lenvatinib (NCT03418922), Nivolumab + Cabozantinib (NCT03299946), Nivolumab + Bevacizumab (NCT03382886), Pembrolizumab + Regorafenib (NCT03347292), Pembrolizumab + Sorafenib (NCT03211416), Avelumab + Axitinib (NCT03289533), and others<sup>[28]</sup>.

There are ongoing early-phase studies with a combination of ICIs with other therapeutic agents such as the DNA methyltransferase (DNMT) inhibitor Guadecitabine (NCT03257761), the anti-OX40 mAb INCAGN01949 (NCT03241173), the anti-phosphatidylserine mAb Bavixumab (NCT03519997) and others<sup>[16]</sup>.

## **COMBINATION WITH LOCAL THERAPY**

Strategies to improve the potential efficacy of ICIs in HCC are being investigated in several ongoing clinical trials by including the addition of other conventional therapies such as TACE, RFA and other local therapies. Radiotherapy has been demonstrated to provide synergistic effect in combination with PD-L1 or CTLA-4 inhibitors<sup>[61,62]</sup>.

### **Nivolumab (anti-PD-1) + local therapy**

Nivolumab in combination with TACE using drug-eluting beads is under study to assess the safety of this combination in a phase I trial (NCT03143270)<sup>[63]</sup>.

### **Pembrolizumab (anti-PD-1) + TACE**

A phase I/II study of Pembrolizumab post TACE is evaluating safety and efficacy of the combination therapy (NCT03397654)<sup>[28]</sup>.

### **Tremelimumab (anti-CTLA4) + RFA or TACE**

Tremelimumab was also examined in combination therapy with RFA or TACE to test if tumor necrosis could induce antigenic stimulation and systemic immune response enhanced by immune checkpoint blockade (NCT01853618)<sup>[16,64]</sup>. This study resulted in partial response in 5 patients (26%) out of 19 evaluable patients and 12 patients (63%) had stable disease with time to progression 7.4 months and median overall survival of 12.3 months<sup>[16,64]</sup>.

### **Other combinations with local therapies**

In addition to above mentioned trials, there are several other clinical studies ongoing to assess the combination of ICIs with local therapies including Nivolumab plus radioembolisation using yttrium-90 (NCT03033446), Durvalumab + Tremelimumab combined with radiotherapy (NCT03482102), Pembrolizumab with the oncolytic viral preparation Talimogene Laherparepvec (NCT02509507) and others<sup>[28,65,66]</sup>. These studies suggest another therapeutic option for treating chemoresistant cancer may become available.

## **OVERCOMING THE LIMITATIONS OF IMMUNE CHECKPOINT BLOCKADE THERAPY**

Despite the clinical success with immune checkpoint blockade therapy, there have been several limitations. One of the major limitations of using ICIs is the associated significant adverse events from

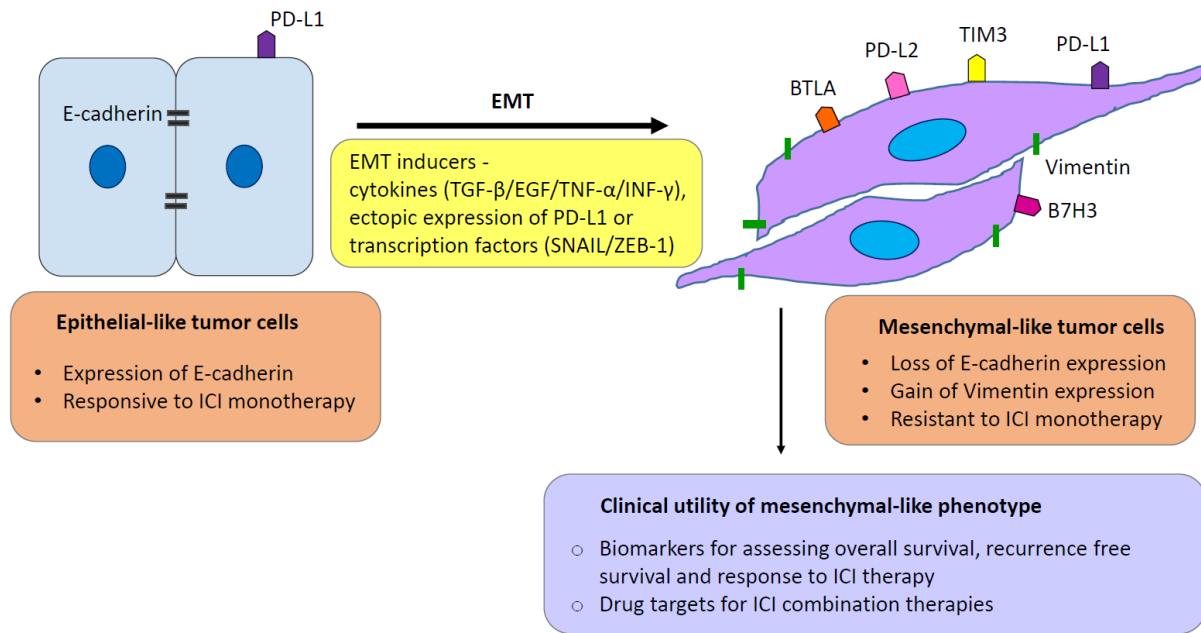
the therapy<sup>[28,52,67]</sup>. Johnson *et al.*<sup>[68]</sup> have reported two cases of lethal myocarditis in melanoma patients treated with combination of Nivolumab and Ipilimumab. In HCC, ICI monotherapy has shown some tolerable adverse effects such as fatigue, rash, pruritus and increase of serum transaminases that could be managed either by steroid therapy or discontinuation as there were no fatal adverse effects<sup>[20,35,52]</sup>. Several other immune-related adverse events such as pulmonary, gastrointestinal, cardiac, rheumatologic, renal, endocrine, neurologic and dermatologic toxicities have been reported in various cancers treated with ICIs<sup>[69]</sup>. The ideal management of adverse events is to identify these adverse events early with careful monitoring and use of respective treatment options<sup>[69]</sup>. Gastrointestinal toxicities including diarrhoea have been managed with anti-motility agents such as loperamide, diphenoxylate/atropine or higher fibre intake<sup>[69]</sup>. Similarly, the possible liver toxicities post ICI therapy can be managed through liver function tests prior to therapy followed by steroids and mycophenolate mofetil if necessary<sup>[70]</sup>. These toxicities can be rare in incidence but clinicians should monitor these events and act promptly for proper management<sup>[69]</sup>.

Another important limitation of immune checkpoint blockade therapy is poor response to ICI therapy whereby the patient fails to respond after the initial therapy or the patient develops resistance to ICI following initial response<sup>[67]</sup>. In hepato-pancreatic-biliary cancers, a majority of patients fail to respond to ICI therapy<sup>[71]</sup>. The failure of ICI therapy can result from three factors: (1) mutations of the immunogenicity of cancer itself leading to variable expression of immune related components; (2) redundancy due to expression of other immune checkpoint molecules besides the targeted molecule; and (3) decreased T-cell infiltration<sup>[46,72,73]</sup>. A study by Gopalakrishnan *et al.*<sup>[74]</sup> reported that the gut microbiome altered melanoma patient response to anti PD-1 ICIs. Similarly, another study in liver cancer revealed that the gut microbiome utilizes bile acid to regulate immune responses<sup>[75]</sup>.

The expression of immune checkpoint molecules varies among individuals suggesting the need for predictive biomarker to improve the efficacy of ICI therapy<sup>[28]</sup>. The expression of PD-L1 and tumor-infiltrating lymphocytes has been reported to be associated with success of ICI therapy<sup>[28]</sup>. The FDA has approved an IHC test for PD-L1 expression as a predictive biomarker<sup>[76]</sup>. However, some patients with low PD-L1 expression responded well to Nivolumab<sup>[77]</sup>. There is need for more robust predictive biomarkers for ICI therapy besides PD-L1 expression. Tumor mutation burden (TMB), a measure of the overall number of mutations in the tumor specimen, has also been reported as a potential predictive biomarker in ICI therapy<sup>[25]</sup>. Moreover, overexpression of alternative immune checkpoint molecules such as TIM-3 and LAG-3 following anti-PD-1 therapy has been reported<sup>[72]</sup>. In a clinical study of 422 HCC patients, although PD-L1 expression alone lacked predictive power, combining PD-L1 expression with epithelial-to-mesenchymal transition (EMT) phenotype marker expression was associated with poor overall survival and recurrence-free survival<sup>[25]</sup>. As EMT has also been implicated as a resistance mechanism in patients undergoing ICI treatments, a better understanding of this process may aid in overcoming resistance to ICI therapies. **Figure 1** summarizes the association between EMT and immune checkpoint regulation and also depicts the EMT process as a main resistance mechanism to immune checkpoint blockade therapy.

## ROLE OF EMT IN IMMUNE CHECKPOINT BLOCKADE THERAPY

EMT is a complex cellular process that enables epithelial cells to gain mesenchymal features resulting in aggressive and motile phenotype<sup>[78]</sup>. The EMT process enables cells to move distances and participate in the formation of internal organs, while the reverse process mesenchymal-to-epithelial transition (MET) enables cells to settle, proliferate and differentiate into different organs once they reach the destination<sup>[79-81]</sup>. EMT is regulated by several factors including transcription factors such as Snail, Twist, zinc-finger E-box-binding transcription factor, ZEB and others<sup>[78,82]</sup>. EMT is often induced by various cell signalling pathways such as TGF- $\beta$ , Wnt, STAT and NOTCH pathways<sup>[83]</sup>. The process of EMT induces epithelial carcinoma cells to transition to metastatic tumor cells such that tumor cells spread from their primary site to a new



**Figure 1.** Interconnection between EMT and immune checkpoint based immunotherapy. The diagram illustrates the transition of epithelial-like tumor cells toward a mesenchymal phenotype is associated with immune checkpoint regulation. EMT is induced by several factors including cytokines, upregulation of transcription factors and immune checkpoint molecule PD-L1. EMT is accompanied by the modulation of well-known EMT markers, the loss of epithelial marker E-cadherin and gain of mesenchymal marker Vimentin. Mesenchymal-like tumor cells with elevated expression of different immune checkpoint molecules are more resistant to ICI therapy compared with epithelial-like tumors. The coexistence of features of EMT and expression of immune checkpoint molecules opens the possibility of a mechanistic link between these processes and EMT markers in combination with immune checkpoint molecules can be studied in a prognostic or therapeutic context. PD-L1: programmed death protein ligand -1; EMT: epithelial-to-mesenchymal transition

secondary site where the reverse phenomenon MET enables the metastasized tumor cells to proliferate and differentiate to form secondary tumors<sup>[84,85]</sup>. Accumulating evidence implicates the process of EMT in promoting immune evasion of cancer cells<sup>[78,86]</sup>.

Several *in vivo* patient and animal model studies have shown that the activation of EMT in HCC promotes tumor progression and metastasis<sup>[87]</sup>. *In vitro* studies have shown that TGF- $\beta$ -induced EMT activates CXCR4/CXCL12 which in turn contributes to HCC tumor progression<sup>[88,89]</sup>. Another study in a mouse model has reported that miR-181, regulated by TGF- $\beta$ , is upregulated in HCC and promotes carcinogenesis<sup>[90]</sup>. The association of EMT and HCC has also been reported in several clinical studies. A study of 123 HCC patient samples reported that the majority of clinically aggressive HCC samples had decreased E-cadherin expression, a marker of EMT status<sup>[91]</sup>. In addition, the study also reported that EMT transcription factors Snail and Twist were associated with poor prognosis in HCC with increased invasive and migratory potential<sup>[91]</sup>. Another study reported that HCC patients with mesenchymal tumor phenotype showed earlier recurrence compared to patients with epithelial phenotypes<sup>[92]</sup>. Moreover, the study also showed that patients with epithelial tumor phenotype were more responsive to Sorafenib<sup>[92]</sup>. Collectively, these studies have demonstrated the pivotal role of EMT in HCC progression.

Accumulating evidence shows that cancer cells undergoing EMT can influence the components of the TME and facilitate immune escape by tumors<sup>[86,93]</sup>. The immune components within the TME are comprised of immunosuppressive cells including MDSCs, cancer-associated fibroblasts, tumor-associated macrophages and Treg cells<sup>[94]</sup>. EMT facilitates immune evasion of tumor cells by influencing these immunosuppressive TME cells. For instance, EMT promotes an immunosuppressive TME by recruitment of tumor-associated macrophages through regulation of cytokines<sup>[95]</sup>. EMT also contributes to immunosuppression through

regulation of immune checkpoint molecules as reported in several instances earlier in this review. EMT is also known to promote immune resistance to NK cell-mediated lysis<sup>[94]</sup>. An EMT inducer, TGF- $\beta$ , promotes immunosuppression by several mechanisms including impaired maturation, differentiation or activation of innate and adaptive immune cells, inhibition of cytotoxic T-cell functions and dysregulating cytokine production<sup>[94]</sup>. The association of EMT and immunosuppression in tumor cells has also been reported in HCC. A study reported that hypoxia-induced EMT promotes overexpression of CCL20 resulting in reduced CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation along with increased immunosuppressive Treg cells<sup>[96]</sup>. A study has reported that Snail-induced EMT is associated with immunosuppression in cancer patients<sup>[86]</sup>.

In addition, a study has shown that there is an association between EMT score of tumor cells and expression of immune checkpoint molecules such as PD-1, PD-L1, PD-L2, B7-H3 and others<sup>[97]</sup>. Several lung cancer studies have reported the association between EMT and immune checkpoint molecules. One of the earlier studies in lung adenocarcinoma reported that EMT was strongly associated with upregulation of multiple targetable immune checkpoint molecules such as PD-1, PD-L1, PD-2, CTLA-4, BTLA, B7-H3 and TIM-3<sup>[98]</sup>. Similarly, another study in lung adenocarcinoma demonstrated that EMT phenotype was related to PD-L1 overexpression<sup>[99]</sup>. Notably, a significant correlation between mesenchymal phenotype with expression of immune checkpoint molecules such as PD-1, PD-L1, CTLA-4, OX40L and PD-L2 was confirmed in lung cancer<sup>[97]</sup>. MUC1-C has been reported to simultaneously induce EMT and the expression of PD-L1 in non-small cell lung cancer<sup>[93]</sup>. In lung cancer cell lines, induction of EMT through downregulation of miR-200s and ZEB1 overexpression resulted in increased PD-L1 expression<sup>[100]</sup>. A very interesting study by David *et al.*<sup>[101]</sup> utilized M7824, a bifunctional fusion protein, inhibiting PD-L1 and TGF- $\beta$  to demonstrate that TGF- $\beta$ -induced immunosuppression in non-small cell lung cancer was mediated by PD-L1 upregulation. Chae *et al.*<sup>[102]</sup> reported reduced infiltration of immune cells with antitumor functions and increased infiltration of immune cells with immunosuppressive functions in mesenchymal non-small cell lung cancer. This study further reported increased expression of immune checkpoint molecules CTLA-4 and TIM-3 in mesenchymal lung adenocarcinoma and lung squamous cell carcinoma<sup>[102]</sup>.

Furthermore, PD-L1 expression was closely related with EMT as higher PD-L1 expression was observed in oral squamous cell carcinoma cells co-cultured with mesenchymal phenotypes<sup>[103]</sup>. In breast cancer, Noman *et al.*<sup>[104]</sup> revealed that ZEB-1/miR200 or Snai1 simultaneously induced EMT and upregulated the expression of PD-L1. Chen *et al.*<sup>[105]</sup> demonstrated that EMT positive human esophageal cancer tissues had higher PD-L1 expression compared to an EMT negative subgroup. Similar studies have shown an association between EMT and immune checkpoint expression in several cancers including thymic carcinoma<sup>[106]</sup>, melanoma<sup>[107]</sup>, adeno cystic carcinoma<sup>[108]</sup>, extrahepatic cholangiocarcinoma<sup>[109]</sup> and renal cell carcinoma<sup>[110]</sup>.

Many studies have reported several pathways involved in the regulation of PD-L1 by EMT. PD-L1 expression in non-small cell lung carcinoma was regulated by DNA methylation in a TGF- $\beta$ 1 dependent manner and by NF- $\kappa$ B/IKK $\epsilon$  signalling pathway in a TNF- $\alpha$  dependent manner<sup>[111]</sup>. Another study in lung cancer demonstrated that *p*-Smad2 dependent TGF- $\beta$  signalling is involved in PD-L1 overexpression<sup>[101]</sup>. Epidermal growth factor also induced EMT and PD-L1 expression in breast cancer and salivary adenoid cystic carcinoma cells<sup>[108,112]</sup>.

In HCC, a significant association of EMT phenotype with PD-L1 expression was reported in 422 HCC patients<sup>[25]</sup>. The study confirmed that high risk HCC patients had significantly higher expression of mesenchymal marker *Vimentin* and lower expression of the epithelial marker *E-cadherin* along with elevated expression of *PD-L1*<sup>[25]</sup>. Moreover, the combined coordinate expression of *PD-L1* with *E-cadherin* and *Vimentin* was associated with poor overall survival and recurrence-free survival<sup>[25]</sup>. This study suggested that patients with an EMT phenotype may benefit from PD-1/PD-L1 blockade therapy. *In vitro*



studies demonstrating the direct link between EMT and immune checkpoint expression in HCC are currently lacking.

A few studies have examined the regulation of immune checkpoints in HCC with cytokines that are known to induce EMT, but no EMT markers were evaluated in these studies. One such study in HCC identified that blocking PD-L1 and TGF- $\beta$  enhanced the immune response against tumor suggesting the combination approach of ICIs and TGF- $\beta$  inhibitor drugs<sup>[113]</sup>. The crosstalk between cytokines interferon (IFN)- $\gamma$  and TNF- $\alpha$  was shown to synergistically regulate PD-L1 expression in HCC cells<sup>[114]</sup>. Brown *et al.*<sup>[115]</sup> reported that resistance to ICI therapy in HCC was dependent upon overexpression of an immune checkpoint molecule, IDO-1. The authors demonstrated that IDO inhibitors could improve the efficacy and response to ICI therapy<sup>[115]</sup>. Although a few studies have explored the role of EMT in regulating immune checkpoints in HCC, further studies are warranted in this area. A better understanding of how EMT confers resistance to HCC cells treated with ICIs will enable us to develop more effective treatments for HCC.

Immunotherapy, in particular ICI therapy, has revolutionized the treatment approach in several cancers including HCC. ICI treatment is the best alternative in advanced HCC where other curative treatments are not applicable and when systemic therapies fail<sup>[20,25]</sup>. Several ICI clinical trials are underway for HCC, the majority of them target PD-1, PD-L1 and CTLA-4 as monotherapy or in combination with other ICIs or molecular targeted agents<sup>[28]</sup>. Recent studies have identified several novel immune checkpoint molecules that can be potential targets in HCC<sup>[25,116,117]</sup>.

Despite the clinical breakthrough of ICIs in HCC treatment, the response rate is unsatisfactory with a few adverse effects<sup>[28]</sup>. In addition, the resistance to ICI therapy has also limited the use of ICIs in a large patient population<sup>[72]</sup>. The challenge with ICI therapy is to increase the proportion of patients who may gain clinical benefits from this therapy<sup>[38]</sup>. The use of combination therapy with other ICIs may prove to be beneficial as studies report the emergence of alternative checkpoint molecules reduce the response to ICI therapy<sup>[72]</sup>. The efficacy of ICI therapy can be improved by early identification and management of adverse events<sup>[118]</sup>. The selection of patient population who might respond to ICIs is another challenge of ICI therapy<sup>[119]</sup>. Several predictive biomarkers have been utilized such as expression of immune checkpoint molecules (PD-L1 expression by IHC) and TMB<sup>[25,120]</sup>. However, there are limitations to these biomarkers. Studies have shown that PD-L1 negative patients also respond to anti-PD-1 and anti-PD-L1 treatments<sup>[120,121]</sup>. In addition, it has been reported that patients with a lower number of mutations also benefited from ICI therapy along with patients with higher mutational load<sup>[120,122]</sup>. Thus, there is an urgent need for better predictive biomarkers to improve efficacy of ICI therapy.

Recent studies in several cancers have identified the role of EMT in regulation of immune checkpoint expression. The association between EMT and immune checkpoint expression suggests the utility of EMT status as a potential predictive biomarker in ICI therapy. In addition, EMT inhibitors in combination with ICI may be a potential combination therapy to improve efficacy of ICI therapy in HCC. A few studies have investigated the potential benefits of targeting both EMT and immune checkpoint molecules by utilizing a fusion protein or antibodies targeting TGF- $\beta$  and PD-L1<sup>[101,123]</sup>. Drugs such as Silimarin and Apatinib have been identified that could block both PD-L1 expression and EMT in non-small cell lung cancer and osteosarcoma suggesting similar potential in HCC<sup>[114,124]</sup>. Collectively, inhibiting the EMT process could increase the sensitivity to ICI treatments and both *In vitro* and *in vivo* HCC studies in this area will lay the foundation for future clinical trials.

## CONCLUSION

The emergence of ICIs has provided much hope in improved cancer therapy in several malignancies including HCC. The majority of clinical studies for HCC are based on a few ICIs either as monotherapy or



combination therapy. There have not been sufficient studies exploring novel immune checkpoint molecules and predictive biomarkers for ICIs in HCC. The relationship between EMT and immune checkpoint molecules presents a promising combinatorial approach for the treatment of HCC.

## DECLARATIONS

### Authors' contributions

Made substantial contributions to conception and design of the study, manuscript writing: Shrestha R, Jayachandran A

Manuscript revision and interpretation: Shrestha R, Bridle KR, Crawford D, Jayachandran A

Final approval of manuscripts: Shrestha R, Bridle KR, Crawford D, Jayachandran A

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Commentary

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## Comment on “APOBEC3B interaction with PRC2 modulates microenvironment to promote HCC progression”

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Accounting for 75%-85% of all primary liver cancer cases, hepatocellular carcinoma (HCC) is nowadays one leading cause of cancer-related mortality worldwide<sup>[1]</sup>. More than half of HCC patients are diagnosed at the advanced stage, for which limited treatment options are available and no curative ones exist so far, leading to poor prognosis<sup>[2]</sup>. The main risk factors for HCC, such as infection with HBV and HCV, excessive alcohol consumption, obesity, and diabetes, all contribute to chronic liver inflammation, which leads to the formation of an altered liver microenvironment. In turn, an altered liver microenvironment can reciprocally reprogram the immune cells and hepatocytes involved in inflammation, together setting the stage for progression to cirrhosis and eventually to HCC<sup>[2-4]</sup>.

It has been demonstrated that tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) are the most abundant immune cell populations infiltrated in the tumor microenvironment of HCC. As pivotal players in cancer-related inflammation, TAMs and MDSCs promote hepatocarcinogenesis by stimulating angiogenesis and inducing immunosuppression and correlate with inferior prognosis<sup>[5-7]</sup>. Thus, it is of crucial importance to gain an in-depth look at the interplay between hepatocytes and immune cells, especially TAMs and MDSCs, during the development of HCC.

Recently Wang *et al.*<sup>[8]</sup> presented a remarkable study unraveling the functional significance of hepatocyte-intrinsic apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3B [APOBEC3B (A3B)] in



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promoting HCC progression by recruiting TAMs and MDSCs to the tumor microenvironment, thus inhibiting CD8<sup>+</sup> T cell function and further facilitating immune escape.

In this issue, the authors first demonstrated elevated abundance of A3B in HCC patients due to overactivation of the non-classical NF- $\kappa$ B pathway and direct transcriptional regulation by RelB. Taking advantage of both immunocompetent and immune-deficient mouse HCC models, they revealed that A3B activated HCC initiation through modulation of the immune system as A3B exerted its carcinogenic function only in mice with complete immune system, which was accompanied by increased secretion of CCL2, IL-34 and BMP7 and the subsequent accumulation of TAMs, MDSCs and Programmed cell death 1(PD1)<sup>+</sup> CD8 T cells.

After establishing the impact of hepatocyte-intrinsic A3B on immunological environment in HCC development, Wang and his colleagues performed a series of analyses to investigate the molecular basis of this phenomenon. They found out that A3B inhibited PRC2 activity through both interference of its binding affinity and attenuating its enzymatic activity, while PRC2 has been reported to be indispensable in the methylation of H3K27 and regulate chemokine expression<sup>[9,10]</sup>. Bioinformatic analyses showed a highly overlapping cohort of target genes, whose expression levels altered in inverse correlation upon exogenous A3B expression and H3K27me3. Experiments further demonstrated H3K27me3 sites at the promoter regions of CCL2, IL-34 and BMP7. Taken together, upregulated A3B suppressed occupancy of H3K27me3 on the promoter of chemokines CCL2, IL-34 and BMP7 by inhibiting PRC2 activity.

In last decade, immunotherapy dramatically revolutionized the therapeutic landscape in oncology and was announced as Breakthroughs of the Year by Science in 2013. However, the progress of introducing either chimeric antigen receptor (CAR)-modified T cells or checkpoint inhibitors into HCC therapy is rather slow. In 2017, Nivolumab was approved as the only anti-PD-1/L1 antibody for the treatment of HCC patients<sup>[11]</sup>. However, the response rate reached only about 20%<sup>[12]</sup>. One major cause of such low effectiveness lies in the immunosuppressive tumor microenvironment and immune escape. In addition to immunotherapy, epigenetic therapy has drawn much attention in recent years as well. However, outcome of pre-clinical and clinical trials of epigenetic drugs in HCC was rather disappointing, indicating other molecular mechanisms involved in epigenetic modulation<sup>[13]</sup>. This work by Wang and his colleagues discovered a crucial role of A3B in promoting HCC initiation by modulating immunological microenvironment via inhibition of H3K27 methylation, revealing A3B as a novel therapeutic target in immunotherapy of HCC, explaining partially the current failure of epigenetic drugs, and demonstrating the significance of combined therapy targeting both innate and acquired immune systems in future HCC treatment.

## DECLARATIONS

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The author contributed solely to the article.

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Review

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# The use of cell free DNA in the diagnosis of HCC

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## Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and is associated with high mortality. The currently used methods for diagnosing HCC, including imaging modalities and liver biopsy, detect tumors at a relatively advanced stage or are invasive. Non-invasive biomarkers are urgently needed to facilitate screening and early diagnosis of HCC, as well as treatment monitoring and detection of tumor recurrence. Liquid biopsy, the analysis of blood or other body fluids to obtain genetic and epigenetic information, has historically been applied to other types of cancer including breast and prostate cancer. Over the past few decades, liquid biopsy analysis has shed significant insights on genetic and epigenetic aberrations in HCC detectable in peripheral blood. Aberrations in nucleic acids found circulating freely in body fluids or contained within extracellular vesicles such as exosomes or microvesicles show potential clinical utility as non-invasive biomarkers. In this review, we present available literature on cell-free nucleic acids in the diagnosis of HCC.

**Keywords:** Hepatocellular carcinoma, liquid biopsy, cell free nucleic acid, cell free DNA, exosomes, microvesicles, biomarkers

## INTRODUCTION

Hepatocellular cancer (HCC) has become the second leading cause of cancer deaths worldwide<sup>[1]</sup>. Unfortunately, most cases of HCC are undetected until late stage due to absence of symptoms in early stage HCC, and the lack of sensitive and convenient methods of screening. Previous estimates showed that the



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one-year survival for HCC in the United States is less than 50%, while the five-year survival is 10%<sup>[2]</sup>. With the advent of potent therapy for chronic hepatitis C virus (HCV) infection, the overall global incidence of HCC may plateau or decrease as a result of decreased HCV-associated HCC, however these gains appear to be threatened by the increasing incidence of nonalcoholic fatty liver disease (NAFLD)-associated HCC and the persistently high levels of hepatitis B virus (HBV)-associated HCC.

Diagnosis of HCC can be made by imaging studies such as multiphasic computed tomography scan or magnetic resonance imaging. However, tissue biopsy remains the gold-standard for HCC diagnosis especially in non-cirrhotic patients or those with nonspecific imaging studies. The risks of biopsy include procedure-related complications such as pain, bleeding, and perforation of adjacent organs, as well as tumor seeding along the needle track and sampling errors resulting in false negative results. Aside from these risks, technological advancements over the past few decades have led to a better understanding of the high heterogeneity and dynamic evolution of HCC tumor cells, and a time- and location-constrained tissue biopsy is inadequate in reflecting these dynamic changes. These shortcomings have led to increased interest in the application of liquid biopsy analysis to HCC. Although the concept of liquid biopsy has been in existence for several decades, the term gained traction in the early 2000's, with one of its first uses pertaining to the capture of circulating tumor cells (CTCs) for biomarker analysis in breast cancer patients<sup>[3]</sup>.

Liquid biopsy generally refers to the analysis of blood or other body fluids to obtain genetic or epigenetic information which can be applied in screening, diagnosis, prognostication, treatment monitoring or disease recurrence<sup>[4]</sup>. The major advantage of liquid biopsy is non-invasiveness which makes it attractive for frequent analysis to track mutations and other molecular changes over time. The most commonly used HCC serum biomarker is serum alpha-fetoprotein (AFP), together with its fucosylated glycoform (AFP-L3). AFP is normally produced during gestation by the fetal liver and yolk sac, and levels decline rapidly after birth. Regeneration of liver cells leads to AFP production, as can be seen in chronic liver disease and in HCC. Other types of malignancy, for instance testicular or ovarian cancer, can also cause AFP elevation. The AFP-L3 glycoform, named for its ability to bind *Lens culinaris* agglutinin, is a relatively new test developed in 1992 that is more specific for HCC, compared to AFP<sup>[5]</sup>. Serum AFP concentration can be normal even in advanced HCC<sup>[6]</sup>. In two studies of approximately 1800 patients, AFP was found to have about 60% sensitivity and 80% specificity in detecting HCC using a cut off level between 10 to 20 ng/mL<sup>[7,8]</sup>. Higher serum AFP levels are associated with greater specificity and less sensitivity, for instance AFP > 400 ng/mL implies HCC until proven otherwise. However, fewer than 20% of HCC cases have such elevated AFP levels<sup>[9]</sup>.

Serum and plasma biomarkers detectable through liquid biopsy show promise in the early detection of HCC either alone or in combination with AFP. These markers have the potential to be adjunctive or superior to conventional methods of HCC diagnosis. Several of these markers, however, are still in preclinical development and testing and none of them has of yet been recommended for HCC diagnosis. Here, we provide an updated summary of cell-free nucleic acid (cfNA) analysis in the diagnosis HCC, with emphasis on cell-free DNA (cfDNA).

## LIQUID BIOPSY FOR HCC

Liquid biopsy specimens contain genetic information in CTCs or in the form of cfNAs released by apoptotic cells or living cells. cfNA can be found circulating freely in body fluids or are taken up by extracellular vesicles such as exosomes and microvesicles. The various types of cfNA include cfDNA, mRNA (cfRNA), and noncoding RNAs including miRNAs (cfmiRNA). Other noncoding RNAs including long noncoding RNA, small nuclear RNA, small nucleolar RNA, and piwi-interacting RNA may also be present in liquid biopsy specimens and could potentially serve as biomarkers although there are currently very few studies on these subtypes.

The first report of cfDNA derived from human peripheral blood was published by Mandel and Metais<sup>[10]</sup> in 1948, however its significance was not realized until several decades later in 1977 when it was discovered that serum and plasma from cancer patients carry higher concentrations of cfDNA compared to healthy individuals<sup>[11]</sup>. About a decade later, Vasioukhin showed that cfDNA can have cancer characteristics, suggesting that cancer cells can release DNA into peripheral blood<sup>[12]</sup>. This notion was soon confirmed by other investigators<sup>[13,14]</sup>, and cfDNA released by cancer cells into circulation has been subsequently referred to as circulating tumor DNA (ctDNA).

Analysis of plasma and serum cfRNA is limited by the very small quantities present in circulation as well as degradation by ribonuclease (RNase). Incorporation of cfRNA into extracellular vesicles protects them from degradation. Over the past decade, several groups have shown that cfRNA can potentially be applied in HCC detection and monitoring<sup>[15-17]</sup>. A recent study by Xu *et al.*<sup>[18]</sup> showed that serum mRNA levels of exosomal hnRNPH1 in patients with primarily HBV-associated HCC were significantly higher than in patients with chronic hepatitis B, liver cirrhosis, or healthy control<sup>[18]</sup>. Exosomal hnRNPH1 levels also associated with TNM stage, Child-Pugh classification, portal vein embolism and lymph node metastasis.

Non-coding RNA, especially cfmiRNAs were first demonstrated as a promising biomarker in patients with solid cancers in 2008<sup>[19]</sup>. Since then, there have been several studies on non-coding RNAs in different types of cancer including HCC<sup>[20]</sup>. A recent article mapped the differential expression of non-coding RNAs in normal liver tissue and in various stages of liver disease leading to HCC; each liver phenotype was found to demonstrate a unique RNA signature<sup>[21]</sup>. Induction of exosomal miR-21 and miR-10b in HCC was found to promote cancer cell proliferation and metastasis, potentially serving in prognostication and therapy for HCC<sup>[22]</sup>. Several other cfmiRNAs have been studied, including miR-1<sup>[23]</sup>, miR-16<sup>[24-26]</sup>, and miR-122<sup>[23,27-29]</sup>. An in-depth review of circulating miRNA signatures in HCC is beyond the scope of this article, and the reader is referred to a recent article by Mirzaei *et al.*<sup>[30]</sup> for further information.

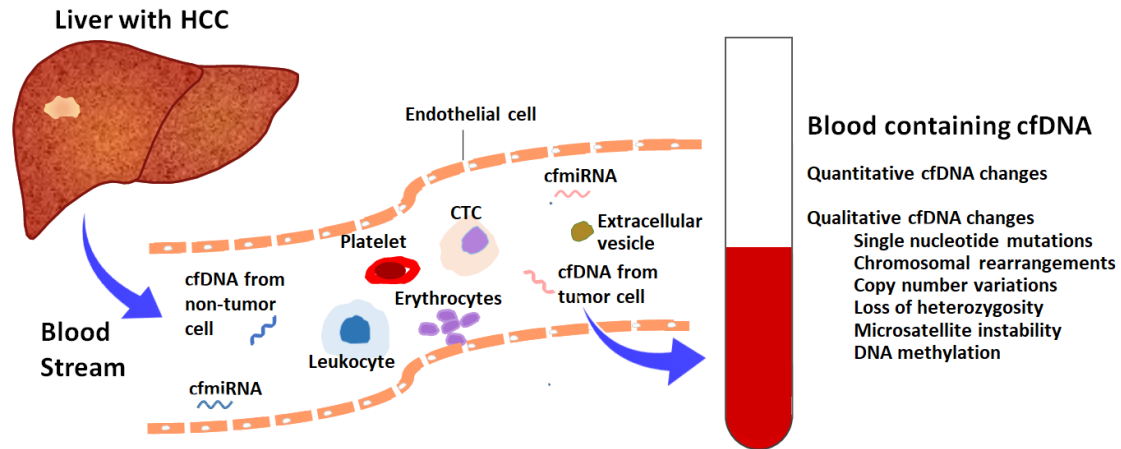
Liquid biopsy analysis in HCC has significantly expanded over the past decade, providing substantial information on different HCC tumors and their microenvironment, and the potential application of such information to disease diagnosis and monitoring.

### Isolation of cell-free DNA in liquid biopsy samples

There are several challenges in the isolation of cfDNA in general and ctDNA in particular, including DNA lysis as a results of blood clotting in collection tubes, DNA contamination during processing or DNA loss during isolation. Thus, the right sample collection tube and optimal processing methods are crucial to the success of isolation and to the accuracy of the sample obtained. A 1 mL volume of blood typically yields 10 ng of cfDNA, and in cancer patients, about 0.01% to 1% of cfDNA comprises ctDNA. Several methods have been used in the isolation of ctDNA, including targeted methods involving polymerase chain reaction (PCR) based on known genetic mutations, for instance digital PCR; bead, emulsion, amplification and magnetics (BEAMing) PCR; and amplification-refractory mutation system-PCR. Alternatively, a variety of untargeted methods can be employed to sequence millions of DNA fragments, including Sanger sequencing and next-generation sequencing techniques such as targeted amplification sequencing or targeted capture sequencing<sup>[31,32]</sup>.

### HCC-ASSOCIATED QUANTITATIVE CHANGES IN CELL-FREE DNA

Cancer is associated with both quantitative and qualitative changes in cfDNA detectable by liquid biopsy<sup>[33-37]</sup> [Figure 1]. In patients with HCC, total cfDNA concentration is significantly higher than in those without HCC<sup>[33,34]</sup>. Although non-specific, cfDNA increase in association with HCC has potential utility in screening for HCC, as well as in monitoring of treatment response and in predicting HCC



**Figure 1.** Liquid biopsy for hepatocellular carcinoma. Tumors release a number of molecules into circulation including tumor cells, cell-free DNA, different circulating RNA classes, proteins and extracellular vesicles including exosomes and microvesicles. Cell-free DNA can be isolated from blood or other body fluids and analyzed to determine genetic and epigenetic changes present in circulation which are reflective of changes occurring in tissues, potentially avoiding the need for invasive tissue sampling. cfDNA: cell free DNA; cfmiRNA: cell free microRNA; CTC: circulating tumor cell; HCC: hepatocellular carcinoma

recurrence<sup>[34,38-40]</sup>. A recent manuscript by Yan *et al.*<sup>[41]</sup> analyzed cfDNA and AFP levels from 24 patients with HCC and 62 patients with chronic hepatitis B with varying degrees of fibrosis (F0 to F6). Using multivariate analysis, the authors found that age and cfDNA were independent predictors of HCC, while AFP was not an independent predictor. They developed a combination model including cfDNA level, age and AFP, collectively referred to as HCC index, for HCC diagnosis by backward logistic regression analysis. The HCC index showed an area under the receiver operating characteristic curve (AUROC) of 0.98 (95% confidence interval 0.92-1.00), a sensitivity of 87% and specificity of 100% for the diagnosis of HCC at a cut-off value of 0.61<sup>[41]</sup>, proving superior to cfDNA alone or AFP alone in the diagnosis of HCC<sup>[41]</sup>. As shown by Yan *et al.*<sup>[41]</sup>, combination of cfDNA level with other protein or genetic biomarkers holds promise as a liquid-biopsy based clinical tool in the early diagnosis of HCC.

## HCC-ASSOCIATED QUALITATIVE CHANGES IN CELL-FREE DNA

Genetic or epigenetic alterations in cfDNA in association with HCC are detectable by liquid biopsy and are reliable indicators of changes occurring in tumor tissues. In general, these changes are grouped into single nucleotide mutations<sup>[35,42]</sup>, variations in DNA copy number<sup>[35,36]</sup>, chromosomal rearrangements, loss of heterozygosity, microsatellite instability, and changes in methylation pattern<sup>[37]</sup>.

### Single nucleotide mutations

Indepth genomic analysis of HCC tumor tissue to the base pair level has shown that no two tumors carry the same cadre of somatic mutations<sup>[43]</sup>. There is considerable variability in the number of mutations even among patients with advanced stage HCC, as demonstrated by analysis of three patients with advanced HCC which showed 7.2, 15 and 7,910 mutant fragments per 5 mL of plasma<sup>[43]</sup>. Tumor-specific somatic mutations in several genes have been identified in the peripheral blood of HCC patients, including *TP53*<sup>[44]</sup>, *HCK*<sup>[45]</sup> and *TERT*<sup>[46]</sup>.

The three most frequent somatic mutations in HCC are *TERT* promoter activating mutations which are found in 40%-60% of HCC patients; and the mutually exclusive *TP53* and *CTNNB1* mutations which are found in 30-50% of HCC cases<sup>[47-49]</sup>. Digital droplet PCR to interrogate the single nucleotide mutations *TERT* c.-124C>T, *TP53* c.747G>T (p.R249S), *CTNNB1* c.121A>G (p.T41A) and c.133T>C (p.S45A) in the



peripheral blood of patients with predominantly HBV-positive and BCLC stage A showed that 56% of the patients harbored ctDNA containing these mutations<sup>[46]</sup>. *TERT* promoter mutations, however, have been observed in both HCC patients as well as non-HCC cirrhotic patients, suggesting limited utility as a biomarker for HCC<sup>[50-52]</sup>. On the other hand, the *TP53* p.R249S mutation appears specific for HCC and has been identified in plasma, serum and urine samples obtained from cancer patients<sup>[53-57]</sup>. The *TP53* p.R249S mutation is more common in HBV- and aflatoxin-associated HCC, compared to HCC associated with other etiologies. ctDNA in 14 patients with advanced HCC using next generation sequencing showed that somatic *CTNNB1* mutations were the second most common mutation and occurred in 29% of the patients studied<sup>[58]</sup>.

The significant heterogeneity of HCC genetics in association with different etiologies (for instance alcohol related liver disease vs. HBV vs. HCV vs. NAFLD) has posed a major challenge to the development of a universal biomarker panel for detecting HCC. This challenge necessitates the integration of multiple genes and multiple loci within a given gene, as well as combining a vast array of protein and genetic biomarkers. CancerSEEK is a recently developed blood test which detects eight tumor-associated protein biomarkers and mutations (including single base substitutions) in 1933 distinct genomic positions<sup>[59]</sup>. The test was used to query peripheral blood derived from 812 healthy controls and 192 non-metastatic cancers of the breast, colorectum, esophagus, liver, lung, ovary, pancreas and stomach<sup>[59]</sup>. Among 44 patients with HCC, the test showed 98% sensitivity and 99% specificity in cancer detection. Overall, the test detected five cancer types with sensitivities ranging from 69% to 98%, and with over 99% specificity. The performance of CancerSEEK in differentiating HCC patients from other high risk patients, for instance those with advanced fibrosis or cirrhosis, is yet to be studied.

### Chromosomal rearrangements

Genomic sequencing has identified a number of chromosomal rearrangements in HCC. Ono *et al.*<sup>[60]</sup> determined cancer-associated genomic rearrangements in HCC tumors through whole-genome sequencing. Subsequently, they validated some of these rearrangements by means of PCR using ctDNA isolated pre-operatively from peripheral blood of HCC patients and primers designed to detect the breakpoints of chromosomal rearrangements seen in tumor tissue. The authors found that pre-operative ctDNA from 7 HCC patients showed several deletions, inversions, tandem duplications and translocations seen in HCC tumor tissue<sup>[60]</sup>. Chromosomal rearrangements can lead to copy number variations and other genetic aberrations, potentially serving as an early noninvasive marker for HCC.

