REVIEW

Liquid Biopsy Using Cell-Free or Circulating Tumor DNA in the Management of Hepatocellular Carcinoma

Xueying Lyu,* Yu-Man Tsui,* Daniel Wai-Hung Ho, and Irene Oi-Lin Ng

Department of Pathology and State Key Laboratory of Liver Research, The University of Hong Kong, Hong Kong

SUMMARY

Plasma circulating tumor DNA (ctDNA) offers a noninvasive means for the detection and clinical management of liver cancer. The different aspects of plasma ctDNA have been intensively explored in recent years. These include the mutational landscape such as single-nucleotide variations, copy number variations, methylation landscape, end motif/coordinate preference, hepatitis B virus integration, and mitochondrial DNA mutations. The clinical utility of ctDNA depends on the concordance of the genetic information between the plasma and the tumor tissue in hepatocellular carcinoma (HCC). The use of ctDNA in clinical management of HCC is discussed in regard to the detection and diagnosis, prognosis, drug treatment guidance, and disease monitoring of HCC. The barrier to the implementation of liquid biopsy for cell-free DNA profiling and the coping strategies by standardization of relevant procedures also is discussed.

Liver cancer (hepatocellular carcinoma [HCC]) is a fatal cancer worldwide and often is detected at an advanced stage when treatment options are very limited. This drives the development of techniques and platforms for early detection of HCC. In recent years, liquid biopsy has provided a means of noninvasive detection of cancers. By detecting plasma circulating tumor DNA (ctDNA) released from dying cancer cells, the presence of HCC can be detected in a noninvasive manner. In this review, we discuss the molecular characteristics of ctDNA and its various molecular landscapes in HCC. These include the mutational landscape, single-nucleotide variations, copy number variations, methylation landscape, end motif/co-ordinate preference, hepatitis B virus integration, and mitochondrial DNA mutations. The consistency between the plasma ctDNA and the tumor tissue genomic DNA mutational profile is pivotal for the clinical utility of ctDNA in the clinical management of HCC. With strategic use of genetic information provided from plasma ctDNA profiling and procedure standardization to facilitate implementation in clinical practice, better clinical management would become permissible through more efficient detection and diagnosis of HCC, better prognostication, precision-matched treatment guidance, and more reliable disease monitoring. *(Cell Mol Gastroenterol Hepatol 2022;13:1611–1624; https://doi.org/10.1016/j.jcmgh.2022.02.008)*

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Liver cancer (hepatocellular carcinoma [HCC]) is a prevalent and lethal cancer worldwide.[1] HCC often presents at advanced stages and hence is inoperable. Although liver resection is the major curative therapy, the recurrence rate even after surgery is high.[2] Therefore, early detection is pivotal to better clinical management and important to support recurrence surveillance, identify relevant molecular-targeted drugs, and predict drug response for patients.

Circulating cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA) are noninvasive and promising strategies to assay the circulating DNA in the bloodstream. Together with other circulating biomarkers, they are referred to collectively as *liquid biopsy*. It relies on the detection of intrinsic molecular properties to distinguish the specific DNA originating from tumor cells (ie, ctDNA). ctDNA should share the same molecular alterations as their tumor source, and this makes ctDNA an ideal alternative to tissue biopsy. This review summarizes the most recent information about ctDNA characteristics, detection methods, genetic variation profiles, and its clinical applications for HCC to provide an overview of adopting liquid biopsy in the clinical management of HCC. Finally, possible implementation barriers, the coping procedure standardization, and future perspectives are discussed. Note that cfDNA refers to the input sample of assays while ctDNA represents the specific subset of cfDNA that carries specific molecular alterations; they may be used interchangeably in this article.

Molecular Characteristics of ctDNA and Their Detection Technologies

ctDNA is a short DNA fragment of approximately 120 bp released from necrotic or apoptotic tumor cells. Although

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normal nontumor cells also shed cfDNA into the bloodstream, the cfDNA from tumor cells (ie, ctDNA) only accounts for less than 1% of total cfDNA in the blood.[3,4] The short half-life of ctDNA[3] and the difficulty in distinguishing ctDNA from cfDNA released from normal cells complicates the clinical utility of ctDNA. Specialized tubes can be used for blood sample collection to reduce the chance of white blood cell rupture and genomic DNA contamination from the damaged white blood cells.[5–8] Plasma extracted then can be subjected to a column-based cfDNA extraction kit, which already has been approved by the US Food and Drug Administration for use in routine clinical practice. However, to date, there are no extraction methods that specifically enrich or harvest the tumor cfDNA fraction.[8] Therefore, tumor-specific genetic alteration information has to be extracted from the ctDNA by next-generation sequencing (NGS) methods, among which the often used ones include whole-genome sequencing, whole-exome sequencing, and target-panel sequencing. The former is more exploratory in nature, while the latter requires a known predesigned gene panel with respective probes to help enrich the specific regions of interest for targeted library constructions and sequencing procedures. With regard to NGS, the sensitivity, specificity, and cost efficiency for detection of the tumor-specific genetic alterations in ctDNA need to be balanced. Raising sequencing coverage depth of ctDNA beyond the most optimal level may not improve the sensitivity of detecting tumor-specific genetic alterations further and becomes economically inefficient.[9] In addition to NGS, other methods for detecting tumor-specific genetic alterations in ctDNA include droplet digital polymerase chain reaction (PCR) and quantitative real-time PCR, which are relatively cheaper and convenient detection methods but require prior knowledge of known tumor-specific genetic alterations for detection and are of relatively lower throughput (Table 1).

