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Precision diagnosis of hepatocellular carcinoma

Zhenxiao Wang[1], Hanjiao Qin[2], Shui Liu[1], Jiyao Sheng[1], Xuewen Zhang[1]

[1]Department of Hepatobiliary and Pancreatic Surgery, Second Hospital of Jilin University, Changchun, Jilin 130041, China;
[2]Department of Radiotherapy, Second Hospital of Jilin University, Changchun, Jilin 130041, China.

Abstract

Hepatocellular carcinoma (HCC) is the most common type of primary hepatocellular carcinoma (PHC). Early diagnosis of HCC remains the key to improve the prognosis. In recent years, with the promotion of the concept of precision medicine and more in-depth analysis of the biological mechanism underlying HCC, new diagnostic methods, including emerging serum markers, liquid biopsies, molecular diagnosis, and advances in imaging (novel contrast agents and radiomics), have emerged one after another. Herein, we reviewed and analyzed scientific advances in the early diagnosis of HCC and discussed their application and shortcomings. This review aimed to provide a reference for scientific research and clinical practice of HCC.

Keywords: Hepatocellular carcinoma; Precision medicine; Serum markers; Liquid biopsy; Molecular diagnosis; Radiomics

Introduction

At present, surgery remains the mainstay treatment for HCC to achieve long-term survival,[1] but the postoperative 5-year survival rate is merely 40–50%.[2] Owing to a lack of typical manifestations of early stage HCC, the majority of patients have already progressed to moderate or advanced stages by the time they are diagnosed, and only 30–40% of the patients are potential candidates for surgical treatment. Consequently, to improve the 5-year survival rate of HCC patients, the screening of high-risk patients and early identification of HCC are major priorities.

For early stage HCC screening, the European Association for the Study of the Liver (ECSL) and the American Association for the Study of Liver Diseases (AASLD) recommend an abdominal ultrasound (US) (with or without alpha-fetoprotein [AFP] monitoring) every 6 months in high-risk HCC patients with chronic liver disease or cirrhosis.[3,4] However, the overall sensitivity of US to HCC is only 46%. On top of this, sensitivity for lesions smaller than 2 cm is only 21%, and it is substantially lower for lesions than 1 cm.[5] In the early stage of the Barcelona Clinic Liver Cancer staging system (BCLC) and the Milan standard, sensitivity was only 47% and 27.3%, respectively.[6,7] Indeed, US is highly dependent on the level of expertise of the operator and the patient's own condition. Moreover, traditional US has a low display rate of fine and low-velocity blood flow, which limits the ability of US to distinguish benign from malignant tumors.[8] However, AFP is only expressed in 60–80% of HCC patients, and serum AFP levels may remain within the normal range.[9] Additionally, increased AFP levels can also be detected in benign liver diseases (such as hepatitis and cirrhosis) and pregnant women.

Magnetic resonance imaging (MRI) and computed tomography (CT) can diagnose HCC with an overall sensitivity of 65% and 72%, respectively.[5] Nonetheless, in HCC with lesions smaller than 2 cm, the sensitivity of CT and MRI is only 48% and 62%, respectively,[10] and are even less sensitive for <1 cm lesions.[11] Therefore, based on the concept of precision medicine, the development of novel laboratory and imaging diagnoses is a key strategy for improving the early diagnosis of HCC.

Precision medicine is a new medical concept and model based on "individualized medicine", with the rapid advancement of genome sequencing technology and the cross-application of bioinformation and data science, including the prediction of health risks and the precision diagnosis of diseases. Therefore, precision medicine may be a promising direction for improving the early diagnostic rate and accuracy of HCC. New discoveries have recently been made to gain a deeper

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Correspondence to: Xuewen Zhang, Department of Hepatobiliary and Pancreatic Surgery, Second Hospital of Jilin University, Changchun, Jilin 130041, China.
E-mail: zhangxw@jlu.edu.cn;
Jiyao Sheng, Department of Hepatobiliary and Pancreatic Surgery, Second Hospital of Jilin University, Changchun 130041, China.
E-mail: shengjiyao@jlu.edu.cn

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understanding of the intricate signaling regulatory network in HCC, and the diagnostic relevance of these molecular markers and metabolites in HCC has been continuously validated, which can be used in conjunction with traditional pathology to further improve the accuracy of HCC diagnosis. Employing techniques such as gene sequencing and omics technologies to classify HCC at the molecular level is indeed an inevitable trend in the evolution of precision medicine. Moreover, rapid progress in biocompatible nanomaterials, computer artificial intelligence, machine learning, data depth mining technology, and interdisciplinary collaboration has provided robust support for radiomics and novel contrast agents, which further improves the precision medicine imaging. In light of the aforementioned reasons, new diagnostic methods, including emerging serum markers, liquid biopsies, molecular diagnosis, and imaging techniques (eg, novel contrast agents and radiomics), were reviewed to provide innovative ideas for the early diagnosis and accurate typing of HCC to improve prognosis.

Emerging Serum Markers for HCC

In recent years, numerous novel serum biomarkers have been studied[12-21] [Table 1], including lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), des-gamma-carboxy prothrombin (DCP), Golgi protein 73 (GP73), Glypican-3 (GPC3), and so on. When detected alone or in combination, along with related scores, they have tremendous potential for the diagnosis of early stage HCC.

