Liquid biopsy for early detection and therapeutic monitoring of hepatocellular carcinoma

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Advances in our knowledge of the molecular characteristics of hepatocellular carcinoma (HCC) have enabled significant progress in the detection and therapeutic prediction of HCC. As a non-invasive alternative to tissue biopsy, liquid biopsy examines circulating cellular components such as exosomes, nucleic acids, and cell-free DNA found in body fluids (e.g., urine, saliva, ascites, and pleural effusions) and provides information about tumor characteristics. Technical advances in liquid biopsy have led to the increasing adoption of diagnostic and monitoring applications for HCC. This review summarizes the various analytes, ongoing clinical trials, and case studies of United States Food and Drug Administration-approved in vitro diagnostic applications for liquid biopsy, and provides insight into its implementation in managing HCC. (2022 Sep 21 [online ahead of print])

Keywords: Hepatocellular carcinoma; Liquid biopsy; Cell-free nucleic acids; Early diagnosis; Therapeutic drug monitoring

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common liver cancer and the fourth leading cause of cancer-related deaths worldwide in 2020. The 5-year survival rate of patients with HCC is 15%, making it the second most lethal tumor after pancreatic cancer. In South Korea, the incidence of HCC was 21.2 per 100,000 person-years (crude incidence) and 13.9 per 100,000 person-years (age-standardized incidence) in 2018. Moreover, its mortality rate in 2019 was 20.6%, which is the second highest mortality rate among the most common cancers in South Korea in 2021.

The etiologies of HCC include hepatitis B virus (HBV)/hepatitis C virus (HCV) infection, non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis, and alcohol abuse. Each year, HCC reportedly affects 2-7% of patients with active HCV- or HBV-related cirrhosis worldwide. NAFLD has recently become a leading cause of HCC and is predicted to affect more than 10 million adults in the United States by 2030. The prevalence of NAFLD is approximately 25%, and the annual incidence of HCC among patients with NAFLD was 1.06% in a large cohort study conducted in the United States. Based on the evidence described above, the Clinical Practice Guidelines of the Korean Association for the Study of the Liver recommend that high-risk patients undergo biennial HCC screening by ultrasound imaging, with or without alpha-fetoprotein (AFP) levels. In a long-term follow-up study on the efficacy of HCC screening, the survival rate was better in patients who underwent regular
assessment than in those who did not. However, ultrasound screening for HCC demonstrated a sensitivity of 63%, which was even lower (47%) than that for patients with cirrhosis.

Advances in our knowledge of the molecular characteristics of HCC, combined with the development of new liquid biopsy technologies, have enabled significant progress in the early detection and therapeutic monitoring of HCC using blood. As a non-invasive alternative to tissue biopsy, liquid biopsy provides information about tumor characteristics through assays involving body fluids. Liquid biopsy has advantages over traditional tissue biopsy: sample collection is minimally invasive and liquid biopsy also allows repeated sampling, which enables real-time monitoring of the molecular characteristics. Moreover, tissue biopsy is affected by intra-tumor heterogeneity, whereas liquid biopsy can provide a heterogeneous molecular profile.

Circulating cellular components, such as exosomes, nucleic acids, cell-free DNA (cfDNA), cell-free RNA (cfRNA), and
cancer tumor cells (CTCs), found in body fluids (e.g., urine, saliva, ascites, and pleural effusions) of cancer patients are used for tumor detection and monitoring. Exosomes are extracellular vesicles released from cells and are known to carry bioactive molecules that facilitate cancer growth. CTCs are cancer cells that are detached from the primary tumor and are known for their role in metastasis. cfDNA and ctDNA are DNA fragments that exist in the naked form during cellular processes such as necrosis and apoptosis. Similarly, cfRNA is shed into the blood from both cancerous and non-cancerous cells (Fig. 1).

Current liquid biopsy applications include early diagnosis, minimal residual disease detection, therapeutic selection guidance, and prognostic prediction. The most common application for liquid biopsy is companion diagnostics, which is a test that provides the applicability of a therapeutic drug to a specific person (Fig. 2). The United States Food and Drug Administration (FDA)-approved liquid biopsy tests include CancerSEEK, Guardant360 CDx, FoundationOne Liquid CDx, Cologuard, and EpiColon. CancerSEEK detects mutations in 16 cancer-related genes and eight proteins to identify the presence of early stage cancers. Whereas CancerSEEK is designed to detect multiple cancers, Cologuard and EpiColon specifically detect colorectal cancer (CRC). Guardant360 CDx and FoundationOne Liquid CDx are companion diagnostic tests that detect panels of mutations and can be used to guide treatment. Guardant360 CDx is designed to identify mutations in non-small cell lung cancer. In contrast, FoundationOne Liquid CDx detects a high blood tumor mutational burden, high microsatellite instability, and tumor fraction values in solid tumors.

Commercially available approaches to liquid biopsy, in either LDT or FDA-approved kit, are used in current clinical practice for tumor tests and tests for circulating tumor cells or DNA. This review focuses on ctDNA and cfDNA testing with a brief description of other approaches. The biomarkers used in ctDNA tests include DNA mutations, copy number alterations, transcriptome signatures, proteins, DNA methylation, and metabolic abnormalities. This review describes the modalities of liquid biopsies as well as their applications in the diagnosis of HCC and monitoring of therapeutic efficacy in affected patients.

