

Review

Genomic Landscape of Liquid Biopsy for Hepatocellular Carcinoma Personalized Medicine

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Abstract. *Hepatocellular carcinoma (HCC) is the sixth most frequently diagnosed cancer and the third leading cause of cancer-related deaths worldwide. Advanced-stage HCC patients have poor survival rates and this requires the discovery of novel clear biomarkers for HCC early diagnosis and prognosis, identifying risk factors, distinguishing HCC from non-HCC liver diseases, and assessment of treatment response. Liquid biopsy has emerged as a novel minimally invasive approach to enable monitoring tumor progression, metastasis, and recurrence. Since the liquid biopsy analysis has relatively high specificity and low sensitivity in cancer early detection, there is a risk of bias. Next-generation sequencing (NGS) technologies provide accurate and comprehensive gene expression and mutational profiling of liquid biopsies including cell-free circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and genomic components of extracellular vesicles (EVs) including micro-RNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs). Since HCC is a highly heterogeneous cancer, HCC patients can display various genomic, epigenomic, and transcriptomic patterns and exhibit varying sensitivity to treatment options. Identification*

of individual variabilities in genomic signatures in liquid biopsy has the potential to greatly enhance precision oncology capabilities. In this review, we highlight and critically discuss the latest progress in characterizing the genomic landscape of liquid biopsy, which can advance HCC personalized medicine.

Hepatocellular carcinoma (HCC) is the most prevalent form of liver cancer, which represents the sixth most frequently diagnosed cancer and the third leading cause of cancer-related death worldwide (1). HCC early detection, surveillance status, and curative treatment are associated with significant improvements in patients' overall survival (OS) (2). However, the HCC incidence rate is growing every year and its early diagnosis and accurate staging remain challenging (3). An additional challenge is HCC risk assessment and the prevention of cancer recurrence along with monitoring the patients' postoperative status and treatment response (4).

From 70% to 90% of all HCC cases develop as a consequence of liver cirrhosis that, in turn, can be caused by inflammation associated with hepatitis B virus (HBV) or hepatitis C virus (HCV), exposure to toxins such as alcohol abuse and aflatoxin B1 (AFB1), congenital disorders, and metabolic syndrome (5). Since a large proportion of patients with HCC are accompanied by cirrhosis, it is considered as an important factor in liver injury, which leads to liver cancer. Therefore, the discovery of minimally invasive biomarkers that could enable precise HCC risk prediction and differentiating HCC from non-HCC diseases are important to identify the early stages of HCC (6).

Alpha-fetoprotein (AFP) is the only biomarker recommended for clinical usage in HCC; however, it

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demonstrates relatively low sensitivity and specificity and limited diagnostic performance (7). Combined usage of AFP with its glycoform, AFP-L3, and with des-gamma carboxyprothrombin (DCP) significantly improves the performance of HCC detection. Other efficient candidate biomarkers and biomarker panels and models for detecting HCC at early stages are also being proposed (8, 9). With the progress in next-generation sequencing (NGS) and the development of multi-omics technologies, individual variability in gene expression patterns can be assessed (10). This is especially important in the case of HCC, cancer with a high degree of intra- and inter-tumoral heterogeneity and a variety of molecular subtypes (11-13). Multi-omics-based biomarker screening has a great potential in enabling personalized treatment or risk-stratified management of HCC patients (14-16).

Personalized medicine is an emerging field, which provides novel approaches to disease's early diagnosis, prevention, prognosis, and treatment response based on individual variabilities in gene expression profiles, environmental factors, lifestyle, and diet (17). If a disease such as cancer has complex pathogenesis, patients can display various genomic, epigenomic, and transcriptomic patterns and can have different sensitivity to treatment options (18, 19). The whole-genome analysis enables identifying somatic mutations and gene amplification and greatly enhances precision oncology capabilities. This can be achieved through the usage of both inclusionary and exclusionary biomarkers that can improve the quality of patient care (20).

