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REVIEW ARTICLE

Advances in the early diagnosis of hepatocellular carcinoma

Weiyi Wang, Chao Wei*

Xiamen Amplly Bio-engineering Co., Ltd, Xiamen, PR China

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Abstract Hepatocellular carcinoma (HCC) is one of the most prevalent cancers globally. In contrast to the declining death rates observed for all other common cancers such as breast, lung, and prostate cancers, the death rates for HCC continue to increase by ~2–3% per year because HCC is frequently diagnosed late and there is no curative therapy for an advanced HCC. The early diagnosis of HCC is truly a big challenge. Over the past years, the early diagnosis of HCC has relied on surveillance with ultrasonography (US) and serological assessments of alpha-fetoprotein (AFP). However, the specificity and sensitivity of US/AFP is not satisfactory enough to detect early onset HCC. Recent technological advancements offer hope for early HCC diagnosis. Herein, we review the progress made in HCC diagnostics, with a focus on emerging imaging techniques and biomarkers for early disease diagnosis.

Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death globally. At-risk individuals are advised to undergo periodic HCC screening. If identified at an early state, surgical resection offers a favorable prognosis, with 5-year survival rates of more than 70%.[1] However, most patients have advanced disease at the time of diagnosis with 5-year survival rates <12.5% in China. The lack of surveillance and insufficient early diagnostic precision are blamed for the poor prognosis and high mortality of HCC.

Currently, ultrasonography (US) tests are recommended by all guidelines with or without serum alpha-fetoprotein (AFP) assessments for early HCC detection. Cross-sectional imaging techniques are further recommended for liver nodules ≥1 cm. However, the sensitivity and specificity of the methods used clinically for HCC diagnosis remain unsatisfactory, particularly during early disease stages.[2] With greater efforts, advanced imaging techniques are in development to enhance HCC detectability and improve

* Corresponding author. Xiamen Amplly Bio-engineering Co., Ltd, 10 Yangguang Street, Haicang District, Xiamen, PR China.
E-mail address: weichao@amplly.com (C. Wei).
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the characterization of HCC nodules. In addition, interest surrounding important HCC biomarkers with potential clinical value has emerged. A large number of studies have identified serum/plasma protein biomarkers, as well as circulating DNA/RNA markers that display promising diagnostic abilities to facilitate HCC surveillance and/or detection. This review will summarize current clinical and experimental studies to highlight progress as well as some of the challenges of early HCC diagnosis.

Imaging

Unlike other malignancies, HCC can be diagnosed based on imaging features alone. Imaging-based diagnosis is recommended by nearly all HCC guidelines.[3–11] US is the most commonly accepted method for HCC surveillance and early diagnosis due to its wide availability and cost-effective/non-invasive nature that does not require excessive radiation. Early diagnosis provides survival benefits to those with HCC[12–15] but US screening lacks sensitivity with an ~84% accuracy for all HCC stages[16,17] with an accuracy ranging from 47 to 63% in cirrhotic HCC patients at the early disease stages.[17,18] Confounding liver diseases and obesity contribute to the lack of sensitivity of US. Moreover, US imaging is operator-dependent and challenges with its standardization remain. At present, for lesions $<$1 cm, routine screening is recommend via repeat US at 3- to 6-month intervals, according to various guidelines.[8,9,19] In lesions $\geq$1 cm, magnetic resonance imaging (MRI) imaging, Computed Tomography (CT), and other cross-sectional imaging techniques provide a definitive diagnosis. According to the guidelines set by the US—Liver Imaging Reporting and Data System (US-LIRADS), US examinations should be classed as severe, moderate or no limitations.[20]

Multiphasic MRI and CT show a similar performance for HCC diagnosis. These techniques have a higher accuracy for early HCC than US. Although Gadoxetic acid-enhanced MRI (dynamic contrast-enhanced CT as alternative) screening is advised for high risk groups as a first-line surveillance tool in Japan[4] and many centers in the USA, these cross-sectional imaging modalities remain too costly for widespread applications.

The use of gadoxetic acid and gadobenate dimeglumine hepatobiliary contrast agents in MRI can enhance sensitivity, but shows limited efficacy for small HCC. A prospective MRI-based surveillance study of patients with cirrhosis showed sensitivities of 86.0%, significantly higher than the US method (27.9%),[21] despite the high costs and time-consuming nature of the procedure. Dynamic contrast-enhanced CT is also readily available for the evaluation of focal liver lesions. For CT-based surveillance, the sensitivity for early detection is 66.7—73%, which is marginally better than US screening.[22–24] CT remains limited by the high cumulative doses of radiation.

