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REVIEW ARTICLE

Circulating cell-free DNA in clinical management of hepatocellular carcinoma: Updates on detection, diagnosis, treatment, and recurrence

Zita Hoi-Ying Chan, Xueying Lyu, and Daniel Wai-Hung Ho*

Department of Pathology and State Key Laboratory of Liver Research, School of Clinical Medicine, The University of Hong Kong, Hong Kong SAR, China

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Abstract

The evident concordance between tissue and liquid biopsies in hepatocellular carcinoma (HCC), a prevalent and lethal cancer worldwide, has positioned liquid biopsy as a valuable non-invasive tool for the clinical management of HCC. Among its analytes, circulating cell-free DNA (cfDNA) has recently gained significant attention as a key biomarker for HCC. This review provides an overview of recent advancements in the use of cfDNA for the detection and diagnosis, treatment decision-making, and recurrence surveillance of HCC. The various merits of cfDNA underscore its strong potential for clinical integration in HCC. However, there is also an emerging imperative that arises from the varying cfDNA-related methodologies, which demonstrate disparate outcomes across studies, emphasizing the importance of systematic evaluation and standardization to ensure consistent and equitable patient care.

Keywords: Hepatocellular carcinoma; Circulating cell-free DNA; Machine learning; Tumor burden

***Corresponding author:**
Daniel Wai-Hung Ho
(dwhho@hku.hk)

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1. Introduction

Hepatocellular carcinoma (HCC) is a major subtype of primary liver cancer (PLC), accounting for around 90% of cases.[1,2] HCC is a deadly malignancy, particularly known for its high recurrence and poor prognosis. The high disease burden is partly due to the difficulty of early detection, and most cases are usually diagnosed at advanced stages, where curative treatment options are limited. There are known etiological risk factors for HCC, including chronic inflammation or even cirrhosis related to viral infection (hepatitis B virus [HBV] and hepatitis C virus), excessive alcohol consumption, as well as metabolic dysfunction-associated steatotic liver disease (LD). Those high-risk groups may require regular surveillance using ultrasound or the blood alpha-fetoprotein (AFP) test.[3] Traditional tissue biopsy is rarely performed for screening purposes due to its invasiveness, potential tumor-seeding effect, and the limitation of regional sampling. Given the recent evidence suggesting the concordance between tissue and liquid biopsy in human cancers, including HCC,[4] it is generally believed that liquid biopsy can sensitively and faithfully recapitulate the genuine biological profile in tumor tissue, while also

minimizing the individual drawbacks that exist in tissue biopsy. Hence, liquid biopsy has been widely adopted in various aspects of HCC, ranging from early detection and diagnosis to different perspectives of clinical management, such as monitoring of treatment response and disease recurrence, companion diagnostics, and prognostication.[5]

Circulating cell-free DNAs (cfDNAs) are extracellular DNA molecules found in blood and other body fluids. It has gained significant attention in recent times for its non-invasive clinical values. It serves as a key biomarker in liquid biopsy. Our prior review[6] discussed the concordance of molecular features identified in tumor tissue DNA and those in cfDNA. These molecular features include single-nucleotide variants (SNVs), copy number variations (CNVs), DNA methylation aberrations, preferred end motifs or coordinates, and HBV integrations, which hold significant clinical relevance and utility.

This current review will offer new insights into the latest advancements regarding the utilization of cfDNA in the detection and diagnosis, treatment decision-making, and recurrence surveillance of HCC. Specifically, it will delve into the recent efforts to translate the cfDNA molecular features into diagnostic score models through the application of machine learning (ML) techniques. Moreover, it will also discuss various efforts aiming at improving early detection. Given that HCC is often diagnosed only in advanced stages, improving early detection can facilitate clinical management, ultimately resulting in better survival outcomes. In addition to detection and diagnosis, the application of cfDNA can be extended to treatment, including predicting patients' responses to drugs to ensure optimal benefits, as well as monitoring the cfDNA levels to assess treatment effectiveness in patients. Finally, this review will explore the cfDNA-based assessment of HCC recurrence.

2. Detection and diagnosis perspective

HCC, if diagnosed in the early stages, can be managed effectively clinically. Unfortunately, HCC is often only diagnosed at advanced stages due to the limitations of routine ultrasound and AFP surveillance. For ultrasound surveillance, it falls short in detecting tumor nodules <1 cm. AFP surveillance relies on an elevation in AFP levels (>20 ng/mL) in HCC patients, but ~40% HCC patients do not exhibit an elevation in AFP levels.[5,7,8]

These limitations of routine surveillance methods stressed the need for alternative surveillance methods. In the search for alternatives, cfDNA released from tumor cells has emerged as a promising surveillance molecule for detecting early-stage HCC. Our prior review unveiled efforts to profile cfDNA characteristics (including SNVs, CNVs, DNA methylation aberrations, preferred end motifs or coordinates, and HBV integrations) prevalent in HCC patients.[6] In more recent studies, efforts have been made to translate these molecular characteristics into diagnostic score models through the application of ML techniques. However, these models have not reached full sensitivity in early detection. In several recent studies, efforts had also been made to enhance early detection with study design-, biomarker-, and technology-based approaches.

2.1. Diagnostic score models with ML techniques

In general, an HCC diagnostic scoring model is established using ML on a discovery cohort. This process incorporates cfDNA biomarkers and occasionally blood protein biomarkers to establish the diagnostic scoring model. Subsequently, this model is validated in a separate cohort. Further details are shown in Table 1.

Yu and Lei[9] incorporated both cfDNA biomarkers, SNVs, genomic integrations of HBV, and methylations, as well as blood protein biomarkers, AFP and protein induced by vitamin K absence or antagonist-II (PIVKA-II), into an unspecified ML algorithm to develop the methylation, mutation, and protein-HCC (M2P-HCC) score. The discovery cohort consisted of 313 individuals at high or very high risk for HCC as defined in the Guideline for Stratified Screening and Surveillance of PLC.[10] This resulted in a classification of three groups where a score of 1–85 indicates a negative (NEG) result, 86–96 indicates a borderline risk (BR) for HCC, and 97–157 indicates a positive (POS) result for HCC. There were 258, 39, and 16 cases classified as NEG, BR, and POS, respectively. Ideally, this model should be validated in a separate cohort, but the validation was conducted on the same cohort instead. Patients underwent enhanced follow-up every 3 months for 6–8 months to observe for signs of HCC. Subsequently, patient outcomes (41 diagnosed HCC and 272 diagnosed non-HCC) during the follow-up period were compared with their initial predictions using diagnostic scores. M2P-HCC test exhibited an area under the curve (AUC) value of 0.88 with a cutoff value of 83 (sensitivity of 82.9% and specificity of 85.7%). In contrast, the combination of AFP and ultrasound only yielded an AUC value of 0.76 (sensitivity of 58.5% and specificity of 94.1%). It is noteworthy that in this validation approach, the clinical diagnosis was not conducted concurrently with the cfDNA collection, which likely explained the discrepancy between the score model results and the observed outcomes (16 POS and 39 BR vs. 41 HCC). Furthermore, the receiver-operating characteristic (ROC) curve analysis that produced the AUC for the M2P-HCC test used a different cutoff of 83, in contrast to the cutoff of 86 in the score model. Therefore, the performance was not truly reflected

Table 1. Summary of studies that have developed HCC diagnostic score models through machine learning techniques