**BIOMARKERS FOR LIQUID BIOPY**

cfDNA is a fractionated portion of circulating nucleic acids (150-200 base pairs) that originates from various cellular events (e.g., apoptosis, necrosis, pyroptosis, and autophagy). cfDNA was first discovered by Mandel and Metais in 1948. Subsequently, Leon et al. revealed that elevated levels of cfDNA were present in the serum of patients with cancer. The cfDNA concentration in patients with cancer varies widely from 0 ng/mL to >1,000 ng/mL, whereas the mean concentration is 30 ng/mL in healthy individuals. Concordantly, higher cfDNA concentrations have been observed in patients with advanced cancer than in those with early stage cancer. Notably, cfDNA concentrations correlated with tumor size. cfDNA concentrations vary according to the cancer type. Patients with liver cancer were shown to have the highest cfDNA levels among various cancers, suggesting that HCC is an appropriate type of cancer for detection via cfDNA analysis. As the amount of cfDNA in the blood reflects complex tumor biology (e.g., tumor burden, tumor metabolism, and apoptosis) and non-cancerous blood cell death associated with high inflammation, identifying the cfDNA source is essential for accurately assessing cancer. The characteristics of ctDNAs have been extensively studied to improve the identification of tumor-derived cfDNAs. ctDNA constitutes 0.01-90% of cfDNA and has a half-life of 1-2.4 hours. The length of ctDNA is 50-150 bp, which is shorter than that of most cfDNAs. The presence of ctDNA in cfDNA can be identified through assays that detect tumor-related biomarkers with high specificity. The concentration, integrity, mutations, and methylation of cfDNA are among the tumor-related biomarkers that can be measured using liquid biopsy. These tumor biomarkers are detected by various means, including droplet digital polymerase chain reaction (PCR), quantitative PCR (qPCR), whole-genome sequencing, whole-exome sequencing, targeted sequencing, and methylation-sensitive high-resolution melting analyses. Although each of these technologies has unique advantages and limitations, a common requirement for liquid biopsy is
the ability to detect low levels of ctDNA within cfDNA (usually with a variant allele frequency <1%).

1. Genomic alterations

Genomic alterations such as single-nucleotide variations, copy number variations, fragmentation, and viral integration have been used as diagnostic biomarkers. Analytical platforms for mutation detection include qPCR, targeted sequencing, whole-genome sequencing, and whole-exome sequencing. qPCR and targeted sequencing are limited to known or designed mutations; thus, they may not detect clonal evolution involving mutations absent from the assay design. Whole-genome sequencing and whole-exome sequencing can be expanded to cover unknown mutations; however, they are costly and require manual review for variant analysis. The standard approach in clinical practice involves the exploration of mutations using droplet digital PCR or targeted sequencing. The advantages of gene-based genomic alteration biomarkers include the detection of mutant allele fractions ≥0.001%, provision of druggable information, and facilitation of real-time patient monitoring. A notable challenge in mutation-based ctDNA detection is that genetic alterations primarily occur in the late stages of cancer. Consequently, early detection of cancer using mutations generally has low sensitivity. In addition, mutations do not provide information concerning the tissue of origin. In addition to mutations, various genomic events such as genomic rearrangements, mutational signatures, and copy number changes can be used as liquid biopsy biomarkers. The DELFI method (i.e., DNA evaluation of fragments for early interception) can detect abnormalities in cfDNA through the analysis of fragmentation patterns. Compared to non-cancer cfDNA, cancer cfDNA has a higher fragmentation profile. In addition, fragmentation analyses provide tissue-specific information required for diagnosis using blood. However, the main applications of cfDNA mutations are to guide clinical treatment, monitor therapeutic responses, and predict the risk of getting cancer. For example, HCC patients with RAS mutations have a higher clinical response to refametinib combined with sorafenib. Another example is oncogenic mutation of the Wnt/-catenin (CTNNB1) pathway. -catenin mutations are associated with T cell rejection and immunotherapy resistance, suggesting adverse outcomes in patients treated with immune checkpoint inhibitors.

2. Epigenetic markers

Significant epigenetic and genetic modifications have been observed in early preneoplastic liver tissues, which are presumed to drive tumorigenesis. Global hypomethylation and hypermethylation of promoters and CpG islands are common phenomena in diverse cancers. Well-established HCC methylation biomarkers include septin 9 (SEPT9), vimentin (VIM), fibulin 1 (FBLN1), tissue factor pathway inhibitor 2 (TFPI2), G protein-coupled bile acid receptor 1 (TGR5), homeobox A1 (HOXA1), empty spiral homeobox 1 (EMX1), TSPY-like 5 (TSPYL5), metallothionein 1M (MT1M), and metallothionein 1G (MT1G).

