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Review Article

Current status and new directions for hepatocellular carcinoma diagnosis

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ABSTRACT

Liver cancer ranks as the sixth most common cancer globally, with hepatocellular carcinoma (HCC) accounting for approximately 75%–85% of cases. Most patients present with moderately advanced disease, while those with advanced HCC face limited and ineffective treatment options. Despite diagnostic efforts, no ideal tumor marker exists to date, highlighting the urgent clinical need for improved early detection of HCC. A key research objective is the development of assays that target specific pathways involved in HCC progression. This review explores the pathological origin and development of HCC, providing insights into the mechanistic rationale, clinical statistics, and the advantages and limitations of commonly used diagnostic tumor markers. Additionally, it discusses the potential of emerging biomarkers for early diagnosis and offers a brief overview of relevant assay methodologies. This review aims to summarize existing markers and investigate new ones, providing a basis for subsequent research.

1. Introduction

Primary liver cancer, primarily known as hepatocellular carcinoma (HCC), has emerged as a significant global health issue. In 2022, approximately 865,269 new cases and 757,948 fatalities were documented.[1,2] Indeed, liver cancer is the sixth most frequently diagnosed cancer and the third leading cause of cancer-related deaths worldwide. The prognosis for liver cancer remains grim, with a low 5-year survival rate of approximately 20%.[1] HCC accounts for 75%–85% of liver cancer cases, while intrahepatic cholangiocarcinoma (ICC) represents 10%–15%, along with other rare subtypes, including HCC and ICC mixed types. Consequently, HCC is often used interchangeably with liver cancer in general discussions.

Pathogenic sequences of HCC usually encompass liver injury, chronic inflammation, fibrosis, cirrhosis, and liver cancer.[1,2] Notably, the main risk factors for HCC include liver flukes (*e.g.*, endemic in northeastern Thailand and the Southeastern Asian liver fluke *Opisthorchis viverrine*), abnormal metabolic conditions (including obesity, diabetes, and metabolic-associated fatty liver diseases (MAFLD)), excessive alcohol consumption, and hepatitis B and C virus (HBV and HCV, respectively) infection.[3,4] Among various factors, simple fatty liver, steatohepatitis, and fatty liver

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cirrhosis are all interrelated conditions classified as fatty liver diseases. The common histopathological features of clinical MAFLD include inflammatory changes, such as steatosis, ballooning degeneration of hepatocytes, Mallory body formation, and necrosis.[5,6] Approximately 10%–20% of patients with MAFLD have steatohepatitis, making it a common complication. Unlike viral hepatitis, some forms of fatty liver can be reversible. Individuals with coinfections with HBV and HCV, as well as those with a single infection with hepatitis D virus (HDV) in endemic regions, are at the highest risk for developing HCC. Additionally, an acute inflammatory response typically resolves once the external stimuli are eliminated. However, unresolved chronic inflammation may lead to liver fibrosis and eventually cirrhosis, which is often followed by HCC.[7] Although preventive interventions to halt the progression to cirrhosis are crucial in decreasing the incidence of HCC, infections such as HBV and HCV, as well as conditions like hemochromatosis, can synergistically lead to HCC without triggering cirrhosis, indicating that cirrhosis is not the sole pathogenic inducer of HCC.[8] The aforementioned main pathogenic factors for HCC may demonstrate various roles in different regions. In high-risk areas, the key determinants of HCC development are chronic HBV infection, aflatoxin exposure, or a combination of both. Conversely, in other areas, HCV infection may be the main cause of HCC.[9] The global cancer data reveals a complex picture, with 21%–55% of HCC cases worldwide attributed to chronic HBV or HCV infections.[2] In high-risk regions such as China and East Africa, chronic HBV infection and aflatoxin exposure are the primary risk factors. In contrast, in Egypt, Italy, and Japan, HCV infection is the leading cause, highlighting the variability of risk factors by region. Moreover, in Mongolia, factors such as HBV and HCV coinfection, HDV infection in HBV carriers, and alcohol consumption contribute to the highest incidence of cholangiocarcinoma in both men and women.[5,6] In summary, liver cancer, particularly HCC, has diverse causative factors, which may differ according to biogeographic regions, environmental factors, and lifestyle habits.

HCC has a complex and varied pathogenesis, necessitating diagnostic methods to adapt to the underlying causative factors and endemic areas. Currently, serological tests and imaging techniques are the most common methods for the early diagnosis of HCC. According to the National Comprehensive Cancer Network clinical practice guidelines, patients at high risk of HCC development (i.e., patients with cirrhosis) must be monitored with abdominal ultrasonography and alpha-fetoprotein (AFP) screening every six months.[10] However, the effectiveness of ultrasonography is limited by its dependence on operator competence and the difficulties in distinguishing between malignant and benign nodules in small cirrhotic livers. A high AFP level is detected in benign liver diseases such as hepatitis and cirrhosis.[11] Thus, early-stage liver cancer must be subjected to an AFP test. However, even with a low cutoff value (10–20 ng/mL), the sensitivity of AFP for diagnosing HCC is approximately 60%, and its specificity needs improvement.[12] In addition, false-negative diagnostic results are based on AFP, and approximately 15%–30% of patients with advanced HCC demonstrate a normal AFP level in the blood.[13] As a result, the American Association for the Study of Liver Disease practice guidelines committee no longer recommends AFP as a marker for the early detection of HCC.[14] Consequently, scientists have developed several AFP variants as more sensitive and accurate diagnostic biomarkers, including des-gamma-carboxyprothrombin (DCP), Golgi protein 73 (GP73), and glypican-3 (GPC-3). However, the diagnostic reliability of each serum marker is insufficient, and combinations of multiple markers must be monitored for an appreciable accuracy of 80%–90%.[15–17] The accuracy of marker testing for HCC varies according to different pathogenic factors, such as HBV and HCV infections, hemochromatosis, MAFLD, α1-antitrypsin deficiency, and cirrhosis. This variability can also be influenced by regional and ethnic differences, alcohol consumption, lifestyle factors,[18–21] and pathogenic factors that do not adhere to the typical progression of liver injury, chronic inflammation, fibrosis, cirrhosis, and liver cancer.[22,23] The HCC size also plays a significant role in diagnosis. According to international standards, HCCs measuring >5 cm are classified as large, whereas those measuring <2 cm are termed small. Large HCCs exhibit considerable genomic, proteomic, metabolomic, and bacteriologic variations. Patients with large HCCs are typically symptomatic and can be easily diagnosed through imaging, whereas diagnosing small HCCs is relatively challenging. Tumor size is closely linked to tumor markers, emphasizing the importance of early detection through blood tests for prevention. In recent years, more new potential markers, such as tumor-educated platelets (TEPs), cancer stem cells (CSCs), cytokines, and innovative diagnostic assays such as liquid biopsy and mass spectrometry, have emerged.[24–29] In this review, HCC formation is briefly described, and the diagnostic markers of HCC are summarized to evaluate the clinical contributions of existing markers and the application of novel potential markers in early HCC detection.

2. Origin and development of HCC

The pathogenesis of HCC is a complex, multistep process and involves genetic susceptibility, interactions between viral and nonviral risk factors, cellular microenvironment and various immune cells, and underlying chronic liver disease. These are considered the origin of the malignant transformation of hepatocytes and early HCC development. The severity of these factors and an altered microenvironment are key contributing features to cancer and are involved in all stages of malignant progression, from the initial transformation stage to invasion and ultimately metastasis.[30]

The cellular origin of HCC remains elusive, probably because of the heterogeneity of liver cancers within the same tumor and among different tumor cells. In an animal study, Mu *et al.*[31] used complementary fate-tracking approaches and determined that HCC in mice originated exclusively from hepatocytes rather than from the progenitor or biliary compartment. Interestingly, some mouse models of HCC support the likelihood of mature hepatocytes, which dedifferentiate into juvenile cells and regain self-renewal ability, as the cellular source of HCC.[32] Furthermore, hepatocyte nuclear factor 4α (HNF4α), a core component of the liver-specific regulatory network, was reported to play a vital role in hepatocyte establishment and maintenance.[33] Notably, the aberrant expression of HNF4α contributes to the proliferation and loss of epithelial morphology, exacerbates dedifferentiation, and induces HCC progression in rodents and humans.[34]

The blockage of stem cell (SC) differentiation is proposed to be a crucial driver of tumor formation. However, unlike most other organs, the liver lacks a well-defined SC population. Currently, most opinions revolve around two types of hepatic SCs, namely, endogenous and exogenous hepatic SCs, both of which may play potent roles in HCC formation. Endogenous hepatic SCs are further subdivided into hepatic oval cells, small hepatocytes, and embryonic hepatocytes. Among these subtypes, hepatic oval cells are believed to play a dominant role in HCC development.[35,36] As illustrated in Fig. 1 A and B, the central vein serves as the focal point of the hepatic lobule, around which the hepatic plates, hepatic blood sinusoids, and bile ducts are radially distributed. Parenchymal cells make up most hepatocytes, mainly hepatocytes and biliary epithelial cells (BECs) or cholangiocytes, which are also known as