As MRI and CT, contrast enhanced US (CEUS) assesses the hepatic blood supply and plays an increasingly important role in the management of HCC.[25] CEUS is modestly more sensitive than either MRI or contrast-enhanced CT for HCC diagnosis.[24,26] CEUS can thus be used as a substitute test for patients with contraindications to iodinated or gadolinium-based contrast agents. Using Sonazoid as a contrast agent, CEUS was reported as a cost-effective modality in HCC surveillance for liver cirrhosis patients.[27] Although CEUS is not advocated as the sole imaging tool for HCC screening, it is recommended by several societies for the evaluation of nodules in cirrhotic patients.[28] However, the lack of availability of US contrast limits its widespread use. In addition, CEUS fails to differentiate HCC and intrahepatic cholangiocarcinoma (ICC).

With the rapid development of advanced techniques, imaging modalities have increased the sensitivity of HCC detection. For HCC surveillance, abbreviated MRI (AMRI) is more cost-effective.[29,30] In a cohort of 174 patients, AMRI was performed to reduce scan times and costs by 30.7—49.0%, whilst with clinically acceptable sensitivity (80.6%) and specificity (96.1%) were maintained.[31] In other studies performed in cirrhotic HBV-infected patients, AMRI conferred high per-lesion sensitivity (85.2%) and negative predictive values compared to US, with comparable positive prediction values and per-lesion sensitivity to complete dynamic contrast-enhanced MRI.[32]

Advancements in MRI techniques have improved both sensitivity and specificity. The perfusion MRI technique has been used not only for the detection and characterization of malignancy, but for the monitoring of anti-angiogenic therapeutic responses and survival in HCC patients.[33,34] For diagnosis, the vascular characteristics induced by tumor angiogenesis can be monitored via dynamic contrast-enhanced MRI perfusion. Quantitative digital subtraction angiography (QDSA) techniques permit the accurate measurement of arterial flow that can distinguish small HCC from cirrhotic livers.[35] Functional MRI techniques including diffusion weighted imaging (DWI) can evaluate the liver in the absence of intravenous contrast agents.[36] Changes in hepatic tissue and vascular alterations can be assessed through combined DWI and Gd-EOB-DTPA during the different stages of tumorigenesis. A meta-analysis showed the combination significantly improves both the diagnostic accuracy (AUC value 0.983 vs. 0.96) and specificity (96% vs. 89%) for chronic liver disease-associated HCC compared to Gd-EOB-DTPA enhanced MRI alone.[37] Other MRI based methods include MR elastography (MRE) that permits the evaluation of hepatic stiffness and fibrosis. MRE was studied for the detection liver fibrosis and for the differentiation of focal liver lesions, including HCC.[38,39] Malignant tumors show greater stiffness on MRE which is lower in cases of small HCC,[38,40] which limits the application of early HCC detection. Apart from gadolinium compounds, superparamagnetic iron oxide (SPIO)/ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles have shown prospects as MRI contrast agents due to their high-relaxivity, tissue-specific nature and excellent biocompatibility. SPIO/USPIO-enhanced MRI can improve the differentiation of HCC and other focal hepatic lesions.[41–43] Tailored SPIO/USPIO nanoparticles can also specifically target tumor cells through their coupling with aptamers, peptides and antibodies.[44–47] Recently, a bi-specific USPIO imaging probe was developed for HCC diagnosis by simultaneously conjugating AFP and GPC3 antibodies. The system has already shown higher sensitivity and specificity than single antibody-labeled or unlabeled USPIO *in vitro*.[48]

In CT, perfusion imaging provides quantitative information regarding arterial perfusion for the assessment of

hemodynamic changes in early HCC. Perfusion CT shows higher specificity and sensitivity for HCC detection in cirrhotic livers.[49,50] Dual-energy CT was applied to improve diagnostic performance and allows the simultaneous acquisition of information in the same anatomical region using two different x-ray energy levels to reconstruct monoenergetic images with higher image quality and high contrast-to-noise ratios.[51] With monoenergetic reconstruction algorithms[52,53] for a lower image to noise, dual-energy CT is a promising and sensitive modality for small HCC detection.