Lens culinaris agglutinin-reactive fraction of AFP

The diagnostic value of AFP-L3 in HCC

The quantitative detection method of AFP commonly used in clinics is the sum of AFP-L1, AFP-L2, and AFP-L3 heteroplasms. At present, AFP-L1 is primarily utilized to detect benign liver diseases such as chronic hepatitis and liver cirrhosis (LC). On the other hand, AFP-L2 is chiefly produced by the yolk sac and is generally detected in pregnant women.[22] AFP-L3 is the lens culinaris agglutinin (LCA)-bound fraction of AFP and can be detected in patients with HCC.[23] The concentration of AFP in healthy subjects typically does not exceed 10 ng/mL, and if <20 ng/mL, the patient is judged to be AFP negative. Therefore, the reliability of AFP levels for HCC diagnosis is compromised by numerous factors.[24] Studies have previously reported that AFP-L3 has superior diagnostic sensitivity in the early diagnosis of HCC compared to total AFP.[25] Moreover, AFP-L3% (the ratio of AFP-L3 concentration to total AFP concentra-

Table 1: Studies on emerging serum markers for HCC diagnosis.

Serum markers	Cut-off values	Sensitivity (%)	Specificity (%)	Patient characteristics	References
AFP-L3%	4%	55	90	LC *vs.* HBV-HCC	[12]
DCP	20 mAU/mL	48	86	LC *vs.* HBV-HCC	[12]
AFP + AFP-L3%	5 ng/mL, 4%	79	87	LC *vs.* HBV-HCC	[12]
AFP + DCP	5 ng/mL, 20 mAU/mL	62	78	LC *vs.* HBV-HCC	[12]
AFP + AFP-L3% + DCP	5 ng/mL, 4 %, 20 mAU/mL	83	75	LC *vs.* HBV-HCC	[12]
hs-AFP-L3%	5%	41.5	85.1	ANHC AFP < 20 ng/mL *vs.* LC	[13]
hs-AFP-L3%	5%	36.2	88.5	ANHC AFP < 10 ng/mL *vs.* LC	[13]
AFP-L3%	5%	7	98.5	ANHC AFP < 20 ng/mL *vs.* LC	[13]
DCP	40 mAU/mL	20.2–78.1	/	ANHC *vs.* LC	[13]
hs-AFP-L3% + DCP	5%, 40 mAU/mL	44.9–90.6	/	ANHC *vs.* LC	[13]
DCP	42 mAU/mL	73	81	HCC *vs.* LC	[14]
DCP	40 mAU/mL	73	79	HCC *vs.* LC	[14]
AFP	5.5 ng/mL	61	50	HCC *vs.* LC	[14]
AFP	10 ng/mL	36	71	HCC *vs.* LC	[14]
DCP/NX-DCP	1.15 (DCP > 35 mAU/mL)	69.2	75.9	HCC *vs.* HL	[15]
DCP	128 mAU/mL	74	92	HCC *vs.* LC	[16]
GPC3	>5%	70.7	94	HCC *vs.* HL	[17]
GPC3	>5%	57.5	95	HCC *vs.* LC	[18]
GPC3	>5%	77	96	HCC *vs.* non-HCC	[19]
GP73	10 RU	69	75	HCC *vs.* LC	[20]
GP73	8.5 RU	74.6	97.4	HCC *vs.* non-HCC	[21]
GP73 + AFP	8.5 RU, 35 ng/mL	89.2	85.2	HCC *vs.* non-HCC	[21]

AFP: Alpha-fetoprotein; AFP-L3%: The ratio of AFP-L3 to total AFP; AFP-L3: Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein; ANHC: Alpha-fetoprotein-negative hepatocellular carcinoma; DCP: Des-gamma-carboxy prothrombin; GP73: Golgi protein 73; GPC3: Phosphatidyl alcohol proteoglycan-3; HBV-HCC: Hepatitis B virus-related HCC; HCC: Hepatocellular carcinoma; HL: Healthy liver; hs-AFP-L3%: The ratio of highly sensitive AFP-L3 to total AFP; LC: Liver cirrhosis; non-HCC: Non-hepatocellular carcinoma; NX-DCP: Novel des-gamma-carboxy prothrombin.

tion) is routinely used for the diagnosis of AFP-negative HCC (ANHC), with a sensitivity of 12% and a specificity of 98%, respectively.[26] Moreover, AFP-L3% can be utilized to diagnose HCC recurrence 5.4±2.6 months earlier than imaging modalities.[27]

Challenges and related advancements in the detection of AFP-L3

At present, AFP-L3% has not been extensively used in clinics, mainly attributed to constraints such as its low accuracy. Liquid-phase binding assay (LiBA) is a clinical standard for the measurement of AFP-L3, whose micro-total assay system (μTAS; WakoTM i-30 autoanalyzer) was approved by the FDA (Food and Drug Administration) for *in vitro* diagnosis in February 2011.[25] However, when the concentration of AFP is lower than 0.3 ng/mL, it is difficult to measure the concentration of AFP-L3 by μTAS, resulting in false negatives. Therefore, existing methods for quantifying AFP-L3 in the serum lack the analytical sensitivity for accurate diagnosis.[13] Kim *et al*[25] designed an analytical sensitive multiple reaction monitoring-mass spectrometry (MRM-MS) assay to quantify serum AFP-L3 levels that could not be detected by the μTAS automatic analyzer. The latter had a sensitivity and specificity of 81.0% and 89.5%, respectively, while the sensitivity of the μTAS automatic analyzer was only 61.5%, and there was no significant difference in specificity (90.0%). Wei *et al*[28] constructed an electrochemical analytical method based on non-interference electrochemical oxidation signals of two kinds of nanoprobes (MPA-CuNPs and LCA-AgNPs). The detection range was 50–100 ng/mL, while the limit of detection was 40 pg/mL. A highly sensitive AFP-L3 (hs-AFP-L3) detection was thus achieved.