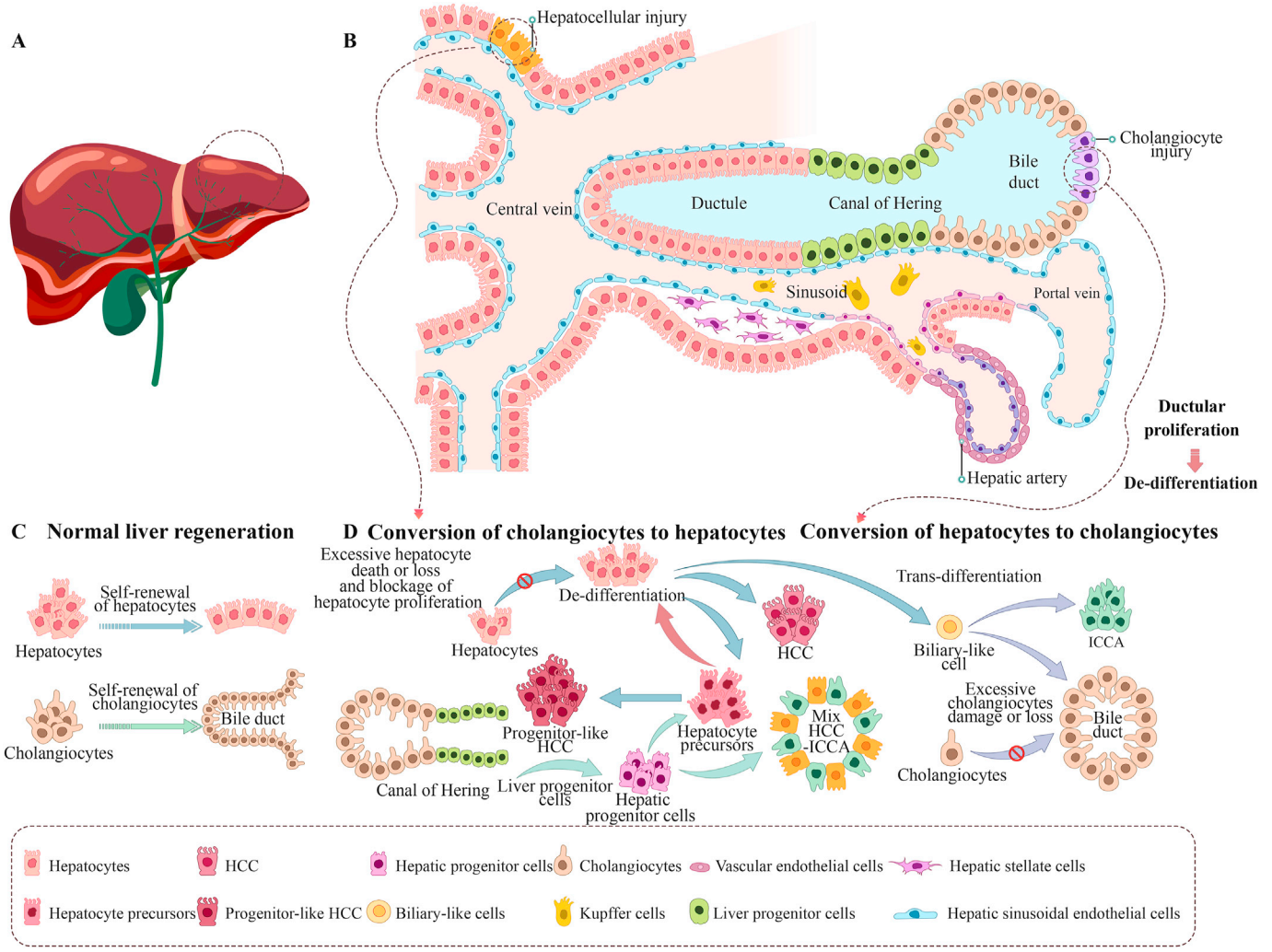

Fig. 1. Structure and regeneration of the hepatic lobule. **(A)** Diagram of the liver structure. **(B)** The hepatic lobule is the fundamental unit of the liver structure and function, exhibiting a multifaceted and prismatic nature. It is responsible for vital functions, including metabolic regulation, detoxification, and bile production. The focal point of the hepatic lobule structure is the central vein, around which the hepatic plates, hepatic blood sinusoids, and bile ducts are radially distributed. These components consist of hepatic parenchymal (predominantly hepatocytes and BECs) and nonparenchymal cells (*e.g.,* fibroblasts, stellate cells, Kupffer cells, and endothelial cells). **(C)** Liver regeneration includes the regeneration of liver parenchymal cells and the reconstruction of the liver tissue structure. Typically, phenotypic fidelity characterizes the regenerative activity in liver regeneration: that is, the proliferation and self-renewal of the parenchymal cells, including hepatocytes and cholangiocytes. **(D)** When hepatocyte proliferation is impaired due to excessive cell death, liver progenitor cells with hepatobiliary characteristics, derived from bile duct cells, may gradually differentiate into hepatocytes. Conversely, if cholangiocyte proliferation is inhibited by excessive cholangiocyte damage or loss, periportal hepatocytes can also be transformed *in situ* into cholangiocytes, mimicking similar transformations that occur during embryonic development. These retrotransformation processes have the potential to contribute to tumorigenesis. Abbreviations: BECs, biliary epithelial cells; HCC, hepatocellular carcinoma; ICCA, intrahepatic cholangiocarcinoma.

bile duct cells and are arranged in the biliary tree to alter the hepatocyte-secreted bile. The remaining small proportions of liver cells are nonhepatocytes, such as fibroblasts, stellate cells, Kupffer cells, and endothelial cells.[37] As shown in Fig. 1C, the regenerative activity in liver regeneration is characterized by phenotypic fidelity: hepatic epithelial cells (hepatocytes and cholangiocytes) proliferate, resulting in more identical cells.[38] If the proliferative capacity of liver cells is severely impaired (Fig. 1D), liver SCs are activated. These cells, also known as oval cells, possess a high nucleoplasmic ratio and are located in the periportal region of the Herring canal, where they are considered bipotential progenitor cells. In cases of excessive hepatocyte death or inhibited hepatocyte proliferation, progenitor cells with hepatobiliary characteristics originate from bile duct cells and gradually differentiate into hepatocytes. If bile duct cell proliferation is inhibited, periportal hepatocytes transform into bile duct cells *in situ*, mimicking similar transformations that occur during embryonic development and are used for the structural and functional reconstructions of the liver.[39–41] These retrotransformation processes may ultimately lead to tumor cell production.

The investigators utilized diphtheria toxin receptor and green fluorescent protein knockout mice to examine leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5)-positive SCs. They monitored the behavior of these SCs in the liver under physiological conditions and in response to carbon tetrachloride (CCl_4)-induced liver injury. Additionally, the researchers isolated SCs from mouse liver and subjected them to an organoid formation test. The results indicate that LGR5-expressing SCs are only detectable after CCl_4-induced liver injury but not at any stage of the normal mouse lifespan.[42] Another study reported that the LGR5/R–Spo1/Wnt3a axis promotes the stemness of “hepatoblast-like” HCC cell lines.[43] In the study, LGR5 expression was explicitly observed in a specific subset of HCC cell lines that displayed a hepatoblast-like appearance, along with the expression of liver fetal or progenitor markers. Furthermore, Cao *et al.*[42] identified a static or slow-circulating cell population called the label-retaining cells (LRCs), located in the

mouse gastrointestinal tract and gallbladder.[44,45] The isolated single LRC can form organoids, carried and expanded in culture for several months, and differentiated into hepatocytes and bile duct cells *in vitro*.

Exogenous hepatic SCs primarily include bone marrow-derived hematopoietic SCs, mesenchymal SCs (MSCs), as well as pancreatic and adipose SCs in the blood circulation. Under conditions of liver injury, bone marrow SCs can migrate to the liver portal area and differentiate into hepatic parenchymal or bile duct epithelial cells.[46] This theory has been further supported by an *in vitro* study demonstrating that CCl_4-induced liver injury serum treatment induced MSC differentiation into hepatocyte-like cells.[47] To date, several surface markers and side populations of HCC have been isolated, including EpCAM, CD133, CD44, CD13, CD90, CD24, CD47, and OV6. They identify the presence or absence of CSCs by their markers, rather than by a defined cell lineage, with the most common method of identifying hepatic CSCs being the use of the cell surface antigens CD133 and CD44 as surface markers of liver SCs to label isolated cells.[48–50]

HCC can also originate from the accumulation of somatic mutations or epigenetic alterations. Although most HCCs occur in "passenger" genes, some are considered "driver factors" for the activation of key signaling pathways that may lead to hepatocarcinogenesis.[51] For instance, the mutation of telomere reverse transcriptase (TERT) promoter is barely detected in normal human hepatocytes but is quite common in dysplastic nodules and patients with HCC.[52,53] These alterations are the most frequent recurrent somatic mutations identified in low-grade dysplastic nodules (LGDN) (6%), high-grade dysplastic nodules (HGDN) (20%), and HCC (60%).[54–56]

During HCC development, patients typically experience chronic hepatitis, liver fibrosis, and cirrhosis. Approximately 70%–80% of liver cancer cases occur in the presence of cirrhosis, which is a complex process driven by a series of molecular events.[30,57] The onset of hepatitis can be triggered by various factors such as HBV or HCV, alcoholism-induced alcoholic liver disease (ALD), metabolic dysfunction-associated steatohepatitis (MASH), drug-induced liver injury, and other lifestyle factors. A long-term inflammatory response due to persistent disease may cause sustained damage and result in liver fibrosis and cirrhosis. Ultimately, the process terminates with HCC with characteristic precancerous cirrhotic nodules, commonly known as LGDNs. With the aggravation of symptoms, LGDNs progress to HGDNs, which are then transformed into early- or later-stage HCC.[58–61] Importantly, in approximately 20%–30% of cases without cirrhosis, HCC may primarily develop in the presence of both HBV and HCV infections, MASH, or adenomas.[62] In general, liver cancer is an irreversible process; therefore, early detection and subsequent targeted therapies are the only ways of increasing patient survival.