Currently, epigenetic biomarkers are used in two commercialized liquid biopsy products for HCC, Oncoguard Liver (Current Procedural Terminology Test Code: 81599) and HelioLiver (Current Procedural Terminology Test Code: 16222). Oncoguard Liver uses an algorithm based on multiple markers, such as sex, age, HCC methylation (HOXA1, EMX1, and TSPYL5), and AFP. During clinical validation for detecting early stage HCC, Oncoguard Liver demonstrated a sensitivity of 72% and a specificity of 88% in a study population that included 159 HCC samples and 250 control samples. HelioLiver uses a pre-specified diagnostic algorithm that measures cfDNA methylation levels in 28 genes (77 CpG sites), three protein tumor markers (AFP, AFP-L3%, and des-gamma-carboxy prothrombin), and patient demographic characteristics (age and sex). HelioLiver demonstrated sensitivities of 85% for HCC at any stage and 76% for early stage HCC, with a specificity of 91% in a study population that included 122 HCC samples and 125 control samples.

APPLICATIONS OF cfDNA/ctDNA IN THERAPEUTIC MONITORING

Several studies have monitored cfDNA concentration in relation to treatment outcomes. In a previous study, cfDNA
was quantified on admission and after treatment. Of the 48 patients, 17 were controlled without complications, 16 experienced recurrence, and 15 developed post-operative complications. The investigators measured the copy numbers of beta-2-microglobulin (B2M) and peptidyl-prolyl cis-trans isomerase A (PPIA) in cfDNA. Before surgery, the cfDNA concentrations did not differ between the groups; after surgery, the PPIA copy number was higher among patients who developed complications. Analysis of resectable liver metastases of CRC revealed that the presence of postoperative ctDNA in RAS mutant–positive patients is significantly associated with a lower recurrence-free survival rate. Similarly, a study monitored changes in cfDNA, ctDNA (telomerase reverse transcriptase [TERT] mutations), and AFP before (D-1) and after (D+2 and M+1) transarterial chemoembolization (TACE) treatment. Treatment responders showed a significant increase in the baseline and post-treatment (M+1) concentrations of cfDNA and ctDNA, whereas the AFP level did not correctly predict tumor response. Moreover, the mutation burden of ctDNA and cfDNA in patients with recurrence is higher.

An investigator-initiated phase 2 study of pembrolizumab immunological response evaluation (INSPIRE) evaluated the genomic and immunological landscape of peripheral blood from solid tumors after pembrolizumab treatment. The results showed that genomic features from the blood, including fold changes in the numbers of CD4+ T cells and Ki67+ programmed cell death protein 1 (PD-1)+CD8+ T cells, were significantly correlated with the clinical response to pembrolizumab.

APPLICATIONS OF ctDNA/cfDNA IN THE MULTI-CANCER EARLY DETECTION (MCED)

Another application of liquid biopsy is the detection of multiple cancers with a single test that can provide information concerning the tissue of origin. Some obstacles to this application include the potential for inaccurate tissue-of-origin predictions owing to alterations not exclusive to any specific cancer. The analysis of epigenetic changes is superior to the study of genetic changes in predicting tumor tissues of origin. CancerSEEK is an FDA-approved in vitro diagnostic test that examines 16 gene-based and eight protein-based biomarkers for ovarian, stomach, liver, esophageal, pancreatic, colorectal, breast, and lung cancers. Clinical validation studies have demonstrated 33-98% sensitivity, with a specificity of 99% and a median tissue of origin accuracy of 63.

Galleri is a pan-cancer test for >50 types of cancer developed by Grail, LLC. Grail enrolled >134,000 participants in the Galleri. In a circulating cell-free genome atlas study, the Galleri test detected signals from 50 types of cancer with a specificity of 99.5% and tissue of origin accuracy of 89%; sensitivity varied according to the type of cancer, ranging from 11.2% to 93.5%. Galleri has not acquired FDA approval and has been commercialized as a laboratory-developed test. Grail’s laboratory is certified by the Clinical Laboratory Improvement Amendments of 1988. The extensive clinical research conducted by Grail aimed to prove clinical effectiveness by separating clinical designs according to the population with a long follow-up period and the clinical utility of Galleri for screening for multiple cancers.

cfRNA AND EXOSOMES FOR EARLY DIAGNOSIS OF HCC

Other analytes for liquid biopsy include cfRNA, exosomes, and circulating tumor cells. Various types of cfRNAs are present in the blood, including messenger RNA (mRNA), microRNA (miRNA), long noncoding RNA (lncRNA), piwi-interacting RNA, and transfer RNA. cfRNA is produced during necrotic and apoptotic processes and is then released into the bloodstream. Notably, analyses of cfRNA can correctly identify the tumor tissue of origin using cell-type decomposition or cell-type-specific RNA markers. Transcriptome-wide characterization of cfRNAs in cancer has identified mRNAs specific to lung and breast cancers. Analysis of cfRNA can improve the detection rate in patients with low levels of ctDNA and allow for the prediction of tumor tissue of origin and the identification of cancer subtypes. MicroRNAs (e.g., miR-1, miR-122, miR-21, miR-26a, miR-29a, miR-155, miR-96, and miR-99a) have been associated
with survival in patients with HCC.\textsuperscript{64-66}