Nuclear imaging techniques such as Positron Emission Tomography (PET) are valuable in the clinical setting for the staging, diagnosis and prognostic assessment of anticancer therapeutic responses. ^{18}F-fluorodeoxyglucose (^{18}F-FDG) is the most commonly used metabolic tracer for PET. However, primary HCC exhibits broad ^{18}F-FDG uptake, thus FDG-PET has a reported sensitivity of 30—70%,[54] despite providing prognostic value for liver surgery, transplantation and palliative treatment. As opposed to ^{18}F-FDG, PET with ^{11}C-acetate shows promise as a HCC diagnostic with higher sensitivities of 50—93%.[54] The main drawback of ^{11}C-acetate is its short half-life (~20 min). Dual-tracer PET/CT combined with ^{18}F-FDG with ^{11}C-acetate PET/CT performs to a higher level for HCC staging and patient selection for liver transplantation.[55] ^{18}F-fluoropropionic acid (^{18}F-FPA), an ^{11}C-acetate acetate analog, mimics ^{11}C-acetat and as such, the combination of ^{18}F-FPA and ^{18}F-FDG provides an alternative for HCC detection.[56] In addition, PET with different radiolabeled choline, such as ^{11}C-choline, ^{18}F-fluorocholine and ^{18}F-fluoroethylcholine, have been tried as HCC probes in PET imaging. The reported diagnostic sensitivity ranged from 63% to 100% in well differentiated HCC.[54] The combination of ^{11}C-choline and ^{18}F-FDG PET holds utility for the accurate diagnosis of HCC subtypes with 93% sensitivity in those with chronic liver disease.[57] To overcome the weakness of low spatial resolution, near-infrared fluorescence (NIRF) has been recently integrated into PET.[58] MHI-148, an NIRF dye with specific tumor accumulation capabilities, has been conjugated with ^{68}Ga for PET tracing. The NIRF/PET dual-modality imaging probe showed improved diagnostic effects on deep-tissue small HCC in mouse and rabbit models. The clinical value of PET modalities in HCC diagnosis however, requires further assessments.

As a non-ionizing cutting-edge modality, Terahertz (THz) spectroscopy has potential for the early diagnosis of digestive cancers.[59] In HCC, THz absorption properties can distinguish tumors from normal tissue and continuous-wave THz digital holography contributes to the diagnosis of early-stage HCC.[60] Besides, the feasibility of THz imaging has been proven for discrimination between tumor tissue and normal liver tissue according to the differences in transmission rates.[61]

Protein biomarkers

In addition to imaging techniques, serum biomarkers hold value as HCC diagnostics. Biomarker assessments are more objective and reproducible than imaging based modalities, and are considered adjunct tools, encompassing dysregulated proteins or mRNAs. In recent decades, alpha-fetoprotein (AFP) has emerged as a most promising and well-studied biomarker candidate. Dysregulated levels of AFP in the plasma strongly correlate with HCC malignancy.[62,63] Serum biomarker AFP assessments for HCC surveillance, in combination with US are recommended as diagnostic tools according to the Asian HCC guidelines.[3,4,10] However, AFP testing remains far from satisfactory for early HCC, owing to insufficient sensitivity and specificity. Using 20 ng/mL as a cut-off, the reported sensitivities of AFP for HCC at any stage in cirrhotic patients ranged from 41% to 65%, with specificities ranging from 80% to 94%.[64] Whilst during the early stages of HCC progression, detection rates were as low as 1/3.[65] The reason of AFP limited in practice is that 80% of small HCC cases do not show elevated serum AFP. On the other hand, false-positives that compromise the specificities among HCC and other liver related disorders, including cirrhosis and acute hepatitis, are always challenging. Efforts have been made to optimize AFP related methods or to identify novel protein biomarkers for early HCC diagnosis.

AFP

From multicenter assessments, a cut-off value of 10.9 ng/mL for AFP shows a sensitivity of 66%.[66] The accuracy of AFP can be further improved by threshold optimization based on liver etiology. AFP levels $\geq$59 ng/mL can accurately detect HCC in those with HCV-mediated cirrhosis; whilst $\geq$11 ng/mL can identify HCC in the absence of HCV infection.[67] Other patient factors, including platelets, alanine aminotransferase (ALT),[68] age and gender[69] can be incorporated into AFP algorithms to improve HCC detection rates. Recent Chinese cohort studies highlighted how HCC risk predictions could be enhanced through genetic assessments and AFP serum monitoring. When accounting for single nucleotide polymorphisms (SNPs) (rs12506899 and rs2251844), the genetic corrected AFP levels could increase HCC prediction efficiencies.[70] However, to significantly improve HCC diagnosis, more straightforward methods and biomarkers that can be combined with AFP are required.