Molecular Landscapes of ctDNA in HCC

Genetic and epigenetic aberrations were considered as important factors that drive HCC initiation, progression, and metastasis. With an overview of HCC-associated molecular landscape in ctDNA, it provides us with a better understanding of hepatocarcinogenesis and facilitates the mechanistic investigation of the underlying pathologic mechanism in HCC. Molecular alterations in the ctDNA of HCC mainly include single-nucleotide variation (SNVs), copy number variations (CNVs), DNA methylation aberrations, preferred end motifs or coordinates, and hepatitis B virus (HBV) integration (Table 2).

SNVs

In the plasma of HCC patients, single-nucleotide mutations could be detected at variable proportions in HCC patients, ranging from 35% to 96%, and may be related to the size of the target gene panel examined and their treatment status.[10–16] By analyzing recent cfDNA studies of HCC (Figure 1A), the most commonly altered genes were hitting several pathways, including the PI3K/AKT/mTOR signaling pathway (PTEN, PIK3CA, KRAS, NF1, TSC2), Wnt/β-catenin signaling pathway (CTNNB1, AXIN1, APC), p53/cell-cycle pathway (TP53, ATM, RB1, CDKN2A), and chromatin remodeling (ARID2, ARID1A, NCOR1).[17,18] TP53 and CTNNB1 are 2 of the most frequently mutated genes that were identified in every aforementioned study. On the other hand, telomerase reverse transcriptase (TERT) promoter mutations are also highly and recurrently detected.[10–16,19–25] These genes play important roles in various aspects of HCC development, with impacts particularly on DNA repair, apoptosis, proliferation, and cell division.[26–28]

CNVs

Apart from SNVs, CNVs also can be detected in the cfDNA of HCC patients. CNVs are larger-scale structural variations (amplification or deletion) caused by genomic instability and usually affecting particular chromosomes or chromosomal segments. At chromosomal level, 1q and 8q amplifications are detected frequently, while the deletion of 1p, 4q, and 8p also typically are observed (Figure 1B).[22,29–33] As for the gene level, amplification of CDK6, epidermal growth factor receptor (EGFR), MYC proto-oncogene, bHLH

Table 1. A Brief Summary of the Technologies for ctDNA Detection in HCC

Method	Sensitivity	Coverage	Variation	Advantage	Limitation
ddPCR	High	Specific and known regions	SNV, CNV, Meth	Rapid, sensitive	Relatively lower throughput; does not detect novel targets
qPCR	High	Specific and known regions	SNV, CNV, Meth	Cheaper	Relatively lower throughput; does not detect novel targets
WGS	Moderate	Whole genome	SNV, CNV, HBV, EM	Multiplex capabilities; detects novel variations; high-throughput detection	Relatively high cost; needs bioinformatics analysis support
WES	Moderate	Whole exome	SNV, CNV, HBV, EM	Multiplex capabilities; detects novel variations; high-throughput detection	Relatively high cost; needs bioinformatics analysis support
TS	Relatively high	Panel size	SNV, CNV, HBV, EM	Multiplex capabilities; detects novel variations; high-throughput detection	Relatively high cost; needs bioinformatics analysis support

ddPCR, droplet digital PCR; EM, end motif; HBV, HBV integration; Meth, methylation; qPCR, quantitative real-time PCR; TS target-panel sequencing; WGS, whole-genome sequencing; WES, whole-exome sequencing.

Table 2. A Summary of the Studies on the Various Types of Molecular Landscape of ctDNA in HCC

Reference	Variation	Cohort	Application	Mutation rate	Consistency	Sample source for cfDNA extraction (volume, mL)	Detection method
45	SNV, PEC	90 HCC, 67 H, 36 C, 32 NC	D	–	–	Plasma (4)	WGS
49	SNV, CNV, HBV	481 HCC, 517 C	D	–	–	Blood (10)	WGS, HBV
11	SNV, CNV	26 HCC	G, M	89%	50%–100%	Whole blood (20)	68-gene TS/70-gene TS
13	SNV, CNV	206 HCC	D	88%	–	Whole blood (10)	54-gene/68-gene/70-gene TS
15	SNV, CNV	24 HCC	P	96%	–	Plasma (2)	74-gene TS
20	SNV, CNV	34 HCC	P, M	100%	–	Plasma (–)	TS, WGS
22	SNV, CNV	187 HCC	G, P	–	–	Plasma (–)	TS
25	SNV, CNV	14 HCC	G, P	100%	–	Whole blood (20)	68-gene TS, ddPCR
58	SNV, HBV	65 HCC, 70 NC	D	–	–	Plasma (2)	TS
10	SNV	48 HCC	D	56%	22%	Plasma (1)	ddPCR, SS
12	SNV	51 HCC, 10 C	D	35%	29%	Plasma (1)	7-gene TS
14	SNV	26 HCC, 10 C, 10 H	D, P	96%	89%	Plasma (0.6–1.8)	354-gene TS
16	SNV	59 HCC	P	56%	97.3%–100%	Blood (10)	69-gene TS, ddPCR
19	SNV	41 HCC	P	20%	–	Plasma (0.72)	3-gene TS
21	SNV	37 HCC	D	–	52%–84%	Blood (10)	TS
23	SNV	77 HCC, 8 C	G	83%	83%	Plasma (5), serum (1)	25-gene TS, ddPCR, SS
24	SNV	27 HCC	G	96%	–	Plasma (–)	–
51	SNV	8 HCC	D	75%	71%	Plasma (5), serum (1)	58-gene TS
65	SNV	895 HCC	P	20%–42%	92%	Whole blood (10)	ddPCR, 1-gene TS
66	SNV	81 HCC	P	–	–	Plasma (–)	ddPCR, SS
48	Meth, HBV	45 HCC, 18 C, 18 H, 36 NC	D, M	–	–	Whole blood (10)	WGBS
38	Meth	104 HCC, 174 NC, 95 at-risk disease	D, P	–	–	Venous blood (10)	MSP
39	Meth	25 HCC, 35 C or H, 20 NC	D, M	92%	–	Plasma/serum (0.4)	MSP
40	Meth	237 HCC	D, M	37%–63%	–	Plasma (0.25)	Pyrosequencing, MSP
41	Meth	50 HCC, 50 NC	D	22%–70%	–	Blood (20)	MSP
42	Meth	36 HCC, 17 C, 38 NC	D	–	–	Plasma (2)	MCTA-sequencing technique
43	Meth	80 HCC, 40 C, 40 H, 20 NC	D	34%	-	Serum (0.4)	MSP
55	Meth	28 HCC	D	89%	68%–89%	Plasma (–)	MSP
59	Meth	116 HCC, 60 C	D	–	–	Plasma (>1)	MSP
61	Meth	144 HCC, 106 C	M	–	–	Plasma (1)	BS
62	Meth	97 HCC, 46 H, 80 NC	D	–	–	Plasma (1.2–1.5)	ddPCR
67	Meth	1098 HCC, 835 NC	D, P	–	–	Plasma (1.5)	BS
68	Meth	68 NC, 66 H, 96 C, 109 HCC	D, M	–	–	Plasma (–)	MSP, BS