Recently, a new noninvasive approach, liquid biopsy, to detect and monitor tumors has been developed as an alternative approach to conventional tissue biopsy. A liquid biopsy enables repeated analysis of biomolecules collected from the blood circulation and other body fluids such as cerebrospinal or pleural fluids, saliva, and ascites (21). Such an approach allows analyzing cell-free circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and circulating non-coding RNAs (ncRNAs) (22, 23). Recent systematic reviews and meta-analyses showed that genomic profiling of liquid biopsy is a promising strategy in the identification of individual mutational patterns of HCC patients. However, the examination of liquid biopsy has relatively low sensitivity and high specificity in the early detection of HCC and there is a risk of bias (24-26). Therefore, accurate phenotyping and genotyping of liquid biopsy are of crucial importance.

Our review highlights and critically discusses the recent advancements in the identification of tumor-specific genomic signatures in ctDNA, CTCs, and exosomal ncRNAs as well as the assessment of their utility of HCC diagnosis and prognosis, and the prediction of treatment efficacy. We focus on the identification of individual variabilities in gene

expression and mutational profiles of HCC patients with the use of liquid biopsy (with the exception of circulating, non-exosomal ncRNAs) for the development of personalized patient management and treatment strategies.

Genomic Signatures of Circulating Tumor DNA

Cell-free ctDNA is derived from small fragments of genomic DNA, which arise in the blood circulation due to cell death. ctDNA can be detected in more than 75% of advanced staged human malignancies including pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and head and neck cancers, but in less than 50% of the primary brain, renal, prostate, or thyroid cancers (27). As Jiang *et al.* demonstrated the size distributions of plasma DNA fragments of HCC patients, HBV carriers with and without cirrhosis and healthy controls have a peak at 166 base pairs (bp) (28). However, a positive correlation has been observed between the proportion of cell-free DNA fragments of less than 150 bp and the tumor DNA concentration in plasma, no correlation if ctDNA length is between 150 and 180 bp, and a negative correlation if ctDNA length is >180 bp. The average fragment size of cell-free DNA was lower than that in tissue extracts indicating that ctDNA originates from tumor cells upon their apoptosis.

ctDNA in HCC diagnosis and prognosis. ctDNA has been identified with the use of deep sequencing in all stages of HCC to allow detection of somatic mutations in key genes, which drive hepatic carcinogenesis. Ng *et al.* showed that somatic mutations in HCC-associated genes are present in the ctDNA of 63% of patients and can be detected without prior knowledge of the mutations present in tissue biopsy in 27% (8/30) of patients (29). However, Howell *et al.* demonstrated high specificity, but low sensitivity of plasma ctDNA for detecting mutations in HCC tissue, since 71% of patients had mutations identified in HCC tissue DNA that were not detected in the matched ctDNA (30). They observed lower plasma ctDNA levels in Barcelona Clinic Liver Cancer (BCLC) stage A as compared to BCLC stage B/C/D (median 122.89 ng/mL vs 168.21 ng/mL) HCC patients. 29 mutations in eight genes (21 unique mutations) were detected in 18/51 patients (35%); the most frequently mutated gene was *ARID1A* (11.7%), followed by *CTNNB1* (7.8%) and *TP53* (7.8%). Additionally, Liao *et al.* found that ctDNA with mutations in HCC driver genes such as *TERT*, *CTNNB1*, and *TP53* can be detected in patients who suffered from vascular invasion predicting a shorter recurrence-free survival (RFS) (31).

The ultra-deep sequencing of all exons in a targeted panel of 58 genes including frequent HCC driver genes revealed 21 somatic mutations in the tumor tissue of 75% of patients (32). 15 of 21 somatic mutations in oncogenes and tumor

suppressors (*TERT* promoter, *TP53*, *CTNNB1*, *JAK1*, *AXIN1*, and *NTRK3*) have been identified in plasma and serum of HCC patients, evidencing that ctDNA allows confidential detection of mutations characteristic for tumor tissue. Additionally, somatic copy number alterations (SCNA)-based machine learning and low-depth whole-genome sequencing of ctDNA in 384 plasma samples allowed early detection of HBV-related HCC and distinguishing cancer-free HBV patients (33).