### Copy number variations

Shotgun massively parallel sequencing (MPS) was used to determine tumor-associated copy number variations in the tumor tissue of 4 HCC patients, and in their plasma pre- and post-resection of tumor, compared to 16 healthy controls<sup>[35]</sup>. Characteristic copy number variations in tumor tissue were reflected in pre-resection plasma samples, and were missing almost entirely in post-resection plasma samples. The pre-resection plasma samples detected approximately 10% to 100% of tumor-associated copy number aberrations seen in their corresponding tumor tissue, with detectability of plasma copy number aberrations strongly correlating with plasma ctDNA concentration<sup>[35]</sup>. In another study, MPS analysis of plasma ctDNA size in 90 HCC patients compared to patients with chronic hepatitis B ( $n = 67$ ), hepatitis B-associated cirrhosis ( $n = 36$ ), and healthy controls ( $n = 32$ ) showed that HCC plasma carried high levels of aberrantly short and long DNA<sup>[36]</sup>. The short ctDNA preferentially carried tumor-associated copy number aberrations. Among the 90 HCC patients, 76 (84%) had at least one chromosomal arm-level copy number aberration on chromosomes 1 or 8. In addition, plasma derived from HCC patients contained high levels of mitochondrial DNA albeit much shorter than nuclear DNA<sup>[36]</sup>. The observation that cfDNA in HCC patients are shorter and more fragmented than in patients without liver disease or with non-malignant liver processes has been made by several other investigators<sup>[36,61]</sup>. This observation is worthy of

further investigation and may have clinical utility in the diagnosis or monitoring of HCC either alone or in combination with other biomarkers.

### Loss of heterozygosity and microsatellite instability

Pang *et al.*<sup>[62]</sup> used three high-polymorphic microsatellite markers located on chromosome 8p, D8S277, D8S298 and D8S1771 to examine loss of heterozygosity (LOH) and microsatellite instability. By analyzing plasma cfDNA and tumor tissues from 62 HCC patients, they examined the features of these aberrations in peripheral blood and determined their concordance with tumor tissue. LOH in one or more of the three examined loci was identified in about 58% of patients, occurring at a higher rate in those with metastatic HCC (63%) compared to those with non-metastatic disease (26%)<sup>[62]</sup>. Majority of patients carried microsatellite instability in plasma samples at the same loci as their corresponding HCC tissues, with a concordance rate of about 73%<sup>[63]</sup>. Their findings suggest that LOH and microsatellite alterations may potentially serve in non-invasive diagnosis of HCC, however these alterations generally occur less commonly than the other genetic alterations discussed, and studies are needed to delineate the clinical applicability of these observations.

### Alterations in DNA methylation

DNA methylation, one of the earliest known and well-studied epigenetic modifications, confers changes in chromatin structure, DNA stability and DNA-protein interactions to modify gene expression. Methylation events occur very early in carcinogenesis hence are often detected in precancerous states. To date, several studies have showed that altered DNA methylation at several genes are associated with the initiation and progression of HCC, including *p15* and *p16*<sup>[64]</sup>, *APC*<sup>[65]</sup>, *SPINT2*<sup>[66]</sup>, *SFRP1*<sup>[67]</sup>, *TFP12*<sup>[68]</sup>, *GSTP1*<sup>[69]</sup> and *RASSF1A*<sup>[70]</sup>. NAFLD-related HCC is associated with hypermethylation of the glycine N-methyltransferase (*GNMT*) promoter, resulting in reduced gene expression<sup>[71]</sup>. Differential DNA hypomethylation has also been seen in HCC. DNA hypomethylation is known to induce several processes leading to transposon activation, chromosomal instability, and the generation of copy number variations. Hypomethylation of repetitive DNA sequences by way of long interspersed nucleotide elements 1 (LINE-1) has been detected in the plasma of patients with HCC<sup>[72]</sup>. Concordance in the methylation profile of several tumor suppressor genes between HCC plasma and tumor tissue has been demonstrated by several studies.

Wong *et al.*<sup>[37]</sup> showed that 25% of patients with *p15* methylation in tissue also demonstrated methylated *p15* in blood samples, and nearly all patients with *p15* and *p16* methylation in tissues demonstrated methylation abnormalities in blood samples. Importantly, patients with *p15* and *p16* methylation developed HCC metastasis or recurrence after treatment, suggesting that analysis of *p15/p16* methylation in cfDNA derived from peripheral blood can serve as a biomarker for predicting the metastasis or recurrence of HCC.

Iyer *et al.*<sup>[65]</sup> analyzed the tumor methylation profile of several tumor suppressor genes including *APC*, *FHIT* and *E-cadherin* through analysis of plasma and corresponding tumor DNA from 28 HCC patients, as well as plasma DNA from age and sex-matched controls. The analysis showed a statistically significant concordance in methylation profile between plasma and corresponding tumor DNA for all genes analyzed. The concordance for *APC* methylation in plasma DNA vs. HCC tumor tissue was almost 82%, with sensitivity and specificity of 78% and 90%. For *FHIT*, the concordance, sensitivity and specificity were all approximately 86%. For *E-cadherin*, concordance was 79%, with sensitivity and specificity of 68% and 100%. As in other studies, *p15* and *p16* methylation patterns were also found to be concordant with sensitivities ranging from 50%-60% and specificities in the 85%-95% range.

*RASSF1A*, a member of the Ras association domain family protein is a tumor suppressor frequently silenced in malignancy by hypermethylation. Serum analysis showed that 90% of HCC patients and 62.5% of HCV patients demonstrate *RASSF1A* hypermethylation, compared to 10% in healthy serum<sup>[73]</sup>.

A biomarker panel based on analysis of a number of genes may serve to better differentiate HCC blood from normal samples, as shown for a combined analysis of the methylation pattern of four genes *APC*, *GSTP1*, *RASSF1A*, and *SFRP1* which showed an AUCROC of 0.933 in identifying HCC from normal samples, compared to 0.800 to 0.881 for the individual genes<sup>[67]</sup>. In another study to evaluate the potential of ctDNA methylation patterns in the diagnosis and prognostication of HCC, Xu *et al.*<sup>[74]</sup> identified a methylation marker panel differentially enriched in HCC tissue compared to blood leukocytes of healthy individuals. In a training data set of 715 HCC samples and 560 normal samples, the sensitivity and specificity of a 10-marker panel based on methylation patterns were 85.7% and 94.3%, respectively, and a combined prognostic score based on these markers significantly correlated with risk of death<sup>[74]</sup>. These studies suggest that methylation changes characteristic of HCC can be reliably identified in peripheral blood samples and potentially serve as biomarkers for diagnosis and prognostication of HCC.

## CONCLUSION

Liquid biopsy analysis of serum and plasma can reliably detect genetic and epigenetic alterations present in HCC tumor tissue, providing a less invasive alternative to the current gold standard of liver biopsy. Due to the significant heterogeneity of HCC, a single biomarker would lack the requisite sensitivity and specificity for HCC diagnosis, hence a panel consisting of multiple genetic and epigenetic alterations, likely in combination with protein biomarkers, would have the best diagnostic utility. One such test is CancerSEEK, which detects eight tumor-associated protein biomarkers and mutations in 1933 distinct genomic positions, with 98% sensitivity and 99% specificity for HCC detection when tested in 44 HCC patients and 812 controls<sup>[59]</sup>. The authors estimated a cost of about \$500 to perform a CancerSEEK analysis<sup>[59]</sup>. Although the test holds promise for diagnosing and monitoring HCC, further studies, including performance of the assay in patients at high risk for HCC such as those with advanced fibrosis or cirrhosis would need to be undertaken. Several other analyses of cfDNA biomarkers either alone or in combination with non-nucleic acid biomarkers for non-invasive diagnosis of HCC are in progress.

## DECLARATIONS

### Authors' contributions

Study concept and design: Banini BA, Sanyal AJ

Literature search: Banini BA

Drafting of the manuscript: Banini BA

Critical revision of the manuscript for important intellectual content: Banini BA, Sanyal AJ

### Availability of data and materials

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### Conflicts of interest

Both authors declared that there are no conflicts of interest. Dr. Sanyal is President of Sanyal Biotechnology and has stock options in Tiziana, Durect, Indalo, Inversago. He has served as a consultant to Medimmune, Astra Zeneca, Nitto Denko, Nimbus, Salix, Tobira, Takeda, Terns, Conatus, Lilly, Poxel, Blade, Surrozen, Birdrock, Siemens, Madrigal, Novartis, Pfizer, Hemoshear, Novo Nordisk, Gilead, Exhalenz, Bristol Myers Squibb, Glympse and Genfit. He has been an unpaid consultant to Intercept, Zafgen, Prosciento, Iqvia, NGM Bio, Echosens, Immuron, Syntlogic, Zafgen, Zydus, Nordic Bioscience. His institution has received grant support from Gilead, Salix, Tobira, Intercept, Merck, Astra Zeneca, Zydus and Novartis.

**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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Case Report

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# Hepatocellular carcinoma in HCV - liver cirrhosis before and after successful DAA treatment

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## Abstract

Chronic hepatitis C virus (HCV) infection is a major cause of liver cirrhosis and hepatocellular carcinoma (HCC) worldwide. The recent advancement of direct-acting Antiviral Agents (DAAs) in hepatitis C therapy, resulted in sustained virological response rates of over 90% in treated patients in different stages of liver fibrosis. The efficacy of DAAs treatment has also been confirmed in real-life cohorts that include subjects with decompensated cirrhosis and therefore seems a promising step to a significant reduction in the recurrence of HCC in patients who achieved complete destruction of the HCC nodules by local therapy. We present a 72-year old patient with HCV-related liver cirrhosis who successfully responded to DAAs treatment after complete destruction of an early HCC nodule.

**Keywords:** Hepatocellular carcinoma, hepatitis C virus infection, Direct-acting Antiviral Agents, hepatocellular carcinoma recurrence

## INTRODUCTION

The clinical implementation of direct-acting Antiviral Agents (DAAs) therapy allowed achieving over 90% sustained virological response (SVR) rate in treated patients with chronic hepatitis C virus (HCV) infection regardless of the presence of liver cirrhosis<sup>[1-3]</sup>. The efficacy of DAAs treatment has also been confirmed in real-life cohorts that include subjects with decompensated cirrhosis and therefore seems a promising step



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to a significant reduction in the recurrence of hepatocellular carcinoma (HCC) in patients who achieved complete destruction of the HCC nodules by local therapy<sup>[4-6]</sup>.

An unexpectedly high recurrence rate of HCC after DDAs therapy was reported by the initial studies<sup>[7,8]</sup>, however not confirmed later on<sup>[9,10]</sup>. Given the ambivalent results, any data regarding long-term DDAs therapy in the referred patient cohort are of particular interest.

## CASE REPORT

A 72-year old female patient with chronic hepatitis C infection and related liver cirrhosis was successfully treated with DDAs following complete destruction of HCC nodule. The patient in question has been followed-up:

- More than 18 years after liver cirrhosis was diagnosed.
- 68 months after early HCC was detected.
- 52 months after complete HCC destruction and DDAs therapy.
- 49 months after DDAs treatment.
- 46 month after achieving SRV12.

### HCV liver cirrhosis

The patient presented with chronic hepatitis C infection (genotype 1b), positive anti-HBc total antibodies, but neither positive hepatitis B surface antigen nor detectable HBV DNA and anti-HIV. No history of alcohol and drug abuse was present. She had a compensated type - II diabetes mellitus and moderate arterial hypertension.

Liver cirrhosis has been diagnosed during cholecystectomy in 2001. At that time there were no complications due to portal hypertension. The patient was declared as a primary non-responder after IFN/RBV administration in 2002, therefore received supportive treatment in the following 13 years. In that period the chronic liver disease remained compensated. The viral load remained low (HCV RNA < 800,000 IU/mL), with normal or slightly elevated ALT < 2× upper limit of normal.

### HCC during HCV, before DAAs treatment

#### *HCC development*

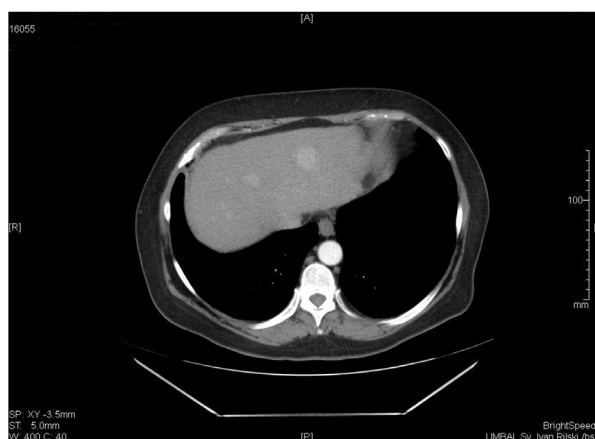
In September 2013 a nodule 14 mm × 8 mm in size was detected in the third segment of the liver following abdominal ultrasound examination and contrast enhanced CT-scan. US-guided percutaneous biopsy diagnosed Barcelona Clinic Liver Cancer (BCLC) stage A HCC. By the time the patient had symptomatic portal hypertension with grade 2 esophageal varices and thrombocytopenia. Hence, local therapy was indicated.

### HCC complete destruction

In December 2013 transarterial chemoembolization (TACE) was performed. However residual follow-up CT scan showed residual nodule in the third hepatic segment [Figure 1]. Therefore there sessions of radiofrequency ablation (RFA) were performed in 2014 and early 2015. CT-scan demonstrated complete ablation of the lesion after the last session, with a zone of ablation-induced necrosis 36 mm × 47 mm in size [Figures 2 and 3].

### DDAs treatment and follow-up

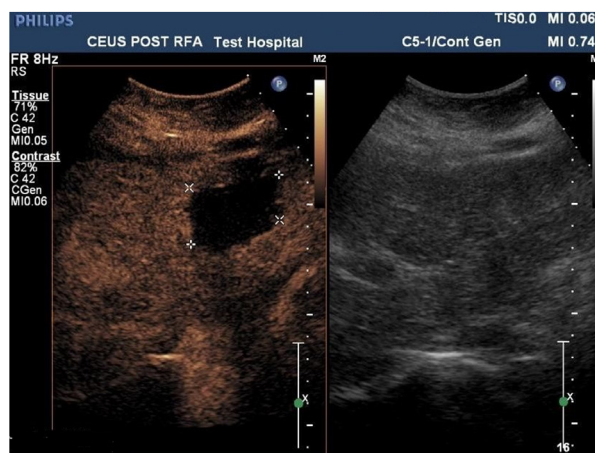
January 2015 DDAs therapy 12-week regimen with Ombitasvir, Paritaprevir/Ritonavir, Dasabuvir and Ribavirin was initiated. At week 2 HCV RNA levels became undetectable, remained so until the end of the therapy, as well as at week 12 and 24. Complete normalization of aminotransferases was observed. Transitory anemia, fatigue, jaundice due to direct hyperbilirubinemia were observed during the therapeutic period. No signs of decompensation of the chronic liver disease were present<sup>[11]</sup>.



**Figure 1.** Contrast enhanced CT before first RFA (2014). CT: computed tomography; RFA: radiofrequency ablation



**Figure 2.** Contrast enhanced CT check after last RFA (2015). CT: computed tomography; RFA: radiofrequency ablation



**Figure 3.** CEUS check after last RFA (2015). CEUS: contrast enhanced ultrasound; RFA: radiofrequency ablation

No signs of HCC recurrence were observed within the 24-week post treatment follow-up. A decrease in the alfa-fetoprotein levels (77 UI/mL to 4 UI/mL) was observed by week 12 (April, 2015), remained so in the following months (July-October 2015). Contrast-enhanced ultrasound showed reduction in the size of the zone of interest to 26 mm (September, 2015).

### HCC recurrence after SVR

February 2018, 37 months after RFA and 34 months after SVR12, the patient was admitted for clinical follow-up. Using contrast enhanced ultrasound and contrast-enhanced computed tomography (CT)-scan a recurrent HCC nodule, 25 mm × 20 mm in size, was detected in the same liver segment. The chronic liver disease was compensated, but with signs of progression of the portal hypertension with grade 3 gastroesophageal varices. Serum HCV RNA and HBV DNA were undetectable.

In March, 2018 microwave ablation and percutaneous ethanol injection were performed in the detected vital HCC nodule. Following CT-scan confirmed absence of vital tissue in the malignant nodule, with axial dimensions of the ablated zone 56 mm × 49 mm.

One year later, due to progression Sorafenib therapy was initiated.

### DISCUSSION

The initial data that indicated increased risks of HCC recurrence after DAAs therapy have not been confirmed by subsequent studies, but the discussed problem is still a matter of debate<sup>[12]</sup>. In our case report, the late HCC recurrence is more likely to be a result of the evolution of long-lasting liver cirrhosis, rather than to the performed DAAs treatment. The discussed subject was with history of cirrhosis for more than 18 years and HCC initially occurred prior to DAAs therapy. Although, HCC was diagnosed in early stage (BCLC-A) complete destruction prior DAAs therapy was achieved by multiple sessions of local therapy (TACE and RFAs). Patients who underwent more than one HCC treatment had a higher recurrence rate than those treated only once<sup>[12-15]</sup>.

In another case, series of cirrhosis associated with HCV done in Bulgaria, more advanced HCCs were successfully treated with percutaneous thermal ablation ( $n = 17$ ) or resection ( $n = 1$ ). Subsequently, all patients were treated with DAA for HCV infection. HCC recidivism (local or distal intrahepatic) was observed in 13 patients (72%) (18, personal communication). Of particular importance is that subject was anti-HBc positive. Anti-HBc has also been shown to be prognostic factor in HCC recurrence and recurrence free survival after curative resection<sup>[16,17]</sup>. On the other hand, HCC recurrence was detected relatively late - 37 months post initial complete destruction of HCC.

The natural history of viral liver cirrhosis includes HCC development. Suppression of viral replication reduces the rate of HCC occurrence. The local therapy is successful in the early stage of HCC, but did not eliminate the risk of HCC recurrence. The new DAA therapy is very short. Some communications reported an unexpectedly high rate of HCC reoccurrence after DAA therapy, but this was not confirmed by further studies. In our case the delayed HCC recurrence might be associated not only with HCV eradication but long term disease and past HBV.

### DECLARATIONS

#### Authors' contributions

Study concept and design, literature search, drafting the manuscript: Krastev Z, Jelev D, Krasteva D, Genov J, Komitova T

#### Availability of data and materials

Not applicable.

#### Financial support and sponsorship

None.

## Conflicts of interest

All authors declared that there no conflicts of interest.

## Ethical approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

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Original Article

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# Tumor growth rates and recurrence-free survival in chronic viral hepatitis patients with hepatocellular carcinoma

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## Abstract

**Aim:** Survival in patients with hepatocellular carcinoma (HCC) is impacted by stage of liver disease, tumor characteristics, and HCC surveillance in high-risk individuals. Factors associated with HCC tumor growth rate (TGR) and its influence on recurrence-free survival after treatment was investigated.

**Methods:** TGR was calculated in 164 HCC patients with chronic viral hepatitis who had two consecutive magnetic resonance imaging or computed tomography scans  $\geq 30$  days apart prior to treatment and who were followed prospectively to determine the rates of recurrence-free survival.

**Results:** The median TGR in 164 patients was 17.8% per month (mean 33.3% per month). Regression tree analysis indicated that the top three predictors of TGR were alpha-fetoprotein (AFP) levels ( $\geq 16.7$  ng/mL), platelet counts ( $\geq 140,000$  mm<sup>3</sup>), and serum albumin level ( $< 3.55$  g/dL). The regression tree identified patient groups with TGRs ranging from 0.65% to 39.4% per month. At a median follow-up of 22 months, the overall recurrence-free survival was 53.8%. The Cox model with backwards AIC search identified TGR (HR = 1.34,  $P = 0.029$ ), age  $> 56$  years (HR = 1.08,  $P = 0.072$ ), hepatitis C virus (HR = 1.44,  $P = 0.091$ ), macrovascular invasion (HR = 1.94,  $P = 0.092$ ), and the most definitive treatments (orthotopic liver transplantation, HR 0.14,  $P < 0.001$ ; surgical resection, HR = 0.54,  $P = 0.072$ ; radiofrequency ablation, HR = 0.58,  $P = 0.060$ ) as independent predictors of recurrence-free survival. For all treatment modalities, slow



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TGR was significant for prolonged survival ( $P = 0.029$ ). The poorest survival rates were observed in patients with fast TGRs treated by transarterial chemoembolization.

**Conclusion:** The TGR correlated with AFP, platelet count, and albumin level. Patients with fast TGRs had shorter recurrence-free survival after HCC treatments. TGR is a potential imaging biomarker to predict clinical outcomes in HCC.

**Keywords:** Liver cancer, growth rates, hepatitis B, hepatitis C, hepatocellular carcinoma treatments

## INTRODUCTION

Worldwide, hepatocellular carcinoma (HCC) is the fifth most frequently encountered malignancy and is the third leading cause of cancer-related deaths<sup>[1]</sup>. In the United States, the incidence of HCC has significantly increased and is projected to be among the top three causes of cancer-related deaths by 2030<sup>[2]</sup>. In addition, the financial burden of HCC in the United States has continued to increase over the last decade<sup>[3]</sup>. Numerous studies showed that the most common etiologies are chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), with HBV accounting for at least 42% and HCV accounting for at least 27% of HCC cases globally<sup>[4]</sup>. The remaining cases are associated with excessive alcohol intake and non-alcoholic steatohepatitis.

Over the last two decades, improvements in HCC survival have been made by advances in HCC treatments in surgery and interventional radiology. Furthermore, the implementation of surveillance protocols in high-risk populations has resulted in early HCC detection and improved post-treatment survival<sup>[5]</sup>. Additional factors that predict HCC survival include the degree of liver dysfunction as well as the initial tumor size and number of tumors.

Another potential factor is the tumor volume doubling time (TVDT) which is assessed by two serial radiologic imaging studies prior to HCC treatments. Initially, TVDT was used to determine suitable screening intervals for early HCC detection. Previous imaging studies reported TVDTs ranging from a median of 117 days to a mean of 127 days, and suggested intervals of 4 to 5 months for HCC screening<sup>[6,7]</sup>. Other reports showed that shorter TVDTs were correlated with earlier deaths after hepatectomies as well as higher recurrence rates after surgical resection and radiofrequency ablation<sup>[8-10]</sup>.

These papers on TVDT highlight its potential value as a prognostic tool for predicting HCC survival rates. Nevertheless, some of these studies were limited by early imaging technology, variations in screening intervals, and small sample sizes. Further, a recent report indicated that the TVDT is a less suitable variable for tumor growth rate because (1) mean TVDT estimates are not accurate if the time interval measurements are short; (2) the TVDT is not defined if the consecutively estimated volumes are similar; and (3) the asymmetrical frequency distribution of the TVDT makes it less suitable for statistical analysis<sup>[11]</sup>. In contrast, the mean tumor growth rate (TGR) gives a more correct value for average growth rate and has a symmetrical frequency distribution. Thus, an improved understanding of tumor growth, as measured by TGR, may help in guiding prognostic evaluations and aid in determining treatment options for patients with HCC. In the report herein, we assessed factors associated with TGR in 164 patients with chronic viral hepatitis and HCC. In addition, we evaluated the potential value of TGR as a factor in predicting recurrence-free survival after HCC treatment in these patients.

## METHODS

### Patient population

Between 1984 and 2014, 357 patients with HCC were evaluated at the Liver Center in Pasadena, California. A database was created to collate and anonymize patient records, including laboratory tests, tumor size,

HCC treatments, and current status. Amongst the 357 patients, 24 individuals were excluded from this study due to diffuse appearing tumor in which the size could not be determined (22 patients) or due to an HCC diagnosis made within six months of final patient entry into the database (2 patients). Of the remaining 333 patients, 169 who began HCC treatment prior to a second tumor size measurement were also excluded. The remaining 164 patients had two consecutive imaging studies prior to HCC treatment and are the subjects in the present study. HCC lesions were detected via surveillance in 113 patients with alpha-fetoprotein (AFP) testing and US scans. The remaining 51 patients were either diagnosed by their referring physicians or during their first visit to our Liver Center. The number and size of lesions, as reported by CT scan or MRI, were recorded. The diagnostic criteria for cirrhosis were by imaging findings of a nodular surface, platelet count < 140,000 mm<sup>3</sup>, presence of esophageal varices or ascites, or by liver biopsy.

The TGR was determined for all 164 patients. The diagnosis of HCC by MRI or CT scan were according to AASLD criteria from their 2005 and 2011 recommendations<sup>[12,13]</sup>. Prior to that time period, imaging criterion for HCC diagnosis relied on findings of a hypervascular lesion, elevated levels of AFP, tumor growth on subsequent imaging, and biopsy of the lesion if the above criteria were not clear. The dates and corresponding tumors sizes from the first and second imaging studies (CT or MRI) were recorded. The time interval between the first and second images were  $\geq 30$  days (median time 92 days), and all were prior to any treatments for HCC<sup>[5]</sup>.

### Baseline laboratory tests

Baseline laboratory tests were obtained from all patients. These included platelet counts, serum albumin, total bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, and AFP. For HBV patients, virus genotype, HBV DNA levels, precore mutation, basal core promoter mutation, and HBeAg were recorded. For HCV patients, virus genotype and HCV RNA levels were recorded. Sera from patients whose HCC was diagnosed prior to 1991 were retrospectively tested for anti-HCV antibodies and HCV RNA.

### HCC treatments

Of the 164 patients followed in this study, 113 received definitive treatments, 7 received chemotherapy, and 44 were offered supportive care. HCC patients were referred to academic centers for surgical and/or locoregional therapies. Treatment options included orthotopic liver transplantation (OLT), surgical resection, RFA, transcatheter arterial chemoembolization (TACE), or percutaneous ethanol injection (PEI). If a patient had multiple treatments, they were assigned to the most definitive treatment category. OLT, surgical resection, and RFA were considered to be the most definitive treatments. Patients who did not receive one of the above treatments were given chemotherapy or supportive care.

### Post-treatment outcomes

Patients who returned for regular follow-up care were continuously screened with imaging studies and laboratory tests. In order to calculate dates of recurrence-free survival, dates of diagnosis, initial treatment, recurrence, and latest follow-up or death were recorded.

### Statistical analyses

#### *Tumor growth rate calculation*

The TGR was calculated using Schwartz's equation:  $TGR = \log(V/V_0)/(T - T_0)$  where  $T - T_0$  indicates the time interval between the two measurements and  $V_0$  and  $V$  represent the tumor volumes ( $V = 4/3\pi R^3$ ) at the two measurement points<sup>[14]</sup>. The Schwartz equation assumes early, exponential stage growth with the TGR reported in % per month. In the analyses below,  $\log_{10}$  TGR is used since  $\log$  TGR has a distribution closer to the normal distribution.

### *Predictors of tumor growth rate*

Bivariate analysis - The bivariate analysis for assessing each categorical predictor vs. log TGR was computed using *t*-tests/analysis of variance. The correlation between log<sub>10</sub> TGR and continuous variables was computed via the Spearman correlation ( $r_s$ ).

Multivariate analysis - The multivariable regression tree (binary partition) analysis was used to determine the simultaneous association between log<sub>10</sub> TGR and 19 potential predictors, including age, gender, ethnicity, HCC surveillance, serum albumin, serum AFP, platelet count, cirrhosis, diabetes, initial tumor size, HBV or HCV infection, and antiviral treatment. For hepatitis B patients, HBV genotype, HBV DNA, precore mutation, basal core promoter mutation, and HBeAg values were included. For hepatitis C patients, HCV RNA and genotype were included. In this tree model, every value of each predictor variable was considered. Patients with slow vs. fast TGRs were separated via a progression of binary splits (partitions). The best split was determined by the impurity criterion, a reduction of the residual sum of squares due to the binary split (GINI criterion). Missing values were allowed. Each split resulted in one parent node and two child nodes. Child nodes, in turn, were split until further splits did not significantly improve the predicted TGR. The final result was an intuitive and interpretable decision tree<sup>[15]</sup>. A  $P < 0.07$  was considered statistically significant.

### *Predictors of recurrence-free survival*

Predictors of HCC recurrence-free survival were analysed. The outcome (event) was HCC recurrence or death. The primary predictor was log<sub>10</sub> TGR. The other 9 potential predictors were age, gender, ethnicity, HCV or HBV, diabetes, cirrhosis, macrovascular invasion, HCC surveillance, and the most definitive treatments (OLT, surgical resection, RFA, PEI, TACE, chemotherapy, or supportive) for a total of 10 potential predictors. There were 125 events, 39 HCC recurrences and 85 deaths with no recurrence.

Bivariate analysis - Hazard ratios (HR) for each potential predictor, ignoring the other 9 predictors, were computed along with its 95% confidence bounds and *P*-values. Restricted cubic splines were used to determine if the relation between a continuous predictor vs. the log hazard ratio was linear.

Multivariate analysis - The 10 potential predictors simultaneous to the event rate were assessed using a Cox proportional hazard model. A backwards minimal AIC search was used to determine which of the potential predictors were significant, with the restriction that log<sub>10</sub> TGR was included in all models. For the final model, all possible two-way interactions were evaluated. Statistical significance was taken as  $P < 0.07$ . Model accuracy was assessed using Harrell's C concordance statistic with values of C ranging from 0.50 (worse) to 1.0 (best).

## **RESULTS**

The baseline characteristics of 164 HCC patients who had two consecutive imaging studies with either MRI or CT scans prior to treatments are listed in Table 1. The average age was  $64.48 \pm 10.38$  years, 64.6% were male, and the majority were Asian (64.0%), followed by white (18.3%), Hispanic (14.0%), and African American (3.70%). Hepatitis B infection was detected in 39.6% of patients, Hepatitis C infection in 59.8%, and the remaining patients were co-infected with both viruses. In the HBV infected HCC patients with measurable tests, 21.5% were HBeAg positive, 29.2% were genotype C, 30.8% had basal core promoter mutations, 23.1% had precore mutations, and the mean HBV DNA level was  $2.41 \times 10^6$  IU/mL (IQR:  $1.00$ - $1.23 \times 10^5$ ). In the HCV infected HCC patients with measurable tests, 45.9% had genotype 1 and the mean HCV RNA was  $1.44 \times 10^6$  IU/mL (IQR:  $594.5$  -  $1.27 \times 10^6$ ). The mean albumin level was  $3.80 \pm 0.66$  g/dL, platelet count was  $138,000 \pm 75,600$  mm<sup>3</sup>, and AFP level was  $45.2 \pm 11.8$  ng/mL. Of 164 HCC patients, 68.9% were detected by surveillance. 19.5% had diabetes, 78.7% had cirrhosis, and 5.50% had macrovascular invasion.

**Table 1. Baseline characteristics of 164 patients with hepatocellular carcinoma**

Characteristic	Number (%) or Mean $\pm$ SD
Age at diagnosis (years)	64.48 $\pm$ 10.38
Gender	
Female	58 (35.4)
Male	106 (64.6)
Ethnicity	
African American	6 (3.70)
Asian	105 (64.0)
Hispanic	23 (14.0)
White	30 (18.3)
Virology	
HBV	65 (39.6)
HCV	98 (59.8)
HBV + HCV	1 (0.60)
HBV genotype	
A	3 (4.62)
B	10 (15.4)
C	19 (29.2)
F	1 (1.54)
Missing	32 (49.2)
HBV precore mutation	
Yes	15 (23.1)
No	18 (27.7)
Missing	32 (49.2)
HBV basal core promoter mutation	
Yes	20 (30.8)
No	8 (12.3)
Missing	37 (56.9)
HBeAg	
Negative	42 (64.6)
Positive	14 (21.5)
Missing	9 (13.8)
HBV DNA (IU/mL) <sup>*</sup>	2,411,000 (IQR: 1.00-123,400)
HCV genotype	
1	45 (45.9)
2	17 (17.3)
3	6 (6.12)
6 or 7	7 (7.14)
Mixed	2 (2.04)
Missing	21 (21.4)
HCV RNA (IU/mL) <sup>*</sup>	1,442,000 (IQR: 594.5-1,270,000)
Antiviral treatment	
Yes	50 (30.5)
No	108 (65.9)
Missing	6 (3.60)
Albumin (g/dL)	3.80 $\pm$ 0.66
Total bilirubin (mg/dL)	1.30 $\pm$ 1.20
Alkaline phosphate (U/L)	128 $\pm$ 129
AST (U/L)	78.2 $\pm$ 59.2
ALT (U/L)	70.1 $\pm$ 54.3
Platelet count ( $\times 10^3$ mm <sup>3</sup> )	138 $\pm$ 75.6
AFP (ng/mL)	45.2 $\pm$ 11.8
Surveillance	
Yes	113 (68.9)
No	51 (31.1)
Diabetes	
Yes	32 (19.5)
No	125 (76.2)
Missing	7 (4.27)

Cirrhosis	
Yes	129 (78.7)
No	35 (21.3)
Macrovascular invasion	
Yes	9 (5.50)
No	149 (90.9)
Missing	6 (3.60)
Initial tumor size (cm)	3.62 ± 2.27

\*Interquartile range (IQR) opted over mean ± SD for accuracy. HBV: hepatitis B virus; HCV: hepatitis C virus; HBeAg: hepatitis B e-antigen; AST: aspartate aminotransferase; ALT: alanine aminotransferase; AFP: alpha-fetoprotein

### The tumor growth rate

The mean initial tumor size was  $3.62 \pm 2.27$  cm. The TGR in 164 HCC patients ranged from 0% per month to 440.2% per month with a median growth rate of 17.8% per month and a mean growth rate of 33.3% per month. Bivariate predictors of log TGR are shown in Table 2. HCC patients with serum albumin levels  $\leq 3.50$  g/dL had a median TGR of 27.0% per month while those with albumin levels of  $> 3.50$  g/dL had a median growth rate of 14.2% per month ( $P = 0.016$ ). HCC patients with AFP levels  $\leq 10.0$  ng/mL had slower average growth rates compared to patients with AFP levels of 11.0-191 ng/mL and  $> 191$  ng/mL (11.1%, 18.7%, and 30.3% respectively,  $P = 0.029$ ). Also, HCC patients with diabetes had slower growth rates compared to those without diabetes (10.5% and 21.6% respectively,  $P = 0.051$ ).

Of the 19 variables evaluated, the regression tree model identified  $\text{AFP} < \text{or} > 16.7$  ng/mL as the best single discriminator between slow and fast growing tumors expressed in log TGR. The next best predictor of TGR in the high AFP node was platelet counts  $< \text{or} > 140,000$   $\text{mm}^3$ . For the node with patients having an  $\text{AFP} \geq 16.7$  ng/mL and a platelet count  $< 140,000$   $\text{mm}^3$ , an albumin level  $< \text{or} > 3.55$  g/dL provided additional prognostic value. Further analysis identified age at  $< \text{or} > 56$  years and ethnicity as other significant variables. As shown in Figure 1, an AFP value of  $< \text{or} > 16.7$  ng/mL best discriminated slow and fast-growing tumors (10.9% per month and 23.8% per month respectively,  $P = 0.050$ ). Within the  $\text{AFP} \geq 16.7$  ng/mL node, platelet counts of  $< \text{or} >$  than 140,000  $\text{mm}^3$  next discriminated TGRs of 21.0% per month and 39.4% per month respectively ( $P = 0.085$ ). In the platelet count  $< 140,000$   $\text{mm}^3$  node, albumin level  $\geq 3.55$  g/dL identified the slower growing tumors with a TGR of 9.15% per month, while those with albumin level  $< 3.55$  g/dL had a TGR of 31.4% per month ( $P = 0.0004$ ). Within the albumin level  $< 3.55$  g/dL node, ethnicity other than Hispanic had a faster TGR (36.4% per month vs. 11.4% per month,  $P = 0.005$ ). Finally, within the albumin  $\geq 3.55$  g/dL node, age  $< \text{or} >$  than 56 years had TGRs of 0.65% per month and 15.7% per month respectively.

### The survival outcomes

The overall recurrence-free survival for 164 HCC patients is shown in Figure 2. At a median time of 22 months, 53.8% of the HCC patients were alive and recurrence-free. A bivariate analysis which included 10 potential predictors showed that the TGR ( $\text{HR} = 1.27$ ,  $P = 0.061$ ), age ( $\text{HR} = 1.02$ ,  $P = 0.006$ ), HCV ( $\text{HR} = 1.42$ ,  $P = 0.061$ ), surveillance ( $\text{HR} = 0.70$ ,  $P = 0.065$ ), and the most definitive treatments (OLT,  $\text{HR} = 0.13$ ,  $P < 0.0001$ ; surgical resection,  $\text{HR} = 0.40$ ,  $P = 0.004$ ; RFA,  $\text{HR} = 0.50$ ,  $P = 0.010$ ) were significant predictors of tumor free survival [Table 3].

Multivariate analysis using the Cox model with backward AIC search identified TGR ( $\text{HR} = 1.34$ , 95%CI: 1.03-1.74,  $P = 0.029$ ), age  $> 56$  years ( $\text{HR} = 1.08$ , 95%CI: 0.99-1.18,  $P = 0.072$ ), HCV ( $\text{HR} = 1.44$ , 95%CI: 0.94-2.20,  $P = 0.091$ ), macrovascular invasion ( $\text{HR} = 1.94$ , 95%CI: 0.90-4.18,  $P = 0.092$ ), and the most definitive treatments (OLT,  $\text{HR} = 0.14$ ,  $P < 0.0001$ ; surgical resection,  $\text{HR} = 0.54$ ,  $P = 0.072$ ; RFA,  $\text{HR} = 0.58$ ,  $P = 0.060$ ) as simultaneous independent risk factors for recurrence-free survival. To evaluate the effect of the same level of slow or fast growth rate on recurrence-free survival, the 164 patients were divided into equal-sized groups below and above the median TGR (17.8% per month). The median TGR in the slower group was 4.58%

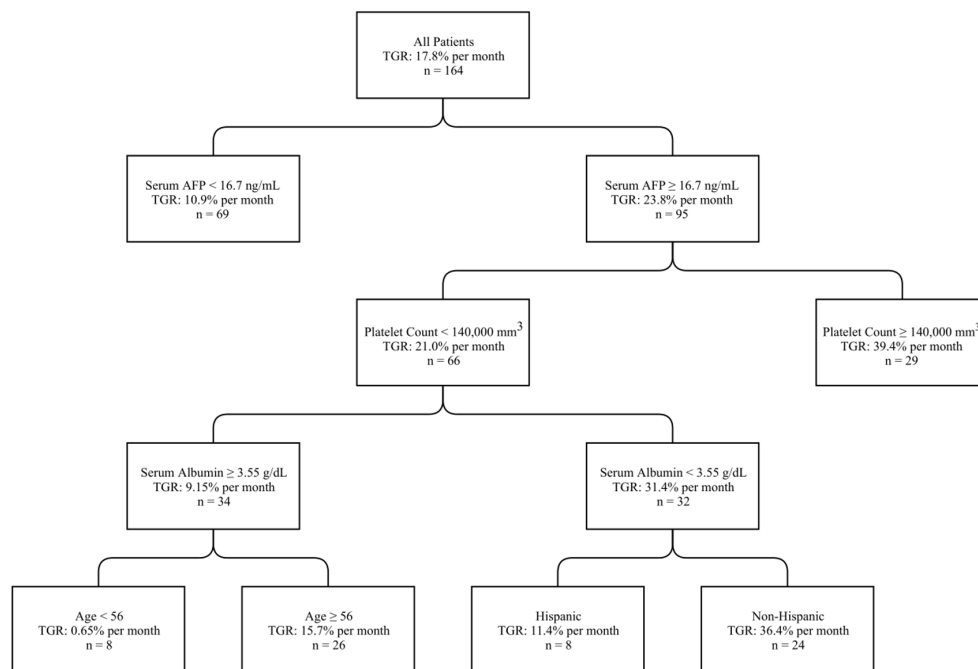
**Table 2. Bivariate predictors of tumor growth rate**

	<i>n</i>	Median TGR (%/mo)	Q1-Q3 (%/mo)	<i>P</i> -value
Gender				
Female	58	15.6	4.60-35.9	0.6560
Male	106	19.3	4.90-40.4	
Ethnicity				
African American	6	9.10	0.70-19.3	0.2526
Asian	105	18.0	5.40-38.4	
Hispanic	23	11.8	2.40-34.4	
White	30	26.3	6.00-47.9	
Virology				
HBV	65	23.2	5.40-42.4	0.3284
HCV	98	16.6	4.10-33.4	
HBV + HCV	1	2.20	--	
HBV genotype				
A	3	33.0	4.60-59.6	0.5253
B	10	17.9	3.30-38.5	
C	19	21.8	9.60-51.7	
F	1	--	--	
HBV precore mutation				
Yes	15	28.7	8.10-54.0	0.4053
No	18	22.5	11.3-76.8	
HBV basal core promoter mutation				
Yes	20	22.5	10.3-50.4	0.7229
No	8	17.8	6.30-41.1	
HBeAg				
Negative	42	20.5	4.90-37.8	0.3877
Positive	14	30.8	7.70-68.5	
HBV DNA				
Negative	3	42.4	5.30-64.9	0.7686
Positive	54	21.1	5.20-35.6	
HCV genotype				
1	45	12.9	2.30-28.1	0.1408
2	17	22.0	9.20-42.3	
3	6	29.3	23.5-44.3	
6/7/mixed	9	3.00	2.20-32.5	
HCV RNA				
Negative	15	14.4	4.70-26.2	0.3802
Positive	61	15.2	3.10-28.5	
Antiviral treatment				
Yes	50	18.6	2.30-37.8	0.8826
No	108	17.8	7.10-36.8	
Albumin (g/dL)				
≤ 3.50	55	27.0	8.00-46.3	0.0161
> 3.50	109	14.2	2.60-32.5	
AFP (ng/mL)				
≤ 10.0	54	11.1	2.50-28.3	0.0294
10.0-191	66	18.7	7.40-34.8	
> 191	40	30.3	4.20-75.2	
Platelet count (× 10 <sup>3</sup> mm <sup>3</sup> )				
≤ 75.0	34	23.7	7.70-39.5	0.2834
75.0-150	62	15.4	2.50-31.7	
> 150	64	20.4	7.10-41.8	
Surveillance				
Yes	113	18.0	4.30-38.4	0.5565
No	51	15.2	5.00-37.6	
Diabetes				
Yes	32	10.5	2.00-28.1	0.0506
No	125	21.6	5.60-42.3	
Cirrhosis				



Yes	129	17.7	4.10-38.4	0.5418
No	35	17.9	8.10-36.9	

TGR: tumor growth rate; HBV: hepatitis B virus; HCV: hepatitis C virus; HBeAg: hepatitis B e-antigen; AFP: alpha-fetoprotein

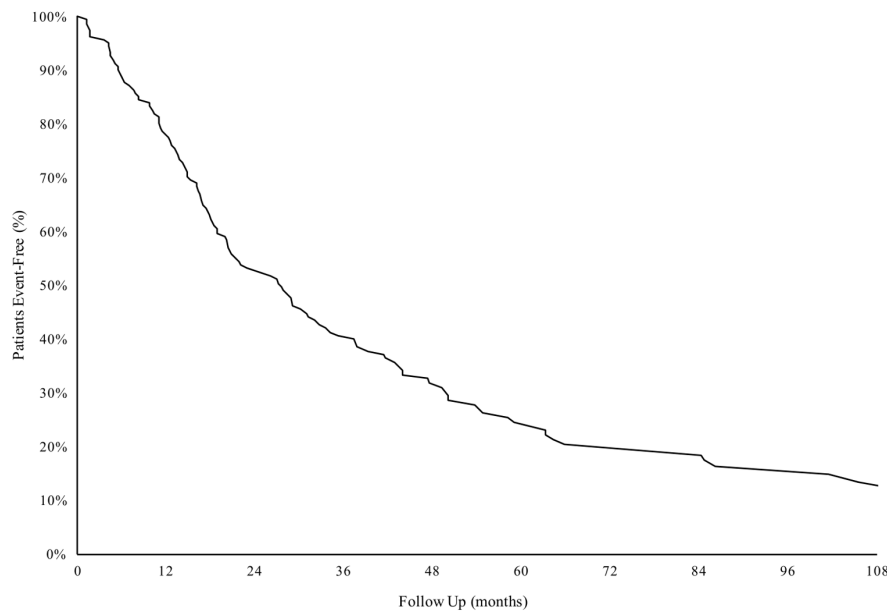


**Figure 1.** Regression tree analysis: predictors of hepatocellular carcinoma tumor growth rates. Each node is based on available data for each predictive variable presented. TGR is reported as a median. TGR: tumor growth rate; AFP: alpha-fetoprotein

per month and the median TGR in the faster group was 38.9% per month. As illustrated in Figure 3, the recurrence-free survival in patients who received OLT, surgical resection, or RFA was significantly longer in patients with slow TGRs for each treatment modality ( $P = 0.029$ ). Patients who received OLT who had slow TGRs had the longest recurrence-free survival. Those HCC patients who received surgical resection or RFA had similar survival rates in both the slow and fast TGR groups. The poorest recurrence-free survivals were observed in the TACE treated or supportive care patients with fast TGRs.

