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Liquid biopsy - a review

Weronika Rutkowska-Kawalec
Tytus Chałubiński Regional Hospital in Zakopane
https://orcid.org/0009-0002-4283-6458
Karolina Michalczuk
V Wojskowy Szpital Kliniczny z Polikliniką w Krakowie
https://orcid.org/0000-0002-4427-9133
Dariusz Fabian
Specjalistyczny Szpital Wojewódzki w Ciechanowie
https://orcid.org/0009-0006-7241-7245
Marek Kurowski
Szpital MSWiA w Łodzi
https://orcid.org/0009-0008-2831-3312
Elżbieta Leszczyńska-Knaga
Wojewódzki Szpital Specjalistyczny MEGREZ w Tychach
https://orcid.org/0009-0007-5584-8614
Natalia Jakubczyk
Szpital MSWiA w Łodzi
https://orcid.org/0009-0008-3190-4658
Paweł Moczydłowski
Szpital Miejski Specjalistyczny im. G. Narutowicza w Krakowie
https://orcid.org/0009-0007-1555-2958
Monika Ryglewicz
Szpital Bielański Warszawa
https://orcid.org/0009-0006-1063-2265
Anna Gliwa

Uniwersytecki Szpital Kliniczny we Wrocławiu
https://orcid.org/0009-0006-0251-0591
Karolina Kuczapska
https://orcid.org/0000-0002-5921-1094

Abstract

Introduction and objective:
Hepatocellular carcinoma (HCC) is a leading cause of cancer-related deaths worldwide. Early detection is crucial for better outcomes. Traditional diagnostic methods, such as imaging and biopsies, often fail to detect HCC in early stages. Liquid biopsy, based on circulating free DNA (cfDNA) analysis, offers a promising, non-invasive approach, allowing frequent testing, addressing tumor heterogeneity, and reducing costs.

Review methods:
This article's databases were accessed through the WHO website, PubMed, and Google Scholar.

A brief description of the state of knowledge:
Early detection of HCC significantly improves survival. Biomarkers from cfDNA, including DNA fragment patterns, methylation markers (e.g., USP44), 5-hydroxymethylcytosine (5hmC), and digital PCR analysis, have shown potential in early-stage detection. Advanced cfDNA fragmentomics identifies tumor-specific DNA fragmentation patterns. Techniques like DELFI demonstrate high sensitivity (94%) and specificity (98%). Machine learning enhances cfDNA analysis by integrating multiple markers, improving accuracy in distinguishing cancerous from precancerous states. Combining methylation analysis with machine learning further addresses challenges of tumor heterogeneity.

Summary:
Studies highlight the high sensitivity and specificity of cfDNA biomarkers for HCC diagnosis, especially in high-risk groups like individuals with cirrhosis. Integrating technologies like 5hmC analysis and machine learning enables early diagnosis and treatment monitoring. These advancements represent a transformative step in cancer diagnostics, offering effective tools to improve patient outcomes.

Keywords

Hepatocellular carcinoma, early-stage HCC, liquid biopsy, diagnosis

INTRODUCTION

The most common form of primary liver malignancy is hepatocellular carcinoma (HCC), which develops in over 90% of cases in the context of chronic liver disease, with cirrhosis being the strongest risk factor [1]. HCC accounts for 80% of all liver cancer cases and is the third most common cause of cancer-related deaths worldwide [2]. Moreover, it ranks as the sixth most commonly diagnosed cancer. According to estimates, 841,000 new cases of HCC and 782,000 deaths related to this cancer were reported globally in 2018 [1] . By 2020, these numbers increased to 906,000 new cases and 830,000 deaths. Furthermore, in 2024, it is projected that 41,630 new liver cancer cases will be diagnosed in the United States alone, with 29,840 deaths expected [3].

In 2023, the North American Association of Central Cancer Registries estimated 41,210 new HCC cases in the United States, indicating a threefold increase in incidence over the past four decades, although this rise has stabilized since 2015 [4] . Notably, the incidence rate

among women has been increasing by 2% annually, while in men, it has remained stable since 2015. Mortality rates in women increased by 1% per year between 2017 and 2021, but decreased by 1% annually in men during the same period [2,3]. The global burden of liver cancer continues to rise, and the World Health Organization projects that more than 1 million people will die from liver cancer by 2030.

The primary risk factors for HCC include hepatitis B virus (HBV) or hepatitis C virus (HCV) infections, exposure to aflatoxin B1 (AFB1), excessive alcohol consumption, metabolic dysfunction-associated steatohepatitis (MASH), and demographic factors such as gender (men are at a higher risk of developing liver cancer than women, with a global male-to-female HCC incidence ratio of 2.8:1 [5]), race, and age [1]. Racial and geographic disparities in HCC incidence are also significant. For example, in Asia and Africa, the high prevalence of HCC is associated with HBV and HCV infections. In contrast, the incidence in the United States and Western Europe has risen over the past decade, driven by the maturation of the hepatitis C epidemic and the increasing role of non-alcoholic fatty liver disease (NAFLD) as a risk factor.

