Liquid biopsy in the clinical management of hepatocellular carcinoma

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Received 8 April 2020
Revised 21 July 2020
Accepted 27 July 2020
Published Online First
3 September 2020

To cite: von Felden J, Garcia-Lezana T, Schulze K, *et al*. *Gut* 2020;**69**:2025–2034.

ABSTRACT

With increasing knowledge on molecular tumour information, precision oncology has revolutionised the medical field over the past years. Liquid biopsy entails the analysis of circulating tumour components, such as circulating tumour DNA, tumour cells or tumour-derived extracellular vesicles, and has thus come as a handy tool for personalised medicine in many cancer entities. Clinical applications under investigation include early cancer detection, prediction of treatment response and molecular monitoring of the disease, for example, to comprehend resistance patterns and clonal tumour evolution. In fact, several tests for blood-based mutation profiling are already commercially available and have entered the clinical field.

In the context of hepatocellular carcinoma, where access to tissue specimens remains mostly limited to patients with early stage tumours, liquid biopsy approaches might be particularly helpful. A variety of translational liquid biopsy studies have been carried out to address clinical needs, such as early hepatocellular carcinoma detection and prediction of treatment response. To this regard, methylation profiling of circulating tumour DNA has evolved as a promising surveillance tool for early hepatocellular carcinoma detection in populations at risk, which might soon transform the way surveillance programmes are implemented. This review summarises recent developments in the liquid biopsy oncological space and, in more detail, the potential implications in the clinical management of hepatocellular carcinoma. It further outlines technical peculiarities across liquid biopsy technologies, which might be helpful for interpretation by non-experts.

Liquid biopsy for early detection of hepatocellular carcinoma (HCC) in patients at risk

- Methylation profiling from plasma DNA has proven useful to discriminate patients with early stage HCC from patients at risk.
- Clinical trials testing the performance of these biomarkers in cirrhotic patients against standard surveillance with ultrasound±alpha fetoprotein (AFP) are ongoing.
- Also, early studies suggest that composite biomarkers of mutations in circulating tumour DNA and tumour markers, such as AFP and des-γ-carboxy-prothrombin, are able to identify HCC.

Potential future applications of liquid biopsy for the clinical management of HCC

- Prognostication, for example, by molecular analyses of circulating tumour cells, circulating tumour DNA and/or extracellular vesicles.
- Predicting risk of recurrence or detection of minimal residual disease after resection, for example, by detection of circulating tumour cells and/or circulating tumour DNA analyses. These markers might improve clinical trial design for testing adjuvant therapies.
- Prediction of response to systemic therapies, for example, by molecular analyses of extracellular vesicles and/or mutations from circulating tumour DNA.
- Monitoring treatment response/tumour burden, and identification of emerging clones of resistance to systemic therapies, for example, by mutation analyses from circulating tumour DNA.

INTRODUCTION

The incidence of hepatocellular carcinoma (HCC), the most frequent form of primary liver cancer, is on the rise worldwide.[1] The WHO projects more than 1 million deaths due to HCC in 2030. Unlike most solid tumours, HCC generally develops in the background of chronic liver disease, mainly cirrhosis, as a result of viral hepatitis (B or C), alcohol use disorder or non-alcoholic fatty liver disease. The annual risk of HCC in patients with cirrhosis is between 2% and 4%,[2] which renders cancer surveillance in these patients cost-effective.[3] Thus, clinical practice guidelines recommend bi-annual surveillance in patients at high risk with abdominal ultrasound (US) and serum alpha fetoprotein (AFP).[3 4] Surveillance aims at identifying tumours at early stages, when patients can be potentially cured with surgical therapies.[5] This has shown to increase survival in uncontrolled studies.[5] However, the accuracy of US and AFP for early HCC detection is suboptimal. A recent meta-analysis of pooled data including 13 000 patients found an aggregate sensitivity of this approach of 63% for the detection of early stage HCC.[6] This would miss close to 40% of potentially curable HCC patients. Besides suboptimal performance, the implementation of surveillance programmes among patients at high risk is very low. In the USA, less than 25% of patients at

risk are enrolled in HCC surveillance,[7] with significant differences if patients are followed in primary or subspecialty care. Overall, there is an urgent clinical need to both improve the accuracy of tools for early HCC detection and to increase its implementation rate.

Major advances have occurred in the management of HCC patients at advanced stages. After 10 years of negative clinical trials following sorafenib's approval,[8] six systemic therapies have shown clinical efficacy in the context of randomised phase 3 trials both in first line (combination of atezolizumab and bevacizumab,[9] lenvatinib[10]) and second line (regorafenib,[11] cabozantinib[12] and ramucirumab[13]). However, most of these trials were conducted in parallel, which precluded direct face-to-face comparisons. As a result, the optimal sequence of therapies that a patient should receive with maximal efficacy and minimal toxicity remains unclear. Only AFP higher than 400 ng/dL is a bona fide biomarker of response to ramucirumab after an ad hoc clinical trial.[13] The efficacy of immune checkpoint inhibitors (CPI) was also evaluated in monotherapy, and despite promising results in phase 2 trials with objective response rates (ORR) nearing 20%,[14 15] phase 3 trials failed to meet the primary endpoints.[16 17] Combination of the programmed cell death ligand 1 (PD-L1) inhibitor atezolizumab with the vascular endothelial growth factor monoclonal antibody bevacizumab significantly increases survival compared with sorafenib in first line, with ORR close to 30%.[9] Despite being repeatedly challenged, this is the first time that a drug outperforms sorafenib in first line and underscores the clinical efficacy of CPI in HCC. There are no biomarkers able to identify these patients who will benefit from this combination, which could spare unnecessary toxicity to those unlikely to respond. An additional limitation in HCC is the limited access to tissue samples for biomarker studies since most patients are diagnosed with imaging. Thus, liquid biopsy has emerged as a novel tool for biomarker development to specifically address these two clear-cut clinical problems: early HCC detection and prediction of treatment response. Another crucial advantage of liquid biopsy is its simplicity in terms of sample procurement, which could favourably impact implementation of surveillance and enables sequential sampling; this opens the door for real-time molecular monitoring of HCC.