AFP-L3

The AFP-L3 isoform is specifically increased in HCC[71,72] and in 2015 was accepted as an FDA approved HCC biomarker. Despite its high specificity, AFP-L3 is limited by low sensitivity. In an MA comparing 12 HCC studies, its sensitivity as a HCC detection agent was 48.3% with a specificity of 92.9%.[73] For early stage HCC diagnosis, AFP-L3 tests individually showed a low sensitivity of 28%.[66] Despite the detection rates improving through the use of immunoassays that are highly sensitive for AFP-L3% (hs-AFP-L3%) in Japanese studies,[74] AFP-L3 must be combined with other biomarkers to increase the detectability of small HCC.

DCP (PIVKA II)

Protein induced by vitamin K absence II (PIVKA II) also known as Des-γ-carboxy-prothrombin, was identified as a serum biomarker for HCC and is FDA approved in East Asia.

Studies have shown that serum DCP has high diagnostic specificity and can differentiate HCC from non-malignant chronic liver disease. DCP shows comparable diagnostic efficacy for HCC cases that are AFP-positive or negative,[75] making it a useful supplement to AFP assessments. However, the lack of sensitivity of the DCP assay makes it controversial as a surveillance biomarker. From a meta-analysis performed in 2018,[76] serum DCP showed a relatively higher diagnostic specificity for primary HCC and DCP combined with AFP, and exhibited improved sensitivity for HCC than any of the biomarkers used in isolation. For early HCC diagnosis, the combination trials of DCP and AFP are continuously reported to display higher sensitivity (47.5—94.0%), than any individual biomarker.[75,77—79]

Combination of AFP, DCP and AFP-L3 measurements is already recommended by the JSH to increase sensitivity.[11] Early HCC diagnosis using triple biomarkers was intensely investigated and exceeded that of each individual biomarker. A serum-based tool model "GALAD" based on objective measures of sex, age, and the aforementioned biomarkers was developed.[80,81] An analysis from Germany based on this diagnostic algorithm revealed a superior specificity of 93.3% and sensitivity of 85.6% for early HCC diagnosis.[82] A multi-national study with of over 6000 patients confirmed the ability of GALAD to discriminate patients with HCC from those with chronic liver disease (AUC>0.90) with superior sensitivity and specificity.[83] Moreover, the GALADUS score (GALAD + US) further enhanced the performance of the GALAD score.[84] Currently, the development of equipment for the detection of AFP, AFP-L3, and PIVKA II, such as the μTASWako i30 immunoanalyzer, facilitates the application of these biomarkers.

GPC3

Glypican-3 (GPC3) belongs to the glypican family. It is not detectable in healthy or nonmalignant livers of adults but is overexpressed in most HCC tumor tissues.[85,86] As a specific diagnostic biomarker, GPC3 was positively expressed in ~63—91% of patients in surgical HCC tissues, especially in HCV-induced HCC cases.[87] Moreover, GPC3 is an immunotherapeutic target for HCC.[88] Peptides or antibodies targeting GPC3 have been used in HCC contrast agent-based imaging techniques to recognize the lesion site and reduce the requirement for liver biopsies.[89] The serum levels of GPC3 correlate with tissue expression, enabling potential non-invasive HCC screening, although the results from many studies differ due to variable cut-off values, detection methods, and HCC etiologies. The majority of studies suggest that in HCC patients, GPC3 levels in the serum are upregulated, which as a biomarker is comparable to AFP. An MA showed that the pooled sensitivity and specificity for GPC3 were 56% (95%CI: 53—59%) and 89% (95%CI: 87—90%), respectively.[90] For early diagnosis, another MA reported the pooled sensitivity and specificity of serum GPC3 as 55.1% (95%CI: 47.9—66.2%) and 97.0% (95%CI: 95.2—98.2%), respectively.[91] Although the diagnostic accuracy of serum GPC3 for early HCC remains unsatisfactory, its co-assessment with AFP can enhance the sensitivity of detection.[91—93]

GP73

Golgi protein-73 (GP73) is a type II epithelial cell Golgi-resident protein. Serum GP73 levels increase during liver disease, particularly in those with HCC.[94] From a study including 443 serum samples from healthy, chronic hepatitis, liver cirrhosis or HCC patients, serum GP73 levels significantly increased in patients with liver disease, especially in HCC patients.[95] An MA showed that the sensitivity and specificity of GP73 were 77% (95% CI: 75—79%) and 91% (95% CI: 90—92%) during HCC diagnosis.[96] Accordingly, GP73 is now thought to have surpassed serum AFP as an early diagnostic for HCC.[95,97—99] However, the discrimination ability of GP73 in HCC and liver disease is controversial as serum GP73 levels in liver cirrhosis patient's decrease during HCC progression.[100,101] If used in combination with other markers, GP73 represents a powerful early diagnostic tool for high-risk populations. Indeed, the simultaneous detection of GP73 and AFP showed a sensitivity of 87—94% and a specificity of 85—99% for HCC diagnosis.[96,98,102]