Discovery cohort	Validation cohort	Sample source (s)	Biomarker (s)	Blood collection tube	cfDNA extraction platform	cfDNA profiling method	Machine learning algorithm (s)	Score model	References
313 HR	No validation cohort	Plasma	cfDNA: SNV, HBV INTG, METH BP: AFP, PIVKA-II	EDTA blood collection tubes	MagMAX cell-free DNA isolation kit (Thermo Fisher Scientific)	SNV, HBV INTG, and METH: Race-seq	UNK (algorithm name was not specified)	NEG: 1–85 BR: 86–96 POS: 97–157	9
TRAIN: 490 HLTY, 344 LC, 345 HCC TEST: 145 LC, 151 HCC	88 LC, 112 HCC	Plasma	cfDNA: SNV BP: AFP, AFP-L3, PIVKA-II	Cell-free DNA blood collection tubes (Streck)	MagMAX cell-free DNA isolation kit (Thermo Fisher Scientific)	SNV: cSMART (targets 931 regions across 21 genes)	XGBoost	POS: >0.54	11
122 HLTY, 191 PLC, 149 CRC, 146 LUAD	121 HLTY, 190 PLC, 149 CRC, 146 LUAD	Plasma	FSC, FSD, EDM, BPM, CNV	EDTA blood collection tubes (Becton Dickinson)	QIAamp circulating nucleic acid kit (Qiagen)	FSC, FSD, EDM, BPM, and CNV: WGS	GLM, GBM, RF, DL, XGBoost	POS: >0.39	12
Plasma: 13 HLTY, 22 LC, 22 HCC, 51 UNK Tissue: 91 HLTY, 94 AATD, 67 LO, 94 LC, 405 HCC	Tissue: 501 HCC, 191 UNK	Plasma and tissue	METH	EDTA blood collection tubes	13 HLTY and 51 UNK: QIAsymphony liquid handling robot (Qiagen) 22 LC and 22 HCC: QIAamp circulating nucleic acid kit (Qiagen)	METH: Infinium 450K or EPIC arrays (Illumina)	LinearSVC	UNK (score cutoffs were not specified)	13
195 HLTY, 54 LD, 168 HCC	84 HLTY, 22 LD, 73 HCC	Plasma	METH	cfDNA blood collection tube (Zhixuan Biotech)	Plasma cell-free DNA extraction kit (Concert)	METH: enzymatic methyl-seq (targets 1595 HCC-associated CpG sites)	GDBT, LR	POS: >0	14

Abbreviations: AATD: Alpha-1 antitrypsin deficiency; AFP: Alpha fetoprotein; BP: Blood protein; BPM: Breakpoint motif; BR: Borderline risk; cfDNA: Circulating cell-free DNA; CNV: Copy number variation; CRC: Colorectal cancer; DL: Deep learning; EDM: End motif; EDTA: Ethylenediaminetetraacetic acid; FSC: Fragment size coverage; FSD: Fragment size distribution; GBM: Gradient boosting machine; GDBT: Gradient boosting decision tree; GLM: Generalized linear model; HBV INTG: Hepatitis B virus integration; HCC: Hepatocellular carcinoma; HLTY: Healthy; HR: High risk; LC: Liver cirrhosis; LD: Liver disease; LinearSVC: Linear support vector machine classifiers; LO: Liver obesity; LR: Logistic regression; LUAD: Lung adenocarcinoma; METH: Methylation; NEG: Negative; PIVKA-II: Protein induced by vitamin K absence II; PLC: Primary liver cancer; POS: Positive; RF: Random forest; SNV: Single-nucleotide variation; TEST: Testing cohort; TRAIN: Training cohort; UNK: Unknown; WGS: Whole genome sequencing.

and evaluated. Particularly, the overlapping cohorts used for discovery and validation, the lack of concurrent clinical diagnosis at the time of cfDNA collection, and the use of a different cutoff in ROC analysis collectively emphasize the need for additional validation. Future work should be conducted in independent cohorts, with clinical diagnoses synchronized with cfDNA collection, and consistent score cutoffs applied to strengthen findings.[9]

Wu *et al.*[11] also incorporated both cfDNA and blood protein biomarkers in their large-scale, multicenter study, involving 490 healthy (HLTY) individuals, 577 liver cirrhosis (LC) patients, and 608 HCC patients. Specifically, SNVs were utilized as cfDNA biomarkers, and serum AFP, AFP-L3, and PIVKA-II were used as blood protein biomarkers. For the discovery cohort, this study divided it into two cohorts: training (490 HLTY, 344 LC, and 345 HCC) and testing (145 LC and 151 HCC), where SNVs were first filtered in the training cohort. The SNVs were required to meet the following criteria: the *p*-value calculated with Fisher's exact test was <0.05, found in <10 LC patients, and found in more than 10 HCC patients. The SNVs that met the criteria were then incorporated with the blood protein biomarkers in the testing cohort to build the diagnostic scoring model with the XGBoost ML algorithm. This yielded a diagnostic scoring model where a score >0.54 indicates a POS result for HCC. Subsequently, this model was validated in a separate cohort (88 LC and 112 HCC). The accuracies for this model in Barcelona Clinic Liver Cancer (BCLC) stages 0–C were 78.95%, 80.56%, 81.90%, and 84.14% respectively. Conversely, the accuracies for AFP surveillance were 87.37%, 80.56%, 80.17%, and 82.07%, respectively. Notably, the ML model's accuracy only exceeded AFP surveillance in the BCLC stage B and above. This is potentially attributable to the uneven distribution of cases at different stages (stage 0: 29 cases, stage A: 94 cases, stage B: 123 cases, stage C: 249 cases), with a limited number of BCLC stage 0 cases in the discovery cohort. This limitation of the sample cohort impairs the ML algorithm's ability to learn early-stage HCC features, thereby restricting its effectiveness in detecting early-stage HCC. Increasing early-stage sample sizes in future studies is essential to developing ML diagnostic models that can enable more effective early-stage HCC detection.[11]

Bao *et al.*[12] investigated multi-cancer detection, which differentiated cancer and non-cancer cases. Furthermore, upon detecting cancer, it also enabled cancer origin differentiation, naming PLC, colorectal cancer (CRC), or lung adenocarcinoma (LUAD). The study developed multi-cancer detection and cancer origin models based on 122 HLTY, 191 PLC, 149 CRC, and 146 LUAD. These models incorporated cfDNA biomarkers, including fragment size coverage, fragment size distributions, end motifs, breakpoint motifs, and CNVs. The study built these models through a stacked ensemble ML approach, which incorporated algorithms including the generalized linear models, gradient boosting machines, random forests, deep learning, and XGBoost. This yielded a diagnostic scoring model where a score >0.39 indicates a POS result for one of the aforementioned cancers. This model was then evaluated in the validation cohort (121 HLTY, 190 PLC, 149 CRC, 146 LUAD). The multi-cancer detection achieved a sensitivity of 95.5% and a specificity of 95.0%. Subsequently, in cancer origin differentiation conducted on cancer patients that were correctly detected in multi-cancer detection, the accuracies for PLC, CRC, and LUAD were 98.5%, 93.6%, and 91.6%, respectively.[12]

Among the studies included in this review, only Gonçalves *et al.*[13] integrated plasma (n = 108: 13 HLTY, 22 LC, 22 HCC, 51 individuals with unknown [UNK] health status) and tissue samples (n = 751: 91 HLTY, 94 alpha-1 antitrypsin deficiency [AATD] patients, 67 liver obesity [LO] patients, 94 LC, 405 HCC) for diagnostic score model construction. Because of the unequal numbers of plasma and tissue samples, they adopted an ML workflow: randomly selecting tissue samples to match the number of plasma samples, followed by training linear support vector machine classifiers. This workflow was iterated 1000 times, with the same set of plasma samples trained alongside a different set of tissue samples in each iteration. After all iterations, the differentially methylated regions (DMRs) were incorporated one at a time, from the most frequent in all iterations to the least frequent. The incorporation process continued until no further improvements in precision and recall were observed. Ultimately, a total of 38 DMRs, including 118 hypermethylated and 74 hypomethylated sites, were identified. These 38 DMRs yielded a mean precision of 96% and a recall of 86% in the validation cohort. It is also worth noting that this study validated their model solely using tissue samples (501 HCC, 191 UNK).[13]

Guo *et al.*[14] utilized cfDNA methylation biomarkers and applied the gradient boosting decision tree ML algorithm to the discovery cohort (195 HLTY, 54 LD patients, 168 HCC) to construct two models: one for HCC versus HLTY, and another for HCC versus LD. These models identified 37 and 264 biomarkers, respectively. Then, a combined set of 283 unique biomarkers from the two models was subsequently used to construct a stacked model using a logistic regression algorithm. This yielded a diagnostic score model in which a score >0 indicated a POS result for HCC. This model was then evaluated in a validation cohort (84 HLTY, 22 LD, 73 HCC), achieving an AUC of 0.957 (sensitivity of 90% and a specificity of 97%).[14]

Overall, these studies employed various molecular features to generate diagnostic scoring models. The performance varied, highlighting the necessity for continued systematic evaluation and standardization for feature inclusion and model building in the future. Nonetheless, these studies also demonstrated that diagnostic score models developed using ML techniques hold promise in translating HCC biomarkers into practical tools for clinical diagnostic purposes.