AN OVERVIEW OF LIQUID BIOPSY

The concept of liquid biopsy refers to the release and molecular analysis of tumour components, mostly nucleic acids, circulating tumour cells (CTCs) and extracellular vesicles (EVs), which are released by tumours to the bloodstream or other body fluids[18] (figure 1). EVs are membrane-embedded nanovesicles that are actively released by all types of cells and function in cell-to-cell communication.[19 20] The presence of circulating nucleic acids in the blood has been known for decades,[21] but its application in clinical settings, particularly in oncology, significantly increased over the last 5 years.[22] This technology was initially developed in prenatal medicine to test for chromosomal aberrations in the unborn child.[23–25] The coincidental detection of DNA aberrations in cell-free DNA coming from undiagnosed cancers of pregnant women[26] has further underscored the potential for its application in oncology. In 2016, the Food and Drug Administration (FDA) approved the first diagnostic tool to detect druggable *EGFR* mutations in plasma of lung cancer patients,[27] thus directly impacting treatment decisions. With the new revolution in immuno-oncology, an obvious need is to identify patients who respond to these treatments. Besides PD-L1 expression on tumour tissue, tumour mutational burden has been confirmed in several phase 3 clinical trials as a predictive biomarker to immune checkpoint inhibition in some tumour types.[28] Tumour mutational burden is assessed by genome-wide mutation analysis of the tumour tissue. This is less feasible in cell-free DNA, because tumor-derived DNA represents a much smaller fraction of cell-free DNA compared with tumour tissue, which results in a much higher sequencing depth needed to confidently call mutations. A recent study in advanced lung cancer revealed that a targeted gene panel of 150 frequently mutated genes was enough to accurately determine the tumour mutational burden found in corresponding tumour tissue.[29] Further, the tumour mutational burden derived from plasma DNA was predictive of response to immune checkpoint inhibition,[29] thus removing the need for a tissue biopsy. Similarly, PD-L1 expression on EVs (ie, exosomes), which reportedly correlates with the cell surface of the tumour cell of origin, predicted better response to immune checkpoint inhibition in a small study on patients with melanoma.[30] In long-term responders to immune checkpoint inhibition (>24 months), detection of circulating tumour DNA (ctDNA) was able to identify patients with minimal residual disease who progressed later on, thus facilitating personalised treatment decisions in these patients.[31]

However, the implications of liquid biopsies are not limited to patients with advanced stage disease. Lately, several prospective biomarker studies have also confirmed the utility of sequential sampling to detect minimal residual disease after curative treatments in patients with colorectal or lung cancer[32–34] (level of evidence 2, figure 2). Interestingly, the detection of ctDNA after surgery preceded the radiological detection of recurrence by 3–5 months in non-metastatic colon cancer and localised lung cancer, respectively.[33 34] In locally advanced colorectal and rectal cancer, prospective sequential sampling of ctDNA was able to discriminate patients with very high risk of recurrence after resection from those with low risk, thus identifying patients who might benefit from adjuvant therapy[35 36] (level of evidence 2, figure 2). These studies indicate potential clinical applications of liquid biopsies even at earlier cancer stages. However, if patients with detectable ctDNA before or after surgical treatments benefit from adjuvant treatments is currently under investigation, for example, the phase 3 CIRCULATE study for patients with colon cancer UICC stage II (ClinicalTrials.gov Identifier: NCT04089631).

Nevertheless, one of the major goals in oncology remains early cancer detection when patients are potentially curable. A large study including >1000 patients with different non-metastatic cancer types (70% AJCC stage I or II) and >800 healthy controls, found high rates of cancer detection using a combined panel of mutations in plasma DNA and known protein markers (ie, tumour markers) with sensitivity ranging from 69% to 98% and a specificity of 98%[37] (level of evidence 3, figure 2). In addition, the test was able to identify the anatomic site of the tumour in the majority of patients based on the profile of plasma DNA mutations and tumour markers.[37] Similarly, a genome-wide approach on cell-free DNA was able to identify cancer-specific DNA fragmentation patterns that discriminate patients with or without cancer with sensitivities of detection ranging from 57% to more than 99% among the seven cancer types tested and a specificity of 98%.[38] This machine learning based algorithm also adequately predicted the tissue of origin in 75% of cases.[38] Aneuploidy has recently been suggested as a context-dependent, cancer-type-specific oncogenic event that could have clinical relevance as a prognostic marker.[39] The application of repeated element sequencing on cell-free DNA of >800 non-metastatic cancer