3. Common clinical biomarkers of HCC

Current clinical practice guidelines recommend regular screening for HCC in patients with liver disease due to hepatocyte regeneration.[63] The "China guideline for liver cancer screening (2022, Beijing)" and the "Expert consensus on early screening strategies for liver cancer in China" emphasize the importance of accurate liver cancer screening and effective early screening strategies for liver cancer,[64,65] similar to the "pyramid" hierarchical screening model and focus on high-risk groups. The European Association for the Study of the Liver (EASL) – European Organization of Research and Treatment of Cancer (EORTC) HCC Clinical Practice Guidelines are the first comprehensive HCC management guidelines.[66] These guidelines emphasize the importance of early screening and prevention and recommend monitoring and diagnostic strategies for at-risk populations. Blood biomarkers, primarily proteins, cytokines, enzymes, and isoenzymes, are still of great significance for the early diagnosis of liver cancer. Although many biomarkers associated with liver cancer pathology are known, none of them, individually, can provide sufficient evidence for liver cancer detection. Therefore, the diagnostic accuracy and specificity can be greatly improved by the joint detection of multiple markers (Table 1).[67–84]

3.1. AFP

AFP, replaced by albumin 2–3 months after birth and encoded by the q-arm 25th region of chromosome 4, is the most commonly used marker for diagnosing HCC. AFP is a member of the albumin family and a glycoprotein composed of 591 amino acids with a molecular weight of 68 kDa, mainly produced by the fetal liver and yolk sac during pregnancy.[85,86] Individuals with HCC often exhibit high AFP levels in their bloodstream. Varying degrees of elevation may precipitate cirrhosis and liver damage.[87] In addition, several physiological factors during pregnancy and gonadal-derived malignant tumors, such as gastric cancer, can increase the circulatory levels of AFP.[88]

AFP is produced in endoderm-derived tissues during embryogenesis, and solid cells produce and secrete AFP in the early stages of development. Notably, the blood level of AFP gradually falls to a minimal amount of <10 ng/mL in neonates within approximately 300 days, and this occurs along with further development and maturity of the host tissues and organs.[89] Consequently, under normal conditions, the blood levels of AFP in adults are ≤20 ng/mL.[90] In other words, endoderm-like cells initiate AFP synthesis and secretion, which gradually increases during post-embryonic development. In contrast, AFP secretion gradually diminishes as the fetus reaches a later stage of development. When a liver tumor occurs and hepatocytes are damaged, malignant cells lose their ability for terminal differentiation and remain at a particular stage of cell development. The proliferation and secretion of products of immature cells turned out to be abnormal. Thus, AFP is not a tumor-specific protein but a product of early immature cells with a more active AFP gene.[91] Relying only on the AFP level may not be sufficient for diagnosing liver cancer, as the exact blood level of AFP is also influenced by tumor size. Moreover, the sensitivity of AFP is 52% in patients with liver cancer with a tumor diameter of >3 cm, but only 25% in those with a <3 cm-diameter tumor.[92] Approximately 80% of patients with early HCC having small tumors demonstrate no significant increase in the blood AFP level.

Furthermore, the production of a high blood level of AFP is not always translated to HCC development. Benign liver lesions, such as those arising from the repair process after various liver injuries, must be first excluded before the possibility of endodermal tumors is considered.[93,94] For instance, the process of recovering from liver injury caused by acute and chronic hepatitis leads to increased AFP production owing to the regeneration of hepatocytes.[95,96] However, the resulting AFP level rarely exceeds 400 ng/mL in the circulation, and blood AFP levels gradually return to normal over 3 months as the regenerating cells differentiate and mature.[97]

High AFP levels in patients are associated with HCV-positive liver disease. The normalization of AFP levels is observed in patients with HCV undergoing interferon therapy. This phenomenon indicates an inflammatory response in hepatocytes, attributed to degeneration and necrosis, followed by controlled cellular regeneration.[98] Approximately 10%–20% of patients with MAFLD have steatohepatitis, and AFP levels are usually increased in these patients, with increasing levels of AFP paralleling the extent of hepatic steatosis.[99] AFP testing is frequently used to detect HCC recurrence, as many patients experience early recurrence after treatment, which must be closely monitored. Despite the low sensitivity of

Table 1
Common clinical biomarkers used for HCC diagnosis.

Tests	Biomarkers	Sensitivity (%)	Specificity (%)	Sources	Advantages	Limitations
Individual serologic tests	AFP	54–63	82–89	HCC; cHCC-CC	Most widely used	Nonspecific and may yield false negative
	AFP-L3%	10% –35%:71	10%–35%: 63	HCC(AFP-L3% with total serum AFP of 10–200 ng/mL)	High specificity	No physiological threshold
		>35%: 33	>35%: 100			
	GP73	69	75	HCC	Identifying benign diseases	Difficulty in excluding extraneous sources
	DCP	86	93	Differentiating HCC from cirrhosis	Distinguishing between HCC and cirrhosis	Short half-life and affected by vitamin K
	GPC-3	55	58	HCC	Distinguishing between HCC and ICC	Highly expressed in gastrointestinal tumors
	AFU	64	67	Early-stage HBV-HCC and HCV-HCC	HCC detected earlier than ultrasonography	Congenital lack of AFU
	OPN	71	80	HCC	Sensitivity to HBV and correlated with pathologic features; Monitoring patients with HBV	Expressions were affected by HBV infection
Multiple combined serologic tests	AFP/AFP-L3%/DCP	79	87	HCC	Improving the sensitivity of the diagnosis	Inefficient application of individual indicators
	DCP and AFP	Tumor diameter: 2–3 cm: 70	–	HCC	Exclusion of the effects of vitamin K	Low specificity
		Tumor diameter: 3–4 cm: 80				
	AFU and AFP	53	92	Early-stage HCC	Suitable for early diagnosis	Both may be elevated in pregnant women
	OPN and AFP	82	77	HCC	More suitable as a prognostic indicator	Affected by HBV infection

Abbreviations: AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive α-fetoprotein; AFU, α-L-fucosidase; AUC, area under the receiver operating characteristic curve; cHCC-CC, combined hepatocellular-cholangiocarcinoma; DCP, diethyl chlorophosphate; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; OPN, osteopontin.

AFP, no perfect combination of reliable biomarkers currently exists to accurately detect early HCC recurrence.[100]

Although AFP plays an important role in monitoring HCC recurrence, its reliability as an early diagnostic biomarker remains controversial. A review based on several studies indicates that the sensitivity of AFP for HCC diagnosis only ranges from 54% to 63%, and the specificity ranges from 82% to 89%. Additionally, 30%–40% of patients with HCC exhibit low AFP levels in their blood.[67–71] Retrospective studies conducted in different regions have identified the role of different pathogens in the missed diagnosis of patients with HCC for whom AFP was used as the targeted biomarker.[72] Therefore, a sensitive and specific test must be performed in regions where certain pathogens are endemic to determine how these pathogens may affect serum AFP levels, potentially compromising the reliability of AFP as a biomarker for the early diagnosis of liver cancer.

To date, AFP plays a pivotal role in HCC and has demonstrated new potential applications. Recent studies have suggested its potential as a target for HCC immunotherapy.[101] One such approach is AFP lentiviral priming combined with AFP499 peptide enhancement, which has shown enhanced therapeutic effectiveness in c-MYC/myeloid cell leukemia 1 and c-mesenchymal-epithelial transition factor/beta-catenin mouse models of HCC. The combined use of the AFP vaccine and anti-programmed death ligand 1 (PD-L1) antibody has shown promising results, significantly obstructing HCC progression and representing a hopeful advancement in HCC treatment.

3.2. AFP variant (AFP-L3)

AFP variants refer to the lectin lens culinaris agglutinin (LCA)-binding AFP (AFP-L) proteins. Different sugar chains bind to AFP and form AFP-L1, AFP-L2, and AFP-L3 variants.[102] AFP-L1 is the nonLCA-bound fraction that constitutes the major glycoform of AFP detected in the blood of patients with chronic hepatitis and liver cirrhosis. AFP-L2 is mainly derived from yolk sac tumors and can be detected in mothers during pregnancy. AFP-L3, also known as AFP heterogeneity 3, is an abnormal glycosylated AFP, where the special AFP is produced particularly by liver cancer cells in fragment binding with LCA.[103] In addition, an increase in AFP-L3 levels can be caused by active hepatitis, pregnancy, and female reproductive tumors.[104,105]

The ratio of AFP-L3 to total AFP in serum, referred to as the AFP heteroplasmy ratio or AFP-L3%, is used clinically. In 2005, the United States Food and Drug Administration approved the monitoring of AFP-L3% for liver cancer as an early warning indicator. When an AFP-L3% threshold value of $\geq$10% is used, it serves as a highly specific marker for diagnosing liver cancer.[73] Therefore, AFP-L3% is proposed as a diagnostic and prognostic marker for HCC.[74] A retrospective study of 272 patients (166 with HCC and 106 with benign liver diseases) showed that AFP-L3% was not reported for a total AFP <10 ng/mL and that all patients with an AFP level >200 ng/mL had HCC.[75] In patients with a total serum AFP of 10–200 ng/mL, an AFP-L3% >10% has a sensitivity of 71% and a specificity of 63% for HCC diagnosis.[75] In contrast, an AFP-L3% >35% has a reduced sensitivity of 33% but an increased specificity of 100%.

Furthermore, a retrospective study analyzed 689 patients with liver cirrhosis and HBV infection from a prospective study to determine the diagnostic effectiveness of AFP, AFP-L3, DCP, and combined markers in early HCC detection. AFP, AFP-L3, and DCP levels were compared between the case and control groups at 6 and 12 months, respectively, after HCC detection. At the optimal threshold (AFP, 5 ng/mL; AFP-L3,4%), the sensitivity and specificity of the combination of AFP and AFP-L3 were 79% and 87%, respectively, and were not further improved by the addition of DCP.

Interestingly, AFP-L3 levels began to significantly increase at 6 months in 42 HCC cases but remained unchanged in the control group.[76] Importantly, AFP-L3% is more sensitive and specific than AFP in patients with minor or early-stage HCC and can more exactly reflect tumor characteristics such as poor differentiation and malignant invasion.[77] Despite its appreciable sensitivity and specificity, AFP-L3% is still only used as a recommended test. Although AFP-L3 is closely associated with HCC, it can only be detected as a percentage within the AFP pool and shares with no physiological threshold. Therefore, the combination with AFP-L3 testing is a promising method for early HCC detection.