DCP

The diagnostic value of DCP in HCC

DCP is also known as prothrombin induced by vitamin K absence II (PIVKA II). During the malignant transformation of hepatocytes, the function of the vitamin K-dependent carboxylase system is impaired, which induces the production of DCP. Some studies have established that DCP is more accurate than AFP in the diagnosis of HCC in patients with LC, with a sensitivity and specificity of 74% and 92%, respectively. Although AFP, combined with DCP, is more effective in the diagnosis of liver cancer, there is little difference compared with DCP or AFP alone.[16] In a case–control study on the diagnosis of early stage HCC, Poté *et al*[14] determined that the sensitivity and specificity of DCP were 77% and 82%, respectively, which were significantly superior to those of AFP.[29] This implies that DCP can be used as an alternative marker for screening early stage HCC. In addition, a large-scale, multicenter study determined that the sensitivity and specificity of DCP for the diagnosis of ANHC were 76.3% and 89.1%, respectively. Furthermore, DCP can distinguish between ANHC and AFP-positive benign liver diseases (sensitivity: 76.3%, specificity: 76.0%), which will assist the clinical differential diagnosis of HCC.[30]

The value of DCP in evaluating microvascular invasion (MVI)

Vascular invasion, including macrovascular and MVI, is an aggressive manifestation of tumors. Macrovascular invasion can be identified by the naked eye or imaging examination, while MVI is mainly diagnosed by pathology and is difficult to be detected before operation. MVI is one of the main risk factors for the early recurrence of HCC within 2 years after an operation.[31] DCP has a certain potential for predicting MVI. Clinical studies have exposed that a preoperative DCP level >90 mAU/mL is an independent predictor of MVI, and the combined measurement of MVI with DCP serum level and DCP tissue immunostaining was more accurate than DCP serum level alone; sensitivity increased from 70% to 87%, while specificity increased from 63% to 90%.[14] Even though early stage HCC is clinically characterized as a solitary tumor measuring <3 cm and is generally considered less malignant, some locally advanced HCC with MVI ranging from 18.1% to 37.0% still exists. Clinical studies have found that at least one among the three factors (tumor diameter ≥2 cm, AFP ≥200 ng/mL, or DCP ≥40 mAU/mL) can predict MVI in HCC ≤3 cm.[32]

GPC3

GPC3 is a member of the glycosylphosphatidylinositol-anchored heparan sulfate proteoglycans and plays a pivotal role in cellular growth, differentiation, and migration. It is absent in the hepatocytes of healthy individuals and hepatitis patients but highly expressed in fetal liver, HCC tissues, and most HCC cell lines. The first report on the diagnostic value of serum GPC-3 in HCC, published in 2003, uncovered that it had a high specificity and sensitivity of 53% for the diagnosis of HCC.[33] Therefore, GPC3 can be used as a biomarker for the diagnosis of ANHC and early HCC.[34] According to earlier studies, the area under curve (AUC) and sensitivity of serum GPC3 for the diagnosis of HCC were 0.879 and 79.52%, respectively. When GPC3 was combined with AFP, the AUC and sensitivity were increased to 0.925 and 88.10%, respectively. In addition, in BCLC stage 0 and A HCC patients, the sensitivity of GPC3 (76.43%) was significantly higher than that of AFP (64.33%), and an increase in serum GPC3 levels could be detected in 63.2% of AFP-negative patients.[35] Additionally, a recent meta-analysis summarized the diagnostic value of serum GPC3 in HCC. The results showed that the overall sensitivity and specificity of serum GPC3 for the diagnosis of HCC were 68% and 92%, while diagnostic odds ratio (DOR) and AUC were 23.53 and 0.87, respectively. GPC3 has been proposed as a supplementary serum marker to be used in conjunction with AFP for the diagnosis of HCC.[36]

GP73

GP73 is usually present in the Golgi apparatus. Although the serum level of GP73 is elevated in hepatitis, LC, HCC, and non-liver malignant tumors, it is significantly increased in HCC patients. A recent meta-analysis validated that the diagnostic values of GP73, GPC-3, and AFP

were significantly higher than those of GP73, GPC-3, and AFP, alone or in pairs. The sensitivity and specificity of combined detection were 91% and 84%, respectively, while the DOR was as high as 58, significantly higher than that of AFP alone (DOR = 14).[37] Marrero *et al*[20] discovered that when 10 RU was used as the cut-off value, the sensitivity of serum GP73 in ANHC patients was 62%, implying that GP73 has a certain application value in the diagnosis of ANHC. Another multicenter study involving 4217 participants reported that the sensitivity and specificity of serum GP73 for the diagnosis of HCC, based on a threshold of 8.5 RU, were 74.6% and 97.4%, while the sensitivity and specificity of the combined detection of GP73 and AFP were 89.2% and 85.2%, respectively.[21] Therefore, GP73, combined with other serum markers, has a high diagnostic value of HCC.

Research progress of combined serum markers for the diagnosis of HCC

In recent years, studies on emerging serum markers ways have prompted efforts to transform valuable biomarkers into clinical models and form a unified standard. In several studies, combined detection of multiple indicators appears to be superior to that of individual indicators.

Clinical studies exposed that the combination of AFP, GPC3, and GP73, three serum markers for the diagnosis of early HBV-related HCC, had an AUC of 0.843, a sensitivity of 84.1%, and a specificity of 71.7%, which was superior to any index alone.[38] A recent meta-analysis evaluated the predictive value of combined AFP, AFP-L3%, and DCP for the diagnosis of HCC. The results showed that the overall sensitivity and specificity of the combined markers for the diagnosis of HCC were 88% and 79%, respectively. Moreover, subgroup analysis demonstrated that the overall sensitivity and specificity for the differential diagnosis of HCC and LC were 81% and 82%, respectively, suggesting that the combination of the three markers may be useful for the diagnosis and screening of HCC.[39] In addition, the GALAD score, a grading system based on these three serum markers, has been used to screen for HCC in patients with chronic liver disease.[40,41] Indeed, its sensitivity for early HCC diagnosis is 80.2%, which is higher than that of AFP, AFP-L3, and DCP alone. In clinics, given that non-alcoholic steatohepatitis (NASH) patients are typically obese, their special body shape considerably reduces the sensitivity of ultrasonic detection of HCC; hence, early HCC monitoring in NASH patients is particularly difficult.[41] In another instance, Best *et al*[40] evaluated the diagnostic ability of the GALAD score in patients with non-alcoholic steatohepatitis related hepatocellular carcinoma (NASH-HCC). For the Milan standard early NASH-HCC, the GALAD score has a cut-off value of -0.63, a sensitivity of 68.0%, and a specificity of 95.2%. Overall, its sensitivity is also higher than that of AFP, AFP-L3, DCP, and other markers for the individual diagnosis of all stages of NASH-HCC,[40] implying that it has a favorable diagnostic value for HCC, which is challenging to detect with US or cannot be detected using other biomarkers.