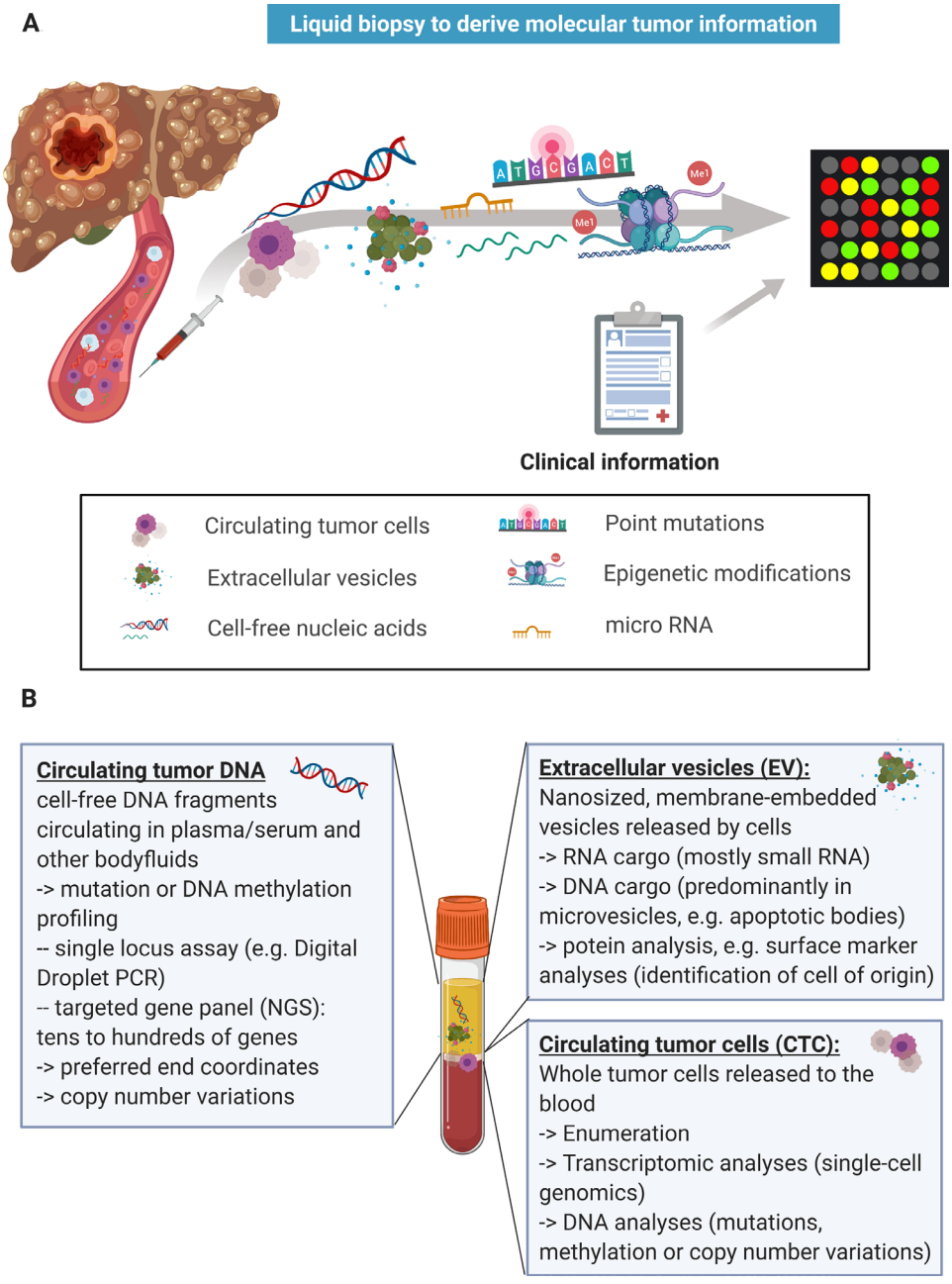

Figure 1 Schematic concept and analytical properties of liquid biopsy. (A) Tumour components, such as circulating nucleic acids, tumour cells and extracellular vesicles are released by tumours to the bloodstream. These are readily available for further molecular analysis. (B) Summary of each component and molecular analysis that can be performed. NGS, next-generation-sequencing.

patients identified aneuploidy in 49% of liquid biopsies.[40] Combining these with somatic mutations and tumour markers yielded a median sensitivity of 80% across eight cancer types at a specificity of 99% to discriminate cancer patients from healthy controls.[40] However, caution needs to be taken in the analysis of somatic mutations from cell-free DNA as a recent study has confirmed that a high number of mutations in cell-free DNA have features from clonal hematopoiesis (~81% in controls and~53% in patients with cancer).[41] Further, commercially available tests for plasma mutation profiling might render discordant results.[42] These findings underscore the need for standardised isolation protocols to avoid preanalytical contamination, as well as validated in silico analytical pipelines.

CTDNA ANALYSIS IN HCC

Mutation profiling of ctDNA

Given the limited access to tissue specimens for HCC patients not treated with resection, cell-free DNA analysis might be particularly helpful for the management of HCC as it provides access to tumour molecular alterations using a blood sample. It could also allow serial monitoring of tumour DNA over time, including the identification of emergent mutations driving acquired resistance, and capturing intratumoral heterogeneity (ITH). A recent study in gastrointestinal cancers shows how profiling of ctDNA reflects the acquired resistance and ITH better than single-lesion tumour biopsies.[43] This study evaluated 42 patients with stable disease or partial response to targeted therapy, including 23 patients with tumour biopsy at the time of progression.[43] In fact, 78% of the

