

REVIEW

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Biomarkers for diagnosis and therapeutic options in hepatocellular carcinoma

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Abstract

Liver cancer is a global health challenge, causing a significant social-economic burden. Hepatocellular carcinoma (HCC) is the predominant type of primary liver cancer, which is highly heterogeneous in terms of molecular and cellular signatures. Early-stage or small tumors are typically treated with surgery or ablation. Currently, chemotherapies and immunotherapies are the best treatments for unresectable tumors or advanced HCC. However, drug response and acquired resistance are not predictable with the existing systematic guidelines regarding mutation patterns and molecular biomarkers, resulting in sub-optimal treatment outcomes for many patients with atypical molecular profiles. With advanced technological platforms, valuable information such as tumor genetic alterations, epigenetic data, and tumor microenvironments can be obtained from liquid biopsy. The inter- and intra-tumoral heterogeneity of HCC are illustrated, and these collective data provide solid evidence in the decision-making process of treatment regimens. This article reviews the current understanding of HCC detection methods and aims to update the development of HCC surveillance using liquid biopsy. Recent critical findings on the molecular basis, epigenetic profiles, circulating tumor cells, circulating DNAs, and omics studies are elaborated for HCC diagnosis. Besides, biomarkers related to the choice of therapeutic options are discussed. Some notable recent clinical trials working on targeted therapies are also highlighted. Insights are provided to translate the knowledge into potential biomarkers for detection and diagnosis, prognosis, treatment response, and drug resistance indicators in clinical practice.

Keywords Hepatocellular carcinoma, Biomarkers, Cancer diagnosis, Cancer surveillance, Cancer treatment, Therapeutic option, Tissue biopsy, Liquid biopsy, Serological biomarkers, Circulating tumor cells, Cell-free DNA, Circulating tumor DNA, Non-coding RNAs, Methylation, Tumor-derived vesicles, Metabolomics, Surgical resection, TACE, HAIC, Local ablation, Liver transplantation, Targeted therapies, Immunotherapies, Artificial intelligence, Clinical trials

Introduction

Liver cancer is the second most lethal cancer worldwide, with hepatocellular carcinoma (HCC) being the most prevalent subtype, accounting for 90% of cases. Common risk factors include viral infections such as hepatitis B/C virus (HBV/HCV) infections, chronic liver diseases (CLDs) like fatty liver or cirrhosis, alcohol abuse, and metabolic diseases including diabetes. Other less common risk factors are hemochromatosis, autoimmune hepatitis, and exposure to certain environmental toxins. HCC is often challenging to diagnose due to the absence

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or ambiguity of symptoms. Early-stage symptoms typically encompass abdominal pain, weight loss, and fatigue, while later stages may involve jaundice, ascites, and fever. The insidious progression of chronic liver conditions further complicates the monitoring of liver diseases. Moreover, the heterogeneity of HCC at the molecular and histological levels contributes to the complexity of diagnosis and treatment. Apart from the apparent hepatitis virus, the presence of somatic alterations due to prolonged hepatic damage, aging, or inherited genetic susceptibility can result in hepatocarcinogenesis. Since patients typically do not undergo genetic testing until suspicion of liver diseases arises, these markers hold less significance in predicting HCC. Instead, they play a more substantial role in prognostic predictions and disease management. While this review does not delve into the genetic changes that lead to the development of HCC, they have been discussed extensively in other articles [1–7].

Consequently, surveillance programs and early detection tests are critical for the timely diagnosis and treatment of HCC. Screening efforts primarily target populations with multiple risk factors, such as known carriers of the hepatitis virus, individuals with cirrhosis, or those with a family history of HCC. Meta-analyses have supported the benefits of surveillance on HCC to improve patients' survival and clinical outcomes [8, 9]. HCC can often be diagnosed using non-invasive imaging, like computer tomography (CT) / magnetic resonance imaging (MRI), due to its characteristic radiographic features, such as arterial hyperenhancement, venous wash-out, and capsule enhancement [10]. The Liver Imaging Reporting and Data System (LI-RADS) is an established scoring system for HCC diagnosis among HBV-infected and cirrhotic patients, with a 67% sensitivity and 93% specificity [11] for detecting tumor nodules larger than 1 cm in diameter [12]. Risk stratification is currently solely determined by LI-RADS. However, a biopsy is still necessary for patients with LR-4 or above observations without known risk factors for HCC or those with atypical imaging findings. The NCCN recommends core-needle biopsy for highly suspicious lesions for HCC that do not meet imaging criteria or specific patient categories [13]. Furthermore, the utilization of biomarkers, such as AFP, prothrombin induced by vitamin K absence or antagonist-II (PIVKA-II) (or named as des-gamma-carboxy prothrombin (DCP)), and glypican-3 (GPC3), has shown promise in improving the early detection of HCC. Only about half of the patients can be diagnosed through surveillance at a curative stage of the disease. Patients with early or intermediate-stage HCC may undergo curative therapies such as surgical resection, locoregional ablation, or liver transplantation. However,

advanced-stage HCC patients are limited to chemotherapy or systemic treatments, such as chemoembolization, sorafenib, and nivolumab. Even though around half of HCC patients receive systemic therapies, the median overall survival (OS) remains limited due to the lack of effective therapeutic options.

Given the challenges in treating advanced-stage HCC, even with the advent of novel systemic therapies, it is imperative to explore improved methods for early detection, diagnosis, and prevention. To this end, significant insights into disease biology have been gleaned from genomic, transcriptomic, and epigenomic studies. These studies have led to identifying several molecular subclasses of HCC that exhibit distinct prognostic and therapeutic implications. This review aims to analyze various biomarkers that can offer valuable information on early detection, disease management, and therapeutic options. Additionally, clinical trials targeting some of these markers will be highlighted. For example, developing personalized medicine approaches, such as immunotherapy and targeted therapy, has shown potential in improving HCC treatment outcomes. Immune checkpoint inhibitors (ICIs), such as pembrolizumab and atezolizumab, have demonstrated promising results in clinical trials, while targeted therapies, such as lenvatinib and regorafenib, have exhibited significant improvements in OS. Furthermore, advancements in the understanding of the tumor microenvironment and the role of non-coding RNAs (ncRNAs), such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), have opened new avenues for the development of novel diagnostic and therapeutic strategies. The application of liquid biopsies, mainly circulating tumor DNA (ctDNA) and extracellular vesicles, has also garnered interest as a non-invasive method for the early detection and monitoring of HCC.

In summary, developing numerous detection methods aims to gain insights through various biomarkers, enabling patients with HCC to benefit from early detection and diagnosis of the disease. This, in turn, can enhance their therapeutic regimens and increase survival probability. Consequently, this review will discuss current findings of biomarkers for early screening, detection, and diagnosis, as well as treatment options and response. Ongoing clinical trials targeting specific biomarkers will also be covered.

Current and promising diagnostic biomarkers for HCC

Current diagnostics using imaging and tissue biopsy

The American Association for the Study of Liver Diseases suggests that patients with a high risk of HCC perform ultrasound liver surveillance twice a year [14]. Although ultrasound is the most handy routine surveillance, its

sensitivity is only 50%, while specificity is more than 90%. However, its detection is limited to early lesions and tumor nodules, which may reduce the therapeutic window [15]. CT and MRI are much more sensitive for diagnosing HCC, with over 90% sensitivity and specificity for tumors larger than 2 cm in diameter [16]. Despite its high sensitivity and accuracy, the cost of this cross-sectional imaging is high, and it is only feasible to be included in routine surveillance once evident signals appear.

Current stratification standards for HCC patients are based on tumor burden and cancer-related characteristics, including liver function and physical status [17]. Imaging techniques, including ultrasound, CT, and MRI, are commonly used to determine the size and number of tumor nodules in the liver. The modified Child-Pugh classification (CPC) proposed by Pugh et al. in 1973, which includes ascites, hepatic encephalopathy, prothrombin time, serum bilirubin, and albumin levels, has been used for many years to assess the remaining hepatic function in HCC-diagnosed patients [18, 19]. Still, its accuracy for use in HCC has been questioned in recent years [20, 21]. With the advancement of detection methods, improved anti-viral therapies to maintain liver function, and more prevalence of non-viral-associated HCC, more patients have been diagnosed with CPC A liver function recently, and it is no longer providing enough information for determining staging and prognosis in most cases [20, 22]. In addition to other technical limitations, it is suggested that the albumin-bilirubin (ALBI) grade is more potent in differentiating the HCC prognosis in most patients [21, 23–25]. The 2022 BCLC staging strategy recommended the ALBI score and AFP concentration for hepatic function assessment regardless of tumor burden [26]. The importance of portal hypertension in HCC is also underlined, and it often correlates with advanced conditions such as prior variceal bleeding, which should be considered, especially in patients with cirrhosis before surgical resection [27, 28].

A tissue biopsy is not usually performed unless the imaging can only provide marginal evidence of the diagnosis. For example, suppose the lesion is small and cannot be identified by imaging, or in the cases of non-cirrhotic liver disease, in that case, tissue biopsy can help make a precise diagnosis [29]. A biopsy can also help differentiate less common liver cancer subtypes from HCC and provide genetic mutation information directly from the tumor tissues [30]. It is essential in the design of precision medicine. However, tissue biopsy is an invasive method, which is potentially causing pain and bleeding in addition to liver damage. Also, there is a slight risk of inducing intrahepatic metastasis when tumor cells detach from the primary site and move to other sites along the wound [31].

After all, tissue biopsy remains a crucial tool for diagnosing HCC, as it can provide a definite diagnosis through histological evidence. Pathologists can more accurately perform tumor staging, which benefits the patient. Tissue biopsies can be used to monitor treatment response and provide prognostic information. Furthermore, it helps us know more about cancer biology, new biomarkers, and the creation of new treatments. The biopsy tissue is valuable for many pre-clinical studies to look for potential biomarkers or drug targets before the enrolment of actual patients in clinical studies. Nevertheless, due to the limitations of tissue biopsy, it is not feasible to utilize this method for surveillance. This is precisely the juncture where the potential of liquid biopsy comes to the fore, demonstrating its unique value and advantages. Details of ultrasound, imaging, and tissue biopsy are beyond the scope of this review, and the remaining sections will focus on the molecular biomarkers of HCC.

Current research in liquid biopsy diagnostics

Using liquid biopsy to screen for cancer and detect treatment response is critical as it can provide in-time information with minimal invasion to the patients [32, 33]. The most common fluid obtained in clinical settings is blood. Still, there are also other body fluids such as urine, ascites fluids, bile, saliva, etc. [34]. Current research directions aim to push forward the ability of liquid biopsy to obtain as much valuable information as a typical biopsy (Fig. 1). Large-scale HCC surveillance programs for high-risk individuals are limited to ultrasound with or without serum alpha-fetoprotein (AFP). AASLD is against the recommendation of using biomarkers to diagnose HCC, provided that the accuracy is insufficient until more solid validations in phase III and IV biomarker studies emerge [14, 35]. The following will discuss the current and potential biomarkers in various aspects of HCC to develop a more systematic and comprehensive panel for economical but sensitive HCC screening and diagnostic methods to improve patient outcomes.

Serological diagnostic biomarkers

Unless the doctor strongly suspects that the patients are likely to develop HCC, non-invasive measures, such as imaging and liquid biopsies, are typically performed in standard clinical practice for patients with an elevated risk of HCC. The most prevalent serum protein marker is AFP, whose expression is increased during tumorigenesis. AFP is a fetal glycoprotein, the analog of albumin in the fetus, and its expression should be reduced after birth [36]. For a long time, serum AFP levels have been considered the “gold standard” for screening tests when used in conjunction with ultrasonography [37, 38]. However, the

Biomarkers of Hepatocellular Carcinoma Diagnosis

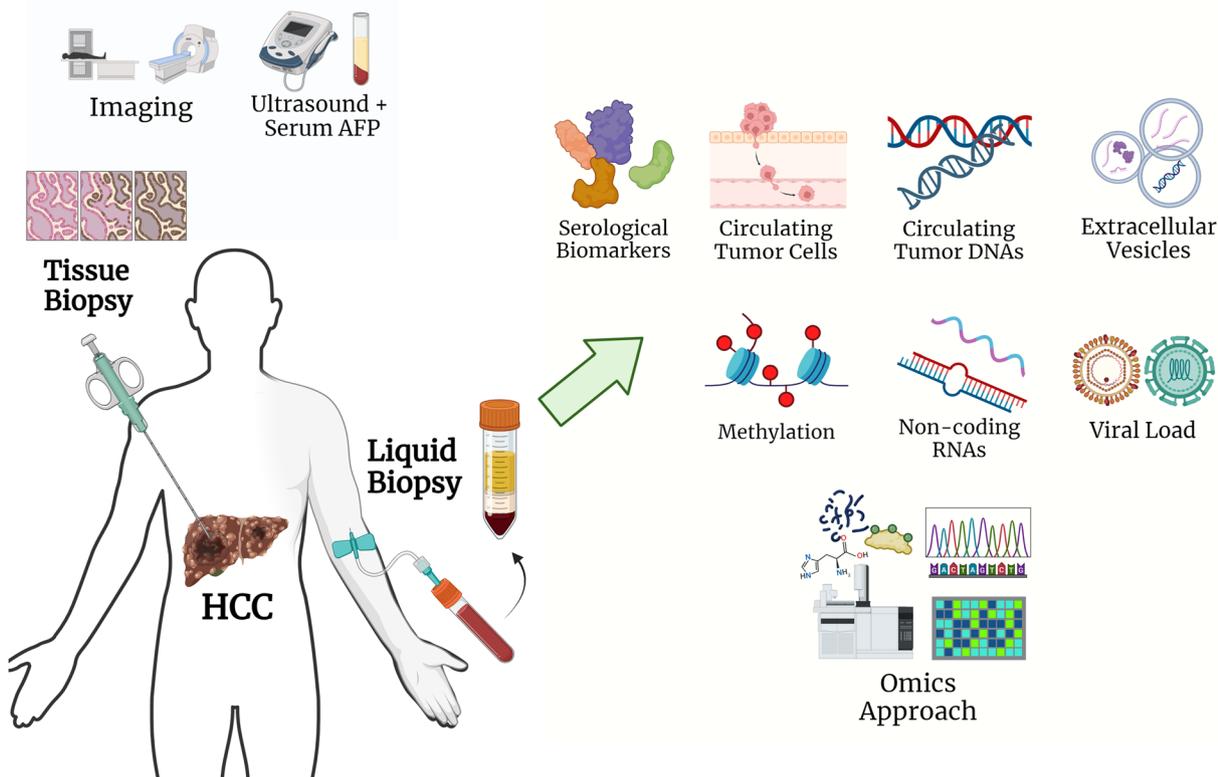


Fig. 1 Biomarkers of HCC diagnosis. The current surveillance methodology for Hepatocellular Carcinoma (HCC) primarily involves ultrasound and serum AFP measurements, sometimes supplemented with imaging techniques. In instances where diagnosis or treatment options are ambiguous, tissue biopsy is often employed to confirm the histological structure of the tumor. Liquid biopsy offers a minimally invasive alternative for gaining insights into tumor heterogeneity. Recent research has identified potential biomarkers from diverse sources, including serological components, genetic materials, cells, and vesicles. Among these candidate biomarkers, circulating tumor DNAs and omics stand out due to their ability to reflect the complexity of HCC tumors. These technologies offer great promise in understanding the intricacies of HCC and developing more effective and personalized treatment strategies

level of AFP fluctuates a lot among patients with liver diseases. The cut-off for AFP in diagnosing HCC is generally considered to be 20 ng/mL [39]. AFP alone has a sensitivity of about 60% when the cut-off value is at 20 ng/mL, but it decreases quickly to below 50% when the value is raised to 50 ng/mL [40, 41]. It decreases to around 40 to 50% in patients with cirrhosis, while specificity remains relatively the same [15, 42]. Tumors of size smaller than 3 cm in diameter also reduce the sensitivity of AFP to about 25%, compared to more than half when > 3 cm [43]. However, this value may vary depending on the findings from the corresponding ultrasound examination. Therefore, it is generally recommended to be used with imaging techniques due to its variable sensitivity and specificity [29]. Also, the sensitivity and specificity both decrease in patients with acute and chronic liver diseases, especially hepatitis patients or HBV carriers [37, 41,

44]. Notably, elevated ALT can also impair the specificity of AFP in detecting HCC [45]. The serum ALT levels were correlated with AFP levels even in patients without HCC, and it became a confounding factor for the diagnosis. On the other hand, early-stage or small tumor HCC may not elevate AFP levels [36], so the combination with ultrasound is still likely to provide a false negative result. Consequently, in 2018, the AASLD guideline for liver diseases no longer recommended ultrasound plus AFP tests every 6 months for high-risk individuals [46]. Instead, ultrasound with or without AFP tests is suggested, as they cannot determine if AFP tests in surveillance can improve survival.