Liquid Biopsy for HCC

To optimize diagnostic methods for HCC, approaches are no longer restricted to the detection of protein levels but can also focus on trace components released by tumors during the tumorigenesis of HCC. These components, such as circulating tumor non-coding RNA (ct-ncRNA), cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), circulating tumor cell (CTC), or extracellular vesicles (EVs), are released into the bloodstream or other body fluids, including urine, sputum, pleural effusion, ascites, and cerebrospinal fluid,[42] putting forward the concept of fluid biopsy. Cells, proteins, molecules, nucleic acids, and vesicles in body fluids can be isolated through various analytical methods to collect as much information as possible to assist in the early diagnosis and monitor disease progression[43-54] [Table 2].

CTCs

The diagnostic value of CTCs in HCC

CTC, which exists in the blood and originates from primary tumors and metastases, can be detected by liquid biopsies to provide vital information about tumor progression, metastasis, and recurrence. There is tremendous potential for the precision medicine treatment of HCC patients. In a controlled study of HCC patients with and without chronic liver disease, the sensitivity, specificity, and AUC of CTC for HCC were determined to be 72.5%, 95.0%, and 0.88, respectively, while those of AFP (when 20 ng/mL was used as the cut-off value) were 57.0%, 90.0% and 0.77, respectively. In terms of sensitivity, CTC detection was significantly superior to AFP monitoring.[43] Some studies combined CTC and AFP for the diagnosis of HCC and discovered that, relative to the healthy control group, the AUC for the diagnosis of HCC was 0.857, with a sensitivity of 73% and a specificity of 93.4%, which was higher than any index alone.[55]

Issues and related progress in the detection of CTC

At present, the CellSearch® system is the standard analytic method of CTC.[56] Through the positive selection of CTC epithelial markers such as cytokeratin (CKs), epithelial cell adhesion molecule (EpCAM), or the asialoglycoprotein receptor (ASGPR), CTC is isolated, screened, and then counted using flow cytometry.[57] It is worth pointing out that the HCC-CTC detection method, based on EpCAM, has been approved by the FDA.[58] For EpCAM-positive screened CTC, especially in patients with serum AFP level ≤400 ng/mL, a preoperative 7.5 mL blood CTC ≥2 is a strong predictor of postoperative tumor recurrence in patients with HCC.[59] However, the level of HCC-CTC in the blood is extremely low, and tumor cells are prone to epithelial–mesenchymal transition, leading to the loss of EpCAM. Presently, the detection rate of EpCAM+ CTCs is reported to be between 30% and 80%.[59] Therefore, there is an urgent need for a sensitive and specific detection technique to expand the

Table 2: Studies regarding the performance of liquid biopsy for the diagnosis of HCC.

Liquid biopsy strategy	Sensitivity (%)	Specificity (%)	Patient Groups	References
CTC panel (EpCAM$^+$, CD90$^+$, CD133$^+$, CK19$^+$)	72.5	95	HBV-HCC *vs.* non-HCC	[43]
RP11-85G21.1 (lnc85)	80.0	74.5	ANHC *vs.* non-HCC	[44]
miRNA7™ panel (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, miR-801)	83.2	93.9	HL *vs.* HBV-HCC	[45]
	79.1	76.4	CHB *vs.* HBV-HCC	[45]
	75.0	91.1	LC *vs.* HBV-HCC	[45]
circRNA panel (has_circ_0000976, has_circ_0007750, has_circ_0139897)	83	87.3	Small-HCC (solitary, ≤ 3 cm) *vs.* non-HCC	[46]
	76.2	93.8	ANHC *vs.* non-HCC	[46]
	75	93.8	Small ANHC (solitary, ≤3 cm) *vs.* non-HCC	[46]
ctDNA methylation diagnostic model (GSTP1)	69.2	93.3	HCV-HCC *vs.* CHC	[47]
cfDNA methylation diagnostic model (RASSF1A, miR-203, etc.)	84.3	83	HBV-HCC *vs.* CHB	[48]
cfDNA genome-wide 5hmC, 32-gene diagnostic model	82.7	67.4	Early stage HCC (BCLC 0/A) *vs.* non-HCC	[49]
Model combined EpCAM + CTCs and AFP	73	93.4	HCC *vs.* non-HCC	[50]
Total CTCs	85.7	80.7	Metastatic HCC *vs.* non-metastatic HCC	[51]
exo-miR-10b-5p	90.7	75.0	Small HCC (<2 cm) *vs.* non-HCC	[52]
exo-miR-4661-5p and exo-miR-4746-5p	81.8	91.7	Small HCC (<2 cm) *vs.* non-HCC	[53]
circRNA panel (hsa_circ_0004001, hsa_circ_0004123, hsa_circ_0075792)	90.5	78.1	HCC *vs.* HL	[54]