Identifying major predisposing conditions for HCC enables monitoring of high-risk groups through screening. Curative therapeutic options such as liver transplantation (LT) or surgical resection are only available in the early stages of the disease. However, recent years have witnessed significant advances in locoregional treatments and systemic therapies for advanced HCC. Approximately 80% of HCC cases occur in sub-Saharan Africa and East Asia, reflecting the high prevalence of chronic hepatitis B carriers in these regions [5,6].

As emphasized earlier, the incidence of HCC continues to rise. Without appropriate action, an additional 2.1 million cancer cases are expected by 2030. The growing burden of mortality and cancer incidence will persist for generations, with a projected 45 million additional cancer cases by 2050 [3]. Early detection of HCC through surveillance of high-risk populations, primarily using non-invasive methods such as ultrasonography or serum alpha-fetoprotein (AFP) assessment, can not only save lives but also yield positive socioeconomic impacts, particularly in countries with high HCC incidence [1]. Currently, due to tumor heterogeneity, AFP and DCP, which are commonly used in clinical practice, achieve only 41%-70% and 50%-80% diagnostic efficacy for HCC, respectively [7]. Another promising diagnostic and screening tool is liquid biopsy, which offers the potential to detect HCC at an early stage, enabling timely treatment and preventing disease progression.

Barcelona Clinic Liver Cancer (BCLC)

The BCLC system is widely used for staging hepatocellular carcinoma (HCC) and guiding treatment decisions. In this scale, four stages (A-D) are distinguished, and depending on the stage, an appropriate treatment strategy can be chosen. Basic information is showed in table1. Therefore, it is crucial to detect HCC as early as possible and apply appropriate treatment, ideally tailored to the individual patient [8].

Genomic landscape

In a healthy liver, approximately 30–40 somatic mutations occur annually due to genotoxic stress or replication errors. Hepatocytes are particularly vulnerable to mutagenesis in the context of chronic liver diseases and inflammation. Mutagenesis can involve cell proliferation induced by mitochondrial damage, oxidative stress, and endoplasmic reticulum stress, as well as viral DNA integration, such as in HBV-related HCC. HCV promotes hepatocellular carcinoma (HCC) primarily by inducing double-strand DNA breaks, leading to missense mutations. In contrast, somatic mutations in HCC associated with NAFLD, ALD, and exposure to toxins are typically caused by direct DNA damage due to chronic inflammation and reactive oxygen species [9].

HCC is a complex and heterogeneous disease that involves both genetic aberrations within cancer cells and changes in the tumor's immune microenvironment. Comprehensive multiomic profiling has revealed this diversity, which could facilitate the development of individualized therapeutic strategies and biomarkers [10].

In HCC, common genomic alterations include mutations in the promoter region of the TERT gene, Wnt pathway disruptions, mutations in the TP53 gene, ARID domain-containing genes, MYC oncogene activation, and deregulation of the TGFβ pathway. TERT mutations, which are the most frequent genetic changes in HCC, occur early in the disease and are associated with poorer relapse-free survival, suggesting their potential use in early detection and risk stratification [10].

Clinically significant mutations in HCC are related to the following pathways: tumor suppressor genes (TP53, ARID1/2, RB1, TSC1/2), the PI3K/Akt/MTOR pathway, the MYC pathway, the JAK/STAT pathway, telomerase (TERT/TERC), the WNT-beta catenin pathway (CTNNB1, AXIN1, AXIN2), and the MET pathway [10].

The TP53 gene, which is responsible for apoptosis and angiogenesis, is altered or inactivated in 30–50% of HCC cases. In aflatoxin B1-related HCC, TP53 mutations occur in 80% of cases, in 45% of HBV-related HCC, and in 13% of HCV-related HCC. TP53 mutations, especially in advanced stages of HCC, correlate with an aggressive disease phenotype, poor overall survival, and tumor recurrence. [10] However, recent studies on cfDNA mutations in HCC have shown low detection efficacy, with genes like TP53 and CTNNB1 detected in only 5% of cases. [11] This low detectability may be due to the low frequency of these mutations in circulating tumor DNA (ctDNA) or the presence of allele variants at very low levels in the bloodstream.