AFP-L3 is a glycosylated isoform of AFP, which reacts strongly with lens culinaris agglutinin. AFP-L3 is typically used in triple tests for pregnancy, but it also has a vital role in diagnosing HCC. AFP-L3 is usually reported as

the percentage of AFP-L3 in total AFP, and 10% to 15% is the recommended cut-off [39, 47]. AFP-L3% is approved by the FDA for HCC stratification but not in surveillance programs due to its low sensitivity alone. Among patients with HCV-associated HCC, the sensitivity and specificity for AFP-L3% alone is 37% and 84.9% to 92%, but when combined with AFP, the numbers increase to 69.5% to 77% and 80.2% to 86.6% [47, 48].

In addition to AFP-L3%, PIVKA-II is introduced to the liquid biopsy test for diagnosis of HCC, especially in AFP-negative patients. PIVKA-II is detected in almost 90% of patients with HCC [49], and it is recommended that the Asia-Pacific region be included in routine surveillance programs for HCC [50]. However, similar to AFP-L3%, it is only for risk stratification but has not yet been approved by the FDA for HCC surveillance. The suggested cut-off value for PIVKA-II combined with AFP in small tumor HCC is 40 mAU/mL [43, 51]. In the Hepatitis C Antiviral Long-Term Treatment Against Cirrhosis (HALT-C) Trial, AFP combined with PIVKA-II can have a sensitivity and specificity as high as 91% and 74%, respectively. In comparison, the numbers reduce to 73% and 71% when tested 12 months before diagnosis [43]. Additionally, AFP-L3% and DCP levels are not associated with increased total AFP, making them good candidate markers for combined use with AFP for detecting HCC [47, 52].

As a result, algorithms with multiple biomarkers, such as GALAD score and HCC early detection screening (HES), are coming on stage. GALAD is calculated using gender, age, AFP, AFP-L3%, and DCP [53]. Longitudinal GALAD, which calculated the weighted average of multiple screening tests of an individual, demonstrated an area under the receiver operating characteristic (ROC) curve (AUC) of 0.85 and 0.83, even for early-stage HCC [54]. HES is a predictive model that includes results of AFP, ALT, platelets, and the age of the AFP test [55]. In the improved HES V2.0, AFP-L3% and DCP are included in the algorithm, which outperformed the sensitivity of GALAD in a cohort study in patients with hepatic cirrhosis by about 7% [55].

Additionally, several other clinically utilized biomarkers warrant mention. GPC3, a surface proteoglycan similar to AFP, usually is not detected in healthy adult liver but only in fetal liver. However, GPC3 is overexpressed in patients with HCC and is detectable in both tumor tissue and the serum [56]. It can also differentiate HCC from AFP-negative nodules, which makes it a potential biomarker on top of AFP [57, 58]. The mRNA and protein levels of GPC3 in viral-related HCC are high [59] while absent in healthy livers. However, the expression of GPC3 in non-viral HCC still needs to be clearly illustrated. The serum level of GPC3 was measured using ELISA, but the

percentage of positive patients ranged from 36 to 95% in different studies [60, 61]. However, GPC3 levels may not show significant differences in early-stage HCC [62].

Besides, osteopontin (OPN) has also been reported to be a promising serological biomarker for HCC diagnosis. OPN is important in hepatic inflammation under acute or chronic liver diseases [63]. In early studies, OPN had limited or similar diagnostic value compared with AFP, but later on, it was reported to possess superior performance over AFP, with AUC ranging from 0.7 to >0.9 [64–66]. OPN performs much better than AFP in small tumor HCC, with AUC of 0.85, sensitivity and specificity of 79.2% and 79.6% [67]. It also has an excellent diagnostic value in AFP-negative samples. The combination of OPN with AFP outperforms AFP alone.

Heat shock protein 70 (HSP70) is an oncogene that promotes tumor cell survival and proliferation and possesses anti-apoptosis effects [68]. HSP70 is located on the membrane of primary HCC cells, but it is also released into the circulation and is significantly higher than normal controls [68, 69]. The level of HSP70 can differentiate precancerous lesions with early HCC ($p < 0.001$) or early HCC with progressed HCC ($p < 0.001$), which demonstrated that it could be a sensitive biomarker for the detection and staging of HCC [70]. A recent study in 2022 suggested that when HSP70 is combined with GPC3, they have an absolute sensitivity and specificity of 100% [71]. However, the scale of this research is not large, and using HSP70 as a surveillance protein target is still not convincing.

Golgi membrane protein 1 (GOLM1, also known as Golgi protein-73, GP73) is a transmembrane protein on the Golgi complex. The level of GP73 is found to be significantly higher in patients with HCC, and it has a sensitivity and specificity of 69% and 75% for predicting HCC, which is much better than AFP (62% vs. 25%) in the same cohort to detect early HCC [72]. Its level is independent of serum AFP level [72]. Although the level of GP73 in HBV carriers and cirrhosis patients is elevated to a small extent, its sensitivity and specificity in detecting HCC remain as high as 74.6% and 97.4% when the cut-off is at 35 ng/ml [73]. The meta-analysis also reported that GP73 has the best diagnostic value for HCC among different biomarkers [74]. GP73 is also a promising therapeutic target for HBV-associated HCC patients [75].

Circulating tumor cells to predict HCC

Circulating tumor cells (CTCs) are cells isolated from tumors that travel in the circulation. Some CTCs can survive the attack by immune cells and then reach distant metastatic sites. Well-characterized CTCs are direct proof of the presence of cancer, but the isolation of CTCs is not easy. Standard techniques to isolate CTCs

in related research utilize cell size, density, and immunogenicity to carry out positive and negative enrichment [76]. Since there is no universal surface marker for CTCs due to their heterogeneity, there is not yet a “golden standard” method for isolating CTCs [77]. Some CTC purification platforms allow the capturing of CTCs and then proceeding for subsequent downstream analysis, such as NGS and in situ hybridization [78].

Efforts to isolate CTCs for cancer diagnosis have been ongoing for decades. A study conducted by [79] revealed that CTCs were only present in patients with HCC but were not found in patients with CLD, cirrhosis, or healthy individuals [79]. The sensitivity is about 52%. With the advancement in the CTC capturing technology, a group from China can capture CTCs from 90.18% of patients from a cohort of 112 HCC patients with a new RNA-in situ hybridization-based platform [80]. Among the patients with HBV, two patients were detected with CTCs, which eventually developed into HCC within half a year, demonstrating its potential for diagnosing undetected tumor nodules. A study using nine candidate genes as a multi-marker panel detecting CTCs with stem-like properties in peripheral blood [81]. CD45 cells were depleted by negative enrichment, RNAs were extracted directly from the remaining sample, and qRT-PCR was performed to measure the gene expression levels. The CTC panel has an AUC of 0.88 and a sensitivity of 72.5% for detecting HCC, while the performance is also good considering early-stage HCC or AFP-negative HCC.

The current consensus on CTC isolation focuses on EpCAM⁺/CK⁺/CD45⁻ cells [82]. EpCAM-based detection of CTCs has been employed in HCC diagnosis. Techniques like CellSearch[™], an immunoaffinity-based CTC enrichment platform, have isolated CTCs in HCC patients. EpCAM⁺ CTCs are detected in two-thirds of HCC patients [83]. In 2014, a platform was developed by negative enrichment of CTCs, followed by quantitative PCR. It showed a three-fourth consistency with the CellSearch[™] system [84].

CTC profiling is an essential aspect of liquid biopsy; nevertheless, its importance in cancer diagnosis is not as high as its role in recurrence, therapeutic response, or oncology research [85]. Very few CTCs are found in the bloodstream, and their quantity is proportional to the tumor size. This makes detecting CTCs in early-stage diseases quite challenging [86]. It is almost not practical to isolate CTCs in clinical settings for the diagnosis of cancer. Instead, specific cancer-related genes in CTCs, or CSCs, are amplified and detected by reverse transcription PCR as the easiest method. The main objective of current CTC research is to enhance the sensitivity and specificity of CTC detection to achieve accurate and comprehensive molecular characterization. CTCs are not exact copies of

each other but come from different tumor parts and can change under various conditions. Tumor cells in different parts of the body can also vary. Liquid biopsy using CTCs is not just a less invasive biopsy but a new way to understand tumor cells throughout the body. Since CTCs are released from the primary or distant metastatic tumors, the genetic makeup of these cells reveals information about the heterogeneity, mutations, and potential resistance to treatments. Research has shown that primary and metastatic tumors are processing high heterogeneities [87, 88]. CTC detection and isolation can help precision medicine by finding molecular characteristics and markers for targeted therapy. Furthermore, CTCs can be isolated from patients' peripheral blood and cultured in vitro to become organoids. The tissue culture can be applied for drug response testing, checking the cellular signaling, and stimulating therapeutic strategies. It is also beneficial for new drug development.

Using cell-free DNA / circulating tumor DNA to detect HCC

Cell-free DNAs (cfDNAs) refer to nucleic acid content in the circulation, usually released from dead cells as regular cell turnover or pathologies [89, 90]. CfDNA is present in normal humans and majorly comes from blood cells [91]. Different from CTC analysis, before cfDNA analysis, all cells are removed from the peripheral blood. In patients with cancer, tissues and organs undergo uncontrolled cell deaths, which will release large amounts of nucleic acids into the circulation, contributing to the ctDNA. Since ctDNA originated from tumor cells, analysis of ctDNA content can show a collection of heterogeneous tumor cells that carry the mutations and epigenetic patterns of the tumor. Also, macrophages engulf some CTCs, releasing the nucleic acids to be cfDNA. So, ctDNA is usually regarded as fragmented DNA from tumor tissues and as part of the cfDNA content. The proportion of ctDNA in total cfDNA can differ substantially, from less than 1% to more than 90% [92]. In general, patients with HCC have higher ctDNA levels in the circulation, which is also an indicator of tumor burden. More published research has provided molecular profiling of HCC ctDNA, and its role in the screening and early detection of HCC is an emerging trend. A systematic review analyzed 15 studies with 3,686 patients, and the pooled sensitivity and specificity of using cfDNA to predict HCC is 0.83 and 0.90, while the AUC is 0.93 [93]. The following part will discuss the applications and pros and cons of ctDNA as a detection tool.

Peripheral blood is the most frequent source for extracting cfDNA, although it can also be detected in other fluids such as saliva, ascites, and urine. There is an ongoing discussion about whether to extract cfDNA from serum or plasma, with some research indicating

higher concentrations in serum due to contamination from blood cell lysis. However, plasma is generally the preferred sample in most investigations [94, 95]. Storing and extracting cfDNA necessitates specialized methods because of its short half-life, with plasma separation recommended within six hours using EDTA tubes [96]. The technology of capturing cfDNA from circulation has been improved. Theoretically, ctDNA can carry the whole genome from tumor cells, providing valuable information about the mutational profiles and heterogeneity. The FDA approved a recently marketed product that analyzes patients' blood samples for alterations in 47 genes [97]. This product is designed to detect genetic mutations that may potentially lead to certain cancers, including HCC.

Detecting cfDNA requires highly sensitive and specific techniques, which can identify various DNA-based changes that reflect the DNA status in cancer cells. The most simple tool to check the presence of ctDNA or carried mutations is quantitative PCR (Table 1). However, this is limited to relatively higher concentrations of cfDNA, and the heterogeneity of HCC may affect the significance. Bisulfite conversion is widely adapted to convert unmethylated cytosines to uracil to detect methylation on the HCC-associated genes, resulting in a difference in the DNA sequence between methylated and unmethylated regions. While digital droplet PCR offers high precision and sensitivity, it cannot cover an extensive range of sequences, making NGS more appropriate for multiple gene profiling [98, 99]. Target sequencing, whole exome sequencing [100], or ultra-deep sequencing are usually adapted in molecular biology research to study the profiling or mutation patterns within the cfDNA. Despite this, the sensitivity and specificity of NGS may be hindered by the mistakes of DNA polymerase and amplification reactions, so molecular barcoding approaches are used to identify the correct gene alterations [101] accurately. Since the cost of utilizing NGS to screen for ctDNA is high, custom-designed sequencing panels or low-depth whole-genome sequencing approaches appear to facilitate the screening [101, 102].

In particular, the TERT promoter is the most common mutation in HCC. Many studies have reported that the TERT promoter mutation is associated with HCC occurrence, recurrence, and poorer survival [98, 109, 112, 125]. Apart from TERT promoter, other commonly reported mutations such as CTNNB1, TP53, AXIN1, KRAS, ARID2, etc., are also detected in cfDNA, and their presence and association with HCC development are almost the same as somatic cell mutations [101, 106, 109, 112, 123]. Besides single gene mutations, abnormal methylation on oncogenes or tumor suppressor genes is also related to oncogenesis. For example, a study uses a newly developed methylation-sensitive high-resolution

analysis (MS-HRM) platform to detect the methylation levels of RNF135 and LDHB [119]. The sensitivity of the combined MS-HRM is 57%, which is better than 45% of AFP from the same cohort. When combined with AFP, the sensitivity is as high as 70%. The details of detectable methylations are discussed in the later section.

In addition, several research groups have reported single-nucleotide variants (SNVs) and copy number variants (CNVs) related to HCC in cfDNAs. Cai et al. utilized low-coverage whole-genome sequencing to perform a targeted sequencing that can check the genetic profiles of the cfDNAs from HCC patients [105]. They found that SNVs and CNVs information from ctDNA and corresponding serological biomarkers, including DCP at the cut of 20 ng/mL, can predict clinical outcomes and detect HCC with satisfactory sensitivity ($p < 0.0001$).

Besides, DNA integrity has also been considered a biomarker for HCC in recent research. DNA with shorter lengths preferentially carrying the tumor-associated CNVs. Also, it is more likely to detect high levels of mitochondrial DNA in the cfDNA from HCC patients [145]. Huang et al. also reported similar findings, that cfDNA integrity is reduced in patients with HCC, and it is promising to be a biomarker for detecting HCC in surveillance with a sensitivity of 43.4% and AUC of 0.705 [114]. The cfDNA integrity is lower than those with benign nodules and healthy controls. Also, patients with HCC or other types of liver cancer have comparable cfDNA integrity ($p = 0.7356$) as those without tumors ($p = 0.9138$). CfDNA fragmentation is a nonrandom process where cfDNA fragments with specific genetic endings likely originate from HCC [146]. Detection of the tumor-associated endings, which are prevalent across the genome, may provide a simple but effective method for identifying the presence of tumors through serological surveillance.