2.2. Initiatives toward early detection

While diagnostic score models hold promise for translational purposes, some of these models showed varying diagnostic sensitivities across different HCC stages, with lower sensitivities for early stages and higher sensitivities for more advanced stages.[11,12] Importantly, HCC can be managed more effectively when diagnosed at an early stage, highlighting the need to improve sensitivity for early detection. Here, we outline studies that showcase study design-, biomarker-, and technology-based strategies to improve sensitivity for early detection.

2.2.1. Study design-based approach

Recent studies have increasingly adopted prospective designs instead of relying on retrospective samples, addressing bias toward advanced-stage HCC cases that characterized earlier research. This approach involves identifying high-risk non-HCC individuals and tracking their outcomes, allowing the collection of early-stage HCC cases for profiling and improving early detection sensitivity.

Yu and Lei[9] collected cfDNA samples from 313 high-risk individuals upon enrollment, and classified them into NEG (258 cases), BR (39 cases), or POS (16 cases) for HCC with an unspecified ML algorithm. Meanwhile, these individuals were reviewed every 3 months in the 6–8 months review period to identify potential early-stage HCC cases. If AFP, PIVKA-II, and ultrasound surveillance indicated a likelihood of potential early-stage HCC, enhancement tests, such as ultrasound enhancement, computed tomography enhancement, and magnetic resonance imaging, would be promptly conducted within a month. Ultimately, at the end of the review period, these individuals either were diagnosed with HCC (41 cases) or remained free of it (272 cases).[9]

Similarly, Lin *et al.*[15] collected cfDNA samples from 35 high-risk individuals upon enrollment and reviewed them. However, there was no enhanced follow-up on these individuals. At the end of a follow-up period of ≥1 year, 21 individuals (3 BCLC stage 0 and 18 BCLC stage A) were diagnosed histologically with early-stage HCC in accordance with the American Association for the Study of LDs (AASLD) HCC Management Guidelines,[16] while 14 individuals were diagnosed as non-HCC. For those who were diagnosed, they also collected cfDNA samples at the time of diagnosis. Then, the cfDNA samples collected from early-stage HCC patients at diagnosis and from non-HCC individuals at enrollment were subjected to targeted sequencing (TS). The TS used a panel targeting the exons of 23 genes (*ACVR2A*, *ALB*, *APC*, *ARID1A*, *ARID1B*, *ARID2*, *ATM*, *AXIN1*, *CDKN1A*, *CDKN2A*, *CTNNB1*, *ERBB2*, *JAK1*, *KEAP1*, *KRAS*, *NFE2L2*, *NRAS*, *PIK3CA*, *PTEN*, *RB1*, *RPL22*, *RPS6KA3*, and *TP53*) known for their recurrent mutations (>1%) and potential driver functions in HCC. Their study revealed that three genes were more frequently mutated in early-stage HCC patients compared to non-HCC individuals: *ARID1A* (p=0.011), *CTNNB1* (p=0.005), and *TP53* (p=0.019). In early-stage HCC patients, the mutation rates for these genes were 85.7%, 42.9%, and 100%, respectively. In contrast, in non-HCC individuals, the respective mutation rates were 42.9%, 0%, and 71.4%, respectively. While these three genes achieved an AUC of 0.844, it is crucial to note that this study focused on capturing the early-stage molecular landscape in HCC with no validation cohort, underscoring the necessity for further validation.[15]

Comparing the cfDNA collection and follow-up approaches of the two studies, Yu and Lei[9] solely collected cfDNA samples at enrollment, while Lin *et al.*[15] collected cfDNA samples both at enrollment and at diagnosis. Although Lin *et al.*[15] ultimately did not analyze HCC patients' cfDNA samples collected at enrollment, their inclusion would have enabled a more comprehensive exploration of the molecular landscape. On the other hand, Yu and Lei[9] had a more comprehensive follow-up approach than the other study. Given that Lin *et al.*[15] depended on histological data for diagnosis, this likely constrained the collection of samples from very early-stage cases, resulting in a smaller number of very early-stage samples (3 BCLC stage 0) compared to early-stage samples (18 BCLC stage A). A combination of Yu and Lei's[9] follow-up approach and Lin *et al.*'s[15] cfDNA collection approach may yield a comprehensive strategy to improve sensitivity for early detection.

Another recent large-scale study by Fan *et al.*[17] utilized multi-center prospective observational cohorts of 13,728 patients (chronic hepatitis B and cirrhosis) to predict HCC risk. With low-pass whole genome sequencing data, they derived novel multimodal HCC prediction models (aMAP-2 and aMAP-2 Plus) that integrated longitudinal data (aMAP score[18] and AFP) without or with cfDNA signatures (nucleosome status, fragment profile, and end-motif), respectively. Longitudinal discriminant analysis

model involving only longitudinal data demonstrated an AUC of 0.74–0.83 across cohorts. The performance also gradually improved with increasing longitudinal timepoints. Given the inferior performance (AUC < 0.8) of different models (including aMAP-2) for predicting patients with cirrhosis, they developed the aMAP-2 Plus score that displayed improved performance (AUC of 0.89). Notably, the 1-year cumulative incidence of HCC among aMAP-2 Plus-defined high-risk patients (13.1%) was significantly higher than that of the low-risk counterparts (0.6%). This is particularly useful as a regular surveillance measure on high-risk patients for HCC. Taken together, Fan *et al.*[17] proposed the stepwise application of aMAP scores (aMAP > aMAP-2 > aMAP-2 Plus) to achieve superior patient stratification and HCC surveillance among high-risk individuals. The same group later performed a multi-center cross-sectional study on HCC surveillance.[19] They derived a PreCar score that integrated five features of cfDNA (nucleosome footprint, 5-hydroxymethylcytosine, fragment size, end-motif, and CNV) using a support vector machine to screen for early HCC. PreCar score achieved significantly higher sensitivity for early HCC (BCLC stage 0/A) than the routine ultrasound or AFP method. They also propose the combined use of the PreCar score and ultrasound for routine HCC surveillance.

2.2.2. Biomarker-based approach

There were also developments in identifying novel biomarkers, particularly methylation biomarkers, which may potentially enhance their sensitivity for early detection. Kim *et al.*[20] used single CpG sites in the development of a methylation-sensitive high-resolution methylation (MS-HRM) assay. They first identified differentially methylated sites present in both the Cancer Genome Research Center cohort (125 HLTY, 180 HCC) and the Cancer Genome Atlas cohort (50 HLTY, 379 HCC). Following this, methylated sites with high methylation levels in 507 whole blood samples, 684 pan-normal tissues, and 7296 pan-cancer tissues were excluded. This led to the identification of HCC-specific markers: cg16579555 and cg13204512 within *RNF135*, and cg02659794 within *LDHB*. These markers were then integrated into the MS-HRM analysis, generating melting curve slopes that indicate the methylation levels to facilitate diagnostic utility. The validation was conducted using plasma samples from 202 HLTY, 211 BR, and 313 HCC. The accuracies of the MS-HRM, AFP, and the combined MS-HRM+AFP were 78.1%, 72.9%, and 81.7%, respectively.[20]

Gonçalves *et al.*[13] employed clustered CpG sites to mitigate overfitting that may arise from the imbalance between the number of CpG sites and samples. They defined clustered CpG sites as regions that span at least three CpG sites, where consecutive sites should not be more than 500 base pairs apart. In brief, a total of 38 DMRs (118 hypermethylated and 74 hypomethylated) were identified in the discovery cohort (108 plasma [13 HLTY, 22 LC, 22 HCC, 51 UNK] and 751 tissue [91 HLTY, 94 AATD, 67 LO, 94 LC, 405 HCC]), yielding a mean precision of 96% and a recall of 86% in the validation cohort (501 HCC, 191 UNK).[13]

While Kim *et al.*[20] and Gonçalves et al.[13] investigated genome-wide methylation, Zhang *et al.*[21] focused specifically on ribosomal DNA (rDNA) methylation. rDNA encodes ribosomal RNA, with hundreds of copies located on chromosomes 13, 14, 15, 21, and 22. They first characterized rDNA methylation patterns in 24 tissues or cells. The characterization divided rDNA into five methylation zones, in which zones 1, 3, and 5 exhibited relatively low but variable methylation levels, whereas zones 2 and 4 exhibited relatively high and stable levels. Then, they further characterized rDNA methylation patterns in colon cancer, esophageal cancer, HCC, lung cancer, and prostate cancer. Their findings revealed that zones 2 and 4 exhibited hypomethylation and variable methylation levels, which were likely associated with cancer. This was validated in plasma-derived cfDNA from 26 HCC patients compared to 32 HLTY individuals and 8 HBV carriers, yielding an AUC of 0.91. Additionally, this was also validated in plasma-derived cfDNA from 12 HCC patients vs. 7 cirrhosis patients, yielding an AUC of 0.96. Both validations outperformed AFP, which exhibited an AUC of only 0.83.[21]

Although Gonçalves *et al.*[13] and Zhang *et al.*[21] did not specifically show an increase in sensitivity for early-stage detection,[13,21] the development of methylation biomarkers, from single CpG sites to clustered CpG sites and rDNA CpG sites, holds promise for advancing early detection efforts.