3.3. GP73

GP73, also known as Golgi membrane protein 1 or Golgi phosphoprotein 2, is a Golgi transmembrane glycoprotein whose complementary DNA was cloned by differential screening of a complementary DNA library extracted from the livers of adult patients with giant cell hepatitis.[106,107] GP73 is located in the cis and medial Golgi cisternae and consists of the cytoplasmic, transmembrane, and Golgi lumen domains.[106] The N-terminal features a hydrophobic structure containing a single transmembrane region with a signal peptide cleavage site, whereas the C-terminal has five glycosylation sites, an α-helix structural domain, and an acid tail consisting of and acting as a functional protein–protein interaction structural domain.[108] GP73 is expressed in various epithelial cells, including ciliated columnar epithelial cells, distal convoluted tubules, collecting duct epithelial cells, prostate epithelial cells, and bile duct epithelial cells in the lungs, kidneys, prostate, and liver, respectively.[109] After the cleavage by preprotein convertase, mature GP73 is released from the Golgi and secreted into the bloodstream.[110]

Studies on various liver diseases, such as HBV, HCV, autoimmune hepatitis, and ALD, have shown that while no significant differences exist among affected liver tissues, the expression level of GP73 in diseased tissues is approximately 70 times higher than in normal tissues.[111] Furthermore, a study involving 352 patients found that the serum GP73 levels in patients with HCC were significantly higher than that in patients with liver cirrhosis.[78] In the same study, the sensitivity and specificity of GP73 for the diagnosis of HCC were reported to be 69% and 75%, respectively.

Moreover, temporal expression trends of GP73 and glucose-regulated protein 78 (GRP78) are similar under pressure from the endoplasmic reticulum (ER) stress. It is a fundamental cellular stress response that maintains cellular protein homeostasis in response to endogenous or exogenous stimuli.[112] As an example of endogenous factors, the accumulation of natural mutations in the HBV genome during viral infection can produce several mutant HBV-encoded proteins, mainly including three S open reading frame (ORF) small, medium, and large, and one C ORF protein HBC core antigen (HBcAg).[113] The accumulation of the mutant proteins stimulates ER stress in infected hepatocytes. As a required heterodimer partner, GP73 deficiency causes a significant reduction of intracellular GRP78. In contrast, the protein level of GRP78 is strongly induced by GP73 overexpression.[114] In addition, a mouse study found that GP73 secretion into the bloodstream is rapidly upregulated under ER stress.[115] The high levels of GP73 increase the opportunities for interaction with GRP78, facilitating subsequent signal transmission to the target cells. However, in the absence of GP73, ER stress is not transmitted among cells, confirming that GP73 is a key ER stress transmission factor. More importantly, GP73 likely affects the microenvironment in viral hepatitis, MASH, and ALD-induced tumors by promoting the activation and transmission of intracellular ER stress.[116–119] Taken together, these molecular events mediated by GP73 are collectively deposited for tumorigenesis and metastasis, which highlights the possibility and reliability of GP73 as an HCC biomarker.

However, GP73 may not serve as a reliable diagnostic biomarker for gastrointestinal tumors unless it is detected in combination with other polypeptide markers, such as AFP or DCP.[120,121] The reason for this is that GP73 is expressed in the epithelial cells of many organs or tissues, except for the liver. Therefore, when using GP73 as a biomarker for early HCC detection, it is challenging to exclude contributions from other tumor tissues located outside the liver.

3.4. DCP

DCP is a novel serological biomarker for HCC and is induced by the absence of protein induced by vitamin K or by the action of the antagonist-II. As an abnormal prothrombin moiety, DCP is produced by the carboxylation defect of the prothrombin precursor after translation, enabling its occurrence in HCC cells.[122] Moreover, because the production of normal prothrombin is tied to vitamin K sufficiency, DCP is released under physiological conditions with insufficient vitamin K levels. Four functional domains exist within DCP: a gamma-carboxyglutamic acid (GLA) domain, two annular domains, and a catalytic domain. Among these domains, 10 GLA residues are vitamin K-dependent at the amino terminus of the peptide chain. Under sufficient serum vitamin K conditions, all 10 residues are converted into GLA, forming a normal prothrombin.[123,124] In contrast, vitamin K deficiency induces the formation of the abnormal prothrombin DCP. In addition, the anticoagulant drug warfarin, a potent vitamin K antagonist, can block the redox cycling of vitamin K, causing a significant increase in DCP levels and a corollary increase in the levels of DCP-inducible proteins.[125,126]

Vitamin K deficiency not only directly affects redox cycling, but also significantly increases blood DCP levels in affected patients. Furthermore, because vitamin K is well absorbed by the small intestine only in the presence of bile salts, patients with intrahepatic cholestasis, in which decreased bile salt is a common disease feature, experience interference of vitamin K absorption, leading to increased DCP levels.[127,128] Currently, the specific mechanism by which vitamin K interferes with prothrombin/thrombin homeostasis in patients with HCC remains incompletely understood. Additionally, local synthesis of prothrombin may be compromised in the presence of liver damage, resulting in the production of abnormal prothrombin DCP.[129–131]

As a tumor marker, DCP, unfortunately, has a half-life of approximately 40–72 h in the blood, which is 3–5 days shorter than that of AFP, stressing its potential curative effects on HCC more expeditiously.[132] In addition, monitoring AFP and DCP levels in patients with HCC after treatment reveals that the 5-year and long-term survival rates are significantly higher in the group with normal DCP levels post-treatment than in the group with abnormal DCP levels. Comparatively, DCP is more valuable in assessing the prognosis of patients with HCC; however, its diagnostic specificity is much lower than that of AFP.[133]

Within the context of HCC diagnosis, DCP is barely monitored alone as a diagnostic marker but is more usually assessed alongside AFP and AFP-L3, particularly in areas with a higher incidence of HBV infection, such as East Asia and Africa. Notably, DCP is significantly more effective than either AFP or AFP-L3 in differentiating HCC from cirrhosis, with 86% sensitivity and 93% specificity.[79] More importantly, the combined application of DCP and AFP can improve the diagnostic sensitivity for HCC by 80% for tumors with diameters of 3–4 cm and by 70% for tumors with diameters of 2–3 cm.[80] Because it arises from vitamin K imbalance or deficiency with unknown mechanisms, DCP is poorly specific as a sufficient tumor marker for detecting HCC. Thus, similar to GP73, the use of DCP as an HCC diagnostic biomarker requires its combination with other

biomarkers to enhance diagnostic accuracy and achieve reliable tumor detection.

3.5. GPC-3

GPC-3, also called OCI-5, DGSX, GTR2-2, MXR7, SDYS, SGB, SGBS, and SGBS1, is a protein that was first identified in the rare undifferentiated epithelial cell OCI-5.[134] GPC-3 belongs to the heparin sulfate (HS) proteoglycan family and is composed of a core protein and two HS glycosaminoglycan chains. Outside the cell membrane, GPC-3 is anchored through glycosylphosphatidylinositol (GPI), the main component mediating cell interactions with the extracellular matrix.[135] After being digested from the GPI anchor site on the outer surface of the cell membrane by the lipase Notum, soluble GPC-3 is released into the bloodstream.[136] GPC-3 is abundantly expressed during early embryonic development and takes on essential roles in tissue morphogenesis and growth. Notably, GPC-3 expression is undetectable in healthy adult livers but is significantly elevated in most human liver cancers compared to colorectal cancer cells.[137–139]

GPC-3 plays a role in early tumorigenesis by activating the classical Wnt signaling through autocrine and paracrine manners, resulting in the high expression of Wnt target genes. In addition to functioning as a Wnt ligand, GPC-3 acts as a coreceptor or storage site interacting with other ligands, such as fibroblast growth factors (FGF), via the HS side chains. GPC-3 facilitates ligands to bind to their receptors and initiate the signaling pathways involved in HCC development and invasion.[140,141] Studies have shown that soluble GPC-3, which is cleaved at the GPI anchor domain and diffuses from the cell membrane, can block Wnt signaling and extracellular signal-regulated protein kinase 1/2 (Erk1/2) and protein kinase B (Akt) phosphorylation in Huh6-, Huh7-, and HepG2-derived tumors.[142,143] Antiangiogenic effects have also been observed in those tumors. Thus, the firmness of the GPC-3 attachment to the cell membrane positively correlated with the detectability of early HCC.[144]

GPC-3 is expressed explicitly in HCC liver tissues but not in normal liver tissues, cirrhosis, or benign lesions and thus is widely used to distinguish HCC from ICC.[145,146] Impressively, GPC-3 levels correlate with HCC stages, with higher levels observed in moderately and poorly differentiated HCC compared to well-differentiated HCC, enabling both classification and assessment of the HCC staging.[147] Notably, Li *et al.*[148] showed that GPC-3 can distinguish AFP-negative HCC from benign liver nodules, underscoring the potential discriminating quality of GPC-3 as a veritable biomarker for the detection of HCC detection.