5hmC: 5-hydroxymethylcytosine; AFP: Alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer staging system; cfDNA: Cell-free DNA; CHB: Chronic hepatitis B; CHC: Clathrin heavy chain; circRNA: Circular RNA; CTC: Circulating tumor cell; ctDNA: Circulating tumor DNA; EpCAM: Epithelial cell adhesion molecule; GSTP1: Glutathione S-transferase pi-1; HBV-HCC: Hepatitis B virus-related HCC; HCC: Hepatocellular carcinoma; HL: Healthy liver; LC: Liver cirrhosis; lncRNA: Long non-coding RNA; miRNA: MicroRNA; non-HCC: Non-hepatocellular carcinoma; NX-DCP: Novel des-gamma-carboxy prothrombin; RASSF1A: RAS association domain family 1A.

clinical applicability of CTCs. Xia *et al*[60] designed a near-infrared fluorescent agent named MLP that can bind to aminopeptidase N (APN), which is overexpressed in tumor cells. Double-targeted magnetic fluorescent nanorods (MB-MLP-EpCAM) composed of the antibody EpCAM and a fluorescent group (MLP) were synthesized on Fe_3O_4 magnetic nanorods (MB). EpCAM is used as a routine biomarker to bind and enrich HCC-CTCs. In addition, the fluorescent group (MLP), driven by the targeted protease (APN), not only improves the labeling efficiency of HCC-CTCs but also improves the detection purity with higher resolution. Hence, the combination of overexpressed EpCAM and APN as two specific targets can significantly increase the CTC capture rate (>85%) without compromising the cell survival rate (>90%) and without interfering with false-positive signals caused by a single target.[60] In addition, Guo *et al*[43] developed an optimized qPCR-based detection platform to determine whether a panel comprising four putative stem cell biomarkers (EpCAM, CD90, CD133, and CK19) was superior to EpCAM alone in diagnosing HCC, with AUC values of 0.88 and 0.93 in the training and validation cohorts, respectively. The diagnostic value of ANHC was likewise satisfactory (AUC: 0.89, sensitivity: 77.7%, specificity: 95.0%).[43]

ct-ncRNA

Ct-ncRNAs can be released into the bloodstream by tumor and host cells through apoptosis/necrosis, active secretion by EVs, or binding to plasma proteins. With the rapid progress of ncRNA research in recent years, ncRNA regimens have gradually emerged for clinical HCC monitoring. circRNAs can be used as biomarkers of HCC; a large-scale multicenter study found that plasma circRNA kits (circpanel) of hsa_circ_0000976, hsa_circ_0007750, and hsa_circ_0139897 circRNAs can be used to detect hepatitis B virus-associated HCC. The study determined that it has higher accuracy than AFP in distinguishing HCC from non-HCC patients (sensitivity 83% *vs.* 59.1%, specificity 87.3% *vs.* 88.6%) and has superior performance in diagnosing ANHC (sensitivity 76.2%, specificity 93.8%) and small ANHC (sensitivity 75%, specificity 93.8%).[46] Currently, the majority of studies pertaining to ct-ncRNAs are still in the preclinical phase, although these related diagnostic panels have been greatly promoted in China. Through the study of plasma miRNA expression in healthy, LC, chronic hepatitis B (CHB), and HBV-related HCC patients, the JiaFan team postulated that plasma miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801 were potential circulatory markers for the diagnosis of HCC. Compared with AFP, their sensitivity for HCC can be increased by 30%. This miRNA panel

(miRNA7 ™) has been applied in clinical settings and distinguishes HCC with high accuracy from the healthy liver (sensitivity: 83.2%, specificity: 93.9%), CHB (sensitivity: 79.1%, specificity: 76.4%), and LC (sensitivity: 75.0%, specificity: 91.1%).[45]

cfDNA and ctDNA

The diagnostic value of cfDNA and ctDNA in HCC

In 1948, Mandel and Métais first reported the existence of cfDNA in human blood, which is mainly derived from cellular rupture and active DNA release mechanisms such as the vesicular transport of nucleic acids (such as exosomes, virions, and Argonaute2).[61] The concentration of cfDNA is very low in healthy people (about 10–15 ng/mL on average), while the overall level of cfDNA in cancer patients is higher than that in non-cancer patients.[62] Meanwhile, the ctDNA produced by necrosis, apoptosis, and the active release of DNA is part of cfDNA, and its proportion ranges from <0.1% to > 10%.[63] There is currently no method to specifically isolate ctDNA specifically from cfDNA. Only when tumor genome-specific mutations, such as single-nucleotide variation (SNV), copy number variation (CNV), and epigenetic changes, are detected in cfDNA can the existence of ctDNA be confirmed.[47] A study theorized that 73.0 ng/mL is the optimal cut-off value of cfDNA level that can distinguish between HCC and HCV carriers, with a sensitivity of 69.2% and a specificity of 93.3%, significantly higher than AFP. The combination of cfDNA and AFP can further distinguish between HCC and non-HCC patients, with a sensitivity of 95.1% and a specificity of 94.4%.[47] Cai *et al*[64] described that the levels of plasma CNV and SNV were dynamically correlated with the tumor load of HCC patients through SNV and CNV analyses of plasma ctDNA from 34 patients with long-term HCC follow-up. It can not only evaluate the postoperative minimal residual disease but also diagnose tumor recurrence 4–6 months earlier than the serum markers AFP, AFP-L3%, and DCP.