2.2.3. Technology-based approach

In addition to developments in methylation biomarkers, notable advancements were also seen in methylation sequencing approaches. Bisulfite sequencing (BS-seq) is considered the gold standard for methylation sequencing, as it enables the quantification of methylation levels at single-base resolution. However, bisulfite treatment damages the DNA, which restricts the use of BS-seq for low-input samples, particularly when cfDNA concentrations are limited in early stages compared to advanced stages. The recent enzymatic methyl-seq (EM-seq) achieves a similar conversion to bisulfite treatment without damaging the DNA, as in BS-seq.[14]

A comparison by Guo *et al.*[14] demonstrated that methylation levels obtained using BS-seq and EM-seq were

generally comparable. For hypermethylated CpG sites, the methylation levels were consistent between the two methods, whereas for hypomethylated CpG sites, EM-seq yielded significantly lower methylation values, likely due to incomplete conversion. To alleviate such noise, it was suggested to filter reads with ≥3 CH sites (CC, CT, or CA) in EM-seq to reduce noise and yield a comparable performance to BS-seq. They also further validated EM-seq in plasma-derived cfDNA from 84 HLTY, 22 LD, and 73 HCC. In brief, this validation achieved an AUC of 0.957 (sensitivity of 90% and a specificity of 97%). While this validation did not conclusively indicate enhanced sensitivity for early-stage detection, it demonstrated the promising potential of EM-seq.[14] Given that EM-seq does not cause DNA damage like BS-seq, there is a potential for EM-seq to enhance sensitivity, particularly in the context where cfDNA concentrations are lower in the early stages.

3. Treatment perspective

In addition to its role in detection and diagnosis, cfDNA applications can be extended to treatment, including guiding therapeutic decisions and monitoring treatment effectiveness.

3.1. Guiding treatment decisions

When it comes to the use of cfDNA for guiding HCC treatment decisions, a key focus is predicting patients' drug response to guarantee their benefits. A recent study by Cheung *et al.*[22] investigated cfDNA biomarkers that can predict patients' response to pre-operative nivolumab treatment. They recruited 20 intermediate and locally advanced HCC patients to receive nivolumab followed by hepatectomy. Of the 19 patients (one patient withdrew) who completed the treatment, seven achieved major pathologic response (MPR), and 12 patients were non-MPR. Pre-treatment plasma cfDNA was collected and used to develop a nivolumab response score, calculated using CNV intervals significantly correlated with the degree of pathological response. This score was validated in a separate cohort (tissue samples from 37 nivolumab-treated HCC patients). The plasma and tissue cohort achieved an AUC of 0.802, indicating the cfDNA test may have satisfactory predictive power on the treatment effect.[22]

A separate study conducted by Coto-Llerena *et al.*[23] highlighted the potential of cfDNA in guiding treatment decisions for multinodular HCC. The study enrolled seven patients with multinodular HCC. Tissue biopsies were obtained from multiple nodules before treatment and subjected to whole-exome sequencing (WES). The mutations of the nodules were used to differentiate between intrahepatic metastasis (IM) and multicentric occurrence (MC) nodules (five patients were IM and two patients were MC). Corresponding cfDNA were collected and subjected to TS (*TERT* promoter and exons of 75 genes recurrently mutated in HCC). The mutations detected in cfDNA were compared with those found in tissue biopsies. Among the five patients with IM nodules, four had tumor mutations that were all detectable in cfDNA, while one patient had only 25% of tumor mutations detectable in cfDNA due to low sequencing depth. Meanwhile, of the two patients with MC nodules, one had 83.3% and the other had 100% tumor mutations detectable in cfDNA, respectively. This demonstrates that cfDNA can capture tumor heterogeneity in multinodular HCC. Nonetheless, none of the patients with MC nodules showed a significant correlation between tumor and cfDNA variant allele frequencies (VAFs), with correlations of only 0.12 and 0.44, respectively.[23] Similarly, Fu *et al.*[24] reported that neither tumor size nor tumor number correlated with cfDNA abundance, which was estimated based on the total mutated genes and the average VAF.[24] Nevertheless, these results suggest that cfDNA can capture tumor heterogeneity but may be insufficient to distinguish the different forms of multinodular HCC based on VAFs.

Overall, further research is essential to develop the capabilities of cfDNA in predicting responses to drug prescriptions and distinguishing the different subtypes of HCC, and ultimately to advance its capability to guide treatment decisions in other relevant areas.

3.2. Monitoring treatment effectiveness

In addition to guiding treatment decisions, cfDNA can also serve to monitor treatment effectiveness. An important concept in monitoring treatment effectiveness is tumor burden, which refers to the amount of cancer present in a patient. It reflects the amount, size, and/or extent of tumor lesions. An effective treatment should reduce the tumor burden, which should ideally be indicated by a reduction in the amount of cfDNA. This will allow cfDNA to be used as a surveillance modality to monitor treatment effectiveness. In this section, we present a few recent studies that demonstrate the use of cfDNA to monitor treatment effectiveness (Table 2).

Xia *et al.*[25] recruited 20 resectable HCC patients for a trial with camrelizumab/apatinib, a combination of immunotherapy (IMT) and anti-angiogenesis therapy (AAT). Such a treatment combination has recognized clinical value in unresectable HCC patients, and this trial sought to investigate its value as neoadjuvant therapy in resectable patients. For the 18 patients who completed neoadjuvant therapy, 16.7% and 33.3% patients reached objective response rate (ORR) based on Response Evaluation Criteria in Solid Tumors (RECIST) V.1.1 and

Table 2. Summary of studies that have utilized cfDNA to monitor treatment effectiveness

Cohort	Treatment	Biomarker (s)	Sample timepoint (s)	References
20 HCC (2 WD PRE, 1 WD POST-NEO, 4 WD POST-HT)	NEO (camrelizumab 200 mg q2w 3cyc, apatinib 250 mg 21 d)+HT+ADJ (camrelizumab 200 mg q3w 8cyc, apatinib 250 mg)	SNV, CNV, INDEL, FUS	PRE, POST-NEO, POST-HT, POST-ADJ	25
30 HCC (13 WD POST-NEO, 13 WD POST-HT)	NEO (sintilimab 200 mg q3w, bevacizumab 15 mg/kg q3w)+HT+ADJ (sintilimab 200 mg q3w, bevacizumab 15 mg/kg q3w)	SNV	PRE, POST-NEO	26
96 HCC (4 POST)	NEO (atezolizumab/bevacizumab or nivolumab)+HT	SNV	PRE, POST	27
Part 1: 7 HCC (1 WD POST-TACE) Part 2: 19 HCC (10 WD POST-TACE)	TACE+ADJ (pembrolizumab 200 mg q3w)	CONC	PRE, ON-ADJ, POST-ADJ	28
21 HCC	HT or LT or SYS (nivolumab or lenvatinib or atezolizumab/bevacizumab) or Y90	METH	HT: PRE, POST*2 SYS, Y90: ON*17	29

Abbreviations: ADJ: Adjuvant therapy; cfDNA: Circulating cell-free DNA; CNV: Copy number variation; CONC: Concentration; cyc: Cycles; d: Days; FUS: Fusion; HT: Hepatectomy; INDEL: Insertion and deletion; LT: Liver transplantation; METH: Methylation; NEO: Neoadjuvant therapy; ON: On treatment; ON-ADJ: On adjuvant therapy; POST: Post-treatment; POST-ADJ: Post-adjuvant therapy; POST-HT: Post-hepatectomy; POST-NEO: Post-neoadjuvant therapy; POST-TACE: Post-transarterial chemoembolization; PRE: Pre-treatment; q2w: Every 2 weeks; q3w: Every 3 weeks; SNV: Single-nucleotide variation; SYS: Systemic therapy; TACE: Transarterial chemoembolization; WD: Withdrew; Y90: Y90 radioembolization.