Table 2. Continued

Reference	Variation	Cohort	Application	Mutation rate	Consistency	Sample source for cfDNA extraction (volume, mL)	Detection method
47	HBV	50 HCC	D, M	88%	–	Plasma (1)	TS
50	CNV, PEC, SNV	10 NC, 10 H, 10 HCC	D	–	100%	Plasma (2)	WGS, TS
30	CNV, EM	63 HCC, 187 H	D	94%	–	Plasma (–)	WGS
46	CNV, EM	34 HCC, 17 H, 38 NC	D, M	–	–	Plasma (4)	BS
29	CNV	151 HCC	G, P	27%	–	Plasma (1.5)	WGS
31	CNV	31 HCC, 8 H or C	D	42%	–	Plasma (–)	–
32	CNV	76 HCC, 274 NC	D, P	57%	–	Plasma (2)	WGS
33	CNV	90 HCC, 67 H, 36 C, 32 NC	D	84%	63%	Plasma (3–4.8)	WGS
34	CNV	117 HCC	P	–	–	Plasma (–)	WGS
74	CNV	1 HCC	G	–	–	Plasma (–)	–
64	5hmC, EM	2250 C, 508 HCC, 476 NC	D	–	–	Plasma (–)	5hmC-sequencing, WGS
57	5hmC	1204 HCC, 392 H or C, 958 NC	D	–	–	Peripheral blood (5–10)	5hmC-seal profiling

BS, bisulfite sequencing; C, cirrhosis (irrespective of etiology); D, detection and diagnosis; ddPCR, droplet digital PCR; EM, end motif; G, guiding drug administration; H, hepatitis (irrespective of etiology); HBV, HBV integration; M, monitoring; MCTA, Methylated CpG tandems amplification; Meth, methylation; MSP, methylation-specific PCR; NC, normal control; P, prognosis; PEC, preferred ends coordinate; SS, sanger sequencing; TS, target-panel sequencing; WGBS, whole-genome bisulfite sequencing; WGS, whole-genome sequencing; 5hmC, 5hmC modification; –, not available.
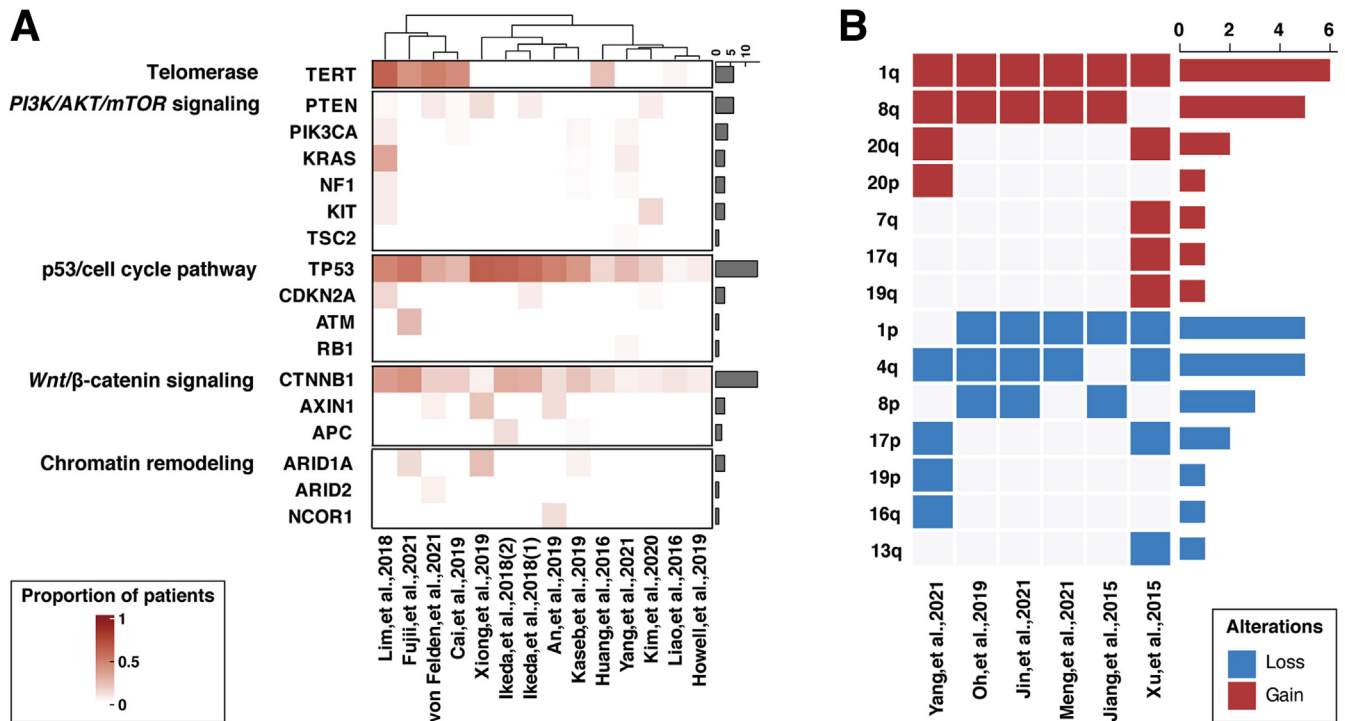
Figure 1. A summary of genetic aberrations of SNVs and CNVs reported from previous studies in HCC. (*A*) Proportions of HCC patients with recurrent SNVs in related pathways reported by 14 previous studies. (*B*) CNVs (Gain or Loss) at chromosome level found in 6 studies.