Application of ctDNA methylation for the diagnosis of HCC

In recent years, the "methylation mode" of ctDNA has been the most concerned research hotspot. DNA methylation is an epigenetic regulation of gene expression, which usually leads to gene silencing, whereas tumor suppressor gene methylation is an early event in various tumors; therefore, the detection of early tumor DNA methylation can provide a reliable diagnostic approach for the early diagnosis of tumors. Wong *et al*[65] detected p15 and/or p16 methylation in 92% of HCC patients for the first time, and 87% (20 of 23 cases) of HCC tumor methylation patients had aberrant methylation in peripheral blood, which laid the foundation for the early non-invasive diagnosis and disease surveillance of small HCC in high-risk populations. In addition, RAS association domain family 1A (RASSF1A) promoter methylation was detected in 80–90% of HCC tissues. Zhang *et al*[66] found that hypermethylation of p16, p15, and RASSF1A could be detected 1–8 years, 1–5 years, and 1–9 years before clinical diagnosis, respectively. 5-hydroxymethylcytosine (5hmC) is a rich epigenetic marker generated by the oxidation of 5-methylcytosine by 10–11 translocation enzymes. The modification of 5hmC is related to the pathobiology of cancer[67]; the JiaFan team used the 5hmC-Seal technique to acquire the map of 5hmC in the whole genome of 2554 Chinese patients with cfDNA and developed a diagnostic model of 32 tumor-related genes. This model accurately distinguished early HCC (stage 0a) from non-HCC in Barcelona. The sensitivity and specificity for early HCC and CHB/LC were 82.7% and 67.4%, respectively. Conversely, the sensitivity and specificity of AFP were only 44.8% and 76.1% (cut-off value = 20 ng/mL).[49]

EVs

EVs are mainly composed of microvesicles (MVs)/microparticles (MPs) and exosomes (Exs). On the one hand, EVs are nanosized membranous vesicles produced by most kinds of cells. On the other hand, EVs are abundant in various body fluids, which contain not only DNA and mRNA but also a large amount of ncRNA. EVs regulate and participate in multiple physiological and pathological processes, including carcinogenesis. Lastly, EVs fall under the category of fluid biopsy, and several studies on circulating ncRNAs are based on the detection of exosomal ncRNAs.

EVs are promising biomarkers for the diagnosis of early HCC. A number of EVs and exosomal proteins, lipids, and ncRNAs can provide new biomarker information on HCC. Zhang *et al*[68] quantified >1400 exosomal proteins by super-SILAC-based MS analysis on the exosomes secreted by three human HCC cell lines, including the non-motile Hep3B cells and the high-motile MHCC97H (97H) and MHCCLM3 (LM3) cells. It was found that 469 and 443 exosomal proteins were differentially expressed in 97H/Hep3B and LM3/Hep3B, respectively. These proteins are involved in glycolysis I, gluconeogenesis I, and pentose phosphate pathways, suggesting that metastatic HCC cells tend to output more glucose metabolism-related proteins through exosomes. Sohn *et al*[69] detected the expression level of miRNAs by extracting serum exosomal miRNAs via qRT-PCR. They evinced that the exosomes miR-18a, miR-221, miR-222, and miR-224 from HCC patients were significantly higher than those in patients with LC, whereas the levels of miR-101, miR-106b, miR-122, and miR-195 were lower than those in patients with LC, signaling that serum exosomal miRNAs can be used as new serological markers of HCC. Furthermore, several EV-related studies have demonstrated excellent application prospects in the screening and recurrence detection of HCC. With improvements of detection technology, EVs are likely to become an integral part of clinical fluid biopsies.

Molecular Diagnosis of HCC Based on Fine-Needle Aspiration (FNA) Biopsy

As biomedicine gradually enters the era of precision medicine, the diagnosis of HCC should also follow the principles of individualization and precision, necessitating a more detailed and microscopic diagnosis of

HCC. The molecular diagnosis of HCC specifically involves using high-throughput sequencing techniques, such as whole-genome sequencing, exome sequencing, and single-cell sequencing, to type HCC at the molecular level.

For hepatic space-occupying lesions undiagnosed via laboratory and imaging tests, the AASLD and ECSL guidelines recommend the use of liver biopsies as additional testing. However, at present, diagnosis via pathological examination may be challenging in some cases.[3,4] For instance, liver adenoma and high-grade atypical nodules are characterized by cytological atypia and structural alterations, but this is insufficient to diagnose HCC and is readily mistaken for highly differentiated HCC. Molecular analysis using the acquired tissues will become an effective means to assist the diagnosis of HCC.

FNA biopsy is a safe, effective, minimally invasive, and low-cost method of pathological examination that permits exploration in different directions. Based on FNA sampling, gene detection, molecular diagnostics, and single-cell sequencing have become significant precision medicine tools and foundations. According to earlier LC studies, GPC3, HSP70, and GS as a panel of three immunohistochemical markers could clinically distinguish high-grade dysplastic nodules from highly differentiated HCC; when any two of the three markers were positive, the sensitivity and specificity were 71.88% and 100%, respectively.[70] The inclusion of Annexin A2 (ANXA2) in the panel further enhanced the diagnostic accuracy of highly differentiated HCC; when three of the four markers were positive, the sensitivity and specificity were 74% and 100%, respectively.[71]

Imaging for HCC

When monitoring high-risk HCC patients, CT or MRI can be used to confirm the diagnosis of HCC in patients with suspected HCC but with negative AFP or insufficient ultrasonographic evidence.[4] However, for the diagnosis of early stage HCC and micrometastasis, as well as the evaluation of MVI, the specificity and sensitivity of previous multiphase CT or MRI are relatively low. Consequently, early accurate detection is intricate to achieve. The application of contrast media can improve diagnostic efficiency. The enhanced MRI of Gadolinium-ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA), a liver-specific contrast agent, has been recommended by multiple guidelines as an effective method for detecting and characterizing liver lesions. Nevertheless, its diagnostic accuracy for small HCC is not ideal. Actually, the diagnostic sensitivity is merely 45–80%, and the sensitivity for tumors smaller than 1 cm is as low as 46%.[72]