modified RECIST (mRECIST), respectively. Furthermore, throughout the study, cfDNA samples were collected at various timepoints (some patients had incomplete timepoints): pre-treatment, post-neoadjuvant therapy, post-hepatectomy, and post-adjuvant therapy. Xia *et al.*[25] defined a detectable tumor burden as the presence of cfDNA, characterized by detecting at least one somatic mutation, SNVs, CNVs, insertions and deletions, or gene fusions. For patients who achieved an MPR, all had detectable tumor burden pre-treatment (2 out of 2 MPR), which decreased to 50% (1 out of 2 MPR) post-neoadjuvant therapy, and ultimately dropped to 0% (0 out of 2 MPR) post-hepatectomy and post-adjuvant therapy. Similarly, for non-MPR patients, all had detectable tumor burden pre-treatment, which decreased to 80% (8 out of 10 non-MPR) post-neoadjuvant therapy, then to 33.3% (3 out of 9 non-MPR) post-hepatectomy, and 55.6% (5 out of 9 non-MPR) post-adjuvant therapy.[25]

Sun *et al.*[26] recruited 30 patients with resectable HCC for a trial with sintilimab/bevacizumab. This IMT/AAT combination has been approved in China for treating unresectable HCC, so their trial focused on its value in perioperative use in resectable HCC. Over the course of the study, cfDNA samples were collected pre-treatment and post-neoadjuvant therapy (some patients had incomplete timepoints) to assess the tumor burden based on the detected maximum somatic VAFs. The maximum somatic VAF showed a decline from pre-treatment to post-neoadjuvant therapy in 88% (7 out of 8) of patients who achieved a partial response, 60% (9 out of 15) of patients who remained stable, and 33% (1 out of 3) of patients who progressed.[26]

Raj *et al.*[27] conducted a study to demonstrate the use of cfDNA to monitor tumor burden. They recruited 96 patients with advanced HCC. These 96 patients were first either administered the preferred prescription combination (atezolizumab/bevacizumab) or the alternative (nivolumab), in cases of refractoriness or intolerance to atezolizumab/bevacizumab. The detectable tumor burden was defined as the presence of at least one somatic mutation. Out of the 96 patients, 11.5% (11 patients) showed a complete response based on mRECIST criteria, of which four successfully downstaged patients continued to receive hepatectomy. For the four patients who received hepatectomy, cfDNA samples were collected from them pre- and post-treatment. All (4 out of 4) of them had detectable tumor burden pre-treatment, which decreased to 25% (1 out of 4) post-treatment.[27]

Pinato *et al.*[28] conducted a trial involving transarterial chemoembolization (TACE) followed by adjuvant pembrolizumab IMT. In the first part of their trial, seven HCC patients were recruited to assess the safety of TACE followed by pembrolizumab. One patient withdrew after TACE; no dose-limiting toxicities were observed. In the second part of their trial, another 19 HCC patients were recruited; 10 patients withdrew after TACE. Therefore, a total of 15 patients (seven BCLC A and eight BCLC B) completed the treatment of TACE plus pembrolizumab in both parts of the trial. Out of these 15 patients, 53.3% achieved ORR 12 weeks after TACE. CfDNA samples were collected at multiple timepoints (some patients had incomplete timepoints): pre-treatment, on-adjuvant therapy, and post-adjuvant therapy. Tumor burden was determined based on the cfDNA concentration (ng/mL).

For responders, the cfDNA concentration significantly decreased from a median of 0.15 ng/mL pre-treatment to a median of 0.07 ng/mL while on-adjuvant therapy (p=0.0048). Conversely, for non-responders, the cfDNA concentration significantly increased from a median of 0.06 ng/mL pre-treatment to a median of 0.08 ng/mL post-adjuvant therapy (p=0.003).[28]

Angeli-Pahim *et al.*[29] conducted a study on HCC patients undergoing either surgical (5 received resection, while another 5 received transplantation) or non-surgical treatments (9 received systemic therapy, whereas 2 received Y90 radioembolization). CfDNA samples were obtained from these patients at three timepoints: pre-treatment, 1–60 days post-treatment, and 61–230 days post-treatment. The assessment of tumor burden was based on a tumor methylation score (TMS), calculated from the difference in the number of methylated molecules per 1000 genomic equivalents between the plasma and the buffy coat. There was a significant reduction in tumor burden at 1–60 days post-treatment (p=0.008) and further at 61–230 days post-treatment (p=0.031). Meanwhile, among those who underwent non-surgical treatments, cfDNA samples were collected at 17 different timepoints, and tumor burden was evaluated based on the difference in TMS (ΔTMS) between timepoints. The ΔTMS was ≤0 in patients who responded to the treatment, 1–222 in patients who remained stable, and >222 in patients who progressed.[29]

Collectively, these studies demonstrate that cfDNA could reflect tumor burden, showing a trend of decreased tumor burden in patients who responded to the treatment[25-29] and an increased tumor burden in those who progressed.[28,29] This shows that cfDNA can be a valuable tool for monitoring treatment effectiveness. However, the varying methodologies used to measure tumor burden in these studies limit the ability to generalize the findings broadly, as inconsistent measurement approaches can lead to variability in results and interpretations. Consequently, the current evidence underscores the need for future systematic evaluations to standardize measurement protocols. Establishing uniform guidelines would enhance comparability across studies and improve the reliability of cfDNA as a universal biomarker for tumor burden assessment, thereby increasing the generalizability of conclusions and facilitating its integration into clinical practice.

4. Recurrence surveillance perspective

After treatment for HCC, post-treatment management is also crucial due to its high recurrence rates. Even in early-stage HCC patients who received curative surgical resection, the 1-year recurrence rate exceeds 10% and the 5-year recurrence rate reaches 70–80%. Hence, identifying HCC patients with recurrence is crucial to post-treatment management.[30] Here, we summarize a selection of recent studies that have collected pre-treatment, initial post-treatment, and/or serial post-treatment cfDNA samples (Table 3), suggesting that cfDNA could potentially serve as a predictive or monitoring biomarker for HCC recurrence.

In particular, these studies have primarily focused on early HCC recurrence (≤2 years), which commonly originates from minimal residual disease (MRD). Recurrence occurs when disseminated or circulating tumor cells (CTCs) derived from the primary lesion colonize distant sites and form new tumors; therefore, the recurrent tumor inherits mutations from the excised tumor. Consequently, some studies have opted for a tumor-informed approach, which tracks tumor mutations in cfDNA. While this approach improves sensitivity, it may potentially miss other crucial mutations. As a result, some studies have adopted a tumor-uninformed approach.[31,32]

4.1. Likelihood of recurrence assessment

4.1.1. Before treatment

Multiple recent studies have established correlations between pre-treatment cfDNA characteristics and the likelihood of post-treatment recurrence. Fu *et al.*[24] collected pre-treatment cfDNA from 258 HCC patients within a week before they received hepatectomy. Employing a tumor-uninformed approach, they conducted TS on the cfDNA with a universal panel that covers 150 common oncogenes. Through survival analysis, the study revealed that patients with somatic mutations in *APC*, *ARID1A*, *CDKN2A*, *FAT1*, *LRP1B*, *MAP3K1*, *PREX2*, *TERT*, and *TP53* have higher recurrence rates. The 1-year recurrence-free survival (RFS) rates, based on the number of mutated genes in the aforementioned list, are 88.2% for no mutated genes, 68.6% for 1–2 mutated genes, and 31.0% for >2 mutated genes.[24]

Huang *et al.*[31] collected pre-treatment cfDNA from 74 HCC patients on the day immediately preceding their liver transplantation. While WES was performed on the tumor (primarily to evaluate consistency between tumor and cfDNA samples), this study adopted a tumor-uninformed approach by also performing WES on the cfDNA. The study revealed that patients with more than two mutated genes have a higher recurrence rate of 31.7%, compared to those with less than two mutated genes, who have a rate of 11.5% (p=0.0002).[31]

Hu *et al.*[33] collected pre-treatment cfDNA from 88 HCC patients prior to hepatectomy. They employed a tumor-informed approach, where WES was first performed on the tumor. The common hotspots and up to 50 somatic mutations detected in the tumor were used to design a

Table 3. Summary of studies that have established correlations between cfDNA and the likelihood of recurrence