Mutations in human telomerase reverse transcriptase (*TERT*) gene promoter are the most prominent in HCC tumor tissue. A recent study showed that about 55% of 130 HCC patients are positive in ctDNA *TERT* promoter mutations, and this correlates with large tumor size and high DCP levels, followed by significantly shorter OS (34). Moreover, a triple diagnostic panel consisting of ctDNA *TERT* promoter mutations (C228T and C250T), miR-122, and AFP showed the best performance in distinguishing HBV-related HCC from non-HCC diseases such as chronic hepatitis B (CHB) and liver cirrhosis (35). Additionally, a positive correlation between the presence of *TERT* promoter mutation in plasma and an advanced tumor-node-metastasis (TNM) stage and vascular invasion has been observed in 42/95 HCC patients (36).

The exposure to dietary AFB1 induces DNA damage and mutations in *TP53* gene (*TP53* R249S). HBV exerts a synergistic effect with the AFB1 exposure to promote hepatocarcinogenesis (37). A somatic mutation in *TP53* that causes the R249S substitution has been found in ctDNA of HCC patients with or without hepatectomy (38). In operable HCC, *TP53* is the most common ctDNA mutant gene (50%), while R249S is the most recurrent mutation (19.2%) (39). HCC patients with a detectable mutation in postoperative plasma had a poor disease-free survival (DFS) than those without mutation (17.5 months *vs* 6.7 months), and postoperative ctDNA status can serve as an independent risk factor for the disease recurrence.

ctDNA epigenetic profiling also represents a promising approach for non-invasive cancer detection, since the inclusion of ctDNA methylation patterns enhances ctDNA diagnostic and prognostic value. For example, hypomethylation near HBV integration sites was detected in HCC patients, but not in patients with hepatitis or cirrhosis (40). Methylation profiles of tumor DNA and ctDNA have been shown to highly match each other, while diagnostic specificity and sensitivity of methylated ctDNA correlate with tumor stage and progression as well as treatment response. The methylated CpG island tandem amplification and sequencing (MCTA-Seq) showed that the liver is a major non-hematopoietic tissue contributing to plasma ctDNA level in healthy adults (41). A meta-analysis performed using both quantitative or qualitative analysis of ctDNA with the involvement of ctDNA methylation profiling and 33 papers

with 4,113 patients showed that ctDNA displays promising diagnostic potential in HCC. However, ctDNA cannot serve as an independent tool for HCC detection being only complementary to AFP (42).

ctDNA in predicting treatment response. Curative treatment with the use of surgical resection, liver transplantation (LT), trans-arterial chemoembolization (TACE) and selective internal radiotherapy (SIRT) are applicable only at the early stages of HCC and in less than 30% of cases (43, 44). However, ctDNA level can serve to predict patients' survival and the disease recurrence in operable HCC. For example, Long *et al.* observed that the ctDNA-low and ctDNA-high HCC patients had median recurrence times of 19.5 months and 14.0 months, respectively (45). Multivariate analysis showed that postoperative ctDNA, tumor number and microvascular invasion were independent risk factors for recurrence in operable HCC.

About 70%-80% of HCC patients are diagnosed at the advanced stages and can receive only systemic therapy with the use of multi-kinase inhibitors (MKIs), CDK4/6 inhibitors or immune checkpoint inhibitors (ICIs) (46-48). Selecting a treatment strategy is very complicated and dictates the necessity of biomarkers with clinical significance for predicting treatment efficacy and safety and for monitoring individual susceptibility to treatment. Targeted ultra-deep sequencing of 25 genes and Digital Droplet PCR of the *TERT* promoter in ctDNA have been exploited to identify predictors of primary resistance of advanced-staged HCC patients to systemic therapies. The most frequent mutations in the ctDNA of patients with advanced HCC were *TERT* promoter (51%), *TP53* (32%), *CTNNB1* (17%), *PTEN* (8%), *AXIN1*, *ARID2*, *KMT2D*, and *TSC2* (6% each) (49). *TP53* and *CTNNB1* mutations were mutually exclusive. MKI-treated patients with mutations in genes encoding the components of phosphatidylinositol-3-kinase (PI3K)/mechanistic target of rapamycin (mTOR) pathway had significantly shorter progression-free survival (PFS) than those without these mutations. Treatment with ICIs had no effects on PFS time of advanced-staged HCC patients with mutations in the PI3K/mTOR signaling pathway.