Novel contrast agents for HCC imaging

In recent years, biocompatible nanoparticle contrast agents have proven to be an effective means for MRI to improve the diagnostic rate of small HCC, achieving accurate imaging diagnosis and enhancing the diagnostic performance of HCC lesions measuring <1 cm. Gdofullerenes nanoparticles (GFNPs) (such as Gd@C82 and Gd3N@C80) have excellent superparamagnetism and acceptable biocompatibility and have been widely used as MRI contrast agents. Under alkaline conditions, GFNPs (GF-OH) are synthesized from H_2O_2 and solid Gd@C82 by a simple solid–liquid nucleophilic reaction. In the H22 mouse orthotopic liver cancer model, GF-OH can specifically disseminate in liver tissues and recognize 0.5 mm-sized liver tumors. In addition, the imaging window is more than 3–6 hours, which is 6–12 times that of common contrast agents. Superparamagnetic iron oxide nanoparticles (SPIONs) are commonly used as T2 contrast agents. However, traditional SPIONs (e.g., Feridex and Resovist) have a low transverse relaxation rate (R2), reducing the sensitivity of the contrast medium.[73] Furthermore, adverse side effects (back pain) and long-term accumulation of large large-diameter SPIONs in the liver when used as a contrast agent limit their application.[47] Sridharan *et al*[74] developed a superior reticular endothelial nano-contrast agent, namely, iron-doped nano-calcium phosphate (nCP: Fe-CA), based on the natural biological mineral calcium phosphate which can detect liver tumor lesions with a diameter <0.25 cm and multiple cirrhotic nodules with a diameter <0.2 cm. Compared with SPIONs, nCP: Fe-CA degrades and has a high clearance rate. Under identical conditions, the hepatobiliary clearance rate of nCP: Fe-CA is 72 h, while the retention time of SPIONs is 30 days, and the biosafety of the former is relatively superior to that of the latter.[74]

Application of radiomics for the early diagnosis and differential diagnosis of HCC

The limitations of traditional imaging and reporting methods, such as insufficient depth of interpretation of image features and subjectivity of the observer (doctor's knowledge base, diagnostic expertise, and working status), cannot meet the needs of modern precision medicine. The term "radiomics" was coined by Lambin *et al*[75], which aims to use "advanced feature analysis" to extract quantitative data from medical images and explore the correlation between research methodology and clinical outcomes. It is an objective approach to translating imaging features into digital data as an auxiliary diagnostic means to effectively improve the error tolerance rate of imaging doctors.[75] Tumor segmentation, feature extraction, data preprocessing, dimension reduction, modeling, and model evaluation are all components of radiomics analysis. Firstly, the region of interest (ROI) of the imaging data is delineated (tumor segmentation), then tools such as "PyRadiomics package" are employed to extract imaging markers (feature extraction), convert, and normalize the collected data to obtain preprocessed data. Thereafter, the dimension of the preprocessed data is reduced using approaches such as least absolute shrinkage and selection operator (LASSO), neighborhood rough set (NRS), and so on. K-nearest neighbors (KNN), support vector machine (SVM), random forest (RF), back-propagation neural network (BPNet) and other methods are subsequently employed to construct the model. Finally, the model is internally or externally validated.[76]

Radiomics analysis is conducive to the early diagnosis, differential diagnosis, and evaluation of MVI in HCC[77-83] [Table 3]. Dankerl *et al*[84] reported that radiomics methods can outperform imaging doctors in predicting the nature of the lesion (benign and malignant), with an accuracy of 75.1%, whereas the range of imaging doctors is between 52% and 74%, depending on their level of experience. Due to atypical imaging features, 30% and 20% of the cases diagnosed via CT and MRI, respectively, are challenging to be confirmed by FNH. The hepatobiliary phase (HBP) of MRI (Gd-EOBDTPA-MRI) provides valuable diagnostic information for distinguishing FNH from HCC, but the differential diagnosis is also difficult because of their overlapping features. Ding *et al*[77] developed a radiology model to distinguish HCC from non-cirrhotic FNH using MRI (Gd-EOBDTPA-MRI). The accuracy of the model was 95.6% and 94.1% in the training cohort and validation cohort, while the AUC was 0.984 and 0.972, respectively. In recent years, multiple studies have been conducted on the diagnosis of MVI by radiomics approaches. Several MVI diagnostic models have thus been established using different dimensionality reduction and modeling methods. One study compared the diagnostic performance of different models established by each method. The results showed that, the LASSO + GBDT method had the highest accuracy (84.48%), the LASSO + RF method had the highest sensitivity (92.59%), and the LASSO + BPNet method had the highest specificity (87.50%). This also suggests that different image modeling methods have contrasting clinical values for the diagnosis of MVI.[85]

Deficiency and Prospect

At present, challenges in the diagnosis of early stage HCC and missed diagnosis of micrometastases remain the dominant factors for the poor prognosis of HCC. When exploring the diagnostic and screening methods of HCC, we should not only assess their sensitivity but also consider the invasiveness of the examination. In recent years, studies on "precision diagnosis" of different types of HCC have emerged one after another [Figure 1]. Although emerging serum markers complement AFP and have potential advantages in early HCC diagnosis, a further comprehensive evaluation is required to evaluate its detection complexity, cost, and accuracy. With improved detection ability and the accumulation of clinical data, emerging serum markers are anticipated to be widely used in clinics. In recent years, liquid biopsy has become a research hotspot for the diagnosis of HCC. The samples can be easily acquired and, combined with sequencing methods, accurately diagnose HCC at the molecular level. Additional related panels have been widely used in clinics, but the difficulty in liquid biopsies lies in the separation of detection components and the selection of sequencing targets. Molecular diagnosis is the embodiment of accurate diagnosis in precision medicine. When imaging and routine tissue microscopic examination are unable to reach a diagnosis, FNA-based molecular approaches can diagnose HCC with minimal invasion, representing one of the future directions for the development of precision medicine in the differential diagnosis of HCC. Nonetheless, imaging remains the primary approach for clinical HCC diagnosis. Contrast agent studies are gradually focusing on nano-metal

Table 3: Studies of radiomics in the differential diagnosis of HCC.