transcription factor (*MYC*), *BRAF*, and *CCNE1* are reported frequently, whereas deletion events are less consistent among studies.[11,13,15,25] The earlier-described chromosomes and genes are the common and conserved CNV signatures possessed by HCC patients and readily revealed by the CNV landscape obtained from patients' cfDNA. Because there is only a scanty amount of ctDNA present in the bloodstream for HCC patients, this limits the detection of CNVs in cfDNA. To overcome the limitation, Jin et al[30] attempted to improve the detection of the CNV signal by applying fragment-size selection of less than 150 bp in a cohort of 197 HCC patients. Because CNV generally influences a larger fraction of the genome compared with SNV, copy number analysis and the detection of alterations usually are performed and evaluated at a relatively large scale. Different studies used and/or developed algorithms, aiming to accurately estimate the genomic regions potentially affected by CNV. At the genome-wide level, statistics such as tumor fraction, prediction score, and stability score are calculated as indicators for estimating the overall likelihood of CNV. Similarly, metrics at a smaller scale (eg, arm or bin level) also have been developed to measure CNV in the cfDNA of HCC patients.[34]

Methylation

Considering the conservation of tissue-specific methylation patterns in tumors, liquid biopsy–based methylation can be used in a cancer diagnosis without a priori knowledge of somatic mutations or copy number aberrations.[35]

Methylated *septin 9* (*SEPTIN9*) is an approved blood-based biomarker for colorectal cancer screening. Septins are the guanosine triphosphate–binding proteins that participate in cell division, cytoskeletal organization, and membrane remodeling processes.[36] Promisingly, *SEPTIN9* methylation in cfDNA has been shown to serve as a noninvasive and effective indicator for HCC diagnosis as well.[37] In particular, when combined with the serum α-fetoprotein (AFP) level, the sensitivity of HCC detection can be improved from 82.7% to 91.3%.[38] In addition, *CDKN2A*, *CDKN2B*, *RASSF1A*, *STEAP4*, *TBX2*, *VIM*, and *ZNF154* are genes reported by previous studies that their serum DNA methylation was associated with HCC development and progression.[39–41] Wen et al[42] found that 4 genes, *RGS10*, *ST8SIA6*, *RUNX2*, and *VIM*, are hypermethylated in HCC, which could be applied in HCC detection clinically. On the other hand, a previous study reported that the hypomethylation of the *UBE2Q1* gene promoter is a prospective biomarker for HBV-associated HCC.[43]

Preferred End Motif or Coordinate

Preferred ends in DNA molecules refer to the certain base positions in the genome (coordinate) or specific base composition (motif) at the end of DNA fragments. Because fetal-specific DNA molecules with preferred ends exist in the plasma of pregnant women, it is likely that the plasma of cancer patients also carries tumor DNA with similar preferred ends that distinguish them from the remaining cfDNA molecules of nonmalignant origins.[44] This hypothesis

has led to subsequent investigations based on the end motifs and coordinates of ctDNA in HCC. To this end, Jiang et al[45] identified the tumor-associated and non–tumor-associated preferred end coordinates by comparing the cfDNA end coordinate profile of a HCC patient with that of a chronic HBV patient. Moreover, ctDNA in plasma-carrying, tumor-associated DNA ends were significantly shorter than nontumor-associated DNA. In addition, the ratio of tumor- to nontumor-associated cfDNA preferred end was correlated positively with tumor DNA concentration. Furthermore, they also found that there was a specific pattern of 4-mer end motifs among HCC patients. HCC patients especially were found to have a lower abundance of DNA motif CCCA in their plasma compared with that in non-HCC cases.[46] Along this direction, Jin et al[30] found 139 preferential end motifs that were significantly related to the fragment size in HCC and HBV-infected patients.