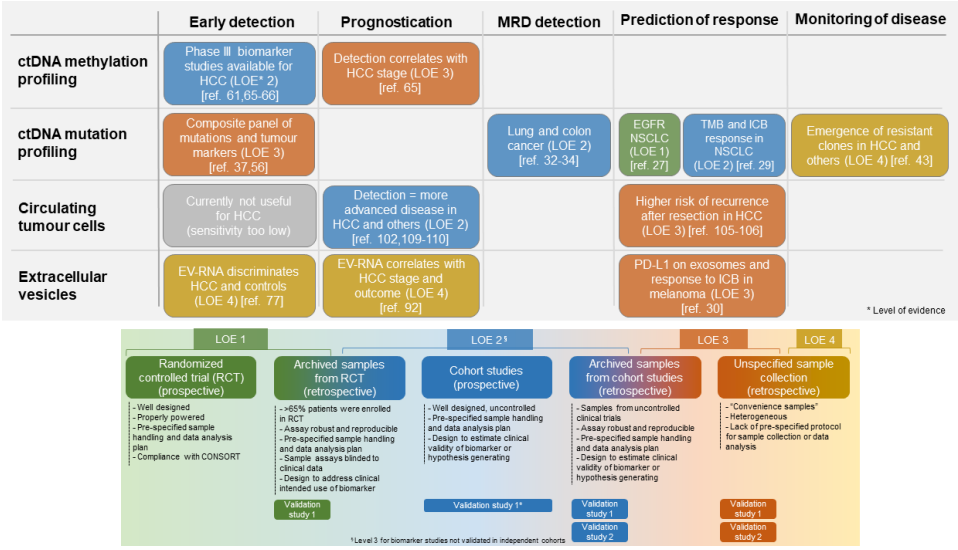

Figure 2 Landscape and level of evidence of available liquid biopsy studies across different clinical implications. Upper panel shows examples for available liquid biopsy studies in HCC or other cancer entities with level of evidence (LOE) as defined in the lower panel (adapted from Simon *et al*[69]). ctDNA, circulating tumour DNA; EGFR, extracellular growth factor receptor; EV, extracellular vesicle; HCC, hepatocellular carcinoma; ICB, immune checkpoint blockade; MRD, minimal/molecular residual disease; NSCLC, non-small cell lung cancer; PD-L1, programmed cell death ligand 1; TMB, tumour mutational burden.

patients had at least one resistance mutation identified in ctDNA after progression, while these mutations were only detected in matched biopsies in 48% of the patients.[43]

Detection of ctDNA is challenging not only because it requires sensitive and specific techniques but also because special precautions must be taken during sample collection to ensure cell-free DNA stability. Technologies for ctDNA analysis can be broadly classified into single-target assays (eg, Digital Droplet PCR) for detection of aberrations limited to a single nucleotide of interest (eg, point mutations or methylation changes) or next-generation-sequencing (NGS) based assays which include whole-genome or whole-exome sequencing (WES) or targeted sequencing of a gene panel of interest. Importantly, the larger the gene panel of interest, the higher the sequencing effort needed to meet the required lower limit of detection of mutations (ie, the lowest variant allele frequency (VAF) at which a variant can be detected with enough confidence as opposed to an inherent sequencing error). Therefore, the sensitivity to detect low frequency variants is inversely proportional to the size of the gene panel for a given sequencing effort (ie, sequencing costs) (figure 3A). On the other hand, small gene panels obviously limit the number of patients with detectable mutations (eg, *TP53* mutations are only present in ~30% of patients with HCC[1]). This partially explains the reported differences in performance and accuracy for ctDNA studies, and represents a key element in proper study design depending on the outcome of interest.

One of the first studies exploring cell-free DNA in HCC was specifically focused on the determination of Ser-249 mutation of *TP53*, a well-defined hotspot in HCC on Aflatoxin exposure in West African populations, using restriction fragment length polymorphism.[44] In a follow-up study in the same cohort, authors found a concordance of 88% between plasma and matched tumour pairs,[45] confirming the feasibility of ctDNA analysis in HCC. With the broader implementation of NGS technologies, a better understanding of the mutational landscape of HCC has allowed a more comprehensive analysis of ctDNA. A series of proof of concept studies addressed, with different approaches, the performance of ctDNA to accurately capture the mutations present in HCC tissue. The analysis of 32 multiregional HCC tissue specimens from five patients together with matched ctDNA by WES and targeted deep sequencing (TDS) showcases the challenges of ctDNA analysis.[46] Due to the relatively small fraction of tumour DNA among all cell-free DNA, and the relatively high sequencing effort needed for whole exome approaches, this study was able to validate only 18% of the mutations detected in tissue when using WES on ctDNA (median sequencing depth 226×). This number increased to 84% when applying TDS with a smaller panel of genes and higher sequencing depth (median 1807×).[46] On the other side, only 47% of mutations that were detected in ctDNA with TDS could be validated in corresponding tissue, which could be due to a high rate of false positives or ITH.[46] Another exploratory approach, including 30 patients, defined somatic mutations in cell-free DNA and HCC tissue independently.[47] To detect mutations at low frequencies, authors examined the data 'by interrogation' for all mutations detected in the biopsy/cell-free DNA counterpart. By using a targeted panel of 46 genes, they captured at least one mutation in ctDNA in 27% of the cases, and a total of 63% including the ones detected by interrogation.[47] We evaluated 24 multiregional tissue samples from eight early stage HCC patients and matched blood samples by ultra-deep sequencing using a targeted panel of 58 genes.[48] Twenty-one somatic mutations were found in HCC tissue in six out of the eight patients, of which 71% were also detected in cell-free DNA.[48] A more recent study, using a much smaller targeted panel of only eight genes, analysed ctDNA from 51 patients with HCC and found mutations in 35% of patients.[49] In eight patients with paired tissue available, all mutations found in ctDNA were confirmed in paired tissue.[49] However, 71% of tissue mutations were not found in ctDNA, indicating poor sensitivity for this approach.[49]

A recent study has reported the detection of recurrent mutations in non-tumoral adjacent tissue of HCC patients.[50] This has raised concerns regarding the certainty that mutations detected in plasma are actually derived from the tumour. However, there are two points that should be considered when interpreting the data: (1) The VAF of detected mutations was generally very low