Exosomes are extracellular vesicles with phospholipid bilayers 30-150 nm in size. Since the identification of exosomes in the late 1980s, they have been shown to carry diverse cargoes, including long noncoding RNA, mRNAs, and proteins. In addition to their diverse cargo transport abilities, exosomes have good stability in all body fluids, low immunogenicity, and biocompatibility. Thus, they have attracted attention for use in various applications (e.g., liquid biopsy and drug delivery).\textsuperscript{67} Exosome targets in HCC include proteins, mRNA, miRNAs, lncRNAs, circular RNA, and DNA.\textsuperscript{68} Exosome levels have been correlated with tumor size and aggressiveness,\textsuperscript{69,70} and exosomes have been reported to transport signaling molecules involved in angiogenesis and tumorigenesis. In a currently available diagnostic test, the combined analysis of extracellular vesicle mRNA showed a sensitivity of 94% and specificity of 75%.\textsuperscript{71} On the other hand, combined analyses of extracellular vesicle miRNA and AFP showed a sensitivity of 86% and specificity of 88%.\textsuperscript{72} Currently, cfRNA and exosome analyses have not yet been implemented in clinical practice and require assay standardization and appropriate guidelines for in vitro diagnostic governance.


circulating tumor cells (CTCs) and early diagnosis and monitoring of HCC

CTCs are defined as tumor cells from primary or metastasized tumors that leave the site of origin to the peripheral blood system.\textsuperscript{73} Since they were first described in 1869, CTCs have often been described as seeds of metastatic tumors.\textsuperscript{74} Several studies have focused on the use of CTCs to monitor disease progression and predict prognosis.\textsuperscript{75} In addition to the epithelial to mesenchymal transition phenotype, morphological changes in the blood to withstand biological events such as shear stress and immune attacks add metastatic potential.\textsuperscript{76,77} Thus, CTCs are separated and captured using various techniques based on their altered biophysical characteristics.\textsuperscript{78} Separation using physical properties includes size-based and density-based separation.\textsuperscript{79} On the other hand, separation based on biological properties uses immunoaffinity and immunomagnetic separation. To this end, epithelial markers (EpCAM/CK8,18,19\textsuperscript{80,81}), mesenchymal markers (vimentin and Twist\textsuperscript{82}), and HCC-specific markers (GPC3 and ASGPR) were used to capture CTCs in patients.\textsuperscript{83,84} Because of the association of CTCs with their role in metastasis, numerous studies have been conducted to monitor and predict therapeutic interventions. EpCAM+ CTCs have been shown to have a worse prognosis and a higher rate of recurrence in HCC.\textsuperscript{85,86} Consistently, increases in CTCs were correlated with an increased risk of tumor recurrence and extrahepatic metastasis in liver resection and TACE.\textsuperscript{87-90} The advantage of CTCs is their further utilization in single-cell, transcriptome, and genomic analyses to identify therapeutic targets and molecular characteristics.\textsuperscript{91} The drawback is the heterogeneity of biomarkers, lack of sensitivity due to 20-35% of HCC patients with EpCAM expression,\textsuperscript{92} and low signal-to-noise ratio in early stage disease.\textsuperscript{93,94} Nevertheless, continuous studies using CTCs in monitoring and predicting therapeutic responses would elucidate the metastatic mechanism of HCC as the previous researches had.

review of current ongoing clinical studies

Although different analytes and methods have been used in liquid biopsies, the search for suitable biomarkers is still being actively pursued in many clinical studies. A review of ongoing clinical studies can provide insights into the implementation of liquid biopsies in clinical settings. For this purpose, we performed a search for clinical trials involving HCC through June 23, 2022, on clinicalTrials.gov, using the terms “liquid biopsy OR cfDNA OR ctDNA”, which yielded 39 results (16 recruiting, four not yet recruiting, six completed, six with unknown status, two terminated, three active/not recruiting, and two enrolling by invitation only). Of the 39 clinical trials, ten focused on diagnostic feasibility, seven on changes in ctDNA as an outcome for a drug, five on biomarker exploration (i.e., observational analysis), and 17 on therapeutic monitoring (Supplementary Table 1). Therapeutic responses were monitored after sequential sorafenib-regorafenib treatment, transplantation, TACE, transarterial
radioembolization, liver resection, immune checkpoint inhibitor treatments, combined immunotherapy, targeted therapy (PD-1 or sorafenib), and tyrosine kinase inhibitor treatments. In addition, ctDNA analysis has been included as an additional outcome measure in interventional clinical trials of new treatments such as GT90001+nivolumab combination therapy, chiauranib (phases 1 and 2), pembrolizumab or KEYTRUDA® for pediatric HCC, nivolumab+yttrium Y-90 combination therapy, itacitinib (phase Ib), and other open-label studies (Supplementary Table 2). The incorporation of ctDNA analyses in the early phases of clinical trials indicates the importance of stratifying patient groups at the molecular level. Active clinical evaluations of liquid biopsy include a study of the diagnostic accuracy of SEPT9 promoter methylation, the diagnostic accuracy of gender, age, AFP-L3%, AFP, des-gamma-carboxy prothrombin (GALAD) score, and a study without disclosed biomarkers (Supplementary Table 3). Additionally, to develop an adequate liquid biopsy for HCC, studies on novel biomarkers, actionable alterations, and concordant ctDNA markers with tumor tissues are actively pursued (Supplementary Table 4).