Imaging modality	Radiomics features	Algorithm	Clinical application	Performance	References
MRI	2260	mRMR, LASSO, RF, multivariable LR	Differentiation between HCC and FNH in non-cirrhotic liver	Accuracy: 95.6%, AUC: 0.984 (in training cohort); Accuracy: 94.1%, AUC: 0.972 (in validation cohort)	[77]
US	5324	LASSO, LR	Differentiation of HCC from other primary liver cancers	AUC: 0.854 (in training cohort); AUC: 0.775 (in validation cohort).	[78]
MRI	1029	LASSO, DT, KNN, RF, LR,	Differentiation between HCC and hepatic hemangioma	AUC: 0.86, sensitivity: 76%, specificity: 78% (in training cohort); AUC: 0.89, sensitivity: 82.2%, specificity: 71.4% (in validation cohort);	[79]
CT	3376	RFE	Preoperative prediction for pathological grade of HCC	AUC: 0.8378 (in training cohort); AUC: 0.7579 (in validation cohort).	[80]
MRI	656	LASSO, LR	Preoperative prediction for pathological grade of HCC	AUC based on T1WI images: 0.712 AUC based on T1WI images: 0.722 AUC based on the combined T1WI and T2WI images: 0.742 (in validation cohort).	[81]
MRI	GALAD	SVM, LASSO	Prediction of small HCC (<2 cm)	AUC: 0.93, sensitivity: 87.50%, specificity: 85.71% (in intra-group validation cohort); AUC: 0.97, sensitivity: 94.12%, specificity: 94.12% (in inter-group validation cohort);	[82]
MRI	837	LASSO, LR	Differentiation of small HCC (≤3 cm) from benign nodules in cirrhotic liver	AUC: 0.917, sensitivity: 93.8%, specificity: 86.4%.	[83]

AUC: Area under curve; CT: Computed tomography; DT: Decision tree; FNH: Focal nodule hyperplasia; GALAD: Consisting of gender, age,AFP-L3, AFP, and des-carboxy-prothrombin (DCP); HCC: Hepatocellular carcinoma; KNN: K-nearest neighbors; LASSO: Least absolute shrinkage and selection operator; LC: Liver cirrhosis; LR: Logistic regression; MRI: Magnetic resonance imaging; RF: Random forest; RFE: Recursive feature elimination; SVM: Support vector machine; US: Ultrasound.

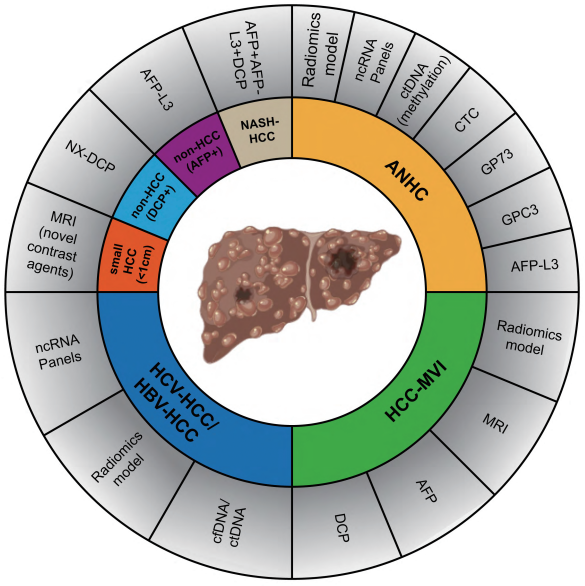

Figure 1: Precision diagnostic methods of different types of HCC. AFP: Alpha-fetoprotein; AFP-L3: Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein; ANHC: AFP-negative HCC; cfDNA: Cell-free DNA; ctDNA: Circulating tumor DNA; CTC: Circulating tumor cell; DCP: Des-gamma-carboxy prothrombin; GP73: Golgi protein 73; GPC3: Phosphatidyl alcohol proteoglycan-3; HBV-HCC: Hepatitis B virus-related hepatocellular carcinoma; HCC: Hepatocellular carcinoma; HCC-MVI: Hepatocellular carcinoma related microvascular invasion; HCV-HCC: Hepatitis C virus-related hepatocellular carcinoma; MRI: Magnetic resonance imaging; NASH-HCC: Non-alcoholic steatohepatitis related hepatocellular carcinoma; ncRNA Panels: Non-coding RNA panels; non-HCC (AFP^+): Non-hepatocellular carcinoma (Alpha-fetoprotein positive); non-HCC (DCP^+): Non-hepatocellular carcinoma (Des-gamma-carboxy prothrombin positive); NX-DCP: Novel des-gamma-carboxy prothrombin; small HCC (<1 cm): small hepatocellular carcinoma (tumor size <1 cm).

particles through interdisciplinary research. Through enzyme and/or biocompatible nanomaterials, targeted clusters are gathered in HCC, which improves sensitivity and specificity and can detect millimeter-level early HCC lesions. However, clinical trials are scarce, and their safety and clinical applicability are still under investigation. Through the construction of an HCC diagnosis or differential diagnosis model, radiomics can be used as a decision-making tool for clinicians and effectively reduce the error rate of radiologists. However, there are still obstacles and limitations in the clinical application of radiology, such as consistency in imaging quality and quantity, the standardization of radiomics analysis, and the retrospective rather than prospective nature of most research methods. We postulate that with a better understanding of the molecular mechanism underlying the biological behavior of HCC and continuous advances in related disciplines and technologies, these examination methods will be continuously optimized and improved, and new detection methods will emerge to address the bottleneck in the early diagnosis of HCC, and eventually substantially improve its prognosis.

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Conflicts of interest

None.

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