Enzyme-linked immunosorbent assay showed no significant difference in sensitivity, specificity, or accuracy from reverse transcription-polymerase chain reaction (RT-PCR) in detecting GPC-3 in tumor tissues and blood.[149] A Meta-analysis yielded 55% sensitivity and 58% specificity, which are less promising compared with AFP. Consequently, GPC-3 is often used in combination with AFP, GP73, and DPC to predict HCC and is appropriate for the assessment of the HCC prognosis.[81,82]

3.6. α-L-fucosidase

Alpha-L-fucosidase (AFU) is a lysosomal acid hydrolase that is widely distributed in mammalian tissues and body fluids and is mainly involved in the catabolism of macromolecules, such as glycolipids, glycoproteins, and mucopolysaccharides containing fucose groups.[150] It can be broadly detected in organs and tissues such as the placenta, fetal tissues, brain, liver, lungs, pancreas, kidney, serum, urine, saliva, and tears. As a valuable biomarker for HCC diagnosis, the HCC group demonstrated higher blood AFU levels than the cohort group with benign hepatic disease.[151] In approximately 85% of HCC cases, AFU detection precedes ultrasound findings by 6 months.[152] Notably, the study shows that the area under the receiver operating characteristic curve (AUC) for AFU was 0.68, with 56.1% sensitivity and 69.2% specificity at a cutoff value of 24 U/L. In contrast, the AUC for AFP was 0.83, with 58.2% sensitivity and 85.2% specificity at a cutoff value of 20 ng/mL.[83] Another study indicated that in the detection of early-stage HBV-HCC and HCV-HCC in the test group, AFU sensitivity was 63.9% and specificity was 67.1%. In this study, a clinical investigation involving 921 participants (including 298 HCC patients, 154 chronic hepatitis patients, 122 liver cirrhosis patients, and 347 healthy controls) demonstrated that the combined diagnosis using AFP and AFU achieved a sensitivity of 52.5% and a specificity of 91.6% for detecting early-stage HCC. This combined approach showed better overall performance compared to using AFP alone, which had a sensitivity of 44.3% and a specificity of 93.7%.[72] In addition to HCC, blood AFU levels increase within weeks of pregnancy. Interestingly, after delivery, the blood AFU level decreases rapidly and returns to normal levels in 5 days.[153,154] Furthermore, AFU detection is also affected in patients with fucosidase accumulation because the absence or reduction of AFU activity in congenital tissues, organs, and body fluids results in glycoprotein or glycolipid metabolism disorders.[155]

An increase in AFU activity in patients with liver cirrhosis reflects the diagnostic value of AFU, particularly for the detection of smaller tumors.[156] In contrast, AFP is not appropriate for the diagnosis of smaller tumors. Therefore, it may be of great translational achievement to apply and popularize AFU as a new diagnostic index for HCC, particularly more significant in diagnosing AFP-negative and small-cell liver cancers, and more suitable for men and women with non-ovarian tumors.

3.7. OPN

Osteopontin (OPN), a protein that undergoes extensive modification and can bind to integrins in the extracellular matrix, is expressed in various cell types, including immune, epithelial, smooth muscle, osteoblasts, and tumor cells.[157] This diversity underscores the wide-ranging implications of the associated research. OPN plays a critical role in restructuring body tissue following an inflammatory response and acts as an inflammatory chemokine, particularly in triggering the inflammatory response in liver cells after an injury.[158,159] Like other cytokines, interactions of OPN with integral and CD44 family proteins are vital in determining the oncogenic potential of different cancers.[160]

OPN is commonly used in combination with other biomarkers; however, its individual diagnostic sensitivity and specificity are 71% and 80%, respectively. When OPN was used in combination with AFP, the overall diagnostic sensitivity and specificity reached 82% and 77%, respectively.[84] Moreover, OPN can significantly enhance the diagnostic sensitivity and specificity of AFP in patients with viral hepatitis-induced HCC.[161] The expression level of OPN is also closely related to the clinicopathological characteristics of HCC, such as envelope infiltration, vascular invasion, lymph node metastasis, and clinical stage. Although the diagnostic sensitivity is significantly lower in other liver diseases, particularly ALD, OPN demonstrates a striking suitability for early HCC detection in individuals with a high HBV infection rate, particularly in Asia.[162–164] However, the concentrations of OPN in the blood may be increased by HBV infection; consequently, the association between OPN and HCC may not be attributable to tumor development but to HBV infection. Therefore, OPN is more applicable as an indicator of tumor staging and prognosis and monitoring patients with HBV-related HCC.

3.8. DNA methylation

DNA methylation is an epigenetic mechanism involving the transfer of a methyl group onto the 5' carbon position of the cytosine to form 5-methylcytosine, which regulates gene expression by recruiting proteins involved in gene repression or inhibiting the binding of transcription factors to DNA.[165]

Among epigenetic mechanisms, methylation of cytosine-phosphate-guanine (CpG) islands in gene promoters is considered a common transcriptional regulation.[165,166] CpG islands are DNA fragments approximately 1000 base pairs long that have a higher CpG density than the rest of the genome but are usually not methylated. Most gene promoters (approximately 70%) are located in CpG islands,[165] particularly the promoters of housekeeping genes. It is associated with the occurrence and development of certain cancers such as HCC,[167–169] which therefore makes aberrant DNA methylation in gene promoters a promising biomarker for early diagnosis.[170,171]

The pathogenesis of HCC is triggered by the interactions of the living environment, biogenetic, and epigenetic factors,[172] in which epigenetics is associated with HCC pathogenesis,[173] and abnormal DNA methylation serves as a major mediator of epigenetic changes in HCC.[174] As described above, DNA methylation catalyzed by DNA methyltransferases (DNMTs, mainly including DNMT1, DNMT3a, and DNMT3b) occurs through the coupling of methyl groups to the 5' carbon position of the cytosine ring. In cancer, the expression of tumor-suppressor genes is frequently silenced by the hypermethylation of CpG islands in the promoter region.[175] That is, DNMT upregulation promotes cancer development,[176] which is mainly manifested by significantly higher mRNA levels of DNMT in HCC than in nontumor liver tissues.[177] In addition, DNMT-mediated epigenetic changes regulate metastasis, invasion, progression, and development of HCC.[178]

In $CD133^{+}/CD44^{+}$ cells, an HCC subpopulation with CSC characteristics, OPN enhanced HCC metastasis by regulating DNA methylation. Suppressing DNMT1 expression to inhibit migration by knocking down OPN in $CD133^{+}/CD44^{+}$ cells reduced the methylation of tumor-suppressor genes, such as *RASSF1*, *GATA4*, and *CDKL2*, and delayed tumor initiation in the $CD133^{+}/CD44^{+}$ subpopulation of HCC cells.[179]

DNMT3 is involved in the epigenetic regulation of metastasis-associated protein 1 (*MTA1*, associated with tumor invasion, angiogenesis, metastasis, and survival) gene during HCC metastasis and invasion.[180] In particular, in HBV-induced HCC, HBV X proteins enhance MTA1 expression through epigenetic regulation, as demonstrated by the molecular mechanism by which HBV X proteins induce MTA1 transcription by increasing promoter methylation and recruiting DNMT3a and DNMT3b to release p53.[181]

Thus, the DNA methylation status and DNMT levels may be potential HCC biomarkers and attractive therapeutic targets for HCC treatment.

4. New potential biomarkers

Blood protein biomarkers are crucial for HCC detection because of their high molecular weight and stability. Recent scientific and technological advancements have deepened our understanding of the molecular mechanisms underlying HCC, leading to the discovery of diverse potential biomarkers. This opens up the possibility of exploring innovative strategies for the early and precise detection of HCC. Although not exhaustive, several new potential markers, along with their characteristics, are summarized in Table 2.

Table 2
Overview of new potential biomarkers for HCC detection.

Categories	Biomarkers	Specificities	Limitations
Cells	CSCs	Maintain stemness, self-renewal ability, and long-term dormancy.	Difficulty in identification and uniqueness to nontumors.
	CTCs	Shedding at the tumor, circulating throughout the body, underwent aggregation and breakdown in the bloodstream.	Short half-life, low blood concentration, and low sensitivity.
Nucleic acids	ctDNA	Released by tumor cells, unique methylation patterns, and high methylation stability.	False Negative, missed diagnoses, and lacking driver genes.
	TEPs(carried noncoding RNA)	Absorb and carry various substances, specific tumor components within the thrombus, and participate in the metastasis of tumor cells.	Low sample content, lack of standardized test, and potential for false positives.
	miRNA	Specific expression profile, ability to circulate in body fluids, and has higher chemical stability.	Regulation by transcription factors; potential for overexpressed, mutated, or deleted; and modulation by hypoxia.
	CircRNA	Higher stability and ease of detection.	Expression levels are minimal, may be nonfunctional, and undergo complex post-transcriptional chemical modifications.
Cytokines	TGF-β and IL-1	Involved in the immune response and can lead to immune inflammation and disease.	Ineffective cytokine therapy and low utilization efficiency of cytokines.

Abbreviations: CircRNA, circular RNA; CSCs, cancer stem cells; CTCs, circulating tumor cells; ctDNA, circulating tumor DNA; IL-1, interleukin-1; miRNA, microRNAs; TEPs, tumor-educated blood platelets; TGF-β, transforming growth factor-β.

4.1. CSCs

Because of the unsatisfactory early diagnosis and predictive treatment of liver cancer, more biomarkers must be collected to improve the therapeutic outcomes. With advancements in technology, more accurate and specific biomarkers are expected for the diagnosis and treatment of liver cancer. Among them, increasing evidence shows that CSCs may be involved in multiple steps of tumorigenesis, progression, and recurrence.[182]

Some surface markers that have been identified include CSCs, such as CD44, CD133, epithelial cell adhesion molecule (EpCAM), CD90, and leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5).[183–185] As a small subset of tumor cells, CSCs can maintain stemness and self-renewal to induce resistance to external stimuli and can remain dormant for a long time in cancer. CSCs are the main cause of cancer metastasis and recurrence and are therefore a key factor in the failure of cancer treatment.[182] Thus, CSCs take on an important role in tumorigenesis and progression and are closely associated with tumor invasion, metastasis, drug resistance, and recurrence after treatment.[186] Identifying specific phenotypic markers for CSC populations in HCC may contribute to the development of new and effective therapies for this type of cancer. As described above, the origin of the liver SCs is currently unclear. Similarly, liver tumor SCs have not been identified. CD44, a multistructural and multifunctional transmembrane glycoprotein, serves as a receptor for hyaluronic acid, also known as hyaluronan, a major component of the extracellular matrix, and acts as a coreceptor for many growth factors and cytokines.[187] CD44 has attracted widespread attention for its important role in mediating intercellular and cell-matrix interactions and its association with malignant processes, particularly in the context of cancer dissemination.[188] In contrast, CD44 is the most common CSC surface marker, crucial for communication with the microenvironment and regulating stemness properties.[189] Other common CSC surface markers for HCC include CD90 and CD133.[190–192]

In HCC, a high CD44 expression promotes high serum AFP concentrations, which emphasizes the potential of CD44 as an HCC biomarker.[193,194] However, the surface antigen proteins are often used for immunohistochemistry, evaluation, and staging of the prognosis rather than for early HCC detection.[195] The reason for this is that the surface antigen proteins, including CD44, are difficult to detect and obtain under normal circumstances.