Cohort	Treatment	Tumor -informed or -uninformed	Tumor biomarker (s)	Tumor target panel	cfDNA timepoint (s)	cfDNA biomarker (s)	cfDNA target panel	References
258 HCC	HT	UN	-	-	PRE	SNV	3D Medicines Plasma ctDNA Detection Kit (150 common oncogenes)	24
74 HCC	LT	UN	SNV, CNV	Agilent SureSelect XT Human All Exon V5 (whole exome)	PRE, POST 2-WK, POST-F/U	SNV, CNV	Agilent SureSelect XT Human All Exon V5 (whole exome)	31
88 HCC	HT	IN	SNV	PredicineWES+ (whole exome)	PRE, POST	SNV	PredicineBEACON (common hotspots and up to 50 personalized targets)	33
8 HCC	HT	IN	SNV	SureSelectXT Clinical Research Exome (whole exome)	PRE	SNV	TSO500 (523 common oncogenes)	34
DISCOV-1: 493 HCC DISCOV-2: 24 HCC VAL: 20 HCC (INC. PTA and PTB)	DISCOV-1: UNK DISCOV-2: 14 HT, 10 HT, and TACE VAL: 20 HT	UN	-	-	DISCOV-1: UNK DISCOV-2: POST 1-4-WK VAL: POST 1-WK VAL_PTA: POST 1-WK, POST 8-MTH, POST 9.6-MTH VAL_PTB: POST 1-WK, POST 6-MTH, POST 11-MTH	SNV	3D Medicines (DISCOV-1: 285 381-PANEL, 208 733-PANEL DISCOV-2: 24 381-PANEL or 733-PANEL VAL: 20 381-PANEL or 733-PANEL)	32
66 HCC	HT or LT	Both	SNV	Agilent SureSelect XT Human All Exon V6 (whole exome)	POST	SNV	IN: ~15-20 personalized targets UN: HBV and HCC hotspots include *TP53*, *CTNNB1*, *AXIN1*, and *TERT*	35

Abbreviations: cfDNA: Circulating cell-free DNA; CNV: Copy number variation; ctDNA: Circulating tumor DNA; DISCOV-1: Discovery cohort 1; DISCOV-2: Discovery cohort 2; HT: Hepatectomy; IN: Tumor-informed; INC. PTA and PTB: The validation cohort includes patients A and B; LT: Liver transplantation; MTH: Months; POST: Post-treatment; POST-F/U: Post-treatment at each follow-up; PRE: Pre-treatment; SNV: Single-nucleotide variation; TACE: Transarterial chemoembolization; UN: Tumor-uninformed; UNK: Unknown; VAL: Validation cohort; VAL_PTA: Patient A from the validation cohort; VAL_PTB: Patient B from the validation cohort; WK: Week (s).

personalized TS panel for cfDNA. A plasma sample was categorized as MRD-POS if it contained either ≥3 low-confidence tumor-specific mutations or ≥2 tumor-specific mutations with at least one mutation detected in both strands. In the end, 77 patients were MRD-POS and 11 were MRD-NEG. Of the 77 MRD-POS patients, 12 patients with a tumor fraction ≥10% have a higher recurrence rate (p=0.035). The median RFS for those with a tumor fraction ≥10% stood at 12.2 months, in contrast to 74.3 months for those with a tumor fraction <10%.[33] There was no significant correlation observed between pre-treatment MRD status and RFS. This observation does not align with the findings by Fu *et al.*,[24] who employed a tumor-uninformed approach and analyzed 1-year RFS rates. In comparison, Hu *et al.*[24,33] used a tumor-informed approach with follow-up for more than 6 years. Given that late HCC recurrence (>2 years) is usually *de novo* tumors that emerge in a carcinogenically predisposed microenvironment, these tumors do not carry mutations from the excised tumor.[32] Hu *et al.*'s[24,33] tumor-informed approach may not be well-suited for follow-up periods exceeding 2 years. Reducing the follow-up period in their study might increase the likelihood of identifying statistically significant associations between MRD status and early HCC recurrence (e2 years).

Pommergaard *et al.*[34] collected pre-treatment cfDNA from eight HCC patients before hepatectomy. While the study also utilized a tumor-informed approach, it differed from Hu *et al.*[34] by using a universal panel rather than a personalized one. They focused on analysis-based approaches to identify tumor-specific somatic mutations in cfDNA, which could avoid prolonging turnaround time from designing personalized panels.[32-34] It was found that the only patient with tumor-specific somatic mutations (*TERT* c.124C>T; *CTNNB1* c.101G >T, p.G34V; *EPHA3* c.250A>G, p.T84A; *FLT4* c.187G>T, p.G63*) detected in cfDNA experienced recurrence.[34]

Altogether, patients with somatic mutations (an indication for the presence of circulating tumor DNA [ctDNA], which is a minor subset of cfDNA that originates from tumor cells) detected in pre-treatment cfDNA, that is, those who are pre-treatment ctDNA-POS, demonstrate a trend toward higher recurrence rates.[24,31,33,34] While additional validations are needed, this potentially suggests that being ctDNA-POS before treatment could be beneficial for the detection and diagnosis of HCC. However, it may also correlate with a higher likelihood of recurrence, making it less favorable for overall survival.

4.1.2. After treatment

Several recent studies have also assessed the likelihood of recurrence with either the initial post-treatment cfDNA sample or a series of post-treatment cfDNA samples. They serve to indicate the value of cfDNA, indicating or predicting HCC recurrence. Huang *et al.*[31] also collected an initial post-treatment sample 2 weeks after liver transplantation, along with serial post-treatment samples at each subsequent follow-up (bi-weekly in the first 3 months and every 2–3 months thereafter). The median follow-up period was 16.9 months. Similar to the pre-treatment samples mentioned earlier, these post-treatment samples also underwent tumor-uninformed WES. Based on the initial post-treatment sample, patients with more than two mutated genes have a higher recurrence rate of 46.2%, compared to those with fewer than two mutated genes, who have a rate of 21.3% (p<0.001). Similarly, based on the serial post-treatment samples, patients with more than two mutated genes detected at any time point exhibit a higher recurrence rate of 44.4% compared to those with less than two mutated genes, who have a rate of 14.9% (p<0.001).[31]

Xu *et al.*[31] conducted a study comprising two discovery cohorts and one validation cohort. The first discovery cohort consists of 493 HCC patients with samples collected at an unspecified time point. In the second discovery cohort, 24 HCC patients underwent either hepatectomy (14 patients) or hepatectomy followed by TACE (10 patients), with samples obtained 1–4 weeks post-treatment. They remained recurrence-free for >2 years. The validation cohort includes 20 HCC patients who underwent hepatectomy, with samples collected a week post-treatment. In addition, two patients in the validation cohort also had samples collected at 8 months and 9.6 months, or 6 months and 11 months. These samples underwent TS with a 381- or 733-gene panel. Their study identified frequently mutated genes, occurring in more than 5% and 10% of cases in the first and second discovery cohorts, respectively. Given that the first discovery cohort represents the overall HCC population, and the second discovery cohort represents HCC patients who exhibited persistent treatment effect, the discordant gene list specifically present in the first discovery cohort likely indicates recurrence-related targets. This results in a final marker gene list (*TP53*, *TERT*, *CTNNB1*, *APC*, *RMB10*, *NTRK3*, *NOTCH1*, *NOTCH2*, *NF1*, *CREBBP*, *GLI3*, *CDKN2A*, and *EZH2*) for further evaluation. In the validation cohort, six and 14 patients were categorized as MRD-POS and MRD-NEG, respectively, based on whether a somatic mutation was detected in any of the genes in the final marker list. Ultimately, all patients in the MRD-POS category and two patients in the MRD-NEG category experienced HCC recurrence (sensitivity of 75% and specificity of 100%). Moreover, for the two patients with additional post-treatment samples at later time points, both of them experienced recurrence. One patient

was POS at all time points, while the other patient was first NEG and then became POS at the later time points.[32]

Hu *et al.*[33] collected pre-treatment cfDNA from 88 HCC patients, as mentioned earlier. Their study also collected a post-treatment sample within 1 week after hepatectomy. These post-treatment samples also underwent tumor-informed personalized TS (common hotspots and up to 50 tumor-specific mutations). A sample was regarded as MRD-POS if it contained either ≥3 low-confidence tumor-specific mutations or ≥2 tumor-specific mutations with at least one mutation detected in both strands. Ultimately, there were 36 MRD-POS and 52 MRD-NEG cases. The median RFS for MRD-POS patients was 17.1 months, whereas it was not reached for MRD-NEG patients (p=0.0013). Furthermore, among the 36 MRD-POS patients, 12 patients with a tumor fraction ≥0.1% have a higher recurrence rate (p=0.0016). The median RFS for those with a tumor fraction ≥0.1% is 7.3 months, in contrast to 25.4 months for those with a tumor fraction <0.1%.[33]