HBV Integration

Similar to HBV integration detected in HCC tumor tissues, recurrently integrated genes detected in cfDNA of HCC patients include *TERT*, *KMT2B*, *MLL4*, and *CCNA2*. HBV integration into the *TERT* promoter is commonly observed and has a frequency of 36.4%.[47] In addition, by using low-pass, whole-genome bisulfite sequencing, a significant enrichment of hypomethylation of cfDNA near HBV integration sites could be found in HCC patients, but not in patients with hepatitis or cirrhosis.[48] Notably, the number of HBV integration events in cfDNA of HCC patients is correlated with ctDNA concentration.[49] The plasma level of virus–host chimera DNA generated by HBV integration into the human genome is associated positively with tumor size. Therefore, HBV integration detected in cfDNA could be a circulating biomarker for prognostic prediction before tumor resection as well as monitoring residual or recurrent tumor after resection.[47]

Mitochondrial DNA

Mutations in mitochondrial DNA (mtDNA) have been implicated in HCCs and noninvasive detection of plasma circulating cell-free mtDNA (ccf-mtDNA) may offer a potential tool for finding tumor biomarker for detection of HCC.[50] In a study cohort of 10 HCC patients, 10 colorectal cancer patients, 10 healthy patients, and 10 hepatitis controls, it was found that ccf-mtDNA has a biased distribution of fragment size at approximately 90 bp. With capture-based deep NGS in the ccf-mtDNA, matched tumor and nontumor tissues, and peripheral blood mononuclear cells of 5 HCC patients, HCC-specific mtDNA mutations were specifically identified in the plasma samples of HCC patients. Furthermore, variants with unknown originations resulting from intratumor heterogeneity also were found in plasma ccf-mtDNA, signifying the sensitivity of using ccf-mtDNA mutations to detect HCC and the underestimation of tumor burdens by using single-biopsy profiling.

Consistency Between Plasma and Tumor Tissue in HCC

An et al[14] discovered that the concordance of detected mutations between HCC tissue and matched plasma cfDNA ranged from 52% to 84%. In addition, Labgaa et al[51] used targeted deep sequencing and detected 21 somatic mutations in the tumor tissues in a cohort of 8 HCC patients, with representative mutations affecting genes including *TERT* promoter, *TP53, CTNNB1, JAK1,* and *AXIN1*. Among these mutations, 15 (71%) of them were identified in paired plasma or serum samples. A study by Ng et al[52] reported 63% of the studied HCC patients carried HCC-associated somatic mutations. Importantly, 81% and 97% of the mutations detected in cfDNA, in nonhypermutator and hypermutator cases, respectively, were detected independently in the tissue counterparts. Regarding cases with tumors of 5 cm or larger having metastasis, cfDNA and tissue DNA were shown to capture similar proportions of somatic mutations (87% and 95%, respectively), suggesting that, in most HCC patients with substantial tumor burden, somatic mutations can be detected confidently in cfDNA to reflect the mutation landscape in their corresponding tumor tissues. On the contrary, consistency is relatively low for cases having smaller tumor size.[53] In fact, not all reported studies suggested high concordance between cfDNA and HCC tumor tissue. In the study by Huang et al,[10] with a cohort of 48 patients, 27 (56%) were detected with designated HCC-related mutations (*TERT, TP53, CTNNB1*) in cfDNA by droplet digital PCR. However, only 6 of them had matched mutations in tumor tissues. In another extreme case,[12] although all mutations detected in plasma cfDNA could be confirmed in the corresponding tumor tissues, as many as 71% of the patients carried mutations that were identified only in the tumor tissues, indicating the low sensitivity of detection by using cfDNA. In addition, for some studies, some key driver mutations could be found in both cfDNA and tumor tissue (eg, *TP53, CTNNB1*, and *ARID1A*). On the other hand, several mutations were detected only in either cfDNA or tumor tissue.[11,14]

Apart from somatic mutations, the concordance of CNV, DNA methylation pattern, and HBV integration between tumor tissue and cfDNA also has been investigated but to a lesser extent. For CNV, comparable bin and chromosomal-arm level CNV patterns were discovered between cfDNA and tumor tissue.[34] As reported by previous studies, tumor-associated CNV could be found in the plasma cfDNA.[54,55] For methylation, their alterations at *APC, FHIT, CDKN2B, CDKN2A*, and *CDH1* genes showed relatively high consistency between HCC tissue and matched plasma cfDNA, ranging from 68% to 89%,[56] but it was reported to be lower (43%) at the *RASSF1A* promoter region.[57] In another study on a cohort of 26 HCC patients, a correlation based on 5-hydroxymethylcytosine (5hmC) modification in the top 30 variant genes was calculated to estimate 5hmC modification origination. The correlation between cfDNA and tumor/adjacent tissue was higher than that among 26 individuals'

cfDNA as background (0.88 vs 0.73), indicating 5hmC in cfDNA was related to the tissue origin.[58] For HBV integration, most of the mismatch breakpoints were regarded as random and sporadic events, except *TERT* and *KMT2B* breakpoints, which showed high concordance between HCC tissue and plasma cfDNA.[49]

Use of cfDNA in the Clinical Management of HCC

Here, we describe the use of cfDNA as liquid biopsy in HCC. By utilizing the aforementioned molecular alterations to distinguish the ctDNA, different biomarkers have been derived for translational applications in different perspectives of clinical management of HCC.

Detection and Diagnosis

Somatic mutations detected in cfDNA could be used as biomarkers for HCC diagnosis. One study reported that the number of mutations, the maximal variant allele frequency (VAF), and ctDNA concentration in the plasma of HCC patients could distinguish malignant lesions from benign ones with an area under the curve (AUC) of 0.8760, 0.8019, and 0.8712 respectively, which are greater than that for AFP alone (0.7827).[14] ctDNA mutations in combination with AFP level,[21] or with HBV insertion events and protein markers,[59] were shown to have promising sensitivity and specificity for HCC detection. Simultaneous detection of HBV integration landscape and mutations in cfDNA by circulating single-molecule amplification and resequencing technology–based targeted deep sequencing showed a higher AUC value when combined with AFP than that with AFP alone, indicating superior detection or diagnostic power for HCC.[49] Plasma cfDNA CNV discovered by shallow whole-genome sequencing, when combined with fragment size and AFP, facilitates early detection of HCC by achieving 92.6% accuracy and better performance than other predictors.[32]