4.2. CTCs

Circulating tumor cells (CTCs) are predominantly composed of epithelial cells in tumor-efferent vessels; however, they can be transformed from an epithelial to a mesenchymal phenotype through SMAD family member 2 (Smad2) and β-catenin-mediated signaling pathways.[196] CTCs propagate through the portal vein and systemic circulation and undergo dynamic processes of aggregation and disaggregation and changes in shape and size in the bloodstream.[197] Usually, CTCs have a half-life of 1–2.5 h in the blood circulation, after which they are eliminated by the immune system; however, a small fraction can survive and seed distant metastatic sites.[198]

At present, CTCs can be used as an alternative biomarker for early HCC detection, whereas epithelial cell adhesion molecule (EpCAM), as the most widely used CTC biomarker, also has some limitations.[199] Regarding CTC separation techniques, they are broadly categorized into two types. One is mainly through the physical properties of CTCs, such as filter machine size, density, migration ability, and deformability.[200] The latter relies primarily on antigen–antibody binding overlying EpCAM.[201] Because EpCAM is not expressed in blood cells, it can be used to isolate CTCs from epithelioid HCC, which has become a common way to capture circulating cancer cells, such as immunomagnetic bead sorting, flow sorting, and microfluidic capture CTCs.[202,203] However, only 35% of HCC cases are positive for the marker, which significantly reduces the sensitivity of the method.[204] Only 0–86 CTCs were detected in 5 mL of blood. These findings raise questions about the appropriateness of using EpCAM as a CTC marker for diagnosing HCC progression.[205] However, the development of next-generation sequencing (NGS) enables the complete genome of CTCs to be deciphered, paving the way for finding new and stable CTC markers.

4.3. ctDNA

Somatic mutations detected in cell-free DNA (cfDNA) could be used as biomarkers for HCC diagnosis. Tumor cell-derived circulating tumor DNA (ctDNA) refers to DNA fragments carrying certain characteristics, including mutation, deletion, insertion, rearrangement, copy number abnormality, and methylation, from the tumor genome, which is constantly flowing through the human blood circulation system. ctDNAs mainly consist of necrotic or apoptotic tumor cells, CTCs fragmented by shear stress, and exosomes secreted by tumor cells.[206] ctDNA may show great diagnostic value in HCC, with higher sensitivity and better clinical correlation.[207] For example, ctDNA methylation is a hot direction for early diagnosis, which may be because the methylation pattern is unique for each cell and remains highly stable under physiological or pathological conditions. Therefore, identifying different methylation patterns may be a discriminatory tool for HCC detection and diagnosis; thus, changes in methylation may offer the best hope for early cancer detection. Aberrant methylation was found in serum DNA in cases diagnosed 1–9 years before the clinical diagnosis of HCC.[208] Ras association domain family protein1 isoform A (RASSF1A) had the highest hypermethylation rates, with at least one positive sample in 35 (70%) cases, compared with 22 (44%) and 12 (22%) cases for p16 and p15, respectively, and had an overall predictive accuracy of 89%, sensitivity of 84% and specificity of 94%. If ctDNA is used in combination with traditional HCC biomarkers, it may have a better effect.[209] However, ctDNA has limitations, including false negatives, missed detections, and absent relevant driver genes, which pose significant challenges for testing.

4.4. TEPs carrying noncoding RNAs

Platelets are produced by megakaryocytes in the hematopoietic tissue in the bone marrow. Multifunctional hematopoietic SCs differentiate directionally to form primitive megakaryocytes, which then mature into mature megakaryocytes.[210,211] Many depressions form on the surfaces of mature megakaryocyte membranes and extend into the cytoplasm. The adjacent stressed cell membranes fuse in the deep part of the depression such that part of the cytoplasm of the megakaryocytes is separated from the parent.[212] Finally, these components, separated from the cytoplasm of megakaryocytes and surrounded by cell membranes, are further severed from megakaryocytes to allow their entry into the bloodstream through the blood sinuses in bone marrow hematopoietic tissue to become platelets.[213,214]

Platelets can absorb and carry proteins, nucleic acids, and other substances, including messenger RNAs (mRNAs), pre-mRNAs, microRNAs (miRNAs), long noncoding RNAs, circular RNAs (circRNA), and mitochondrial RNAs throughout their life cycle.[215,216] During tumor occurrence and development, tumor cells directly or indirectly affect the RNA and protein levels of platelets through various signaling molecules or receptors, giving rise to what is known as TEPs.[217] Its concept is rooted in Theodor Billroth's

discovery in 1878, which found that thrombi rich in specific tumor components are involved in tumor cell metastasis.[218] Further research on the interaction among megakaryocytes, platelets, and cancer has contributed to the development of the TEP concept.[219] Consequently, TEPs do not act directly in HCC. In a liquid biopsy study examining platelet RNA, Ras homolog gene family member A, β-catenin, and serine peptidase inhibitor Kazal type-1 were found to be significantly up-regulated, with increases of 3.2-fold, 3.3-fold, and 3.18-fold, respectively, in patients with HCC compared to those with cirrhosis. In contrast, interferon-inducible transmembrane protein 3 and serpin family D member 1 showed increases of 2.24-fold. This also implies that the expression levels of TEPs mRNA could be a valuable tool for early diagnosis of HCC in patients with underlying cirrhosis.[220]

4.5. miRNA

Transcribed from endogenous genes, miRNAs are a class of noncoding single-stranded RNA molecules with a length of approximately 20–24 nucleotides that are currently considered the highest constituent of TEP RNA.[221] In addition to their wide expression in various animals, plants, and bacterial viruses, miRNAs also impressively demonstrate many critical functions such as binding to mRNA, blocking the expression of protein-coding genes, controlling gene expression, and cellular processes by negatively regulating target mRNA expression and modulating tumor occurrence and development.[222]

Notably, miRNA-21, one of the most abundant miRNAs in blood circulation, is considered one of the earliest miRNAs associated with tumorigenesis. miRNA-21 is highly expressed in most solid cancers (*e.g.,* HCC) and acts as an antiapoptotic and prosurvival agent in tumor cells.[223] For example, the roles of miRNA-21 as an effector of tumor-suppressor genes, programmed cell death factor 4, phosphatase, and tensin homolog deleted on chromosome 10, and tissue inhibitor of metalloproteinase 3 have been extensively studied.[224–226] Furthermore, miRNA-21 is more specifically produced by the liver, and its regulatory function in liver development and pathology has also been established. In addition to miRNA-21, previous studies found that the expression of miRNA-122 is significantly reduced in the liver tissues of patients with HCC; however, the circulatory level is enhanced in the patient's blood.[227,228] Thus, miRNA-21 and miRNA-122 may have excellent prospects for tumor prediction.

4.6. CircRNA

CircRNAs are a class of RNAs with a conservative structure, stable sequence, and rich circulating content and can regulate gene expression at the posttranscriptional level.[229] CircRNAs have great potential as cancer biomarkers in patients with HCC for several reasons: Firstly, circRNAs are easy to detect owing to their stability, abundance, and retention in human cells. Secondly, circRNAs often show specific expression in tissues, particularly at the developmental stage. Lastly, circRNAs are widely present in the serum, plasma, and other body fluids, making them accessible for extraction in humans.[28,230] Because containing complementary only contains binding sites for miRNAs, circRNAs act as a sponge for miRNA in the cytoplasm, where circRNAs competitively sequestrate miRNAs and interfere with the biological functions of miRNAs.[231] Among circRNAs, circRNA-7 (ciRS-7), broadly expressed in many tissues particularly enriched in the brain, is the first family member proven to contain >70 conservative binding sites for miRNA-7.[232] The following study further indicates that like miRNA-7, ciRS-7 also plays an essential role in HCC occurrence and development.[233] Thus, ciRS-7 has been recommended to serve as a potential biomarker for HCC diagnosis.

4.7. Cytokines

Cytokines are mostly low-molecular-weight (6–70 kDa) soluble proteins secreted by lymphocytes, macrophages, natural killer cells, mast cells, and stromal cells. As crucial signaling molecules, cytokines can work with various cell types and regulate biological activities, including immune system mediator activities. Cytokines are involved in the immune response and play an important role as mediators in the communication network of the immune system. The altered levels of cytokines may cause immune-inflammatory storms and diseases.[234,235] Thus, irregular dynamics in cytokines in various biological fluids such as blood, feces, urine, and sweat can provide valuable information for the diagnosis, staging, and prognosis of multiple diseases.[236] Common cytokines, including transforming growth factor-β (TGF-β), FGF, hepatocyte growth factor, and vascular endothelial growth factor, have been identified as valuable biomarkers for HCC detection.[237–239] For example, TGF-β signaling acts as a master regulator for immune cell proliferation, differentiation, development, and survival.[240] In addition, TGF-β activity has been linked to the activation of cancer-associated fibroblasts.[241]

Another recent animal study reported that lenvatinib-treated recurrent tumors had lower expression of PD-L1 and regulatory T-cell (Treg) infiltration than primary tumors. PD-L1 down-regulation by lenvatinib-initiated blocking of FGF receptor 4 (FGFR4)-glycogen synthase kinase 3β enhances proteasomal degradation of the PD-L1 axis and HCC cell sensitivity to T-cell killing.[242] Conversely, IL-2 is increased after the treatment with anti-PD-1; however, IL-2-mediated Treg differentiation is blocked by lenvatinib by inhibiting FGFR4 signaling pathways, including phosphorylations of signal transducer and activator of transcription 5.[243] This illustrates the potential of monitoring FGFR4 expression as a parameter for assessing the treatment effects in patients with HCC using lenvatinib plus anti-PD-1 therapy. Collectively, these studies support that cytokines are critical in HCC pathogenesis.