Zhao *et al.*[35] recruited 59 HCC patients who underwent hepatectomy and 21 HCC patients who underwent liver transplantation. Out of a total of 80 patients, an initial post-treatment sample was collected from 66 patients. These samples were subjected to CTCs and ctDNA detection. Samples were CTC-POS if CTCs could be positively selected by the asialoglycoprotein receptor in a microfluidic system and identified using pancytokeratins. For ctDNA detection, samples were subjected to both tumor-informed (~15 somatic mutations from tumor WES) and tumor-uninformed (HBV and HCC hotspots include *TP53*, *CTNNB1*, *AXIN1*, and *TERT*) TS. For the tumor-informed approach, samples were ctDNA-POS if ≥1 tumor-specific mutation was detected. For the tumor-uninformed approach, samples were considered ctDNA-POS if ≥ 1 somatic mutation or HBV integration was detected. CTC-POS, tumor-informed ctDNA-POS, or tumor-uninformed ctDNA-POS were all significantly correlated with worse RFS (all: p<0.0001, CTC-POS: sensitivity of 75% and specificity of 86.8%, tumor-informed ctDNA-POS: sensitivity of 70.4% and specificity of 93.8%). The double-NEG (CTC-NEG and tumor-informed ctDNA-NEG) patients had better RFS than single-POS or double-POS patients (p<0.0001). The median RFS for CTC-POS, ctDNA-POS, and double-POS patients were 19.2, 6.57, and 5.25 months, respectively, whereas the median RFS for double-NEG patients was not reached.[35]

Altogether, patients with somatic mutations in the initial post-treatment sample, those who are ctDNA-POS, demonstrate a trend toward a higher recurrence rate[31,33] or worse RFS.[33,35] While the initial post-treatment sample was informative, the significance of serial post-treatment samples should not be underestimated. Huang *et al.*[31] demonstrated that patients tested ctDNA-POS at any time point exhibit a higher recurrence rate. Xu *et al.*[32] also reported that a patient who was first tested ctDNA-NEG, and later tested ctDNA-POS, was diagnosed with HCC recurrence. Future investigation should focus more on serial post-treatment samples and standardize aspects, such as sample collection intervals and clinical management guidelines.

5. Future perspectives

To date, there is no Food and Drug Administration (FDA)-approved liquid biopsy test for HCC, highlighting a significant gap in non-invasive diagnostic and monitoring options for this disease. In this section, we will review insights from FDA-approved liquid biopsies in other cancers. Furthermore, we will also discuss future directions in cfDNA research for HCC, highlighting potential strategies to advance this field.

5.1. Food and Drug Administration-approved liquid biopsies in other cancers

The United States FDA has approved several liquid biopsy tests, including the cobas EGFR Mutation Test v2, FoundationOne Liquid CDx, Guardant360 CDx, Epi proColon, and Shield (Table 4). Notably, the first three tests were companion diagnostics, which were approved alongside drugs targeting specific genetic alterations. The cobas EGFR Mutation Test v2 was approved for the detection of *EGFR* exon 19 deletions (19del) or exon 21 substitution mutations (L858R) to identify non-small cell lung cancer (NSCLC) patients eligible for treatment with TARCEVA (erlotinib).[36] The FoundationOne Liquid CDx was approved to detect genetic alterations in NSCLC, prostate cancer, breast cancer and solid tumors: *ALK* rearrangements, *EGFR* 19del and L858R, *EGFR* 20ins, *MET* Δ14, *ROS1* fusions, *BRCA1* and/or *BRCA2* alterations, *BRCA1* and *BRCA2* and/or *ATM* alterations, *PIK3CA* (C420R, E542K, E545A, E545D, E545G, E545K, Q546E, Q546R, H1047L, H1047R, and H1047Y) alterations, and *NTRK1/2/3* fusions. These identified genetic alterations make patients eligible for treatment with ALECENSA (alectinib), *EGFR* tyrosine kinase inhibitors approved by the FDA, EXKIVITY (mobocertinib), TABRECTA (capmatinib), ROZLYTREK (entrectinib), RUBRACA (rucaparib), LYNPARZA (olaparib), PIQRAY (alpelisib), and ROZLYTREK (entrectinib), respectively.[37] The Guardant360 CDx was approved to detect genetic changes in NSCLC and breast cancer: *EGFR* 19del and L858R and T790M, *EGFR* 20ins, *ERBB2* activating mutations, *KRAS* G12C, and *ESR1* missense mutations between codons

Table 4. Summary of Food and Drug Administration-approved liquid biopsy tests

Test	Cancer type	Specimen	Genetic change (s)	Corresponding drug	References
cobas EGFR Mutation Test v2 (Roche Molecular Systems)	Non-small cell lung cancer	Plasma or FFPE tissue	*EGFR* 19del or L858R	TARCEVA (erlotinib)	36
FoundationOne Liquid CDx (Foundation Medicine)	Non-small cell lung cancer	Plasma	*ALK* rearrangement	ALECENSA (alectinib)	37
			EGFR 19del and L858R	*EGFR* tyrosine kinase inhibitors approved by FDA	
			EGFR 20ins	EXKIVITY (mobocertinib)	
			*MET*Δ14	TABRECTA (capmatinib)	
			ROS1 fusions	ROZLYTREK (entrectinib)	
	Prostate cancer		*BRCA1* and/or *BRCA2*	RUBRACA (rucaparib)	
			BRCA1, *BRCA2*, and/or *ATM*	LYNPARZA (olaparib)	
	Breast cancer		*PIK3CA* C420R, E542K, E545A, E545D, E545G, E545K, Q546E, Q546R, H1047L, H1047R, and H1047Y	PIQRAY (alpelisib)	
	Solid tumors		*NTRK1/2/3* fusions	ROZLYTREK (entrectinib)	
Guardant360 CDx (Guardant Health)	Non-small cell lung cancer	Plasma	*EGFR* 19del, L858R, and T790M	TAGRISSO (osimertinib)	38
			EGFR 20ins	RYBREVANT (amivantamab-vmjw)	
			ERBB2 activating mutations	ENHERTU (fam-trastuzumab deruxtecan-nxki)	
			KRAS G12C	LUMAKRAS (sotorasib)	
	Breast cancer		*ESR1* missense mutations between codons 310–547	ORSERDU (elacestrant)	
Epi proColon (New Day Diagnostics)	Colorectal cancer	Plasma	Methylated *SEPT9*	-	40
Shield (Guardant Health)	Colorectal cancer	Plasma	Integrating genomics, epigenomics, and proteomics	-	39

Abbreviations: EGFR: Epidermal growth factor receptor; FFPE: Formalin-fixed paraffin-embedded.

310–547. These genetic changes qualify for treatment with TAGRISSO (osimertinib), RYBREVANT (amivantamab-vmjw), ENHERTU (fam-trastuzumab deruxtecan-nxki), LUMAKRAS (sotorasib), and ORSERDU (elacestrant), respectively.[38] These three tests underscore the importance of aligning detection and treatment to ensure actionable outcomes for the patient.

On the other hand, Epi proColon and Shield were approved for screening individuals at moderate risk for CRC as second-line options, indicating that they are recommended for patients who declined the colonoscopy option. While colonoscopy is considered the gold standard option, some individuals may be reluctant to undergo the procedure due to its invasive nature, the time-consuming preparation (including a full day of preparation and post-sedation recovery), and associated discomfort.[39-41] Hence, second-line options were introduced to promote screening efforts. Epi proColon was approved for detecting *SEPT9* methylation, while Shield was approved for integrating genomics, epigenomics, and proteomics to identify abnormal cancer signals. Importantly, if the results of these tests are POS, individuals are still required to undergo a colonoscopy.