Mutations in ARID genes (found in 3–5% of HCC cases) are associated with advanced tumors with high vascularity. ARID1A and ARID1B genes, which are components of the SWI/SNF complexes involved in DNA repair by chromatin remodeling, frequently mutate in later stages of HCC, particularly in alcohol-related and HBV-related cases. ARID mutations are independent risk factors and are linked to increased biological complexity of the tumor, making it harder to treat [10,12]

Telomeres, which serve two key functions—providing a docking site for the DNA polymerase complex during replication and protecting chromosomes from degradation or fusion—shorten with each round of DNA replication. When telomeres shorten beyond a critical length, the telomerase complex is activated, leading to their elongation. A mutation in the TERT promoter is one of the most common genetic alterations in HCC, occurring in 30–60% of cases, and plays a role in the early stages of hepatocarcinogenesis [13].

A new genetic factor, TERT rs2242652:A, has been identified as associated with HCC development in the ARC (alcohol-related cirrhosis) population and further confirms the importance of PNPLA3 and TM6SF2 as risk factors for HCC in this group [14].

Histological features can also provide clues about genetic changes and oncogenic pathways, aiding in patient prognosis. For example, poorly differentiated, proliferative tumors often contain TP53 mutations, FGF19 amplifications, or activation of TGFβ, RAS/MAPK, and PI3K/AKT pathways, whereas non-proliferative tumors with well-differentiated phenotypes tend to have CTNNB1 mutations (encoding β-catenin), Wnt and JAK/STAT pathway activation, and show microtrabecular and pseudoglandular histological patterns with less immune infiltration [10,15].

Ultrasound in screening for HCC has a sensitivity of 63%, which is even lower (47%) in patients with cirrhosis. Recent advances in understanding the molecular characteristics of HCC, combined with the development of liquid biopsy technology, have enabled significant progress in detecting early-stage HCC and monitoring therapy using blood samples.

Although advanced single-cell sequencing technologies are crucial for understanding tumor biology, tissue samples obtained from serial biopsies rarely capture the full genomic complexity of the tumor and its metastatic sites. Therefore, liquid biopsy offers an advantage over tissue biopsy by capturing both intra- and inter-tumor heterogeneity, especially in the metastatic setting [7, 8]. Currently, cfDNA, of which ctDNA is a small fraction, is the most commonly studied analyte in liquid biopsies and is increasingly integrated into clinical practice.

Circulating free DNA (cfDNA) is a fraction of circulating nucleic acids originating from processes such as apoptosis or necrosis. Elevated cfDNA levels have been observed in cancer patients. ctDNA analysis provides a non-invasive method for initial diagnosis and monitoring treatment response, enabling the capture of tumor heterogeneity and resistance patterns. The concentration of cfDNA varies depending on the stage and type of cancer, reaching its highest values in patients with HCC. Thus, cfDNA analysis holds promise as a method for detecting HCC, reflecting tumor burden, metabolism, and apoptosis [16,17].

Significant epigenetic and genetic modifications have been observed in early preneoplastic liver tissues, likely driving carcinogenesis. Global hypomethylation and promoter CpG island hypermethylation are common in many cancers. In HCC, several well-established methylation biomarkers have been identified, including SEPT9, VIM, FBLN1, TFPI2, TGR5, HOXA1, EMX1, and others.

Epigenetic biomarkers are currently used in two commercial liquid biopsy tests for HCC: Oncoguard Liver (sensitivity 72%, specificity 88%) that measures methylation biomarkers for HCC (HOXA1, EMX1, TSPYL5) and AFP (alpha-fetoprotein), and elioLiver (sensitivity 85% for HCC at any stage, sensitivity 76% for early-stage HCC, specificity 91%), which measures cfDNA methylation levels in 28 genes (77 CpG sites) and three tumor protein markers (AFP, AFP-L3%, des-gamma-carboxy prothrombin) [16].

Liquid biopsy

Liquid “biopsy” technology, which permits the molecular interrogation of liquid samples (typically blood), has advanced with amazing pace, permitting its routine clinical application in patients with cancer, and fast expanding research capabilities that are unveiling the basis of malignant growth [18] . Liquid biopsies offer a technique for extracting tumor-derived information from body fluids and are minimally invasive. [19] Numerous body fluids, including blood, urine, cerebrospinal fluid, and pleural fluid, can be biopsied. Blood is the most widely utilized fluid [20]. Different biological matrices, including circulating tumor cells (CTCs), cell-free nucleic acids, exosomes, or tumor-derived platelets, are used to make the diagnosis [21].

Circulating tumor cells detection in Liquid Biopsy

CTCs, first observed in the 1860s, are shed by primary tumors, travel through the bloodstream, and contribute to metastasis, despite their rarity (about one CTC per million leukocytes). Their morphology varies by tumor type and stage, and they can form protective aggregates with fibroblasts or platelets to evade immune defenses and spread more effectively [22].

CTCs are valuable for real-time tumor monitoring, offering a less invasive alternative to tissue biopsies. They dynamically reflect tumor status, outperforming traditional blood biomarkers in accuracy and serving as indicators of treatment response—lower CTC levels correlate with improved survival, especially in breast cancer. Additionally, CTCs have shown promise in early cancer diagnosis, such as differentiating between benign and malignant pulmonary lesions, even in patients with lung disease [22].