5. Techniques for biomarker discovery

Early cancer detection is crucial in reducing cancer mortality and saving patient outcomes. Therefore, investing in new technologies for exploring HCC markers is imperative. Cancer biomarkers encompass various biochemical entities, including nucleic acids, proteins, sugars, small metabolites, cytogenetic and cellular kinetic parameters, and whole tumor cells in body fluids. These biomarkers can be utilized for risk assessment, diagnosis, prognosis, and prediction of treatment outcomes. Current advancements in cancer biomarkers are outlined, and their suitability for early HCC diagnosis was assessed using multiple biomarkers.

With the aid of multidisciplinary technology, cancer diagnostic tests are undergoing a revolution to identify biomarkers at the cellular and genetic levels. Based on the existing experience of researchers, utilizing mouse-intubated animal models provides valuable insights for the discovery of novel cancer biomarkers and for addressing ongoing challenges in the field. To overcome these obstacles, a concerted and substantial effort is required, necessitating collaboration from diverse talents, including chemists, biologists, clinicians, materials scientists, and engineering and technology researchers. Conceivably, successful exploration will soon enable the reliable, sensitive, and noninvasive detection of cancer, facilitating immediate diagnosis and individualized treatment. Various biotechnologies were listed for finding tumor markers, including second-generation sequencing, metabolomics, intestinal flora, and

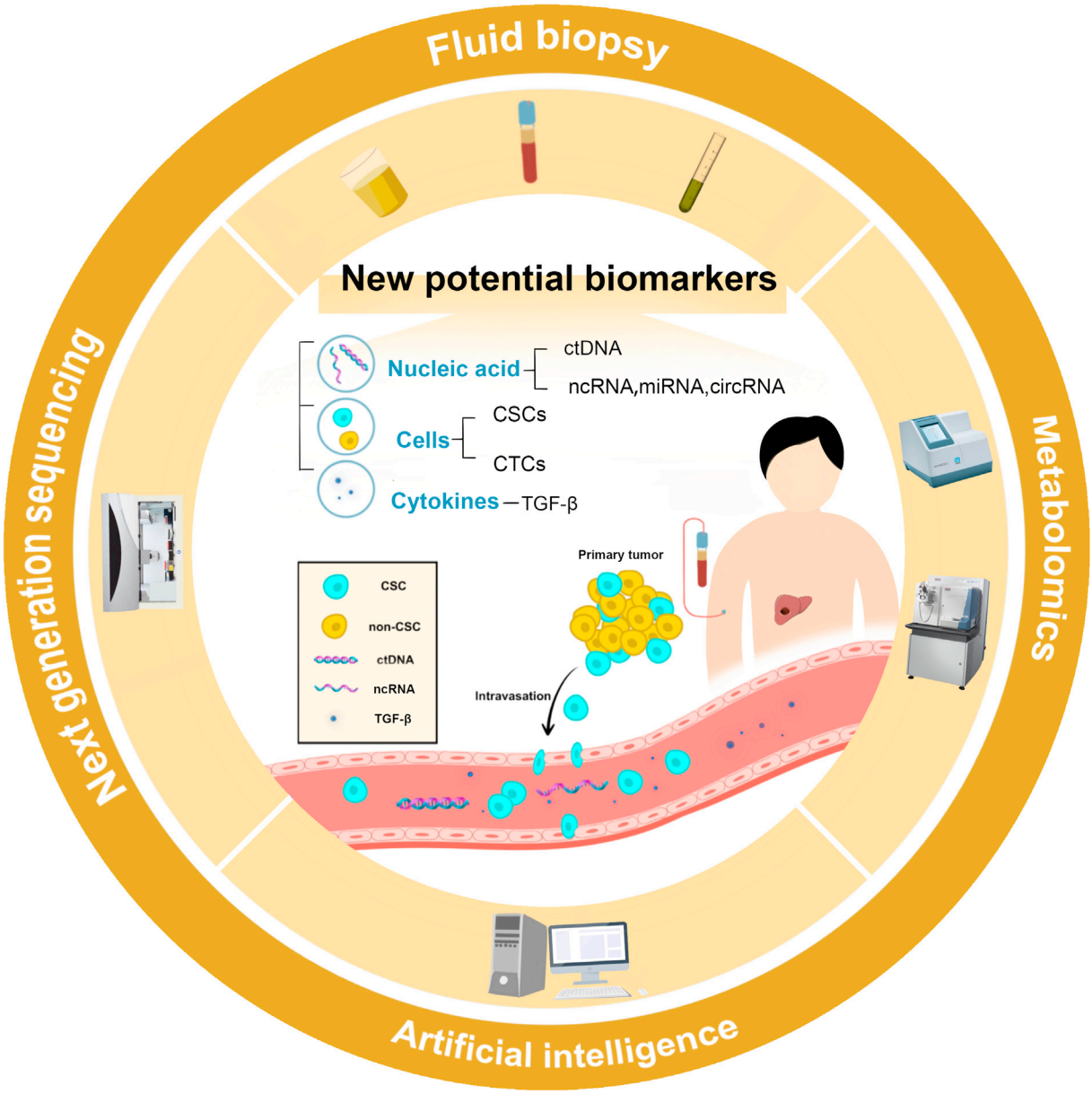

Fig. 2. New potential biomarkers and early diagnostic techniques for HCC. Sources of potential biomarkers include nucleic acids, cells, and cytokines, which are investigated using various biotechnologies to identify possible HCC tumor markers for early diagnosis. Abbreviations: circRNAs, circular RNAs; CSCs, cancer stem cells; CTCs, circulating tumor cells; ctDNA, circulating tumor DNA; HCC, hepatocellular carcinoma; miRNAs, microRNAs; ncRNAs, non-coding RNAs; TGF-β, transforming growth factor β.

artificial intelligence (AI) prediction, with a brief description of their rationale and scope of application, to discover potential HCC tumor markers through these technological pathways (Fig. 2).

5.1. NGS

The emergence of NGS technologies has revolutionized the field of genomics, enabling the rapid and cost-effective generation of genome-scale sequence data with precise resolution and accuracy. Currently, NGS technologies are used in various applications, such as the discovery of rare variants by whole-genome resequencing or targeted sequencing, transcriptome analysis of cells, tissues, and organisms, or identifying epigenetic markers for disease diagnosis.

NGS technologies, including RNA sequencing, whole-exome sequencing, and whole-genome sequencing, form the foundation of today's discovery-based genomics research.[244] DNA sequencing of bulk HCC tumors has revealed a median of 50–70 protein-altering mutations and an average of 2–6 driver mutations across all tumor stages.[55] The most prevalent driver gene mutations are found in the TERT promoter, TP53, CTNNB1, AXIN1, ARID1A, and ARID2, which cause changes in the activation of several pathways, including telomere maintenance, P53 cell regulation, Wnt/β-catenin, Akt/mTOR, MAP kinase, and oxidative stress.[245,246] Most driver mutations tend to occur simultaneously, proposing a synergistic effect of multiple mutations during tumorigenesis and progression. However, not all synergistic effects are promotive; for example, AXIN1 and TP53 mutations are mutually exclusive, suggesting the redundant or deleterious effects of these concurrent mutations. These findings highlight the different mutations and combinations of mutations present in different tumors, which may contribute to the heterogeneity of patients with HCC.[55,247] Although the heterogeneity of tumor tissues results in limited

tissues obtained by biopsy, which does not cover the entire tissue, the assay results do not fully represent the expression of genetic information throughout the tumor tissue. Admittedly, NGS has paved the way for the development of several HCC classification systems. A study showed that almost all individuals contain distinct genotype B and genotype C HBV sequences.[248] Previous studies have shown that co-infection of HBV with different genotypes occurs, and that inter-genotypic HBV co-infection has been widely reported as a prerequisite for the occurrence of HBV recombination. These findings certainly emphasize the importance of using NGS to study the distribution of different genotypes within individuals.[248] However, these classification systems have not yet been approved for clinical use, and the journey toward exploring diagnosis, treatment, and prediction remains daunting.

5.2. Metabolomics

Metabolomics is the analysis of metabolites in biological fluids, cells, and tissues and is commonly used for biomarker discovery. Thanks to innovative developments in informatics and analytical techniques, metabolomics analyses can now be expanded to understand the system-level effects of metabolites.

The main approaches to metabolomics are nontargeted (global) and mass spectrometry-based targeted metabolomics. Nontargeted metabolomics intends to measure the widest range of metabolites in extracted samples, and due to differences in analytical methods, produces a complex dataset requiring certain methods to identify and correlate metabolites in different samples and study their interconnectedness in metabolic pathways with phenotypic or aberrant processes.[249] In contrast, targeted metabolomics is analyzed based on prior information and therefore has higher sensitivity in analyzing specific metabolites and metabolic pathways.[250]

An untargeted metabolomics analysis using liquid chromatography-mass spectrometry collected serum (portal and central venous), liver tissue (HCC tumors, adjacent nontumor, and normal livers), and fecal samples from 102 patients in the discovery cohort (52 patients with HCC and 50 healthy controls) and 100 participants in the fecal samples for the independent validation cohort (50 patients with HCC and 50 healthy controls).[251] Detailed metabolomics assessments revealed different clusters of metabolites when comparing the serum, liver tissue, and fecal samples from patients with HCC and control individuals. Among them, patients with HCC had significantly higher levels of portal serum and HCC tissue metabolites, including DL-3-phenyl lactic acid, L-tryptophan, glycopyrrolate, and 1-methylnicotinamide than healthy controls, which were associated with impaired liver function and poor survival. On the contrary, fecal samples from patients with HCC had lower levels of linoleic acid and phenol in the portal vein than in healthy controls. This may imply that metabolites depleted in the portal are protective against HCC *in vitro*.