5.2. Limitations and prospects of HCC liquid biopsy in clinical translation

Based on the previous section, there are two categories of FDA-approved liquid biopsies in other cancers: those designated as companion diagnostics and those designed to increase screening motivations. Companion diagnostics link genetic alterations to the corresponding targeted treatments. However, current HCC treatments do not rely on genetic information to guide treatment decisions. According to the clinical guidelines from the AASLD and the European Association for the Study of the Liver, patients with early to intermediate-stage HCC are recommended to undergo treatments, such as ablation, resection, transplantation, or locoregional therapies. These treatment options primarily consider several factors,

such as the number of tumor nodules, tumor size, and liver function. Systemic therapies are recommended for patients with advanced unresectable HCC. Several first-line systemic therapy options are available, including atezolizumab/bevacizumab, tremelimumab/durvalumab, sorafenib, or lenvatinib. The selection of first-line systemic therapy depends on various factors, such as treatment availability, potential contraindications (e.g., autoimmune disorders and liver transplantation), and potential risks (e.g., gastrointestinal or esophageal bleeding).[42,43] The selection of first-line systemic therapy does not currently factor in genetic alterations, likely because, unlike other cancers with actionable genetic alterations, HCC lacks well-defined, highly recurrent, and druggable targets. The main mutation drivers in HCC, such as *TERT*, *CTNNB1*, and *TP53*, are still considered undruggable at present.[44] Overall, current HCC treatment options do not target genetic alterations. Therefore, while liquid biopsies can detect genetic alterations and inform treatment decisions in other cancers, they cannot be seamlessly integrated into the treatment routine of HCC.

Cost-effectiveness regarding the choice of technological platform in the assay is another important issue to be considered during the translational application of liquid biopsy. For instance, while EM-seq may represent a promising technology compared to the traditional BS-seq for detecting DNA methylations in cfDNA, the higher initial costs of specialized reagents and enzymes make EM-seq a more expensive option. Unless there is more consolidated evidence supporting the potentially superior performance of EM-seq on a limited amount of cfDNA, the marked difference in the costs between technological platforms may limit their practical applicability.

Detection of false POSs is also a common but inevitable issue that frequently happens in cfDNA studies. Molecular alterations may arise at different disease stages, and background noise from non-HCC stages may lead to false detection or diagnosis. This raises an important issue of identifying truly HCC-specific cfDNA biomarkers that pinpoint a specific HCC stage. Moreover, large-scale studies with multi-center and multi-ethnicity validation are also awaited.

Nevertheless, some studies in this review have explored the feasibility of leveraging cfDNA to guide treatment decisions for HCC. Cheung *et al.*[22] investigated cfDNA biomarkers that can predict patients' response to pre-operative nivolumab treatment, while Coto-Llerena *et al.*[23] investigated the potential of cfDNA in guiding treatment decisions for multinodular HCC. Although these investigations revealed promising potential, further research is crucial to develop toward routinely and effectively using cfDNA to inform and guide treatment decisions. In particular, to explore its capabilities in predicting responses to drugs and differentiating forms of multinodular HCC.

Another valuable perspective gained from FDA-approved liquid biopsies in other cancers is their capacity to enhance screening motivations. In the context of HCC, traditional tissue biopsies are seldom conducted for screening due to their invasive nature, risk of tumor seeding, and the constraints of regional sampling. Recent findings indicate the correlation between tissue biopsies and liquid biopsies in various human cancers, including HCC,[4] suggesting that liquid biopsies can accurately and sensitively mirror the true biological characteristics found in tumor tissue. Moreover, liquid biopsies have the potential to mitigate the inherent limitations associated with tissue biopsies. This advantage undoubtedly can facilitate the integration of liquid biopsies into clinical practice.

5.3. Current trends and future perspectives in liquid biopsy for HCC

Building on the role of liquid biopsy as a companion diagnostic and an enhanced screening motivation tool, as demonstrated by FDA-approved tests in other cancers, the studies involved in this review have showcased additional promising HCC-related applications. These include early-stage detection, treatment monitoring, and surveillance for recurrence. Given that HCC is frequently diagnosed at advanced stages due to the limitations of routine ultrasound and AFP surveillance, several studies[9,11-14] have endeavored to leverage cfDNA for early-stage diagnosis. Studies in this area are trending toward integrating multiple genetic biomarkers (such as somatic mutations, HBV integrations, fragmentomics, and methylation patterns) alongside protein biomarkers (such as AFP, AFP-L3, and PIVKA-II) to develop HCC diagnostic scoring models. However, as previously mentioned, no single approach has demonstrated full sensitivity in early-stage diagnosis; sensitivities tend to be lower for early stages and higher for more advanced stages.[11,12] Nevertheless, a range of study designs,[9,15] biomarkers,[13,20,21] and technology based[14] strategies have been employed to optimize early detection of HCC. However, conclusive evidence demonstrating their genuine effectiveness in enhancing early detection remains elusive. Therefore, further validation of these strategies is imperative.

Multiple studies within this review have indicated that cfDNA can mirror tumor burden, revealing a pattern of reduced tumor burden in patients who exhibited a POS response to the treatment[25-29] and increased tumor burden in those who progressed.[28,29] This demonstrates

the potential of cfDNA as a valuable tool in monitoring treatment effectiveness. Furthermore, it is crucial to highlight that these studies adopted varying approaches to measure tumor burden for monitoring treatment effectiveness. While two studies defined tumor burden through the detection of at least one somatic mutation,[25,27] one determined it based on the difference in maximum somatic VAFs,[26] others determined it based on cfDNA concentration,[28] and the difference in the number of methylated molecules between the plasma and the buffy coat.[29] The optimal measurement of tumor burden remains uncertain, emphasizing the need for future systematic evaluation to standardize measurement and establish guidelines for treatment management based on these measurements.

Finally, several studies in this review have demonstrated a higher recurrence rate associated with the presence of ctDNA from pre-treatment or any post-treatment samples.[24,31-34] This underscores its capacity to inform recurrence. Nonetheless, the sample collection intervals for monitoring recurrence vary across studies. This poses challenges in maintaining consistent patient follow-up and highlights the crucial need to standardize collection intervals, ensuring uniform follow-up procedures.

5.4. Advancing standardization of liquid biopsy in HCC

Overall, the promising potential of liquid biopsy in HCC management hinges on the establishment of systematic evaluation and standardized protocols. The varying biomarkers and ML algorithms used in building diagnostic scoring models, and the different thresholds used for defining HCC positivity across models, pose significant challenges to generalizing conclusions. Variability in measurements of tumor burden to monitor treatment and differences in cfDNA sample collection intervals to monitor recurrence further contribute to inconsistencies across studies. These variabilities hamper the ability to generalize and reproduce results, which in turn limits the integration of liquid biopsy into routine clinical practice.

To address these challenges, the development of standard guidelines is critical. Standardization would enhance comparability across studies, improve the reliability of cfDNA as a universal biomarker in pan-cancer management. Recognizing this need, organizations, such as the International Liquid Biopsy Standardization Alliance (ILSA), have emerged. ILSA and its members recognize that the diverse technologies and goals in the field of liquid biopsy may confound the field and obscure crucial advancements. One of its members, the International Society of Liquid Biopsy (ISLB), aims to establish clear criteria, standard operating procedures (SOPs), reference standards, and guidelines for the design, development, and validation of studies, ensuring that the clinical applications of liquid biopsy are supported by strong evidence.[45] Moreover, the ISLB also emphasizes that multicenter comparison studies are essential for developing robust workflows and establishing SOPs that address variability in clinical applications.[46] The guidelines may enhance the reliability and reproducibility of liquid biopsy technologies, ultimately accelerating their adoption into pan-cancer management. They should be considered in future research to ensure continued advancement and standardization in the field.

6. Conclusion

In conclusion, we anticipate that the future clinical integration of HCC liquid biopsy will be facilitated by its non-invasive nature and resemblance to tissue biopsies. Nevertheless, expanding its clinical utilization will require additional research on HCC cfDNA, in particular, systematic evaluation and standardization. This is essential due to the diversity in methodologies: varying biomarkers and ML algorithms used in building the diagnostic scoring models, different thresholds used for defining HCC positivity across models, distinct approaches to measuring tumor burden for treatment monitoring, and inconsistent cfDNA sample collection intervals for monitoring recurrence across studies. The most optimal methodologies remain to be determined.

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Conflict of interest

Daniel Wai-Hung Ho is an Editorial Board Member of this journal and serves as the Guest Editor of this special issue, but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Separately, other authors declared that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Author contributions

Conceptualization: Zita Hoi-Ying Chan, Daniel Wai-Hung Ho
Supervision: Daniel Wai-Hung Ho
Writing–original draft: Zita Hoi-Ying Chan
Writing–review & editing: Xueying Lyu, Daniel Wai-Hung Ho

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