Nakatsuka *et al.* found that significant increase in ctDNA levels associate with clinical stage, and a poor prognosis in HCC (50). Moreover, ctDNA levels increased significantly a few days after treatment with molecular-targeted agents such as lenvatinib, and there was a correlation between post-treatment ctDNA levels and therapeutic response. Furthermore, the rate of *TERT* mutations increased from 45% to 57% in post-treatment ctDNA, suggesting dynamic changes in mutation rate and their predictive roles in HCC treatment.

Dynamic quantitative and qualitative changes in ctDNA after treatment can also be observed with a serial assessment

of ctDNA concentrations and mutational landscape. Ikeda *et al.* used serial NSG to identify mutational changes including single nucleotide variants (SNVs), amplifications, fusions, and specific insertions/deletions in genomic DNA and ctDNA after treatment with the pyrimidine antagonist capecitabine (51). The concordance levels between tissue DNA and ctDNA for the three most commonly altered genes, *TP53*, *CTNNB1*, and *ARID1A*, were 50.0%, 100.0%, and 90.0%, respectively. However, 4 of 5 patients with metabolic syndrome, 10 of 17 patients with HCV, and one patient with HBV had a *TP53* mutation. Data on ctDNA mutational profiles implicated in HCC are summarized in Table I.

Development of ctDNA tests. Frequently, ctDNA and tissue DNA show different mutational profiles. This can be explained by the facts that (i) tissue and blood samples are collected at different time points; (ii) ctDNA originates from multiple sites, while tissue DNA originates from the biopsied site; and (iii) ctDNA clones are suppressed during therapy. Dynamic changes in ctDNA SNVs and CNVs in long-term follow-up HCC patients correlated with tumor burden has been observed (52). The assessment of ctDNA mutational profile showed superior performance in combination with serum protein biomarkers such as AFP, AFP-L3%, and DCP, as well as tumor imaging for personalized tumor profiling and the assessment of OS and RFS after surgical resection and other adjuvant therapies (such as TACE, SIRT, and targeted treatment).

The use of combined detection of ctDNA alterations and protein biomarkers such as AFP is a feasible approach to identify early-stage HCC. The liquid biopsy assay, HCCscreen, for the early detection of the surface antigen of hepatitis B virus (HBsAg)-positive asymptomatic people with unknown HCC status has been developed by Qu *et al.* (53). The test has allowed identifying a total of 24 positive cases, among which four had confirmed HCC developed at a clinical follow-up for 6-8 months. The assay showed 100% sensitivity, 94% specificity and 17% positive predictive value in the validation cohort. Additionally, Kotoh *et al.* developed a sensitive methylated *SEPT9* assay as a liquid biopsy test for HCC diagnosis with sensitivity and specificity of 63.2% and 90.0%, respectively (54). The assay identified the median copy number of methylated *SEPT9* of 0.0, 2.0, and 6.4 in the healthy donors, chronic liver disease, and HCC groups, respectively, with significant differences among the groups.

Phenotyping and Genotyping of Circulating Tumor Cells

CTC isolation, enumeration and profiling. CTCs originate from primary tumor tissue or metastatic sites and can be detected in the peripheral blood. They represent intact and

viable cells, which can be distinguished from normal cells by their physicochemical properties, genotype profiles, and cell surface antigens. Immunoaffinity enrichment methods enable the identification of CTC surface antigens such as epithelial cell adhesion molecule (EpCAM) and asialoglycoprotein receptor (ASGPR) for CTC quantitative and qualitative assessment (55). For example, EpCAM-based immunomagnetic enrichment followed by multiparameter flow cytometric and immunocytochemical analysis has been developed for precise quantification of CTC number in cancer patients (56). Various anti-EpCAM and anti-ASGPR monoclonal antibody (mAb)-based platforms for phenotypic profiling and isolation of HCC-derived CTCs in HCC patients have been proposed (57, 58).