Technologies like EPISPOT (EPithelialImmunoSPOT) and CellSearch system are used to detect viable circulating tumor cells (CTCs) for tumor analysis. The EPISPOT assay, which uses antibodies against EpCAM (CD326), can detect CTCs down to a single cell and expand them in vitro or in vivo, aiding in the study of protein secretomes in breast cancer. Similarly, the CellSearch system employs antibody-coated magnetic beads to isolate CTCs with epithelial markers like EpCAM, linking CTC counts to survival predictions in prostate cancer. However, CellSearch has limitations, as not all CTCs express EpCAM, and fixed CTCs cannot be cultured or analyzed further [22,23].

The AdnaTest, another immunomagnetic-based assay, enhances CTC detection by combining EpCAM-labeled magnetic beads with PCR to identify tumor-specific mRNA transcripts. For prostate cancer (PCa), it detects markers like PSA and PSMA and can identify tumor-specific splice variants such as AR-V7, a ligand-independent androgen receptor variant linked to aggressive disease, poor prognosis, and resistance to drugs like enzalutamide and abiraterone. Studies suggest AR-V7+ patients respond better to non-AR therapies like cabazitaxel. Additionally, CTCs isolated via CellSearch targeting markers such as estrogen receptor, BCL-2, EGFR2, and Ki67 contribute to the CTC-Endocrine Therapy Index, aiding endocrine therapy predictions in breast cancer [22,24].

Circulating tumor DNA in Liquid Biopsy

Underhill et.al. study identified tumor-specific mutations, such as in KRAS, in ctDNA from plasma. ctDNA, comprising 0.1–10% of circulating cell-free DNA (cfDNA), can transfer genetic material via apoptotic bodies, promoting metastasis. While normal cfDNA levels range from 10–100 ng/ml and can rise due to non-cancerous factors like inflammation or exercise, ctDNA levels correlate with tumor load, stage, and treatment response. Clinically, ctDNA analysis in precision medicine reveals its shorter length (20–50 base pairs) compared to general cfDNA, aiding in cancer detection and monitoring [22,25].

Challenges in ctDNA analysis stem from non-standardized methods for sample handling and preanalytical factors like clotting, DNA leakage, and freeze-thaw cycles, which impact reliability. Targeted approaches, such as droplet digital PCR (ddPCR) and BEAMing, detect specific mutations with high sensitivity, while methods like PARE identify tumor-specific rearrangements for disease monitoring. NGS-based tools like TAm-Seq and CAPP-Seq enable broader genomic screening, detecting low-frequency mutations and correlating ctDNA levels with tumor burden and treatment response. Untargeted approaches, including whole-genome sequencing, provide comprehensive tumor profiling, aiding precision oncology through the identification of copy number and chromosomal alterations [22,26,27].

Extracellular vesicles in Liquid Biopsy

Extracellular vesicles (EVs), small membrane-bound vesicles (30–100 nm) found in body fluids like plasma, urine, cerebrospinal fluid, and saliva, have emerged as critical candidates for liquid biopsies. Once thought to be cellular waste products, EVs are now recognized for their role in cell-to-cell communication, carrying diverse biomolecules such as DNA, RNA, and proteins. Tumor-derived EVs are particularly significant due to their roles in promoting tumor growth, metastasis, immunosuppression, and angiogenesis. Tumors shed EVs abundantly, and their high plasma levels in cancer patients, along with their cargo (tumor-derived DNA, mRNA, ncRNAs, and proteins), offer valuable insights into tumor monitoring, prognosis, and therapeutic response [22,28].

Various techniques effectively isolate extracellular vesicles (EVs) from cellular debris, leveraging their physical and biochemical properties. Preparative ultracentrifugation, the most common method, separates EVs by density, shape, and size, with variations like differential and isopycnic centrifugation improving purity. Size-based methods, including ultrafiltration

and size exclusion chromatography (SEC), separate EVs but may affect vesicle integrity; newer approaches like nanomembrane ultrafiltration and flow field-flow fractionation enhance isolation efficiency. SEC combined with ultracentrifugation improves EV yields for biomarker detection, such as in nephrotic diseases. Immunoaffinity-based methods, using antibodies targeting EV surface markers like CD63, achieve high sensitivity and purity, with techniques like magneto-immunocapture outperforming conventional methods. Polymer-based precipitation, employed in kits like ExoQuick PLUS, isolates EVs rapidly for downstream analyses, while lectin-based approaches target EV membrane oligosaccharides. Microfluidics devices such as ExoChip integrate EV capture and cargo analysis, using antibodies or ciliated micropillars to enrich EVs from small samples. Despite these advances, challenges in isolation efficiency, standardization, and exosomal cargo yield remain. Nevertheless, EV isolation technologies hold significant promise for tumor diagnosis, monitoring, and therapy as minimally invasive tools [22,29,30].