## DISCUSSION

Previous reports have utilized MRI or CT to find potential biomarkers to predict clinical outcomes in patients with HCC. Using MRI, one report showed that patients with fat-containing HCC had less tumor progression, less distant metastases, and a longer time to tumor progression when compared to patients with non-fat containing HCC<sup>[16]</sup>. Another report showed that patients with complete tumor encapsulation on MRI had lower AFP levels, an absence of vascular invasion, more patients in Child-Pugh class A, and significantly longer survivals<sup>[17]</sup>. Further, the authors also noted that the rates of downstaging and eventual liver transplantation were significantly higher. However, recognition of these imaging features depends on the expertise of the interpreting radiologist and may be challenging to implement as a practical clinical tool. Nevertheless, efforts to standardize imaging reporting (i.e., Organ Procurement and Transplantation Network/United Network for Organ Sharing (OPTN) and Liver Imaging Reporting and Data System (LI-RADS) criterion) may allow incorporation of additional important imaging biomarkers for tumor prognosis<sup>[18,19]</sup>. As tumor size is already a basic measure reported with all detected tumors, the calculation of TGR is feasible when serial imaging is available and, thus, may be considered as another potential imaging biomarker.



**Figure 2.** Recurrence-free survival of 164 patients with hepatocellular carcinoma

In the report herein, we first attempted to determine factors associated with tumor growth rate in patients with HBV- and HCV-related HCC. By regression tree analysis of 19 variables, AFP levels  $<$  or  $>$  16.7 ng/mL best discriminated between slow and fast growing tumors respectively [Figure 1]. In a previous report, the initial AFP levels did not correlate with tumor growth rate but, in those patients with repeated AFP values which showed an exponential increase in AFP, the AFP doubling time was closely related to the tumor doubling time<sup>[6]</sup>. Other studies comparing AFP values  $>$  100 ng/mL,  $>$  200 ng/mL, and  $>$  400 ng/mL showed that each of the AFP levels correlated with faster tumor doubling times<sup>[10,11,20]</sup>. These findings indicate that elevated AFP levels are significant indicators of tumor doubling time.

In patients with AFP  $\geq$  16.7 ng/mL, the next best discriminator was platelet counts  $<$  or  $>$  140,000 mm<sup>3</sup>. Within the platelet count  $\geq$  140,000 mm<sup>3</sup> node, the mean platelet count was 201,345 mm<sup>3</sup> (median 192,000 mm<sup>3</sup>). There were only two patients with thrombocytosis (385,000 mm<sup>3</sup> and 420,000 mm<sup>3</sup>). In previous reports, thrombocytosis was noted in 2.70% to 8.20% of HCC patients and was associated with overproduction of thrombopoietin by liver cancer cells<sup>[21,22]</sup>. In these studies, thrombocytosis was associated with larger tumor volumes and higher levels of serum AFP in Asian HCC patients, and with larger tumor sizes, younger patients, and less cirrhosis in European HCC patients. One possible explanation for large tumor sizes in patients with higher platelet counts or in cirrhotic patients with “higher than expected” platelet counts is that platelets are a source of a number of HCC growth stimulants including vascular endothelial growth factor, platelet-derived growth factor, serotonin, and fibroblast growth factor<sup>[22]</sup>. In the study herein, patients with platelet counts  $\geq$  140,000 mm<sup>3</sup> had a faster mean TGR compared to those with platelet counts  $<$  140,000 mm<sup>3</sup> (39.4% per month vs. 21.0% per month, respectively).

There have been few reports on the relationship between serum albumin levels and tumor doubling time. In earlier studies, Child-Pugh scores did not influence tumor doubling times<sup>[6,20]</sup>. A recent study showed that Korean HCC patients with tumor doubling times  $<$  2 months had significantly lower mean albumin levels than those with tumor doubling times  $>$  2 months (3.20 g/dL vs. 3.50 g/dL,  $P = 0.003$ )<sup>[23]</sup>. In our report, the TGR of patients in the platelet count  $<$  140,000 mm<sup>3</sup> node were further discriminated into fast and slow TGR by albumin levels  $<$  or  $>$  than 3.55 g/dL (31.4% per month vs. 9.15% per month). This finding suggests that cirrhosis patients with poor liver synthetic function have less ability to confine the growth of HCC.

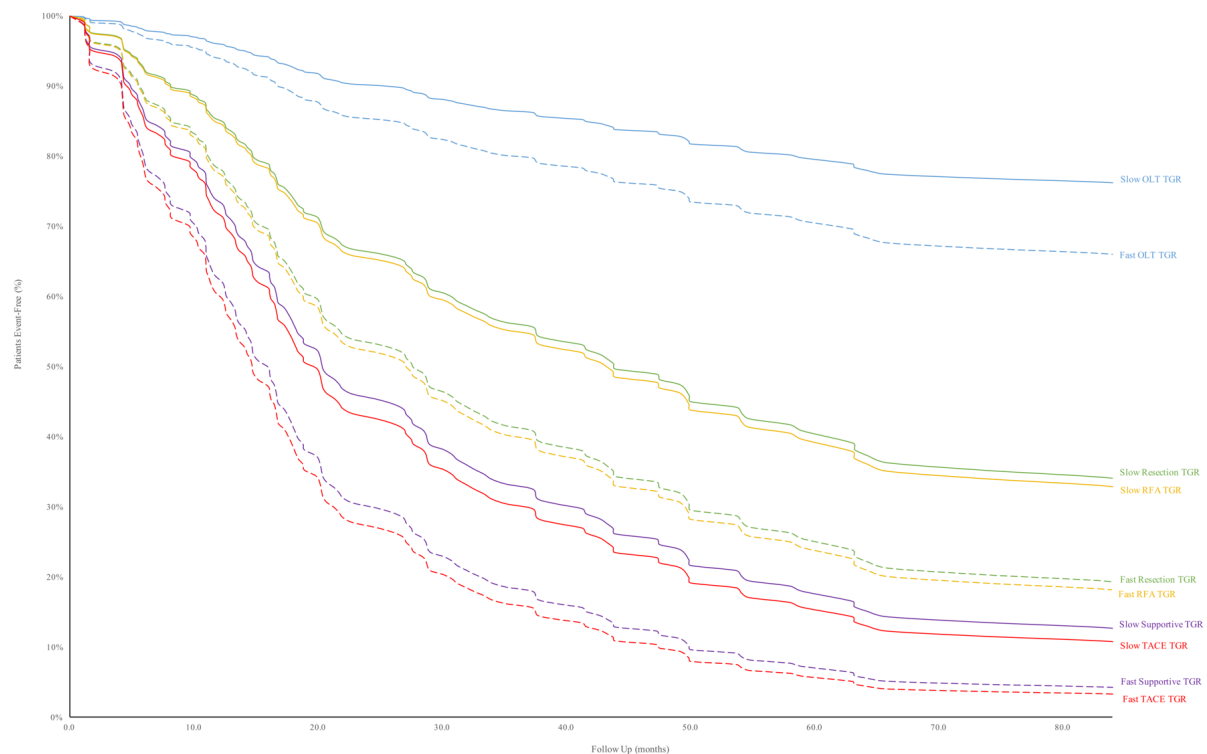
**Table 3. Bivariate and multivariate analysis of recurrence-free survival**

Bivariate analysis					Multivariate analysis			
	<i>n</i>	HR	95%CI	<i>P</i> -value		HR	95%CI	<i>P</i> -value
TGR (%/mo)	164	1.27	0.99-1.63	0.0612	TGR (%/mo)	1.34	1.03-1.74	0.0289
Age (years)	164	1.02	1.01-1.04	0.0059	Age (years)	1.08	0.99-1.18	0.0717
Hepatitis virus					Hepatitis virus			
HBV	65	ref			HBV	ref		
HCV	98	1.42	0.98-2.06	0.0606	HCV	1.44	0.94-2.20	0.0905
HBV + HCV	1	--	--		HBV + HCV			
Sex								
Female	58	ref						
Male	106	0.87	0.60-1.25	0.449				
Ethnicity								
African American	6	ref						
Asian	105	1.57	0.50-4.97	0.4441				
Hispanic	23	2.31	0.67-7.90	0.1826				
White	30	1.78	0.53-5.92	0.3502				
Diabetes								
No	125	ref						
Yes	32	0.90	0.57-1.42	0.6410				
Macrovascular invasion					Macrovascular invasion			
No	149	ref			No	ref		
Yes	9	1.64	0.80-3.38	0.1789	Yes	1.94	0.90-4.18	0.0916
Cirrhosis								
No	35	ref						
Yes	129	1.08	0.69-1.67	0.7429				
Surveillance								
No	51	ref						
Yes	113	0.70	0.48-1.02	0.0647				
Treatment					Treatment			
Supportive	44	ref			Supportive	ref		
Chemotherapy	7	1.78	0.79-4.00	0.1654	Chemotherapy	3.00	1.28-7.01	0.0112
OLT	26	0.13	0.06-0.27	0	OLT	0.14	0.07-0.30	0
Resection	21	0.40	0.21-0.74	0.0039	Resection	0.54	0.28-1.06	0.0716
RFA	29	0.50	0.29-0.84	0.0099	RFA	0.58	0.33-1.02	0.0596
PEI	7	0.59	0.25-1.40	0.2324	PEI	0.67	0.25-1.79	0.4249
TACE	30	0.92	0.57-1.50	0.7464	TACE	1.15	0.68-1.93	0.6056

TGR: tumor growth rate; HBV: hepatitis B virus; HCV: hepatitis C virus; OLT: orthotopic liver transplantation; RFA: radiofrequency ablation; PEI: percutaneous ethanol injection; TACE: transarterial chemoembolization

In our study, TGR significantly influenced recurrence-free survival in patients who received OLT, surgical resection, or RFA. In each of these treatments, recurrence-free survival was significantly longer in patients with slow TGRs. Prolonged recurrence-free survival was observed in patients with slow TGRs who received OLT. The recurrence-free survival was similar in patients with slow or fast TGRs who received surgical resection or RFA. Also, survival was similar in patients who had TACE or supportive care, regardless of TGRs. The poorest recurrence-free survival was observed in patients who received either of the latter two treatments and who had fast TGRs. These findings indicate that TGRs may be a useful biomarker when evaluating HCC patients for treatments and in predicting outcomes to therapies.

While this study strongly supports TGR as a simple imaging-based prognostic biomarker, we should comment that both OPTN and LI-RADS use 6 month threshold growth of 50% as an ancillary criteria for HCC diagnosis, largely based on expert opinion from the OPTN imaging committee<sup>[20,21]</sup>. We believe that this diagnostic definition may be too restrictive in patients with fast TGRs and may possibly affect prognosis since potential HCCs with a fast TGR may be left untreated for an extended period if the OPTN and LI-RADS criterion is used. Therefore, measurement of TGR may also be of use in establishing criteria



**Figure 3.** Recurrence-free survival in hepatocellular carcinoma patients with slow vs. fast tumor growth rate by treatment category. TGR: tumor growth rate; OLT: orthotopic liver transplantation; RFA: radiofrequency ablation; TACE: transarterial chemoembolization

for diagnosis of early HCC. Future studies using TGR along with other imaging criteria will assist in this endeavor.

There are limitations to our study. This was a retrospective analysis of HCC patients from a single community specialty clinic. However all HCC treatments were performed at a university center where multi-disciplinary subspecialties were active in the care of these patients. This scenario is much more representative of the real world setting since issues of long-term follow-up, financial constraints, and day to day care all came into play. Also, patients who did not have a second imaging study prior to HCC treatments were excluded from our analysis which may have biased patient selection. We did not compare the clinical outcome between patients who did or did not have a second imaging study, which may have clarified this issue. Also, we excluded patients with diffuse tumors since the diameter of the tumor could not be determined. However, these patients are usually not eligible for surgical or interventional radiologic treatment and have much shorter life expectancies. Further, only HCC patients with HBV or HCV were evaluated in this report. As such, additional studies should include other disease entities such as alcohol-related and nonalcoholic fatty liver disease-related HCC cases.

In summary, our findings suggest that TGR is influenced by AFP, platelet counts, and albumin levels. TGR significantly influenced recurrence-free survival and response to surgical and locoregional treatments and may be another potential imaging biomarker to predict clinical outcomes in patients with HCC.

## DECLARATIONS

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Jeffery Gornbein, Ph.D., assisted in the statistical analysis of the data; Lori Tong, RN, MSN, participated in the care of these patients; Alex Rosinski, B.S., M.A., assisted in the collection of data in this study.

### Authors' contributions

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All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Review

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# Updates in immunotherapy for hepatocellular carcinoma

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## Abstract

Hepatocellular carcinoma (HCC) carries an unfavorable prognosis and novel therapeutic strategies are needed. Until now, only few systemic agents have improved survival in patients with advanced stage disease. Immunotherapy changed the landscape in several tumor types by producing unprecedented clinical outcomes with a favorable safety profile. Liver presents a particular immune-suppressive microenvironment and HCC develops in a background of chronic inflammation in the vast majority of cases. In this regard, immunotherapy may be a suitable strategy. Preliminary research focused on therapies involving immune cells and anti-tumor immune response for HCC has shown encouraging preliminary results. Immune checkpoint inhibitors, such the anti-PD-1/PD-L1 monoclonal antibodies, have provided durable responses in patients with advanced stage disease, although the pioneers phase III trials did not confirm survival superiority over the available agents. Cancer vaccines, adoptive cellular therapies and combinations of local modalities with immunotherapy are promising approaches under active research.

**Keywords:** Hepatocellular carcinoma, immunotherapy, immunosuppression, prognosis, immunology, antibody, vaccine



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## INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related morbimortality and represents a major health problem worldwide. HCC is ranked as the sixth most incident neoplasm and the fourth cause of cancer-related death<sup>[1]</sup>.

Remarkable progresses have been achieved in prevention, early detection and diagnosis. Concurrently, new therapeutic strategies have been developed both in localized and advanced stages, what is leading to a more favorable outcome for HCC patients comparing to the past decades. However, the field is continuously evolving, and important barriers need to be surpassed.

In a significant proportion of cases, HCC is diagnosed at later stages and more than half of the patients with localized disease will develop disease recurrence after locoregional treatments<sup>[2]</sup>. At present, systemic treatment options are limited. Only a few drugs have showed survival improvement according to phase III trials. In 2008, sorafenib was the first drug that proved survival benefit according to the results of two pivotal trials<sup>[3,4]</sup>. A decade later, lenvatinib<sup>[5]</sup> proved non-inferiority to sorafenib as a first-line option. In the second-line setting, regorafenib<sup>[6]</sup>, cabozantinib<sup>[7]</sup> and ramucirumab<sup>[8]</sup> demonstrated positive results in placebo-controlled studies. Despite the incorporation of these new agents, the median survival of advanced HCC patients treated with systemic treatment still remains around 2 years<sup>[9]</sup> and there exists an unmet need for innovative approaches.

One of the most notable advances in oncology over the last years is the use of immunotherapy, alongside with an increasing knowledge on how the immune system behaves during carcinogenesis and tumor progression. The idea to harness the immune system against cancer comes from the late 19th century. Based on observations that some patients presented tumor remission after developing erysipelas, William Coley proposed to inject a mixture of live and inactivated bacteria into *in vivo* tumors. Although there was not a clear explanation at that time, anti-tumor responses were achieved in different types of tumors with this intervention<sup>[10]</sup>. In 1957, Thomas and Burnet proposed the theory of immunosurveillance, in which lymphocytes played a role of sentinels to detect and destroy transformed cells<sup>[11]</sup>. Afterwards, in the decade of 1970s, the use of tuberculosis vaccine with Bacille Calmette-Guérin was effective in preventing recurrence of urothelial carcinomas and is still applied nowadays<sup>[12]</sup>. In 1990s, interleukin (IL)-2 was approved for treatment of kidney cancer and melanoma. In the same decade, the first anti-CD20 monoclonal antibody, named rituximab, was approved for non-Hodgkin lymphoma<sup>[13]</sup>.

Currently, we are experiencing emerging trends in immunotherapy for treatment of several solid tumors, including HCC. The scope of this review is to summarize the current landscape, updates and future perspectives of immunotherapy in HCC.

## RATIONALE FOR IMMUNOTHERAPY IN HCC

HCC arises in a chronically inflamed background in the vast majority of cases. An underlying liver disease derived from a viral infection or from a non-infectious condition occurs in around 90% of HCC patients<sup>[14]</sup>. The immunologic composition of the liver is crucial for the role of this organ in the entero-hepatic circulation as it exerts an immunologic control function under physiological condition<sup>[15]</sup>.

Liver has an important function in host defense and self-tolerance by the coordinated activity of a diverse immune-cell repertoire. Liver sinusoidal endothelial cells regulates the effector immune response by inhibiting CD4+ and CD8+ T lymphocytes, thus preventing an immune reaction against bacterial antigens coming from the gut. Moreover, these cells express high levels of Program death receptor ligand 1 (PD-L1), which is a transmembrane immunosuppressive protein that inactivates the adaptative immune system by binding to the inhibitory lymphocyte receptor PD-1<sup>[16]</sup>.

Kupffer cells, which are stationary macrophages in the liver sinusoids, contribute to immune tolerance by producing inhibitory cytokines such as IL-10 and prostaglandins and by expanding inhibitory regulatory T cells. Kupfer cells also plays a major role in the clearance of gut-derived endotoxins from the portal circulation<sup>[17]</sup>.

Tolerance-inducing cells, such as inhibitory CD4+CD25+FoxP3+ T regulatory (Treg) cells, are increased in tumor tissue and peripheral blood of HCC patients<sup>[18]</sup>. Tregs can impair the effector function of intra-tumoral CD8+ T cells by several mechanisms, such as IL-10 and transforming growth factor (TGF) beta production<sup>[19]</sup>.

Neutrophils have been shown to induce tumor cell proliferation and stimulate angiogenesis through the secretion of cytokines<sup>[20]</sup>. Infiltrating neutrophils have been shown to recruit Tregs in animal HCC models and the number of neutrophils in HCC infiltrate is reported to be a negative prognostic factor in HCC patients submitted to resection<sup>[21]</sup>.

Myeloid-derived suppressor cells (MDSCs) are myeloid progenitor cells that acts as suppressor of Natural Killer (NK) cells and T cell effector function in the tumor microenvironment mediated by the expression of arginase, that depletes arginine, which is essential for T cell proliferation and also by the release of reactive oxygen species<sup>[22,23]</sup>. The MDSC subset has been reported as a prognostic factor for HCC recurrence after local treatment<sup>[24]</sup>.

Besides the activity of these immune cells, the liver micro-environment overexpresses immune checkpoint molecules, which can downregulate immune responses against tumor cells. PD-1/PD-L1 expression is observed not only in Kupffer cells, but also in tumor infiltrating lymphocytes. An association between PD-1 expression by T CD8+ lymphocytes and poor prognosis in HCC patients is reported<sup>[25]</sup>. T-cell immunoglobulin and mucin-domain-containing molecule-3 is expressed by cells from innate and adaptive immune system and interacts with several ligands such as Galectin 3, which is expressed in liver tissue. Evidence indicates that Galectin-9 inhibits T-cell responses, acting as an immunosuppressive factor. Lymphocyte-activation gene 3 (LAG3) is a membrane protein that is expressed in activated T-cells and suppresses dendritic cells function. LAG3 acts symmetrically with PD-1 to promote cancer evasion from immune recognition<sup>[26]</sup>.

The hepatic chemokine profile also plays a substantial role in modulating immune response. It has been demonstrated that immunosuppressive cytokines such as IL-4, IL-5 and IL-10 are upregulated, while some pro-inflammatory cytokines such as TNF and IL-1 are downregulated, what can facilitate HCC progression<sup>[27]</sup>.

Tumor mutational burden is a measurement of mutations carried by tumor cells that seems to be correlated with cytotoxic T cells infiltration and better response to immune-checkpoint inhibitors (ICI)<sup>[28,29]</sup>. The mutational burden typically translates into a higher neo-antigen load, and therefore a higher chance that an antigen capable of stimulating an immune reaction is expressed on the tumor cell surface. Nevertheless, HCC ranks only as a medium mutated tumor, with an average of 5 somatic mutations per megabase, corresponding to approximately 60 non-synonymous substitutions within expressed genes. This accounts for a likely lower neoantigen burden comparing to melanoma, for example<sup>[30]</sup>.

The current research activity on immunotherapy for HCC are mainly based on targeting the above-mentioned mechanisms. The complexity of immune system and the dismal prognosis of HCC are barriers for the translation of basic research from bench-to-bedside. In this regard, the development of cancer vaccines, adoptive cellular therapies, ICI and combinations of immunotherapy with other agents or with different treatment modalities are being developed and tested in the clinical setting.

## CANCER VACCINES

The number of vaccine trials in HCC is limited and the results showed only a modest efficacy. The aim of cancer vaccines is to stimulate the immune cells to recognize and attack tumor cells by managing tumor antigens and matured dendritic cells, which makes the connection between innate and adaptive immune system<sup>[31]</sup>.

In HCC, a restricted number of tumor-associated antigens has been identified. Research in HCC vaccines mainly focus on alpha-fetoprotein (AFP) because this is usually expressed in this malignancy<sup>[32]</sup>. Initially, a study was conducted based on an AFP-derived peptide and was able to produce T-cell responses. In another study, dendritic cells pulsed with a lysate of HepG2 cell line showed safety and evidence of activity in patients with advanced HCC<sup>[33]</sup>. El Ansary *et al.*<sup>[34]</sup> also observed modest clinical responses, increasing in CD8+ lymphocytes count and in serum interferon concentration using an autologous dendritic cell vaccine<sup>[34]</sup>.

The “Cancer Vaccine development for Hepatocellular Carcinoma” - HEPAVAC project has the goal of developing strategies for a therapeutic peptide-based vaccine for HCC including both “off-the-shelf” and personalized antigens. A multi-epitope vaccine (IMA970A) is currently under evaluation in early/intermediate HCC patients in a phase I/II European multicenter clinical trial (NCT03203005). The actual study start date was September-2017 and the completion date is expected to be on January-2020.

Combination of dendritic cell infusion following trans-catheter hepatic arterial embolization was shown to be safe and to enhance tumor-specific responses more effectively than embolization alone, but recurrence was not completely prevented<sup>[35]</sup>.

The PHOCUS trial, aimed to compare an oncolytic vaccine virus armed with granulocyte-macrophage colony stimulating factor gene (*JX-594*) to sorafenib halted patient enrollment following a planned interim futility analysis that conclude that the trial was unlikely to meet its primary endpoint of overall survival in the final analysis<sup>[36]</sup>. This oncolytic vaccine virus is also in a phase I/II trial in combination with nivolumab in first-line treatment of advanced HCC (NCT03071094).

Up to now, no HCC vaccine is approved for clinical use. Further research on development and applicability of this strategy, together with the completion of the active trials is warranted.

## CELL-BASED IMMUNOTHERAPY

Several approaches involving cell-based treatments are being evaluated in HCC. Briefly, this modality consists of the use of autologous effector cells that are manipulated, expanded and sensitized *ex vivo* before being delivered to the patient<sup>[37]</sup>. Different types of lymphocytes, engineered T cell receptor (TCR) and chimeric antigen receptor (CAR) compose the mainstay of cell-based immunotherapy.

In the late 1980s, Onishi *et al.*<sup>[38]</sup> innovatively reported clinical responses in HCC patients treated with a combination of IL-2 and lymphokine-activated killer (LAK) cells showing a favorable safety profile<sup>[38]</sup>. However, further studies with LAK cells showed conflicting results in terms of efficacy<sup>[39]</sup>.

The post-operative use of isolated and expanded tumor-infiltrating lymphocytes is a promising strategy in HCC. In a study with 12 patients submitted to surgery, this therapy was associated with decreased recurrence rates at 6 and 12 months after resection comparing to a control group<sup>[40]</sup>. Cytokine-induced killer (CIK) cells, which comprises a mixture of T lymphocytes (CD3+/CD56+ cells, CD3-/CD56+ NK cells and CD3+/CD56- cytotoxic T cells) provided longer progression-free survival (PFS) after HCC resection in a randomized trial

involving 150 patients, with an acceptable toxicity profile<sup>[41]</sup>. In another randomized trial, adjuvant CIK cells provided longer time to recurrence, but with no impact in overall survival (OS)<sup>[41]</sup>. An open-label phase III trial tested the efficacy of CIK cells as an adjuvant therapy after resection or percutaneous treatment. The study included 230 patients with HCC to receive CIK cells or no adjuvant treatment. The study met its primary endpoint, with a significant improvement in recurrence-free survival and OS with activated CIK cells<sup>[42]</sup>.

NK cells are also being studied in the HCC field. An ongoing phase II trial (NCT02008929) is focused on safety and efficacy of *ex vivo* expanded allogeneic NK cells in patients with high risk of recurrence after surgical resection and a second phase II trial (NCT02854839) is evaluating the role of NK adoptive cells after trans-arterial chemoembolization (TACE).

One of the most promising cellular therapies consists in the use of CAR T cells, which combines adoptive cellular immunotherapy with targeted therapy throughout receptor proteins that have been engineered to give T cells ability to recognize a specific protein independent of MHC. This approach has shown positive results when targeting CD19 via CAR for B-cell malignancies<sup>[43]</sup>. Currently, there are active clinical trials with CAR T cells in HCC with different target proteins, such as Glypican 3 (NCT02905188; NCT03146234 and NCT02723942). Nevertheless, a phase I/II trial with CAR-T anti-VEGFR2 (NCT01218867) in metastatic tumors (with HCC patients allowed to be enrolled) failed to show a significant clinical activity in preliminary results.

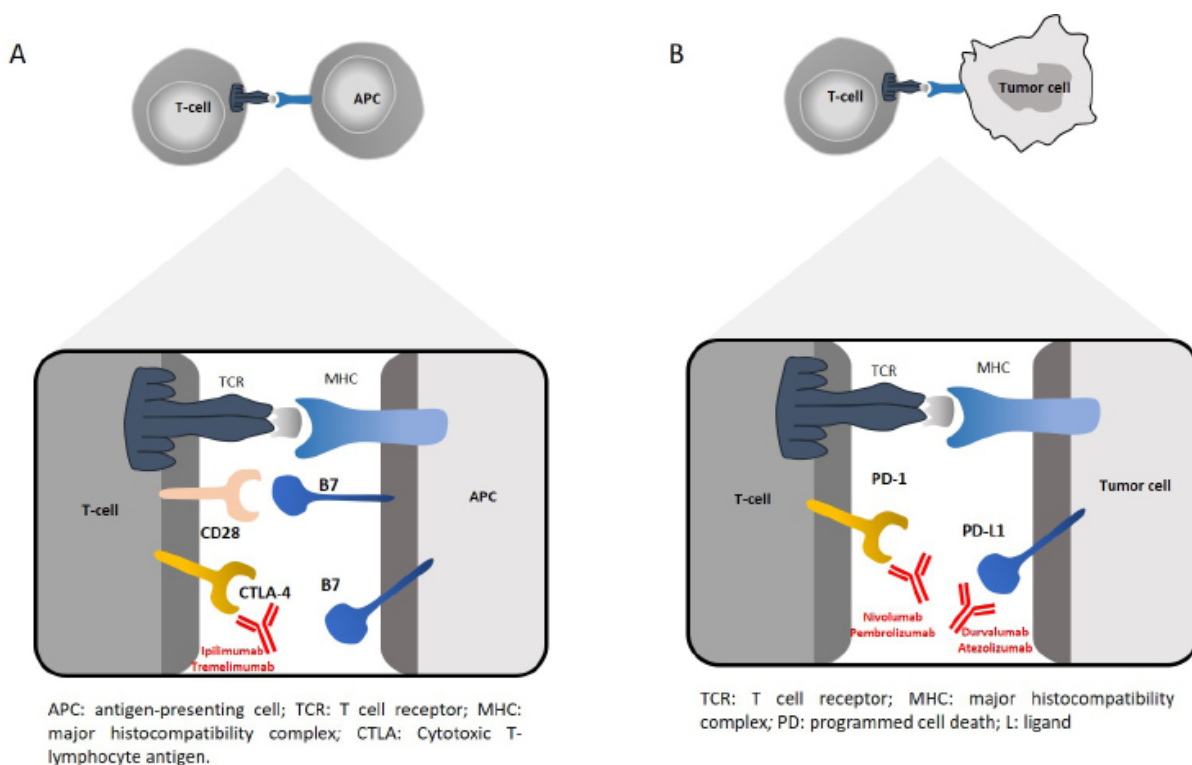
Antigens from hepatitis virus B (HBV) can be potentially used as a target in adoptive cellular therapy, since HBV antigens can be found in both primary tumor and HCC metastasis. In a case report, HCC tumor cells were recognized *in vivo* by lymphocytes engineered to express an HBV-specific TCR. Therefore, this strategy has a potential to control HBV-associated HCC<sup>[44]</sup>. An AFP directed therapy is also being investigated in a phase I trial with autologous T cells with AFP-specific TCR in advanced HCC patients (NCT03132792).

Considering this scenario, adoptive cellular therapies for HCC showed an acceptable safety profile and seems to be an encouraging strategy in the adjuvant setting. However, confirmatory studies are required and there is no sufficient evidence to support its use in clinical practice outside clinical trials.

## IMMUNE CHECKPOINT INHIBITORS

A major boost in the field of immunotherapy for cancer treatment came with the advent of immune checkpoint inhibitors (ICI). This class of drugs provided crucial improvements in the management of several tumors, such as lung cancer<sup>[45]</sup>, melanoma<sup>[46]</sup> and others. The ICIs currently approved for clinical use target either CTLA-4 or PD/PD-L1 pathways. Tremelimumab, which is an anti-CTLA4 monoclonal antibody, was the first ICI evaluated in a clinical trial involving HCC patients. In a phase II study, 21 patients with chronic hepatitis C virus (HCV) infection not eligible for surgery received tremelimumab 15 mg/kg every 90 days for a maximum of 4 doses. A disease control rate of 76.4% was achieved, with 45% of these responses lasting more than 6 months. Few patients presented grade 3/4 adverse events and tremelimumab was considered well tolerated. Besides, a significant drop in HCV viral load was observed among patients enrolled in this trial<sup>[47]</sup>.

Tremelimumab was also tested in a trial combining tumor ablation or TACE considering the hypothesis that tumor destruction by local therapies could enhance antigenic release and stimulate a systemic immune response. Thirty-two patients received tremelimumab every 4 weeks at two dosages (3.5 and 10 mg/kg) for a total of 6 infusions, followed by an infusion every 3 months. The local therapy consisted of TACE in patients



**Figure 1.** (A) Mechanism of action for ipilimumab and tremelimumab. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a negative regulator of T-cell activity. T-cell activation requires two separate stimulatory signals. The first signal occurs when the TCR binds to the major histocompatibility complex (MHC) of an antigen-presenting cell (APC). The second signal, or co-stimulatory signal, occurs when the CD28 receptor of T cells binds the B7 ligand of APCs. CTLA-4 is a naturally occurring T-cell receptor that, when bound by B7 on APCs, prevents the co-stimulation required for T-cell activation and suppresses T-cell activity. Ipilimumab and tremelimumab are monoclonal antibodies designed to bind CTLA-4 and prevent its binding of B7, allowing for T-cell activation and potentiation to occur, allowing for enhanced immune-mediated cytotoxicity. (B) Mechanism of action for immune checkpoint inhibitors: The binding of PD-L1 on tumor cells to PD-1 on T-cells prevents T cells from killing tumor cells. Blocking the binding of PD-L1 to PD-1 with an immune checkpoint inhibitor allows cytotoxic activity of T cells against tumor cells. TCR: T cell receptor; PD: programmed cell death; L: ligand

with intermediate stage and ablation in patients with advanced stage. Partial responses were observed in 26% and stable disease in 63%, with a median time-to-progression of 7.4 months and a median OS of 12.3 months. Again, no signals of serious toxicities were observed. The most common clinical adverse events was pruritus (9%) while increasing transaminases was the most common laboratorial alteration (34%)<sup>[48]</sup>.

Considering the high expression of PD-L1 in HCC cells and also in liver microenvironment components, strategies aimed to the PD1/PD-L1 pathway are been extensively tested in clinical trials. The mechanism of action of ICI directed to PD-1/PD-L1 is showed in Figure 1.

In a small phase I/II trial, the anti-PD-L1 agent durvalumab was evaluated in 40 HCC patients, most of them had been previously treated with sorafenib. An overall response rate of 10% with 20% of grade 3 or 4 toxicities were reported. No treatment-related deaths were registered in this trial<sup>[49]</sup>.

The first data with nivolumab in HCC were published in 2017. The CHECKMATE 040 trial was an open label phase I/II study that included patients with intermediate and advanced HCC, who had progressed on or were intolerant to sorafenib and with HBV, HCV or non-infectious etiology. The first part of this trial evaluated a dose escalation cohort of 48 patients who were given nivolumab in doses ranging from 0.3 mg/kg up to 10 mg/kg every 2 weeks. The dose of 3 mg/kg was chosen for the further step, which consisted of a dose expansion cohort involving 214 patients aimed to test efficacy and safety. Toxicities



did not vary across the different etiologies and the most common events were rash (23%), pruritus (21%) and diarrhea (13%). Less than 2% presented serious adverse events of grade 3 or higher and no treatment-related death was observed. The response rate by RECIST 1.1 was 15% and 20% in the dose escalation and expansion cohorts, respectively, with a median duration of response in the expansion cohort of 9.9 months. The median OS was 28.6 months in the population naïve to sorafenib and 15.6 months in the sorafenib-experienced<sup>[50]</sup>. Based on these results, nivolumab was granted accelerated approval by the US Food and Drug Administration (FDA) in 2017 for patients previously treated with sorafenib<sup>[51]</sup>. However, the phase III trial CHECKMATE 459, which compared nivolumab to sorafenib in the first line was announced to be negative by the company responsible for nivolumab development<sup>[52]</sup>. The results of this trial were presented in the European Society for Medical Oncology 2019 congress: the median OS was 16.4 months for nivolumab and 14.7 months for sorafenib (Hazard ratio 0.85, 95% confidence interval 0.72-1.02,  $P = 0.0752$ ), with a response rate of 15% for nivolumab and 7% for sorafenib<sup>[53]</sup>. Complete results are still pending to be published.

Pembrolizumab, a monoclonal antibody that also targets PD-1, was shown to be active and safe in a phase II trial. The KEYNOTE 224 enrolled 104 HCC patients with intolerance or progression after sorafenib to receive pembrolizumab 200 mg every 3 weeks in a single arm design. The authors recorded a response rate of 17% and 33% of the patients had stable disease. Grade 3 treatment-related adverse events were reported in 24% of the patients, being that the most common were increased transaminasemia and fatigue<sup>[54]</sup>. These results substantiated the accelerated approval of pembrolizumab by the FDA in 2018<sup>[55]</sup>.

The KEYNOTE 240 trial, which was a phase III trial comparing pembrolizumab to placebo in the second-line, were recently presented. The study included 413 patients who were randomized and analyzed for a co-primary endpoint of PFS and OS. The median OS of the pembrolizumab arm was 13.9 months *vs.* 10.6 months for the placebo arm, what did not reach the pre-specified efficacy boundaries for statistical significance<sup>[56]</sup>. Therefore, the trial was not able to confirm the superiority of pembrolizumab over placebo, even though the safety profile was manageable and the clinical difference between the two arms in terms of median OS warrants a further exploration of the role of ICI in HCC.

ICIs combination with ipilimumab and nivolumab in patients with advanced stage disease previously treated with sorafenib yielded a response rate of 31%, including 5% of complete responses, with a median duration of response of 17.5 months. The study randomized patients into 3 arms: nivolumab 1 mg/kg and ipilimumab 3 mg/kg every 3 weeks for 4 cycles followed by nivolumab 240 mg every 2 weeks, nivolumab 3 mg/kg and ipilimumab 1 mg/kg every 3 weeks for four cycles followed by nivolumab 240 mg every 2 weeks or nivolumab 3 mg/kg and ipilimumab 1 mg/kg every 6 weeks. The first arm experienced a median overall survival of 22.8 months, the second and third arms reached 12 and 13 months respectively. Overall, the combination was well tolerated, with 37% of all patients experiencing grades 3-4 treatment-related adverse events<sup>[57]</sup>. Tremelimumab plus durvalumab was associated with 20% of grade 3-4 toxicities with no unexpected safety signals in an analysis from an a phase I/II trial with 40 patients who progressed on or were intolerant to sorafenib<sup>[58]</sup>. An ongoing phase III trial (NCT 03298451) is actually recruiting patients in the first-line setting to sorafenib *vs.* durvalumab *vs.* durvalumab plus tremelimumab. Trials on immunotherapy are listed in Table 1.

A focus is being placed on the potential benefit of ICI as an adjuvant treatment after resection or ablation. In this sense, patients with high risk of recurrence are being enrolled in a phase III placebo-controlled trial with nivolumab in the adjuvant setting. The primary endpoint of this study is recurrence-free survival (NCT03383458). Similarly, pembrolizumab (NCT03867084) and durvalumab with or without bevacizumab (NCT03847428) are also been tested in the adjuvant setting in other phase III trials in patients who achieved complete response after resection or ablation.

**Table 1. Ongoing trials involving immune checkpoint inhibitors in hepatocellular carcinoma**

Drug	Identifier	Phase	n	Setting	Current status
Monotherapy					
Nivolumab	NCT01658878	I/II	42	1L/2L	Completed
Nivolumab	NCT01658878	I/II	214	1L/2L	Completed
Nivolumab	NCT01658878	I/II	200	1L	Completed
Nivolumab	NCT01658878	I/II	262	1L/2L	Completed
Nivolumab	NCT02576509	III	726	1L	Recruiting
Nivolumab	NCT03383458	III	520	Adjuvant	Recruiting
Pembrolizumab	NCT02702414	II	100	2L	Completed
Pembrolizumab	NCT02702401	III	408	2L	Recruiting
Pembrolizumab	NCT03062358	III	330	2L	Recruiting
Pembrolizumab	NCT03211416	I-II	27	1L	Recruiting
Pembrolizumab	NCT03867084	III	950	Adjuvant	Recruiting
Relatlimab	NCT01968109	I-II	168	2L	Recruiting
LY3321367/LY3300054	NCT03099109	I	196	2L	Recruiting
BGB-A317	NCT03412773	III	660	1L	Recruiting
SHR-1210	NCT02989922	II	220	2L	Completed
REGN3767	NCT03005782	I	546	2L	Recruiting
Combination					
Nivolumab/Ipilimumab	NCT01658878	II	620	2L	Completed
Nivolumab/Ipilimumab	NCT03222076	II	45	Neoadjuvant	Recruiting
Nivolumab/Ipilimumab	NCT03510871	II	40	Neoadjuvant	Recruiting
Nivolumab/Pexavec	NCT03071094	II	30	2L	Recruiting
Durvalumab/Tremelimumab	NCT02519348	II	545	1L/2L	Recruiting
Durvalumab/Tremelimumab	NCT03298451	III	1200	1L	Recruiting
Relatlimab/Nivolumab	NCT01968109	I-II	168	2L	Recruiting
REGN3767/REGN2810	NCT03005782	I	546	2L	Recruiting
LY3321367/LY3300054	NCT03099109	I	196	2L	Recruiting
Atezolizumab/Bevacizumab	NCT03434379	III	480	1L	Recruiting
PDR001/FGF401	NCT02325739	II	238	2L	Recruiting
PDR001/INC280	NCT02795429	II	108	2L	Recruiting
Nivolumab/Galunisertib	NCT02423343	II	75	2L	Completed
Regorafenib/Pembrolizumab	NCT03347292	I	40	1L	Recruiting
Cabozantinib/Nivolumab	NCT03299946	I	15	Neoadjuvant	Recruiting
Nivolumab/CC-122	NCT02859324	I-II	50	2L	Recruiting
PDR001/Sorafenib	NCT02988440	II	50	2L	Recruiting
Pembrolizumab/Lenvatinib	NCT03006926	I	104	2L	Recruiting
Nivolumab/TACE	NCT03143270	I	14	2L	Recruiting
Nivolumab/Y90	NCT03033446	II	40	2L	Recruiting

In the neoadjuvant setting, the study PRIME-HCC (NCT 03682276) is recruiting patients to assess safety and activity of nivolumab plus ipilimumab prior to liver resection in HCC in centers from United Kingdom.