The CTCs have a short half-life, which varies from 1 to 2.4 h and during spreading in the bloodstream single CTCs spontaneously aggregate and disaggregate, while undergoing changes in their shape and size. CTCs experience dramatic phenotypic/genotypic modifications, being predominantly epithelial at tumor efferent vessels but further switching to mesenchymal phenotype through Smad2- and β -catenin-mediated signaling (59). The activation of epithelial-to-mesenchymal transition (EMT) correlates with increased CTC number in hepatic veins and is associated with advanced-stage HCC and early tumor recurrence before the appearance of clinically detectable tumor nodules. Exploration of the relationship between the EMT process in CTCs and HCC progression allowed classifying CTCs into three subtypes: epithelial, mixed/hybrid, and mesenchymal CTCs (m-CTCs), which were found in about 53%, 83% and 57% of HCC patients, respectively (60). A CTC amount ≥ 2 per 5 ml has been found in 70.9% of 165 HCC patients, while m-CTCs were significantly correlated with high AFP levels, multiple tumors, advanced TNM and BCLC stage, and tumor earlier recurrence (61).

Since CTCs carry many tumor characteristics and their EMT phenotype correlates with cancer aggressiveness, early detection of CTCs and m-CTCs in the bloodstream of HCC patients provides an efficient tool for tumor monitoring and management. The prognostic significance of phenotypic/genotypic characterization of CTC subpopulations in cancer for accurate identification of tumor stage and size and the assessment of patients' OS has been investigated in many studies. A meta-analysis of 5 studies including 339 patients revealed that the presence of CTCs in peripheral blood significantly increases the risk of HCC recurrence, while a meta-analysis of 18 studies including 1466 patients showed that CTC-positive HCC patients have shorter OS than that of CTC-negative patients (62).

The EMT activation in CTCs can be identified using the expression levels of epithelial markers, EpCAM and E-cadherin, and mesenchymal markers, N-cadherin and vimentin. During EMT, mesenchymal markers are overexpressed in m-

Table I. Findings on circulating tumor DNA mutational profiles in HCC patients.

Mutated genes	Disease status and patients' clinical characteristics observed in the study	References
<i>ARID1A</i> , <i>CTNNB1</i> and <i>TP53</i>	Barcelona Clinic Liver Cancer (BCLC) stages B, C, and D	(30)
<i>TERT</i> promoter, <i>CTNNB1</i> , and <i>TP53</i>	Vascular invasion and a shorter recurrence-free survival (RFS)	(31)
<i>TERT</i> promoter, <i>TP53</i> , <i>CTNNB1</i> , <i>JAK1</i> , <i>AXIN1</i> , and <i>NTRK3</i>	Liver cirrhosis and hepatitis B (HBV) as the main etiologies; a single nodule with a median tumor size of 4.6 cm	(32)
<i>TERT</i> promoter	Large tumor size and high DCP level, poor overall survival (OS)	(34)
	Distinguishing HBV-related HCC from non-HCC diseases such as chronic hepatitis B (CHB) and liver cirrhosis	(35)
	Tumor-node-metastasis (TNM) stage and vascular invasion	(36)
	Resistance of advanced-staged HCC patients to systemic therapies	(50)
<i>TP53</i>	Poor postoperative disease-free survival (DFS) and disease recurrence	(37-39)
<i>TERT</i> promoter, <i>TP53</i> , <i>CTNNB1</i> , <i>PTEN</i> , <i>AXIN1</i> , <i>ARID2</i> , <i>KMT2D</i> , and <i>TSC2</i>	Shorter progression-free survival (PFS); correlation with patients' response to systemic therapies	(49)
<i>TP53</i> , <i>CTNNB1</i> , and <i>ARID1A</i>	Metabolic syndrome, HCV and HBV as the main etiologies; correlation of tumor progression with treatment response	(51)

CTCs and facilitate cancer cell migration, these correlate with portal vein thrombosis and metastasis (63). Additionally, high CTC amounts and a high percentage of m-CTCs are closely related to the expression of cytokeratin 19 (CK19), which is associated with a poor prognosis for HCC patients (64). Mixed CTCs can have a pivotal role in intrahepatic metastasis, while m-CTCs can be predictors of extrahepatic metastasis (65).