Besides circulating isolated nucleic acids, genetic materials in plasma exosomes are also a new potential diagnostic target for HCC [147]. For instance, circular RNA Circ0006602 is one of the five circRNAs found to be up-regulated in the plasma exosomes of HCC patients compared with healthy individuals. As a diagnostic marker, the AUC of exosomal Circ0006602 to HCC patients was 0.907, with a p -value of < 0.001 . The sensitivity is 77.70%, with a high specificity (93.3%) [148]. In another recent study, high-throughput sequencing was used to analyze the exosomal miRNA levels isolated from patients' HCC tissues. After cross-validation with plasma exosomes, miR-483-5p was identified to have the power to differentiate individuals with or without HCC (AUC = 0.898), which is slightly better than the plasma miR-483-5p counterpart (AUC = 0.868) [149]. The sensitivity is, though, similar to AFP, at around 80–85%, with the specificity at 90.38%. Similar research also reported

Table 1 Summary of selected research output on cfDNA related to diagnosis and prognosis of HCC

Marker	Description	Ref.
1 <i>TERT promoter</i>	- Modified droplet digital polymerase chain reaction G > A TERT mutation at -124 bp was found in the blood of 69% of patients. Patients with a mutation have shorter DFS ($p=0.02$). Risk of disease recurrence was about three times higher in patients with the mutation ($p=0.03$).	[98]
2 <i>With liver MRI</i>	- NGS of cfDNA from plasma, combined with liver MRI cfDNA was found in the blood of 36 patients with cirrhosis, and NGS detected 20 different genetic variants. Specific HCC-related gene mutations in cfDNA could help identify patients with LC at increased risk of developing HCC.	[103]
3 <i>139 somatic mutations</i>	- Custom-designed sequencing panel with 354 target genes Number of mutations, variant allele frequency, and concentration of ctDNA linearly correlated with tumor size. Patients with mutations experienced shorter DFS, and increased the risk of recurrence (HR = 7.655, $p < 0.0001$). Status of cfDNA increases risk of recurrence (HR = 10.293, $p < 0.0001$).	[101]
4 <i>5-HMC signature</i>	- Genome-wide 5-HMC sequencing of cfDNA Distribution pattern of 5-HMC regions can differentiate HCC patients, LC patients, and healthy volunteers. HCC score combined with protein biomarkers, achieves 92.75% accuracy in distinguishing HCC patients from LC patients. HCC score is effective in identifying high-risk patients for recurrence after surgery and serves as an independent indicator for RFS ($p=0.00865$) and OS ($p=0.000739$). HCC score values are positively correlated with tumor burden changes during follow-up.	[104]
5 <i>CNVs and SNVs</i>	- Target sequencing and low-coverage whole-genome sequencing Plasma samples before surgery displayed genetic variations that matched the patients' tumor tissues, and these variations changed during follow-up, correlating with tumor burden. A strategy using comprehensive ctDNA mutation profiles was developed to accurately assess tumor burden, showing high consistency with imaging results. This approach detected tumor occurrence an average of 4.6 months earlier than imaging and outperformed serum biomarkers like AFP, AFP-L3%, and Des-Gamma-Carboxy Prothrombin (DCP). It was effective in detecting minimal residual disease (MRD) and predicting patients' outcomes for RFS ($p=0.001$) and OS ($P=0.001$); combining ctDNA with DCP further improved MRD detection sensitivity.	[105]
6 <i>160 SNVs</i>	- Target sequencing and whole exome sequencing 160 subclonal SNVs were identified in tumor tissues, peritumor tissues, and matched PBMCs, with 96.9% of these mutations also found in plasma samples. Two clusters of SNVs are related to cancer progression. Circulating levels of tumor stem somatic mutations could reflect tumor burden and predict prognosis earlier than traditional methods. HCK (p.V174M) mutation was identified as being associated with tumor recurrence and metastasis and was found to promote the migration and invasion of HCC cells.	[100]
7 <i>TP53, CTNNB1</i>	- Customized cfDNA next-generation sequencing panel In 65% of patients, at least one pathogenic variant was found in two major HCC driver genes, TP53 and CTNNB1. There were 16 variants of TP53 and nine variants of CTNNB1 identified. The identified variants in TP53 and CTNNB1 genes are crucial in understanding the genetic basis of HCC and may help in developing targeted therapies.	[106]
8 <i>Mutation variant frequency change (MVFC)</i>	- Unique molecular identifier target sequencing on plasma and PBMCs Plasma mutations with MVFC < 0.2 were found to be enriched for tumor mutations identified in tumor tissues, and their frequency changes correlated with tumor burden. The presence of MVFC-identified tumor mutations after surgery was associated with shorter RFS, indicating MRD. Combining MVFC-identified tumor mutations and AFP improved MRD detection ($P < 0.0001$). The MVFC-based identification of tumor mutations was confirmed to be applicable using a different gene panel, highlighting its potential utility in monitoring and predicting HCC outcomes.	[107]
9 <i>Serum DNA integrity</i>	- Real-time quantitative polymerase chain reaction The Triton/Heat/Phenol method was deemed effective to extract quality DNA. The integrity of serum DNA in HCC patients was significantly higher than in HBV patients or healthy individuals. The integrity of DNA was linked to factors such as tumor size, TNM stage, and the presence of lymph node and distant metastasis.	[108]

Table 1 (continued)

Marker	Description	Ref.
10	<i>TERT promoter, TP53, CTNNB1, ARID1A, TSC2, etc.</i> - NGS assay with 129 validated genes Genomic alterations were found in 92.2% of advanced HCC patients, with common mutated genes including <i>TERT promoter, TP53, CTNNB1, ARID1A, and TSC2</i> . Paired tumor NGS was conducted on 37 patients, showing high concordance in mutations between patient-matched plasma samples. In 27% of tumor-plasma samples, alterations were detected in cfDNA analysis but not in patient-matched tumors. Higher average variant allele fraction correlated with elevated AFP, increased tumor volume, and no previous systemic therapy, but not with OS in treatment-naïve patients.	[109]
11	<i>TERT promoter C228T, TP53, CTNNB1, PIK3CA, NRAS</i> - Droplet digital PCR <i>TERT</i> mutation occurs in all patients with one or more ctDNA mutations. Maximal VAF is linearly correlated with larger tumor size and AFP level. ctDNA correlated with poor OS.	[110]
12	<i>GPBAR1</i> - Methylation-specific polymerase chain reaction Hypermethylation of the <i>TGR5</i> promoter is significantly higher in HCC than CHB and HCs ($p < 0.01$). Increased sensitivity when combined with AFP	[111]
13	<i>TERT promoter, TP53, CTNNB1, AXIN1, ARID1A</i> - Ultra-deep sequencing targeting HCC driver genes Patients who had more than 2 ng/ μ L of cfDNA at diagnosis experienced higher mortality rates ($p = 0.01$). These patients had a higher number of mutated genes and more mutations in cfDNA, and associated with disease recurrence ($p < 0.01$). Patients with more than four mutations detected in cfDNA were at a higher risk of death ($p = 0.042$).	[112]
14	<i>UBE2Q1</i> - Methylation-specific polymerase chain reaction assay Lower methylation frequencies of <i>UBE2Q1</i> from the circulating cfDNA was found in HBV-related HCC patients, compared with LC, CHB and HCs ($p < 0.05$). Combining with AFP increases sensitivity and specificity.	[113]
15	cfDNA integrity - Quantitative PCR of ALU amplicon cfDNA integrity lower in patients with HCC or other liver cancers, but higher in benign diseases and healthy individuals	[114]
16	<i>TP53, CTNNB1, TERT promoter</i> - Droplet digital PCR More than half of the patients were found to have at least one of the four genetic mutations. None of the mutants were detected in adjacent liver tissues among the matched tumour tissues.	[115]
17	<i>INK4A</i> - Pyrosequencing on the CpG sites of the <i>INK4A</i> promoter Hypermethylation on <i>INK4A</i> in HCC than controls detected in the circulating cfDNA.	[116]
18	<i>CTNNB1, TP53, NFE2L2, ARID1A</i> - Targeted sequencing of cancer associated genes Mutations are detected in majority of the HCC patients, and it does not affect the cfDNA concentrations. Higher cfDNA and mutation rates associated with shorter OS ($p < 0.001$).	[117]
19	<i>Methylation fingerprint panels</i> - Genome-wide DNA methylation analysis The study identified three panels: cancer-specific biomarker panels, a pan-GI panel, and a multi-cancer prediction panel, with high AUC values ranging from 0.85 to 0.98 for detecting various gastrointestinal cancers.	[118]
20	<i>RNF135, LDHB</i> - Methylation Sensitive High-Resolution Analysis Combined <i>RNF135</i> and <i>LDHB</i> analysis has a sensitivity of 57% on HCC, compared with AFP at 45%. When combined with HCC, the sensitivity is 70%.	[119]
21	cfDNA concentration and integrity - Real-time quantitative PCR HCC patients have higher level of cfDNA in serum than CLD and healthy controls. Increased DNA fragmentation and poor DNA integrity.	[120]
22	<i>HRNR, TTN</i> - Whole-exome sequencing of cfDNA Single nucleotide variations were present in somatic genes in cfDNA, including in <i>ZNF814, HRNR, ZNF492, ADAMTS12, FLG, OBSCN, TP53, and TTN</i> . Level of cfDNA is higher in patients with HCC than chronic hepatitis patients.	[121]
23	<i>TERT promoter, TP53, NTRK3, JAK1</i> - Ultra-deep sequencing of all exons Mutations are detected in plasma cfDNA from plasma/serum samples of HCC patients.	[122]
24	<i>TERT promoter, TP53, CTNNB1, ARID2, ARID1A, NFE2L2, AXIN1, KRAS</i> - Targeted sequencing with ultra-high coverage and molecular barcoding About half of the HCC patients have at least one mutations, and rates are higher in advanced stages. <i>TP53</i> has the worst survival, whereas <i>CTNNB1</i> and <i>TERT</i> does not affect survival. Combining AFP and prothrombin improved detection rate.	[123]
25	<i>GNB4, Riplet</i> - Methylation-specific polymerase chain reaction Significantly higher CTCs in HCC compared with HBV patients. <i>GNB4</i> and <i>Riplet</i> methylation were different, and is able to diagnosis HCC at 0.98 when combined with CTCs.	[124]

Table 1 (continued)

Marker	Description	Ref.
26	<i>TERT</i> promoter, <i>CTNNB1</i> , <i>TP53</i>	[125]
	- MiSeq for targeted deep sequencing Mutations associated with vascular invasion ($p = 0.04$), predict shorter RFS for HCC patients ($p < 0.001$).	
27	<i>Wnt</i> signaling pathway	[126]
	- QUADAS-2 tool Hypermethylation of <i>Wnt</i> / <i>Beta</i> -catenin signaling pathway is correlated with tumor size, TNM stage, distant metastasis, and HBV infection ($p < 0.05$).	
28	<i>TERT</i> promoter, <i>TP53</i> , <i>CTNNB1</i>	[127]
	- Ultradeep sequencing of cfDNA High levels of cfDNA showed worse PFS and OS. Mutations combined with AFP can stratify the prognosis of HCC patients.	
29	<i>TERT</i> promoter	[128]
	- QuantStudio 3D Digital PCR Mutation is associated with tumor diameter ($p = 0.015$), and tumor volume after TACE ($p < 0.001$). Higher cfDNA levels predict better PFS with TKI initiation ($p = 0.004$).	
30	<i>TERT</i> promoter	[129]
	- qPCR and Droplet digital PCR cfDNA increases with HCC stage, and associated with poor prognosis. Greater cfDNA is associated with better therapeutic response to treatment.	
31	Fragment length profiles	[130]
	- Next generation sequencing The fragment length profiles can differentiate ctDNA from white blood cells, non-tumor cells, and heterogeneous mutations.	
32	ctDNA and mutations	[131]
	- Whole exome sequencing ctDNA increase with disease progression. ctDNA positive predicts recurrence and extrahepatic metastasis ($p = 0.01$), and vascular invasion of portal vein.	
33	<i>CTNNB1</i>	[132]
	- Droplet digital PCR Detection rate of HCC by <i>CTNNB1</i> mutation is 9.5%, and 13.5% when tumor tissues are combined. Mutation is not related to OS.	
34	<i>Alu247</i>	[133]
	- Quantitative PCR HCC cases have higher level of <i>Alu247</i> and RNase P coding DNA than controls (HBeAg-negative CHB patients). No difference in cfDNA concentration, <i>Alu115</i> , integrity index, and mitochondrial DNA copies.	
35	<i>TP53</i> , <i>CTNNB1</i> , <i>AXIN1</i> , <i>TERT</i> promoter	[134]
	- HCC screen Identify HCC from HBeAg+ patients by combining somatic mutations detected in cfDNA with serum AFP levels. Sensitivity 100%, specificity 94%, PPV 17%.	
36	Somatic copy number aberration	[102]
	- Low-depth whole-genome sequencing Machine learning based statistical model to analyse copy number variation in ctDNA, with AUC of 0.92.	
37	<i>TP53</i> R249S	[135]
	- Mass spectrometry and quantitative PCR from cfDNA Seasonal fluctuation in R249S levels, higher concentration in HBeAg+ individuals from April to July.	
38	Aberrant DNA methylation	[136]
	- DNA methylation-based digital droplet PCR Level of cfDNA is higher in patients with HCC than HC. Methylation ratio cut-off at 15.7% has an sensitivity of 78.57% for predicting HCC.	
39	<i>GSTP1</i>	[137]
	- Quantitative PCR 88.5% tumour tissues, 69% non-tumor tissues, 50% ctDNA samples, were exhibiting <i>GSTP1</i> promoter CpG island hypermethylation. No PBMC samples tested positive for methylation.	
40	Mutation and methylation panel	[138]
	- Mutation Capsule Plus 90% sensitivity with 94% specificity, detected four out of five HCC cases from a cohort of 311 asymptomatic HBV carrier with normal ultrasound diagnosis and AFP level.	
41	931 mutation sites across 21 genes	[139]
	- Circulating Single-Molecule Amplification and Resequencing Technology Sensitivities of 81.25% for all stages and 66.67% for early HCC. High accuracy in differentiating AFP-negative, AFP-L3-negative, and PIVKA-II-negative HCC.	
42	Panel of HCC mutations	[99]
	- Next generation sequencing AUC 0.92, sensitivity 65%, specificity 100% regardless of AFP status. Rate of mutations is greater in recurrent HCC than non-recurrent ($p < 0.05$).	
43	Methylation, mutations and protein-HCC	[140]
	- M2P-HCC M2P-HCC is an independent predictor of HCC risk. AUC 0.88, combining AFP and ultrasound, yield inferior performance.	
44	<i>TP53</i> , <i>CTNNB1</i> , <i>TERT</i> promoter, <i>RASSF1A</i>	[141]
	- Urine cfDNA detection by quantitative PCR Significant quantities of ctDNA detected in urine samples. ctDNA marker detection independent of serum AFP. Combining AFP yield AUC of 0.904.	

Table 1 (continued)

Marker	Description	Ref.
45 <i>NLRP7, NLRP2, NLRP3</i>	- Whole-genome bisulfite sequencing Hypomethylation of programmed-cell death genes (PRGs) including NLRP7, NLRP2, and NLRP3 in HCC tissues; NLRP3 methylation levels correlated with expression level ($r=0.51$). Hypomethylated PRGs can discriminate between early HCC patients and healthy controls in cfDNA analysis (AUC = 0.94), and associated with poor HCC prognosis.	[142]
46 <i>Intergenic and repeat regions of HBV integration sites</i>	- Low-pass genome-wide bisulfite sequencing Significant enrichment of cfDNA in intergenic and repeat regions, especially in HBV integration sites. Hypomethylation only in HCC but not in hepatitis and cirrhosis patients. AUC value > 0.85 and a prediction performance of 0.954 in the validation cohort.	[143]
47 <i>GNB4, Riplet</i>	- Whole genome bisulphite sequencing Combining methylation of GNB4 and Riplet in plasma cfDNA, sensitivity, specificity, and AUC are 84%, 92%, and 0.92, superior to AFP.	[144]

that other miRNA candidates (miR-122-5p, let-7d-5p, and miR-425-5p) had HCC early diagnostic power (AUC at 0.660 to 0.808) [150]. These studies suggest that serum exosome-derived small RNAs are potential biomarkers for HCC diagnosis in clinical settings.

Similar to CTCs, the utilization of ctDNA in clinical applications encounters obstacles. Identifying ctDNA among a large pool of cfDNA originating from other cell types presents technical difficulties. Furthermore, it remains uncertain if ctDNA can precisely reflect the genetic composition of fast-growing and highly mobile tumor cells. This is because ctDNA may overrepresent DNA from “weakened” tumor cells that are more susceptible to cell death, subsequently releasing their genetic materials into the blood. Further studies are still required in this field to optimize the full use of ctDNA as a surveillance tool.