CLINICAL CASE–STUDY OF FDA–APPROVED DIAGNOSTICS

Since no FDA-approved liquid biopsy for HCC diagnosis exists, the most well-established cancer diagnostic test, Cologuard, developed by Exact Sciences, is discussed to understand the clinical study considerations required for approval. Cologuard is the first and only FDA-approved stool-based DNA screening test for CRC. The safety and effectiveness of Cologuard were assessed in a prospective, cross-sectional, multicenter, pivotal study (“Multi-Target Colorectal Cancer Screening Test for the Detection of Colorectal Advanced Adenomatous Polyps and Cancer: DeeP-C Study”). This study involved 12,776 patients aged 45 years and older with an average risk of CRC who were enrolled at 90 sites from June 2011 to February 2013. Patient stool samples were analyzed using the Cologuard test and a fecal immunochemical test (FIT). After 90 days, all enrolled patients underwent colonoscopy. The results of the Cologuard and FIT analyses were compared with colonoscopic and histopathological findings. The overall sensitivity and specificity of Cologuard were 92% and 87%, respectively. In this clinical study, Cologuard demonstrated robust performance in two co-primary endpoint analyses: sensitivity in terms of patients diagnosed with CRC (>65% sensitivity for CRC) and specificity in terms of patients without CRC or advanced neoplasia (AN) (>85% specificity for AN). Secondary endpoint analyses showed that Cologuard was non-inferior to the FIT in terms of sensitivity for CRC and superior to the FIT in terms of sensitivity for advanced adenoma (McNemar test P-value=0.0018). In addition, they conducted in silico simulations to show clinical utility in large population settings. Cologuard screens adults 45 or older at an average risk of CRC and does not replace diagnostic colonoscopy.95

Based on the above clinical studies, validation of liquid biopsy for HCC should consider how new diagnostic methods could be incorporated into the current HCC diagnosis workflow, including the identification of appropriate clinical support. According to the 2022 HCC clinical guidelines, the diagnostic workflow proceeds from the surveillance of patients at high risk for HCC to first-line imaging, principal imaging, and ancillary/second-line imaging.11 Current clinical studies suggest that liquid biopsies should be incorporated into surveillance and ancillary imaging (e.g., positron emission tomography-magnetic resonance imaging+ctDNA). Additionally, trial participants should be representative of the target population to prove their clinical effectiveness and utility.96 For example, patients at high risk for HCC (e.g., patients with chronic HBV infection, chronic HCV infection, or cirrhosis) should be included in clinical trials if the intended indication involves the surveillance of patients at high risk for HCC. Moreover, considerations of the primary and secondary endpoints to prove the effectiveness of the test should include a comparison with current standard methods (AFP assessment and ultrasound) and clinical sensitivity/specificity boundaries. Furthermore, risk-benefit analyses should be included, as in the Cologuard study, to determine whether a particular benefit justifies the risk of the new diagnostic method.
DISCUSSION AND PERSPECTIVES

Many challenges must be addressed for liquid biopsy to become a standard component of clinical practice, including ensuring cost-effectiveness, evaluating clinical utility, establishing regulations, and building a testing laboratory infrastructure. The lack of pre-analytical and analytical standards due to variability in liquid biopsy analytes and the absence of cost-effectiveness validation studies have hindered the incorporation of liquid biopsy into clinical practice.97 The pre-analytical factors to consider include the selection of blood collection tubes, timing of sample transit, use of plasma or serum, guidelines for storage conditions, methods for purification/quantification of cfDNA, and protocols for sample preparation.98 Analytical factors to explore include the lack of performance standards in validation studies (e.g., limits of detection/quantitation/blanks), the need for reproducibility, the need to reduce interference, and the need to establish thresholds of analytical sensitivity and specificity.

The United States and China are conducting large-scale clinical trials to promote the incorporation of liquid biopsy into clinical practice. The factors involved in their approaches include well-established laboratory-based tests, robust funding environments, and the potential for large-cohort studies. As liquid biopsy platforms range from PCR to next-generation sequencing, there is a need for collaboration among regulatory bodies, diagnostics development companies, and other research facilities to establish pre-analytical and analytical standards that consider platforms and specimens. Basic research institutions and diagnostics development companies should confirm biomarker feasibility in independent settings, assess whether each biomarker is sufficiently representative of multiple molecular types of cancer, and clearly define the potential representative populations for biomarkers. The relevant standards should be developed through numerous clinical trials with input from patients, healthcare providers, and grant coordinators. Finally, although most clinical trial outcome data are unavailable for acceptable reasons, data sharing and communication among scientists is essential for incorporating this novel technology into clinical practice. The primary concern of these stakeholders should be the scientific advancement of next-generation diagnostics and the well-being of patients who may benefit from liquid biopsy.

Conflicts of Interest
The author declares no conflict of interest.

Ethics Statement
This article is fully based on the articles that were already published and did not involve additional patient participants. Therefore, IRB approval was not necessary.

