

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^[1]. 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^[2]. 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/ β -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^[3]. 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- γ carboxy prothrombin (DCP) are also used as important biomarkers for HCC determination^[4]. 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]^[5]. 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^[6]. According to American Association for the Study of Liver Disease (AASLD) guideline however, liver lesions smaller

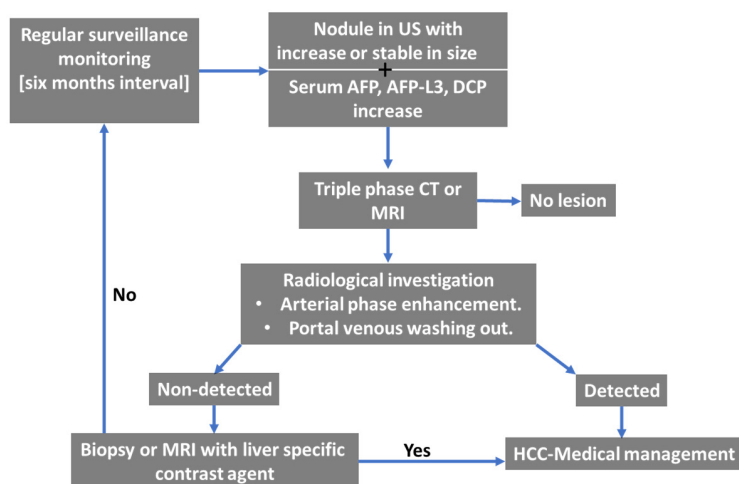


Figure 1. Current clinical practice for hepatocellular carcinoma (HCC) surveillance and diagnosis. AFP: alpha-fetoprotein; DCP: des- γ carboxy prothrombin; MRI: magnetic resonance imaging

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^[7]. 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^[8]. 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^[9,10].

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^[11].

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⁺/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^[12].

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^[13]. 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^[14].

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^[14]. However, AFP-L3 is not typically detected when AFP levels are < 20 ng/mL^[8] 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 γ -carboxy glutamate (GLA) unit which is crucial for binding 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^[15]. 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^[16]. 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^[17]. 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^[18]. 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^[19]. 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^[20].

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^[21,22].

Cell secreted small membrane-enclosed spheres, present in biological fluids are known as extracellular vesicles (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^[23]. 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^[24]. 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^[25]. Reduced expression of another EV cargo, miR-718 is highly correlated with increasing tumor size, recurrence and poor histological differentiation of HCC cells^[26]. 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^[27]. 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^[28]. 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^[29].

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]^[30].

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^[31,32]. 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^[33]. 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^[34-40].

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^[41].

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^[42,43]. 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^[44]. 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^[45].

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.

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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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