Presence of non-coding RNAs in HCC patients

Aside from genetic modifications found in genomic DNA or cDNA, ncRNAs circulating in the bloodstream can also possess diagnostic significance. MiRNAs are short ncRNAs at about 22 bases long, and their function is to regulate gene expression in *cis* or *trans*. Given their involvement in genetic regulation, profiling circulating miRNAs holds promise as a non-invasive marker for human cancers.

In a study recruiting 513 subjects, miRNA profiles were obtained by NGS. A thirteen miRNA-based biomarker panel can differentiate clearly between HBV, HCV, and HBV-positive HCC cases [151]. Two miRNA candidates, miR-375 and miR-92a, are novel identified targets as HBV-specific miRNAs. A trio biomarker miR-25, MiR-375, and let-7f can significantly discriminate HCCs from controls, where miR-375 alone has an AUC of 0.96. Another study by Chen et al. demonstrated that the dual

biomarker of miRNA-30b-5p and MINPP1 can detect HBV-positive HCC [152]. The level of MINPP1 signifies the tumorigenesis of HBV-positive patients, whereas miRNA-30b-5p can regulate the expression of MINPP1.

Sometimes, a single miRNA might not differentiate between progressive liver diseases and HCC. An analysis of miRNA expression in 168 subjects revealed intriguing miRNA expression trends in patients infected with HCV. MiR-484 demonstrated a decrease in advanced fibrosis compared to mild fibrosis and HCC, but it increased HCC relative to cirrhosis [153]. In addition, miR-524-5p showed an increase in both cirrhosis and HCC, while miR-615-5p was found to be increased in the cirrhotic group relative to the control group. Notably, miR-524 displayed considerable potential in distinguishing between cirrhosis and fibrosis. These miRNAs under study may potentially be helpful in the staging and diagnosing of HCV hepatic progression, providing a significant understanding of the disease.

In a recent scientific publication, investigators presented a process that employs DNA molecular markers to convert gene expression profiles into clinical assessments [154]. Remarkably, this approach precisely categorized HCC patients and healthy individuals using only five miRNAs obtained from blood specimens, including miR-1290, miR-4370, miR-1203, miR-221-3p, and miR-4258.

Recent findings revealed that circular RNA (circRNA) is also associated with tumor development [155, 156]. A study delved into the expression patterns of circRNAs in HCC, identifying circMTO1 as a significantly down-regulated circRNA with implications for patient survival [157]. The author concluded that circMTO1's function in suppressing HCC progression positions it as a potential drug target in HCC treatment, and its decreased presence may serve as a prognostic marker for poor patient outcomes. Besides, circSETD3 is recognized as a novel

tumor suppressor of HCC and is a significant prognostic biomarker [157]. CircSETD3 can suppress the growth of HCC through the MAPK signaling pathway, and its downregulation is associated with unfavorable outcomes of HCC.

lncRNA, on the other hand, has a quite different role in tumorigenesis. They are RNA molecules with more than 200 nucleotides and have no protein-coding or potentials or only peptide-coding. They interact with oncogenes or tumor suppressor genes through epigenetic regulation and transcriptional activation or as sponges to silence miRNAs. Some lncRNAs also have a role in post-transcriptional regulation or modulating signaling pathways. It is a relatively new field to use lncRNAs as biomarkers of HCC detection, but some research articles also have significant findings. A review article 2018 discussed some findings on lncRNA as a biomarker of HCC [158]. There are indeed some reports worth mentioning in recent years. For instance, Liu et al. utilized an ultrasensitive strategy, an electrochemical nucleic acid sensor, to detect the expression of a lncRNA Highly up-regulated in liver cancer (HULC) [159]. HULC is highly expressed in patients with HCC, and its level can differentiate between HCC and control individuals [160]. Its level is also different between the HBV-positive group and the control group. Besides, research is now focusing on extracellular vesicle-derived lncRNAs as biomarkers of HCC. EV-LINC00853 displayed outstanding diagnostic ability for all-stage HCC, with higher sensitivity and specificity than AFP, particularly in early-stage and AFP-negative HCC, suggesting its potential as a novel diagnostic biomarker [161]. Another study indicated that serum small EV-derived lncRNAs, specifically EV-DLEU2, EV-SNHG1, EV-MALAT1, and EV-HOTTIP, show promising diagnostic potential for initial-stage HCC, with combined panels achieving the best results [162]. With the appearance of EV-lncRNA sequencing technology, bioinformatics approaches were introduced to analyze the potential lncRNA that can be HCC detection biomarkers. A panel of eight EV-lncRNA was identified as potential biomarkers [163]. However, further experiments are needed to validate those candidates.

Due to the lack of extensive clinical validation, ncRNAs are not being used as a detection biomarker in HCC. However, it is a valuable future research direction in diagnosing cancers because miRNA is closely related to regulating genes, especially cancer-associated genes. This can provide additional insights into the genetic makeup of the potential HCC tumors in patients with liver diseases.

Altered methylation patterns related to HCC occurrence

Apart from typical genetic mutation patterns that traditional sequencing or PCR methods can detect, epigenetic

regulations on the genome also have an essential role in tumor development. Methylation is a process by which methyl groups (-CH₃) are added to the DNA molecule, typically at the cytosine-guanine (CpG) dinucleotide sequences. This can change the activity of a DNA segment without changing the sequence itself. The most crucial aspect of methylation in regulating gene expression is the hypermethylation of tumor suppressor genes. And hypomethylation of oncogenes. Abnormal DNA methylation patterns can contribute to the development of cancers. Therefore, the methylation status of specific genes and their significance as a cancer biomarker has been heavily studied in recent years, with the advancement of detection technology [164] (Table 2).

Hypermethylation of Ras Association Domain Family Member 1A (RASSF1A) is one of the most commonly identified methylation markers for HCC. RASSF1A is a tumor suppressor gene that regulates cell progression by apoptosis, microtubule stability, and cell proliferation. The CpG island of the RASSF1A promoter is usually highly methylated in HCC patients, ranging from 40 to 93%, varying from different studies [168, 197]. This hypermethylation of RASSF1A in HCC patients can be detected in both tumor tissues [166] and liquid biopsy using circulating cfDNA [168]. When combined with serum AFP, methylation of RASSF1A is a better diagnostic biomarker for HCC, especially in patients with hepatitis infection, with a sensitivity and specificity of 65.0% to 85.0% and 70.0% to 100%, respectively [167, 182]. Notably, methylation is also detected on the RASSF1A gene of cirrhotic liver tissues, but not as high as the tumor tissues [175]. Among the cirrhosis cases, RASASF1A could be considered a prediction biomarker for developing HCC [175]. A study has demonstrated that the methylation extent of RASSF1A increases during CLD development, from chronic hepatitis B to cirrhosis and finally into HCC [173]. Consistently, a cohort study with patients recruited in a cancer screening study from Zhang et al. revealed that abnormal methylation of RASSF1A can be detected as early as 1 to 9 years before HCC is diagnosed [222]. Therefore, screening RASSF1A methylation status is considered an effective measure for early detection of HCC in patients with high cancer risk, such as CLD or HBV carriers [180, 222, 223]. However, there are also challenges to validating RASSF1A hypermethylation as a cancer biomarker due to contradictory data on methylation frequency in various malignant diseases for cfDNA and gDNA. Differences in the CpG sites analyzed could cause these discrepancies, the cancer stages of the patients, and the timing of RASSF1A methylation in tumorigenesis. Furthermore, inconsistencies in the association between RASSF1A methylation

Table 2 Summary of selected research output on detection of HCC using methylation patterns

Marker	Blood-based	Description	Ref.
1 <i>RASSF1A</i>	+	Mean rate of 30% and 65% methylation in HCV-related cirrhosis and HCC tissues respectively	[165]
2 <i>RASSF1A</i>		Mean rate of 26.1% and 59.1% methylation in cirrhosis and HCC tissues respectively	[166]
<i>DOK1</i>		Mean rate of 19.6% and 56.0% methylation in cirrhosis and HCC tissues respectively	
3 <i>RASSF1A</i>	+	Mean rate of 70% methylation on top of HCV-associated cirrhosis, with serum AFP combined to improve sensitivity	[167]
4 <i>RASSF1A</i>	+	Hypermethylated sequences in 93% of HCC, 58% of HBV carriers, and 8% of the healthy individuals High <i>RASSF1A</i> at diagnosis or 1-year after resection relates to poor prognosis ($p < 0.01$) <i>RASSF1A</i> increase after cancer diagnosis ($p < 0.014$)	[168]
5 <i>CHFR, VASH2, GRID2IP, CCNJ, F12</i> CpG sites	+	HCC detection sensitivity was 84.5% at 95% specificity and 0.94 AUC using the count of methylated reads on combined specific genes.	[169]
6 <i>WISP1</i>	+	Hypomethylation, increased plasma soluble <i>WISP1</i> improved diagnostic power combining with AFP	[170]
7 Six hypermethylated CpGs sites		A combination of six hypermethylated HCC-specific CpGs sites has a 92% sensitivity predicting HCC, and 98% specificity differentiating from normal liver or other cancers	[171]
8 Long interspersed element-1 (LINE-1)	+	Global hypomethylation measured in LINE-1 repeats in blood leukocytes DNA correlates with elevated risk of HCC ($p = 0.004$)	[172]
9 <i>RASSF1A</i> sequence	+	Higher methylation in HCC (64.2%) than patients with LC (17.5%), CHB (5.0%) and healthy individuals (0). Associated with a worse OS ($p < 0.05$)	[173]
10 <i>CDH1, DNMT3b, ESR1</i> promoter	+	Higher methylation in HBV-related HCC compared with LC, CHB and NC. Combined methylation is a better diagnostic marker than AFP.	[174]
11 <i>RASSF1A, E-Cadherin, RUNX3</i>	+	Hypermethylation in HCC higher than cirrhosis and healthy group ($p < 0.001$). <i>RASSF1A</i> and E-Cadherin were predictors of HCC within cirrhosis cases.	[175]
12 Methylated p16	+	Methylated p16 is higher in HCC than CHC, cirrhosis and healthy subjects Higher in patients with normal AFP than higher AFP	[176]
13 <i>TLX1, GALR1, ZNF154</i>	+	Multi-cancer methylation biomarkers combining three methylation markers have a sensitivity and specificity of 37.3% and 83.3% on HCC respectively.	[177]
14 38 DNA methylation regions	+	Machine learning method for systematic analysis with a 96% of sensitivity and 98% of specificity in an independent training cohort.	[178]
15 Enzymatic methyl sequencing	+	Sequencing utilizing enzymatic conversion of unmethylated bases as a screening model to distinguish HCC patients from non-HCC individuals	[179]
16 <i>TGR5</i>	+	Hypermethylation of the <i>TGR5</i> promoter is significantly higher in HCC than CHB and HCs ($p < 0.01$) Increased sensitivity when combined with AFP	[111]
17 <i>RASSF1A</i> promoter		Hypermethylation of <i>RASSF1A</i> in HCC, but not NC. Reduced <i>RASSF1A</i> is related to TNM stage, metastasis, AFP, portal vein embolus, capsular infiltration, and multiple tumor nodes.	[180]
18 <i>UBE2Q1</i>	+	Hypomethylation of <i>UBE2Q1</i> in HCC than LC ($p = 0.026$), CHB ($p = 0.006$), and HCs ($p = 0.011$). Negatively associated with TNM stage. Increase sensitivity with AFP combined.	[113]
19 <i>SEPT9</i>	+	Increase methylation of <i>SEPT9</i> in HCC than at-risk and healthy individuals ($p < 0.0001$)	[181]
20 <i>INK4A</i>	+	Hypermethylated <i>INK4A</i> in HCC than controls	[116]
21 <i>ELF, RASSF1A, p16, GSTP1</i>		Hypermethylation in tumor than non-tumor tissues ($p < 0.05$), and also combined markers ($p < 0.001$). Increase sensitivity with AFP combined	[182]
22 <i>APC, GSTP1, FASSF1A, SFRP1</i>	+	Hypermethylation in all four genes in HCC than normal or benign controls. Methylated <i>RASSF1A</i> is a prognostic marker of overall survival.	[183]
23 <i>APC</i>		Methylation status of <i>APC</i> complement with AFP to predict HCC	[184]
24 <i>MT1M and MT1G</i>	+	Hypermethylated <i>MT1M</i> and <i>MT1G</i> in HCC than CHB and NC group ($p < 0.001$), with a specificity of 94.6%. <i>MT1M</i> promoter methylation positively correlates with tumor size ($p < 0.001$), and metastasis when combined with <i>MT1G</i> .	[185]

Table 2 (continued)

Marker	Blood-based	Description	Ref.
25 <i>P16</i>	+	P16 methylation increases from benign liver disease to HCC progression.	[186]
26 <i>HOXA1, TSPYL5, B3GALT6</i>	+	The multi-target HCC blood test utilized three methylation markers and demonstrated high concordance (> = 97%) to predict HCC without significant interference observed.	[187]
27 <i>Methylation Fingerprint Panel</i>	+	The study identified three panels: cancer-specific biomarker panels, a pan-GI panel, and a multi-cancer prediction panel, with high AUC values ranging from 0.85 to 0.98 for detecting various gastrointestinal cancers.	[118]
28 <i>RNF135, LDHB</i>	+	Combined RNF135 and LDHB methylation level analysis has a sensitivity of 57% on HCC, compared with AFP at 45%. When combined with HCC, the sensitivity is 70%.	[119]
29 <i>SEPT9</i>	+	A significantly higher copy number of methylated SEPT9 was observed in the HCC group than in the control group ($p < 0.001$)	[188]
30 <i>SEPT9</i>	+	SEPT9 methylation pattern is a better predictor than serum AFP for diagnostic performance.	[189]
31 <i>IGFBP7</i>	+	Frequency of serum IGFBP7 promoter methylation is higher in HCC than in CHB and HC controls ($p < 0.001$).	[190]
32 <i>GNB4, Riplet</i>	+	Circulating tumor cells combined with methylation patterns have a sensitivity of 88.2% and an AUC value of 0.98.	[124]
33 <i>CCND1</i>	+	The methylation status of the <i>CCND1</i> promoter outperforms serum AFP in both AUC and specificity to predict HBV-HCC versus CHB.	[191]
34 <i>SOX1, VIM</i>	+	Higher frequency of SOX and VIM promoter methylation than LC, CHB, and HC subjects ($p < 0.001$).	[192]
35 <i>LINE-1, RASSF1A</i>	+	LINE-1 was hypomethylated in 66.7% and RASSF1A promoter was hypermethylated in 73.3% of HCC serum. Associated with HBsAg positivity, tumor size, AFP, and lymph node metastasis.	[193]
36 <i>BARD1, MAGEB3, BRUNOLS, FXYD6, TET1, TSPAN5, DPPA5, KIAA1210, and LSP1</i>	+	Neighboring CpG sites on 9 genes are predictable for prospective HCC development from HBV-negative cirrhotic patients. <i>DPPA5, KIAA1210, LSP1</i> are hypermethylated, while <i>BARD1, MAGEB3, BRUNOLS, FXYD6, TET1, TSPAN5</i> are hypomethylated, compared with controls.	[194]
37 <i>EXO1</i>		DNA methylation status in five CpG islands of the EXO1 gene was associated with the prognosis of HCC	[195]
38 <i>Wnt/beta-catenin signaling pathway</i>	+	Hypermethylation of Wnt/Beta-catenin signaling pathway is correlated with tumor size, TNM stage, distant metastasis, and HBV infection ($p < 0.05$).	[126]
39 <i>SEPT9</i>	+	Serum methylated <i>SEPT9</i> test has a high diagnostic accuracy for HCC on cirrhotic patients (AUROC 0.944, $p < 0.0001$), and is the only variable associated with HCC diagnosis in this cohort.	[196]
40 <i>RASSF1A, SOCS1</i>	+	<i>RASSF1A</i> and <i>SOCS-1</i> methylation were detected in 40% and 38% of HCC patients. <i>RASSF1A/SOCS-1</i> /AFP panel detects HCC at sensitivity of 86% and specificity of 75%.	[197]
41 <i>CCND2</i>	+	<i>CCND2</i> methylation is significantly higher in HCC patients ($p < 0.001$). Advanced HCC is associated with higher <i>CCND2</i> methylation and lower <i>CCND2</i> mRNA levels than early-stage disease.	[198]
42 <i>GSTP1</i>	+	Higher GSTP1 promoter region methylation frequency in pre-ACHBLF patients compared to CHB and HCs. Lower GSTP1 mRNA levels in pre-ACHBLF patients. Increased ACHBLF incidence in pre-ACHBLF patients with methylated GSTP1.	[199]
43 <i>MAGE-A1, MAGE-A3</i>		Different methylation patterns in several CpG sites among the MAGE-A1 and MAGE-A3 promoters in HCC cells. Clinical sensitivity and specificity were 91.2% and 100%.	[200]
44 <i>KLHL35, PAX5, PENK, and SPDYA</i>		These genes are significantly more highly methylated in HCC than in non-cancerous liver tissue, irrespective of the hepatitis virus status. LINE-1 hypomethylation was prevalent in HCC.	[201]
45 <i>A1AT, SERPINA1</i>	+	More fully-methylated SERPINA1 promoters in control than HCC samples, and higher hemimethylation in stage I compared to stage II and III HCC. Higher AFP and A1AT levels in hemimethylated patients ($p < 0.001$).	[202]
46 <i>TFPI2</i>		Methylation of TFPI2 gene was detected in 44.9% of primary HCC samples, 10.7% of the corresponding non-tumorous liver samples, and 5.0% of the normal liver samples. Lower expression of TFPI2 is correlated with TNM stage, and methylation is associated with poorer prognosis ($p < 0.001$).	[203]