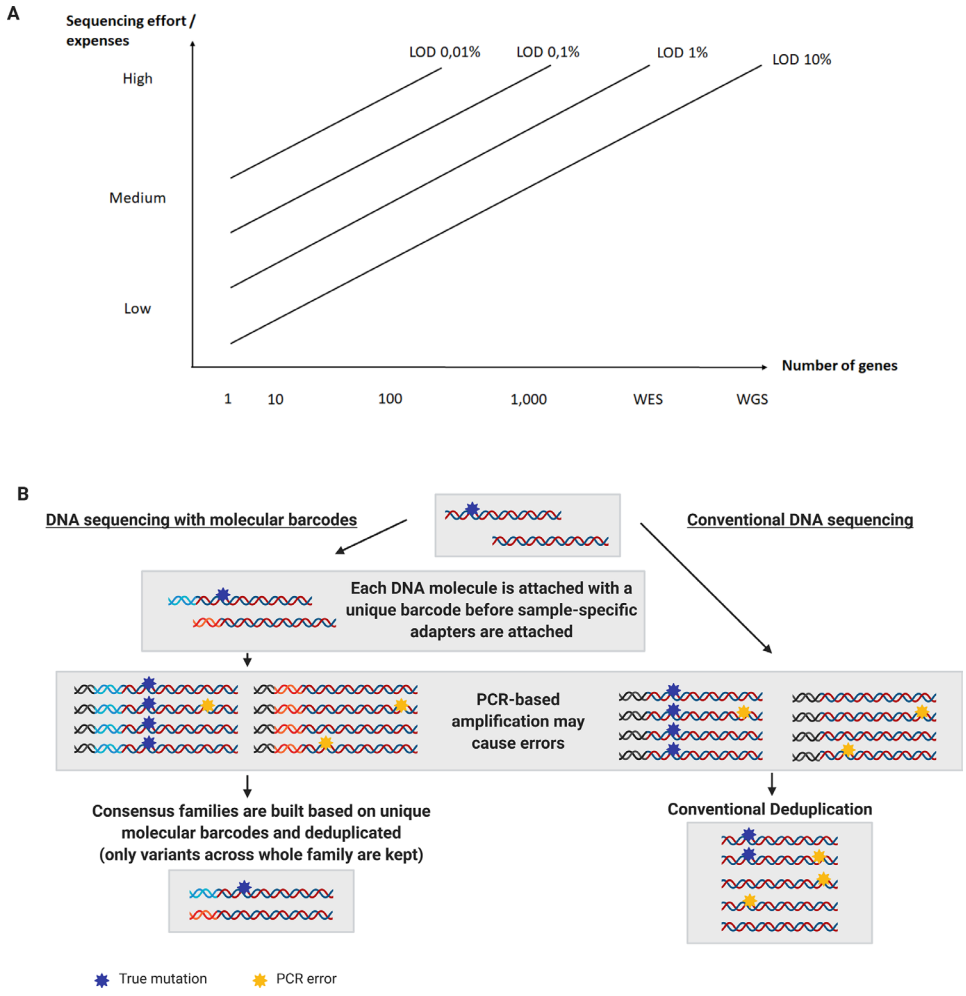

Figure 3 Considerations for the analysis of circulating tumour DNA. (A) The size of the assessed gene panel correlates with the sequencing effort for a given lower limit of detection. (B) Schematic overview of cell-free DNA mutation analysis with the incorporation of unique molecular barcodes (MBC) (ie, dual-index sampling) compared with conventional analysis with multiplex-indexing only. MBC allow for the aggregation of consensus families for each DNA molecule, which drastically reduces the number of sequencing errors that might occur during amplification. LOD, limit of detection; WES, whole-exome sequencing; WGS, whole-genome sequencing.

in non-tumoral tissue (median of 8.7% in WES and 2.2% in ultra-deep targeted sequencing).[50] This is in strong contrast compared with tumorous tissue with VAF between 35% and 80% or higher depending on purity of the tissue and other factors. (2) The most frequently detected mutations reported by the authors (PKD1: 12.9% of patients, KMT2D: 9.7%, STARD9: 9.7%, APOB: 9.7%) are not commonly observed in liver cancer. And the small number of mutations that are known in HCC (eg, TP53, ARID1A, ARID2) were detected with particularly low VAF of around 1%–5%. In a pilot study, mutations with lower VAF were less likely to be detected in plasma compared with higher VAF.[48] However, more data are needed to determine factors that impact the likelihood of mutation detection in plasma DNA because most studies comparing tissue and ctDNA mutation profiling have been focussing on driver mutations in tissue with high VAF.

Notably, all these pilot studies were conducted using conventional NGS technologies. A known limitation of this approach in the context of liquid biopsy is the high number of false positive variant calls. These false positive calls can be introduced during PCR-based amplification steps, and negatively impact the calling of variants with low VAFs of 2%–3% and below. This is a fundamental difference to tissue-based analysis where tumour variants represent the predominant allele fraction (roughly 35%–80% or higher, depending on tumour type and purity among other factors) and thus PCR errors (which occur in the range of 1% and below) can easily be tagged and disregarded. Besides, patient-specific characteristics (eg, anaemia, comorbidities, performance status and so on) and DNA extraction yields impact the amount of blood volume and tumor-derived DNA molecules that can be retrieved. Fortunately, many refinements both in library preparation techniques and computational analysis, such as integrated digital error correction,[51] have improved the accuracy of mutation calling from cell-free DNA. Specifically, the implementation of unique molecular identifiers, that are attached to each DNA molecule before amplification, allows the aggregation of consensus families for deduplication and thus a more robust approach to calling variants with low allele frequencies (figure 3B) as reported in recent contemporary studies.[43 52 53]

Somatic mutation profiling of ctDNA has also been explored in serial samples to capture real-time tumour dynamics, disease progression or response to therapies. In the first of a pair of consecutive studies, sequential plasma samples of three HCC patients and paired multiregional tumour and peritumoral tissue were included.[54] Here, a custom gene panel containing 574 cancer-associated genes was used to identify subclonal mutations in HCC tissue. Analysis of corresponding ctDNA reflected