Osteoponin

Osteopontin (OPN) is a multifunctional phosphorylated glycoprotein implicated in a range of pathological and disease states, including cancer.[103] In patients with HCV or HBV-induced liver disease, serum OPN levels are significantly elevated.[104—106] In a large population-based cohort in Europe, plasma OPN strongly correlated with HCC incidence.[107] The performance of OPN for the early diagnosis of HCC outperformed AFP, highlighting its utility as a biomarker, particularly amongst high-risk group of patients. From an MA that included 12 studies, OPN outperformed AFP for HCC diagnosis, showing a sensitivity upon pooled analyses of 81.3% vs. 63.9%. The combination of AFP and OPN (85.6%) enhanced the sensitivity of diagnosis for early HCC,[108] and represents a predictor for HCC development.[107] In addition, AFP, OPN, combined with DKK1 can enhance early HCC diagnostic capacity.[109]

DKK1

Dickkopf-1 (DKK-1) inhibits Wnt signaling, a well-characterized mediator of cancer cell metastasis. In recent years, DKK-1 has been widely implicated in carcinogenesis with its overexpression demonstrated for a range of human cancers.[110] For HCC, the serum levels of DKK1 show huge potential to improve diagnostics, most notably in patients that are AFP-negative and in the early stages of HCC.[111] Recently, in a novel bioanalytical approach, namely an aptamer-based ELISA assay following a single SELEX procedure, DKK1 showed potential clinical application for the diagnosis of early-stage HCC.[112] DKK1 combined with DCP and MDK,[78] or AFP and OPN[108] were studied to improve the diagnostic performance.

To-date, no single protein biomarkers have shown sufficient levels of accuracy for their sole use as an early HCC diagnostic. As proteomics technology develops, a larger number of biomarkers for HCC have been identified. MDK, VEGF, SCCA and many others play roles in HCC tumorigenesis but show less than satisfactory early diagnosis value.

Although more effective biomarkers are needed, the combination of a series of HCC-related biomarkers can improve diagnostic performance. Despite the promise of several of these panels, their utility in clinical practice still requires confirmation.

miRNAs

MicroRNAs (miRNAs) are non-coding RNAs of approximately 22 nucleotides that function as post-transcriptional gene regulators through epigenetic silencing. A group of aberrant miRNAs identified in HCC target genes that ultimately contribute to tumorigenesis, proliferation, apoptosis, DNA repair, invasion and metastasis.[113–115] These findings have led to the exploration of miRNAs as potential HCC diagnostics[116–118] given their abundance in the plasma[119] and ease of detection.

Numerous studies have characterized the circulating miRNA profiles in HCC patients. Many deregulated miRNAs have been identified but the candidates and their corresponding diagnostic accuracy differ amongst studies, despite most of the miRNAs being detected during late but not early HCC. Multiple miRNAs can improve the diagnostic power of HCC. Based on a recent MA[120] that referred to 5125 HCC patients and 6561 controls in 59 studies, circulating miRNAs possess promising HCC diagnostic value. Single miRNAs such as miR-130b, 150, 182, 215 and 96, represent prime candidates amongst all miRNAs. Panels of miRNAs such as miR-10a combined with 125b,[120] miR-155, miR-96 combined with miR-99a,[121] show higher diagnostic accuracy compared to single miRNAs. From the aspect of early diagnosis, circulating miRNAs have valuable diagnostic potential. Several miRNA panels show a satisfactory performance with both high sensitivity and specificity. An MA of 50 studies identified 19 circulating miRNAs as biomarkers of early stage HCC,[122] amongst which miR-21 and miR-122 were the most promising miRNA pairs that could independently predict HCC. Mir-21, mir-122, and mir-192 showed high HCC diagnostic accuracy in HBV patients, even when low AFP levels are measured.[123] For HCV-induced HCC, serum miR-214–5p, miR-375, miR-1269 and miR-125b can be considered early biomarkers to discriminate HCC patients from late fibrotic patients.[124] However, a consensus for the optimal combination of miRNAs for early HCC diagnosis are currently lacking, as their utility is dependent on a range of other factors including genetic differences in specific ethnic populations and the varying genetic causes of HCC induced cancer. The combination of miRNAs and AFP show improved diagnostic accuracy than the use of either as a stand-alone diagnostic,[120,123,125,126] suggesting their utility as an integrated diagnostic algorithm for early HCC detection in future clinical practice.