## COMBINATION STRATEGIES

Besides ongoing trials with immunotherapies, there has been active research on combination of immunotherapy with other treatments such as tyrosine kinase inhibitors, ablative therapies, anti-VEGF antibodies and combination of different ICI. Some of these modalities can potentially improve treatment outcomes due to synergistic effects caused by tumor cell death on immune response. T cell activation and pro-inflammatory cytokines release are described to occur few weeks after locoregional therapies for HCC<sup>[59]</sup>.

There are ongoing trials testing the combination of ICI with Yttrium-90 (NCT 03033446 and NCT 02837029), TACE (NCT 03143270) and after liver resection or ablation (NCT03847428). The dual

combination of ICI (anti-CTLA-4 and PD1/PD-L1) is also being evaluated in clinical trials, for example, tremelimumab plus durvalumab (NCT 02119348) and ipilimumab plus nivolumab (NCT01658878).

Encouraging results of the combination of bevacizumab (an anti-VEGF antibody) with atezolizumab (an anti-PD-L1 ICI) were reported in a phase Ib study that included 68 HCC patients. It was reported a response rate of 34%, with 19 of the 23 responses lasting longer than 6 months. Grade 3-4 adverse events were seen in 25% of the patients<sup>[60]</sup>. This combination was granted a breakthrough designation therapy by the US FDA in 2018<sup>[61]</sup> and a phase III trial aimed to evaluate atezolizumab and bevacizumab vs. sorafenib is under recruitment (NCT0343479).

An open-label phase Ib trial that assessed the efficacy of lenvatinib plus pembrolizumab in 94 patients has been recently reported. The combination induced a confirmed response rate of 26.9%, with a median PFS of 9.69 months. Sixty percent of the patients had dose interruptions or reductions, 5 patients had serious adverse events and there were 2 treatment-related deaths<sup>[62]</sup>. Avelumab with axitinib also showed encouraging activity in a trial with 22 patients, although a higher rate of grade 3 hypertension (50%) and hand-foot skin reaction (22.7%) were reported<sup>[63]</sup>. Table 2 summarizes main results on ICI in HCC.

## CHALLENGES AND FUTURE DIRECTIONS

While final results on immunotherapy for HCC are awaited, some relevant issues are to be taken into account when interpreting the available and upcoming data.

ICIs are associated with atypical patterns of response and progression. The traditional radiologic criteria used to evaluate tumor response in oncology trials with cytotoxic chemotherapy may not be accurate enough to detect clinical benefit or treatment failure with immune oncology agents. For example, a radiological increasing in tumor burden without worsening in disease burden, called pseudoprogression, can be explained by an immune-cell infiltration and do not represent treatment failure<sup>[64]</sup>. Patients with an initial progressive disease followed by a later radiologic response may experience a nonconventional survival benefit comparing to those patients with the same initial behavior<sup>[65]</sup>. Therefore, treatment with ICI warrants specific radiologic criteria to assess benefit or the emergence of resistance.

Around 10%-30% of the patients with other solid tumors submitted to ICI treatment present long-term disease control. This finding suggests the existence of a subgroup of patients that probably presents a sensitive tumoral phenotype or a specific predictive biomarker. Mutational burden, tumor-infiltrating lymphocytes and immune gene signatures are also being investigated as potential tools<sup>[66]</sup>. For HCC, the PDL-1 expression seems to be around 20%-25%, but no correlation between PDL-1 expression and better response has been established so far<sup>[67]</sup>.

Immune-related adverse events induced by ICIs are also a major concern in HCC. A wide range of events are described in immunotherapy trials (dermatologic, endocrinologic, gastrointestinal and others), but hepatotoxicity is of particular interest in this context. In clinical trials with anti-PD1 inhibitors, liver enzymes elevations were typically mild. However, HCC patients often present underlying cirrhosis with a limited liver reserve, what increase the risk of decompensation even in mild liver alterations.

In conclusion, the incorporation of immunotherapy in the HCC landscape is still under development. The recently announced negative results of the phase III trials with pembrolizumab<sup>[56]</sup> and nivolumab<sup>[52]</sup> somehow disappointed the initial hope placed in this strategy. Immunotherapy for HCC seems to be a tougher road comparing to what we are experiencing with other tumors. However, huge effort is being made in the search for predictive biomarkers and development of novel strategies to deliver better outcomes for HCC patients.

**Table 2. Data of immune checkpoint inhibitors in advanced hepatocellular carcinoma**

Agent (mechanism)	Trial	Phase	Design	n	Target population	Response rate		Median survival months		Grade 3/4 AEs
						ORR	DCR	OS	PFS	
Monotherapy										
Nivolumab (anti-PD1)	CHECKMATE 040 (NCT 01658878)	I/II	Cohort 1 (dose escalation)	48	Advanced HCC: HCV, HBV or non-infected; sorafenib-naïve or treated	15%	58%	15	NA	25%
			Cohort 2 (dose expansion)	214		20%	64%	NR	4	19%
Pembrolizumab (anti-PD1)	KEYNOTE 224 (NCT02702414)	II	Non-randomized, single-arm	104	Advanced HCC: sorafenib-treated	17%	62%	12.9	4.9	25%
Tremelimumab (anti-CTLA4)	NCT01008358	II	Non-randomized, single-arm	21	Inoperable HCC: Naïve or previously treated	17.6%	76.4%	8.2	NA	45%
Durvalumab (anti-PDL1)	NCT01693562	II	Non-randomized, single arm	40	Stage III or IV Fail, ineligible, refusal or progression to first-line	10.3	NA	13.2	2.7	20%
Combination										
Atezolizumab (anti-PDL1) + Bevacizumab (anti-VEGF)	NCT02715531	Ib	Non-randomized, single-arm	103	Unresectable HCC: Non-previously treated; HBV, HCV or non-infected	32%	96%	NA	14.9	28%
Lenvatinib (kinase inhibitor)+ Pembrolizumab (anti-PD1)	NCT03006926	Ib	Non-randomized, single arm	30	Unresectable HCC: sorafenib-naïve or treated; HCV, HBV or non-infected	46%	92%	NA	9.69	60%
Avelumab (anti-PDL1) plus axitinib (kinase inhibitor)	NCT03289533	Ib	Non-randomized, single arm	22	Unresectable HCC: sorafenib-naïve or treated; HCV, HBV or non-infected	13.6%	68.2%	12.7	5.5	72.7%
Durvalumab (anti-PDL1) plus Tremelimumab (anti-CTLA4)	NCT02519348	I/II	Randomized, three arm	40	Unresectable HCC: sorafenib-naïve or experienced; HCV, HBV or non-infected	25%	57.5%	NA	NA	20%
Nivolumab (anti-PD-1+ Ipilimumab (anti-CTLA4) arm A	NCT01658878	I/II	Randomized, three arms	50	Unresectable HCC: Previous sorafenib; HCV, HBV or non-infected	32%	54%	22.8	NA	37%

AEs: adverse events; ORR: overall response rate; DCR: disease control rate; PFS: progression-free survival; PD1: programmed cell-death; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HBV: hepatitis B virus; NR: not reached

## DECLARATIONS

### Authors' contributions

Discussion, writing and editing of the manuscript: da Fonseca LG

Discussion, writing and editing of the manuscript: Carrilho FJ

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Not applicable.

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None.

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Both authors declared that there are no conflicts of interest.

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Not applicable.

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Original Article

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# Ten-year survival and recurrence of hepatocellular cancer

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## Abstract

**Aim:** Long-term survival after hepatocellular cancer (HCC) is difficult to achieve likely related to recurrence. This study aimed to identify factors that were predictive of 10-year survival after the diagnosis of HCC.

**Methods:** In a prospectively collected database of 1374 HCC cases (1993-2019), we identified 70 patients who survived over 10 years regardless of treatment. We then identified 164 patients in the entire cohort who either had liver resection or transplant, and died before 10 years. Demographics, tumor characteristics, treatment, recurrence and treatment of recurrence were compared.

**Results:** Of the 10-year survivors, 36 underwent transplant, 27 had liver resection and 7 patients had only locoregional therapy. Compared to the non-survivors, the 10-year survivors were younger and had fewer comorbidities or recurrence, smaller tumor size, lower AST, ALT, AFP, platelets, neutrophil-to-lymphocyte ratio. Multivariate analysis showed only age and diabetes to be negative predictors. Recurrence occurred in 24 survivors (34.3%) with mean time to recurrence with standard deviation  $57.1 \pm 42.6$  months compared to 80 non-survivors (48.7%) with mean time to recurrence of  $15.3 \pm 14.8$  months. For hepatic resection, 10-year survivors had longer time to recurrence compared to non-survivors (median: 31.3 months).

**Conclusion:** Long-term survivors mostly occur after resection or transplant, but 10% of our cohort survived 10 years with only locoregional therapy. Underlying health status maybe an important predictor of 10-year survival for



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patients receiving liver resections. Recurrence of HCC occurs in both 10-year survivors and non-survivors, but later recurrence with aggressive treatment of the recurrence may allow for 10-year survival.

**Keywords:** Hepatocellular cancer, 10-year survival, liver transplantation, hepatic resection

## INTRODUCTION

Hepatocellular cancer (HCC) is the fourth leading cause of cancer mortality in the world and the incidence and mortality has been increasing in the USA<sup>[1,2]</sup>. Survival for HCC has been prolonged by curative therapies which include liver transplantation, hepatic resection and ablation<sup>[3]</sup>. The overall 5-year survival for patients with HCC is quite dismal and estimated at 10%-12%, however this is improved in patients with localized HCC (30%) and those who undergo liver transplantation (70%-75%)<sup>[4,5]</sup>. Efforts have been made to promote early detection of HCC with surveillance programs as this can contribute to improved survival by allowing patients to qualify for these curative therapies<sup>[6]</sup>.

Despite these efforts and potentially curative therapies, recurrence occurs in about 54% of patients who undergo resection and 8%-17% of those who undergo liver transplantation<sup>[7-10]</sup>. Recurrences have been treated with repeat liver resections, salvage liver transplantation after resection and locoregional therapies, however these recurrences are likely responsible for compromised long-term survival. While much of the literature focuses on 5-year outcome, less is reported about longer term survival beyond 5 years. Late recurrence, which occurs after 5 years, has been described in patients after resection or transplant<sup>[11-13]</sup>. Others have suggested that underlying liver function as measured by albumin-bilirubin (ALBI) grade correlated with recurrence free survival<sup>[14,15]</sup>.

There are few studies that report or critically evaluate 10-year survival from HCC. This is often difficult as patients relocate, have other illnesses, are lost to follow-up or are no longer followed by the tertiary center that performed the curative therapy. This study reviews a 26-year experience of patients in Hawaii with HCC who have been followed by a group of physicians and the state's only Liver Center and characterizes patients with at least 10-year follow-up from curative therapies. Specifically, we identified 10-year survivors and compared them to patients who received similar therapies who died before 10 years.

## METHODS

### Patients

Utilizing prospectively collected database of 1374 HCC patients from 1993 to 2019, there were 575 patients who had at least 10 years of follow up. We identified those patients who survived at least 10 years regardless of treatment. We then selected a comparison group of all patients who underwent liver transplantation or liver resection and did not survive 10 years. We excluded patients who had liver transplantation or resection who were still alive but did not have at least 10 years of follow up. This comparison group also excluded patients who had non-surgical therapies and did not survive 10 years as this was a large heterogeneous group of patients with more advanced HCC and/or severe cirrhosis who received locoregional therapy or supportive care. This database is based on Hawaii's only tertiary liver center and liver transplant program and also includes patients from the American territories of the Pacific Basin. Approximately 60%-70% of the HCC patients in Hawaii were referred to this center and included in this database. The diagnosis of HCC was made histologically or based on contrast-enhanced computed tomography scan or magnetic resonance imaging with typical HCC features based on guidelines published by the American Association for the Study of Liver Disease<sup>[3]</sup>. This study was approved by the university of Hawaii at Manoa institutional review board.

## Data collected

We obtained demographic (age, sex and ethnicity), anthropometric information [height, weight and body mass index (BMI)], comorbidities, etiology of HCC, tumor size/characteristics, laboratory values, staging, therapeutic modalities, recurrence and survival information. Ethnicity was categorized as “Caucasian”, “Asian”, “Pacific Islanders”, and “Others”. Comorbidity data collected include diabetes mellitus, smoking status, hyperlipidemia, and hypertension.

Significant alcohol use was defined as at least 2 alcoholic beverages daily for 10 years. Positive smoking history included both past and present use of cigarettes. Laboratory values include creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, bilirubin, prothrombin time with international normalized ratio, platelet, neutrophil, lymphocytes, hepatitis B virus (HBV) and hepatitis C virus (HCV) serologies and pre-treatment alpha feto protein (AFP). We defined normal AFP as less than 20 ng/dL. Based on these values, we calculated model for End-Stage Liver Disease (MELD) score<sup>[16]</sup>, fibrosis-4 (FIB-4) index for liver fibrosis<sup>[17]</sup>, AST/platelet ratio index (APRI) and neutrophil-lymphocyte ratio (NLR). The American Joint Committee on Cancer (AJCC) staging system was incorporated with tumor size, number, and location of tumor. Tumor size was categorized by the largest diameter by  $\geq 5$  cm or  $< 5$  cm. Status on underlying cirrhosis, rupture at presentation, and macrovascular invasion on imaging was also noted. Cirrhosis was determined with imaging when tissue was not available. Therapeutic modalities included liver transplantation, hepatic resection and locoregional therapies (radiofrequency ablation (RFA), cryosurgery, transarterial chemoembolization (TACE), percutaneous ethanol injection and yttrium90 transarterial radioembolization). Liver resection was performed on patients with Childs-Turcotte-Pugh (CTP) A and early B with CTP of 7 without ascites or encephalopathy. Patients who received liver transplantations had unresectable tumor who met Milan criteria<sup>[18]</sup> or prior liver resection with recurrence of HCC which met Milan criteria. Single tumors size less than 6.5 cm that were down staged to meet Milan criteria was also evaluated for liver transplantation since 2007.

## Statistical analysis

Statistical analysis was conducted using statistical package for social services (SPSS) (version 23.0. IBM Corp., Armonk, NY, USA), R version 3.4.1 (The R foundation for Statistical Computing, Vienna, Austria) as well as EZR version 1.36 (Division of Hematology, Saitama Medical Center, Jichi Medical University, Japan<sup>[19]</sup>). Primary objective of this study is to elucidate the factors associated with 10-year survival. Secondary objective of this study is to elucidate management strategies for HCC recurrence that allows for long term survival. Comparison of binary variables were accomplished by chi-square test. Continuous variables were analyzed by T test to obtain mean, standard deviation (SD) and standard error of mean (SE). Likelihood ratio was calculated for binary variables. Nominal regression was used to create a regression model. Variables included in this model as followings: age was categorized as a binary variable with  $< 65$ -year-old and  $\geq 65$ -year-old. Demographic data, etiology of HCC, BMI, comorbidity, AFP as binary variable (normal vs abnormal), tumor size as a binary variable, Milan criteria, rupture status, and therapeutic modalities. Multivariable regression model was also created to analyze these variables with  $P < 0.1$  on univariate analysis. We also conducted differences between 10-year survivors and non-survivors on time to recurrence for transplant and hepatic resection. Kolmogorov-Smirnov test was used to test for normal distribution. Mann-Whitney U test was used to compare time to recurrence and recurrence free survival between 10-year survivors and non-survivors.  $P < 0.05$  is considered as statistically significant.

## RESULTS

### Baseline characteristics

This study included 234 patients: 70 patients who survived 10 years after the diagnosis of HCC and 164 patients in the entire cohort who either had liver resection or transplant and died before 10 years. Baseline

**Table 1. Baseline characteristics for all patients**

	Locoregional therapy ( <i>n</i> = 7)	Resection ( <i>n</i> = 163)	Liver transplant ( <i>n</i> = 64)	<i>P</i> value	Total ( <i>n</i> = 234)
Age ≥ 65 years (%)	2 (28.5)	75 (46.0)	4 (6.3)	< 0.001	153 (65.4)
Sex (Males) (%)	5 (71.4)	115 (70.6)	55 (85.9)	0.05	175 (74.8)
BMI ≥ 25 (%)	5 (71.4)	73 (44.8)	48 (75.0)	0.002	126 (53.8)
Diabetes (%)	1 (14.3)	47 (28.9)	18 (28.1)	0.70	66 (28.2)
Hepatitis B (%)	1 (14.3)	68 (41.7)	21 (32.8)	0.48	64 (27.4)
Hepatitis C (%)	5 (71.4)	43 (26.4)	38 (59.4)	< 0.001	86 (36.8)
Hyperlipidemia (%)	1 (14.3)	41 (25.2)	7 (10.9)	0.04	49 (20.9)
Hypertension (%)	5 (71.4)	88 (54.0)	28 (43.8)	0.12	121 (51.7)
AJCC Stages				0.08	
Stage I	7 (100)	109 (66.9)	49 (76.6)		165 (70.5)
Stage II	0 (0)	17 (10.4)	13 (20.3)		30 (12.8)
Stage IIIa	0 (0)	1 (0.6)	0 (0)		1 (0.4)
Stage IIIb	0 (0)	11 (6.7)	0 (0)		11 (4.7)
Stage IIIc	0 (0)	5 (3.0)	2 (3.1)		7 (3.0)
Stage III NOS	0 (0)	1 (0.6)	0 (0)		1 (0.4)
Stage IV	0 (0)	18 (11.0)	0 (0)		18 (7.7)
Single tumor	7 (100)	131 (80.4)	51 (79.7)	0.42	189 (80.8)
Cirrhosis	7 (100)	68 (41.7)	63 (98.4)	< 0.001	138 (60.0)
Normal AFP (%)	3 (42.9)	74 (45.4)	29 (45.3)	0.99	106 (45.3)
Rupture (%)	0 (0)	18 (11.0)	0 (0)	0.01	18 (7.7)
Size ≥ 5 cm (%)	3 (42.9)	98 (60.1)	6 (9.4)	< 0.001	107 (45.7)
Vascular invasion (%)	0 (0)	6 (3.7)	2 (3.1)	0.86	8 (3.4)

BMI: body mass index; AJCC: American Joint Committee on Cancer; NOS: not otherwise specified; AFP: alpha feto protein

characteristics are shown in [Table 1](#). In the entire cohort, mean age was 60.2 years (SD: 10.6) with 153 patients (65.4%) older than 65-year, 175 (74.8%) were male, and 126 (53.8%) had BMI over 25. Ethnic distribution was as follows: 160 (68.4%) were Asian, 42 (17.9%) were Caucasian, 24 (10.3%) were Pacific Islanders, and 8 (3.4%) were mixed or another ethnicity. The incidence of risk factors included: 36.8% had prior HCV, 27.4% had prior HBV, 34.6% has alcohol usage, and 12.8% had NASH/NAFLD. For comorbid conditions, 125 (53.4%) had a smoking history, 66 (28.2%) had diabetes, 49 (20.9%) had hyperlipidemia, and 121 (51.7%) had hypertension. For tumor characteristics, 107 (45.7%) had tumor size ≥ 5 cm, 106 (45.3%) had normal AFP, and 108 (46.2%) met Milan criteria for liver transplantation. For treatment, 64 (27.4%) received liver transplantation, 163 (69.7%) had resection, and 7 patients had only locoregional therapy. For each treatment modalities, age, BMI ≥ 25, HCV, hyperlipidemia, tumor rupture, and size ≥ 5 cm had statistically significant difference among curative therapies. Of note, six patients had salvage transplant. More than half of the patients who were transplanted received locoregional therapy prior to transplant.

### 10-year survivors vs. non-survivors

[Tables 2 and 3](#) summarize the characteristics of 10-year survivors vs. non-survivors. There was no difference in ethnic distribution between the groups. As shown in [Tables 2 and 3](#), 10-year survivors were younger and had a smaller tumor size and lower AFP, AST, ALT, platelets and NLR compared to non 10-year survivors. Univariate analysis showed that 10-year survivors were less likely to be age ≥ 65 years or to have diabetes, hypertension or tumors ≥ 5 cm [[Table 4](#)]. Multivariate analysis showed only age and diabetes to be predictive of survival. Of the 10-year survivors, 36 underwent transplant, 27 had liver resection and 7 patients had only locoregional therapy. We performed separate analysis for transplantation and hepatic resection to compare 10-year survivors and non-survivors. Details are shown in [Tables 5 and 6](#). For liver transplantation, HCC found with surveillance, hypertension and recurrence were significantly different in the univariate analysis. However, in the multivariate analysis, only the presence of recurrence was predictive of not surviving 10 years. For liver resection, Age ≥ 65-year, Hepatitis B, BMI ≥ 25, diabetes, hypertension, and smoking status had significant difference between two groups on the univariate analysis. Only BMI ≥ 25 and smoking were predictive of not surviving 10 years in the multivariate analysis.

**Table 2. Patient characteristics of 10-year survivors vs. non-survivors**

	10-year survivors (n = 70)	10-year Non-survivors (n = 164)	P value
Age in years	55.5 ± 7.5	62.2 ± 11.0	< 0.001
Age ≥ 65 years (%)	12 (17.1)	69 (42.1)	< 0.001
Sex (Males)	57 (81.4)	118 (72.0)	0.17
BMI ≥ 30 (%)	12 (17.1)	31 (18.9)	0.77
Diabetes (%)	9 (12.9)	57 (34.8)	0.001
Hypertension (%)	31 (44.3)	90 (54.9)	0.001
Hyperlipidemia (%)	11 (15.7)	38 (23.2)	0.24
Smoking (%)	35 (50.0)	90 (54.9)	0.56
Alcohol use (%)	27 (38.6)	54 (32.9)	0.50
Hepatitis B (%)	30 (42.9)	60 (36.6)	0.12
Hepatitis C (%)	28 (40.0)	58 (35.4)	0.62
AJCC stages			0.09
Stage I	56 (80.0)	109 (66.4)	
Stage II	11 (15.7)	19 (11.6)	
Stage IIIa	1 (1.4)	10 (6.3)	
Stage IIIb	0 (0)	7 (4.3)	
Stage IIIc	0 (0)	1 (0.6)	
Stage III NOS	0 (0)	1 (0.6)	
Stage IV	2 (2.9)	16 (9.8)	
Single tumor	58 (82.9)	131 (79.9)	0.73
Cirrhosis	51 (72.9)	87 (53.0)	0.01
HCC found with surveillance (%)	12 (17.1)	27 (16.5)	1.00
AST (IU/L)	64.3 ± 45.5	73.6 ± 58.9	0.03
ALT (IU/L)	60.5 ± 38.5	64.0 ± 52.4	0.03
Platelets ( × 10 <sup>3</sup> /cc)	149.6 ± 77.3	190.5 ± 101.2	0.03
FIB-4	4.32 ± 3.16	4.11 ± 3.66	0.07
APRI	0.62 ± 0.63	0.57 ± 0.75	0.94
Creatinine (mg/dL)	0.88 ± 0.21	1.01 ± 0.59	0.03
Neutrophil/Lymphocyte ratio	2.33 ± 1.88	4.20 ± 3.50	0.002
MELD	9.10 ± 3.3	9.28 ± 3.2	0.62
AFP (mg/dL)	2479 ± 14,355	13787 ± 81,011	0.049

Numerical values expressed as ± standard deviation. BMI: body mass index; AJCC: The American Joint Committee on Cancer staging system; NOS: not otherwise specified; HCC: hepatocellular cancer; AST: aspartate aminotransferase; ALT: alanine aminotransferase; FIB-4: fibrosis-4 Index; APRI: AST/Platelet Ratio Index; MELD: Model for End-stage Liver Disease Score; AFP: alpha feto protein

**Table 3. Tumor characteristics and treatment of 10-year survivors vs. non-survivors**

	10-year survivors (n = 70)	10-year Non-survivors (n = 164)	P value
Mean tumor size (cm ± SD)	4.0 ± 2.4	6.7 ± 4.7	< 0.001
Tumor size ≥ 5 cm (%)	23 (35.4)	84 (51.2)	0.01
Single tumor (%)	58 (82.9)	131 (79.9)	0.73
Rupture (%)	2 (2.9)	16 (9.8)	0.12
Vascular invasion (%)	0 (0)	8 (4.9)	0.14
Met Milan Criteria (%)	40 (57.1)	68 (41.5)	0.04
Treatment			< 0.001*
Transplantation (%)	36 (51.4)	28 (17.1)	
Resection (%)	27 (38.6)	136 (82.9)	
Locoregional therapy (%)	7 (10.0)	0 (0)	
Recurrence (%)	24 (34.3)	80 (48.8)	0.04

\*There was also significant difference between transplantation and resection ( $P < 0.001$ ). SD: standard deviation

## Recurrence

Of the 10-year survivors, recurrence occurred in 24 patients (34.3%) with mean time to recurrence with SD, 57.1 ± 42.6 months days and 23 of these patients had treatment for their recurrence. In 164 non 10-year survivors, 136 had liver resection and 28 had liver transplant. Recurrence occurred in 80 patients



**Table 4. Predictors of 10-year survival (all patients)**

	Univariate odds-ratio (95%CI)	Multivariate odds-ratio (95%CI)
Age $\geq$ 65	<b>0.29 (0.14-0.57)</b>	<b>0.33 (0.15-0.72)</b>
Sex (Males)	1.71 (0.86-3.41)	
Hepatitis B	1.30 (0.74-2.30)	
Hepatitis C	1.21 (0.68-2.15)	
Alcohol history	1.28 (0.72-2.29)	
NASH/NAFLD	0.43 (0.16-1.17)	1.07 (0.32-3.52)
HCC found with surveillance	1.05 (0.50-2.21)	
BMI $\geq$ 25	0.84 (0.48-1.50)	
BMI $\geq$ 30	0.84 (0.40-1.75)	
Smoking	0.81 (0.46-1.42)	
Diabetes mellitus	<b>0.28 (0.13-0.60)</b>	<b>0.28 (0.12-0.68)</b>
Hyperlipidemia	0.60 (0.29-1.27)	
Hypertension	<b>0.36 (0.20-0.66)</b>	0.66 (0.33-1.31)
Normal AFP	0.67 (0.38-1.18)	
Size $\geq$ 5 cm	<b>0.47 (0.26-0.84)</b>	0.52 (0.14-1.91)
Met Milan criteria	<b>1.86 (1.06-3.28)</b>	0.69 (0.20-2.41)
Rupture	0.27 (0.06-1.22)	0.36 (0.07-1.85)

Significant values are in bold. NASH: non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease; HCC: hepatocellular cancer; BMI: body mass index; AFP: alpha feto protein

**Table 5. Predictors of 10-year survival after transplant**

	Univariate odds-ratio (95%CI)	Multivariate odds-ratio (95%CI)
Age $\geq$ 65	2.45 (0.24-25.0)	
Sex (Males)	3.0 (0.66-13.3)	
Hepatitis B	1.41 (0.49-4.10)	
Hepatitis C	0.53 (0.19-1.48)	
Alcohol history	0.92 (0.34-2.49)	
NASH/NAFLD	0.76 (0.14-4.08)	
HCC found with surveillance	<b>0.29 (0.09-0.98)</b>	0.29 (0.07-1.21)
BMI $\geq$ 25	0.71 (0.22-2.26)	
BMI $\geq$ 30	0.86 (0.27-2.74)	
Smoking	1.40 (0.52-3.78)	
Diabetes	0.37 (0.12-1.14)	0.51 (0.12-2.20)
Hyperlipidemia	1.04 (0.21-5.09)	
Hypertension	<b>0.32 (0.10-0.98)</b>	0.28 (0.06-1.34)
Normal AFP	0.55 (0.20-1.50)	
Size $\geq$ 5 cm	1.62 (0.28-9.58)	
Single tumor	1.13 (0.33-3.84)	
Recurrence	<b>0.29 (0.09-0.98)</b>	<b>0.19 (0.03-1.02)</b>

Significant values are in bold. NASH: non-alcoholic steatohepatitis; NAFLD: non-alcoholic fatty liver disease; HCC: hepatocellular cancer; BMI: body mass index; AFP: alpha feto protein

of non 10-year survivors (48.7%) with mean time to recurrence of  $15.3 \pm 14.8$  months and 61 (76.3%) had treatment of the recurrence. Recurrence rate was 23.4% after transplant, 50.9% after resection and 85.7% after just locoregional therapy. For the liver transplant patients, 73.3% of recurrences received the following treatments: resections-5, RFA-2, external radiation-2 and systemic therapy-2. In the patients who received liver resection, 80.7% of recurrences were treated with the following; RFA-19, systemic therapy-15, TACE-14, repeat resection-11, radiation-3, Yttrium-90 radioembolization-2, and cryotherapy-1. Thirty-five liver resection patients had more than one recurrence and received: chemotherapy-17, RFA-7, TACE-7, repeat resection-2 and Yttrium-90 radioembolization-1. Of the 7 patients who had only locoregional therapy, 5 patients had RFA and 2 patients had TACE as their initial treatment. One patient had RFA for a 1.0 cm lesion and died 14 years later from cardiac problems. The other 6 patients had recurrences 3-11 years after their initial LRT and had subsequent procedures. Predictors of recurrence included alcohol abuse, HCV,

**Table 6. Predictors of 10-year survival after hepatic resection**

	Univariate odds-ratio (95%CI)	Multivariate odds-ratio (95%CI)
Age ≥ 65	<b>0.35 (0.14-0.88)</b>	0.27 (0.43-4.50)
Sex (Males)	0.99 (0.40-2.45)	
Hepatitis B	<b>2.35 (1.01-5.45)</b>	2.14 (0.75-6.10)
Hepatitis C	0.43 (0.139-1.32)	
Alcohol history	1.16 (0.48-2.79)	
NASH/NAFLD	0.20 (0.03-1.55)	
HCC found with surveillance	1.59 (0.53-4.76)	
BMI ≥ 25	<b>0.27 (0.10-0.72)</b>	<b>0.32 (0.10-1.02)</b>
BMI ≥ 30	0.36 (0.08-1.64)	
Smoking	<b>0.33 (0.13-0.80)</b>	<b>0.25 (0.09-0.74)</b>
Diabetes	<b>0.08 (0.01-0.57)</b>	0.15 (0.02-1.22)
Hyperlipidemia	0.82 (0.31-2.22)	
Hypertension	0.36 (0.15-0.85)	0.63 (0.22-1.82)
Normal AFP	0.65 (0.28-1.53)	
Size ≥ 5 cm	0.96 (0.41-2.22)	
Met Milan criteria	1.03 (0.43-2.49)	
Rupture	0.60 (0.13-2.78)	
Single tumor	1.09 (0.38-3.14)	
Vascular invasion	< 0.01 (0-inf)	
Recurrence	0.88 (0.38-2.00)	

Significant values are in bold. NASH: non-alcoholic steatohepatitis; NAFLD: non-alcoholic fatty liver disease; HCC: hepatocellular cancer; BMI: body mass index; AFP: alpha feto protein

screenable diagnosis, symptoms at the diagnosis, size ≥ 5 cm, treatment modalities (transplantation: 23.4%, resection: 50.9%, LRT: 85.7%). Age ≥ 65-year, AJCC staging, hypertension, hyperlipidemia, normal AFP, ethnicity, tumor rupture, presence of single tumor, or vascular invasion were not significant predictors of 10-year survival. For transplantation, there was significant difference on tumor recurrence with 13.9 % had recurrence for 10-year survivors and 35.7% had recurrence on non-survivors ( $P = 0.05$ ). However, hepatic resection did not have significant difference on recurrence ( $P = 0.92$ ). There was no difference between 10-year survivors and non-survivors regarding treatment status of recurrence for both transplant and hepatic resection. For transplantation, time to recurrence did not have significant difference between 10-year survivors and non-survivors. However, hepatic resection had significant difference ( $P < 0.001$ ) between 10-year survivors [median: 938, interquartile range (IQR): 730-2155] and non-survivors (median: 357, IQR: 155-514). There was significant difference ( $P > 0.001$ ) between 10-year survivors (median: 4065, IQR: 2,678-5,762) and non-survivors (median: 453, IQR: 174-1315) for recurrence free survival.

## DISCUSSION

### Characteristics of 10-year survivors vs. non-survivors

Survival after HCC has generally been related to the therapies that patients receive and which therapy they receive is mainly dependent on tumor characteristics and underlying liver function. Because HCC is such a heterogeneous neoplasm, the underlying liver function is further influenced by etiology of liver disease and external factors such as alcohol, smoking, and metabolic factors. When all things were considered in this study, patients who survived 10 years after diagnosis were more likely to be younger. Ten-year survivors also had smaller tumor size and fewer of them exceeded 5 cm. They may also have better underlying liver function as evidenced by lower liver enzymes and higher platelet count however fibrosis markers (FIB-4 and APRI) did not seem to differ between survivors and non-survivors. Previous studies have suggested differences in long term survival based on etiology of chronic liver disease with a better prognosis in those with viral hepatitis B or C compared to those with NASH or ALD<sup>[20]</sup>. Others have shown that underlying liver function can prognosticate long term survival<sup>[14]</sup>. Wu *et al.*<sup>[21]</sup>, in an evaluation of 8450 HCC patients long-term, determined that 10-year survival was dependent on the number of lesions, the presence of

cirrhosis, child pugh classification and the time elapsed before first recurrence or metastasis. In this study, however, proportion of previous HBV or HCV infection did not differ in 10-year survivors and non-survivors. Non-survivors were more likely to have metabolic factors of diabetes and hypertension. Obesity, smoking and alcohol use did not seem to differ between survivors and non-survivors. However, after multivariate analysis, 10-year survivors were younger and less likely to have diabetes. Hypertension, size  $\geq 5$  cm and meeting Milan criteria were no longer significant after multivariate analysis.

### Treatment modality and survival

This current study attempted to characterize all 10-year survivors as previous studies have described 10-year survival after a particular modality: resection, transplant or RFA. Our study showed long-term survivors mostly occur after resection or transplant, but 10% of our cohort survived long-term with only locoregional therapy. Baseline characteristics in these three groups differed because of requirements for each of the therapies. To avoid confounding factors and bias, we conducted separate analyses for liver transplantation and resection.

Selection of patients for liver transplantation varies depending on the transplant center but generally requires AJCC stage I or II and the absence of macrovascular invasion, tumor rupture, high AFP, morbid obesity and severe medical comorbidities. In our center, we specifically require patients to have BMI 35 or less and AFP  $< 1000$  ng/mL. In this study, only recurrence was a predictor of 10-year survival after transplantation. Surveillance, hypertension were no longer significant after multivariate analysis.

Previous studies have suggested that 10-year survival after liver resection was primarily dependent on tumor characteristics. Zheng *et al.*<sup>[22]</sup>, in 212 patients who underwent liver resection for HCC, reported 23% 10-year survival and predictors of survival included tumors  $< 5$  cm, solitary tumors and the absence of vascular invasion. However, more than 20% of 10-year survivors had microvascular invasion, poor tumor differentiation, AFP greater than 1000 ng/mL and tumor size greater than 10 cm. Long-term survival may also be influenced by surgical expertise, as Chapman *et al.*<sup>[23]</sup> reported that centers with high volumes of resections for HCC had significantly improved 10-year survival after hepatic resection. In our study, however, the univariate analysis on hepatic resection suggested that Age  $\geq 65$ , HBV, BMI  $\geq 25$ , smoking, and diabetes were associated with 10-year survival. With the multivariate analysis, only lower BMI and smoking were predictive of non-survival and all of the other tumor characteristics and recurrence did not affect survival.

This study did not compare 10-year survivors versus nonsurvivors in locoregional therapy specifically because we had a large heterogeneous group of patients who underwent locoregional therapy. Previous studies have demonstrated 10-year survival after ablative therapies, but these have typically involved patients with small tumors. Chen *et al.*<sup>[14]</sup> in 271 patients with BCLC stage 0 patients with tumors  $< 2.0$  cm, reported a 56.4% 10-year survival. While Shiina *et al.*<sup>[24]</sup> used a more liberal criteria of ablating up to 5 cm tumors and noted a 10-year survival of 27.3% in 1170 patients.

### Recurrence

Recurrence of HCC occurred in both 10-year survivors and non-survivors, but later recurrence with aggressive treatment may have allowed for 10-year survival. Zheng *et al.*<sup>[22]</sup> in 212 patients who underwent liver resection showed that 77% of the short term survivors developed recurrence within 2 years while 42% of the 10 year survivors developed recurrence most of whom had intrahepatic recurrences that were treatable. In a study of 878 patients with HCC, Lee *et al.*<sup>[25]</sup> reported a 19.8% recurrence after transplant compared to a 64.9% recurrence after resection and suggested that transplant may have a protective effect against late recurrence of early stage HCC. Risk factors for recurrence included multiple tumors, tumor size, histologic features (grade, extent, vascular invasion) and preoperative AFP. Our study also showed

lower recurrence rate after liver transplantation compared to hepatic resection and the importance of tumor recurrence on 10-year survival, especially after transplantation. Later recurrence was also associated with 10-year survival after liver resection. Tumor size  $\geq 5$  cm was associated with recurrence probably because these patients were ineligible for liver transplantation. Unlike previous reports, our study also included detailed information on comorbidities and risk factors and we also found that alcohol consumption was a predictor of recurrence.

### Limitations

This study was limited in that it was a single center study in a unique and diverse patient population in the Pacific which may limit generalizability. Our population had a large proportion of Asian and Pacific Islanders compared to a typical US study. We also had a high proportion of noncirrhotic HBV-related HCC patients, who were more likely to be candidates for resection. Geographic isolation of the entire state and smaller remote islands may also have limited access to care in different ways than larger states or countries. Finally, we reported all-cause mortality, so it is unclear if the non-10-year survivors died from HCC related issue or another problem.

In conclusion, long-term survivors mostly occur after resection or transplant, but 10% of our cohort survived 10 years with only locoregional therapy. Recurrence of HCC occurred in both 10-year survivors and non-survivors, but later recurrence with aggressive treatment of the recurrence may have allowed for 10-year survival. Finally, long-term survival and recurrence after HCC may be influenced by other comorbidities such as diabetes, smoking and alcohol use which may affect both the tumor and the overall health of the individual but larger studies would be needed to further investigate this.

### DECLARATIONS

#### Authors' contributions

Research design, statistical analysis, manuscript drafting, editing: Sempokuya T

Research supervision, research design, statistical analysis, manuscript drafting and editing: Wong LL

#### Availability of data and materials

Not applicable.

#### Financial support and sponsorship

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#### Conflicts of interest

Dr. Wong is on the speakers bureau for Eisai.

#### Ethical approval and consent to participate

This study was approved by the university of hawaii at manoa institutional review board.

#### Consent for publication

Not applicable.

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Original Article

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# CT of hepatocellular carcinoma in non-alcoholic fatty liver disease: imaging characteristics and inter-rater agreement

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## Abstract

**Aim:** To determine the computed tomography (CT) features of non-alcoholic fatty liver disease (NAFLD) associated hepatocellular carcinoma (HCC).

**Methods:** In this institutional review board approved study, we reviewed 38 patients with NAFLD (68.4% male; mean age 63 years) with histology confirmed HCC and triphasic liver CT. CT images were independently reviewed by four readers blinded to clinical and pathology data. The reviewers assessed HCC for arterial phase hyper enhancement (APHE), portal venous phase washout (PVWO), delayed phase washout (DPWO), and enhancing capsule. Features of cirrhotic morphology and portal hypertension (PH) were also evaluated. The final CT features were determined by majority and a fifth reader reviewed cases lacking majority. Inter-rater agreement was determined by prevalence-adjusted kappa.

**Results:** Mean HCC size was  $3.6 \pm 2.8$  cm (range, 1.1-16.0 cm). The HCCs showed APHE in 92.1%, PVWO in 55.3%, DPWO in 81.6%, and enhancing capsule in 44.7%. Cirrhotic morphology was present in 65.8% and PH in 63.2%. Inter-rater agreement was moderate to almost perfect for APHE (0.74-1.0), cirrhosis (0.79-0.89), and PH (0.79-0.95), weak to perfect for DPWO (0.47-0.95) and poor for PVWO (0-0.42).



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**Conclusion:** NAFLD associated HCC demonstrate less frequent portal venous washout on CT which may affect their imaging diagnosis.

**Keywords:** Hepatocellular carcinoma, computed tomography, fatty liver, inter-rater agreement, non-alcoholic fatty liver disease

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the second most common cause of cancer related death worldwide, with increasing mortality rates in Europe, North America, South America and Africa<sup>[1,2]</sup>. Imaging plays a pivotal role in management of HCC and is an established method for diagnosis with radiological hallmarks on contrast enhanced multiphase computed tomography (CT) or magnetic resonance imaging (MRI). The imaging hallmark features include arterial phase hyper enhancement (APHE), portal venous phase washout (PVWO) and/or delayed phase washout (DPWO), and presence of enhancing capsule. Based on some or all of the three features, several guidelines have been developed for the non-invasive imaging diagnosis and standardization in reporting of observations suspicious for HCC such as European Association for the Study of the Liver (EASL), European Organization for Research and Treatment of Cancer, Organ Procurement and Transplantation Network (OPTN), American Association for the Study of Liver Diseases (AASLD) and Liver Imaging Reporting and Data System. It should be noted that EASL does not recognize capsule as a major imaging feature of HCC. However, these guidelines have only been validated in patients with most commonly recognized risk factors for development of HCC including, alcoholic cirrhosis and chronic viral hepatitis and not in non-alcoholic fatty liver disease (NAFLD)<sup>[3-8]</sup>.

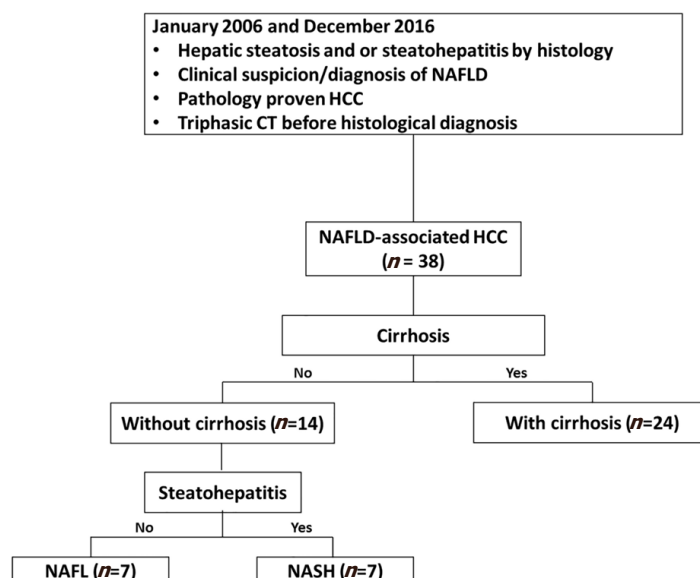
Although most HCCs (75%-90%) develop in cirrhosis resulting from chronic hepatitis B or C infections and alcoholic injury<sup>[9]</sup>, an estimated 4%-22% of HCC occur in the setting of NAFLD<sup>[10-12]</sup>. NAFLD has now become the most common cause chronic liver disease in developed countries<sup>[13,14]</sup>. Given its increasing prevalence worldwide, NAFLD may become the most common chronic liver disease associated with HCC.