Identification of differentially methylated sites helps build statistical models or biomarker signatures for HCC diagnosis, prognosis, and recurrence monitoring. Logistic regression and random forest algorithms were used in the discovery cohort model training and validation in another cohort. Models based on cfDNA methylation for HCC detection could achieve a typical sensitivity of 91%–97% and specificity of 85%–92%.[42,60,61] Combining methylation markers such as *HOXA1, EMX1,* and *TSPYL5* with other biomarkers such as AFP or AFP-L3 can improve the performance for early stage HCC detection.[62–64]

In addition, conserved 5hmC modification shows the potential of early HCC detection by genome-wide 5hmC modification.[58] 5hmC, nucleosome footprint, 5'-end motif, and fragmentation of plasma cfDNA can be profiled to derive a HIFI (5-Hydroxymethylcytosine/motIf/Fragmentation/nucleosome footprInt) score to help diagnose HCC patients with high sensitivity and specificity to differentiate HCC from liver cirrhosis.[65] The differentiation power for HCC vs liver cirrhosis by the cfDNA-derived HIFI showed an AUC (0.995–0.996) superior to AFP alone (0.826–0.845).

Interestingly, the diagnostic performance of the HIFI method was independent of cfDNA concentration and may overcome the problem of varying cfDNA concentrations across individuals.

These plasma cfDNA profiling platforms may help screen out HCC patients in a noninvasive and relatively convenient manner and are likely superior to sequencing single-tumor tissue biopsy by better representation of the heterogeneous HCC tumor. However, in cancers with low tumor burden or early stage tumors, mutations present in the tumor tissues may not be detected easily in cfDNA and it still is recommended to consider both tissue and cfDNA sequencing when making clinical judgments.[9]

Prognostication

Numerous studies have shown the possibility that SNVs in ctDNA can serve as potential markers for HCC prognostic evaluation. In the study by Cai et al,[20] the dynamic changes in SNV and CNV of cfDNA correlated nicely with patient tumor burden. The overall mutation profiles accurately evaluated patients' tumor occurrence in advance of medical imaging for an average of 4.6 months and showed superior performance than other serum biomarkers in revealing HCC incidence and detecting minimal residual disease. Importantly, the mutational burden reflected by cfDNA could be translated into predicting patients' prognostic outcomes in terms of relapse-free survival and overall survival (OS). Similarly, HCC patients with detectable mutations in their postoperative plasma had poorer disease-free survival than those without (17.5 vs 6.7 mo), and, in addition, postoperative cfDNA status informed the risk of recurrence.[14] Intriguingly, even the detection of *TP53* R249S hotspot mutation in cfDNA could significantly predict worse overall and progression-free survival in HCC patients, irrespective of their hepatectomy status.[66] In a recent study by Kim et al,[16] by jointly considering the presence of m*utL homolog 1 (MLH1)* SNV and increased cfDNA level, they achieved a better prediction in identifying HCC patients with worse OS.

VAF or mutant allele frequency (MAF) is also an independent risk factor for microvascular invasion and recurrence postoperatively. HCC patients with increased MAF had a relatively shorter disease-free survival and OS in comparison with those with decreased MAF.[67] Moreover, reduction in mean VAF after 4 weeks of lenvatinib treatment was associated with better progression-free survival, indicating the subset of patients who are more responsive to the treatment.[15] HCC patients with mutations related to the *PI3K/mTOR* pathway had notably lower progression-free survival than those without these mutations, while it was not associated with prognostic outcomes for those subjected to immune checkpoint inhibitor (ICI) treatment.[51]