98%–99% of those subclonal mutations.[54] Of note, changes in those subclonal mutation frequencies over time were related to patient tumour burden.[54] The follow-up study included 34 tissue resection specimens from HCC patients that were sequenced by WES and matched ctDNA was analysed by a custom target panel including all mutations previously identified in tissue.[55] In total, this study analysed 168 sequential plasma samples, with an average of 54.1 single nucleotide variants (SNV) in preoperative ctDNA samples and similar SNV frequencies between ctDNA and tissue samples.[55] The levels of SNV and copy number variations in ctDNA decreased after surgery and showed a subsequent increase in cases with tumour recurrence, indicating the feasibility to monitor treatment response, or even detect minimal residual disease, with ctDNA.[55]

A recent study has investigated an approach to combine mutation analysis of ctDNA with the tumour markers AFP and des-γ-carboxy-prothrombin for early HCC detection.[56] The test yielded 85% sensitivity and 93% specificity in a training cohort, which consisted of 65 HCC and 70 non-HCC cases.[56] When applying the test to a cohort of 331 asymptomatic HBsAg-seropositive individuals, who had negative HCC screening with AFP and US, the test scored positive in 24 individuals, of whom four developed HCC with a follow-up of 6–8 months (positive predictive value of 16.7%).[56] None of the patients with a negative test developed HCC.[56]

All this evidence confirms the feasibility of mutation detection in HCC using liquid biopsy. With recent technological refinements, it is likely that future studies will achieve even better accuracy and ultimately allow to test circulating mutation profiling in additional clinically scenarios (eg, prediction of treatment response, detection of emerging mutations of resistance and so on).

Assessment of epigenetic changes in ctDNA

DNA methylation changes play a key role in cancer development and progression.[57] The landscape of most frequent methylation changes has extensively been characterised in HCC using tissue samples.[58 59] Some of these genes have been tested in plasma DNA to discriminate HCC from controls and were deemed potentially useful for early HCC detection.[60] Using a single-target approach, methylation of SEPT9 in plasma DNA yielded high accuracy for the detection of HCC in two independent cohorts (pooled area-under-the-receiver-operating-curve (AUC) of 0.94).[61] This test is also used for the detection of colon cancer,[62 63] which challenges its potential as a truly HCC-specific rather than cancer-specific biomarker. Nevertheless, this test has received a CE Mark enabling the commercialisation for early detection of HCC in Europe,[64] and a prospective, multicentre, case–control study to assess the performance in US cohorts has recently been fully recruited (ClinicalTrials.gov Identifier: NCT03804593). Furthermore, several studies have applied genome-wide methylome sequencing to identify aberrantly methylated genes associated with early stage HCC.[65 66] A large study including>1000 patients with HCC and >800 normal controls found a panel of 10 DNA methylation sites with high diagnostic accuracy for HCC (AUC 0.96), which was independently validated (AUC 0.94).[65] However, a major limitation of this study is that controls were not limited to patients at risk for HCC as defined in clinical guidelines (eg, patients with cirrhosis), who would represent the ideal target population for surveillance tools and early detection of HCC.[3 4] Additionally, eight DNA methylation markers were identified with prognostic capacities for survival.[65] Based on follow-up data,[67] the FDA granted this test breakthrough device designation for early detection of HCC, and a clinical trial for HCC surveillance was initiated (ClinicalTrials.gov Identifier: NCT03694600). Another study performed a discovery, phase I pilot and phase II clinical validation cohort study, and identified a panel of 6 DNA methylation markers with a similar sensitivity of 95% at a specificity of 92% to detect HCC among controls at risk (AUC of 0.94).[66] A follow-up study was recently presented at the ASCO 2020 meeting, including 136 HCC cases (81 early-stage BCLC stage 0/A) and 401 controls. The authors reported a model including sex, AFP and three methylation markers (HOXA1, TSPYL5, B3GALT6) with 70% sensitivity and 89% specificity for the detection of early stage HCC.[68]

In summary, these studies underscore the great potential for methylation profiling of plasma DNA for the detection of HCC (figure 2, level of evidence 2). However, these studies share common major limitations which mostly arise from the study design. Besides lacking adequate controls, the minority of cases are early stage HCC, which would be candidates for curative treatment options, such as resection or transplantation. This is mostly due to the fact that these studies were conducted retrospectively and used so-called 'convenient samples'.[69]

EVS IN HCC

EVs, including microvesicles and exosomes, are nanoparticles enclosed by lipid bi-layers and thus protected from enzymatic degradation.[19 20] EVs are heterogeneous, both in terms of biogenesis and content, and are released by all cells as part of normal physiological function.[70] While larger EVs such as apoptotic bodies (50–5000 nm) or microvesicles (100–1000 nm) mostly contain fragmented DNA, smaller EVs such as exosomes (30–150 nm) are enriched in cell-type specific, non-coding, regulatory small RNAs.[19 20 71] Recent studies indicate that the functional and targeted uptake of extracellular RNA (exRNA) from exosomes and other EVs have a key role in facilitating and controlling intercellular communication. In fact, nucleic acid payloads have been shown to prime receptor cells and actually modify key cellular functions.[19 20] Specifically, they have been shown to actively participate in non-canonical tumour signalling by directly mediating distal seeding of pre-metastatic niches via intercellular communication,[72–75] in part by delivering very specific payloads of proteins and nucleic acids to target cells.[71]

Clear evidence is emerging that HCC-specific exosomal small RNA expression and protein signatures could be valuable biomarkers with unprecedented sensitivity for early malignant transformation from cirrhosis. Traditionally, studies of the small exRNA expression landscape in HCC and other cancer types have focused specifically on micro-RNAs (miRNA) and long-non-coding RNA (lncRNA).[76] Indeed, a recent report found HCC-specific upregulation of exosomal miR-222, miR-18a, miR-221 and miR-224, and HCC specific downregulation of miR-101, miR-106b, miR-122 and miR-192 compared with cirrhotic patients.[77] miR-222 has been associated with HCC cell migration through AKT signalling pathway activation.[78] Previous studies have suggested that miR-18a induced proliferation and development of HCC in women by reducing the level of oestrogen receptor-α,[79] while much more recent work suggests targeting of B-cell lymphoma 2(-like 10) (BCL2(L10)) promotes progression and migration.[80] Similarly, miR-221 contributed to hepatocarcinogenesis through the dysregulation of DNA damage-inducible transcript 4[81] and nuclear factor 'kappa-light-chain-enhancer' of activated B-cells.[82] On the other hand, down-regulation of miR-101 inhibited apoptosis and enhanced tumorigenicity by specifically targeting myeloid cell leukaemia sequence 1, a key anti-apoptotic member of the BCL2 family, in HCC.[83] miR-122