NAFLD is a spectrum of disease ranging simple non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH) that can progress to cirrhosis<sup>[15]</sup>. NAFL is considered to have minimal risk of progression to cirrhosis and liver failure, while NASH can progress to cirrhosis, liver failure and develop HCC. NASH is thought to be a common underlying cause of cryptogenic cirrhosis as the patients with cryptogenic cirrhosis are comprised mostly of patients with metabolic risk factors including obesity, metabolic syndrome and diabetes<sup>[15]</sup>, but other etiologies such as burnt-out autoimmune hepatitis and occult alcoholism may also result in cryptogenic cirrhosis<sup>[16-18]</sup>. HCCs are known to occur in patients with NAFLD in the absence of cirrhosis<sup>[10,19-21]</sup> and these HCCs may not meet the imaging criteria based on the current guidelines including LIRADS<sup>[22]</sup>.

The effect of hepatic steatosis in NAFLD on the imaging features of HCC has not yet been fully explored. For example, hepatic steatosis may decrease the liver attenuation on CT and as a result, washout observation may be absent or less conspicuous which could render the LIRADSv2017 imaging criteria not applicable<sup>[22-25]</sup>. HCCs can also occur in the absence of cirrhosis in patients with NAFLD as mentioned earlier. Therefore based on these premises, the purpose of our study was to determine the major imaging features of HCC on multiphase CT and the inter-observer agreement in patients with NAFLD.

## METHODS

In this institutional review board (IRB) approved (ID: 15-004925), HIPPA-compliant retrospective study. Written informed consent for retrospective review of data was waived.



**Figure 1.** Patient selection flow diagram. CT: computed tomography; HCC: hepatocellular carcinoma; NAFLD: non-alcoholic fatty liver disease; NAFL: non-alcoholic fatty liver; NASH: nonalcoholic steatohepatitis

## Patient selection

We reviewed our institutional pathology and imaging database between January 2006 and December 2016 with key words NAFLD, hepatic steatosis or steatohepatitis and HCC or hepatocellular carcinoma. This yielded a cohort of 400 patients. Among these 400 patients, only 38 patients met the AASLD criteria for NAFLD<sup>[15]</sup> and had a triple phasic CT (late arterial, portal venous and delayed phase) before histological confirmation of HCC. Patients with cryptogenic cirrhosis were excluded due to uncertainty of the underlying etiology. Among the final group of 38 patients, 24 had NASH cirrhosis (cirrhosis with current or past evidence of steatosis or steatohepatitis) and 14 patients had no cirrhosis- 7 NASH (hepatic steatosis  $\geq 5\%$  with inflammation  $\pm$  fibrosis) and 7 NAFL (hepatic steatosis  $\geq 5\%$  without evidence of hepatocellular injury or fibrosis). A flowchart detailing the patient selection and subgroups is shown in [Figure 1](#).

Patient age, sex, height, weight, body mass index (BMI), serum cholesterol, serum triglycerides, presence or absence of obesity, tumor histopathological and clinical management information were obtained from electronic medical records.

Cytological and pathological TNM staging was evaluated according to criteria of the 7th American Joint Committee on Cancer<sup>[26]</sup>. The diagnosis of NAFL, NASH was established at pathology. The time interval between CT and surgical pathology was  $137 \pm 387$  days (range 3 to 1802 days). Twenty-four cases had surgical pathology within 6 months of CT. Histology of the HCC and background liver was evaluated by an experienced pathologist (TM) with expertise in NAFLD. HCCs were graded based on WHO classification and NAFLD was graded based on NAS score.

## CT imaging review

All the CT images were independently reviewed on PACS Workstation (Centricity, GE Healthcare, Waukesha, WI) by four board-certified abdominal radiologists (SPS, ECE, AK, CAB) who were blinded to clinical and pathological findings other than the presence of HCC. Each reader recorded the imaging features of HCC, including size, location, APHE, PVWO, DPWO, and presence capsule. Readers also assessed for findings of cirrhosis-surface nodularity, caudate lobe hypertrophy, left lobe enlargement, widened fissures, widened gallbladder fossa, and portal hypertension (PH), splenomegaly, collaterals

and gastroesophageal varices. In patients with multiple HCCs, only the largest HCC with histological confirmation was assessed. The final imaging features of HCCs were determined by majority. A fifth reader blinded to clinical and pathological findings reviewed cases lacking majority. LIRADs criteria is only applicable in patients with cirrhosis or chronic viral hepatitis and therefore would not be applicable for patients with NAFLD without cirrhosis.

### Statistical analysis

Continuous variables were expressed as mean  $\pm$  standard deviation and categorical data as percentage. Inter-rater agreement was determined by prevalence-adjusted bias-adjusted Cohen's kappa<sup>[27]</sup>. Agreement between cirrhotic on CT and pathology was also determined by prevalence-adjusted Cohen's kappa<sup>[27]</sup>. Inter observer agreement was classified as none (0-0.2), minimal (0.21-0.39), weak (0.40-0.59), moderate (0.60-0.79) and strong (0.80-0.90) and almost perfect ( $> 0.90$ ). Differences between non-cirrhotic and cirrhotic subgroups were compared using an unpaired *t*-test with equal variance assumption for continuous data and Fischer's exact test for categorical data. Statistical significance was assumed for *P* values of less than 0.05. Data were analyzed using JMP 11.0 (SAS, Cary, NC) and Prism 5.0 (GraphPad Software, Inc, La Jolla, CA).

## RESULTS

### Clinical and pathological characteristics

Mean age of subjects was 63 years (range 45-79). Patients were predominantly male ( $n = 26$ , 68.4%), diabetic ( $n = 28$ , 73.7%), and obese (BMI  $\geq 30$ ;  $n = 25$ , 65.8%). Mean  $\pm$  SD total serum cholesterol and triglyceride values were  $151.9 \pm 39.7$  (range: 36-229 mg/dL) and  $130.6 \pm 53.5$  (range: 54-237 mg/dL), respectively. Most of the patients underwent liver transplant ( $n = 21$ , 55.3%) or hepatic resection or segmentectomy ( $n = 12$ , 31.6%). Of the 21 patients that underwent liver transplant, 16 patients received chemoembolization (14) or ablation (2) before transplantation, 4 patients received only ablation or chemoembolization, and 1 patient was lost to follow-up. Of 38 patients, 2 (5.3%) patients developed recurrent HCC. CT studies performed before any treatment was used for imaging analysis.

At pathology, HCCs were well differentiated in 14 patients (36.8%), well to moderately differentiated in 5 patients (13.2%), moderately differentiated in 16 patients (42.1%), moderate to poorly differentiated in 2 patients (5.3%) and poorly differentiated in 1 patient (2.6%). Hepatic steatosis was minimal (26.3%) in 10 patients (26.3%), mild in 26 patients (68.4%), and moderate in 2 patients (5.3%). Steatohepatitis was present in 25 patients (65.8%) and cirrhosis in 24 patients (63.2%). The clinical and pathological characteristics are summarized in Table 1.

### HCC and liver parenchyma imaging characteristics - majority features

Mean HCC size was  $3.6 \pm 2.8$  cm (range: 1.1-16.0 cm). APHE was seen in 92.1%, PVWO in 55.3% DPWO in 81.6% and enhancing capsule in 44.7% [Figures 2 and 3]. Fat within the HCC was present in only one patient. Cirrhotic morphology was present in 25 patients (65.8%) and portal hypertension in 24 patients (63.2%). The imaging features are summarized in Table 2.

### Non-cirrhotic NAFLD vs. cirrhotic NAFLD

Non-Cirrhotic NAFLD (NAFL in 7 and NASH in 7) and cirrhotic NAFLD were present in 14 (36.8%) and 24 (63.2%) patients respectively. Patients with non-cirrhotic NAFLD were older ( $P = 0.03$ ), had larger mean HCC size ( $P = 0.008$ ) and higher degree of hepatic steatosis ( $P = 0.003$ ). PVWO feature was observed significantly more in the non-cirrhotic group as compared to the cirrhotic group (78.6% vs. 41.7%,  $P = 0.04$ ). Portal hypertension features were more commonly seen in patients with cirrhotic NAFLD (91.7% vs. 14.3%,  $P < 0.0001$ ). There was no significant difference between the two groups with respect to gender distribution ( $P = 0.47$ ), BMI ( $P = 0.14$ ), presence of diabetes ( $P = 0.45$ ), cholesterol level ( $P = 0.24$ ), triglyceride level

**Table 1. Clinical-pathological characteristics in patients with NAFLD-associated HCC (*n* = 38; main) by non-cirrhotic (*n* = 14) and cirrhotic (*n* = 24) liver**

Clinical and Pathological findings	Main Cohort ( <i>n</i> = 38)	Non-cirrhotic ( <i>n</i> = 14)	Cirrhotic ( <i>n</i> = 24)	<i>P</i> -value
Age (years) (mean ± SD)	63 ± 7.2	66.2 ± 6.3	60.9 ± 7.2	0.03
Gender				0.47
Female	12 (31.6)	3 (21.4)	9 (37.5)	
Male	26 (68.4)	11 (78.6)	15 (62.5)	
BMI (kg/m <sup>2</sup> ) (mean ± SD)	32.2 ± 4.9	30.6 ± 4.6	33.1 ± 5.0	0.14
Diabetes (%)	28 (73.7)	9 (64.3)	19 (79.2)	0.45
Total Cholesterol (mean ± SD)	151.9 ± 39.7	171 ± 36.4	147.4 ± 39.9	0.24
Triglycerides (mean ± SD)	130.6 ± 53.5	129.2 ± 71.1	131 ± 50.7	0.95
HCC Pathology Source				
Biopsy	5 (13.2)	3 (21.4)	2 (8.3)	< 0.001
Surgical Resection	12 (31.6)	10 (71.4)	2 (8.3)	
Liver Explant at Transplant	21 (55.3)	1 (7.1)	20 (83.3)	
Pathologic Features				
Tumor Grade				
Well differentiated	14 (36.8)	4 (29)	10 (41.7)	0.31
Well-Moderately differentiated	5 (13.2)	1 (7.1)	4 (16.7)	
Moderately differentiated	16 (42.1)	9 (64.3)	7 (29.2)	
Moderate-Poorly differentiated	2 (5.3)		2 (8.3)	
Poorly differentiated	1 (2.6)		1 (4.2)	
Hepatic Steatosis Grade				0.003
Minimal	10 (26.3)		10 (41.7)	
Mild	26 (68.4)	12 (85.7)	14 (58.3)	
Moderate	2 (5.3)	2 (14.3)		
Hepatic Fibrosis Stage				
0	9 (23.7)	9 (64.3)		
1	3 (7.9)	3 (21.4)		
2	2 (5.3)	2 (14.3)		
4	24 (63.2)		24 (100)	

*P*-value for non-cirrhotic group vs. cirrhotic group; continuous variables analyzed using unpaired *t*-test with equal variance assumption; categorical data analyzed using Fischer's exact test. BMI: body mass index; NAFLD: non-alcoholic fatty liver disease; HCC: hepatocellular carcinoma

(*P* = 0.95), tumor size (*P* = 0.67), tumor grade (*P* = 0.31), APHE (*P* = 1.00), DPWO (*P* = 1.00) and enhancing capsule (*P* = 0.09).

### HCC and liver parenchyma imaging characteristics - inter-rater agreement

Inter-rater agreement was moderate to almost perfect for HCC APHE (0.74-1.0), none to moderate for PVWO (0-0.42), weak to almost perfect for DPWO (0.47-0.95) [Figure 4] and none to moderate for capsule (0.05-0.79). The inter-rater agreement was moderate to almost perfect for cirrhosis (0.79-0.89) and portal hypertension (0.79-0.95). Pathology and CT agreement for presence of cirrhosis was strong at 0.84. Inter-rater agreement for imaging features is summarized in Table 3.

## DISCUSSION

In our study of NAFLD associated HCC, many HCCs did not demonstrate major imaging features particularly PVWO and enhancing capsule were absent in nearly half and DPWO was absent in nearly 20% of the patients. Cirrhotic morphology was present in 25 (65.8%) patients. A third (36.8%) of HCC occurring in non-cirrhotic livers would not be eligible for LIRADs classification.

Our study results are in agreement with previous reports in literature that HCC can occur even in the absence of steatohepatitis or fibrosis/cirrhosis<sup>[10,19-21,28-34]</sup>. Also HCCs in non-cirrhotic liver were larger in agreement with existing literature that HCCs in non-cirrhotic liver usually present at a later stage and are



**Figure 2.** A 70-year old male with histologically confirmed hepatocellular carcinoma in non-cirrhotic liver with mild steatosis and no steatohepatitis (non-alcoholic fatty liver). Patient was obese (BMI of 30.7), diabetic and dyslipidemic. On multiphase contrast enhanced CT (A-C), a heterogeneously enhancing mass (arrow) on arterial phase (A) with no washout on portal venous (B) and complete washout on delayed phase (C) images can be seen. In addition, a thin but incomplete capsule (arrowhead) can be seen on delayed phase image (C). No features of portal hypertension were seen on imaging



**Figure 3.** A 73-year old male with histologically confirmed hepatocellular carcinoma in non-cirrhotic liver with severe steatohepatitis (non-alcoholic steatohepatitis). Patient was obese (BMI of 31.1) and diabetic. On multiphase contrast enhanced CT (A-C), a heterogeneously enhancing mass (arrow) on arterial phase (A) showing no washout on portal venous (B) and delayed phase (C) images can be seen

**Table 2. Imaging features of HCC and liver parenchyma at CT by majority consensus in patients with NAFLD-associated HCC ( $n = 38$ ) by non-cirrhotic ( $n = 14$ ) and cirrhotic ( $n = 24$ ) liver morphology**

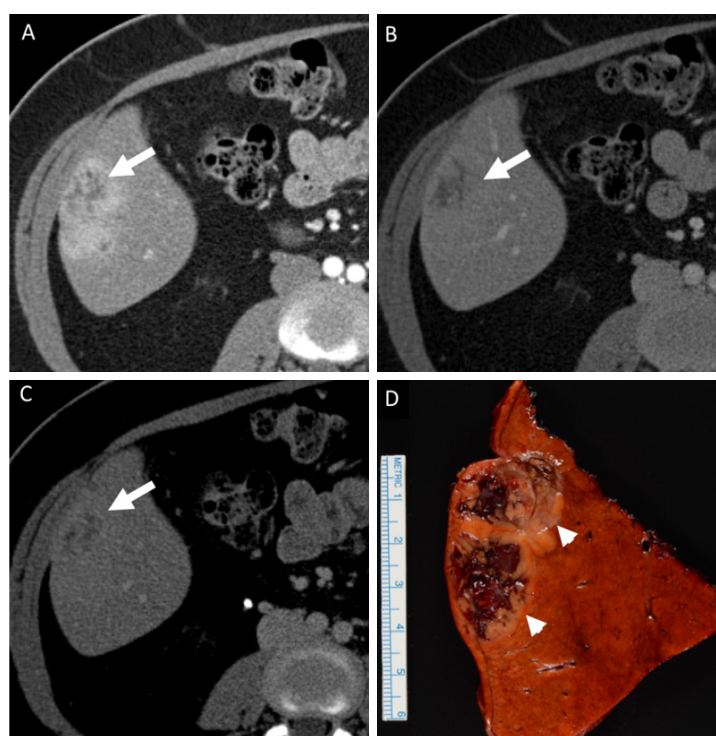
HCC imaging features	Main cohort ( $n = 48$ )	Non-cirrhotic pathology ( $n = 14$ )	Cirrhotic pathology ( $n = 24$ )	P-value
Tumor size (mean $\pm$ SD)	3.6 $\pm$ 2.8	5.1 $\pm$ 3.9	2.7 $\pm$ 1.3	0.008
APHE	35 (92.1)	13 (92.8)	22 (91.7)	1.00
PVWO	21 (55.3)	11 (78.6)	10 (41.7)	0.04
DPWO	31 (81.6)	12 (85.7)	19 (79.2)	1.00
Enhancing "Capsule"	17 (44.7)	9 (64.3)	8 (33.3)	0.09
Cirrhotic Liver Morphology	25 (65.8)	2 (14.3)	23 (95.8)	< 0.001
Portal hypertension	24 (63.2)	2 (14.3)	22 (91.7)	< 0.001
LIRADS Score*				0.29
2	3 (7.9)	0	3 (12.5)	
3	0	0	0	
4	11 (28.9)	3 (21.4)	8 (33.3)	
5	24 (63.2)	11 (78.6)	13 (54.2)	

\* LIRADS score applied to see if HCCs would meet the criteria, however if there is no cirrhosis, LIRADS criteria should not be applied as per guidelines. APHE: arterial phase hyperenhancement; PVWO: portal venous washout; DPWO: delayed phase washout

larger in size at presentation<sup>[20]</sup>. This may be due to lack of screening guidelines in non-cirrhotic patients with NAFLD<sup>[3,28]</sup>. Larger tumor size seen in non-cirrhotic livers at presentation may make them ineligible for transplant based on Milan criteria for transplantation<sup>[35,36]</sup>.

Imaging features of HCC on multiphase CT and MRI are based on sequential changes in the intra-lesional blood supply during hepatocarcinogenesis. Advanced HCCs receive their blood supply predominantly





**Figure 4.** A 47-year old female with histologically confirmed hepatocellular carcinoma (HCC) in non-cirrhotic liver with minimal steatosis and moderate steatohepatitis (non-alcoholic steatohepatitis). Patient was obese (BMI of 30.5) and diabetic. On multiphase contrast enhanced CT (A-C), three of the four readers interpreted this as a heterogeneously enhancing mass (arrow) on arterial phase (A) with no washout on portal venous (B) and delayed phase (C) images. However 1of the four readers interpreted this as a heterogeneously enhancing mass (arrow) on arterial phase (A) with washout on portal venous (B) and delayed phase (C) images. A resected specimen (D) showing HCC (arrow) with background fatty liver can be seen

**Table 3. Inter-observer agreement for HCC features and liver parenchyma morphology at MRI in patients with NAFLD-associated HCC ( $n = 38$ )**

HCC imaging features	R1 vs. R2	R1 vs. R3	R1 vs. R4	R2 vs. R3	R2 vs. R4	R3 vs. R4
APHE	0.74	0.79	0.79	0.74	0.84	1.0
PVWO	0.42	0.00	0.53	0.05	0.32	0.05
DPWO	0.95	0.84	0.74	0.79	0.68	0.47
Enhancing "Capsule"	0.47	0.05	0.42	0.37	0.79	0.37
Cirrhotic Liver Morphology	0.79	0.89	0.89	0.79	0.79	0.89
Portal hypertension	0.74	0.79	0.95	0.84	0.79	0.84

Data are presented as prevalence-adjusted bias-adjusted kappa. R1: reader 1; R2: reader 2; R3: reader 3; R4: reader 4; APHE: arterial phase hyperenhancement; PVWO: portal venous phase washout; DPWO: delayed phase washout

from the anomalous arteries (arterial blood supply)<sup>[37,38]</sup>. This results in high arterial flow which manifests as APHE on dynamic multiphase imaging followed by washout (de-enhancement of a HCC, greater enhancement of the surrounding liver, or a combination of both factors) on portal venous and/or delayed phase imaging<sup>[39-43]</sup>. The washout is attributed to diminished portal venous blood supply of the HCC, high tumoral cellularity with associated small extracellular volume, and expanded extracellular space of the surrounding fibrotic liver<sup>[37,38,43,44]</sup>.

Effect of NAFLD on imaging features on HCC is currently being explored. In our study APHE was present in most (92.1%) of the cases. APHE is a major imaging criterion and has a good sensitivity for detection of HCC, ranging from 65%-96%<sup>[39,43,45]</sup>. The lower sensitivity can be seen in early/well differentiated HCC with partial neovascularization. APHE is more sensitive than other enhancement features but lacks specificity for



HCC. The specificity can be increased by combined assessment of APHE & washout (PVWO or DPWO)<sup>[39,46]</sup>. Sensitivity and specificity of combined APHE & washout for diagnosis of HCC ranges from 43% to 98%, and 81% to 100%, respectively<sup>[43,46-48]</sup>.

For assessment of washout, either portal venous or delayed phase imaging can be used. In this study, PVWO was absent in 17 (44.7%) of the HCCs. In contrast, DPWO was absent in only 7 (18.4%) HCCs. In addition, the interobserver agreement was none to moderate for PVWO and weak to almost perfect for DPWO. These results support the added value of delayed phase imaging in assessment of washout. Triple phase CT is the standard protocol for HCC in chronic liver disease, however some centers may not include the delayed phase to reduce the radiation dose<sup>[4]</sup>. However, this may lead to loss of important diagnostic information (washout characteristics). In a prior study with NAFLD associated HCC, PVWO was absent in 30% cases but DPWO was not reported<sup>[49]</sup>. Ehman *et al.*<sup>[45]</sup> reported lack of washout (portal or delayed) in only 18% (15% on CT and 21% on MRI) of the 184 pathologically proven HCCs with cirrhosis resulting from several different etiologies<sup>[45]</sup>. Washout in NAFLD associated HCC may not be demonstrated well probably due to hepatic steatosis that can lead to liver hypo-attenuation on post-contrast enhanced images, thereby resulting in the appearance of persistent HCC hyper-attenuation or iso-attenuation during the portal venous and delayed phase imaging. Interestingly however in our study PVWO was more commonly seen in subgroup with non-cirrhotic NAFLD, which had higher proportion of cases with mild moderate steatosis in comparison to cirrhotic NAFLD subgroup. These findings suggest that degree of hepatic steatosis may have more determining effect than presence of fibrosis for washout appearance. Future studies with larger number of patients are required to confirm our findings. The findings should be confirmed in studies with larger population of NAFLD associated HCCs.

Capsule appearance is a highly specific but not very sensitive feature of HCC<sup>[43,45,50]</sup>. Capsule is thought to be caused by expansile growth of HCC causing perilesional compression of liver tissue, which appears as enhancing rim around the HCC in portal venous or delayed phase. Capsule is supplied by the portal venous system leading to this delayed enhancement<sup>[51]</sup>. Interestingly, capsule is a recognized major feature of HCC based on the LIRADS, OPTN guidelines but not on the AASLD and EASL guidelines<sup>[7,8]</sup>. In our study, capsule appearance was seen in only 17 (44.7%) of the NAFLD-associated-HCCs but still within the reported range of 27 to 64% in studies including all chronic liver diseases<sup>[43,45]</sup>.

Inter-rater agreement was poor for PVWO, variable for DPWO and capsule suggesting the difficulty in interpretation of washout and detection of capsule in NAFLD associated HCCs [Figure 4]. We had four experienced abdominal radiologists working in tertiary level institute with large exposure to HCCs on a daily basis. Even with this level of expertise, the interobserver agreement for PVWO was poor. We think that this is due to the variable hepatic steatosis in background liver that affects interpretation of the washout particularly PVWO.

The study has some limitations. Despite searching a large patient database containing around 2,500 patients (pathological data for HCC), our final study cohort was relatively small. Due to retrospective nature of the study, the imaging (triphasic CT) technique including scanner, sequence protocol(s) and contrast agent(s) was not uniform over the study period and this may have introduced variability in the phase of image acquisition. Some cases of NAFLD associated HCCs may have been excluded that either lacked pathological assessment or were interpreted as cryptogenic cirrhosis. The readers were aware of the diagnosis of HCC in the lesions which may have introduced bias for imaging features. However this bias of prior knowledge did not inflate interobserver agreement for the imaging features. Degree of hepatic steatosis may have changed from time between histological analysis and radiological assessment, due to systemic interventions or natural progression of NAFLD. This was unavoidable as several patients received locoregional treatment before undergoing surgery or liver transplantation. These treatments may also have

contributed to the changes in the liver parenchyma. However we minimized this variation as most of the studies had histological evaluation within 6 months of CT study.

In conclusion, NAFLD associated HCC may not show portal venous phase washout on CT and may impact the imaging diagnosis of HCC. Our study should be confirmed in studies with larger population of NAFLD associated HCCs. There may be a need for modification of criteria for multiphase CT based diagnosis of NAFLD associated HCCs particularly in the non-cirrhotic patients.

## DECLARATIONS

### Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Garg I, Thompson SM, Mounajjed T, Ehman EC, Venkatesh SK

Performed data acquisition, as well as provided administrative, technical, and material support: Garg I, Thompson SM, Sheedy SP, Mounajjed T, Khandelwal A, Ehman EC, Bookwalter CA, Venkatesh SK

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

None.

### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

In this IRB approved (ID: 15-004925), HIPPA-compliant retrospective study. Written informed consent waived by the IRB.

### Consent for publication

Not applicable.

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Review

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# Hepatocellular carcinoma in alcoholic liver disease: mechanistic considerations and clinical facts

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## Abstract

Alcoholic hepatocellular carcinoma (AHCC) represents a lethal stage, emerging in the course of severe injurious stages of alcoholic liver disease including cirrhosis. AHCC only affects a few alcohol consumers, certainly not all individuals who consume large amounts of alcohol over a long period of time, suggesting a role of yet unknown genetic risk or protection factors. Most likely, hepatic DNA is ultimately involved, attacked by intermediate products derived from reactive oxygen species (ROS) generated from cytochrome P450 2E1 of the NADPH and oxygen dependent microsomal ethanol-oxidizing system whereby ethanol is metabolized. Ethanol and acetaldehyde are activated to procarcinogens, to be promoted to ultimate carcinogens by ROS and causatives for AHCC instead of any other putative chemical contained in alcoholic beverages. Prevention of HCC associated with cirrhosis is best accomplished by early recognition of alcohol abuse at the stage of alcoholic fatty liver rather than alcoholic hepatitis (AH) or alcoholic steatohepatitis (ASH), leading to the advice of consequent abstinence from alcohol. Abstinence early started effectively prevents AHCC development, as opposed to late begin of abstinence that lacks risk reduction. Although drug therapy may partially be effective in AH or ASH, no established drug options are available for a realistic therapy of AHCC. Liver transplantation is controversially discussed and can be considered, but may be an option for only a few patients on a case by case base. In conclusion, AHCC results from a ROS dependent conversion of ethanol and acetaldehyde to procarcinogens as promoters of AHCC.

**Keywords:** Alcohol, alcoholic liver disease, alcoholic cirrhosis, alcoholic hepatocellular carcinoma, microsomal ethanol-oxidizing system, cytochrome P450 2E1, reactive oxygen species, carcinogens



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## INTRODUCTION

The WHO considers cancer as the second leading cause of death globally, responsible for an estimated 9.6 million deaths in 2018, attributing about 1 in 6 deaths to cancer<sup>[1]</sup>. More specifically, the most common causes of cancer death are cancers of lung (1.76 million deaths), colon including rectum (862,000 deaths), stomach (783,000 deaths), liver (782,000 deaths), and breast (627,000 deaths), whereby around one third of deaths from cancer can be traced back to the 5 leading behavioral and dietary risks: high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, and alcohol use<sup>[1]</sup>. Consequently, identifying the cause of liver cancer or more specifically primary hepatocellular carcinoma (HCC) in a patient with this type of cancer can be challenging and is variable among different countries or regions<sup>[2-7]</sup>.

This article discusses current aspects of primary alcoholic hepatocellular carcinoma (AHCC) with focus on potential mechanistic steps and risk factors closely related to alcohol metabolism, reactive oxygen species (ROS), and the gut-liver axis in the context of the intestinal microbiome. In addition, clinical aspects with diagnostic approaches and therapy options including the controversially disputed liver transplantation will be considered.

## LITERATURE SEARCH AND SOURCE

The PubMed database was used to identify publications for the following terms: alcohol, ethanol, DNA, ROS, alcoholic liver injury, and alcoholic hepatocellular carcinoma. Terms were used alone or combined. Limited to the English language, publications of the first 50 hits from each searched segment were analyzed for suitability of this review article. Publications were also derived from the large private archive. The search for publications was completed on 4 October 2019. The final compilation consisted of original papers, consensus reports, and review articles. The most relevant publications were included in the reference list of this review.

## ALCOHOL AS CHEMICAL

Ethanol is a short length chemical and a synonym to the term alcohol that is commonly used in a clinical setting. In the human gastrointestinal tract, alcohol can be produced from sugar with the help of intestinal bacteria and commonly undergoes rapid degradation by ubiquitous enzymes, for which alcohol serves as natural endogenous substrate keeping the respective enzymes active at low levels and prepared for larger amounts of exogenous alcohol eventually consumed as alcoholic beverage. Endogenous ethanol may be of clinical interest as “auto-brewery syndrome”, a drunk-driving defence challenge<sup>[8-10]</sup>, with details provided in a case control study<sup>[10]</sup>.

Clearly, alcohol is otherwise a product found in beverages such as wine prepared from the sugar containing grapes with the help of baker's yeast alcohol dehydrogenase (ADH)<sup>[11,12]</sup>. Interestingly, a different ADH type is also present in humans and partly involved when the alcohol consumed as beverage is oxidized in the human gastrointestinal tract<sup>[13]</sup> and the liver<sup>[8,14,15]</sup>.

## ALCOHOL AND ACETALDEHYDE OXIDATION IN THE LIVER

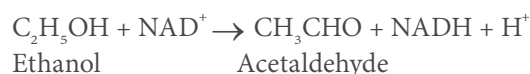
After drinking of alcoholic beverages, alcohol is quickly taken up by the mucosa of the upper gastrointestinal tract<sup>[16]</sup>, as evidenced by the prompt appearance in the blood with higher blood alcohol levels if the alcoholic beverage was consumed before a meal as long as the stomach is empty, as compared to lower blood alcohol levels found if the alcohol is consumed during or right after a meal<sup>[17]</sup>. As an exogenous compound lacking an option of storage within any organ, alcohol must be removed for which several possibilities exist<sup>[16]</sup>. Whereas only small amounts of the ingested alcohol leave the body by exhalation or with the urine, most of the alcohol must undergo enzymatic degradation in the liver<sup>[4,5,8,14-18]</sup>. The human



liver is prepared to oxidize exogenous and endogenous alcohol using two different enzymes: ADH<sup>[5,8,13-17]</sup> and microsomal ethanol-oxidizing system (MEOS)<sup>[8,14-16,18-21]</sup>. Through both reactions, acetaldehyde is produced and its oxidation proceeds via the mitochondrial ALDH<sup>[8,15,16,18]</sup>, requiring NAD<sup>+</sup> as cofactor but it can also be metabolized in the endoplasmic reticulum of the liver<sup>[15]</sup>.

## ADH

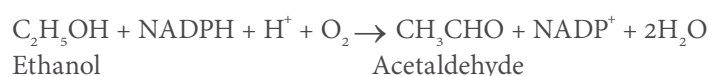
Hepatic ADH is present in the cytosol of human liver cells and metabolizes ethanol according to the following equation:



Additional information of ADH and its role in alcohol metabolism is provided in various other reports<sup>[5,8,13-17]</sup>. Evidence is lacking that in the course of this reaction reactive radicals are generated.

## MEOS

MEOS is a constituent of the endoplasmic reticulum membranes in the human liver that correspond to the microsomal fraction following ultracentrifugation of the liver homogenate<sup>[8,14-16,18]</sup>. The oxidation of ethanol precedes via the following equation:



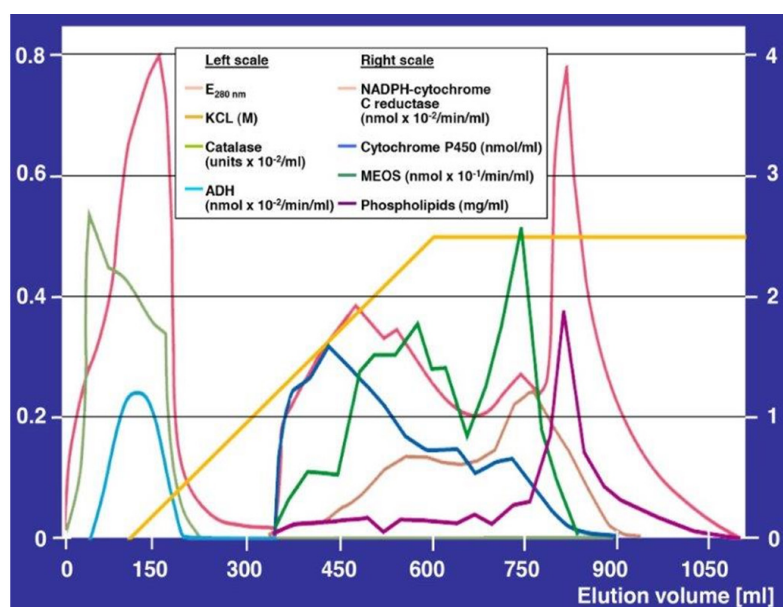
MEOS is different from ADH as well as catalase<sup>[19,20]</sup> and was isolated from these two enzymes using column chromatography<sup>[21-24]</sup>, providing a typical elution pattern [Figure 1]<sup>[21]</sup>.

## MECHANISTIC CONSEQUENCES OF ALCOHOL METABOLISM RELATED TO CARCINOGENICITY

Alcohol has a direct contact to the mucosal cells of the gastrointestinal tract with its MEOS and cytochrome P450 2E1 (CYP 2E1), and generated ROS may increase leakage of endotoxins, chemically known as lipopolysaccharides (LPS), out of the intestinal tract directed to the liver<sup>[8,15,16,25]</sup>, modifying also the intestinal microbiome and the gut-liver axis. There is experimental and clinical evidence that Toll-like receptor 4 (TLR4) could be involved in AHCC development via signaling activation of LPS-TLR4<sup>[5,7]</sup>, whereby coexistence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection could represent a risk factor<sup>[7]</sup>.

Overall, alcohol dependent inflammation of the liver is responsible for non-malignant stages of alcoholic liver disease (ALD) but is a contributory factor of AHCC initiation through hepatocellular DNA damage<sup>[5]</sup>. Specific liver macrophages are involved in AHCC development triggered by activated hepatic Stellate cells (HSCs)<sup>[5]</sup>, which are also known as promoters of liver alcoholic fibrosis<sup>[8,15]</sup>. Ectopic expression of TLR4 in hepatocytes and its activation by LPS triggers AHCC through production of TLR4 and tumor-initiating stem-cell like cells<sup>[5]</sup>. It has been reported that activated HSCs may also promote AHCC development via matrix or soluble factors that help tumor cell survival and growth<sup>[5]</sup>.

Most importantly, ROS can bind to and damage DNA and cause lipid peroxidation with the generation of highly carcinogenic exocyclic etheno-DNA adducts<sup>[5]</sup>. Other DNA adducts of interest include N<sup>2</sup>-ethyl-deoxyguanosine and N<sup>2</sup>-propano-2'-deoxyguanosine, which may modify the DNA integrity, whereas acetaldehyde protein adducts may impair the DNA repair system<sup>[7]</sup>. Consequently, the direct



**Figure 1.** Purification of the MEOS and its separation from catalase and ADH activities. Separation was achieved by DEAE cellulose ion exchange column chromatography after solubilization of liver microsomes. In the void volume eluted up to around 220 mL, the highest peak represents the protein curve assessed as E280 nm, and the peak below that is the catalase peak, whereas ADH presents as the lowest peak. Starting with an elution volume of around 330 mL, microsomal components begin to appear. The first peak represents cytochrome P450, the second peak represents E280 nm, followed by a third peak with two shoulders and by a fourth peak representing MEOS. At around 770 mL, the reductase peak emerges, followed by the phospholipid peak at around 790 mL elution volume. Adapted from the original figure published in a previous report<sup>[21]</sup>. MEOS: microsomal ethanol-oxidizing system; ADH: alcohol dehydrogenase; DEAE: diethyl-amino-ethyl

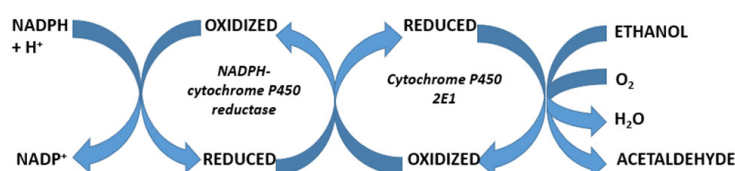
DNA mutagenic effect of acetaldehyde and the indirect carcinogenic effect of adducts may modulate carcinogenesis leading to AHCC<sup>[5,6,26,27]</sup>. Other considerations focus on DNA methylation that modifies the expression of genes: hypomethylation is related to enhanced gene expression, considered as an import factor of cancer development<sup>[6]</sup>. Prolonged alcohol use also causes a reduction of S-adenosylmethionine in the liver, the methyl donor of DNA<sup>[6]</sup>. Reviewing the relevant literature with inclusion of those referenced<sup>[5-7]</sup>, it is obvious that many mechanism have the potential of causing AHCC. In face of multiple proposals and study data, and as expected, not all results are confirmative but even contradictory, not allowing a valid uniform proposal how AHCC develops in the human liver during prolonged alcohol abuse.

### Principles of hepatic carcinogenesis

There is general believe that carcinogenesis of various organs including the liver is triggered by an enhancement of oncogene expression or due to an impairment of cells to improve their DNA quality leading to inappropriate DNA repair associated with DNA mutations, conditions that facilitate oncogenic mutations<sup>[5,6]</sup>. Conditions in the human and experimental setting of AHCC are complex due to a broad range of variabilities. Although these variable DNA related modifications had been observed mostly in animal models or human tissues, respective translation of these meaningful data to humans with AHCC is warranted. The variability of proposed molecules damaging DNA as a result of alcohol consumption and metabolism requires a closer look with focus on alcohol metabolism and more specifically electrophile metabolites, in addition to the concomitant production of ROS to be discussed in general.

### Microsomal components of MEOS

ROS production is closely connected with the degradation of ethanol via MEOS, which is not a single enzyme but represents a system composed of the three microsomal components CYP 2E1, the reductase,



**Figure 2.** Constituents of the microsomal ethanol-oxidizing system

**Table 1. MEOS and its cytochrome P450 isoforms**

Cytochrome P450 isoforms	MEOS activity/cytochrome P450
1A2	10.90
2A6	3.75
2B6	2.89
2D6	0.70
2E1	11.51
A4	3.38

To assess the turnover number, MEOS activity as nmoles acetaldehyde/min is calculated per nmoles cytochrome P450, all expressed per mg of microsomal protein. Compilation achieved by data extraction from a report of Asai *et al.*<sup>[32]</sup>

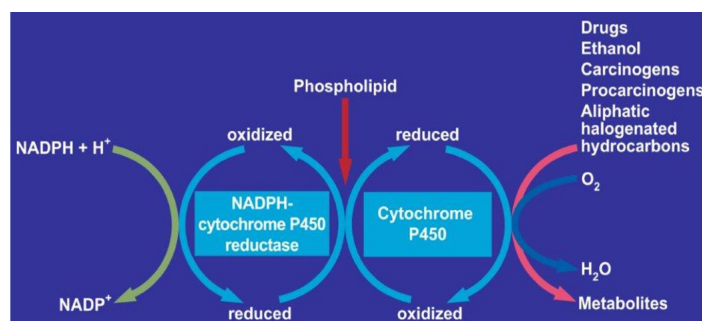
and phospholipids without their yet clearly determined place of interaction [Figure 2], as early recognized during isolation of MEOS [Figure 1]<sup>[21]</sup>, with ROS production and ethanol oxidation carried out by the three components<sup>[8,15,16,18]</sup>. Separated from ADH and catalase activities shown on the left side of the elution pattern, MEOS activity was recovered in eluates on the right side containing all three microsomal constituents: cytochrome P450, NADPH-cytochrome c reductase (better to be analyzed as compared to NADPH cytochrome P450 reductase), and microsomal phospholipids<sup>[21]</sup>.

Using our published elution procedure of a stepwise KCl gradient<sup>[22]</sup>, a subsequent report reaffirmed the validity and reproducibility of our initial results in all details<sup>[28]</sup>, as published before<sup>[22]</sup>. This reassured our proposed participation of special microsomal enzymes and constituents in MEOS and its independence from ADH and catalase. The participation and obligatory role of these three components for MEOS was later verified using purified microsomal constituents in order to reconstitute MEOS<sup>[29,30]</sup>, in support by other studies showing the direct oxidation of ethanol by a catalase-free and alcohol dehydrogenase-free reconstituted system containing cytochrome P-450<sup>[31]</sup>. Therefore, a close association between ROS and MEOS exists, to be further discussed in relation to carcinogenicity and AHCC.

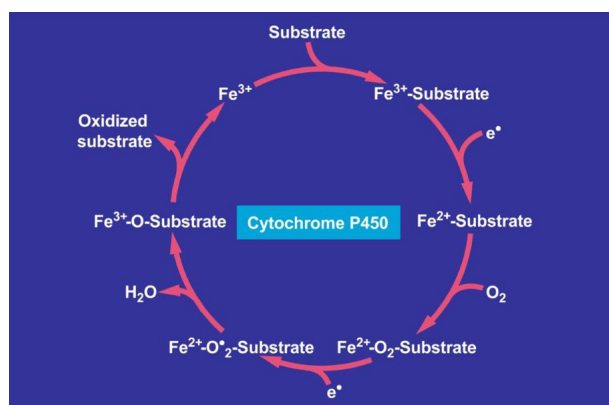
### Reactive oxygen species

New sophisticated analytical techniques were developed that helped characterize MEOS, CYP isoforms with preference of microsomal CYP 2E1, and ROS<sup>[4,8,15,16,18,32-36]</sup>. One of the highlights was the observation in humans that MEOS consists of several CYP isoforms with CYP 2E1 as the most active in microsomal ethanol oxidation<sup>[32]</sup>, with variable turnover numbers of MEOS activity if calculated per CYP isoform content [Table 1]<sup>[32]</sup>.