Table 2 (continued)

Marker	Blood-based	Description	Ref.		
47		<i>RUNX3, p16, RASSF1A, CDH1</i>	+	88%, 100%, 50%, and 13% of HCC patients were detected with hypermethylation of <i>RUNX3, p16, RASSF1A</i> and <i>CDH1</i> . The inclusion of <i>RUNX3</i> in the gene panel can potentiate the detection of advanced cancer.	[204]
48		<i>MAPK10</i>		Methylation of <i>MAPK10</i> detected in 58% of HCC cell lines, and 666% of primary HCC tissues, results in downregulated expression of <i>MAPK10</i> proteins.	[205]
49		<i>SOX11</i>	+	Higher methylation of <i>SOX11</i> promoter in HCC patients (69.4%) compared to CHB patients (13.6%) and healthy controls (10.7%). Significant difference in <i>SOX11</i> promoter methylation between HCC patients with vascular invasion and those without. <i>SOX11</i> methylation demonstrates a 69% sensitivity in distinguishing HCC from CHB, higher than the 57% sensitivity of serum AFP.	[206]
50		<i>HCCS1</i>	+	<i>HCCS1</i> promoter methylation frequency higher in HCC patients compared to CHB patients and healthy controls ($P < 0.001$). <i>HCCS1</i> promoter methylation associated with TNM stage ($P = 0.01$). 62.5% sensitivity for serum <i>HCCS1</i> promoter methylation in discriminating HCC from CHB, compared to 55% for AFP alone, and sensitivity combined with AFP level is 81.7%.	[207]
51		Sat2		Correlation between hypomethylation Sat2 with a breakpoint in chromosome 1 ($p < 0.001$). Sat2 demethylation play a role in early stage of liver carcinogenesis.	[208]
52		Sat2, LINE-1		Sat2 hypomethylation associated with HCC risk. LINE-1 is not associated with HCC risk by age. Decrease in Sat2 methylation and LINE-1 hypomethylation associated with increased risk of HCC for HBsAg carriers.	[209]
53		Sat2, LINE-1	+	Negative relationship between urinary aflatoxin B albumin levels and LINE-1 and Sat2 methylation. It is associated with the risk of HCC development.	[210]
54		<i>SFRP2</i>	+	<i>SFRP2</i> methylation levels significantly higher in patients with HBV-HCC than in those with CHB and healthy controls ($p < 0.001$). <i>SFRP2</i> mRNA level significantly lower in HCC group compared to the others ($p < 0.05$). <i>SFRP2</i> methylation level showed better diagnostic value than AFP.	[211]
55		<i>SGIP1</i>	+	Elevated levels of <i>SGIP1</i> methylation in HCC patients associated with poorer OS, PFS, and MFS compared to those with low levels ($p < 0.05$).	[212]
56		<i>SCAND3, Myo1g</i>	+	<i>SCAND3</i> and <i>Myo1g</i> methylation were high in HCC cell lines and tissues, and serum <i>SCAND3, Myo1g</i> , and <i>SCAND3 + Myo1g</i> methylation values showed better detection and early detection of HCC than AFP alone. In the AFP-negative HCC group, <i>SCAND3</i> and <i>Myo1g</i> methylation also can predict diagnosis.	[213]
57		<i>LZP</i>		Methylation of <i>LZP</i> promoter decreases mRNA expression, and is negatively related to the HCC status.	[214]
58		<i>ATM</i>		Higher <i>ATM</i> promoter methylation in HCC tissues, and associated with <i>ATM</i> expression ($p < 0.001$). Methylation of <i>ATM</i> promoter associated with better outcomes in locally advanced HCC patients who received radiotherapy	[215]
59		<i>IL-6</i>	+	<i>IL-6</i> promoter methylation levels lower in HCC patients than in CHB patients ($p < 0.001$). <i>IL-6</i> promoter methylation level is an independent factor in HCC development, and its diagnostic value is superior to AFP. Combined use of AFP and <i>IL-6</i> methylation level improves AUC (0.773).	[216]
60		<i>CDO1</i>	+	<i>CDO1</i> promoter methylation frequency higher in HBV-related HCC than in LC, CHB, and healthy controls ($p < 0.001$). Higher frequency of <i>CDO1</i> promoter methylation in advanced stages HCC compared to early stages. Improved diagnostic value combined with serum AFP.	[217]
61		<i>CDH13</i>	+	Higher methylation frequency of <i>CDH13</i> promoter in HCC patients compared to NCs and CHB groups ($p < 0.05$). Methylation level of <i>CDH13</i> promoter influenced by TBil, ALB, and AFP. Combined methylated <i>CDH13</i> level and AFP level show better diagnostic score (AUC = 0.796). <i>CDH13</i> methylation is an independent predictor for HCC prognosis ($p < 0.05$).	[218]
62		<i>FES</i>		<i>FES</i> hypermethylation correlated with tumor size, serum AFP, and tumor differentiation ($p < 0.005$). Both <i>FES</i> hypermethylation and protein downregulation associated with PFS and OS of HCC patients.	[219]
63		<i>NLRP7, NLRP2, NLRP3</i>	+	Hypomethylation of programmed-cell death genes (PRGs) including <i>NLRP7, NLRP2</i> , and <i>NLRP3</i> in HCC tissues; <i>NLRP3</i> methylation levels correlated with expression level ($r = 0.51$). Hypomethylated PRGs can discriminate between early HCC patients and healthy controls in cfDNA analysis (AUC = 0.94), and associated with poor HCC prognosis.	[142]

Table 2 (continued)

Marker	Blood-based	Description	Ref.
64 <i>DBX2, THY1</i>	+	<i>DBX2</i> and <i>THY1</i> are hypomethylated in HCC. Diagnostic sensitivity and specificity of <i>DBX2</i> for differentiating healthy controls and early stage HCC were 88.89% and 87.10% respectively. Diagnostic sensitivity and specificity of <i>THY1</i> were 85.19% and 80.65% respectively.	[220]
65 <i>F-box protein 43 promoter</i>	+	F-box protein 43 promoter methylation levels were significantly lower in HCC PBMCs than in chronic hepatitis B and healthy control PBMCs ($P < 0.001$). This was superior to those of AFP levels in the diagnosis of HCC. Combination of F-box protein 43 promoter methylation and AFP levels improved the AUC to 0.888 with sensitivity of 76.42% and specificity of 86.08%.	[221]
66 <i>RASSF1A, p16, p15</i>	+	Abnormal methylation was found in blood samples 1 to 9 years before HCC was diagnosed. <i>RASSF1A</i> had the highest rate of increased methylation (70%), followed by p16 (44%) and p15 (22%). Combined risk factors and methylation markers, the accuracy of predicting HCC was 89%, with 84% sensitivity and 94% specificity	[222]
<i>GNB4, Riplet</i>	+	In tissue validation, <i>GNB4</i> and <i>Riplet</i> had an AUC of 100%, with 100% sensitivity and specificity for detecting any-stage HCC. In blood tests, this combination showed a high sensitivity of 84.39% and specificity of 91.92%, with an AUC of 92.51% for detecting any-stage HCC. The dual-marker panel was more sensitive for detecting stage I HCC than AFP. It had a high sensitivity (70.27%) for detecting a single tumour of size 3 cm or less.	[144]

and clinicopathological parameters suggest that other genes' aberrant methylation may significantly influence tumorigenesis in some cancers [224].

Additionally, methylation of the *CDKN2A*, coding for the tumor suppressor protein p16, is a well-studied HCC detection biomarker. P16, a tumor suppressor protein, is vital for controlling the cell cycle by blocking the actions of CDKs. By inhibiting these enzymes, p16 helps avert uncontrolled cell growth and division, which could lead to tumor formation. Hypermethylation of *CDKN2A* results in a lower level of p16, which is more prevalent in patients with HCC than in chronic hepatic cirrhosis and healthy control people [176]. The levels of p16 increase with HCC progression from benign liver disease [186]. It is also a potent marker as it can help differentiate HCC among patients with normal AFP than higher AFP levels [176]. Like *RASSF1A*, p16 methylation was found in 44% of patients before HCC diagnosis [222].

The septin 9 (*SEPT9*) gene, a crucial regulator of cell division, functions as a tumor suppressor. Its excessive methylation is associated with the progression of liver cancer [225, 226]. The *SEPT9* gene is expressed in all healthy tissues, but its expression is reduced or silenced due to abnormal promoter hypermethylation in liver cancer [227]. An extensive epigenome analysis of 304 liver cancer tissue specimens identified *SEPT9* as a crucial epi-driver gene in liver cancer development, primarily due to the hypermethylation of the *SEPT9*-promoter [225]. Increased methylation of *SEPT9* is found in HCC patients than in healthy individuals [181], and it's a better predictor than serum AFP for

diagnosis of HCC. It is also reported that a higher copy number of *SEPT9* methylation was presented in HCC than in control individuals [188].

Glutathione S-transferase pi 1 (*GSTP1*) is a protein detoxifying harmful substances, and its hypermethylation is often observed in HCC. This hypermethylation reduces the *GSTP1* enzyme's production, impairing detoxification processes and potentially contributing to HCC development and progression [228]. *GSTP1* hypermethylation and AFP level can be a high-sensitivity biomarker for detecting HCC at 93.55% sensitivity [182]. It is also consistent with *RASSF1A* and is hypermethylated in tumors compared to benign control groups [183]. In patients with acute-on-chronic hepatitis B liver failure (ACHBLF), which is a condition of acute regression of liver function due to CLDs such as HBV infection, *GSTP1* methylation frequency was significantly higher than in chronic hepatitis B patients and healthy individuals [199]. The higher methylation and lower expression of *GSTP1* are associated with ACHBLF incidence.

A repetitive DNA sequence known as a retrotransposon, long interspersed nuclear element-1 (*LINE-1*), is found in the genome. In the case of HCC, *LINE-1* exhibits overexpression and hypomethylation. This hypomethylation in *LINE-1* elements can result in genomic instability, higher mutation rates, and changes in gene expression, which can all contribute to the development and progression of cancer. Research has demonstrated a connection between *LINE-1* hypomethylation and unfavorable outcomes in individuals with liver cancer. More than 50% of HCC patients demonstrate an overall hypomethylation in *LINC-1* [201]. This pattern is also observed in cfDNA,

ctDNA, and blood leukocyte DNA [172, 193]. Additionally, satellite 2 DNA (Sat2), located in the 1q12 pericentromeric region, is a sequence with reduced methylation in cancer cells and contributes to chromosomal instability [229]. Recent research has revealed that Sat2 and similar heterochromatic regions can generate ncRNAs [230], which might have a role in responding to stress and facilitating heterochromatin reformation. Nevertheless, the influence of DNA hypomethylation on Sat2 expression remains uncertain, as different studies have produced inconsistent results. Nonetheless, several studies have reported that Sat2 hypomethylation is associated with HCC risk. A significant relationship has been identified between Sat2 sequences with reduced methylation and increased 1q copies having a 1q12 breakpoint. This decrease in methylation is thought to change how CpG-rich satellite DNA interacts with chromatin proteins, causing heterochromatin to decondense, break, and form 1q abnormally. This implies that the demethylation of Sat2 could be involved in the initial phases of the development of liver cancer [208]. Meanwhile, aflatoxin is a well-described carcinogen leading to HCC. Aflatoxin B1 albumin is a metabolite under aflatoxin exposure and can be excreted through urine. A cohort study with 1140 cancer-free participants revealed a reverse association between the aflatoxin concentration and global LINE-1 and Sat2 methylation [210]. This demonstrates that there may be an epigenetic regulation effect of aflatoxin, which results in HCC development. Urinary aflatoxin metabolite level and DNA methylation can be a biomarker of potential HCC occurrence. On the other hand, a prospective case-control study measuring methylation levels in leukocyte DNA suggested that Sat2 hypomethylation is associated with the risk of HCC, but LINE-1 is not associated with HCC risk by age [209]. The association is stronger within HBsAg carrier between Sat2 and LINE-1 hypomethylation and the risk of HCC.

Other reported methylated genes that can be potentially regarded as biomarkers of HCC are listed in Table 2.

Presence of tumor-derived vesicles in the circulation

Extracellular vesicles (EVs) are tiny, membrane-bound capsules containing various organ components [231]. There are three main types of EVs: microvesicles, exosomes, and apoptotic bodies, each varying in size and composition and released from cells in different ways [232]. EVs contain various cellular components such as proteins, DNA, RNA, and lipids [233]. It plays a crucial role in cell-to-cell communication and is involved in essential biological processes like inflammation and tissue remodeling [234]. Various liver cells, such as hepatocytes, hepatic stellate cells, and endothelial cells, are released into the bloodstream. The EVs released by the

tumor cells are tumor-derived vesicles [235]. LncRNAs, which regulate biological and genetic functions, have been found in EVs and linked to numerous diseases. The composition of EVs can change in response to different stimuli, making them potential biomarkers for HCC. Liquid biopsy methods are now widely used to detect the presence of EVs, providing valuable information about the occurrence and progression of HCC [235].

Change in viral load in viral hepatitis-related HCC patients

Pre-treatment serological data were analyzed in a study of 125 HBV-positive HCC patients with chemotherapy. Upon analyzing the virological data alongside conventional clinical variables, factors such as high total bilirubin, HCV infection, and elevated HBV DNA levels significantly influenced survival outcomes [236]. Notably, an exploratory analysis revealed a higher incidence of severe hepatitis during chemotherapy in patients with high pretreatment HBV DNA levels. On the other hand, Choi and the others reported that the association of viral load on patients with HBV but not cirrhosis, with or without prior anti-viral treatment, is non-linear [237, 238]. The risk of developing into HCC is the highest at around 6 to 7 logIU/mL. The risk decreases with increasing baseline viral load. It is worth mentioning that CLDs with viral infection are alone the dangerous risk factor leading to HCC. Therefore, the clinical importance of checking the viral load as a potential prognostic biomarker is crucial. Several viral factors contributing to HCC, including HBsAg, HBeAg, viral DNA levels, viral genotypes, and viral mutations, may be considered integratively and provide essential information for surveillance program development [239].