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Data Availability
Data sharing not applicable to this article as no datasets were generated or analyzed.

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Supplementary Material
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Supplementary Table 1. List of published trials to study the therapeutic monitoring via liquid biopsy in hepatocellular carcinoma

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<th>Estimated enrollment/perspective</th>
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<td>Liquid Biopsy in Hepatocellular Carcinoma (HCCGenePanel)</td>
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<td>30/prospective</td>
<td>First LRT* with or without Sorafenib for HCC, measure ctDNA</td>
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<td>NCT05375370</td>
<td>Prediction of Hepatocellular Carcinoma Recurrence After Curative Treatment by Monitoring Circulating Tumor DNA</td>
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<td>Evolution of tcDNA change at baseline, 4 months, 6 months after surgery</td>
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<td>NCT05390112</td>
<td>Cohort Study of Patients With Hepatocellular Carcinoma and Circulating Tumor DNA Monitoring of Chemoembolization</td>
<td>Recruiting</td>
<td>167/prospective</td>
<td>Radiological response at 1 month according to mRECIST† and ctDNA detection</td>
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<td>NCT03493763</td>
<td>Study on Recurrence Monitoring of Hepatocellular Carcinoma With 5-Hydroxymethylcytosine Test</td>
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<td>Time of hepatocellular carcinoma recurrence, 5 hmc§ level after liver resection/AFP</td>
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<td>NCT03956940</td>
<td>Analysis of cfDNA in Patients With Hepatocarcinoma and Treated by Sorafenib or Regorafenib</td>
<td>Terminated</td>
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<td>Sorafenib-Regorafenib Sequence Treatment Monitoring Study, cfDNA concentration at baseline, after change at baseline, 4 months, 6 months after surgery</td>
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<td>NCT03839706</td>
<td>Relationship Between 18FDG PET/MRI Patterns and ctDNA to Predict HCC Recurrence After Liver Transplantation</td>
<td>Recruiting</td>
<td>20/interventional</td>
<td>18F-Fluorodeoxyglucose Positron Emission Tomography Magnetic Resonance Imaging Patterns and Circulating Tumor DNA to Predict Hepatocellular Carcinoma Recurrence After Liver Transplantation</td>
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<td>NCT02036216</td>
<td>Circulating Cell-free DNA as a Predictive Biomarker for Hepatocellular Carcinoma</td>
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<td>Preoperative, postoperative (2 weeks) and postoperative (1 month), cfDNA mutations for predictive marker</td>
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<td>NCT04965454</td>
<td>Evaluation of Treatment Predictors Reflecting Beta-catenin Activation in Hepatocellular Carcinoma</td>
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<td>Fluorine-18 fluorocholine PET/CT and cell-free DNA mutation profiling monitoring in immune checkpoint inhibitor therapy</td>
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<td>NCT04506398</td>
<td>Heterogeneity and Evolution of hepatocellular Carcinoma in Post-transplant HCC Recurrence</td>
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<td>Molecular-subtype heterogeneity between primary HCC and post-transplant HCC recurrence, molecular subtype</td>
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<td>NCT0499833</td>
<td>HepatoPredict Prognostic Tool for the Decision of Liver Transplant in Hepatocellular Carcinoma</td>
<td>Recruiting</td>
<td>40/prospective</td>
<td>Recurrence after transplantation (6 months after)</td>
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<td>NCT05371873</td>
<td>A Study of Chromosomal Abnormalities as a Predictor of Staging and Prognosis in Patients With Liver Cancer</td>
<td>Enrolling by invitation</td>
<td>250/prospective</td>
<td>cfDNA monitoring after liver resection (4 years), use UCAD¶ pipeline, prediction efficacy testing</td>
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<td>NCT04800497</td>
<td>The Role of Circulating Tumor Cells As Markers Of Advanced Disease And Prognosis In HCC</td>
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<td>Circulating tumor cells and recurrence/survival</td>
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<td>Therapeutic Resistance and Clonal Evolution Assessed With Liquid Biopsy in ICI Treated Primary Liver Cancer</td>
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<td>300/prospective</td>
<td>Immune checkpoint inhibitors at first-line setting (n=200) or second-line setting (N=100), before and after the first cycle of immune checkpoint inhibitor treatment, the dynamic changes of blood Tumor Mutation Burden, ctDNA and the composition of immune cells</td>
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<td>HBV-host cfDNA as Minimal Residual Tumor Marker for HBV-related HCC</td>
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<td>50/prospective</td>
<td>New Biomarker for Detection of Minimal Residual Tumor in Hepatitis-B Virus Related Hepatocellular Carcinoma After Curative Therapies: cfHBV-host Chimera DNA Fragment, 4 weeks after surgery</td>
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<td>NCT04152356</td>
<td>Combined Immunotherapy and Targeted Therapy for Hepatocellular Carcinoma</td>
<td>Unknown status</td>
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<td>Immunotherapy after surgery and control group. 7 CTCs tests before, 7 days after surgery and 1st, 3rd, 6th, 9th, and 12th postoperatively.</td>
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<td>NCT03958669</td>
<td>Fingerprint Characterization Tyrosine Kinase Inhibitors in Advanced HCC</td>
<td>Recruiting</td>
<td>40/prospective</td>
<td>Fingerprint analysis of HCC tumor tissue to predict tyrosine kinase inhibitor therapy responses</td>
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## Supplementary Table 2. List of published trials to study new drugs incorporating liquid biopsy as their other measures in hepatocellular carcinoma