Table 1 Performance of selected liquid biopsy studies across different clinical scenarios in the management of HCC

Clinical scenario	Liquid biopsy strategy	Study	Target panel	Patients	Performance			Comments/limitations
Early detection					**Sensitivity**	**Specificity**	**AUC**	
	ctDNA methylation profiling							
		Oussalah *et al*[61]	SEPT9	98 HCC, 191 cirrhotics	81%–97%	69%–96%	0.94 (pooled)	Single target
		Kisiel *et al*[66]	Six markers	95 HCC, 51 cirrhotics 98 healthy	95% (91% for BCLC 0/A)	92%	0.94	<50% early stage, controls 1/3 cirrhotics
		Xu *et al*[65]	Ten markers	1098 HCC, 835 healthy	83%–86%	91%–95%	0.94–0.97	Across all cancer stages, healthy controls instead of cirrhotic
		Roy *et al*[67]	Not reported	60 liver cancer, 10 benign liver disease, 30 healthy, 30 other cancers	95%	98%	Not reported	Blinded follow-up study for Xu *et al*[65]; across all cancer stages, inadequate controls (ie, small number of cirrhotics)
	ctDNA mutation profiling							
		Qu *et al*[56]	Four genes (TP53, CTNNB1, AXIN1, TERT promoter) plus HBV insertion site, AFP, and DCP	65 HCC, 70 non-HCC (training); 331 at risk patients (validation)	85% (training); 100% (validation)	93 (training); 94% (validation)	Not reported	Positive predictive value 17% in validation cohort
	Extracellular vesicles							
		Sohn *et al*[77]	Eight exosomal miRNAs	20 chronic hepatitis B, 20 cirrhosis, 20 HCC	Not reported	Not reported	Not reported	Validation cohort missing, low level of evidence
Prognostication					**Performance**			
	ctDNA methylation profiling							
		Xu *et al*	Ten markers	1098 HCC	Association with overall survival (training and validation set)			
	CTC detection							
		Ogle *et al*[109]	AFP, EpCAM, glypican 3, and DNA-PKs	69 HCC	Detection of ≥2 CTC correlated with worse overall survival			Validation missing
Monitoring of disease/prediction of response					**Performance**			
	ctDNA methylation profiling							
		Xu *et al*[65]	Eight markers	1098 HCC	Association with tumour response after surgery and tumour burden/stage of disease			External validation missing
	CTC detection							
		Sun *et al*[106]	EpCAM (CellSearch)	123 HCC	Detection of ≥2 CTC correlated with shorter recurrence-free survival after resection			Single centre
		von Felden *et al*[105]	EpCAM (CellSearch)	57 HCC	Detection of any CTC correlated with shorter recurrence-free survival after resection			Single centre

AFP, alpha fetoprotein; AUC, area-under-the-receiver-operating-curve; CTC, circulating tumor cell; ctDNA, circulating tumor DNA; DCP, des-γ-carboxy-prothrombin; DNA-PKs, DNA-dependent protein kinases; EpCAM, epithelial cell adhesion molecules; HCC, hepatocellular carcinoma.

was associated with suppression of overall HCC tumour growth, invasiveness, regulated intrahepatic metastasis via angiogenesis in HCC,[84] and as a predictor of response of recurrence following curative resection.[85] Similarly, miR-122 expressed in adipose tissue increased the antitumoral efficacy of sorafenib on HCC in vivo.[86] Finally, miR-195 suppressed tumorigenicity and regulated the canonical G1/S transition via modulation of cyclin D1, cyclin dependent kinase 6 and E2 promoter binding factor 3 in HCC cells.[87] For HCC-specific exosomal expression of lncRNA, previous reports have indicated that regulator of reprogramming,[88] very low density like receptor,[89] hepatocellular carcinoma up-regulated EZH2-associated long non-coding RNA,[90] cancer susceptibility candidate 9 and long-non-coding RNA Lucat1[91] are all enriched and plausible biomarker candidates related to a spectrum of phenotypes from tumour stemness to recurrence and metastasis. A recent prospective study including 79 patients with HCC found that elevated expression of miR-21 and lncRNA-ATB in serum exosomes was associated with more advanced stage and progression of disease, suggesting a prognostic role.[92] The overall survival and progression-free survival were significantly lower in patients with higher circulating levels of exosomal miR-21 and lncRNA-ATB.[92] However, a secondary cohort was missing and external validation is needed to further confirm these findings (figure 2, level of evidence 4).

It is important to note that other small RNA species are also strongly expressed in small exRNA and may also serve as powerful, completely novel biomarkers, for example, in the context of early HCC surveillance. In fact, multiple small functional non-coding exRNA can arise from transcriptional post-processing of a single larger RNA precursor gene (eg, endogenous siRNAs,[93] miRNA hairpins yielding miRNA*[94] and piRNAs[95]). Utilisation of exRNA processing pipelines from the 'Extracellular RNA Communication Consortium' (https://exrna-atlas.org/), such as exceRpt,[96] can help isolate these particular annotated species of exRNA for prioritisation and separation studies, and also facilitate deconvolution of the admixture of EV carriers (eg, low-density vesicles, lipoprotein and Argonaute2-positive ribonucleoprotein particle carriers and high density vesicles)[71] that are actually co-isolated in any particular EV isolation protocol. This facilitates a de novo association of EV carrier profiles with any particular exRNA signature, flagging samples with potential non-exosomal EV contamination and prioritising high exosomal purity samples for exRNA biomarker mining.