Potential metabolomics clues for HCC diagnosis

On the other hand, metabolic profiling is also an emerging research direction in the biomarkers of HCC diagnosis. With the advancement of Omics technology, metabolic profiles from hepatitis patients and HCC patients were characterized and compared. High classification accuracy models could be derived from these multi-omics data [76]. “Omics” technologies, which include genomics, transcriptomics, proteomics, and metabolomics, provide an in-depth molecular and biochemical analysis of a biological entity, whether it's an organism, tissue, or specific cell type. Metabolomics, which pertains to the exhaustive study of lightweight metabolites within a biological sample, holds several unique advantages. It is more closely aligned with the phenotype, is not restricted to specific species, and involves fewer molecules than other omics technologies. The metabolite profile serves as a comprehensive summary of the preceding omics profiles, which makes

it particularly effective in identifying minor alterations in metabolic pathways and shifts from homeostasis [240]. Techniques such as MS and nuclear magnetic resonance (NMR) spectroscopy have been effectively used in metabolomic analysis.

Gas chromatography (GC)-MS-based metabolomics is commonly used to analyze patients' serum. In a study involving 20 HCC patients and 20 normal subjects, serum metabolome was analyzed. Metabolites, including butanoic acid, L-valine, glycerol, etc., significantly differentiate the two groups. The specificity is 100%, and the total accuracy is 75% [241]. The same group also investigated using urine metabolomics via GC/MS, yielding an AUC of 0.9275 for detecting HCC from control groups combined with AFP [242]. Another metabolic profiling study unveiled 43 serum metabolites and 31 urinary metabolites in HCC patients, which impact crucial metabolic pathways [243]. Specific metabolites, including bile acids, histidine, and inosine, exhibited notable statistical variances and considerable fold changes, hinting at their prospective utility as HCC biomarkers. Nonetheless, it was observed that irregular levels of specific bile acids were linked with conditions such as liver cirrhosis and hepatitis. The research also distinguished HCC patients with low AFP values from healthy subjects, utilizing a set of metabolite markers. Moreover, membrane phospholipids, measured by LC-MS/MS, were shown to discriminate HCC from adjacent liver tissues with an AUC of 0.963 [243].

Although the technology has matured for more than a decade already, the detailed metabolic profiles of HCC patients have not yet been well characterized due to the heterogeneity of this disease. The training and validation cohort have not been large enough to validate the power of specific metabolic panels for HCC diagnosis. With the advancement of analytical tools, the omics approach is inevitably essential to precision medicine.

Omics approach to identify novel biomarkers

The general approach of using omics technologies to diagnose HCC involves analyzing an array of biological molecules, such as proteins, metabolites, and modified RNA sites, to identify specific biomarkers and molecular profiles associated with the disease [244]. This comprehensive and integrative method allows for early detection and improved understanding of HCC pathogenesis, ultimately leading to better patient outcomes and targeted treatment options [245]. By overcoming challenges in data analysis, identification, and accessibility, omics technologies can revolutionize the field of HCC diagnosis and management. Mass spectrometry (MS)-based omics technologies have the potential to diagnose HCC by analyzing biological molecules related to disease

development, such as proteomics techniques that identify specific serum protein panels [246]. However, there are challenges to overcome, such as the rapid identification of metabolites, proteins, and modified RNA sites and the development of feasible in-house databases and powerful bioinformatics tools [247, 248]. Addressing these challenges and making multi-omic strategies accessible to non-specialist groups could lead to a better understanding of HCC pathogenesis and improved human health outcomes.

Additionally, employing bioinformatics analysis on the current dataset is a valuable method for the secondary discovery of potential targets. Establishing prediction models based on cfDNA, methylation profiling, and somatic copy number aberrations data is a viable strategy [102, 249–251]. In recent years, the integration of machine learning algorithms has substantially advanced the field of biomarker discovery [178, 252]. Besides the bioinformatics approach to the studies of epigenetics and ncRNAs [253, 254], spatial proteomics, or transcriptomics [253, 254] can also contribute to the disease prognosis and management. However, it is crucial to recognize that bioinformatics should be utilized as a supplementary tool in biomarker research rather than a primary source due to the absence of experimental evidence. Consequently, further experimental and clinical validations must be conducted to ensure the efficacy of the identified biomarkers.

Taken together, many promising targets can be considered as new liquid biopsy biomarkers for detecting HCC. However, there is still a long way to look for a new universal serological HCC detection biomarker using liquid biopsy to replace AFP completely.

Biomarkers for treatment options and therapeutic response

Some recent review articles have discussed the representative findings of molecular biomarkers to predict HCC prognosis and the current staging system to predict HCC prognosis [32, 35, 255, 256]. The following part highlights the current understanding of biomarkers that can provide insights into treatment options and therapeutic responses (Fig. 2).

Biomarkers related to surgical resection

While the surgical resection guidelines are beyond this review's scope, we will discuss biomarkers for postoperative monitoring and prediction of recurrence. A recent study revealed that serum AFP level greater than 400 ng/mL is a risk factor for OS and recurrence-free survival (RFS) (HR: 1.512) [257], which is consistent with previous studies that AFP decrease after surgery is an excellent prognostic indicator [258, 259]. Another study has

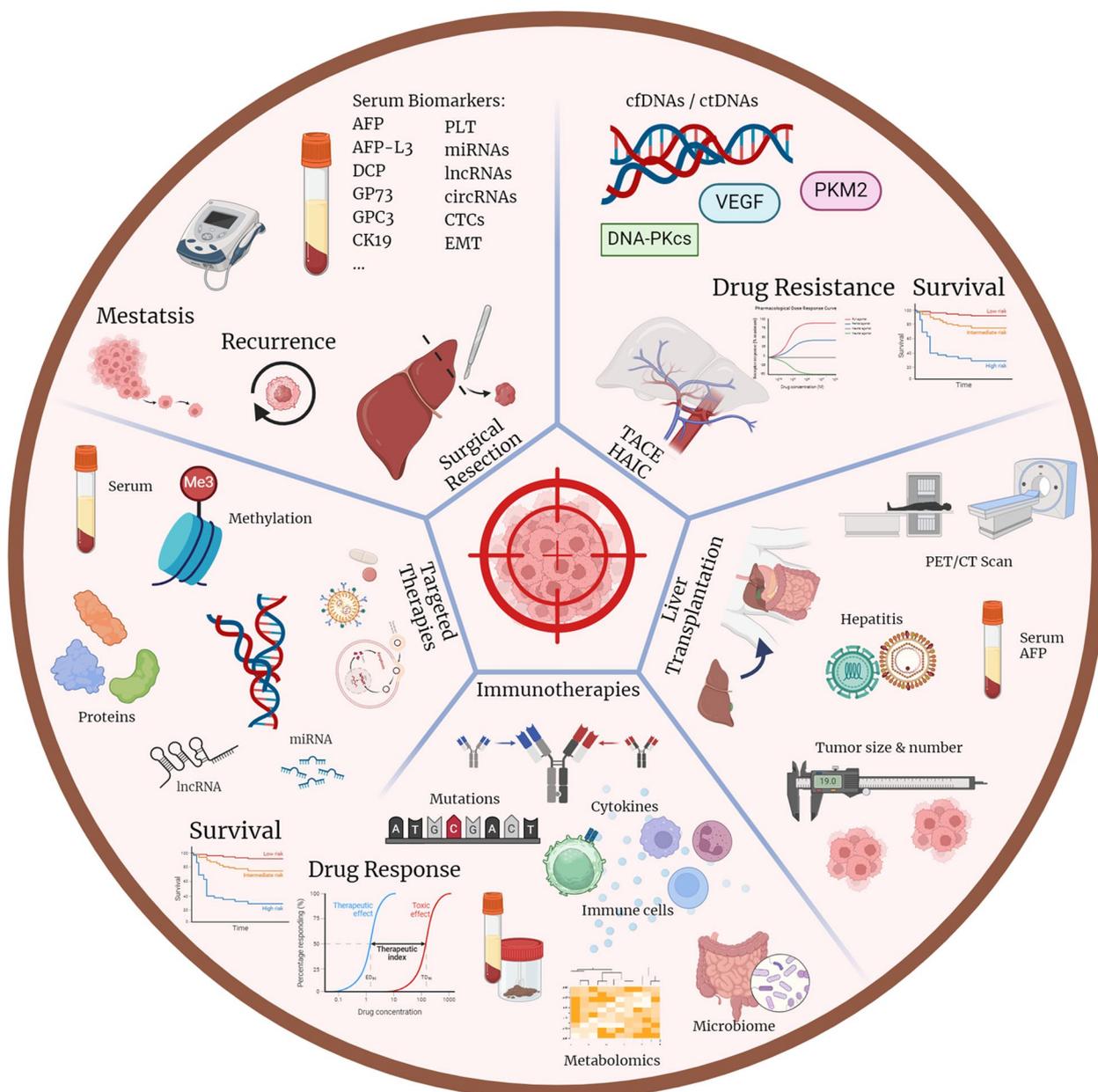


Fig. 2 Biomarkers for therapeutic options. To facilitate the decision on the best treatment for HCC patients, biomarkers predicting therapeutic response to different treatments, including surgical resection, TACE, HAIC, liver transplantation, targeted therapies, and immunotherapies, are being investigated. Some of the significant aspects of the studies are highlighted in the illustration, and the predicted response and correlated clinical outcomes are drawn on the diagram

combined the level of sulfite oxidase with AFP, which can predict postoperative outcomes and recurrence risk at sensitivity and specificity of 93.8% and 95.2%, respectively [260].

On the other hand, Yamamoto et al. compared the predictive values of AFP, AFP-L3%, DCP, and GP73 in postoperative patients [261]. It was found that DCP is

the best prognostic marker for HCC development, but AFP-L3% may be more significant for early recognition of tumor recurrence. Another study investigating the significance of combining these three tumor markers found that the number of elevated markers post-surgery predicts survival and recurrence [262]. A multivariable analysis using large-scale clinical data has identified that males, large tumors, multi-nodules, high

albumin-bilirubin, and high serum AFP are the predictors of early recurrence after hepatectomy [263].

It was shown that GPC3-positive patients with HCC have worse survival than GPC3-negative patients [264]. In general, higher GPC3 expression is associated with poorer prognosis in HCC patients, no matter who is HBV-related, post-operative, or not [265–267]. Similar results were observed for serum GPC3, in which a higher level signifies a shorter OS and disease-free survival (DFS) after surgical treatment [268, 269], although there are also controversial results [270]. Consequently, GPC3 is being investigated for its potential when combined with other biomarkers for the prognostic indication. The expression of GPC3 and OPN in HCC tissue was a negative predictor for patients with HBV-related small HCC tumors who have undergone surgical resection [271]. On the other hand, Feng et al. discovered a link between the expression of GPC3 and CK-19 in HCC tissue and local recurrence in patients with post-surgical resection for HCC [272].

Apart from the conventional serological markers, there are also reports of other potential biomarkers. A study found that platelet count (PLT) and five platelet-related factors can predict HCC recurrence, especially late recurrence, in non-cirrhotic patients [263]. Ascites, PLT levels, alkaline phosphatase levels, and tumor sizes were independently associated with the risk of HCC recurrence. However, larger cohorts are needed to validate these findings. ncRNAs are also within the research scope. The HCCseek-23 panel with eight miRNA showed 81% sensitivity and 83% specificity for early-stage HCC diagnosis and 93% sensitivity for identifying AFP-negative HCC. Combined with serum biomarkers, they were significantly associated with DFS. Furthermore, lncRNA MVIH has also been reported to be a predictor. Overexpression of MVIH is correlated with poor RFS ($p < 0.001$) and OS ($p = 0.007$) of postoperative HCC patients [273]. One study found that circFOXK2 is highly expressed in HCC tissues and correlates with poor outcomes in patients undergoing radical hepatectomy ($p = 0.0005$) [274]. The research revealed that circFOXK2 promotes HCC progression and activates the Warburg effect by upregulating specific proteins and miRNA sponges, making it a potential prognostic marker and drug target for HCC.

Identifying CTCs with specific genes can also help healthcare providers predict patients' responses to treatment. For instance, MAGE3, surviving, and CEA could be used to indicate the effectiveness of cryosurgery on unresectable HCC [275]. CTCs are associated with tumor diffusion and thrombosis, and the presence and number of CTCs are associated with worse survival [79]. A study focusing on the EMT properties of CTCs found that CTC count and mesenchymal characteristics of

CTCs are associated with early recurrence or postoperative DFS [80]. During the process of EMT, the expression of EpCAM is usually down-regulated, and it becomes no longer the only reliable marker for the enrichment of CTCs [276]. Another study has shown consistent results that CTCs with high EMT phenotypes are more likely to be metastatic. More epithelial markers, such as CD133, vimentin, HER2, and PSMA, are provided for the positive enrichment of these stem-like CTCs [277]. Additionally, HCC patients with CTCs detected are associated with vascular invasion, elevated AFP levels, disease progression, recurrence, and poorer prognosis [278–280].

On the other hand, Chen and others performed the unique molecular identifier target sequencing on plasma and PBMCs from HCC patients [107]. They found that mutation variant frequency changes (MVFCs) correlate with shorter RFS, thus suggesting minimum residual disease (MRD). MRD refers to the small number of tumor cells that remain in the primary site after treatment, usually surgical resection. That tiny tumor volume is not generally detectable by conventional detection strategies, such as ultrasound, imaging, or serological tests. MRD is a condition in which patients are susceptible to recurrence before average time and is usually accompanied by shorter RFS and OS. Chen defined the MRD-positive patients as MVFC mutations favorable in ctDNA. The prognostic evaluation is greatly improved when combined with serum AFP and DCP ($p < 0.0001$). The design of their platform is also compatible with different gene panels, so it is theoretically applicable to other samples or diseases.

Biomarkers related to transarterial chemoembolization and hepatic arterial infusion chemotherapy

Transarterial Chemoembolization (TACE) is a medical process where a catheter is used to directly administer chemotherapy, typically doxorubicin, into the tumor's feeding arteries. This is then followed by blocking these arteries [281]. This procedure has a relatively low mortality rate related to treatment, which is less than 5%, and it has been found to extend the median survival rate to somewhere between 11 and 20 months [282]. Notably, this treatment does not yield practical results for over 40% of patients who are left to depend on less potent systemic therapies [283]. Hence, discovering predictive biomarkers for TACE treatment response could greatly benefit patients. This would enable them to transition to alternative therapies promptly without unnecessary delay. Pyruvate kinase muscle isozyme M2 (PKM2) is reported to be associated with resistance ($p < 0.01$) and survival to TACE (27.5 m vs. undefined, $p < 0.0001$) [284]. Elevated PKM2 predicts a poor therapeutic outcome. Furthermore, cfDNA and ctDNA levels are also suggested to be

associated with the failure of the TACE procedure, with a sensitivity of 80% and a specificity of 95.6% [285]. A recent study on 132 patients showed that DNA-dependent protein kinase (DNA-PKcs) is associated with poorer survival (4.5 vs. 16.9 m, $p=0.011$) [286]. The expression of DNA-PKcs is related to doxorubicin resistance.

Like TACE, Hepatic arterial infusion chemotherapy (HAIC) delivers the chemotherapies into the liver, but instead of the arteries, the vein leading to nearby organs will be blocked. A study found that serum VEGF levels in advanced HCC patients can predict the therapeutic response and survival for receiving cisplatin and 5-FU via HAIC [287]. In a recent Phase III clinical trial testing the efficacy of HAIC-oxaliplatin plus fluorouracil (HAIC-FO), a 15-gene mutation panel was discovered that can predict the responsiveness to the treatment. 83% of patients were identified to have a longer OS (19.3 vs. 10.6 months, $p=0.002$) [288].