The above studies have shown that metabolomics has significant advantages in identifying tumor markers; that is, hematology mass spectrometry analysis is quite well established, perhaps a new source of mass spectrometry would be a good direction to go.[67] In HCC, tumor-associated substances may enter the bile earlier, although bile extraction is not an easy task. Several studies have demonstrated the advantages of bile over blood for the analysis of tumor heterogeneity,[252] and it may be feasible to extract bile and identify new tumor markers thanks to a mouse bile duct intubation procedure.[253]

5.3. Liquid biopsy method for obtaining bile

Liquid biopsy of tumors involves the collection of blood or other fluid samples, such as urine, ascites, saliva, and pleural effusion, from patients using a minimally invasive or noninvasive method to detect tumor cells or other disease markers. Currently, liquid biopsies are mainly based on blood samples. The blood tests for patients with cancer mainly focus on detecting CTCs, ctDNA, circulating tumor miRNAs (ctmiRNAs), exosomes, and TEPs, and analyzing related disease information.[206] The liver, being an organ with an abundant blood supply, may be advantageous for detecting tumors and metastases; hence, serological testing techniques are intensive.

Bile, a body fluid that is not readily accessible, remains underexplored and may hold significant secrets. Recently, a case describing the experimental method of bile duct intubation in mice presented a possible way for obtaining bile and mass spectrometry. As shown in Fig. 3, this study highlights the ability to collect bile by artificial bile duct cannulation in mice,[253] raising the possibility of using bile as an alternative liquid biopsy material.

The bile, produced by hepatocytes, is mainly composed of bile salt, pigment, cholesterol, lecithin, potassium, sodium, and calcium but lacks digestive enzymes, and the contents are relatively more stable before flowing into the intestine. Bile is already used as a

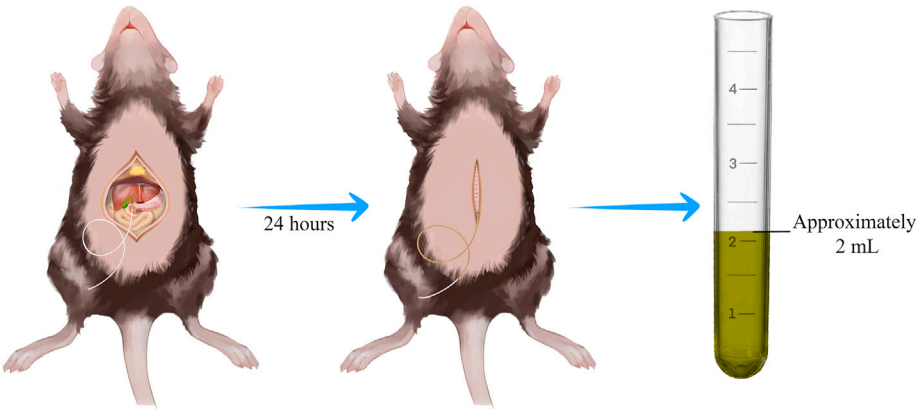

Fig. 3. Mouse bile duct cannulation and bile collection. An approximately 10-week-old C57BL/6 mouse was anesthetized; its abdominal fur was shaved, and the skin was cleaned. After anesthesia, a midline incision was made in the abdomen to expose the liver and duodenum area. A thin catheter was inserted into the bile duct, and the catheter was connected to a tube for bile collection, then fixed with sutures, as shown in the left picture. The incisions in the abdominal muscle and subcutaneous membrane were closed with sutures, and the end of the collection tube was left outside the abdominal muscle. The bile collection tube was placed between the subcutaneous membrane and the abdominal skin. After 24 h, the abdominal skin was reopened, and the collected bile could be observed in the collection tube, as shown in the middle picture. As shown on the right side, approximately 2 mL of dark green bile was collected from an adult C57BL/6 mouse.

valid biopsy sample for ctDNA detection to evaluate biliary tract and gallbladder malignancies.[252,254] Kinugasa *et al.*[255] performed cytology and NGS on 30 patients with gallbladder cancer, analyzing bile and tumor tissue to detect possible mutations in 49 oncogenes from the isolated DNA samples. Of these patient samples, 87.5% of the bile ctDNA samples had identical mutations, illustrating the same percentage of concordance between cytology and bile ctDNA analysis. The frequency of ctDNA mutations is approximately half of that detected in the tumor tissue DNA. However, the concordance between bile ctDNA and tissue DNA samples indicates that mutated tumor DNA can also be detected in bile by NGS, supporting that bile liquid biopsy may be used to diagnose gallbladder cancer and HCC.

However, the use of bile for the biopsy diagnosis of HCC encounters many unpredictable challenges.[256,257] First, no standard collection and analysis protocol has been established based on the bile as a biopsy sample. Second, bile is secreted continuously, although it is mainly concentrated in the gallbladder under conditions of food deprivation. Third, whether the liquid biopsy removal of partial water and other components in bile causes a significant alteration of bile in patients with HCC is unknown. Finally, the greatest challenge is the difficulty in obtaining bile, a process that is nearly impossible without invasion. Despite the current challenges associated with using bile liquid biopsy to detect HCC, advances in biotechnology and the output of mechanistic investigations may help overcome all the barriers and realize the potential use of bile as an important vehicle to detect biomarkers. In the future, the mouse bile duct cannulation model can be further optimized to improve the diagnostic and prognostic values of the innovative surgery model. Although the bile flown out from one branch is collected for all assays, the other branch may collect the flow-out bile to rejoin into the enterohepatic circulation of the mouse. Thus, a long-term dynamic model is hoped to be developed, in which the investigators can not only detect HCC biomarkers at an earlier stage and potentially identify novel markers but also track the post-therapeutic effects of HCC mouse models based on long-term and real-time bile analyses. Owing to the difficulties of bile sampling, this technique may be suitable only for basic research at this time. However, the ability to dynamically display changes in bile will undoubtedly advance the development of hepatic metabolomics.

5.4. Potential of AI in identifying HCC biomarkers

Recent advancements in high-throughput and histological technology and the reduction of application cost, have enabled a transition from cell line studies to individual patient assessments, from tissue analyses to single cell investigations, and from single time point to multi-dimensional time point. In HCC, various related maps are drawn using large amounts of data and fewer abnormally expressed genes, proteins, and metabolites to predict and find possible candidate biomarkers; however, the established prediction and prognosis strategies are still limited.[258] Recently, as a new technical discipline, AI has been applied to improve the accuracy of medical imaging diagnosis of HCC and predict the risk of HCC development in patients with liver disease, making it possible to find a reliable biomarker or a group of biomarkers.[259,260]

According to ClinicalTrials.gov (https://clinicaltrials.gov/), seven studies are undergoing on the relationship between AI and HCC, including AI-based optimization of early treatment of HCC drugs, deep learning (DL), prediction of liver failure after hepatectomy, and accuracy in CT diagnosis of HCC (Supplemental Table 1). A deeper understanding of the HCC phenotypes is essential for improving targeted therapies and clinical translation.

Li *et al.*[261] proposed an extreme learning machine (MFC–CNN–ELM) structure combined with multiple fully connected convolution neural networks for the nuclear grading of HCC. The results show that the MFC–CNN–ELM algorithm has a good performance in the nuclear grading of HCC.

Chen *et al.*[262] used histopathological hematoxylin and eosin images from the public database of genomic data to train the neural network (INSTERATIONV3) for automatic classification, with a performance level close to that of a 5-year experienced pathologist, with an accuracy of 96.0% for distinguishing between benign and malignant tumors, and 89.6% for classifying highly, moderately and poorly differentiated tumors.

Chaudhary K *et al.*[263] proposed a DL-based HCC model and established a DL-based survival sensitivity model on data from 360 patients with HCC, providing two optimal patient subgroups with significant survival differences ($P = 7.13 \times 10^{-6}$) and good model adaptability (C-index = 0.68). The more aggressive subgroups were associated with frequent TP53 inactivation mutations, high expression of dry biomarkers (KRT19 and EpCAM), and tumor marker BIRC5, as well as activated Wnt and Akt signaling pathways.[263]

Liang *et al.*[264] proposed an interpretable human-centered DL guidance framework called PathFinder (pathology–biomarker–discoverer), which can assist pathologists in finding new tissue biomarkers from well-performing DL models. PathFinder can realize the localization, characterization, and verification of potential biomarkers while ensuring the most advanced prognostic performance and verifying their potential in clinical prognosis according to the criteria recommended by the tumor marker prognostic study report. This represents a successful example of introducing DL into clinical practice through knowledge and discovery, which shows the feasibility of AI in identifying biomarkers.[264]

AI provides effective information for biomarkers and specific treatment decisions for HCC and can serve as a decision-making reference for exploring potential biomarkers, predicting prognosis, and clinical treatment of HCC. However, providing sufficient clinical tumor-related effective learning samples for AI has certain limitations.

6. Conclusions

This review compiles a relatively extensive list of HCC markers, detailing their advantages and disadvantages while emphasizing their diagnostic accuracy and specificity, which are essential for diagnosis and prognosis. Currently, commonly used serum biomarkers for early HCC diagnosis include AFP, AFP-L3, and DCP, with emerging potential biomarkers such as AFU, GP73, and OPN, enhancing accurate detection and facilitating early treatment. Furthermore, markers like ctDNA and CSCs are under-investigated because of population differences and various injury mechanisms that may affect diagnostic results.

HCC is a heterogeneous disease with such a complex genomic landscape, making it challenging to understand its occurrence and progression for biomarker identification and targeted therapy development. In clinical practice, imaging and serologic diagnosis often fail to accurately determine the nature of the liver-occupying lesions, particularly in decompensated cirrhosis, complicating the differentiation between HCC and atypical cirrhotic nodules. Consequently, monitoring tumor markers associated with HCC has significant adjuvant value.

By summarizing past results, the review further discusses techniques for identifying new biomarkers, such as metabolomics, NGS, and AI, providing a new direction for exploring tumor markers along with their underlying rationale.

Authors' contributions

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Declaration of competing interest

The company affiliated with the authors did not sponsor this research. Any author' s position at any company has no influence on the study. Personal opinions do not represent the views of the company. The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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