Increasing attention has been given to cancer-derived exosomal miRNA in body fluids. Exosomes are membrane vesicles that play vital roles in reprogramming the tumor microenvironment, the contents of which are stable due to the protection of lipid membrane. Exosomal miRNAs are the most extensively investigated biomarkers. MiR-21 targets several tumor suppressor genes and is overexpressed in many cancers.[127] As mentioned earlier, in HCC patients, higher levels of serum miR-21 are observed than those in chronic hepatitis B or healthy controls. More importantly, miR-21 enriched in exosomes showed a superior sensitivity of detection than that in whole serum.[128] In another study, enriched miR-519d, miR-21, miR-221 and miR-1228 were detected in exosomal fraction but not in exosome-depleted serum fraction in HCC patients.[129] Accordingly exosomal miRNAs in HCC were investigated to improve diagnostic reliability. Serum exosomal miR-18a, miR-221, miR-222, as well as miR-224 in HCC patients were found markedly abundant than those in liver cirrhosis or chronic hepatitis B individuals, whilst the levels of miR-101, miR-106b, miR-122 and miR-195 were downregulated in HCC vs. chronic hepatitis B groups.[130] A total of 8 upregulated exosomal miRNAs (miR-122, miR-125b, miR-145, miR-192, miR-194, miR-29a, miR-17–5p and miR-106a) in HCC were identified as diagnostic markers with an AUC of 0.535–0.850.[131] Unsurprisingly, AFP was chosen to be combined with serum exosomal miRNAs as biomarkers. MiR-122, miR-148a, along with AFP provided a higher AUC of 0.931 to distinguish early HCC from liver cirrhosis.[132]

In summary, miRNAs are attractive HCC early diagnosis biomarkers despite the current restricted research programs and lack of practical translation. Panels of deregulated miRNAs in the serum/plasma and/or exosomes combined with other biomarkers are likely to play a greater role in HCC diagnostics in the future.

LncRNAs

LncRNAs are $\geq$200 nucleotides non-coding transcripts that play regulatory roles in cancer and disease pathogenesis. Increasing evidence demonstrates that lncRNAs promote or inhibit cancer development,[133] and are involved in tumor onset and development, dependent on the tissue/cancer type.[134] LncRNAs are deregulated in various tumors and surprisingly, most show tissue-specific and neoplasma-specific expression, making them promising tumor biomarkers. An increasing number of lncRNAs are now known to possess biological functions associated with various aspects of cancer,[135,136] including development in the early stages. Notably, some lncRNAs are found in blood, urine and other body fluids, which are easily accessible by non-invasive sampling. The diagnostic value of circulating lncRNAs is therefore an attractive and emerging area of HCC studies.

There is preliminary evidence supporting the diagnostic utility of many serum/plasma lncRNAs, such as HULC,[137] UCA1,[138] MALAT1,[139] DANCR,[140] and lncRNA-D16366.[141] Each marker in isolation possesses moderate specificity and/or sensitivity to discriminate HCC from healthy controls or hepatic disease patients, encouraging the discovery of panels of lnRNAs that can improve diagnostic accuracy. Indeed, in an MA performed in 2018, multiple lncRNAs have shown better diagnostic performance than single lncRNA with sensitivity of 89.8% vs. 86.2%, specificity of 88.6% vs. 80.5% and AUC of 0.94 vs. 0.902, including but not limited to the co-assessment of HULC and Linc00152, or RP11-160H22.5, XLOC_014,172, and LOC149086, or uc001ncr and AX800134.[142] Furthermore, lncRNAs may play key roles in clinical diagnosis through a large complex network involving other biomarkers (e.g. AFP). The combination of

LINC00152, RP11-160H22.5 and XLOC014172 with AFP accurately discriminate HCC from either cirrhotic patients or healthy control with the AUC of 0.986 and 0.985, respectively.[143]

Serum exosomal lncRNAs are also biomarker candidates. The levels of exosomal lncRNA-HEIH in HCC patients were found higher than those in HCV-induced cirrhosis patients while the ratio in serum versus exosomes was lower in HCC.[144] Another study demonstrated that both ENSG00000258332.1 and LINC00635 in serum exosomes were able to discriminated HCC from chronic hepatitis B with AUC of 0.719 and 0.750, respectively. Moreover, combination of these two exosomal lncRNAs and serum AFP level (with a cutoff of 20 ng/mL) remarkably improve the discrimination performance with sensitivity of 83.6%, specificity of 87.7% and AUC of 0.894.[145]