CNVs of different sizes also showed their prognostic value. Although different scales of CNV in cfDNA all could advise prognosis of HCC patients, the performance of bin-level CNV was more outstanding.[34] In a recent study investigating cfDNA CNV biomarkers for ICI treatment
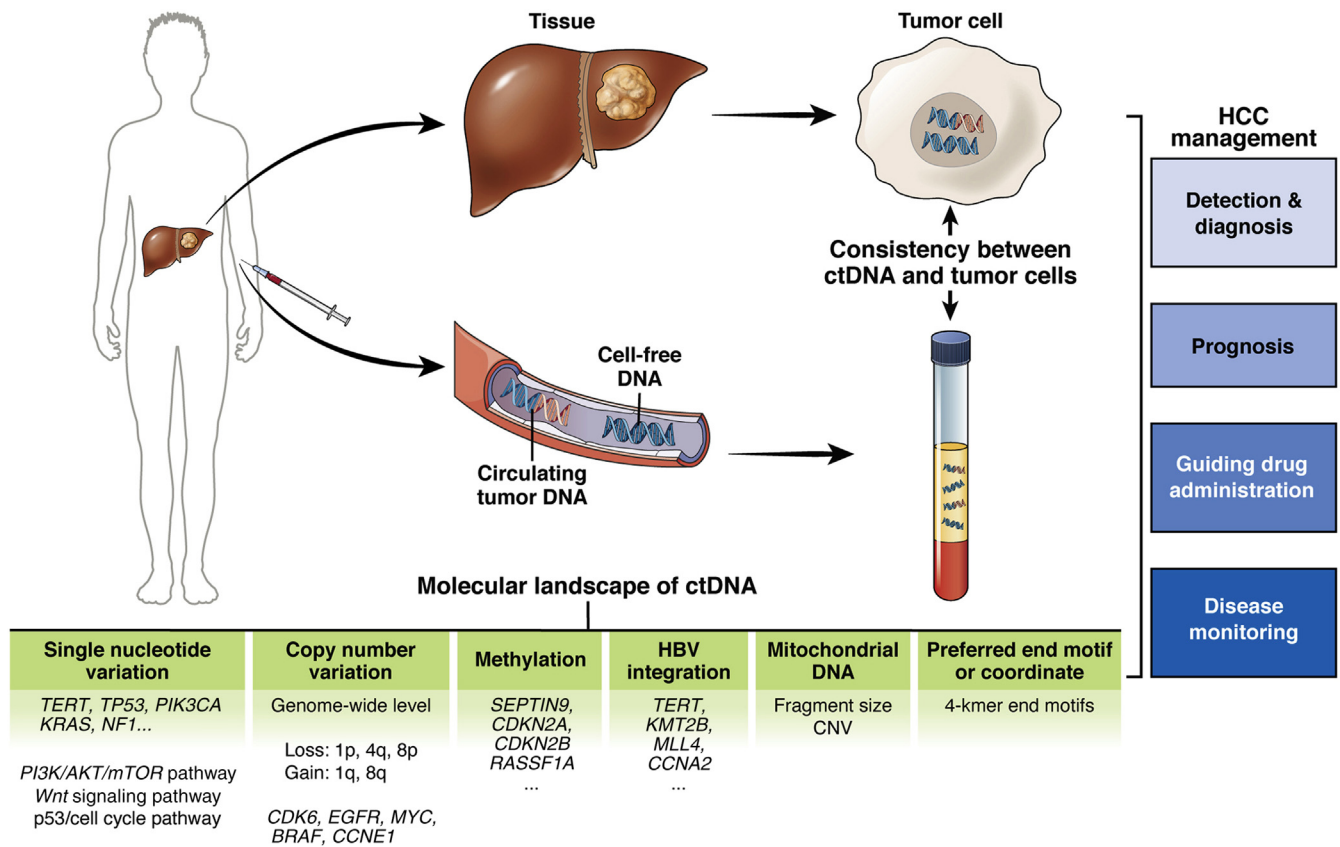
Figure 2. An overview of the molecular landscape of ctDNA and its relevance in the clinical management of HCC. ctDNA originates from the tumor tissue and carries the same genetic aberrations as the tumor cells. With the consistency between ctDNA and tumor cells, ctDNA as a form of liquid biopsy could protentially be used for different aspects in the clinical management of HCC.

response, patients with lower CNV risk scores had better OS and progression-free survival (PFS). Interestingly, it was not associated with prognostic differences in another non-ICI cohort.[22] In another study evaluating the clinical values of cfDNA CNV biomarkers for sorafenib treatment outcome, patients resistant to sorafenib with progressive disease had a significantly higher I-score (a measure of genomic instability), and the high I-score group showed poorer prognostic outcome.[29]

Many studies investigated epigenetic aberrations in relation to HCC prognosis by comparing bisulfite sequencing data between the group with good clinical outcome and the group with poor outcome. Panels of methylation markers were used for prognostic estimation for HCC. For instance, in a study based on 8 methylation markers identified in cfDNA in a cohort of HCC patients, a combined prognosis score was calculated for prognostic prediction in HCC.[68] Moreover, Zhao et al[69] found that the methylation level of *SHISA7, ZNF300, and SLC22A20* closely correlated to different stages of HCC development and was able to distinguish between healthy and diseased individuals. In addition, HBV integration serves as a clinically potential biomarker for early detection of recurrent HCC and 90% of HCC patients carrying virus–host DNA in plasma undergo recurrence within 1 year.[47]

Guiding Drug Administration

In a recent study, Fujii et al[15] retrospectively examined cfDNA by targeted NGS in a cohort of 24 patients with advanced HCC before and 4 weeks after lenvatinib treatment. Surprisingly, novel alterations appeared during lenvatinib treatment when compared with the baseline and post-treatment cfDNA samples. Based on previous reports regarding the association of concerned mutations with responsiveness or resistance to the concerned drugs in other cancers, one can predict and select the appropriate drug to prescribe. For example, in a study conducted on 605 cfDNA samples in multiple cancer types,[9] NGS on a panel of 382 cancer-relevant genes was performed and more than 70% of patients showed clinically druggable mutations. Successful treatment guidance by cfDNA profiling has been exemplified by case reports for diffuse large B-cell lymphoma treated by lenalidomide as guided by possible activation of the NF-κB pathway[70] and metastatic brain tumors from lung adenocarcinoma treated by alectinib (ALK inhibitor), as guided by *(EMAP Like 4-ALK receptor tyrosin kinase) EML4-ALK* fusion.[71] However, studies in cfDNA profiling for treatment guidance are lacking for HCC.

The US Food and Drug Administration recently approved a plasma cfDNA test for *EGFR* mutation as a companion diagnostic for non–small-cell lung cancer patients.[7] A

companion diagnostic is any in vitro diagnostic test providing information necessary for the safe and effective use of a corresponding drug or biological product.[72] However, regarding the use of cfDNA profiling as a companion diagnostic to guide drug treatment, there are several concerns.[8] First, the varying quantity and quality of the input plasma cfDNA across patients are beyond the control of the testing procedure standardization and certification. Second, that mutations are not detected in cfDNA does not absolutely rule out the presence of a mutation; the clinical utility of sole liquid biopsy cfDNA profiling without any biopsy tissue profiling is questionable. Third, clinical utility regarding risk prediction and benefit evaluation for the corresponding drug administration to improve the health outcome[72] and the legal liability and cost effectiveness of using plasma cfDNA genetic profiles to guide treatment also need to be considered. Therefore, the path for establishing the regulatory framework for treatment guidance by liquid biopsy ctDNA profiling may still have a long way to go.