Other candidates for Liquid Biopsy

In addition to plasma and serum, other body fluids like saliva and urine are emerging as significant tools in liquid biopsy (LB). Saliva offers unmatched advantages in accessibility, non-invasiveness, and cost-effectiveness. Salivary diagnostics have shown promise in cancer detection and monitoring. For instance, EFIRM-based technologies have successfully detected EGFR mutations in non-small cell lung carcinoma (NSCLC) and salivary biomarkers like Foxp1 and Gng2 for pancreatic cancer. Non-genomic markers, such as increased salivary porphyrin levels or microbiome changes, have also been linked to oral cancers like OSCC [22,31].

Urine, being completely non-invasive and ideal for repeated sampling, is another strong candidate for LBs. Urine contains cell-free molecular components, such as DNA, RNA, and EVs, useful for monitoring tumors. It has demonstrated utility in both urological and non-urological cancers. Biomarkers like NMP22, PCA3, and lncRNAs (e.g., UCA1, MALAT1) are valuable in prostate and bladder cancer. Additionally, IQGAP3 and UBE2C have diagnostic relevance in bladder cancer, while circular RNAs like PRMT5 are linked to urothelial carcinoma. Urinary LBs also detect epigenetic changes, like DNA methylation in NSCLC, and mutations such as EGFR, comparable in sensitivity to tissue biopsies (~75%). They have shown potential in monitoring responses to drugs like Rociletinib and Osimertinib in NSCLC and tracking tumor recurrence in HCC with markers like TP53 249T and RASSF1A. Ongoing research and clinical trials are further expanding their utility [32].

OBJECTIVES

The aim of this article is to answer the following questions: What is liquid biopsy? What biomarkers can be used to detect HCC in its early stages?

MATERIAL AND METHODS

In this study, the criteria for selecting and excluding source publications have been accurately described, considering their limitations and/or qualities. These criteria were precisely defined to ensure transparency and credibility of the analysis. During the selection process, the following factors were considered: timeframe of publications (from 2021-2024), type of publications (peer-reviewed articles, reports, books, science websites), Language of publications (English and Polish), relevance to the research topic.

The methods of literature search have been thoroughly described, encompassing both electronic databases and journal reviews. There are the details of each method: searched databases: WHO website, PubMed, and Google Scholar platforms, keywords: [Hepatocellular carcinoma, Liquid biopsy, Early stages HCC, Biomarkers].

Table 1. Basic information

	STAGE 0	STAGE A	STAGE B	STAGE C	STAGE D
	very early stage	Early Stage	Intermediate Stage	Advanced Stage	End-Stage
Basic information	Single ≤2 cm • Preserved liver function*, Performance Status (PS) 0	Single tumor or up to three tumors (Single, or ≤3 nodules each ≤3 cm, PS, no vascular invasion, well-preserved liver function (Child-Pugh A)	Multiple tumors (Multinodular, PS 0), no vascular invasion, preserved liver function	Vascular invasion (portal invasion) or extrahepatic spread, regardless of the number of tumors or liver function, preserved liver function, PS 1-2	Severe liver dysfunction and/or systemic deterioration, Any tumor burden, end stage liver function, PS 3-4
1st Treatment option	Curative treatments like liver transplantation or resection	Surgical resection, liver transplantation, or ablation (e.g., TACE).	Transarterial chemoembolization (TACE), liver transplantation or systemic therapy.	Systemic treatments (e.g., sorafenib or other targeted therapies)	Supportive care, palliative treatments
Expected survival	> 5 years		> 2,5 years	> 2 years	3 months

RESULTS

In Chen et al. 2021 3204 people in all were successfully added to the study based on the exclusion criteria, and they were divided into training, validation, and test cohorts randomly. For the purpose of developing and assessing a diagnostic model, they thus obtained cell-free DNA (cfDNA) samples from 2250 patients with liver cirrhosis (LC), 508 patients with HCC, and 476 healthy controls (HC) from 13 hospitals spread across 11 Chinese provinces. These samples were then randomized to training, validation, and test cohorts. They obtained genomewide 5-hydroxymethylcytosine (5-hmc), nucleosome footprint (NF), 5′ end motif, and fragmentation profiles of cfDNAs from all enrolled patients using cutting-edge next-generation sequencing (NGS) technology. With the aid of logistic regression, they created a weighted diagnosis. With a sensitivity of 95.79% and a specificity of 95.00% for HCC in the validation set (95 HCC and 100 LC) and a sensitivity of 95.42% and a specificity of 97.83% in the test set (148 HCC and 1800 LC), the HIFI (5-Hydroxymethylcytosine/motif/fragmentation/nucleosome footprint) method demonstrated a strong diagnostic value in distinguishing HCC from LC. In comparison to AFP (validation: AUC = 0.845 [0.788–0.897], test: AUC = 0.826 [0.785–0.864]), the HIFI method's differentiation power for HCC vs. LC was higher (validation: AUC = 0.995 [0.990–1.000], test: AUC = 0.996 [0.992–0.999]). Furthermore, the HIFI approach demonstrated encouraging diagnostic results in distinguishing HCC from non-HCC (LC + CTRL) and CTRL. The HIFI approach was particularly effective in identifying very early-stage HCC (BCLC: 0, 88.9%) and early-stage HCC (BCLC: A, 94.4%/AJCC: I, 94.7%). The study by Chen et al. reported an overall sensitivity of 82.9% and a specificity of 97.0% for their genome-scale cfDNA profiling approach in detecting hepatocellular carcinoma in cirrhotic patients. These metrics reflect the diagnostic performance across all stages of HCC [33].