To date, lncRNAs are attracting increasing interest as HCC diagnostics. Further validations in larger patient cohorts are now required to investigate their utility as novel biomarkers in clinical practice.

ctDNA

Over recent decades, the concept of "liquid biopsy" has drawn great attention and has dramatically renewed the field of tumor diagnosis. Circulating DNA (ctDNA), along with circulating tumor cells (CTCs) are key measurement parameters for liquid biopsies. ctDNA is a type of cell-free DNA (cfDNA) that is extracellularly expressed and present in the plasma of cancer patients. ctDNA differs to other cfDNA as it reflects the genetic and/or epigenetic mutations that occur during cancer progression and development. In contrast to traditional biopsies, ctDNA can be obtained from the blood (and is therefore minimally invasive) and can reveal important genetic predispositions to cancer, highlighting the genetic landscape of cancer progression.

Methylation is a common epigenetic change during tumor progression. Methylation markers show utility as diagnostic tools in cancer, highlighted by the use of SEPT9 in colorectal cancer. In HCC, commonly over-methylated genes include APC (81.7% of HCC cases), GSTP1 (76.7% of HCC cases), RASSF1A (66.7% of HCC cases), p16 (48.3% of HCC cases), COX-2 (35.0% of HCC cases) and E-cadherin (33.3% of HCC cases),[146] along with p15, SPINT2, SFRP1, p16INK4a, TFPI2, and so on.[147] Amongst these, the hypermethylations of p16, COX-2, and TIMP-3 show a specific association with HCC-associated disease as opposed to non-HCC causes of liver damage including cirrhosis.[146] Alterations to methylation profiles regulate the early events of carcinogenesis,[148–150] showing high diagnostic value in HCC. However, the qualitative analysis of single-gene methylation changes are not recommended.[151] Aberrant RASSF1A, p16, and p15 tumor suppressor genes were identified in the serum DNA 1–9 years prior to clinical HCC diagnosis. The performance of circulating methylated markers for HCC diagnosis was evaluated, either individually or in panels. The hypermethylation the aforementioned target genes showed a predictive accuracy of 89% with 84% sensitivity and 94% specificity.[152] Compared to individual gene assessments, the combination of the methylation profiles of GSTP1, APC, SFRP1 and RASSF1A displayed a higher discriminatory ability to distinguish HCC from healthy or benign controls, and outperformed the individual assessments of each methylated gene.[153] A diagnostic prediction model generated from a large cohort of ten methylation markers yielded high sensitivity and specificity for HCC upon comparison of 715 HCC and 560 normal samples, validated in 383 HCC and 275 normal samples.[154] The assessment of gene methylation patterns obtained from the blood of healthy HCC patients holds promise as a diagnostic and surveillance tool, particularly when combined with AFP assessments.[151]

Due to the advancements in sequencing technologies, the mutational landscape of ctDNA has been investigated for many cancer types. In HCC, three of the most commonly mutated genes include the TERT promoter (mutated in 60% of HCC cases), TP53/p53 (mutated in 35–50% of HCC cases) and CTNNB1/beta-catenin (mutated in 19–40% of HCC cases).[155] Notably, these mutations have been also detected in the peripheral blood of HCC patients.[156] In HCC cases related to aflatoxin exposure, the TP53 p. R249S, c.747G > T mutation is a primary genetic event.[157] However, to-date, no somatic mutations are considered distinctive HCC biomarkers due to the variable specificities encountered. Recently, CancerSEEK, a panel that integrates 61-amplicons within 16 genes and 8 protein biomarkers, had been developed to localize the origin of early cancers and identify at risk individuals.[158] Amongst the eight most important cancer types, the earliest stage of HCC (Stage I) had the highest sensitivity (100%), despite the fact that healthy individuals were used as controls. This specificity may be compromised in practical hepatic disease patients.

The challenge of ctDNA examinations lies in its low expression levels. cfDNA is limited in the peripheral blood and ctDNA only accounts for a small portion (<0.01%) of total cfDNA, particularly during early tumor stages. Robustly sensitive methodologies are required for the application of ctDNA and improvements have been made. Digital PCR (dPCR) increases the sensitivity and precision of detection through massive sample partitioning. The feasibility of detecting four gene loci, namely CTNNB1 (c.121A > G, c.133T > C), TP53 (c.747G > T) and TERT (c.1–124C > T) in the plasma of HCC patients has been proven by ddPCR assay.[159] In addition to PCR-based sequencing, next generation sequencing (NGS) can achieve high throughput in larger panels on the whole genome/exome scale permit the full assessment of the mutational landscape of cancer. With these advanced molecular diagnostic techniques, ctDNA has been extensively investigated for future cancer diagnostics.