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<thead>
<tr>
<th>NCT number</th>
<th>Title</th>
<th>Status</th>
<th>Enrollment/design</th>
<th>Study details</th>
<th>Liquid biopsy relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT03245190</td>
<td>Study of Chiauranib in Patients With Advanced Hepatocellular Carcinoma</td>
<td>Completed</td>
<td>27/single group, open label</td>
<td>Efficacy and Safety of Chiauranib in Advanced Hepatocellular Carcinoma</td>
<td>Screening characteristics of ctDNA measurement (24months)</td>
</tr>
<tr>
<td>NCT03893695</td>
<td>Combination of GT90001 and Nivolumab in Patients With Metastatic Hepatocellular Carcinoma (HCC)</td>
<td>Active, not recruiting</td>
<td>20/single group, open label, two stage study</td>
<td>Safety and tolerability of both GT90001 and nivolumab</td>
<td>Exploratory biomarker discovery (alterations including VEGF and TGF-β pathway)</td>
</tr>
<tr>
<td>NCT04134559</td>
<td>Checkpoint Inhibition In Pediatric Hepatocellular Carcinoma</td>
<td>Recruiting</td>
<td>18/single group, open label</td>
<td>Phase II clinical trials test the safety and effectiveness of an investigational drug</td>
<td>Percent change immune phenotype and circulating DNA</td>
</tr>
<tr>
<td>NCT02837029</td>
<td>Nivolumab and Yttrium Y 90 Glass Microspheres in Treating Patients With Advanced Liver Cancer</td>
<td>Completed</td>
<td>27/single group, open label</td>
<td>Phase I/Ib, maximum tolerated dose (MTD), that is, the highest dose of the study drug nivolumab that does not cause unacceptable side effects, for combination treatment of nivolumab and yttrium Y 90 glass microspheres</td>
<td>ctDNA mutation</td>
</tr>
<tr>
<td>NCT04358185</td>
<td>Itacitinib in Advanced Hepatocellular Carcinoma</td>
<td>Recruiting</td>
<td>25/single group, open label</td>
<td>A Phase Ib Study of Itacitinib, a JAK1 Inhibitor, in Advanced Hepatocellular Carcinoma</td>
<td>Correlation of JAK1 mutation in ctDNA</td>
</tr>
<tr>
<td>NCT03144661</td>
<td>An Open-Label Safety and Tolerability Study of INC062079 in Subjects With Advanced Hepatocellular Carcinoma and Other Malignancies</td>
<td>Terminated</td>
<td>25/parallel assignment, open label</td>
<td>A Phase 1, Open-Label, Dose-Escalation and Expansion, Safety and Tolerability Study of INC062079 in Subjects With Advanced Hepatocellular Carcinoma and Other Malignancies</td>
<td>FGFR4 pathway mutation in ctDNA</td>
</tr>
<tr>
<td>NCT03044587</td>
<td>Nal-IRI With 5-fluorouracil (5-FU) and Leucovorin or Gemcitabine Plus Cisplatin in Advanced Biliary-tract Cancer</td>
<td>Active, not recruiting</td>
<td>93/parallel, open label</td>
<td>Nal-IRI* With 5-fluorouracil and Leucovorin or Gemcitabine Plus Cisplatin in Advanced Biliary-tract Cancer - An Open Label, Non-comparative, Randomized, Multicenter Phase II Trial</td>
<td>Other outcome: exploratory biomarker analysis (cfDNA exome sequencing)</td>
</tr>
</tbody>
</table>

*Nanoliposomal irinotecan.
**Supplementary Table 3.** List of published trials to study the diagnostics via liquid biopsy in hepatocellular carcinoma