Disease Monitoring

cfDNAs contain a pool of genomic DNAs from different tumor clones or tumors from different sites within a patient to provide valuable information for the real-time monitoring of tumor progression on the molecular level, in addition to guiding clinical treatment. In a case report by Wang et al[73] on an advanced HCC, plasma cfDNA level and genetic alteration were assessed and SNVs of *TP53* and *TERT*, insertion or deletion of bases (indels) of *TP53* and *ARID1A*, and amplifications of *vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR),* and *MYC proto-oncogene, bHLH transcription factor (MYC)* were found. Transarterial chemoembolization was administered. Interestingly, although AFP level decreased after treatment, continuous cfDNA monitoring showed the opposite clinical course with portal vein tumor thrombus in abdominal magnetic resonance imaging and new tumor lesions. Because a histologic examination of biopsy tissues showed Programmed Cell Death 1 PD-1 positivity, the patient was treated with anti–PD-1 antibodies. The ctDNA level returned to a normal level later after combination treatment with regression of HCC size and sustained response for the tumor thrombus. This highlights that sensitive detection of ctDNA can benefit disease monitoring. More studies are needed for HCC in the use of cfDNA in monitoring cancer progression, as showcased in lung adenocarcinoma patients.[71]

Barrier of Implementation of Liquid Biopsy by cfDNA Genotyping and Future Perspectives

Currently, plasma cfDNA profiling has not been put into general clinical practice for HCCs. There are several pitfalls that await to be addressed.

Potential Challenges to Implementation

Currently, the exact mechanisms by which cfDNA is released into the bloodstream are unclear. The contributions of cfDNA from apoptosis, necrosis, autophagic cell death, and active release at different time points during disease progression, treatment response, and resistance appearance are poorly understood and will affect interpretation of the clinical observation in cfDNA assays.[3] Other challenges include the difficulty in detecting ctDNA mutations in early stage cancers with lower tumor burden,[74] complex variants of gene fusions,[75] lack of HCC-specific mutation hotspots for detection by NGS panels of insufficient broadness, and unavailability of matching effective therapies for druggable targets.[74–76] The lack of standardization in liquid biopsy procedures (eg, blood collection volume, the types of tubes for blood collection, and sample storage and shipping logistics) resulting from the varying practice for ctDNA profiling in different health care centers also hinders its use in clinical routine practice.[77]

Toward Standardization in Liquid Biopsy of cfDNA Profiling

To allow standardization, some preanalytical factors involved in the early procedures to separate the liquid part of blood from blood cells require investigation[78–80] and global consensus needs to be sought on the assignment of values and standards across multiple clinical centers and diagnostic laboratories through some standardization alliance.[81] Furthermore, to avoid false-negative detection, a lower limit of detection needs to be determined and a standard curve has to be constructed for quantifying the allelic frequencies of the mutants in the cfDNA. These can be accomplished by the spike in reference exogenous DNA with specific known mutations and alterations at different known concentrations into the blood plasma to serve as control for assessing extraction efficiency as well as fragment size bias of the extraction process.[82] Such reference materials for in-house quality control also need to be standardized.[83] An external quality assurance program should be in place to monitor laboratory performance, the proficiency in the cfDNA diagnostic test, and interpretation to identify possible sources of errors in the sample processing and analytical procedures to confer generation of reliable data for clinical decision making.[84]

Further Studies and Future Perspective

Some aspects of cfDNA require further investigation. First, the representation power of different subclones of tumors by different proportions of the relevant ctDNA mutations in the total ctDNA pool needs to be investigated.[72] Second, the parameters of the tumors (eg, tumor vascularization, tumor aggressiveness, metabolic activity, and cell death mechanism) that will affect the dynamics of ctDNA in the bloodstream need to be identified.[85] Third, because cfDNA being actively released from the tumor may have a different meaning from that being released passively from dying cells upon treatment (the former represents treatment-resistant cells/subclones while the latter represents treatment-responsive cells/subclones),[85] changes in cfDNA profiling have to be interpreted carefully before and

after HCC treatment. To better investigate the utility of plasma ctDNA genotyping to complement precision medicine in HCC, randomized trials with large multicenter cohorts and long-term follow-up evaluation for comparing ctDNA-guided decision making against standard treatment without guidance from ctDNA profiling are much awaited amidst the current transition era from the exploratory stage toward the clinical translation stage in research.[3,74,85]

Conclusions

This review provides a summary of the current understanding of cfDNA and ctDNA in HCC. We also discuss the molecular landscapes of ctDNA in HCC, including genomic and epigenetic alterations, and the consistency between plasma and tumor tissue of HCC patients. In addition, we outline the use of cfDNA in the clinical management of HCC (Figure 2). Newly identified mutational and methylation markers when coupled with traditional markers such as AFP and HBV integration detection allows promising detection and diagnosis of HCC. Multiple SNV and CNV markers, methylation markers, mutational burden, and VAF/MAF can help predict the survival of HCC patients in the prognostication of HCC. Although druggable gene mutations can be identified in the plasma cfDNA of HCC, real-life successes for such application in HCC still are scarce; more research as well as standardization in the liquid biopsy of cfDNA profiling are much awaited to address the concerned barriers of its implementation in real clinical practice for the management of HCC. Taken together, liquid biopsy using ctDNA detection and profiling offers a valuable tool in better clinical management of HCC, particularly in the monitoring of HCC progression.

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Correspondence
Address correspondence to: Daniel Wai-Hung Ho, PhD, Department of Pathology, L704, Laboratory Block, Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong. e-mail: waihungh@gmail.com; fax: (852) 2819-5375. or Irene Oi-Lin Ng, MD, PhD, Department of Pathology, Room 7-13, Block T, Queen Mary Hospital, Pokfulam, Hong Kong. e-mail: iolng@hku.hk; fax: 852-28872-5197.