Another study Foda et al. 2023 is based on examination of 724 patients. As a method they used DELFI (DNA Evaluation of Fragments for Early Interceptio) which is Genome-

wide cfDNA Fragmentation Profiles by Low-coverage WGS (whole genome sequencing) and specific target were 473 nonoverlapping 5-Mb regions. In this context, they observed an overall sensitivity for detecting liver cancer, including early-stage disease, was 94%, and the specificity was 98% [34].

In study by Nguyen et al. 2023 from March 2019 to December 2021, 55 healthy volunteers (HC) and 55 patients with HCC verified by imaging diagnostic and histological examination were included in the discovery cohort at the National Cancer Hospital in Hanoi, Vietnam. The mechanism used in this study centered around analyzing the size and fragmentation patterns of cell-free DNA (cfDNA). Tumor-derived DNA fragments (ctDNA) tend to have distinct size profiles compared to non-tumor cfDNA. This characteristic was exploited through ultra-deep sequencing and bioinformatic analysis to identify and quantify ctDNA fragments more effectively. Such methodologies focus on detecting not only mutation-specific signals but also variations in fragment size, which serve as additional biomarkers for cancer. Study have found that sensitivity and specificity of diagnosing HCC by LB were 89% and 82%, respectively. However, it was discovered that patients with stage I and stage II HCC did not significantly differ in their cancer prediction scores [35][32].

The study by Kim et al.2022 demonstrated a blood-based approach for detecting HCC. The method focuses on analyzing methylation levels of the USP44 promoter region in circulating tumor DNA (ctDNA) collected LB method. They included 177 patients. This cohort consisted of individuals with early-stage HCC, cirrhosis, and healthy controls. For early-stage HCC (BCLC stage A), the sensitivity of the MS-HRM assay was reported at **40%**, which improved to **57%** when combined with the alpha-fetoprotein (AFP) test. For all HCC stages, the combined sensitivity increased to **76%**, with a specificity of **95%** when distinguishing HCC patients from healthy individuals. This approach highlights the potential of methylation-specific assays in enhancing early HCC detection, especially when paired with other diagnostic tools [36].

In the study by Cai et al. (2021), the researchers used a liquid biopsy approach combining 5-hydroxymethylcytosine (5hmC) signatures from plasma cell-free DNA (cfDNA) and protein biomarkers for diagnosing (HCC). 413 participants took part. This group included patients diagnosed with HCC, individuals with liver cirrhosis (LC), and healthy controls. The 5hmC profiles were identified through next-generation sequencing (NGS) following specific library preparation techniques. These profiles were integrated with serum protein biomarkers like alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) into a diagnostic model using statistical methods such as sparse Partial Least Squares Discriminant Analysis (sPLS-DA). The combined diagnostic model achieved an overall sensitivity of 94.7% and specificity of 92.8% in distinguishing HCC patients from controls, demonstrating its high diagnostic accuracy for HCC detection in a non-invasive manner [37].

The Wang et al. 2021 study involved patients with liver-related conditions, including 97 individuals with HCC and 46 with chronic hepatitis B/C virus infection (HBV/HCV), along with 80 healthy controls (HC). To extract cfDNA, blood samples were collected. The degree of DNA methylation was measured using a ddPCR platform. The cfDNA levels in the HCC group were significantly higher compared to the healthy control group. For HCC detection, using a cfDNA methylation ratio cut-off of 15.7%, the sensitivity was 78.57% and the specificity was 89.38%. The diagnostic accuracy was found to be 85.27%. The positive likelihood ratio for HCC diagnosis at the 15.7% methylation threshold was 7.40, while the negative likelihood ratio was 0.24 [38].