CTCS IN HCC

CTCs are appealing biomarkers in the context of liquid biopsies. Besides simple detection and enumeration, they offer direct access to intact tumour cells for molecular analyses. For example, the application of single cell genomic technologies to study CTCs has been shown in prostate cancer[97] and also in HCC.[98] In terms of clinical implementation, the CTC enumeration method CellSearch is approved by the FDA.[99–101] CellSearch is a semi-automated device that achieves CTC enrichment using iron-conjugated antibodies against epithelial cell adhesion molecules (EpCAM) and, secondarily, antibodies against cytokeratins 8, 18 and 19. Additional absence of cluster of differentiation 45 ensures adequate exclusion of immune cells. A definitive call of CTC is performed by an investigator via direct visualisation of the candidate using microscopy. Several studies have evaluated the potential role of EpCAM-positive CTCs in patients with HCC. A robust prognostic performance has been shown with the CellSearch system[102] and other systems.[103 104] Additional data reinforced the association between presence of EpCAM-positive CTCs and tumour recurrence after resection.[105 106] However, EpCAM-based CTC calling has some limitations. First, EpCAM is not universally expressed by HCC cells. On average, less than 30% of HCC have significant overexpression of EpCAM when considering genomic data.[107] Thus, only a subset of tumours may be detected using EpCAM-based CTC capture methods. Second, selection for EpCAM results in a relatively low number of detected CTC[102 105 106] compared with other malignancies, such as breast cancer.[108] Thus, current detection rates for EpCAM-based methods have relatively low sensitivity and implementation into daily clinical practice to monitor patients with HCC is not recommended.

Over the past years, additional surface markers and techniques for CTC enrichment have been tested in HCC. An imaging flow cytometry approach detecting AFP, EpCAM, glypican 3 and DNA-dependent protein kinases (DNA-PK) found CTCs in 45/69 HCC patients compared with 0/31 controls.[109] Again, CTC-positivity correlated significantly with the presence of systemic disease, in this case portal vein infiltration, and prognosis with a median overall survival of >34 months for patients with 0 or 1 CTC compared with 7.5 months for patients with≥2 CTC.[109] Glypican 3 has also been tested as a single marker in a prospective study including 85 patients who underwent liver resection. CTCs were purified by density gradient centrifugation and immunomagnetic positive enrichment based on the expression of glypican 3, and finally enumerated with flow cytometry.[110] A median of 3 CTCs were detected in 8 mL blood and presence of ≥5 CTCs correlated with worse outcome.[110] Another study performed CTC nanofiltration and subsequent RNA-ISH analysis targeting epithelial and mesenchymal markers.[103] Herein, the concept included the aim to detect mesenchymal-like CTC (Vimentin- and Twist-positive).[103] Postoperative monitoring of CTC-levels were predictive of tumour recurrence even when imaging modalities were still negative.[103]

The role of CTC analysis in the clinical management of HCC is yet to be defined. Current data does not support a clear role as an early HCC detection tool. In terms of prognosis, there are many studies supporting its role in predicting outcomes, particularly after resection. However, there are some issues that need improvement to allow comprehensive CTC molecular analysis, such as better methods to increase detection yield.

CONCLUSIONS/FUTURE PERSPECTIVES

NGS technologies have revolutionised oncological sciences and expedited the development of precision medicine. In this context, liquid biopsy has already proven as a useful tool for clinical decision making in many tumour types. However, the application of this technology to HCC seems delayed compared with other malignancies that routinely incorporate liquid biopsy analysis in clinical trials. There have been major technological and analytical improvements that have positively impacted accuracy and scalability of liquid biopsy in HCC. To date, the most promising approach for early clinical take-up is DNA methylation profiling of ctDNA for the early detection of HCC in patients at risk (table 1, figure 2). This may soon challenge the long-standing paradigm of AFP and US for HCC surveillance. As for any biomarker research initiative, high standards for the definition of case and control groups are of foremost importance to assess the true utility of liquid biopsy for early detection. Mutation profiling of ctDNA and molecular analysis of CTC are, at least in part, dependent on tumour burden and therefore likely more useful in intermediate or advanced settings when it comes to prognostication or prediction of treatment response. Despite being more immature, the analysis of EVs could provide biomarkers at every stage. In addition, it has the capacity to provide functional information (eg, the interactions between cancer cells and the tumour microenvironment or distant cells).

To overcome the drawback of liquid biopsy in HCC, systematic and standardised sample collections, for example, for patients within clinical trials, are needed. Also funding initiatives for investigator-initiated trials or large collaborative efforts would help well-designed studies and power adequate sample size. This cannot overcome biological disadvantages of HCC compared with other tumour entities, such as paucity of hotspot mutations and druggable mutations in HCC, but it would allow to stratify subgroups of patients by patterns of molecular alterations, who are potentially associated with beneficiary outcome.

In conclusion, liquid biopsy represents a novel, minimally-invasive, powerful tool for biomarker discovery in HCC, with the potential to significantly change decision-making in the short term.

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Contributors JvF and AV outlined the manuscript. JvF, TG-L, KS, BL and AV performed review of the literature and writing of the article. All authors approved the final version of the article.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests AV has received consulting fees from Guidepoint and Fujifilm; advisory board fees from Exact Sciences, Nucleix and NGM Pharmaceuticals and research support from Eisai Pharmaceuticals. The remaining authors have nothing to declare in respect to this manuscript.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not required.

Provenance and peer review Commissioned; externally peer reviewed.

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