Biomarkers related to local ablation

Local ablation involves using electrical equipment to heat and destroy tumor tissues at the local site, typically through radiofrequency. At temperatures exceeding 60 °C, the probe burns the tumor tissues and adjacent inoperable or metastatic tissues. This procedure is sometimes performed via endoscopy, or in the case of larger tumors, a laparotomy may be required. The decision to utilize local ablation for HCC treatment primarily depends on tumor size, ascertained through CT or MRI. The Child–Pugh score is also occasionally considered, with only patients classified as grade A or B deemed suitable for ablation therapies [289]. AFP level is perceived as an indicator of whether patients will experience recurrence following ablation [290]. A comprehensive retrospective study involving nearly 3,000 patients who underwent tumor ablation revealed that additional biomarkers, including tumor size and numbers, serum DCP, platelet counts, Child-Pugh score, and HCV viral load, are associated with distant recurrence [291]. An Italian survey corroborated these findings, reporting that advanced age is an independent predictor of poor OS apart from AFP levels, while ALT can prognosticate recurrence [292]. More studies suggest AFP-L3%, DCP, neutrophil to lymphocyte ratio, ferritin, and specific miRNAs correlate with the OS and DFS after ablation, but further research is awaited to confirm their clinical significance [293].

Biomarkers related to liver transplantation

Liver Transplantation is considered when patients have unresectable HCC tumors by conventional surgery, but the tumors are still not widespread, usually at T1. Patients have to have good health conditions and

normal liver function. As liver transplantation involves the donation of liver tissues from another individual and is a complicated procedure, it is precious. It requires special attention before the clinician and surgeon decide to operate on the patient. Therefore, reliable biomarkers are needed to help determine if a patient is suitable for transplantation and the predicted prognosis after the surgery. AFP levels are usually considered only in current clinical guidelines for therapeutic options. For example, patients with AFP levels above 1000 ng/mL are not considered for liver transplantation due to poor predicted prognosis after operation [46, 294, 295]. Although AFP is one of the most widely used biomarkers for HCC diagnosis, its stability as an indicator of disease progression and the reference range has been controversial. Studies report a positive correlation between AFP mRNA level and tumor size, circulating malignant hepatocytes, and metastases. However, controversial studies have also been reported [296–299], limiting its significance as a prognostic marker for HCC.

Parameters, including tumor sizes, numbers, serum AFP levels, and tumor uptake on FDG PET/CT, are recognized post-transplantation predictors of tumor recurrence in HCC patients [300–303]. A combined set of criteria, including tumor sizes, numbers, serum AFP levels, and maximum tumor-to-background ratio, is the most effective way to predict post-transplant recurrence and select candidates for living donor liver transplantation compared to conventional criteria sets [304]. A study of 103 patients with HCC found that poorly differentiated tumors and high AFP levels were linked with poorer survival ($p<0.05$) and higher recurrence ($p<0.05$) after liver transplantation [305]. The research suggests that these tumor biological characteristics should be taken into account before performing the procedure for patients with HBV-associated HCC. This information can help assess HCC patient survival time after liver transplantation. A 30-year comprehensive study calculated the recurrence rate of 865 HCC patients receiving liver transplantation. Multivariate factors predicting recurrence were developed, including tumor grade, macrovascular and microvascular invasion, tumors outside Milan criteria, radiographic maximum diameter, and specific pre-transplant neutrophil-to-lymphocyte ratios [281]. The situation is highly individual and requires more clinical information to help make decisions.

Biomarkers related to targeted therapies

For patients who have unresectable HCC tumors and who are also not suitable for liver transplantation, systemic therapies are the first-line treatment. Sorafenib is the most commonly used first-line drug, but the development of sorafenib resistance is usually observed. The

study of sorafenib resistance is a hot topic, and many molecular mechanisms leading to sorafenib resistance have been discovered [306]. Therefore, it is crucial to predict the drug response to systemic therapies in HCC patients [307]. In a study using data from the SORAMIC study, researchers utilized EV-based proteomics to predict treatment response [308]. Low GPX3 and high ARHGAP1 in plasma predicted better treatment response to sorafenib with AUC 0.97. From another phase III SHARP trial, the plasma Tregs and CD56^{Dim} NK cells are correlated with shorter OS for patients receiving sorafenib ($p < 0.05$) [309]. When sorafenib is combined with mFOLFOX, plasma sVEGFR21 is associated with shorter time-to-progression in HCC patients. Consistent results were observed that VEGF and HIF in HCC have poor predicted treatment outcomes ($p < 0.001$) and OS ($p < 0.001$) [310]. Apart from typical serological markers, ncRNAs are also at the hot spot. For instance, the lncRNA Linc01056 is found to be downregulated in HCC tumors, where its expression level is related to sorafenib sensitivity [311]. Besides, from a genomic study using patient-derived tissues, a sub-type of β -Catenin with high Male predominance has low miRNA expression and is sensitive to sorafenib [312]. Similarly, the circulating level of another miRNA, miR-518d-5p, is reported to be a potential sorafenib resistance biomarker in BCLC-C HCC patients [313]. On the other hand, the immune microenvironment also affects systemic drugs' therapeutic effect. A study showed that high CD4⁺ T effector/Treg ratios and IFN- γ production by CD8⁺Ki67⁺ T cells are potential biomarkers for improved survival and response to sorafenib therapy [310]. Similarly, pERK⁺/pAkt- CTCs were discovered to be correlated with better prognosis in HCC patients receiving sorafenib treatment [314].

Lenvatinib is sometimes regarded as the second-line therapy after sorafenib resistance has arisen. High expression of FAT1 and LRP1B genes in ctDNA is associated with poor PFS and a higher recurrence rate [315]. Also, serum ST6GAL1, a tumor-secreted protein, is positively correlated with better survival of Lenvatinib [316]. Likewise, a higher level of circMED27 in the serum of HCC patients also correlates with worse prognosis and clinical outcomes when treated with Lenvatinib [317]. Some other biomarkers related to treating HCC with targeted therapies are reviewed in some recent articles [7, 318, 319].

Biomarkers related to immunotherapies

The immune-suppressive environment is one of the reasons for the poor treatment response of HCC. A recent clinical study measured the serum AFP and CRP levels from patients with HCC treated with anti-PD-(L)-1 therapies. The two parameters were identified as independent

predictive markers and developed as the CRAFTY score. The CRAFTY score is associated with survival and therapeutic response in those HCC patients [320]. In 2024, a different study examined the significance of AFP trends in forecasting responses to systemic treatment in HCC patients undergoing bevacizumab combined with immunotherapy [321]. The retrospective evaluation of 536 HCC individuals revealed three unique AFP patterns in both AFP-low and AFP-high groups, with a quick decrease in AFP following treatment associated with improved patient outcomes (HR: 0.2 in AFP-low, and 0.04 in AFP-high class). These results highlight the possibility of using AFP trends as an independent biomarker for predicting clinical results, offering a dynamic prediction tool for systemic therapy prognosis in HCC patients and assisting in clinical decision-making. Weng et al. discovered that SLAMF7 deficiency-caused CCL2 signaling can modulate macrophage repolarization and result in an immunosuppressive response ($p = 0.00034$) [322]. SLAMF7 could be used as a biomarker and drug target to predict patient immunotherapy response. Winograd et al. also found that PD-L1⁺ CTCs could help identify HCC patients likely to respond to immunotherapy [323].

A detailed molecular analysis was carried out on tumor biopsy samples from 60 HCC patients insensitive to sorafenib treatment to understand better the genomic characteristics of HCC patients treating anti-PD-1 [324]. The research found a 10% overall response rate to the therapy, with key contributing factors including female gender, PD-L1 positivity, and a low neutrophil-to-lymphocyte ratio. Notably, CTNNB1 mutations and up-regulation of MET were only detected in those who did not respond to treatment, underscoring their potential as predictive biomarkers. Therefore, these biomarkers merit further exploration to improve our knowledge of their potential clinical application in managing HCC patients receiving immunotherapy. Non-coding RNAs such as RNA methylation and circRNA can also serve as biomarkers to predict the effectiveness of immunotherapies in HCC [325]. For instance, the circuit can inhibit the immune response to HCC tumors and stimulate tumor growth via the Snail/DPP4/CXCL10 axis [326].

Notably, atezolizumab (Anti-PD-L1) combined with bevacizumab (Anti-VEGF) (AtezoBev) is one of the standard therapeutic options for unresectable HCC. However, an integrated molecular analysis from samples of patients enrolled in a clinical trial demonstrated that the pre-existing immunity population, with CD274 expression ($p = 0.0011$), T effector signature ($p = 0.0035$), and density of intratumoral CD8⁺ T cells ($p = 0.053$) as the predictors of better therapeutic response to this combination therapy [327]. Conversely, a high Treg to effector T cells ratio and expression of GPC3 and AFP worsen the

clinical benefit. The serum AFP and albumin-bilirubin (ALBI) levels can be biomarkers to predict the response to AtezoBev [328]. The multivariate analysis established an independent correlation between OS and the ALBI-AFP combination, where patients in the ALBI2-AFP non-responders category exhibited the poorest prognosis.

A stage-specific immune classifier was developed by Shi et al. based on patients' samples [329]. This panel of markers can detect early-stage HCC with a performance superior to serum AFP. It also provides information about whether the patients are resistant to anti-PD-1 monotherapies. Similarly, another study reported that β -catenin activation can result in immune escape of the HCC tumor, and the patients will be more resistant to anti-PD-1 immunotherapies [330]. Bioinformatics analysis also provides potential prognostic biomarkers and therapeutic targets for HCC [331]. Research on novel biomarkers for predicting immunotherapy responses is emerging, and it is anticipated that a more precise understanding will be attained through advanced technologies that analyze the tumor immune landscape, such as single-cell RNA sequencing (scRNA-seq), spatial transcriptomics and multi-omics approaches.

With the help of scRNA-seq, the immunosuppressive microenvironment can be well characterized, and more insight into the interaction between the immune cells can be illustrated. For instance, He et al. utilized the multi-dimensional scRNA-seq to analyze samples from AFP-positive and AFP-negative HCC and adjacent normal tissues [332]. The sequencing results showed that APC-positive tumors are associated with multifaceted immune distortion, including T cell exhaustion and tumor-associated macrophages (TAMs) elevation. When looking further into details, SPP1⁺ TAMs are responsible for the T cell suppression. On the other hand, the CTCs are also regulating the immune microenvironment through intercellular signaling. CCL5 overexpression in CTCs can recruit regulatory T cells, which help the HCC tumor escape from immune response and metastasis [332]. Furthermore, scRNA-seq has offered insights demonstrating that patients showing PR or SD experienced immune shifts toward cytotoxic T cells. In contrast, patients with PD showed increased numbers of CD14⁺ and CD16⁺ monocytes and the upregulation of neutrophil-associated pathways [324]. These observations imply that cytotoxic T cell infiltration, increased activated CD8⁺ T cells during anti-PD-1 treatment, and the suppression of neutrophil-associated markers could be critical markers for predicting and enhancing response to anti-PD-1 therapy in HCC patients.

The microbiome, defined as the collective genomes of the microbiota within a host, is increasingly

acknowledged for its role in immune regulation and responses to various cancer treatments [333, 334]. Significantly, the gut microbiome and the liver interact bidirectionally through the gut-liver axis, enabling gut microbiota and their byproducts to both directly and indirectly impact gene expression in hepatocytes, tumor cells, and non-tumor cells, including immune cells [335–337]. A metagenomic sequencing research study revealed that fecal samples from immunotherapy responders exhibited higher taxa richness and gene counts [338]. It concludes that gut microbiome variations could significantly influence the responses of HCC patients to anti-PD-1 immunotherapy. Interestingly, a meta-analysis involving 1,056 HCC patients revealed that antibiotic-induced reduction in microbiota diversity does not affect OS in HCC patients on ICIs. However, more extensive and better-controlled studies are warranted [339]. A prospective clinical study explores the relationship between the gut microbiome and combined immunotherapy in patients with HCC [340]. Initial findings showed higher gut microbiome diversity in immunotherapy responders, but this difference faded by week 6 of treatment, while certain gut bacteria showed correlations with specific serum metabolites. The study suggests that gut microbiome biomarkers and serum metabolites could help identify HCC patients who would benefit from immunotherapy. Another study reported that antibiotic exposure around the time of treatment initiation has been associated with adverse prognostic implications in patients receiving AtezoBev [341].

In addition to the microbiome, gut metabolites have also been associated with immunotherapeutic responses. In a recent study investigating the connection between gut microbiota and metabolites in predicting outcomes for unresectable HCC patients undergoing treatment with ICIs, researchers discovered significant differences in fecal bacteria between patients with objective response (OR) and those with progressive disease (PD) before starting immunotherapy [342]. The study emphasizes the relationship between fecal microbiota, bile acids, and immunotherapy outcomes for HCC. It showcases the potential of gut microbiota and metabolites as biomarkers for predicting treatment outcomes in ICI-treated HCC patients. Research exploring the impact of the microbiome on immunotherapy responses in HCC patients is still in its early stages. The current consensus suggests that the microbiome modulates the immune environment and response. However, the complex mechanisms that drive this interaction and the potential association between diversity and sensitivity remain unexplored.

Candidate drug targets in HCC and recent clinical trials

Despite ongoing improvements in disease diagnostic platforms, it is crucial that treatment options concurrently evolve to enhance patient prognosis. Notably, conventional targeted drugs, such as sorafenib, Lenvatinib, and regorafenib, have been associated with the rapid development of drug resistance. To combat this, current research in systemic therapies for HCC primarily focuses on combination therapies supplemented with additional targeted inhibitors [343]. Research is exploring the inhibition of multiple molecular targets to boost therapeutic efficacy and longevity, potentially broadening the applicable patient demographic [344]. For example, CD147 is a glycoprotein reported to be highly expressed, and its presence enhances the malignancy of HCC [345, 346]. The signaling machinery involving CD147 is related to the tumor cell properties [346, 347], but efforts have been made to target CD147 using antibodies [348]. On the other hand, GC33 demonstrated anti-tumor activity in both animal models and phase I clinical trials, believed to regulate GPC3 expression in HCC, thus potentially benefiting HCC patients [349]. It was thought to control the GPC3 expression in HCC and has the potential clinical benefit to HCC patients. However, subsequent phase Ib and II studies revealed no clinical benefits within the HCC population, even with concurrent sorafenib treatment [350, 351]. In response to these findings, recent clinical trials have commenced investigations into combinatorial treatments involving other molecular inhibitors alongside targeted drugs. In one phase II trial (NCT01246986), TGF- β RI inhibitor LY2157299 was administered with sorafenib or ramucirumab, a VEGFA inhibitor [352]. The results indicated a correlation between lower TGF- β 1 levels and extended survival in HCC patients. Another avenue of exploration is the ICI AK104 (PD-1/CTLA-4 bispecific antibody), which, when combined with Lenvatinib as a first-line treatment for unresectable HCC patients in a Phase II study (NCT04444167), exhibited promising anti-tumor activity. No toxicities or unforeseen safety issues were reported, suggesting a promising direction for future HCC treatment strategies [353]. Additionally, apart from being the conventional HCC biomarkers, T-cell receptors specific for AFP peptide are also engineered to be tested in clinical trials [354–356]. However, the studies are still in the early stages, and there is yet to validate the safety and efficacy of this TCR-gene transfer therapy.

Indeed, AtezoBev has become a significant focus in HCC research. Numerous recent clinical trials across various phases are investigating the potential of AtezoBev for treating different sub-categories of HCC. In contrast to anti-PD-1 monotherapy, the combination

of atezolizumab and bevacizumab has shown promising responses in intrahepatic lesions, similar to those observed in extrahepatic lesions. This combination may potentially overcome the immune-tolerant hepatic microenvironment in patients with advanced HCC, offering a new avenue for treatment and improved outcomes. Ongoing clinical trials are also examining the application of AtezoBev in different contexts. For example, the Kirros trial (NCT06096779) investigates the impact of Bevacizumab within the AtezoBev combination on patients with advanced or metastatic HCC accompanied by cirrhosis. Moreover, the CCGLC-001 trial (NCT05713994) evaluates the efficacy of AtezoBev in HAIC. In contrast, the CCGLC-008 trial (NCT05717738) investigates the combined therapy of TACE, anti-VEGF antibodies or pan-target anti-angiogenic drugs, and anti-PD-1/PD-L1 antibodies for advanced HCC patients who are not suitable for radical treatment, resection, transplantation, or ablation. Examining AtezoBev in these clinical settings underscores its potential as a promising treatment option for HCC patients, expanding the range of therapeutic approaches and possibly improving patient outcomes. More recent completed and ongoing clinical trials testing on targeted therapies are listed in Table 3.