Metabolites

Metabolomics permits disease-derived metabolite characterization in body fluids. Novel biomarkers can be screened from cancer specific metabolic profiling. In HCC, efforts have been made to identify circulating biomarkers for diagnosis. Aberrant metabolites from different compound classes have been defined, including amino acids, bile acids, lysophosphatidylcholines (LPC) and free fatty acids (FFA). From a recent study, 104 metabolites were found to

be dysregulated in the serum of HCC patients compared to healthy controls. In total, 13 metabolites with the highest AUC (0.785—0.895), sensitivity (65—90%) and specificity (60—100%) were better or comparable to AFP (AUC: 0.808, sensitivity: 80%, specificity: 70%).[160] A 1448 cohort, multicenter study in China screened a serum metabolite panel including phenylalanyl-tryptophan (Phe—Trp) and glycocholate (GCA) by LC-MS based metabolic profiling. The panel was discovered, tested and validated, showing remarkably higher diagnostic performance than AFP to discriminate HCC from cirrhosis (AUC 0.930, 0.892, and 0.807 versus 0.657, 0.725, and 0.650). For small HCC detection, the panel had a satisfactory performance in both test and validation sets (AUC 0.866 and 0.753 vs. 0.682 and 0.676 for AFP). Furthermore, the metabolite panel combined with AFP further improved the detective sensitivity.

In addition to serum, urine is an ideal source of metabolites. Urinary metabolites are differentially expressed in HCC subjects.[161] In West African cohorts, urinary metabolite mapping in HCC patients was distinct to that of cirrhosis, non-cirrhotic liver disease and healthy controls. A panel comprising inosine, indole-3-acetate, galactose, as well as an N-acetylated amino acid (NAA), was suggested as a promising urinary marker with 86.9% sensitivity and 90.3% specificity for discriminating HCC from cirrhosis.[162] Likewise, studies in China revealed the potential of urinary carnitine C4:0 and hydantoin-5-propionic acid as HCC biomarkers.[163]

Other biomarkers in development

High-throughput omics approaches and bioinformatics have been wildly used during the last decade and provide an opportunity to understand the complexity of tumorigenesis. A multi-omics analysis, consisting of mRNA, miRNA, and lncRNA, was performed to identify robust biomarkers for HCC. A network of 23 core mRNAs targeted by 9 miRNAs and 21 lncRNAs in HCC was constructed from three available omics datasets. The levels of YWHAZ, ENAH, and HMGN4 were promising prognosis indicators, whilst other mRNAs, such as LAMA4, GPC3, and LCN2, served as diagnostic markers.[164] A growing number of studies have screened critical genes in HCC as potential biomarkers through bioinformatics assessments.[165—167] A number of hub genes were targeted from protein—protein interaction networks (PPIs) of differentially express genes (DEGs), which were filtered using software from online databases. For instance, integrated bioinformatics analysis derived from 927 HCC tissues and 630 adjacent normal tissues in the Gene Expression Omnibus (GEO) database detected several core genes closely associated to functional pathways involved in the carcinogenesis and progression of HCC, including FOXM1, CCNA2, AURKA, CDKN3, CDC20 and FTCD.[165]

The gut microbiome is considered a non-invasive diagnostic tool for disease diagnosis due to its enormous impact on human health. A study based on faecal microbiota characterization revealed its possible application in early HCC detection. Faecal microbial diversity increased in early HCC vs. cirrhosis livers. The optimal model contained 30 microbial markers and achieved a greater potential (AUC 0.806) for distinguishing early HCC from healthy or cirrhotic controls.[168]

Conclusions

For early HCC diagnosis, US based surveillance is recommended by all clinical guidelines. However, the combination of US with AFP, the most commonly used serum marker, remains unsatisfactory. Novel imaging modalities and non-invasive biomarkers that display favorable specificities and sensitivities have been assessed. Although many have already shown potential diagnostic potential, a larger number of multicenter studies encompassing larger cohorts and longer-term assessments are required to confirm clinical utility. The integration of different methodologies and multi-marker models/algorithms are likely to emerge as the trend for early HCC detection.

Conflict of interests

The authors declare no conflict of interests.

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