<table>
<thead>
<tr>
<th>NCT number</th>
<th>Title</th>
<th>Status</th>
<th>Estimated enrollment/perspective</th>
<th>Study details</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT03162198</td>
<td>Frequency of Circulating Tumor Cells (CTCs) and Amount of Cell-free DNA (cfDNA) in Cirrhotic Patients With Hepatocellular Carcinoma (HCC)</td>
<td>Completed</td>
<td>53/cross-sectional</td>
<td>Frequency of Circulating Tumor Cells (CTCs) and Amount of Cell-free DNA (cfDNA) in Cirrhotic Patients With Hepatocellular Carcinoma (HCC)</td>
</tr>
<tr>
<td>NCT03483922</td>
<td>HCC Screening Using DNA Methylation Changes in ctDNA</td>
<td>Completed</td>
<td>403 participants/retrospective</td>
<td>Chronic hepatitis B, HCC case, healthy; DNA methylation of circulated tumor and PBMC DNA and its Correlation to Development and prediction of HCC</td>
</tr>
<tr>
<td>NCT03311152</td>
<td>Circulating Cell-free DNA-based Epigenetic Biomarker mSEPT9 for Hepatocellular Carcinoma Detection in Cirrhosis</td>
<td>Recruiting</td>
<td>530/cross-sectional biomarker phase II</td>
<td>Diagnostic Accuracy of the Circulating Cell-free DNA-based Epigenetic Biomarker mSEPT9 for Hepatocellular Carcinoma Detection Among Cirrhotic Patients: the SEPT9-CROSS Study, mSEPT9</td>
</tr>
<tr>
<td>NCT05342350</td>
<td>Surveillance and Treatment Of Primary Hepatocellular Carcinoma: An International Prospective Observational Cohort Study of High-Risk Patients for HCC Using Liquid Biopsy</td>
<td>Active, not recruiting</td>
<td>1,600/prospective</td>
<td>High-Risk Patients for HCC Using Liquid Biopsy, GALAD* score performance</td>
</tr>
<tr>
<td>NCT05256459</td>
<td>Clinical Intervention Strategy for High-risk Group of Hepatitis B Related Hepatocellular Carcinoma</td>
<td>Not yet recruiting</td>
<td>1,000/prospective</td>
<td>High risk patients were examined for high-precision HBV-DNA quantification, liver function, AFP, abdominal imaging, monitoring (3-5 years)</td>
</tr>
<tr>
<td>NCT04539717</td>
<td>FAST (Focused Abbreviated Screening Technique)-MRI Study</td>
<td>Enrolling by invitation</td>
<td>820/prospective</td>
<td>HCC screening with Magnetic Resonance Imaging and circulating tumor DNA, α-fetoprotein</td>
</tr>
<tr>
<td>NCT05199259</td>
<td>Multi-analyte Blood Test Clinical Trial</td>
<td>Not yet recruiting</td>
<td>1,200/prospective</td>
<td>Cirrhosis patients, multianalyte blood test</td>
</tr>
<tr>
<td>NCT03517332</td>
<td>Circulating Tumor DNA Exposure in Peripheral Blood</td>
<td>Unknown status</td>
<td>10,000/prospective</td>
<td>Circulating Tumor DNA Exposure in Peripheral Blood Using a Novel Process: A Feasibility Study</td>
</tr>
<tr>
<td>NCT05393102</td>
<td>Auxiliary Diagnosis of Liver Nodules Using cfDNA Whole-genome Signatures</td>
<td>Not yet recruiting</td>
<td>400/prospective</td>
<td>Sensitivity/Specificity of the cfDNA whole-genome signatures-based model in liver nodules diagnosis</td>
</tr>
<tr>
<td>NCT03551951</td>
<td>Tumor Cell and DNA Detection in the Blood, Urine, and Bone Marrow</td>
<td>Recruiting</td>
<td>320/prospective</td>
<td>Circulating tumor cells/disseminated tumor cells/cell free DNA isolation from the blood, urine and bone marrow in various tumor</td>
</tr>
</tbody>
</table>

*Gender, age, alpha-fetoprotein L3% (AFP-L3), AFP, des-gamma-carboxy prothrombin.
**Supplementary Table 4.** List of published trials to other studies (ex. biomarker discovery) via liquid biopsy in hepatocellular carcinoma

<table>
<thead>
<tr>
<th>NCT Number</th>
<th>Title</th>
<th>Status</th>
<th>Estimated enrollment/perspective</th>
<th>Study details</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT03071458</td>
<td>Mutational Landscape in Hepatocellular Carcinoma</td>
<td>Completed</td>
<td>808/prospective</td>
<td>Translating Molecular Classifications and Genetic Alterations of Hepatocellular Carcinoma in Clinical Care, detect mutations</td>
</tr>
<tr>
<td>NCT04484636</td>
<td>PLATON - Platform for Analyzing Targetable Tumor</td>
<td>Recruiting</td>
<td>200/single group, open label</td>
<td>FoundationOne®CDx and FoundationOne®Liquid to find targetable alterations</td>
</tr>
<tr>
<td></td>
<td>Mutations (Pilot-study)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT04445532</td>
<td>Hepatobiliary Tumors Tissue Samples Acquisition</td>
<td>Recruiting</td>
<td>450/prospective</td>
<td>Biomarker study for hepatobiliary tumor patients</td>
</tr>
<tr>
<td>NCT02973204</td>
<td>Circulating Tumor Cells and Tumor DNA in HCC and</td>
<td>Completed</td>
<td>167/prospective</td>
<td>Concordance of CTC/ctDNA mutations, biomarker study</td>
</tr>
<tr>
<td></td>
<td>NET</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT02838836</td>
<td>Tumor Cell and DNA Detection in the Blood, Urine</td>
<td>Recruiting</td>
<td>120/prospective</td>
<td>CTC/DTC numbers measured in blood, urine and bone marrow samples will be correlated with patient outcome, during surgery, CTCs/DTCs and ctDNA isolated from cancer patients will be characterized for genetic alterations and expression of key signaling/proliferation biomarkers and grow in vivo in nude mice.</td>
</tr>
<tr>
<td></td>
<td>and Bone Marrow of Patients With Solid Cancers</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>