With focus on the hepatic microsomal CYP 2E1, a variety of phenomena can now be explained, as illustrated with a few examples<sup>[8,15,16,19-21,28,32-39]</sup>: (1) prolonged alcohol consumption upregulates the NADPH and oxygen dependent MEOS activity<sup>[19-21,37,38]</sup> that adaptively enhances the overall metabolism of ethanol<sup>[36,37]</sup> through an increase of CYP 2E1<sup>[29,33]</sup>; (2) in the presence of NADPH and molecular oxygen, CYP 2E1 metabolizes not only ethanol in the context of MEOS but also many other exogenous substrates with metabolic competitive interactions at the level of CYP [Figure 3]<sup>[16]</sup>; (3) during CYP 2E1 dependent reactions, oxygen split often is incomplete [Figure 4]<sup>[16]</sup>, allowing the generation of various types of ROS and



**Figure 3.** Microsomal metabolism of various chemicals including ethanol. At the level of cytochrome P450, metabolic drug-drug and drug-ethanol interactions are feasible. Figure derived from a previous report<sup>[15]</sup>



**Figure 4.** Hepatic microsomal cytochrome P450 and its interaction with substrates. Cytochrome P450 catalyzes the oxidation of substrates such as drugs and ethanol, which bind to the ferric (3<sup>+</sup>) iron of the cytochrome P450 as the initial metabolic step leading finally to the oxidized substrate. The original figure was published in and derived from a recent article<sup>[15]</sup>

**Table 2. Potentially toxic metabolites resulting from enzymatic degradation of ethanol in the liver**

Selected potentially toxic metabolites and reactive O <sub>2</sub> -species due to hepatic ethanol degradation
Acetaldehyde C <sub>2</sub> H <sub>4</sub> O
Ethoxy radical CH <sub>3</sub> CH <sub>2</sub> O <sup>•</sup>
Hydroxyethyl radical CH <sub>3</sub> C(•)HOH
Acetyl radical CH <sub>3</sub> CHO <sup>•</sup>
Singlet radical <sup>1</sup> O <sub>2</sub>
Superoxide radical HO <sub>2</sub> <sup>•</sup>
Hydrogen peroxide H <sub>2</sub> O <sub>2</sub>
Hydroxyl radical HO <sup>•</sup>
Alkoxy radical RO <sup>•</sup>
Peroxy radical ROO <sup>•</sup>
Lipidperoxides

Derived from original reports and review articles as referenced in previous reports<sup>[15]</sup>

ROS dependent products [Table 2]<sup>[16]</sup>; (4) part of the ROS will be used to metabolize ethanol or acetaldehyde to reactive intermediates, whereby initially ethanol and acetaldehyde per se are not carcinogens but can be classified as procarcinogens that are converted to ultimate carcinogens with the potential of inducing AHCC by attacking DNA<sup>[39]</sup>; and (5) in addition, parts of ROS not used for MEOS would be freely available for direct attack of DNA and could also trigger radical formation of soluble proteins and or phospholipids or those located in structural membranes of liver cells organelles including mitochondria<sup>[8,15,16,18,33-36,39]</sup>.

### Putative procarcinogens and ultimate carcinogens

As a reminder, CYP 2E1 metabolizes not only alcohols but also a bundle of other substrates, some of these are carcinogens or procarcinogens [Table 3], with references for each listed chemical provided in a previous publication<sup>[16]</sup>. Abundant clinical and experimental publications relate to alcohol and liver cancer but further specification is often missing and major questions remain: (1) are alcoholic beverages globally carcinogenic or only specific ingredients like contaminating carcinogens? or (2) is the chemical ethanol per se carcinogenic or is it merely a procarcinogen? or (3) is acetaldehyde as the first metabolite in its original form and per se carcinogenic or is metabolic activation to a reactive intermediate prerequisite to be classified as procarcinogen? or (4) can ROS be considered as carcinogen either alone or perhaps together with an activated membrane protein or a membrane phospholipid that can easily be converted to a reactive lipidperoxide, considering that ROS alone in the absence of alcohol may play a role for HCC in nonalcoholic steatohepatitis (NASH).

Officially, alcohol is now classified as a human carcinogen rather than as a clear procarcinogen by the International Agency for Research in Cancer in a global context<sup>[5,39-41]</sup>, disregarding the known complexity of metabolic events leading to a variability of compounds as outlined above.

### Cocarcinogens

Humans with an alcohol problem are often heavy smokers<sup>[42]</sup> and confronted with potential mutagenic and carcinogenic compounds in the tobacco smoke may enter the body and act as cocarcinogens<sup>[43]</sup>. On theoretical backgrounds but difficult to evaluate in patients, mutagenic and carcinogenic compounds may contaminate alcoholic beverages and could function upon ingestion as cocarcinogens, contributing to initiation and promotion of AHCC<sup>[41]</sup>. However, in the majority of alcoholic beverages mutagenic and carcinogenic chemicals are not found, but if detected, respective amounts are very small and unlikely causing harm the liver. In experimental studies, tumor incidence in the liver caused by dimethylnitrosamine (DMN) is influenced by prolonged alcohol use as evidenced by a specific study protocol<sup>[44]</sup>: rats were pair-fed for 3 weeks a nutritionally adequate liquid diet containing either ethanol (36% of total calories) or isocalorically substituted carbohydrates as control diet. Thereafter, the animals were maintained on laboratory chow and tap water ad libitum for another 2 weeks and received 1.5 mg DMN intraperitoneally per day for the first 5 days. This 5-week cycle was repeated three more times. Chronic pretreatment with the alcohol-containing diet significantly improved the mean survival time of DMN-treated rats compared with identically treated animals fed before with the control diet, but the total number of tumors observed under these experimental conditions and the target organ remained virtually unchanged<sup>[44]</sup>. The partially positive and protective effect on survival in DMN treated animals elicited by prolonged alcohol consumption was unexpected, as was the reduced liver injury by DMN following prolonged pretreatment with the alcohol containing diet<sup>[45,46]</sup>.

### CASCADES OF EVENTS: FROM LIVER INJURY AND ALCOHOLIC FATTY LIVER TO AHCC

The spectrum of ALD is broad and well described in various reports provided by experts in the field<sup>[2,5-8,15-17,47-59]</sup>, starting with the most frequent stage of alcoholic fatty liver (AFL)<sup>[47-49]</sup>, and the transition to alcoholic steatohepatitis (ASH) and alcoholic hepatitis (AH), both of which follow different criteria<sup>[50-53]</sup>, with alcoholic cirrhosis (AC), the most known end stage of ALD<sup>[54-57]</sup> and often precursor of AHCC<sup>[2,5-8,58,59]</sup>. Delineating a 5-hit proposal has the advantage of a clear structure and better overview and has been published previously [Figure 5]<sup>[8]</sup>.

All 5-hit stages are well defined by both, clinical and histology evaluations, but some overlap among the stages is unavoidable. Pathogenic details of all ALD stages have been published in a recent review article<sup>[8]</sup>, some additional details are presented for AFL, ASH, AH, AC and AHCC for a brief overview [Table 4].

**Table 3. Selected substrates of the hepatic microsomal CYP 2E1****Selected substrates catalyzed by CYP 2E1**

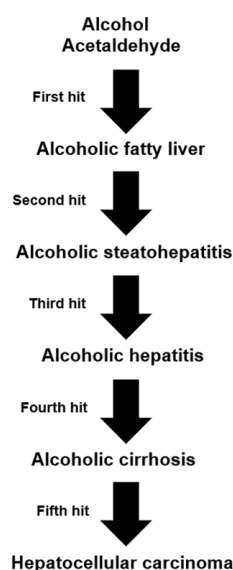

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Acetaldehyde  
 Acetol  
 Acetone  
 Acetaminophen  
 Aniline  
 Benzene  
 Bromobenzene  
 n-Butanol  
 Caffeine  
 Carbon tetrachloride  
 Chloroform  
 1-Chloropropane  
 Chlorzoxazone  
 1,1-Dichloroethane  
 1,2-Dichloroethane  
 1,1-Dichloroethylene  
 cis-1,2-Dichloroethylene  
 trans-1,2-Dichloroethylene  
 Dichloromethane  
 1,2-Dichloropropane  
 1,2-Dibromoethane  
 Diethylether  
 Dimethylformamide  
 Cumene  
 Enflurane  
 Ethanol  
 Ethylbenzene  
 Halothane  
 n-Hexane  
 Isoflurane  
 Methanol  
 Methyl t-butyl ether  
 Methoxyflurane  
 Monochlorobenzene  
 4-Nitrophenol  
 p-Nitrophenol  
 Nitrosamines  
 N-nitrosodimethylamine  
 n-Pentane  
 Phenol  
 n-Propanol  
 Propylbenzene  
 Sevoflurane  
 Styrene  
 1,1,1,2-Tetrachloroethane  
 1,1,2,2-Tetrachloroethane  
 Tetrachloroethylene  
 Toluol  
 Trichloroethylene  
 1,1,1-Trichloroethane  
 1,1,2-Trichloroethane  
 Vinylchloride  
 o,m,p-Xylene

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Listed chemicals are preferred substrates of CYP 2E1. Data are derived from a previous report that provides exact references for each listed substance<sup>[16]</sup>. CYP 2E1: cytochrome P450 2E1





**Figure 5.** The 5-hit working hypothesis in alcoholic liver disease. The 5-hit hypothesis presents various possible steps leading from alcoholic fatty liver, eventually to hepatocellular carcinoma. In clinical practice, some patients with alcoholic hepatitis do not have steatosis/steatohepatitis as a precursor, with additional details provided in Table 4. The original figure was published in an earlier report<sup>[15]</sup>

**Table 4. Alcoholic liver disease and the 5-hit working hypothesis with a tentative cascade of events**

First hit	The first hit is dependent on ADH and occurs at low alcohol levels through the generation not only of NADH + H <sup>+</sup> leading to an increased NADH + H <sup>+</sup> /NAD <sup>+</sup> ratio, which stimulates hepatic fatty acid synthesis <sup>[22]</sup> and increases $\alpha$ -glycerophosphate-trapping fatty acids, but also of acetaldehyde, which impairs hepatic mitochondrial functions including hepatic mitochondrial fatty acid oxidation. This first hit fully explains at least in part the development of alcoholic fatty liver.
Second hit	The second hit is classified as a transition from alcoholic fatty liver to alcoholic steatohepatitis, most likely triggered by the increased production of acetaldehyde via MEOS, and of ROS with its capacity for irreversible covalently binding to cellular macromolecules, including membrane proteins and phospholipids. These injurious alterations at the molecular and cellular level cause some necrosis, apoptosis, and inflammatory cells in the fatty liver, justifying the term alcoholic steatohepatitis, as it includes toxic hepatitis in steatosis. Further stages are characterized by perisinusoidal and pericentral fibrosis due to participation of non-hepatocytes such as Kupffer cells, stellate cells, and sinus endothelial cells. Mediators such growth factors, interferons, interleukins, tumor necrosis factor and endotoxins, as well as hepatic iron, are considered as possible active promoters of liver injury, but considering the multiplicity of proposed mediators, it is difficult to predict how they interact with each other and modify the course of liver injury.
Third hit	The third hit initiates a more severe liver injury stage, whereby alcoholic steatohepatitis is the precursor in most, but certainly not all patients with alcoholic hepatitis. Steatosis is no more a characteristic feature, but is now replaced by necrosis, apoptosis, and inflammation. At this stage, injury becomes more severe and presents with more fibrosis and as a self-perpetuating process, immunity aspects gain additional relevance, because alcohol modifies the innate and adapted immune system, which may explain the individual differences of susceptibility for ALD. With the third hit, the disease may approach a point of no return.
Fourth hit	The fourth hit is dominated by increased fibrosis, due to increased collagen formation. This allows for a clinically unrecognizable transition from alcoholic hepatitis with fibrosis to irreversible cirrhosis. However, AC can also develop without ASH or AH.
Fifth hit	In rare cases, a fifth hit initiates the development of a HCC, mostly occurring in patients with cirrhosis. This final hit scenario of carcinogenesis is triggered by acetaldehyde and ROS through the generation of DNA adducts, which promote mutagenesis, and interference with methylation, synthesis, and repair of DNA. Suggested is a possible role of SIRT1. These overall events will enhance AHCC susceptibility, especially in the absence of an identifiable carcinogen.

Hypothetical steps of the five hits leading to end-stage alcoholic liver disease. Adapted from a previous report<sup>[15]</sup>. MEOS: microsomal ethanol-oxidizing system; ROS: reactive oxygen species; ALD: alcoholic liver disease; AC: alcoholic cirrhosis; ASH: alcoholic steatohepatitis; AH: alcoholic hepatitis; HCC: hepatocellular carcinoma; AHCC: alcoholic hepatocellular carcinoma



**Figure 6.** Macroscopic picture of alcoholic hepatocellular carcinoma

## CLINICAL CHALLENGES OF AHCC

### Characteristic clinical features

AHCC develops mostly in patients with existing AC that may be compensated or not, and the degree of decompensation of AC will determine clinical symptoms superimposing those of AHCC. Clinical symptoms of patients with AHCC have not systematically been investigated and published recently<sup>[5-8,15,58,59]</sup>, but based on own clinical experience, patients with AHCC may have a silent clinical course until decompensation of AC develops. Until that point, patients are either without symptoms or report unspecific signs like fatigue, weakness, loss of appetite and weight, nausea and vomiting, and upper abdominal pain.

### Pathology

#### *Histology*

The histologic findings of all stages in ALD have comprehensively been outlined<sup>[2]</sup>, with some specificities regarding HCC and its variants<sup>[60-62]</sup>: the steatohepatic HCC variant is characterized by a steatotic appearance of > 5% of the tumor, presence of Mallory bodies, fibrosis, inflammation and ballooning of the hepatocytes as in steatohepatitis<sup>[60]</sup>. The inflammatory infiltrate usually consists of neutrophils, plasma cells, and lymphocytes. Microscopically, cells of classical HCC resemble normal liver cells, the similarity to normal liver is most evident in well to moderately differentiated tumors, but liver cell plates change from 1 to 2 cell nuclei to 3 or more<sup>[62]</sup>. In particular, AHCCs with a diameter of 1.5 to 2 cm are usually moderately or well differentiated with a distinct nodular structure, a rate of microinvasion of between 10% and 22%, with satellitosis in around 10%<sup>[6]</sup>.

#### *Gross pathology*

Macroscopically, AHCC is commonly seen in a cirrhotic liver but around 10% of AHCC are found in alcoholic patients without AC or liver fibrosis<sup>[59]</sup>. AHCC nodules may present singular, or multiple as illustrated by the liver of a patient with a long history of alcohol consumption [Figure 6]. The multiple larger nodes of AHCC stretched multifocal over the liver that otherwise presents as typical AC with focal, small regenerative cirrhotic nodules, mostly of the micronodular type, better seen in another picture presenting only AC that shows light splitting in the areas of micronodular regenerating nodules [Figure 7]<sup>[15]</sup>.

### Risk factors

#### *Smoking*

Sufficient evidence supports the view that smoking strikingly increases the risk of AHCC<sup>[43]</sup>, an important issue stressed also in a recent review article<sup>[5]</sup>.



**Figure 7.** Macroscopic picture of alcoholic cirrhosis

#### *Acquired comorbidities*

Risk factors for AHCC include not only AC but also cirrhosis of various other causes and chronic co-infection by hepatitis B or C<sup>[5,59]</sup>. Indeed, a broad range of confounding variables exists that can contribute to the development of AHCC<sup>[5,43,59]</sup>. Among these are diabetes mellitus with the metabolic syndrome, NASH or nonalcoholic fatty liver disease due to overweight or morbid obesity<sup>[5,59]</sup>. In this context, not only ROS generated in the fat tissues and the liver<sup>[5,59,63-65]</sup> but also lipotoxicity in general and more specifically as lipotoxic liver injury<sup>[65]</sup> may represent additional risk factors for causing AC and AHCC<sup>[5]</sup>.

#### *Genetic diseases*

Genetic liver diseases like  $\alpha_1$ -antitrypsin (AT) deficiency leading to storage of AT in the liver, Wilson disease with Cu storage in the liver, or the primary hereditary hemochromatosis (HH) with Fe storage in the liver, all these inherited diseases may lead per se to liver cirrhosis, and HCC are specific risk factors. They are risky due to their existence starting at birth and preexisting mostly for many years before alcohol abuse is initiated. HH is of special interest due to its high Fe content in the liver. From a metabolic aspect, Fe was early recognized in unpublished studies as promoter of MEOS activity, which led to the addition of EDTA to each MEOS assay to capture any Fe in the assay system<sup>[22-24]</sup>. Subsequent studies have confirmed that Fe stimulates not only MEOS activity but also ROS including hydroxyl radical generation<sup>[36]</sup>. This mechanism could explain why the risk of AHCC is increased in patients with HH who abuse alcohol for a long time<sup>[66]</sup>, substantiated by a subsequent analysis of available data, which suggest that iron accumulation in the liver is an independent risk factor for HCC in patients with AC<sup>[67]</sup>. In particular, iron accumulation in the liver is considered to be a co-factor for progression of liver disease, and iron overload can enhance the effects of oxidative stress and influence the natural history of patients with cirrhosis, exposing them to a higher risk of HCC.

#### *Gene polymorphisms*

Enzymes metabolizing ethanol and acetaldehyde in the liver are individually characterized and modified by gene polymorphisms<sup>[7,8,15]</sup>, with abundant studies that addressed the relevance of respective gene polymorphisms for their risk of AHCC<sup>[7]</sup>. However, results were contradictory. With ADH and ALDH studied in a Japanese cohort as an example, gene polymorphisms of *ADH2* and *ALDH2* were found to correlate with AHCC development<sup>[7]</sup>, findings not confirmed subsequently<sup>[7,68]</sup>.

#### *Gender*

Overall prevalence of AHCC is small in women compared with that in men<sup>[7]</sup>. Considering this limitation and a subgroup of consumers who used more than 80 g alcohol per day, the risk of AHCC development was fivefold higher in women than in men<sup>[7,69]</sup>. In general, female alcoholic patients are at a higher risk

for ALD as compared to alcoholic men<sup>[5,8,70,71]</sup>. In particular, women have more advanced liver disease at time of diagnosis, experience a more severe clinical course within a shorter time of alcohol abuse, and had consumed less alcohol compared to men<sup>[72]</sup>, in line with a lower thresholds for development of alcoholic liver injury<sup>[71,72]</sup>. This gender difference can be traced back to higher blood alcohol concentrations in woman compared to men who consume the same amount of alcohol, resulting from a lower proportion of body water in females than in males of equal body weight<sup>[50]</sup> and from a lower ADH dependent first pass metabolism in the gastric mucosa<sup>[73]</sup>. Under discussion are also gender based differences in the sensitivity of hepatic Kupffer cells to endotoxins generated in the gut<sup>[50]</sup>.

### *Immune system*

Little is firmly established related to specific immune reactions that could assist initiate and perpetuate AHCC although immune involvement is most likely<sup>[7]</sup>, as known from other tumors. Alcohol can modify both, the innate immune system (IIS) and the adaptive immune system (AIS)<sup>[8,15]</sup>. More specifically, IIS is promoted by macrophages, Kupffer cells, neutrophils, and natural killer cells, whereby macrophages are prepared to attack antigens of bacterial cell walls and respond by providing cytokines.

### *Amount of alcohol*

An excessive alcohol consumption is a risk factor not only of AC<sup>[7,15]</sup> but also for AHCC<sup>[7]</sup>. For both stages of ALD, a rough linear dose-response relationship between the amount of alcohol consumption and the respective risk is assumed<sup>[7]</sup>. For AHCC, an alcohol use expressed as absolute ethanol is risky at and above > 60-100 g per day, that compares with an odd ratio of 4.52 if the total amount of alcohol consumption during lifetime is considered.

## **Diagnosis of AHCC and surveillance programmes**

As an inexpensive method readily available in clinical settings, ultrasound is commonly used to diagnose AC and AHCC<sup>[74,75]</sup>. Ultrasound parameters for AC include liver size, bluntness of the edge, coarsened of the liver parenchyma, nodularity of the liver surface, and spleen size<sup>[74]</sup>, while data of liver stiffness could be supportive<sup>[5]</sup>. In search for AHCC, ultrasound technique has been improved by introducing the contrast-enhanced ultrasound (CEUS), considered as a major diagnostic breakthrough<sup>[75]</sup>. CEUS is unique in that it allows non-invasive assessment of liver perfusion in real time throughout the vascular phase and may abandon previous methods such as magnetic resonance or computer tomography images. Under imaging guidance, tissue from the suspected HCC may be obtained to verify histologically the diagnosis.

In line with other causes of cirrhosis, periodic screening for AHCC is recommended for patients with AC, who should be included in respective surveillance programmes<sup>[59]</sup>, which offer screenings at intervals of 6 months. This allows early detection of AHCC and implementation of curative procedures. However, contradictory recommendations suggest surveillance by biannual ultrasound<sup>[76]</sup>.

## **Prevention**

Clinical medicine should focus primarily on prevention of AHCC for the sake of the patient at risk, the human society, and financials of the health system. Overall prevention of AHCC is most successful if alcohol abuse can early be stopped. First of all, if physicians suspect that a patient may have an alcohol problem, specific questionnaires could help rule out or confirm the problem, as summarized recently<sup>[53]</sup>. A better and more objective approach would be doing just two laboratory tests in search for a serum quotient AST/ALT that may be > 1.0 if an alcohol problem exists independently from a specific ALD stage including already AFL<sup>[15]</sup>. The ratio is significantly increased in patients with AH and AC ( $2.85 \pm 0.2$ ). This led to the proposal that a serum AST/ALT ratio > 2.0 is highly suggestive of alcoholic hepatitis and cirrhosis<sup>[15,77]</sup>. Values of the AST/ALT ratio have been published for patients with AFL ( $1.64 \pm 1.57$ ), as compared with a corresponding control group consisting of individuals with normal liver histology and normal values of AST and ALT, showing a lower AST/ALT ratio ( $0.72 \pm 0.24$ )<sup>[15,78]</sup>. In search for individuals with severe alcohol abuse, other laboratory data show variable percentages of sensitivity: carbohydrate-deficient

transferrin (CDT; 63%), gamma-glutamyltransferase (GGT; 58%), mean corpuscular volume of erythrocytes (MCV; 45%), aspartate aminotransferase (AST; 47%), alanine aminotransferase (ALT; 50%), and GGT + CDT (90%)<sup>[15]</sup>.

Under the aspect of prevention and associated diagnosis, a liver biopsy to establish the diagnosis of any ALD stage is certainly not recommended under routine conditions, although it was previously considered as diagnostic gold standard. Exemptions now are pretransplantation evaluations or RCTs to test efficacy of drugs for instance in patients with AH, but in most other cases the patient will have no benefit from this invasive procedure. This is also confronted with a fatality rate, though rather low<sup>[5]</sup>.

### Pharmacotherapy option

If AHCC is diagnosed, the patient should abstain from further alcohol use to prevent destruction of the remaining, still functioning liver. While some pharmacotherapy measures are partially effective in patients with severe AH<sup>[53]</sup>, respective options with proven efficacy are not available for AHCC. Instead, palliative measures for pain and symptom relief is the only choice.

### Segment resection, liver transplantation, and tumor ablation

A stringent algorithm for management of AHCC has been presented that should be used as a guideline<sup>[66]</sup>. Options must consider specific criteria and may include segment resection, disputed liver transplantation, locoregional ablation using Sorafenib, transarterial chemoembolization, and radiotherapy. Investigational studies should be performed in the frame of RCTs.

### Prognosis

Presenting mostly as an end stage of the tumor disease, patients with AHCC have a poor prognosis, because overall survival was in a range of 15 to 32 months, which compared to 16 to 47 months in patients who received a curative treatment<sup>[59]</sup>. This again calls for an early recognition of potential individuals with an alcohol problem and associated early stages of ALD. Vague estimates based on four studies<sup>[79]</sup> allow the tentative conclusion that abstinence from alcohol use may reduce AHCC development perhaps by 6% to 7% per year, but a wash out period of 23 years being necessary to achieve the same incidence of AHCC seen in abstinent individuals<sup>[59,79]</sup>. In other words, early abstinence is better than late abstinence.

### EPIDEMIOLOGY AHCC

Worldwide epidemiology data of AHCC suggests that alcohol accounts for around one third of global incident cases of primary liver cancer, with a substantial variability of results among different countries and regions, ranging from 6% in Iran to 61% in Estonia and Moldova<sup>[59,80]</sup>. Details can be derived from a recent large study of the Global Burden of Disease Liver Cancer Collaboration, which considers the burden of primary liver cancer and underlying etiologies from 1990 to 2015 at the global, regional, and national level<sup>[80]</sup>. Accordingly, for virtually all countries data of AHCC and liver cancer due to HBV, HCV, and other causes (OC) are available, for example<sup>[80]</sup>: Australia (AHCC 38%, HBV 9%, HCV 40%, OC 13%), Austria (AHCC 49%, HBV 15%, HCV 30%, OC 6%), Belgium (AHCC 48%, HBV 16%, HCV 28%, OC 8%), China (AHCC 33%, HBV 41%, HCV 8%, OC 18%), France (AHCC 37%, HBV 17%, HCV 36%, OC 9%), Germany (AHCC 44%, HBV 8%, HCV 33%, OC 14%), India (AHCC 21%, HBV 42%, HCV 20%, OC 18%), Japan (AHCC 17%, HBV 8%, HCV 69%, OC 6%), Russia (AHCC 53%, HBV 15%, HCV 24%, OC 8%), United Kingdom (AHCC 36%, HBV 17%, HCV 38%, OC 9%), and United States (AHCC 37%, HBV 9%, HCV 31%, OC 22%)<sup>[80]</sup>. In countries with a high AHCC percentage, a high alcohol consumption is likely. In other countries, HBV and HCV infections are causative for HCC.

### CONCLUSIONS

Ethanol *per se* is not carcinogenic but has to be viewed as a procarcinogen involved in the development of AHCC. This process is triggered by ROS including activated molecules derived from ethanol or

acetaldehyde metabolism attacking hepatocellular DNA. Upregulation of ROS production occurs via CYP 2E1 following prolonged alcohol consumption and represents a major risk factor of AHCC. To prevent AHCC, individuals with an alcohol problems must early be identified in order to achieve alcohol abuse, because only a long period of abstinence will substantially reduce the risk of AHCC initiation, short term abstinence contributes not or little to risk reduction. Concomitant chronic liver diseases are additional risks factors in the context of AHCC and deserve effective treatment. Of special risks are infections by HBV and HCV and genetic liver disease such as hereditary hemochromatosis with its high Fe content in the liver. AHCC represents a late stage of ALD mostly in connection with AC and does not provide early clinical warning symptoms.

## DECLARATIONS

### Authors' contributions

The author contributed solely to the article.

### Availability of data and materials

Not applicable.

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None.

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The author declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Review

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# An update on the histological subtypes of hepatocellular carcinoma

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## Abstract

Hepatocellular carcinoma (HCC) is one of the top-ranking cancers worldwide and in Southeast Asia. The high propensity for tumor recurrence, distant metastasis and chemoresistance remain major hurdles in the treatment of HCC. With advances on genetics and genomics research, molecular targeted therapies are emerging as a hope for better disease control. On the histological perspective, microscopic review of clinical samples has led to subclassification of HCC and establishment of new entities. In this review, latest understanding on macrotrabecular-massive HCC, steatohepatitic HCC, lymphocyte-rich HCC, scirrhous HCC, fibrolamellar carcinoma and combined hepatocellular-cholangiocarcinoma will be discussed, emphasizing on the clinical relevance of these pathological entities. Further delineation of the histological, immunohistochemical, molecular and biological phenotypes of primary liver cancer would further enhance an integrated morphological-molecular classification that better predicts clinical outcome and guides clinical management.

**Keywords:** Liver cancer, subtype, histology

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer. Microscopically, it is characterized by thickened cell plates, malignant tumor cell cytology, capillarization of sinusoids and evidence of invasion. Histological evaluation of HCC specimens plays a key role in tumor staging, and



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in distinguishing HCC from its precursor lesions or other liver nodules. With reference to the multi-step process of hepatocarcinogenesis which could also be observed histologically, some classification systems have been proposed for HCC. For instance, the terms “early HCC” and “progressed HCC” have been defined based on the size and differentiation of tumor<sup>[1]</sup>. In recent years, further investigations have been carried out on the subtyping of HCC specifically referencing the morphological characteristics of tumor cells. The significance of these subtypes was further substantiated by their clinical relevance and the genetic makeup. In the latest 5th edition of the World Health Organization (WHO) Classification of Digestive System Tumors, several histological subtypes were described<sup>[2]</sup>. In this review, we will focus on elaborating the recent understanding on 5 subtypes: macrotrabecular-massive HCC (MTM-HCC), steatohepatic HCC (SH-HCC), lymphocyte-rich HCC, scirrhous HCC and fibrolamellar carcinoma (FLC). In addition, an update on the entity combined hepatocellular-cholangiocarcinoma (cHCC-CCA) will be discussed.

### MTM-HCC

Published in 2018, histological review of clinical samples archive led to the identification of a novel and distinct subtype of HCC defined by the histological features of tumor cells - MTM-HCC. It is defined as the presence of macrotrabeculae of more than 6 cells thick in > 50% tumor, and was identified in 16% on average in 2 large cohorts comprising 237 surgical resection samples and 284 biopsy samples<sup>[3]</sup>. And this subtype was statistically associated with aggressive parameters including tumor size, alpha-fetoprotein (AFP) levels, satellite nodules and vascular invasion. Besides, it was an independent prognosticator for early recurrence (within 2 years) and overall recurrence<sup>[3]</sup>. The prognostic significance was further validated by other group<sup>[4]</sup>. In another study by Jeon *et al.*<sup>[5]</sup>, MTM-HCC, as defined by > 30% of macrotrabecular pattern, was associated with large tumor, hepatitis B virus infection, and less frequent cirrhosis. This subtype was also found to be associated with higher tumor grade, tumor stage, higher AFP level, and a worse recurrence-free survival.

In addition, investigations were carried out to identify specific radiological, immunohistochemical, and genetic features of this entity. Radiologically, MTM-HCC was reported to preferentially demonstrate irregular rim-like arterial phase enhancement on gadoxetate-enhanced magnetic resonance imaging<sup>[6]</sup>. Extending from their initial observation, Calderaro *et al.*<sup>[7]</sup> attempted to identify potential biomarkers for this entity. On analysis of the TCGA dataset, endothelial-specific molecule 1 (ESM1) was identified and validated as a biomarker for MTM-HCC. In addition, angiotensin 2 and VEGFA overexpression was observed in MTM subtype<sup>[8]</sup>. Recent studies also shed light on the genetic composition of MTM-HCC, which was found to be related to cell cycle activation, chromosomal instability, the G3 transcriptomic subgroup<sup>[8]</sup> according to Boyault *et al.*<sup>[9]</sup> and TP53 mutation<sup>[8]</sup>.

### SH-HCC

The steatohepatic subtype was first described by Salomao *et al.*<sup>[10]</sup>. It is characterized by prominent steatotic changes in the tumor cells namely fat accumulation, ballooning degeneration, presence Mallory-Denk bodies and peri-cellular fibrosis. In a study examining HCV-related liver explants, SH-HCC was identified in 35.5% of the cohort<sup>[10]</sup>. According to a follow-up study by the same group in 2012, SH-HCC constituted 14% of HCC explants<sup>[11]</sup>. A more recent paper reported a diagnosis of SH-HCC in around 20% of 96 HCC cases reviewed, and made an observation that SH-HCC was not associated with cirrhosis<sup>[12]</sup>. It was noted that around 60% (14 of 22) SH-HCCs was associated with one more known risk factor for non-alcoholic fatty liver disease (NAFLD)<sup>[10]</sup>. The association with and NAFLD and metabolic syndrome was consolidated in other studies<sup>[13,14]</sup>. While most studies suggested a link of fatty liver disease with this subtype, in 2015 Yeh *et al.*<sup>[15]</sup> looked at a series of SH-HCC and identified a group of patients without any underlying causes for metabolic disease.

Regarding correlation with other histological tumor parameters, there was a lack of microsatellite nodules or microvascular invasion in SH-HCC<sup>[8]</sup>. On the immunohistochemical phenotype, C reactive protein (CRP) expression was frequent<sup>[8]</sup>. Immunohistochemical expression of serum amyloid A and CRP was significantly higher in this subtype than conventional HCC as revealed by another study<sup>[16]</sup>. It was also found that the cancer-associated fibroblasts in SH-HCC more frequently expressed senescence-associated secretory phenotype by immunohistochemical staining<sup>[14]</sup>. On genetic and genomic levels, SH-HCC was shown to associate with IL6/JAK/STAT pathway activation, as well as wild type *CTNNB1*<sup>[8,17]</sup> and *TP53*<sup>[8]</sup>. By multivariate modeling, it was shown to be related to the S1 subclass<sup>[12]</sup> according to Hoshida *et al.*<sup>[18]</sup>.

## LYMPHOCYTE-RICH HCC

Previously known as lymphoepithelioma-like HCC, lymphocyte-rich HCC is characterized by an immune-rich stroma<sup>[19,20]</sup>. Wada *et al.*<sup>[21]</sup> defined this subtype by the presence of more than 100 tumor-infiltrating lymphocytes in 10 high-power fields. Despite a difference in immune cells infiltration, immunohistochemically the tumor cells express epithelial markers and HepPar-1<sup>[22-27]</sup>. The tumor-infiltrating lymphocytes were largely composed of CD3+ T cells<sup>[19-29]</sup>. In contrast to lymphoepithelioma-like carcinoma originating in the nasopharynx, vast majority of lymphocyte-rich HCC were EBER negative<sup>[19,22,23,26,28,30]</sup>. In 2017, Labgaa *et al.*<sup>[31]</sup> published a comprehensive review on a total of 66 lymphocyte-rich HCC cases. In this report, 64% patients were male and liver cirrhosis was present in 46%. While a few studies demonstrated a trend of better survival with this subtype<sup>[19-21,28]</sup>, the prognostic significance of this subtype remains to be clarified due to its rarity. The genomic landscape of 12 lymphocyte-rich HCC was determined by whole-exome sequencing in a recent report<sup>[32]</sup>. Mutations of *CTNNB1*, *AXIN1*, *APC*, *NOTCH1* and *NOTCH2* were less frequently observed in lymphocyte-rich HCC than conventional HCC. Since activation of Wnt/beta-catenin pathway was correlated with poorer clinical response to immune checkpoint inhibitors<sup>[33]</sup>, lymphocyte-rich HCC is possibly more susceptible to immunotherapies. The potential significance in terms of treatment response was in line with in a recent study examining the immunohistochemical expression in 217 HCCs, that a high programmed death-ligand 1 expression was correlated with the lymphocyte-rich subtype<sup>[34]</sup>.

## SCIRRHOUS HCC

Scirrhous HCC shows peculiar histology with small oval cells arranged in nests or trabecular among an abundant fibrous stroma<sup>[35]</sup>. It comprises 0.19% of all HCC from the National Cancer Database from 2004-2015<sup>[36]</sup>. The survival outcome for this subtype remains to be further delineated. Overall survival of patients was found comparable with non-scirrhous HCC in some studies<sup>[36,37]</sup>, while both better<sup>[38-40]</sup> and worse<sup>[35]</sup> survival outcomes were also reported. Furthermore, scirrhous HCC was associated with less frequent HBV infection, lower serum AFP level and less liver cirrhosis when compared with conventional HCC<sup>[37]</sup>. Radiologically, scirrhous HCC was reported to show distinct computed tomography (CT) scan features including presence of washout areas<sup>[41]</sup>. Immunohistochemical analyses revealed expression of stem/progenitor markers in scirrhous HCC; and gene expression profiling highlighted a TGF- $\beta$  signature<sup>[35]</sup>.

## FLC

FLC was first introduced in 1956<sup>[42]</sup> illustrating a primary liver cancer displaying characteristic large eosinophilic tumor cells with prominent nucleoli and pale bodies, and the prominent fibrotic bands traversing the tumor cells in lamellae. The latter feature led to the coining of its nomenclature<sup>[43]</sup>. FLC occurs more often in young adults with a mean age of diagnosis at 25 years<sup>[43-45]</sup>. FLC express CK7 and HepPar-1 immunohistochemical staining<sup>[46]</sup>. From a nationwide study published in 2014 using the SEER data base, the incidence of FLC was 1% among 7225 patients<sup>[47]</sup>. In the same study, it was reported that patients tend to be younger, female, and associated with longer overall survival on univariate analysis. In 2014, it was reported that a chimeric transcript was identified, which was further found to be due to a



deletion in chromosome 19 detected by whole genome sequencing, which in turn leads to the generation of the DNAJB1-PRKACA chimeric protein, with the kinase activity is retained in the latter component<sup>[48]</sup>. This discovery is significant since it provides a pathognomonic genetic feature for this subtype. The tumorigenicity of the fusion transcript was validated by *in vivo* mouse model with hydrodynamic tail vein injection of Crispr/cas9 generated DNAJB1-PRKACA vector<sup>[49]</sup>. Subsequent study revealed an interaction between the fusion kinase and  $\beta$ -catenin<sup>[50]</sup>, suggesting a contributory role of the fusion protein and  $\beta$ -catenin in the pathogenesis of FLC. In addition, analysis of clinical samples suggested the recruitment of heat shock protein 70 by the fusion enzyme and further in phosphoproteomic profiling using cell line models highlights the activation of ERK signaling in DNAJ-PKAC cells<sup>[51]</sup>.

### cHCC-CCA

cHCC-CCA is defined as a primary liver cancer showing unequivocal presence of both hepatocytic and cholangiocytic differentiation in the same tumor<sup>[2,52]</sup>. The 2 components histologically can either be juxtaposed with or intermingled with each other. There is no definite cutoff value as to the minimal proportion of each component present in a tumor to render a diagnosis of cHCC-CCA. In this type of liver cancer, small uniform epithelial cells with scanty cytoplasm and showing CK19, EpCAM, CD56, CD117 or CD133 expression has been observed<sup>[2]</sup>. The radiological feature with CT scan/magnetic resonance imaging was reviewed by a French group<sup>[53]</sup>. In the study, a mixed pattern comprising HCC, intrahepatic cholangiocarcinoma and atypical radiological pattern was observed in cHCC-CCA; and this mixed pattern showed a sensitivity of 48% and a specificity of 81%. Protein expression for of diagnostic purpose of cHCC-CCA has been investigated, and malic enzyme 1 (ME1) was proposed as a potential immunohistochemical marker for cHCC-CCA, in which 77% express ME1<sup>[54]</sup>.

Previous study demonstrated an intermediate clinical outcome of cHCC-CCA between HCC and intrahepatic cholangiocarcinoma (iCCA), when overall survival after resection, disease-free survival after resection, and overall survival after liver transplantation were considered<sup>[55]</sup>. A more recent study comprising 250 cHCC-CCA in the training cohort and 99 cases in the validation cohort demonstrated that the 1-, 2 and 3-year overall survival was 67.7%, 46.8% and 37.9% respectively; and the 1-, 2 and 3-year cancer-specific survival was 73.1%, 52.0% and 43.0%, respectively<sup>[56]</sup>. At times of recurrence or metastasis, as reported by He *et al.*<sup>[56]</sup>, the heterogeneity tends to be retained rendering the clinical behavior of cHCC-CCA recurrence is largely unpredictable<sup>[57]</sup>.

Despite the deviation in clinical outcome, a study on 20 cHCC-CCA samples by capture-based next-generation sequencing revealed similar genomic profiles to conventional HCC. Recurrent alterations in TERT, TP53, cell cycle genes, receptor tyrosine kinase/Ras/PI3K pathway genes, chromatin regulators, *etc.*, were identified in cHCC-CCA, while IDH1, IDH2, FGFR2 or BAP1 mutations were absent<sup>[58]</sup>. On a side note, genomic and genetic profiling of cholangiolocellular carcinoma, which was previously classified as a subtype of cHCC-CCA in the 4th edition of WHO Classification of Digestive Tumors<sup>[59]</sup>, showed that this entity was likely biliary tract origin featuring NCAM expression, chromosomal stability and TGF- $\beta$  activation<sup>[60]</sup>. Consistent findings were reported by Balitzer *et al.*<sup>[61]</sup>. By comparing immunohistochemical expressions, mutational profiles and copy number variation patterns, cholangiolocellular carcinoma was shown to display a highly similar pattern with iCCA, suggesting that the former should instead be classified as a form of well differentiated iCCA.

### FUTURE PERSPECTIVES

In this review, latest understanding on 5 HCC subtypes and the distinct entity cHCC-CCA were discussed. These entities in common demonstrate peculiar pathognomonic histological features. Among these entities, MTM-HCC, lymphocyte-rich HCC and cHCC-CCA are known carry potential prognostic significance.



In addition, lymphocyte-rich HCC may represent a subtype showing relatively favorable response to immunotherapy. SH-HCC may represent a spectrum of HCC arising from specific etiology. Further delineation of the genetic and genomic signatures of FLC and cHCC-CCA may provide insights on the cell of origin and pathogenesis of primary liver cancer.

Apart from defining specific subtypes, some histological features in HCC were found to be closely related to certain genetic alterations. For instance, well differentiated tumors with pseudoacinar pattern, tumor cell cholestasis and lack of immune cell infiltration were associated with *CTNNB1* mutations<sup>[8,62]</sup>. Calderaro *et al.*<sup>[63]</sup> summarized a histological-molecular correlation of liver cancer. In this review, the molecular subclasses<sup>[9,18]</sup> and genetic alterations of histological subtypes including MTM-HCC, SH-HCC, scirrhous HCC, lymphocyte-rich HCC were discussed. Besides, the immune microenvironment of 158 HCC cases was recently characterized by Kurebayashi *et al.*<sup>[64]</sup> using multiplex immunohistochemistry. The accumulating body of information, together with integrated analyses of the expression profiles of HCC at transcriptomic, genomic and proteomic levels may facilitate formulating a classification system of clinical relevance.

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Review

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# Molecular links between non-alcoholic fatty liver disease and hepatocellular carcinoma

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## Abstract

Non-alcoholic fatty liver disease (NAFLD) and its advanced complication, non-alcoholic steatohepatitis (NASH), have become leading causes of hepatocellular carcinoma (HCC) worldwide. In this review, we discuss the role of metabolic, gut microbial, immune and endocrine mediators which promote the progression of NAFLD to HCC. In particular, this progression involves multiple hits resulting from lipotoxicity, oxidative stress, inhibition of hepatic autophagy and inflammation. Furthermore, dysbiosis in the gut associated with obesity also promotes HCC via induction of proinflammatory cytokines and Toll like receptor signalling as well as altered bile metabolism. Additionally, compromised T-cell function and impaired hepatic hormonal action promote the development of NASH-associated HCC. Lastly, we discuss the current challenges involved in the diagnosis and treatment of NAFLD/NASH-associated HCC.