In conclusion, exploring combination therapies represents a promising future in advancing HCC treatment strategies. As research in this field continues to evolve, it holds the potential to substantially improve patient prognosis and redefine the therapeutic landscape for HCC.

Discussions and perspectives

As the prevalence of non-heritable risk factors for HCC continues to increase, it is anticipated that HCC will become more widespread in the coming decades. Despite advancements in targeted drug therapies, the predicted survival rate for HCC patients has not improved significantly. This presents a substantial burden for both the medical and social communities. It is not feasible to perform hepatic CT or MRI for every individual with CLDs or poor lifestyle habits. Consequently, there is an urgent need to identify easily quantifiable biomarkers for early detection or therapeutic indicators through liquid biopsy. This review highlights and discusses significant and recent research findings in studying HCC biomarkers.

As medical knowledge advances, the heterogeneity of HCC has become increasingly apparent to healthcare professionals. It is now widely understood that there is no universal cure for all cancers; instead, personalized medicine is the key to successful treatment. To design the most effective therapeutic regimen for each patient, it is essential to accurately identify their genetic alterations, tumor phenotypes, and immune microenvironment makeup. Ideally, comprehensive diagnostic and

Table 3 Recent clinical trials on targeted drugs from 2020

	Interventions	Trial Name/Identifier	Patient no.	Study Type	Primary Endpoint	Study Status	Ref.
1	SHR-1210 (Anti-PD-1), Apatinib	RESCUE / NCT03463876	190	Phase II	ORR: 34.3%, median PFS: 5.7 m	Completed	[357]
2	Pembrolizumab, MTL-CEBPA (C/EBPa small activating RNA)	TIMEPOINT / NCT04105335	108	Phase I	No AE reported, Anti-tumor activity in 26.7%	Completed	[358]
3	FGF401 (FGFR4 inhibitor), PDR001 (anti-PD1 IgG4 antibody)	NCT02325739	172	Phase I/II	DLT: 8.6%, TTP: 2.63 m (Phase I), ORR: 11.4%	Completed	[359]
4	TACE (Sorafenib)	NCT02504983	200	Phase IV	Time to CR: 14.4 m (TACE+Sora) vs. not reach (TACE only)	Completed	[360]
5	BLU-554 (FGFR4 inhibitor), CS1001 (Anti-PD-1)	NCT04194801	26	Phase Ib/II	ORR: 20%	Completed	[361]
6	Dovitinib	EU-CTR 2011-002445-36	25	Phase II	ORR: 48%	Completed	[362]
7	Tivozanib	NCT01835223	33	Phase Ib/II	PFS: 24 wk, OS: 9 m	Completed	[363]
8	LY2157299 (TGF- β RI inhibitor), Sorafenib, Ramucirumab	NCT01246986	204	Phase II	OS: 7.3–16.8 m TTP: 2.7–4.2 m	Completed	[364]
9	Cabozantinib	CELESTIAL / NCT01908426	707	Phase III	OS: 10.2 m vs. 8 m (placebo)	Completed	[365]
10	Nivolumab, BMS-813160 (CCR2/5-inhibitor), BMS-986253 (anti-IL-8)	NCT04123379	48	Phase II	STN: 8.3%	Completed	[366]
11	Donafenib, Tislelizumab	NCT06232759	56	Phase II	ORR: 80.4%, DCR: 90.2%	Completed	[367]
12	AK104 (Anti-PD-1/CTLA-4), Lenvatinib	NCT04444167	59	Phase II	ORR: 35.5–35.7%, DOR 13.6–13.67 m, PFS: 8.6–9.8 m, OS: 27.1 m	Completed	[353]
13	AtezoBev	NCT04862949	124	Observational	ORR: 29.8%	Completed	[368]
14	TACE: PD-1/PD-L1 inhibitors+ VEGF-TKI/bevacizumab	CHANCE2201 / NCT05332821	474	Observational	OS: 15.9–22.6 m, PFS: 7.4–9.9 m, ORR: 22.9–41.2%	Completed	[352]
15	Atezolizumab, Bevacizumab, UCPVax (anti-telomerase vaccine)	TERTIO / NCT05528952	105	Phase II	ORR	On-going	[369]
16	Chidamide (Histone deacetylase inhibitor), Regorafenib	NCT05770882	46	Phase Ib/II	AE, ORR, PFS	Ongoing	[370]
17	Cabozantinib	CaPture / NCT04767906	40	Phase II	TT	Ongoing	[371]
18	Anti-PD-1 antibody, Lenvatinib	NCT06311929	300	Phase IV	DFS	Ongoing	[372]
19	PTX-9908 (CXCR4 inhibitor)	NCT03812874	50	Phase I/II	AE, DLT	Ongoing	[373]
20	Budigalimab, Livmoniplimab, Lenvatinib, Sorafenib	LIVIGNO-1 / NCT05822752	120	Phase II	BOR	Ongoing	[374]
21	Serplulimab, Bevacizumab	NCT06370065	35	Phase II	ORR	Ongoing	[375]
22	TACE: PD-1/PD-L1 inhibitors+ VEGF-TKI/bevacizumab	CHANCE2202 / NCT05332496	220	Observational	PFS, OS	Ongoing	[376]
23	Zabadinostat (class I HDAC inhibitor), Geptanolimab, Lenvatinib, Sorafenib	NCT05873244	44	Phase II	PFS	Ongoing	[377]
24	ETN101 (mTKI)	NCT06326502	50	Phase I	DLT	Ongoing	[378]
25	Anlotinib hydrochloride, Penpulimab	NCT05862337	480	Phase III	RFS	Ongoing	[379]
26	Camrelizumab, Lenvatinib	NCT04443309	53	Phase I/II	ORR	Ongoing	[380]
27	Ablation: Lenvatinib+ anti-PD-1	NCT05803928	70	Phase I	RFS	Ongoing	[381]
28	Cryoablation: Tislelizumab, Lenvatinib	CASTLE-10 / NCT05897268	25	Phase II	ORR	Ongoing	[382]
29	TACE: Lenvatinib, iodion-125	NCT04967495	171	Phase I	OS	Ongoing	[383]

Table 3 (continued)

	Interventions	Trial Name/Identifier	Patient no.	Study Type	Primary Endpoint	Study Status	Ref.
30	SBRT: AtezoBev	NCT05488522	18	Phase I	DLT	Ongoing	[384]
31	Liver transplantation: Anti-PD-1 inhibitors	NCT05475613	59	Phase II	PFS	Ongoing	[385]
32	Tislelizumab, Sitravatinib	NCT05407519	40	Phase II	RFS	Ongoing	[386]
33	Penpulimab, TQB2618 (TIM-3 inhibitor), Anlotinib Hydrochloride	NCT05975645	40	Phase I	ORR	Ongoing	[387]
34	Tislelizumab, regorafenib	NCT04183088	125	Phase II	AE, ORR, PFS	Ongoing	[388]
35	Adebrelimab, Lenvatinib	HSBRT2401 / NCT06261125	60	Phase II	PFS	Ongoing	[389]
36	Lenvatinib, VIC1911 (Aurora kinase A inhibitor)	NCT05718882	30	Phase II	PFS	Ongoing	[390]
37	Pembrolizumab, Lenvatinib	PLENTY202001 / NCT04425226	192	Phase II	RFS	Ongoing	[391]
38	iNKT Cells, Anti-PD-1, Regorafenib	NCT05962450	84	Phase II	PFS	Ongoing	[392]
39	Oncorine (H101), Tislelizumab, Lenvatinib	NCT05675462	25	Phase I	DLT, MTD, AE	Ongoing	[393]
40	Durvalumab, Tremelimumab	NCT05809869	25	Phase II	BOR, RAE	Ongoing	[394]
41	Regorafenib, ICIs	NCT05573282	50	Observational	OS, PFS	Ongoing	[395]
42	Regorafenib, PD-1 inhibitor	NCT05048017	20	Phase II	PFS	Ongoing	[396]
43	TACE: TKIs	CHANCE-CHESS 2302 / NCT05704192	373	Observational	OS	Ongoing	[397]
44	SBRT: Durvalumab	NCT04913480	37	Phase II	PFS	Ongoing	[398]
45	AtezoBev	Kirros / NCT06096779	120	Phase II	AE	Ongoing	[399]
46	Ipilimumab, Pembrolizumab, Durvalumab, Idarubicin, Bevacizumab	NCT06482801	90	Phase II	AE, PFS, ORR, DOR	Ongoing	[400]
47	Palbociclib	NCT06478927	22	/	PFS	Ongoing	[401]
48	OTX-2002 (mRNA MYC inhibitor), TKIs, ICI	MYCHELANGELO I / NCT05497453	190	Phase I/II	DLT, MTD, AE, ORR, DOR	Ongoing	[402]
49	Atezolizumab, Cabozantinib, Lenvatinib	NCT05168163	122	Phase II	OS, PFS	Ongoing	[403]
50	Tegavivint (TBL1 inhibitor), Pembrolizumab	NCT05797805	108	Phase I/II	AE, DLT,	Ongoing	[404]
51	Toripalimab plus Lenvatinib	NCT04368078	76	Phase II	ORR	Ongoing	[405]
52	Donafenib + Envafohimab	NCT06498622	45	Phase II	RFS	Ongoing	[406]
53	TACE: Sintilimab (IBI308)	NCT04297280	25	Phase II	ORR	Ongoing	[407]
54	HAIC: Tremelimumab + Durvalumab, Lenvatinib	NCT06364007	20	Phase II	ORR	Ongoing	[408]
55	HAIC: Camrelizumab, TKIs	NCT05135364	48	Phase II	PFS	Ongoing	[409]
56	HAIC: AtezoBev, Bevacizumab Biosimilar IBI305 + Sintilimab	CCGLC-001 / NCT05713994	300	Observational	Number of Patients Amendable to Curative Surgical Interventions	Ongoing	[410]
57	Futibatitinib, Pembrolizumab	NCT04828486	25	Phase II	PFS	Ongoing	[411]
58	Lenvatinib	NCT05103904	19	Phase II	ORR	Ongoing	[412]
59	Atezolizumab, Bevacizumab, Durvalumab, Tremelimumab	REFINE-IO / NCT06117891	300	Observational	OS	Ongoing	[413]
60	TACE: Lenvatinib, Anti-PD-1 antibody, Bevacizumab Biosimilar IBI305 plus sintilimab, AtezoBev, apatinib plus camrelizumab, Sorafenib, Donafenib, Regorafenib	CCGLC-008 / NCT05717738	300	Observational	Number of Patients Amendable to Curative Surgical Interventions	Ongoing	[414]
61	Radiotherapy: TKIs, Anti-PD-1	NCT06061445	22	/	OS, ORR	Ongoing	[415]

Table 3 (continued)

	Interventions	Trial Name/Identifier	Patient no.	Study Type	Primary Endpoint	Study Status	Ref.
62	DNAJB1-PRKACA peptide vaccine, Nivolumab, Ipilimumab	NCT04248569	56	Phase I	AE, CD4/CD8 interferon production	Ongoing	[416]
63	HAIC: Lenvatinib, Tislelizumab, Toripalimab, Sintilimab, Camrelizumab	NCT06333561	300	Observational	OS	Ongoing	[417]
64	Durvalumab, Lenvatinib	Dulect2020-1 / NCT04443322	20	/	PFS, RFS	Ongoing	[418]
65	Trametinib (MEK inhibitor), Everolimus (mTOR inhibitor), Lenvatinib	NCT04803318	100	Phase II	AE	Ongoing	[419]
66	Bevacizumab, Atezolizumab, Tislelizumab, Toripalimab, Sintilimab, Camrelizumab	NCT06323382	240	Observational	PFS	Ongoing	[420]
67	Radiation: Durvalumab, Tremelimumab	NCT04430452	21	Phase II	ORR	Ongoing	[421]
68	Microspheres: Chemodrug, IL2, PD1, CTLA4, VEGF antibodies	NCT04770207	100	Phase II	AE	Ongoing	[422]
69	Tislelizumab	HESTIA / NCT05622071	50	Phase II	ORR	Ongoing	[423]
70	Q702 (TKI), Pembrolizumab	NCT05438420	120	Phase I/II	AE, Tumor response	Ongoing	[424]
71	Cabozantinib	NCT04204850	20	Phase II	DCR	Ongoing	[425]
73	Anlotinib hydrochloride, TQB2450 (anti-PD-L1)	NCT04888546	20	Phase I/II	pCR, ORR	Ongoing	[426]

Estimated numbers

AE Adverse Events, BOR Best Overall Response, DCR Disease Control Rate, DFS Disease-Free Survival, DLT Dose Limiting Toxicity, DOR Duration of remission, *m* month, MPR Major Pathologic Response, MTD Maximum Tolerated Dose, mTKI multiple Tyrosine Kinase Inhibitor, OSRR Organ-Specific Response Rate, pCR pathological Complete Response Rate, RAE Rate of Abscopal Effect, RFS Recurrence-Free Survival, STN Significant Tumor Necrosis, TT Time on Treatment, TTP Time to Progression

monitoring information could be obtained from a single blood sample, significantly reducing the need for invasive procedures such as tissue biopsy. This non-invasive approach would greatly benefit patients, eliminating unnecessary discomfort and potential complications associated with more invasive diagnostic methods.

This review does not intend to offer specific clinical recommendations to clinicians; instead, it aims to provide insights into the current research directions of biomarkers that may contribute to patients' well-being. By showcasing the latest findings in biomarker detection using liquid biopsy, we aim to promote a deeper understanding of these innovative techniques and their implications for the future of HCC diagnosis and treatment. Furthermore, continued research and collaboration in this field will be instrumental in advancing personalized medicine and ultimately improving patient outcomes. As our understanding of HCC biomarkers evolves, we anticipate that these advancements will significantly impact the way healthcare professionals approach the management of this complex disease.

Artificial intelligence (AI) is the most promising technological advance in the 21st century. AI and machine learning have been widely applied to nearly every aspect of R&D. In terms of the research in HCC, with improved

computing power, the deep learning models can integrate vast amounts of data, including patients' healthcare records, biochemical test results, imaging, biomarkers, and corresponding clinical outcomes, that are impossible to be analyzed as a whole before. Combining AI-predicted models and histochemical morphological analysis can accurately detect the disease and predict the prognosis and treatment response [427, 428]. Future directions will include validating the stability and interpretability of the AI-generated results and extending the database to a more extensive collection to generate general diagnostic standards.

In conclusion, identifying and validating reliable and easily quantifiable biomarkers for HCC are crucial in addressing the increasing burden of this disease. Using liquid biopsy techniques for detecting these biomarkers offers a non-invasive, cost-effective, and efficient alternative to traditional diagnostic methods. Moreover, understanding the complex interplay between genetic alterations, tumor phenotypes, and immune microenvironments will be essential for developing personalized treatment strategies to improve patient outcomes significantly. As research into HCC biomarkers continues to progress, it is imperative that we also investigate novel technologies and methodologies to enhance

the sensitivity and specificity of these diagnostic tools. This includes exploring the potential of combining multiple biomarkers to improve diagnostic accuracy or using advanced computational methods to analyze complex datasets to identify new biomarkers. By working together, we can overcome the challenges posed by HCC and significantly improve the lives of patients affected by this devastating disease.

Authors' contributions

Y.T.C. conceptualized the study, collected literature, prepared the figures and drafted the manuscript; C.Z. edited the manuscript and figures; J.W., P.L., L.X., H.Y., Y.F. edited the manuscript; Z.C.C. and N.W. conceived the idea, design the study, drafted the manuscript.

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Availability of data and materials

This published article and its supplementary information files include all data generated or analyzed during this study.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare no competing interests.

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