The Lee et al. 2022 study involved 152 HCC patients and 97 individuals in the control group (non-cancer, NC), including 43 patients with liver cirrhosis (LC), 24 with alcoholic liver hepatitis (LA), and 30 healthy controls (HC). The effectiveness of cfDHCC, an

integrated biomarker based on cfDNA, as a diagnostic tool for HCC was assessed. The study also explored cfDNA's ability to evaluate key pathological features of HCC, such as stage, lymphovascular invasion (LVI), tumor size, and multifocality. cfDNA was the only biomarker that effectively classified patients based on their modified International Union Against Cancer (UICC) stage. A machine learning model was used to evaluate the performance of the cfDNA-based biomarker. The sensitivity was 85.7%, and the specificity was 89.4%, both significantly enhancing the accuracy of HCC diagnosis using cfDNA as a biomarker compared to traditional serum biomarkers. Specifically, the cfDHCC score outperformed individual biomarkers like AFP and other serum enzymes in distinguishing HCC patients and demonstrated superior accuracy in determining UICC stages, detecting multifocality and LVI, and estimating tumor size compared to plasma cfDNA or cfAFP-DNA alone. Additionally, cfDHCC more accurately predicted HCC recurrence and survival outcomes than the individual biomarkers assessed in this study [39].

All studies are compered in Table 2.

Table 2. All studies

Study	Method	Patients	Sensitivity	Specificity	Biomarker
Chen et al. 2021	NGS, Genome-wide profiling of circulating cell-free DNA; HIFI methods	2250 (LC), 508 HCC, and 476 (HC)	82.9%	97.0 %	Circulating cell-free DNA (cfDNA)
Foda et al. 2023	DELFI	724	94%	98%	cell-free DNA (cfDNA)
Nguyen et al. 2023	fragment length profiles	55 HC and 55 HCC	89%	82%	length of cfDNA fragments
Kim et al.2022	MS-HRM	177	76%	95%	USP44 promoter methylation
Cai et al. 2021	sPLS-DA	413	94.7%	92.8 %	5-hydroxymethylcytosine (5hmC) signatures in plasma cfDNA. Protein biomarkers in plasma (including AFP or others).
Wang et al. 2021	ddPCR	97 HCC and 46 HBV/HCV, 80 HC	78.57%	89.38%.	Methylation changes in circulating tumor DNA (ctDNA)
Lee et al. 2022	machine learning model	52 HCC, 97 NC, including 43 LC, 24 LA, and 30 HC	85.7%,	89.4%	Cell-free DNA (cfDNA), cfAFP-DNA, mutations, methylation patterns, and other molecular alterations

DELFI- DNA Evaluation of Fragments for Early Interception, ddPCR - Digital droplet PCR platform, sPLS-DA - sparse Partial Least Squares Discriminant Analysis, MS-HRM - Methylation-sensitive high-resolution melting, NGS- next-generation sequencing, LC – patients with liver cirrhosis HCC- patient with HCC, HC- health cohorts, HBV/HCV - with chronic hepatitis B/C virus infection, LA - patients with alcoholic liver hepatitis

DISCUSION

Hepatocellular carcinoma (HCC) is becoming an increasingly serious health threat, not only in regions with high exposure to HCV/HBV viruses but also in developed countries where lifestyle-related diseases like obesity and hypertension are on the rise. This trend is contributing to the growing prevalence of non-alcoholic fatty liver disease, a key risk factor for HCC. Consequently, there is an urgent need for more effective screening methods. Early detection and accurate prognostic assessment are critical for managing HCC, including the monitoring of high-risk patients and optimizing treatment. However, this necessitates long-term, ongoing monitoring.

In comparison with traditional tissue biopsies and imaging techniques, liquid biopsy, which involves blood samples, is a non-invasive procedure that can be performed more frequently and is cost-effective for routine clinical monitoring. This could lead to improved patient adherence to medical recommendations. Existing screening methods such as AFP, DCP, and ultrasound imaging have limited specificity and sensitivity, making them insufficient for reliable clinical application. Traditional tissue biopsy, which provides only a snapshot of a small tumor fragment at a specific moment, does not fully capture the tumor's heterogeneity. Thus, a significant challenge in HCC treatment is the absence of biomarkers that offer clinically reliable diagnosis and prognosis.

In response to this challenge, efforts have been made to identify effective biomarkers for use in liquid biopsy, with cfDNA showing promise. Liquid biopsy using cfDNA analysis can identify mutations or other molecular changes, track treatment responses in real-time, and guide drug selection and dosage for HCC, offering high sensitivity. Furthermore, modern liquid biopsy technologies enable early detection and treatment monitoring using blood samples, providing an alternative to traditional tissue biopsies. Liquid biopsy allows for repeatable, minimally invasive sample collection, making it possible to track the molecular features of the tumor.

Overall, liquid biopsy is a promising, non-invasive method that overcomes the tumor's heterogeneity and allows for real-time monitoring of disease progression, recurrence, and treatment response. The goal of biomarkers used in liquid biopsy is to identify patients who require specialized monitoring and personalized therapy and to assess the value of these tests in clinical decision-making.