**Keywords:** Non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, hepatocellular carcinoma, Gut microbiome, dysbiosis, autophagy, ER-stress, ROS, TNF $\alpha$ , TLR-9, TLR-4, hyperinsulinemia

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the primary form of liver cancer and is a leading cause of cancer-related mortality worldwide<sup>[1]</sup>. It is predominantly known to occur in patients suffering from underlying chronic liver disease and cirrhosis. Hepatitis B and C virus (HBV and HCV, respectively) infections, excessive consumption of alcohol and non-alcoholic fatty liver disease (NAFLD) historically have been



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recognised as the major causes of HCC; however, the incidence of virus-associated HCC is expected to decrease in the near future due to the development of effective and inexpensive vaccines for HBV and potent anti-HCV drugs<sup>[1,2]</sup>. In contrast, the prevalence of non-viral hepatitis continues to rise and has become the major cause for liver transplantation in Europe and the USA<sup>[3]</sup>. The increased prevalence of metabolic disorders, particularly diabetes, NAFLD and obesity, have led to changes in the epidemiology and aetiology of HCC<sup>[4]</sup>. Obesity is considered a risk factor for hepatic complications such as NAFLD and HCC<sup>[5-9]</sup>. Although 17%-33% of the general population is estimated to be affected by NAFLD, it reaches 75% in obese individuals and is even higher in patients with type II diabetes mellitus (T2DM)<sup>[10,11]</sup>. Moreover, T2DM itself is associated with an increased risk of liver damage<sup>[12]</sup>, including HCC<sup>[13-15]</sup>. Chronic damage to liver metabolism caused by alcohol and poor nutrition leads to alcoholic liver disease that can co-exist with NAFLD/non-alcoholic steatohepatitis (NASH), and thereby increases both the progression of NAFLD and the risk for NAFLD/NASH-associated HCC<sup>[2,3]</sup>.

This review focuses on NAFLD-associated HCC, and describes its epidemiology and the clinical, cellular, metabolic, microbiome and endocrine factors that promote the development of HCC from NAFLD. We also examine the molecular pathways that lead to progression from NAFLD to HCC as well as the challenges and future directions for its treatment and prevention.

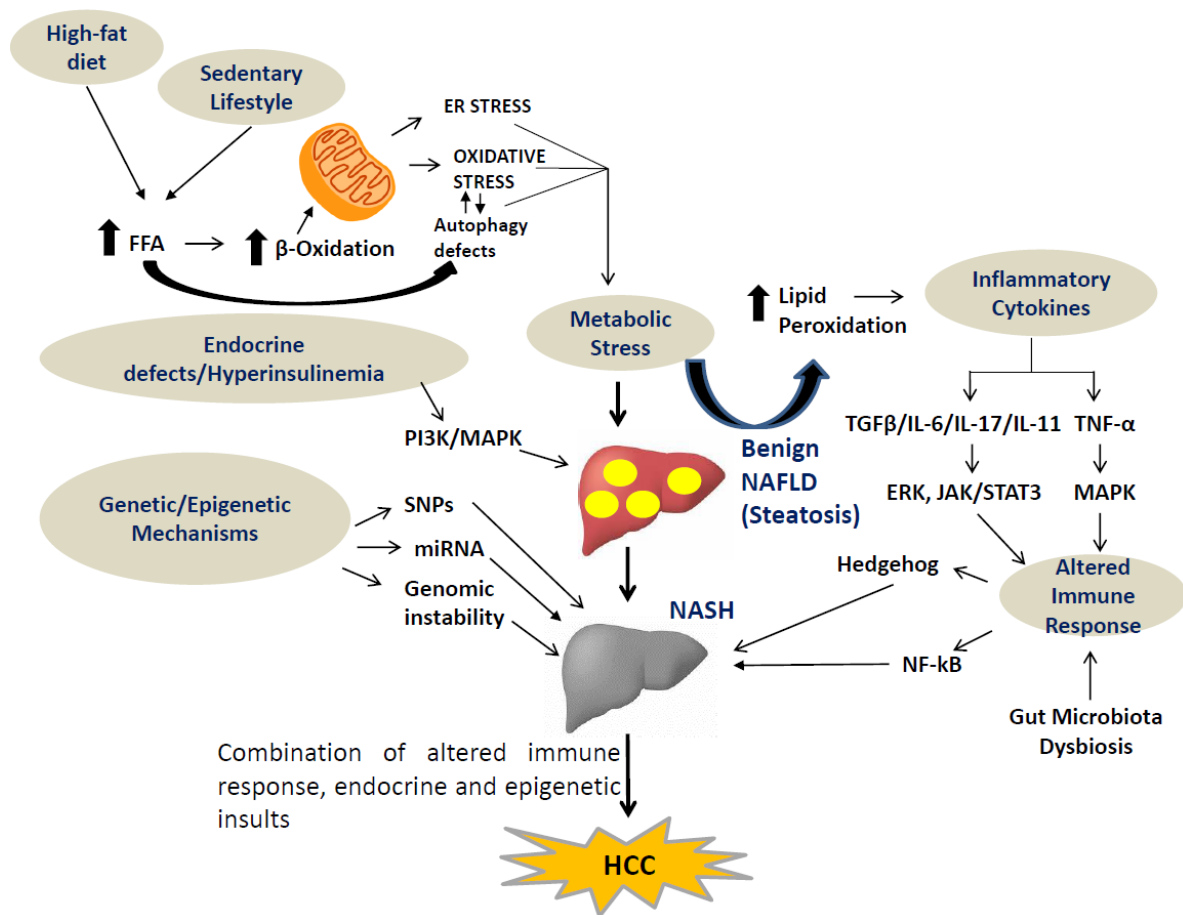
## NAFLD INCREASES THE RISK OF LIVER CANCER

NAFLD encompasses a spectrum of liver pathologies which involve an accumulation of triglycerides in the hepatocytes, hepatocyte apoptosis, liver inflammation and fibrosis termed as NASH, and, in extreme cases, it can progress to cirrhosis and HCC<sup>[16]</sup>. NAFLD is the most common cause of HCC across the globe<sup>[16-28]</sup>. Although the progression of NAFLD to HCC involves NASH and cirrhosis, the direct development of HCC from benign steatosis or non-cirrhotic NASH has also been reported<sup>[29,30]</sup>. The increased prevalence of the underlying liver disease in the general population has led to an increase of 9% in the annual rates of incidence of NAFLD-associated HCC<sup>[31]</sup>. Interestingly, HCC can progress from NASH as well as cirrhosis. In a study cohort based on 756 patients, Piscaglia *et al.*<sup>[32]</sup> reported that 46.2% of the NAFLD associated HCC cases occurred without cirrhosis. Similar results were reported by a Japanese study, in which 49% of NAFLD associated HCC cases arose without cirrhosis<sup>[33]</sup>, and a German study where 41.7% of the cases arose without cirrhosis<sup>[34]</sup>. Furthermore, in animal models, diet-induced NAFLD leads to spontaneous HCC<sup>[35]</sup>.

## CELLULAR MECHANISMS INVOLVED IN NAFLD PATHOGENESIS

NAFLD is a complex disease with multiple modifiers such as diet, lifestyle and gut microbiota which act in a susceptible genetic/epigenetic environment and modulate response to calorific excess<sup>[36,37]</sup>. The role of insulin resistance is central to this pathophysiological process and causes an increase in hepatic fat accumulation by increased deposition of free fatty acids (FFAs)<sup>[38]</sup>. This leads to oxidative stress, protein misfolding, autophagy inhibition and mitochondrial damage within hepatocytes, termed as “lipotoxicity”<sup>[38]</sup>. Chronic lipotoxicity challenges hepatocytes with both oxidative and endoplasmic reticulum (ER) stress. Oxidative stress mediated by reactive oxygen/nitrogen species (ROS/RNS) play a major role in NAFLD/NASH pathogenesis and complications. The high production of ROS causes mitochondrial damage, lipid peroxidation and low-density lipoprotein oxidation culminating into inflammation, activation of hepatic stellate cells (HSCs) leading to fibrogenesis, necrosis, cirrhosis and HCC<sup>[39]</sup>.

ER stress is cell activated to regulate protein synthesis and restore homeostatic equilibrium in response to accumulation of unfolded or misfolded proteins. However, deregulated or insufficient responses to ER stress in liver may lead to lipid accumulation, insulin resistance, inflammation and apoptosis, all of which play important roles in the pathogenesis of NAFLD<sup>[40]</sup>. These events lead to inflammation and fibrosis as macrophage infiltration, hepatic progenitor cell activation and fibrogenesis ensue<sup>[41,42]</sup>. There are multiple



**Figure 1.** Multiple hits lead to onset and progression of NAFLD/NASH to HCC. Diverse signalling pathways involved in metabolic stress such as FFAs ER-stress, cytokine production (IL-6, IL-17, IL-11 and TGF-β), altered immune response, pro-fibrogenic mediators (hedgehog and NF-κB), gut dysbiosis and endocrine defects drive the development of NAFLD/NASH-associated HCC. NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; HCC: hepatocellular carcinoma; FFAs: free fatty acids; ER: endoplasmic reticulum; IL-6: interleukin-6; IL-17: interleukin-17; IL-11: interleukin-11; TGF-β: transforming growth factor β; SNPs: single nucleotide polymorphisms; miRNA: micro RNA; PI3K: phosphatidylinositol 3-kinases; MAPK: mitogen-activated protein kinase; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B-cells; TNF-α: tumour necrosis factor-alpha; ERK: extracellular receptor kinase; JAK: Janus kinase; STAT: signal transducer and activator of transcription

factors that contribute to the pathogenesis of NAFLD and its progression. These include: dysregulated lipid metabolism, oxidative stress, ER stress, mitochondrial dysfunction, altered immune function, and gut-microbiota imbalance acting together in a genetic/epigenetic environment [Figure 1].

## MOLECULAR MECHANISMS LEADING TO PROGRESSION FROM NAFLD TO HCC

Studies on the development of HCC suggest that carcinogenesis in hepatocytes is a consequence of genetic/epigenetic alterations as well as complex changes in energy metabolism, cell growth and proliferation and immune signalling pathways. These changes in the cells lead to inflammation, hepatocyte injury, fibrosis and progression to HCC [Figure 1].

### Genetic/Epigenetic mechanisms

Several single nucleotide polymorphisms (SNPs) have been associated with the occurrence of NAFLD and its progression to advanced fibrosis<sup>[36]</sup>. The patatin-like phospholipase domain-containing protein 3 (PNPLA3) gene polymorphism is associated with the progression of NASH-associated HCC<sup>[43]</sup>. PNPLA3 impairs triglyceride mobilisation from lipid droplets. Patients carrying the PNPLA3 polymorphism are reported to have a three-fold increased risk of developing HCC<sup>[44,45]</sup>. Transmembrane 6 superfamily



member 2 gene (*TM6SF2*) mutations are also prevalent in NASH patients<sup>[46]</sup>. They are believed to be linked to liver injury in the pathogenesis of NASH-associated HCC<sup>[47]</sup>. Recently, membrane bound O-acetyl transferase domain containing 7 (*MBOAT7*) rs641738 variant associated with NAFLD progression has also been linked to HCC susceptibility<sup>[48]</sup> [Figure 1].

In addition to the SNPs, genetic instability is also believed to stimulate the progression of NASH to HCC. Mutations in oncogenic genes, such as the human telomerase reverse transcriptase (*hTERT*) gene which catalyses the addition of nucleotides to the ends of eukaryotic chromosomes, tumour protein p53, cyclin dependent kinase inhibitor 2 A, albumin, catenin beta-1 and axis inhibition protein 1 (involved in Wnt/ $\beta$ -catenin signalling), are prevalent in exome-sequencing analysis of HCC<sup>[49]</sup>. Aberrant DNA methylation is also an important mechanism in NASH progression<sup>[50]</sup>, and can lead to silencing of genes involved in DNA repair, lipid metabolism, glucose metabolism and progression of fibrosis<sup>[50]</sup>. In particular, the epigenetic changes in the gene encoding chromodomain helicase DNA-binding protein 1 are reported to be linked to NASH-associated HCC<sup>[51]</sup>.

The expression of several microRNAs (miRNAs) also is reported to be dysregulated in many types of cancer, including NASH-associated HCC<sup>[52]</sup>. The miRNAs are small noncoding RNAs that down-regulate gene expression by interfering with transcription and/or translation. These miRNAs are involved in cell signalling pathways associated with oncogenesis, such as transforming growth factor (TGF)- $\beta$ , Wnt/ $\beta$ -catenin, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinases (PI3K)/AKT/mTOR pathways, which can be activated in HCC<sup>[53]</sup>. In particular, miRNAs known to target the inhibitors of PI3K/AKT pathway are found in HCC. In this connection, steatosis, hepatomegaly and HCC have been observed in phosphatase and tensin-deficient mice<sup>[54]</sup>. Several miRNAs are differentially expressed in a high fat diet mouse model during the NAFLD-NASH-HCC transitions<sup>[55]</sup>. Hepatic miR-340-5p, miR-484, miR-574-3p and miR-720 were expressed in NAFLD, NASH and HCC, and miR-125a-5p and miR-182 showed early and significant dysregulation during hepatic tissue damage<sup>[55]</sup>.

### Metabolic pathways

The association of obesity, high-fat diet and diabetes to NAFLD/NASH and its progression to HCC suggests the existence of a molecular link between energy metabolism and cell cycle control in the hepatocytes, which may be a key mechanism driving the progression of NASH to HCC. Several animal studies have been conducted to investigate NASH-associated HCC. These studies showed that the progression of NASH-associated HCC may be due to abnormal lipid metabolism, oxidative stress, ER stress and mitochondrial dysfunction acting independently or in tandem<sup>[56,57]</sup> [Figure 1].

It is worth noting that mitochondrial activities such as  $\beta$ -oxidation, electron transfer, ATP production and ROS generation regulate the fat metabolism and energy homeostasis in hepatocytes<sup>[58]</sup>. During the early hepatosteatotic phase of NAFLD when there is fatty acid accumulation in hepatic cells, mitochondria prevent oxidative stress and help facilitate the partition of lipotoxic FFAs into stable triglycerides that can be stored in fat droplet, and thereby prevent oxidative stress<sup>[59]</sup>. However, chronic high-fat or high-fructose diet leads to lipid over-accumulation in the hepatocytes due to cellular metabolic reprogramming and accumulation of toxic metabolites<sup>[60]</sup>. These changes lead to imbalances in hepatic metabolism that result in excessive production of FFAs, which can cause lipotoxicity<sup>[61]</sup>.

The excessive accumulation of these fatty acids increases  $\beta$ -oxidation and ROS production, which can limit mitochondrial function<sup>[62]</sup>. When these mitochondrial abnormalities are accompanied by diminished intracellular antioxidant protection in NASH, pathways of fatty acid metabolism are altered<sup>[63]</sup>, which, in turn, can cause metabolic stress<sup>[63]</sup>. Overproduction of ROS frequently occurs in cancer, and is believed to play an important role in the development of HCC<sup>[64]</sup>. Intriguingly, oxidative stress

and abnormal methylation of tumour suppressor genes are found the livers of NAFLD patients<sup>[65]</sup>. ER stress also contributes to hepatocyte injury and carcinogenesis in NASH<sup>[66]</sup>. Thus, the cross-talk among oxidative stress, ER stress and cell death pathways likely plays a role in the development of NASH and its progression to HCC<sup>[67]</sup>. Similar to oxidative and ER stress, autophagy dysregulation may be involved in the progression of NASH to HCC<sup>[68]</sup>. In this regard, impaired autophagy leads to defective lipid metabolism<sup>[69]</sup>, proteotoxicity<sup>[70]</sup>, mitochondrial dysfunction<sup>[71]</sup> and inflammation<sup>[72]</sup>, all of which can contribute to HCC induction.

Several xenobiotic metabolising genes of the aldo-keto reductase family show parallel induction in NASH and HCC, suggesting a genetic link between NASH and its progression to HCC<sup>[73-76]</sup>. Thus, disturbance in hepatic cell metabolism can lead to increased cell death, DNA damage, immune cell activation and compensatory proliferation<sup>[57]</sup>. These changes in hepatic cells activate HSCs and induce fibrosis. If tumour surveillance and DNA damage repair are impaired in NASH, pre-malignant cells can develop, and, after critical genetic/epigenetic changes, they become clones that progress to HCC. Therefore, the cumulative effects of oxidative stress and proliferative response during inflammation and fibrosis are thought to drive the progression of HCC<sup>[57]</sup>.

### Gut microbiota

“Gut microbiota” refers to populations of bacteria hosted by the adult human intestine, which maintain a symbiotic relationship with the host and have a key role in the host immune system. They perform various functions in the body such as digestion of inaccessible nutrients, synthesis of vitamins and resistance to pathogens<sup>[77]</sup>. They are known to ferment carbohydrates such as cellulose and xylans into short-chain fatty acids (SCFAs). The liver is exposed to gut-derived products by portal circulation, which provides a defence against bacterial toxins. SCFAs improve hepatic autophagy and gut barrier function, and reduce the permeability of bacterial toxins. These gut products can reduce pro-inflammatory pathways and insulin resistance, which are associated with the progression of chronic liver disease<sup>[78,79]</sup>. The gut microbiota composition is dynamic and may be influenced by diet, hygiene and the use of antibiotics<sup>[80]</sup>. The modification of the normal microbiota termed as “dysbiosis” is believed to be associated with the progression of NAFLD and other chronic metabolic diseases<sup>[81-85]</sup>.

Dysbiosis in gut flora has been associated with HCC incidence in humans and animal models<sup>[86]</sup>. Mice kept in germ-free conditions or given antibiotics tend to develop fewer and smaller HCCs<sup>[87,88]</sup>. At the molecular level, dysbiosis of the gut microbiota leads to an increase in secretion of inflammatory cytokines, such as tumour necrosis factor alpha and interleukin-8 (IL-8) along with the activation of toll like receptor (TLR)-4 and TLR-9, resulting in production of IL-1 $\beta$  by Kupffer cells, which are star-shaped (stellate) phagocytic cells located in the liver. IL-1 $\beta$  promotes lipid accumulation and apoptosis in hepatocytes, causing steatosis and inflammation, as well as activation of HSCs to produce fibrogenic mediators, and accelerate HCC establishment<sup>[89-92]</sup>. Furthermore, dysbiosis promotes the development of NAFLD-associated HCC by modifying bile acid metabolism. Specifically, alterations in the composition of the gut microbiota can result in higher levels of deoxycholic acid and the activation of its receptor farnesoid X receptor, which provokes a senescence-associated secretory phenotype in HSCs, resulting in the secretion of various inflammatory and tumour-promoting factors in the liver, thus promoting the development of HCCs<sup>[87,93]</sup> [Figure 1]. To summarise, the intestinal microbiota may promote the development of NAFLD-associated cirrhosis and HCC by increasing inflammatory cytokine secretion, activating TLR-4 and TLR-9 and modifying bile acid metabolism.

### Immunological pathways

Metabolic stress not only leads to increased ROS generation but also triggers the inflammatory responses, which are a pre-requisite for the progression of NASH-associated HCC. Insulin resistance and oxidative

stress are known to stimulate nuclear factor kappa-light-chain-enhancer of activated B-cells pathway, which promotes hepatocyte survival<sup>[94]</sup>. ROS and the products of lipid peroxidation stimulate the release of inflammatory cytokines including tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-6 from hepatic cells<sup>[95]</sup>. TNF- $\alpha$  is reported to promote hepatocellular carcinogenesis by activating hepatic progenitor cells<sup>[67]</sup>. IL-6 activates the signal transducer and activator of transcription 3, an oncogenic transcription factor that induces cell proliferation, inhibits apoptotic pathways and may be involved in the development of NASH-associated HCC<sup>[96]</sup> [Figure 1]. Similarly, other cytokines such as IL-17A and IL-11 have also been implicated in NASH and NASH-associated HCC<sup>[97-99]</sup>.

Cellular injury activates the hedgehog signalling pathway, which is involved in repair and regeneration in the liver and replaces damaged hepatocyte. The Hedgehog signalling pathway is implicated in fibrogenic activation and hepatocellular ballooning<sup>[100]</sup>, features associated with the advancement of NASH. Impairment of the Hedgehog pathway also leads to dysregulation of cell repair mechanisms and promotes the malignant transformation involved in the progression of HCC<sup>[101]</sup>. The TGF- $\beta$  signalling also mediates the progression of fibrogenesis through regulation of cell death and lipid metabolism in NASH<sup>[102,103]</sup> [Figure 1].

The role of CD8+ and CD4+ T lymphocytes in hepatocyte damage and carcinogenesis has been studied in various animal models<sup>[104]</sup>. In mouse models and human samples, dysregulation of lipid metabolism in NAFLD causes a selective loss of intrahepatic CD4+ but not CD8+ T lymphocytes, leading to increased inflammation and accelerated development of HCCs<sup>[105]</sup>. Platelet cargo, platelet adhesion and platelet activation appear to be pivotal for NASH and subsequent hepatocarcinogenesis. In particular, platelet GPIb $\alpha$  is a mediator of hepatic immune cell trafficking and antiplatelet therapy (e.g., aspirin/clopidogrel and ticagrelor) has been demonstrated to prevent NASH and subsequent HCC development<sup>[106]</sup>. One recently published study used a new zebra fish model and reported that a high fat diet promotes non-resolving inflammation in the liver and enhances cancer progression<sup>[107]</sup>. The authors found that metformin inhibits high fat diet-induced HCC progression, by reducing inflammation and restoring tumour surveillance<sup>[107]</sup>.

### Endocrine pathways

Several hormones play important roles in the pathogenesis of NAFLD and its consequent progression to HCC. One of the most crucial in these is insulin resistance and hyperinsulinemia, which is an associated feature of NAFLD<sup>[108]</sup>. Insulin resistance and hyperinsulinemia are known to increase the expression of insulin and insulin-like growth factor-1 (IGF-1). Insulin and IGF-1 trigger signalling cascades by binding to their receptors, namely the insulin receptor and the IGF-1 receptor, to activate the PI3K and MAPK pathways, which are crucial in the pathogenesis of HCC since they induce cell proliferation and inhibit apoptosis<sup>[109]</sup>. In particular, the PI3K pathway mediates the progression of HCC by cyclin D1-dependent control of the cell cycle, mTOR dependent cellular growth and mouse double minute 2 homolog Mdm2/p53-dependent apoptosis<sup>[110]</sup>. Interestingly, there can be cross-talk between other signalling pathways and PI3K-mediated signalling; e.g., hippo signalling suppresses IRS2/Akt-mediated HCC development in rodent models<sup>[111]</sup>. Activation of the MAPK pathway by insulin resistance induces the expression of proto-oncogenes, c-fos, and c-jun, and promotes hepatic fibrosis and carcinogenesis by activating the Wnt/ $\beta$ -catenin signalling cascade<sup>[112]</sup> [Figure 1].

NAFLD is also associated with increased circulating levels of leptin<sup>[113]</sup>. Leptin initiates intracellular signalling of pro-inflammatory cytokines such as TNF- $\alpha$  and IL-6 and activates JAK2/STAT, MAPK and PI3K signalling pathways by binding to its receptor in HCC cells<sup>[114]</sup>. Furthermore, leptins are known to upregulate hTERT expression, leading to the immortalisation of HCC cells<sup>[115]</sup>. NAFLD is also associated with decreased sensitivity to thyroid hormones<sup>[116]</sup> and both NAFLD and HCC are associated with hypothyroidism in humans<sup>[117,118]</sup>. Furthermore, thyroid hormone treatment decreases hepatosteatosis and

the progression of NAFLD in both rodents and humans<sup>[119]</sup>. Several new studies provide evidence for a potential role of androgen and the androgen receptor pathway in the development of NASH-related HCC and in the treatment of HCC<sup>[120]</sup>. Gender disparity exists in the incidence of NAFLD associated HCC<sup>[121]</sup>. Intriguingly, although males are more likely to develop both NAFLD and HCC than females, after the age of 60 this trend is reversed<sup>[122-124]</sup>. This has been attributed to the loss of the protective effects of oestrogen in females<sup>[125]</sup>. Besides hormonal stimuli, deregulation of hepatic circadian clock genes also significantly contributes towards the progression of NAFLD to HCC<sup>[126]</sup>.

## **FUTURE DIRECTIONS AND CONCLUSIONS**

Studies from clinical and basic research have provided a better understanding of the aetiology of NASH-associated HCC. Data from various studies reveal that the co-ordinated actions of genetic instability, impaired lipid metabolism, increased oxidative stress altered lipid metabolism, hepatocyte apoptosis, inflammation, fibrosis and altered hormone signalling contribute to the development of HCC. These pathways likely act simultaneously and in combination to activate genetic and epigenetic mechanisms that cause progression of NAFLD and promote the development of NAFLD/NASH-associated HCC. At the clinical level, currently, it is not possible to determine which patients with NASH are most prone to develop HCC. Further studies are required to identify the patients who are at a risk of developing HCC. The identification of specific biomarkers is essential for predicting the transition from NASH to HCC. Currently, there are no pharmacological therapies for the prevention or treatment of NASH and NASH-associated HCC, thus understanding the mechanisms for the pathogenesis of these conditions may lead to the development of novel therapies. Anti-fibrotic, anti-diabetic, anti-inflammatory, antibiotics/probiotics and lipid-lowering drugs either alone or in combination could hold promise for the treatment for NAFLD/NASH-associated HCC.

## **DECLARATIONS**

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### **Authors' contributions**

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Critical revision of the manuscript for important intellectual content: Raza S, Sinha RA

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### **Conflicts of interest**

All authors declared that there are no conflicts of interest.

### **Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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Review

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# Epidemiology of hepatocellular carcinoma in nonalcoholic fatty liver disease

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## Abstract

Along with the changes in our food culture and lifestyle, conditions such as obesity, diabetes mellitus, and metabolic syndrome have been on the rise, and the incidence of nonalcoholic fatty liver disease (NAFLD), which is closely related to these diseases, has also increased rapidly. Despite being a risk factor for the development of hepatocellular carcinoma (HCC), NAFLD has no established screening method, and HCC originating from NAFLD often tends to be discovered in its advanced and symptomatic stages, which has become an important clinical problem. Even though the carcinogenicity rate among the entire population of NAFLD patients is not high compared to that of patients with viral hepatitis, since HCC also often develops from non-cirrhotic livers, it is difficult to narrow down the cases that need to be under surveillance. Going forward, it will be important to clarify the clinical characteristics and genetic background of NAFLD-related HCC and establish not only a useful surveillance method but also preventive methods.

**Keywords:** Hepatocellular carcinoma, nonalcoholic fatty liver disease, epidemiology

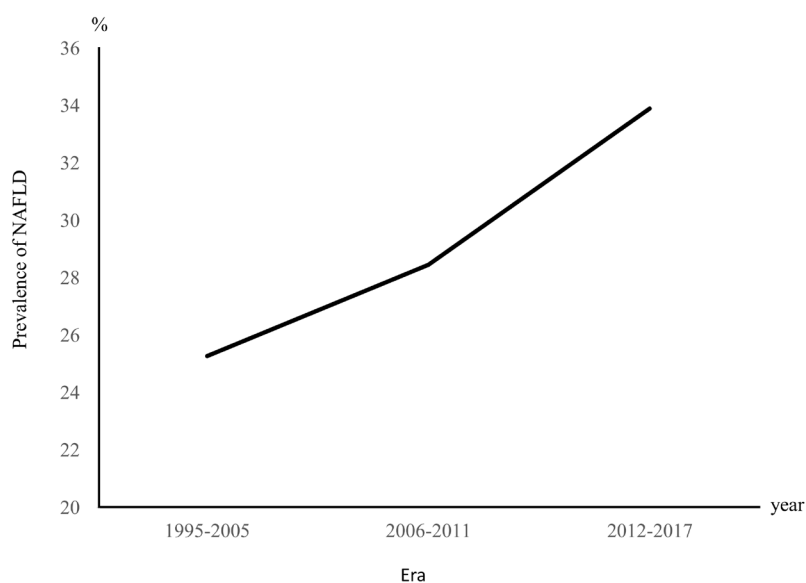
## INTRODUCTION

Hepatocellular carcinoma (HCC) is the seventh most common type of cancer worldwide and the second most common cause of cancer-related death<sup>[1]</sup>. Although the majority of cases are caused by viruses such as



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**Figure 1.** Trends in the prevalence of NAFLD in Asia Pacific regions. NAFLD: nonalcoholic fatty liver disease

**Table 1. Global prevalence of nonalcoholic fatty liver disease**

Region	Prevalence (%)
Global	24
US, middle eastern countries	30
Europe 14 countries	33
Asia-Pacific regions	
China	12.5-24.5
Japan	25
Korea	27.3
Taiwan	11.4

hepatitis B virus (HBV) and hepatitis C virus (HCV)<sup>[2,3]</sup>, there has been a rapid increase in the number of cases of HCC from nonviral causes<sup>[4,5]</sup>.

Although alcohol has been known to be an important nonviral cause of HCC, recent years have seen growing attention to nonalcoholic fatty liver disease (NAFLD) as an important cause of the condition<sup>[6]</sup>. The prevalence of NAFLD is closely related to the increase in the prevalence of obesity<sup>[7,8]</sup>, Type 2 diabetes mellitus (T2DM), hypertension, dyslipidemia, and metabolic syndrome (MetS)<sup>[9-11]</sup> and is increasing in both developed and developing nations, with approximately 30% of the world's population being affected<sup>[12]</sup>.

The prevalence of NAFLD is increasing worldwide [Table 1]<sup>[13]</sup>, and the trends in the Asia-Pacific region are similar [Figure 1]<sup>[14]</sup>. Particularly in the developing countries, the prevalence of NAFLD has recently increased due to an increase in caloric intake and a decrease in exercise owing to the westernization of lifestyles accompanying economic development<sup>[15]</sup>.

Histopathologically, NAFLD can be classified into nonalcoholic steatohepatitis (NASH) or nonalcoholic fatty liver (NAFL). NASH was defined as steatosis with lobular inflammation and ballooning degeneration, with or without Mallory-Denk bodies or fibrosis. Patients with simple steatosis or steatosis with non-specific inflammation were identified as NAFL<sup>[16]</sup>. It is estimated that NASH accounts for 20%-30% of NAFLD cases, and these cases are prone to advance to severe liver fibrosis and liver cirrhosis and have been found to develop into HCC<sup>[17]</sup>.

**Table 2. Summary of clinical features of patients with NAFLD hepatocellular carcinoma**

Incidence rate	NAFLD	Ref.[5,20-23]
	NAFLD with cirrhosis	Ref.[20,77]
	NAFLD without cirrhosis	Ref.[23,77]
	NASH	Ref.[22,77]
Age and sex	Higher incidence rate in older and male patients (compared with HCV-derived HCC)	
Complications	Obesity, type 2 diabetes mellitus, insulin resistance, cardiovascular disease, dyslipidemia, metabolic syndrome, <i>etc.</i>	
Race	Highest incidence rate in Hispanic patients, followed by Caucasian and African American patients	
Genetic elements	<i>PNPLA3</i> rs738409 SNP, <i>H63D</i> polymorphism, and <i>MBOAT7</i> rs641738 variant, <i>etc.</i>	
Other risks	Past history of drinking, iron, <i>etc.</i>	
Clinical features	Detection	Detected more often in the advanced stage and with symptoms outside of surveillance (compared with HCV-related HCC)
	Morphology	Larger tumor size, absence of encapsulation, and a more infiltrative characteristic (compared with HCV-related HCC)
	Tumor marker	Less frequently elevated AFP levels (compared with HCV-related HCC) and often elevated PIVKA-II levels
	Liver function	Relatively well preserved (compared with other etiologies)
	Background	Less advanced fibrosis (compared with HCV-related HCC)
	Prognosis	Controversial
	Prevention and treatment	Metformin, exercise
	One promising approach; prevention of the development of fibrosis: GLP-1 receptor antagonist	

NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; HCV: hepatitis C virus; HCC: hepatocellular carcinoma; PIVKA-II: prothrombin induced by vitamin K absence-II; GLP-1: glucagon like peptide-1; SNP: single-nucleotide polymorphism; AFP: alpha-fetoprotein

Although complete elimination of HBV is difficult, it has been possible to prevent the onset of cancer to some degree by suppressing the viral replication and calming the inflammation using nucleotide and nucleoside analogs<sup>[18]</sup>, while the emergence of direct-acting antiviral agents has made it possible to eliminate HCV in almost all cases, thereby reducing the risk of cancer<sup>[19]</sup>. Based on these clinical advancements, the incidence of viral-related HCC, especially HCV-related HCC, is likely to continue to decrease, while the incidence of NAFLD-related HCC is likely to increase due to the lifestyle changes mentioned above<sup>[4]</sup>.

This paper aims to provide a review of the literature regarding the epidemiology of NAFLD-related HCC and elucidate the problems and challenges in cases of NAFLD-related HCC that have been on the rise.

Table 2 shows a summary of the features of NAFLD HCC.

## INCIDENCE OF HCC IN PATIENTS WITH NAFLD

In recent years, there have been many reports suggesting that NAFLD is an important etiology of HCC. In the US, the Surveillance, Epidemiology, and End Results reported that, between 2004 and 2009, there was a 9% annual increase in NAFLD-related HCC cases<sup>[5]</sup>. The Global HCC BRIDGE Study showed that 10%-12% of cases in North America/Europe and 1%-6% of cases in Asia diagnosed as HCC were caused by NAFLD<sup>[20]</sup>. Moreover, in Japan, the percentage of HCC patients with a nonviral etiology has increased from 10.0% in 1991 to 24.1% in 2010, which consolidates the observation that there is an increase in the number of NAFLD-related HCC cases<sup>[4]</sup>.

The 130-facility cohort of the US Veterans Health Administration showed that the risk of HCC onset in NAFLD cases was 0%-38% over 5-10 years of observation, and showed that, when adjusted for the patients' race and MetS characteristics, NAFLD patients had greater annual risk of developing HCC than the controls [0.21/1000 vs. 0.02/1000 person-years (PYs); hazard ratio (HR): 7.62; 95% confidence interval (CI): 5.76-10.1]<sup>[21]</sup>. Furthermore, the estimated annual incidence rate of HCC derived from NASH, which is an

advanced form of NAFLD, is reported to be 5.29 per 1000 person-years (95%CI: 0.75-37.56)<sup>[22]</sup>, whereas the incidence rate of HCC in NAFLD patients with cirrhosis is reported to be 10.6 people per 1000 PYs<sup>[21]</sup>. In contrast, investigations in Japan revealed that approximately 32% of NAFLD-related HCC cases did not have cirrhosis, which may be a characteristic of NAFLD-related HCC<sup>[23]</sup>.

## CLINICAL FEATURES OF NAFLD-RELATED HCC

Unlike other etiologies, NAFLD-HCC is generally characterized by a large tumor size, moderately to highly differentiated histology, and absence of encapsulation<sup>[24]</sup> and is often discovered in the advanced stages of the disease<sup>[5,25]</sup>. Furthermore, NAFLD-related HCC is more infiltrative than HCV-related HCC, and often tends to be detected outside of surveillance<sup>[26]</sup>.

There have been reports comparing HCV-related HCC and NASH-related HCC that have shown NASH-related HCC occurs at older age than HCV-related HCC<sup>[27]</sup>, and the prevalence of obesity, T2DM, and dyslipidemia is greater in NASH-related HCC<sup>[28]</sup>. Furthermore, although an elevated alpha-fetoprotein (AFP) level is observed in 69.6% of HCV-related HCC patients<sup>[29]</sup>, this occurs in < 1/3 of NASH-related HCC patients<sup>[28]</sup>, and an elevated prothrombin induced by vitamin K absence-II (PIVKA-II) level is relatively common in NAFLD-related HCC patients<sup>[30]</sup>.

Strategies have been provided to treat HCC, regardless of its etiology<sup>[31]</sup>, and there have been various reports related to the treatment results and prognosis. It has been reported that the percentage of patients who were able to receive curative treatments such as liver resection, including liver transplant, was lower for NAFLD-related HCC than HCV-related HCC (NAFLD-related HCC: 21/212 *vs.* HCV-related HCC: 80/275)<sup>[27]</sup> and other etiologies of HCC<sup>[32]</sup>. In contrast, NAFLD-related HCC patients had a low cirrhosis prevalence, liver functions such as the synthetic capacity were relatively well preserved<sup>[32,33]</sup>, and liver resection rates were higher than those of HCV-related HCC<sup>[26,33]</sup>. However, as NAFLD-related HCC occurred at an advanced age and patients often had cardiovascular and metabolic complications, there was no difference in the overall survival rate between NAFLD-related HCC (one year: 56%; three years: 23%) and HCV-related HCC (one year: 58%; three years: 21%)<sup>[27]</sup>. In some reports, the overall survival of NAFLD-related HCC patients was lower than that of HCV-related HCC patients<sup>[29,32]</sup>. Conversely, there are reports suggesting that the relapse-free survival rate was high after curative resection of NAFLD-related HCC<sup>[34]</sup> and that the overall survival was nearly the same or greater than that for HCV-related HCC or alcoholic cirrhosis-related HCC<sup>[35,36]</sup>, thus a consensus has not been obtained.

## Link to obesity and T2DM

NAFLD is strongly related to insulin resistance, MetS, and cardiovascular disease<sup>[9-11]</sup>. In the UK, it was reported that the increase in cancer incidence and cases attributed to NAFLD occur in parallel with the steady increase in MetS incidence observed among HCC patients<sup>[25]</sup>. In particular, T2DM and obesity are closely related to NAFLD/NASH, and there are concerns that HCC will increase in the future<sup>[37]</sup>.

It has been reported that up to 70% of T2DM patients and up to 90% of patients with obesity have NAFLD<sup>[37,38]</sup>. Furthermore, a high percentage of patients with T2DM and obesity have advanced fibrosis<sup>[39-43]</sup>. The emergence of T2DM occurs in parallel with fibrosis, and the increase or decrease in body mass index (BMI) over time is related to the progression or improvement of liver fibrosis in NAFLD patients<sup>[39-41,43]</sup>.

## Obesity

Obesity has been increasing globally for the past several decades along with the changes in the food and lifestyle culture. HCC has been increasing among patients with obesity, and a perspective study involving a US population showed that the relative risk (RR) of death in patients with obesity grade II and I is 4.52 and



1.90, respectively<sup>[35]</sup>. There have been other reports linking HCC and obesity. In a prospective cohort study in Europe, general obesity (RR: 2.19) and abdominal obesity (RR: 2.03) were reported to be related to the risk of HCC<sup>[44]</sup>. Compared to the normal body weight, the RR of HCC was 1.17 (95%CI: 1.02-1.34) in those who are overweight and 1.89 (95%CI: 1.51-2.36) in those who are obese<sup>[45]</sup>.

In terms of the relationship between BMI and HCC, a study cohort in Italy showed that the RR of HCC onset for BMI > 30 kg/m<sup>2</sup> was 1.97 times higher<sup>[46]</sup>. Studies in South Korea showed that it was 1.56 times higher for BMI > 30 kg/m<sup>2</sup><sup>[47]</sup>. Other studies showed, as mentioned above, that it was 1.13 for BMI of 25-29.9 kg/m<sup>2</sup> and increased to 4.52 for BMI between 35 and 39.9 kg/m<sup>2</sup><sup>[35]</sup>. Furthermore, a meta-analysis of 11 cohort studies showed that an increase in BMI by 5 kg/m<sup>2</sup> increases the risk of HCC by 24%<sup>[45]</sup>. Furthermore, the European Prospective Investigation into Cancer reported that the waist-hip ratio and a rough estimate of abdominal fat are good prognostic factors of HCC<sup>[44]</sup> and suggests that the assessment of fat deposition is just as important as assessing BMI. It was also reported that obesity during early adulthood is a risk factor of HCC and that the increase in BMI during early adulthood speeds up the onset of HCC<sup>[48]</sup>.

### T2DM

HCC has been increasing among T2DM patients<sup>[49,50]</sup>. An epidemiological study in the US on the RR of HCC in T2DM patients showed that the risk of HCC increased by 2.87 times (95%CI: 2.49-3.30) due to T2DM<sup>[51]</sup>. A multicenter case-control study in Italy reported that the risk of HCC due to T2DM had an odds ratio of 4.33 (95%CI: 1.89-9.86)<sup>[36]</sup>. Furthermore, examination of non-HCV HCC cases showed that the risk of HCC in T2DM patients is twice as high<sup>[52]</sup>, and the risk of developing HCC due to T2DM when there is no liver cirrhosis is 1.353 times higher (95%CI: 1.249-1.465)<sup>[53]</sup>, whereas a history of T2DM is also a risk factor (HR: 2.14, 95%CI: 1.69-2.71)<sup>[54]</sup>. Furthermore, a meta-analysis showed that HCC prevalence and incidence rates increase by 2.5 times due to T2DM<sup>[55]</sup>.

T2DM is mediated by insulin resistance, and the subsequent inflammatory cascade is thought to be involved in the progression of the condition to NAFLD and HCC<sup>[55-58]</sup>. With respect to the relationship between NAFLD-related HCC and T2DM, it was reported that, while the prevalence of T2DM in HCV-related HCC patients was 24.9%, the prevalence was 73.1% in NAFLD-related HCC patients, which suggests a strong relationship between the two<sup>[59]</sup>.

### Age and sex

Incident rate of HCC is high in men regardless of etiology including NAFLD<sup>[60]</sup>. A study in the US suggests that the incidence rate of HCC is higher in men than in women (0.22 vs. 0.04 per 1000 PYs), whereas the incidence rate of HCC in NAFLD patients was found to be higher in patients aged ≥ 65 years than in younger patients [0.41 vs. 0.01 (< 45 years) and 0.02 (45-64 years) per 1000 PYs]<sup>[21]</sup>. As mentioned above, BMI is related to the onset of HCC, and, although UK studies have shown a positive correlation between BMI and HCC (HR: 1.19, 99%CI: 1.12-1.27), this relationship was reportedly more profound in men, in whom the risk of HCC increases linearly from a BMI of > 22 kg/m<sup>2</sup><sup>[61]</sup>. The severity of NAFLD and the level of progression of fibrosis are risk factors of HCC development. In the young, NASH is more prominent in males, while, in older patients (> 50 years), it is more common in women and the severity of NASH is higher in women as well<sup>[60,62]</sup>. Furthermore, a cross-sectional study on NAFLD reported that increasing age is correlated with the severity of fibrosis in NASH patients<sup>[63-65]</sup>.

### Race and genetic elements

Although it has been reported that there is no difference in the extent of liver damage between Hispanic and Caucasian patients with NAFLD<sup>[66,67]</sup>, the incidence of HCC was highest in Hispanic patients (0.29 per 1000 PYs), followed by Caucasian patients (0.21 per 1000 PYs) and African American patients (0.12 per 1000

PYs)<sup>[21]</sup>. However, US-born Hispanic patients had higher HCC incidence rates than Hispanic patients born outside of the US<sup>[68]</sup>, which suggests the importance of other risk factors such as the environment, lifestyle habits, and MetS, in addition to polymorphism of the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) gene.

*PNPLA3* rs738409 single-nucleotide polymorphism, which is a risk factor of steatosis, NASH, and fibrosis<sup>[69]</sup>, is also a risk factor of HCC (odds ratio: 1.40)<sup>[70]</sup>. Furthermore, the risk allele “G” is observed in 40% of the European population, and it reportedly increases the risk of HCC by approximately 12 times<sup>[71]</sup>. In HCC patients, GG homozygosity is related to early onset (at young age), background liver disease with short cirrhotic history or less fibrosis, diffuse-type HCC, and poor prognosis<sup>[71]</sup>.

In addition, it has been reported that the *H63D* gene, which is a common polymorphism of human hereditary hemochromatosis, is related to the risk of non-cirrhotic HCC incidence in Africans<sup>[72]</sup>, whereas the membrane-bound O-acyltransferase (*MBOAT7*) rs641738 variant is reportedly related to NAFLD-related HCC with non-advanced fibrosis<sup>[73]</sup>.

### Others

Previous alcohol intake and iron level in hepatocytes have been reported as risk factors of HCC in NASH patients<sup>[74,75]</sup>, with the increase in hepatocellular iron levels being related to advanced fibrosis in NAFLD<sup>[76]</sup>. A study in Italy compared 51 NASH cirrhosis-related HCC patients against 102 patients without HCC and found that hepatocytes staining positive to iron were in significantly greater quantity in the HCC cohort than in the non-HCC cohort<sup>[75]</sup>.

### HCC in NAFLD with cirrhosis

As mentioned above, a study that examined 296,707 NAFLD patients showed that 490 patients developed HCC (0.21 per 1000 PYs), and the incidence rate of HCC was significantly higher than that of the control group (0.02 per 1000 PYs, HR: 7.62, 95%CI: 5.76-10.09). Among the NAFLD patients, those with cirrhosis had the highest annual incidence rate of HCC (10.6 per 1000 PYs), and 1.6-23.7 people per 1000 PYs were at risk<sup>[21]</sup>. In another report, the incidence of NAFLD patients with cirrhosis who developed HCC was from 2.4% at seven years to 12.8% over three years<sup>[77]</sup>, which was marginally lower than the 4% annual incidence of cancer observed in cases of cirrhosis caused by HCV<sup>[22,77]</sup>.

With respect to the relationship between the indicators of fibrosis and development of cancer from NAFLD, patients with a cirrhosis diagnosis and a high Fibrosis-4 (FIB-4) score had the greatest risk of HCC (13.5 per 1000 PYs), whereas patients without a cirrhosis diagnosis and a low FIB-4 score had a low risk of HCC development (0.04 per 1000 PYs)<sup>[21]</sup>. A separate study showed that a high NAFLD fibrosis score and a high FIB-4 score were strongly related to the incidence of HCC<sup>[78]</sup>. Furthermore, sex (male), age ( $\geq 70$  years), T2DM, and high blood pressure have been reported as risk factors of HCC in NAFLD patients with cirrhosis<sup>[30]</sup>.

### Relationship between cryptogenic cirrhosis and NAFLD

Liver cirrhosis is the most common cause of HCC, and 80%-90% of HCC patients have had cirrhosis<sup>[79]</sup>. Although viruses are common origins of liver cirrhosis, it has been reported that, in 6.9%-50% of HCC cases, the etiology of liver disease could not be determined<sup>[80-82]</sup>. A prospective US study<sup>[82]</sup> showed that cryptogenic cirrhosis (CC) is responsible for up to 29% of the etiology of HCC. Half of these patients had histological and clinical features of NAFLD, and another retrospective study showed that HCC patients with CC had a greater prevalence of T2DM and obesity than those who developed the condition from a virus or alcoholic cirrhosis<sup>[83]</sup>. Given its similarity to NASH cirrhosis, a strong correlation between CC

and NAFLD was suggested, and many of the CC cases were severely advanced NASH, that is, burned-out NASH<sup>[83-86]</sup>.

Based on the abovementioned data, it is speculated that the role of NAFLD in HCC etiology is greater than the data that have been reported, and the existence of burned-out NASH is a point to be noted in epidemiological research related to NAFLD-related HCC.

### HCC in NAFLD without cirrhosis

Several cross-sectional studies showed that 15%-50% of patients diagnosed with HCC without cirrhosis were patients with non-cirrhotic NAFLD<sup>[35,87-89]</sup>. This suggests the possibility that NAFLD is an independent risk factor of HCC, even in those cases without cirrhosis<sup>[82,90,91]</sup>.

There has been an increase in the number of cases of HCC that developed in NAFLD patients without cirrhosis<sup>[92,93]</sup>. The characteristics of NAFLD-related HCC without cirrhosis include a larger tumor size<sup>[94-96]</sup>, older age, and slightly lower prevalence of T2DM than those of NAFLD-related HCC patients with cirrhosis<sup>[94]</sup>. In a recent meta-analysis of a cohort of NAFLD patients without cirrhosis, the cumulative HCC mortality for the study periods of up to 20 years was between 0% and 3%<sup>[77]</sup>.

In the cohort study mentioned above, approximately 20% of NAFLD-related HCC patients did not have cirrhosis<sup>[21]</sup>, and NAFLD patients without cirrhosis had an annual HCC incidence of 0.08 per 1000 PYs (vs. 0.02 per 1000 PYs in the control group without NAFLD), whereas reports from Japan also suggested that approximately 32%<sup>[22]</sup> to 49% (28% being in stages 1-2 of fibrosis)<sup>[30]</sup> of NAFLD-related HCC cases had no cirrhosis. A separate study reported that 10%-75% of NAFLD-related HCC patients had no cirrhosis in their background<sup>[87,89,97]</sup>, suggesting that NASH itself can promote the development of HCC and that HCC can develop from NASH and simple steatosis without fibrosis<sup>[33]</sup>.

As a mechanism of how HCC develops in NAFLD patients without cirrhosis, the possibility of transformation of hepatocellular adenoma (HCA) comes to mind. NAFLD is strongly correlated to obesity, MetS, and T2DM, among others<sup>[30]</sup>. Furthermore, it has also been reported that there is a relationship between the prevalence of obesity/MetS and HCA (particularly related to the subtype that has a risk of malignant transformation: inflammatory HCA)<sup>[98,99]</sup>, which seems to support this possibility. Furthermore, as a result of a recent study, some of the cases of HCC developing in NAFLD patients show steatosis, ballooning, Mallory bodies, and pericellular fibrosis in its histological presentation, and there is also a characteristic subtype called steatohepatitic HCC that resembles steatohepatitis<sup>[100]</sup>, which suggests that there is a close relationship between non-cirrhotic NAFLD and development of HCC.

### CLINICAL ISSUES AND CHALLENGES IN HCC SURVEILLANCE OF NAFLD PATIENTS

With the increase in NAFLD prevalence, there has been an increase in the prevalence of NAFLD-related HCC, albeit not as high as that of viral-related HCC, which has led to the increasing importance of surveillance.

Even though the AASLD (American Association for the Study of Liver Diseases) and EASL-EORTC (The European Association for the Study of the Liver-The European Organisation for Research and Treatment of Cancer) Guidelines recommend patients with cirrhosis to be screened for HCC every six months<sup>[101]</sup>, HCC surveillance of NAFLD-cirrhosis cases is included under "Other conditions" in the AASLD Guidelines<sup>[102]</sup>, and there are no specific recommendations. In general, screening is performed using ultrasound examinations, but there are limitations of using this approach for patients with obesity<sup>[103,104]</sup>. Although magnetic resonance imaging scans provide excellent lesion detectability, it is difficult to recommend this approach for screening due to its high cost and availability issues.

In contrast, since the carcinogenic risk is not high in non-cirrhotic NAFLD, and because only 23% of all NAFLD-related HCC cases are detected by screening and 62.3% of cases are found already symptomatic<sup>[25]</sup>, the cost-effectiveness of HCC surveillance for non-cirrhotic NAFLD is poor. Furthermore, it is not at a level to be recommended. For this reason, tools and biomarkers that help to narrow down high-risk populations of cancer development is important for HCC surveillance among patients with non-cirrhotic NAFLD.

The problem of HCC surveillance in NAFLD patients is to narrow down those patients who should be screened. The most important approach would be to distinguish whether the patient exhibits severe fibrosis and cirrhosis, which indicate a risk of HCC development. The gold standard for the diagnosis of fibrosis is liver biopsy, but it involves the issue of invasiveness, and, in recent years, the problem of sampling error has also been reported. To narrow down severe fibrosis populations, the use of the aspartate aminotransferase-to-platelet ratio index (APRI)<sup>[105]</sup>, NAFLD fibrosis score<sup>[106]</sup>, and FIB-4 index<sup>[107]</sup> have been reported as a simple approach. There are also reports related to the usefulness of special ultrasound tests such as Fibroscan<sup>[108]</sup>.

In recent years, it has been reported that a scoring system based on age, sex, T2DM or viral hepatitis history, aminotransferase, and AFP is useful regardless of the etiology of the condition<sup>[109]</sup>, and we may need to consider whether it can be introduced in the surveillance of NAFLD patients.

Furthermore, in view of the differences observed between races, it may be useful to actively screen more Hispanic populations, who are at a high risk of developing NAFLD-related HCC. Additionally, although it may be useful to use *PNPLA3* rs738409 polymorphism for screening from a genetic point of view, it would be difficult to introduce this approach to the surveillance procedures at this stage, when we take into consideration the cost<sup>[110]</sup>.

On the other hand, while HCC is often detected using ultrasound and AFP tests, the frequency of high AFP levels in NAFLD-related HCC cases is not as high as that in HCV-related HCC cases, and there are also reports suggesting that there are many cases with high PIVKA-II levels. As such, it may be one approach to introduce the evaluation of PIVKA-II levels to the surveillance process.

## PREVENTION AND TREATMENT

At present, it is important to prevent progression of NAFLD as early as possible by improving lifestyle; this prevents the development of NAFLD-related HCC. It has also been reported that exercise has a preventive effect on the development of HCC<sup>[111,112]</sup> and that exercising for  $\geq 5$  days per week reduces the RR of HCC to 0.56<sup>[113]</sup>. Conversely, certain recent reports have suggested the possibility of therapy with drugs such as GLP-1 receptor antagonists and metformin.

### GLP-1 receptor antagonist

Fibrosis is a risk factor of cancer, and one target should be to prevent the development of fibrosis. The use of drugs targeting dyslipidemia, insulin resistance, oxidative stress, inflammatory cytokines, apoptosis, and the angiotensin pathway, among others, has been explored<sup>[114]</sup>, but, until now, no definite drug therapy has been established. However, reports from a phase 2 trial suggest that glucagon-like peptide-1 (GLP-1) receptor antagonists may prevent the occurrence of HCC by ameliorating liver fibrosis in NAFLD patients<sup>[115]</sup>. Phase 3 trials are expected to be conducted in the future.

### Metformin

The use of metformin is related to the decrease in HCC incidence in T2DM patients<sup>[116-121]</sup>, and a meta-analysis showed that the use of metformin in T2DM patients reduced the risk of HCC by 70%<sup>[122]</sup> or

50%<sup>[119]</sup>. The mechanism of this action is reportedly via AMP-activated protein kinase activation<sup>[123]</sup> and mammalian target of rapamycin inhibition<sup>[124]</sup>. It has also been reported in prospective studies that the use of metformin in patients with cirrhosis increased survival<sup>[125]</sup> and that it improved the outcome of radiofrequency ablation treatments given to HCC patients<sup>[126]</sup>. On the other hand, insulin and sulfonyl preparations have been shown to increase the risk of HCC<sup>[127,128]</sup>.

## CONCLUSION

There has been a dramatic increase in the number of NAFLD patients, which is closely related to obesity, T2DM, and MetS, as our food and lifestyle habits change. Although the risk of HCC development is not as high as that due to viral hepatitis, because of the large population of patients with NAFLD, there has been an increase in HCC developing from NAFLD. NAFLD-related HCC is often discovered in its advanced stages, and, due to problems of low detectability by ultrasound examinations due to patients being obese and the development of the condition in non-cirrhotic livers, there is yet to be an effective method of surveillance. At present, there are no epidemiological data that can help overcome these challenges. In the future, there will be a need for studies focusing particularly on HCC surveillance in non-cirrhotic NAFLD. Immediate efforts toward early detection and improvement of treatment of NAFLD-related HCC will be important, such as enlightenment of and cooperation with medical professionals working on patients with obesity, T2DM, MetS, and cardiovascular disease, in addition to educational activities for NAFLD patients. Moreover, as weight gain during early adulthood is a risk factor of HCC, it is important to raise awareness of this matter through school education. On the other hand, regarding medical treatment, there are currently no therapeutic drugs that have been shown to be effective; however, some drugs have promise. Metformin is associated with a decrease in the incidence of HCC in T2DM, which is closely related to NAFLD. Furthermore, a phase 2 trial has provided evidence that GLP-1 receptor antagonists ameliorate liver fibrosis, which is considered to predispose individuals with NAFLD to developing HCC. The efficacy of GLP-1 receptor antagonists will be determined in phase 3 trials.

## DECLARATIONS

### Authors' contributions

Study concept and design, literature search, drafting of the manuscript: Moriguchi M, Seko Y, Takahashi A  
Supervision of the project: Itoh Y

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Not applicable.

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All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

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Review

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# Technical update on transcatheter arterial chemoembolization

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## Abstract

Transcatheter arterial chemoembolization has become an established drug delivery system for palliative or bridging treatment of hepatocellular carcinoma. Over the last two decades, various research and developments have taken place to improve the transcatheter arterial chemoembolization procedure from both a clinical and a technical perspective. This review article aims to provide an update on the technical developments over the last decade.

**Keywords:** Transcatheter arterial chemoembolization, Doxorubicin, bead, cisplatin

## INTRODUCTION

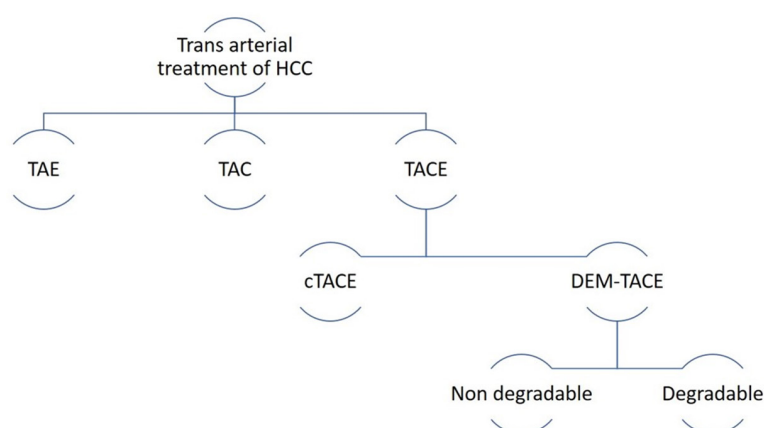
Since its first introduction in the late 1970s, transcatheter arterial chemoembolization (TACE) has become an established drug delivery system for palliative or bridging treatment of hepatocellular carcinoma (HCC)<sup>[1,2]</sup>. Randomized controlled trials have shown a survival benefit in patients treated with TACE, compared to transcatheter arterial embolization (TAE) using bland agents with no additional chemotherapy<sup>[3-5]</sup>. TACE has also replaced trans-arterial chemotherapy (TAC), which delivered chemotherapy in isolation without vessel occlusion.

The liver has a dual blood supply via both the hepatic artery and the portal vein; TACE takes advantage of this dual blood supply. As 80%-90% of HCCs derive their blood supply from the hepatic artery, it therefore, becomes an ideal vessel to access and deliver both an embolic and a chemotherapeutic agent,



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**Figure 1.** A simplified classification of various transarterial image-guided treatment options for HCC. TAE is a bland embolization that is rarely used unless in an emergency for treating ruptured HCC. TAC is currently not used. TACE is the most commonly used technique with cisplatin or doxorubicin. HCC: hepatocellular carcinoma; TAE: transarterial embolization; TAC: transarterial chemotherapy; TACE: transcatheter arterial chemoembolization; cTACE: conventional transcatheter arterial chemoembolization; DEM-TACE: drug-eluting microsphere-transcatheter arterial chemoembolization

leading to tumor ischemia, necrosis, and growth control<sup>[6]</sup>. As most normal hepatocytes are supplied by the portal vein, embolizing via the hepatic artery minimizes collateral ischemic damage and reduction in liver function, and the chemotherapy agent is not affected by the first-pass metabolism, as it would be if administered orally or intravenously.

TACE can be technically classified as conventional (cTACE), which can be selective or less than selective, and drug-eluting microsphere (DEM-TACE), where the treatment is delivered as close to the tumor as possible by super-selective catheterization of the feeding arteries. DEM-TACE can be further subdivided based on the degradable nature of the microsphere [Figure 1].

cTACE is undertaken with lipiodol, a poppy seed oil-based contrast medium, causing transient ischemia, in which chemotherapy agents such as cisplatin, doxorubicin, or mitomycin are suspended as an emulsion. Due to the lack of Kupffer cells in the tumor, lipiodol has the benefit of being retained in the tumor for weeks, thus enabling post-procedural computed tomography (CT) evaluation of the tumor load. However, lipiodol can lead to severe pain requiring strong opioid analgesia. cTACE lacks the benefit of a sustained high drug level in the tumor and can also lead to systemic elevation of the drug levels. Post-embolization syndrome is more common with cTACE<sup>[7,8]</sup>. Due to the above disadvantages, DEM-TACE was introduced in 2006, which produced sustained tumor-selective drug delivery, limited systemic elevation of drug levels, and permanent feeding vessel embolization<sup>[9]</sup>. Fewer courses of TACE are required with DEM-TACE compared to cTACE<sup>[10]</sup>. There is no Level 1 evidence demonstrating superiority in efficacy between the two techniques; however, there are many single-center prospective cohort studies demonstrating a higher complete response and lower rate of progressive disease with DEM-TACE<sup>[11]</sup>.

## CURRENT INDICATIONS AND PATIENT SELECTION

Patient selection for TACE continues to depend on the tumor size, number, extrahepatic spread, liver function, portal vein involvement, and the patient's general performance status. Childs-Pugh score and Barcelona clinic liver criteria are used to select patients for the appropriate treatment<sup>[12]</sup>. A multidisciplinary team approach to consider a patient for TACE and pre-procedure patient counseling are important to ensure ideal patient selection. Table 1 summarizes the indications for TACE. Decompensated liver function, infiltrative HCC, untreatable AV fistula, renal dysfunction, and chemotherapy-related



**Table 1. Indications for transcatheter arterial chemoembolization**

Intermediate stage patients, BCLC-B (asymptomatic, multinodular tumors without vascular invasion or extrahepatic spread)
Patients tumor suitable for curative treatment but not eligible due to performance status
Disease recurrence after curative treatment by surgery or ablation
Bridging or downstaging while patient fulfills criteria for liver transplantation or donor becomes available
Downsizing tumor or reducing circulation to meet criteria for ablation

BCLC: barcelona clinic liver cancer



**Figure 2.** A: an oblique axial CTA multiple intensity projections reformat, showing the vascular path from the coeliac axis to the left lobe tumor; B, C: intraprocedural images pre- and post-embolization. Given the prior delineation of vascular anatomy, only two arterial angiograms were done, reducing contrast load and radiation exposure

contraindication are absolute contraindications. HCC size above 10 cm, portal hypertension with or without untreated varices, portal vein thrombosis, and biliary involvement are relative contraindications. The more infiltrative the tumor is into the vessels and bile ducts, the higher is the risk of complications. Cardiac failure is a contraindication for cTACE but not for DEM-TACE.

### TACE and liver transplantation

Unlike TACE, liver transplantation is curative in a select group of patients with HCC. TACE can be used as a bridging treatment to inhibit tumor progression in patients who are candidates for transplant while awaiting a suitable donor or fulfillment of transplant criteria<sup>[13,14]</sup>.

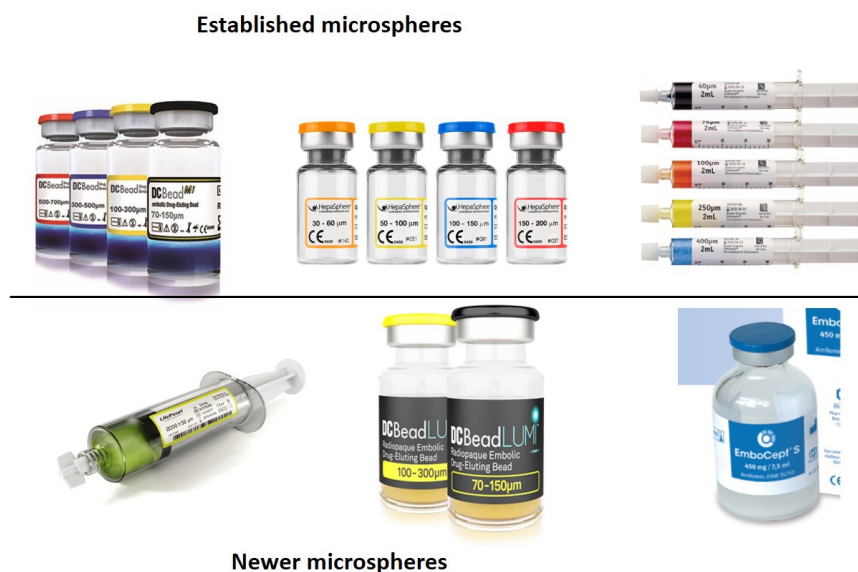
### TACE as an adjunct to other therapies

Increasingly, TACE is being used as an adjunct to reduce tumor size and vascularity to facilitate ablation techniques, such as radiofrequency, microwave, and cryotherapy. These ablation techniques can also be used after TACE for residual disease even if a patient was originally deemed suitable only for TACE<sup>[15-17]</sup>.

### PRE-PROCEDURE PATIENT MANAGEMENT

The preparation of a patient for TACE includes high-quality triple-phase post-contrast CT or magnetic resonance imaging to delineate the arterial anatomy and circulation to the tumor [Figure 2]. Besides, 4D CT can help reduce intra procedural volume of contrast and risk of nephrotoxicity. CIN (contrast-induced nephrotoxicity) is more common in larger tumors measuring above 5 cm in size<sup>[18-20]</sup>.

A review of the patient by the operator ahead of the procedure ensures the patient is being informed of the palliative, curative, or bridging nature of the procedure and its complications. For example, accidental damage to the main hepatic artery during TACE is a rare risk, which can make transplant challenging and rarely impossible.



**Figure 3.** The top row shows the established spheres. The bottom row shows the newer spheres currently coming into clinical use. Image courtesy of Biocompatibles UK Limited, Merit Medical USA and Terumo UK. Images of Embozene and Embocept obtained from free brochures on the Internet

Before the procedure, patients should be well hydrated. This is to reduce the risk of nephrotoxicity from iodinated contrast medium, tumor lysis syndrome, and dehydration due to a lack of fluid intake from post-procedure nausea or vomiting<sup>[19,20]</sup>. Due to the risk of infection and abscess formation, antibiotics for prophylaxis is a routine practice based on the local departmental or hospital rules<sup>[21,22]</sup>. Antibiotics, when used, should cover both Gram-positive, Gram-negative and anaerobic organisms and are recommended for all high-risk patient groups such as diabetics, immunosuppressed, *etc.* A mandatory up to date liver function test should be performed within a week of the TACE given the risk of liver ischemia and failure from the procedure. An echocardiogram of the heart is performed to assess the left ventricular function and to facilitate both patient selection and assess the impact of cytotoxins on the myocardium, especially if multiple episodes of treatment are being considered.

## CHEMOTHERAPY AND EMBOLIC AGENTS UPDATE

### Chemotherapy agents

Cisplatin and doxorubicin remain the routinely used chemotherapy agents for HCC. Other agents such as epirubicin and combinations have been tried with limited advantage<sup>[23,24]</sup>.

### Embolic agents

#### cTACE

Lipiodol is the agent used for cTACE. Lipiodol has a limited embolic property and causes transient ischemia. Further bland embolization with gel foam or Polyvinyl alcohol (PVA) is used to bring arterial flow to stasis. There has been no further development and a clinical alternative to lipiodol is not available. Cisplatin and doxorubicin are the routine chemotherapy agents used with lipiodol.

#### DEM-TACE

DEM-TACE uses a drug-eluting microsphere as embolic agents. The various spheres available and their advantages are listed in Table 2 and depicted in Figure 3. DC bead, HepaSphere, and Embozenes are polyvinyl alcohol-based. Life pearl is polyethylene glycol-based.

**Table 2. Various drug-eluting microspheres currently available in the market and their advantages**

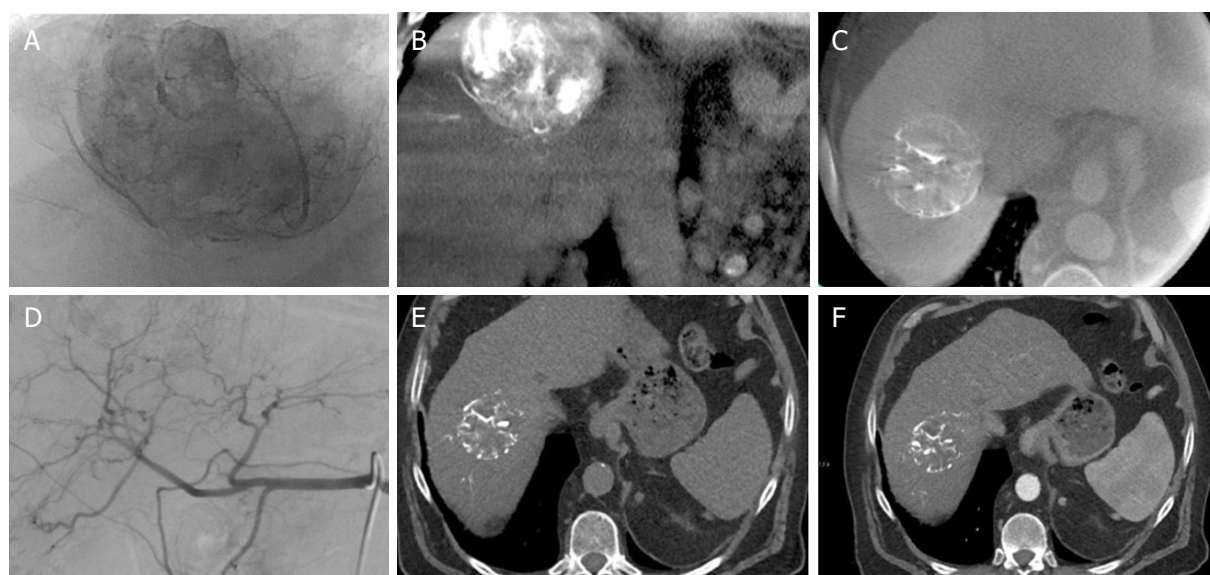
Types	Company	Structure	Available sizes (μm)	Advantages
DC Bead (EU) LC Bead (USA) M1 version is smaller size	BTG, London, UK (Now Boston Scientific)	Polyvinyl alcohol hydrogel modified with sulfonate groups	70-150 100-300 300-500 500-700	Largest data available, can be loaded before embolization and as a secondary action, will elute a local, controlled and sustained dose to the tumor after embolization
DC bead LUMI	BTG, London, UK (Now Boston Scientific)	As above and also, covalently bound radio-opaque moiety	70-150 100-300	Visibility on fluoroscopy and on table cone-beam CT
HepaSphere or QuadraSphere	Merit Medical, South Jordan, UT, USA	Poly (vinyl alcohol-co-sodium acrylate) hydrogel	Dry state 30-60 50-100 100-150 Hydrated state 120-240 200-400 400-600 600-800	Compresses by 80% but returns to shape and size becoming predictable and conformable. Entire sphere loads
Embozene TANDEM Oncozene	Varian Medical Systems, Inc. 3100 Hansen Way, USA	Hydrogel core made of sodium poly (methacrylate) and outer biocompatible shell of poly bis [trifluoroethoxy] phosphazene	Oncozene 40 ± 10 75 ± 15 100 ± 25 Embozene 40- 75 100 250 400 500 700 900	Tightly calibrated to enable more choices for embolization. Less than 5% size change on eluting
LifePearl	Terumo European Interventional Systems, Leuven, Belgium	Hydrogel network of poly ethylene glycol and 3-sulfopropyl acrylate	100 ± 25 200 ± 50 400 ± 50	Wide range of drug loading options Enhanced suspension characteristic. Tight calibration and longer suspension time
DSM – TACE EMBOCEPTc	PharmaCept GmbH, Berlin, Germany	Active ingredient – Amilomer DSM 35/50. Partly hydrolyzed starch, cross-linked and substituted with glycerol ether groups	50	Biodegradable. Tolerated better as less post embolization syndrome. Nonimmunogenic

CT: computed tomography; DSM: degradable starch microsphere; TACE: transcatheter arterial chemoembolization

These microspheres or beads are available in various sizes. A very small size bead usage in a large HCC stands the risk of shunting. Large size bead, on the other hand, can cause proximal occlusion without enough beads reaching the middle of the tumor. The size of the microsphere should be chosen based on tumor circulation<sup>[25,26]</sup>. Routinely, a non-degradable DC bead at 100-300 μm is our preferred size, which shrinks by 20% upon standing.

### DC bead

Consists of polymeric microspheres with the ability to encapsulate chemotherapeutic agents such as doxorubicin, irinotecan, and epirubicin with hydrogen ions, by electron attraction. It is manufactured by free radical polymerization of PVA with modification of sulfonate sodium to enable it to encapsulate the chemotherapeutic agent. DC beads have the most available clinical data and provide a sustained release of the drug. Patients with DC bead DEM-TACE treatments can receive a higher dose of doxorubicin without the undesired systematic circulation of injected drugs in comparison with cTACE<sup>[27]</sup>. Ninety percent of patients with unresectable HCC receiving DEM-TACE do not have hepatic artery damage with one- and two-year survival rates around 70% and 60%, respectively<sup>[23]</sup>.



**Figure 4.** A-C: fluoroscopy and non-contrast CBCT immediately and first LUMI-TACE demonstrate excellent uptake within the lesion with minimal non-target embolization; D: second LUMI-TACE Angiography showing feeding vessels supplying small areas of residual disease; E, F: unenhanced and arterial-phase axial computed tomography images one month following the second LUMI-TACE, demonstrating a complete response. Comparison with the unenhanced imaging is vital. Image courtesy of Dr. Peter Littler - consultant interventional radiologist, Freeman Hospital, Newcastle upon Tyne, UK

#### *HepaSpheres or QuadraSphere*

These microspheres are hydrophilic, calibrated, and can be compressed by 80%, facilitating a smooth transit in a microcatheter. They are small, soft, and easily conform to the vessel lumen for complete occlusion, enabling greater tumor necrosis<sup>[28,29]</sup>.

#### *LifePearl*

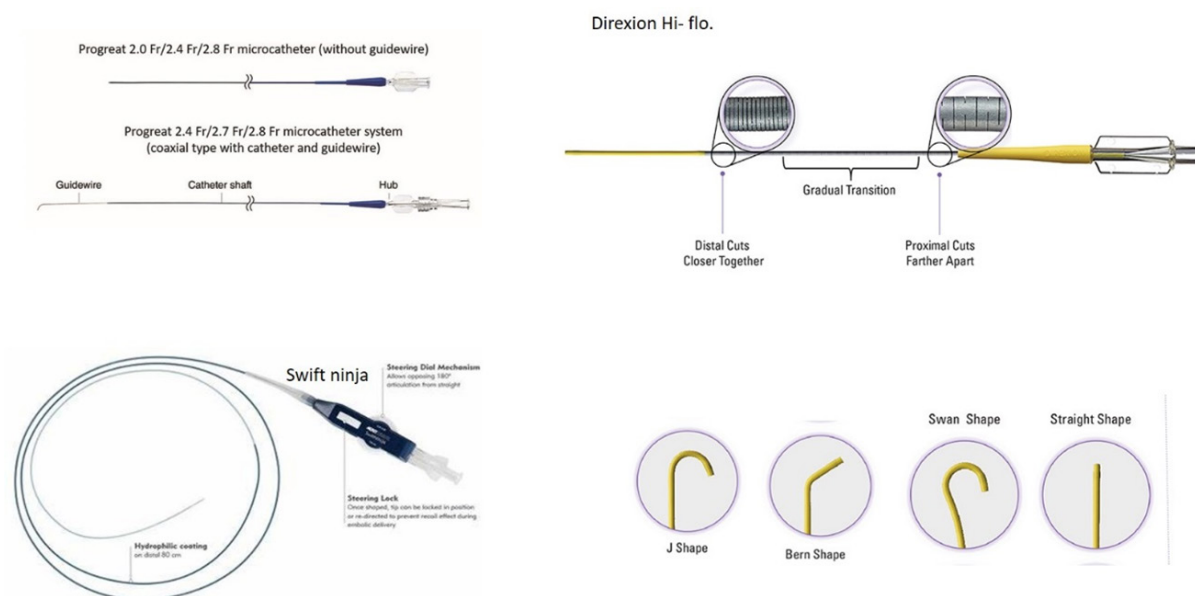
LifePearl is made from polyethylene glycol unlike the preceding three microspheres, which are made from polyvinyl alcohol. Polyethylene glycol offers a longer time in suspension than DC Bead and HepaSphere when loaded with doxorubicin and DC Bead and Tandem when loaded with irinotecan. Longer time in suspension enables a smoother embolization procedure without the need for any interruption to resuspend the microspheres<sup>[30]</sup>.

#### *Radio-opaque microspheres - DC or LC bead LUMI*

Classically, the microspheres or beads, after loading with the chemotherapy agent, are mixed with non-ionic contrast for direct fluoroscopic visualization. These beads do not retain contrast in tumor vessels and are washed out within minutes of the procedure. DC or LC Bead LUMI<sup>TM</sup> microspheres contain covalently bound iodine making them radio-opaque and enabling real-time assessment of the bead deposition in the HCC. The density and distribution of the radio-opaque beads can help accurately identify the embolization endpoint and the degree of flow stasis. Additionally, one can also visualize non-target reflux. Performing an on-table cone-beam non-contrast enhanced CT scan, immediately after embolization with LUMI beads, may provide important information about the completeness of treatment based on contrast retention<sup>[31]</sup>. During follow up imaging, it is essential to compare unenhanced with contrast-enhanced CT images to ensure accurate assessment of response, as shown in [Figure 4](#).

#### *Degradable starch microsphere-TACE*

Degradable starch microsphere (DSM) has an active ingredient called Amilomer, DSM 35/50. The starch microspheres are derived from partly hydrolyzed starch, which is cross-linked and substituted with



**Figure 5.** Various microcatheters with advanced properties such as coaxial wires, shapes, torque ability, and steerability. Image courtesy of Pro great - Terumo UK, Direccion Boston Scientific UK, Swift Ninja Merit Medical USA

glycerol ether groups. The microsphere is non-immunogenic and is prepared in a highly pure form of starch, which undergoes enzymatic degradation by  $\alpha$ -amylase. The degraded material is completely water-soluble. The DSM sphere is small at 50  $\mu\text{m}$  with a half-life of 35 min. There is reduced post-embolization syndrome with less pain and ischemic damage to the tumor-bearing organ. This makes it ideal for large tumors enabling therapeutic benefits for patients with repeated cycles and better tolerance<sup>[32,33]</sup>.

## NEWER INTRAPROCEDURAL ACCESSORIES

### Interventional kit

Compared to the 1980s and 1990s, super-selective catheterization techniques and catheter skills have evolved and become a routine for various transcatheter procedures. Selective catheterization with micro-catheters is routine, with the use of a 2.7 French and 2.4 French micro-catheter. More recently, 2.0 French, angled and steerable micro-catheters with or without coaxial wire systems have become readily available. As shown in Figure 5. Novel techniques of catheterization have also evolved such as side hole access via a balloon occlusion catheter<sup>[34,35]</sup>.

### On-table CT

Development of the hybrid CT/angiography system and C-arm cone-beam CT technology provides cross-sectional imaging as an adjunct to catheter angiography with or without intra-arterial contrast. This can be used with image fusion or co-registration with catheter angiogram to help localize and perform selective TACE<sup>[36-38]</sup>.

The LUMI beads are radio-opaque, enabling fluoroscopic visualization of bead deposition in the tumor, and are ideally suited to be visualized on the cone-beam on-table CT to assess for endpoints and plan further courses of TACE<sup>[31]</sup>.

### Radial access TACE

This approach is gaining popularity as an option for patients to choose between femoral and radial access, as shown in Figure 6. In the past, radial and brachial access TACE were used as alternative access sites in





**Figure 6.** Catheter angiogram before (A) and after (B) transcatheter arterial chemoembolization performed via radial access due to steep downward angulation of the coeliac axis and upward of the hepatic artery

patients with a steeply angled coeliac axis, challenging or occluded iliac and femoral arteries, or due to an unstable catheter position via the femoral access.

More recently, the benefits of early mobilization and superior patient satisfaction via radial artery access have made radial access a routine rather than an alternative<sup>[39]</sup>. Radial access has been studied extensively for coronary intervention with additional benefits in an acute setting<sup>[40]</sup>. The medical device industry also responded by developing longer shaft length catheter systems to reach the tumors in the liver<sup>[41]</sup>. A small risk of posterior fossa stroke and hand ischemia exists, and this should be clearly explained to the patients as part of the informed consent. A Barbeau test is a modification of Allen's test and is a requirement to ensure enough collateral flow via the ulnar artery to the hand. Vasodilators are used to prevent spasm of the radial artery but can be beneficial in the hepatic circulation during catheter manipulation.

## COMPLICATIONS

The incidence of post-TACE complications is unchanged and liver ischemia; infarction and failure continue to be the major risks. However, in comparison to cTACE, the severity of post-embolization syndrome can be less with DEM-TACE due to the highly selective technique of embolization. The newer starch microspheres (DSM-TACE) are biodegradable and better-tolerated, making them ideal in unresectable large HCCs and patients requiring multiple episodes of TACE.

## CONCLUSION

TACE continues to be an important treatment option to improve survival for a chosen group of patients with HCC who are unsuitable for other modern image-guided techniques or are unfit for surgery. It is largely a palliative procedure and to a lesser extent curative. The advances in catheters, embolic technology, and catheter skills over the last two decades have made it a safe, effective, and well-tolerated procedure. Standardization of type of TACE, size of bead, and the type and volume of a chemotherapy agent is not yet available. Magnetic nanoparticle as a carrier is ongoing research<sup>[42]</sup>.

## DECLARATIONS

### Authors' contributions

The author contributed solely to the article.

### Availability of data and materials

Not applicable.



**Financial support and sponsorship**

None.

**Conflicts of interest**

The author declared that there are no conflicts of interest.

**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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# AUTHOR INSTRUCTIONS

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Journal articles not in English	Zhang X, Xiong H, Ji TY, Zhang YH, Wang Y. Case report of anti-N-methyl-D-aspartate receptor encephalitis in child. <i>J Appl Clin Pediatr</i> 2012;27:1903-7. (in Chinese)
Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. <i>BJOG</i> 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
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: <a href="https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm">https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm</a> . [Last accessed on 30 Oct 2017]
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### 2.4.1 File Format

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### 2.4.2 Length

There are no restrictions on paper length, number of figures, or amount of supporting documents. Authors are encouraged to present and discuss their findings concisely.

### 2.4.3 Language

Manuscripts must be written in English.

### 2.4.4 Multimedia Files

The journal supports manuscripts with multimedia files. The requirements are listed as follows:

Video or audio files are only acceptable in English. The presentation and introduction should be easy to understand. The frames should be clear, and the speech speed should be moderate.

A brief overview of the video or audio files should be given in the manuscript text.

The video or audio files should be limited to a duration of 3 min and a size of up to 500 MB.

Please use professional software to produce high-quality video files, to facilitate acceptance and publication along with the submitted article. Upload the videos in mp4, wmv, or rm format (preferably mp4) and audio files in mp3 or wav format.

### 2.4.5 Figures

Figures should be cited in numeric order (e.g., Figure 1, Figure 2) and placed after the paragraph where it is first cited;

Figures can be submitted in format of tiff, psd, AI or jpeg, with resolution of 300-600 dpi;

Figure caption is placed under the Figure;

Diagrams with describing words (including, flow chart, coordinate diagram, bar chart, line chart, and scatter diagram, etc.) should be editable in word, excel or powerpoint format. Non-English information should be avoided;

Labels, numbers, letters, arrows, and symbols in figure should be clear, of uniform size, and contrast with the background; Symbols, arrows, numbers, or letters used to identify parts of the illustrations must be identified and explained in the legend;

Internal scale (magnification) should be explained and the staining method in photomicrographs should be identified;

All non-standard abbreviations should be explained in the legend;

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### 2.4.6 Tables

Tables should be cited in numeric order and placed after the paragraph where it is first cited;

The table caption should be placed above the table and labeled sequentially (e.g., Table 1, Table 2);

Tables should be provided in editable form like DOC or DOCX format (picture is not allowed);

Abbreviations and symbols used in table should be explained in footnote;

Explanatory matter should also be placed in footnotes;

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### 2.4.7 Abbreviations

Abbreviations should be defined upon first appearance in the abstract, main text, and in figure or table captions and used consistently thereafter. Non-standard abbreviations are not allowed unless they appear at least three times in the text. Commonly-used abbreviations, such as DNA, RNA, ATP, etc., can be used directly without definition. Abbreviations in titles and keywords should be avoided, except for the ones which are widely used.

### 2.4.8 Italics

General italic words like *vs.*, *et al.*, *etc.*, *in vivo*, *in vitro*; *t* test, *F* test, *U* test; related coefficient as *r*, sample number as *n*, and probability as *P*; names of genes; names of bacteria and biology species in Latin.

### 2.4.9 Units

SI Units should be used. Imperial, US customary and other units should be converted to SI units whenever possible. There is a space between the number and the unit (i.e., 23 mL). Hour, minute, second should be written as h, min, s.

### 2.4.10 Numbers

Numbers appearing at the beginning of sentences should be expressed in English. When there are two or more numbers in a paragraph, they should be expressed as Arabic numerals; when there is only one number in a paragraph, number < 10 should be expressed in English and number > 10 should be expressed as Arabic numerals. 12345678 should be written as 12,345,678.

### 2.4.11 Equations

Equations should be editable and not appear in a picture format. Authors are advised to use either the Microsoft Equation Editor or the MathType for display and inline equations.

## 2.5 Submission Link

Submit an article via <http://www.oaemesas.com/hr>.



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