This article reviews studies on various biomarkers that can be used in HCC screening to detect the disease in its early stages. Some of these biomarkers can also function as non-invasive systems for diagnosing, predicting, and monitoring the progression of HCC. In the study by Chen et al. (2021), circulating cell-free DNA (cfDNA) exhibited sensitivity 82.9% and a specificity of 97.0%, indicating that the HIFI method holds significant potential as a new strategy for overall diagnosis of HCC. Another study by Lee et al. (2022) found variable expression levels of oncogenes and tumor suppressor genes related to HCC, such as TP53, TERT, and ARID1A, to aid in early detection and monitoring of HCC prognosis. This research utilized a highly sensitive cfDNA detection test using a machine learning-based algorithm to evaluate AFP expression from captured cfDNA (cfAFP-DNA).

Additionally, cfDNA methylation analysis appears to be a valuable biomarker in liquid biopsy, as it provides a reliable and effective method for early diagnosis of HCC. Studies by Kim et al. (2022) and Wang et al. (2021) demonstrated sensitivity of 76% and 78.57%, respectively, and specificity of 95% and 89.38%, respectively, using cfDNA methylation. Combining cfDNA fragment length with other features associated with tumor-derived DNA appears to be an effective strategy to overcome the limitations of using mutations as the only biomarkers for detecting ctDNA and improving the accuracy of early HCC screening, as demonstrated in the study by Nguyen et al. (2023), which achieved sensitivity of 89% and specificity of 82%. Another study by Foda et al. (2023) highlighted the efficiency and cost-effectiveness of cfDNA fragmentomic analysis in detecting HCC, suggesting that this method could become an accessible screening test for HCC, potentially improving screening rates beyond current low levels.

An intriguing aspect of cfDNA analysis specific to HCC is that liver transplantation is the most effective treatment for early and intermediate-stage HCC. Liquid biopsy monitoring of post-transplant patients could play a dual role in tracking recurrences and rejections, with promising results observed in cfDNA studies of these patients.

In summary, liquid biopsy using ctDNA holds strong diagnostic and prognostic potential and could be clinically utilized as a reliable tool for detecting early-stage HCC, aiding therapeutic decision-making, and improving overall survival outcomes.

However, researchers agree that further large-scale clinical studies are necessary to fully confirm the clinical utility and scalability of these liquid biopsy techniques in the routine screening and treatment of HCC. [33].

CONCLUSION

Recent advances in liquid biopsy techniques have shown promising potential in improving the early detection, diagnosis, and prognosis of hepatocellular carcinoma (HCC). The combination of various molecular biomarkers, including cell-free DNA (cfDNA) and its modifications, along with protein biomarkers, offers a non-invasive, cost-effective, and highly sensitive approach for monitoring HCC, especially in its early stages. Studies on cfDNA fragmentomics, DNA methylation, and 5-hydroxymethylcytosine (5hmC) signatures have demonstrated their ability to detect subtle molecular changes associated with HCC that may be missed by traditional diagnostic methods such as imaging or biopsy.

Key achievements, such as the use of USP44 methylation analysis through methylation-sensitive high-resolution melting (MS-HRM) and digital PCR for ctDNA methylation analysis, have shown great potential in detecting hepatocellular carcinoma at an early stage, offering high specificity and sensitivity. The integration of machine learning algorithms with cfDNA biomarkers further enhances diagnostic and prognostic accuracy, overcoming challenges related to tumor heterogeneity and the limitations of single biomarkers.

Profiling circulating cfDNA signatures, particularly in patients with liver cirrhosis, is becoming a valuable tool for the early detection of HCC. These biomarkers not only improve diagnostic accuracy but also provide insights into the molecular landscape of the tumor, which can aid in guiding therapeutic decisions and monitoring treatment response. Moreover, the combination of multiple biomarkers, such as in studies involving 5hmC signatures and protein biomarkers, offers a more comprehensive diagnostic approach, increasing sensitivity and reducing the risk of false-negative results.

In conclusion, the integration of liquid biopsy platforms utilizing cfDNA and associated biomarkers has the potential to revolutionize clinical management of HCC. These methods can provide real-time dynamic information on disease progression, recurrence, and treatment efficacy, making them crucial for improving early detection and developing personalized

treatment strategies for HCC patients. However, further large-scale clinical studies are required to fully validate the clinical utility of liquid biopsy techniques in HCC diagnosis.

AUTHOR'S CONTRIBUTION

Conceptualization: KM, WR-K, DF
Methodology: MK, KM
Software: GB, AN
Check: EL-K, NJ
Formal analysis: WR-K, PM, KM
Investigation: AG, MR, MK
Resources: MK, MR
Data curation: WR-K, NJ
Writing-rough preparation: MK, PM, KM
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