Different approaches for CTC isolation *via* targeting EMT markers have been proposed (66). In addition to CK19 and compared to EpCAM and vimentin, glypican-3 (GPC-3) has been proposed for CTCs separation, since a correlation between the positive count of CTCs using GPC-3 (≥ 5 CTC per 7.5 ml blood) and BCLC stage has been observed (67). Clinical significance of GPC-3-positive CTCs as a prognostic biomarker has been confirmed in multivariate analysis, which showed that preoperative GPC3-positive CTCs ≥ 5 indicate a poor prognosis, and therefore may be a useful biomarker for HCC patients' outcome (68).

Qi *et al.* identified in HCC patients with a high CTC count and a high m-CTC percentage, 67 differentially expressed genes (DEGs) involved in cancer cell adhesion and migration, apoptosis, and angiogenesis (69). One of the DEGs was *BCAT1* that is involved in the resistance of cancer cells to chemotherapeutic drugs (70). Genetic analysis showed that CTCs derived from advanced-stage HCC tissues have *HER-2* amplification and *TP53* deletion. Using the Cancer Genome Atlas (TCGA)-based SCNA profiling, tumor tissue origination was confirmed for 73% of CTCs from 75% of cancer patients (71).

Predictive value of CTCs. A high correlation between the characteristics of CTCs with those of HCC tissue suggests that CTCs have a potential for the assessment of post-surgical

tumor recurrence and prognosis in HCC patients. Zhou *et al.* showed that CTC count ≥ 5 per 5 ml has more predictive value than AFP >400 $\mu\text{g/l}$ and tumor diameter >5 cm (72). The authors found that preoperative CTC amount in the peripheral blood of HCC patients closely correlates with microvascular invasion, while surgical removal of the tumor decreases the CTC burden. CTC persistence at a high level (≥ 5 per 5 ml) after surgery suggested a risk of early recurrence, while CTCs <5 per 5 ml associated with longer OS and PFS.

Sun *et al.* observed that patients with a post-operative CTC amount ≥ 3 exhibit higher extrahepatic metastasis risk and shorter OS than those with lower CTC count (31.25 months *vs* not reached) (73). Low CTC count correlated with AFP levels ≤ 400 ng/ml, absence of vascular invasion, high differentiation, and early tumor stage in HCC patients during postoperative follow-up. In contrast, the postoperative early recurrence of HCC positively correlates with a higher number of CTCs, m-CTCs and mixed CTCs, while a significantly shorter postoperative RFS in patients positive for m-CTCs has been observed (74). The 1-year RFS of CTC-negative and CTC-positive patients after LT were 91.6% and 61.5%, while OS rate was 91.7% and 88.5%, respectively (75). Therefore, the amount of CTCs can be useful in the evaluation of recurrence risk following LT, which is the most effective strategy for HCC treatment decreasing the CTC burden. A post-operative CTC number ≥ 1 per 5 ml has been proposed as a potential marker for predicting tumor recurrence after LT (76).

A recent study by Vogl *et al.* showed that the number of CTCs that are negative for CD45 and positive for ASGPR, CD146, and PD-L1 significantly decreases after intervention with micro-wave ablation, while no significant differences were observed in patients who received conventional trans-

arterial chemoembolization (C-TACE) (77). However, Chen *et al.* observed no relevance of dynamic changes of CTCs and their EMT phenotype to HCC recurrence after curative treatment (78). None of the total CTC or EMT markers correlated with clinical characteristics, such as age, sex, HBsAg, Child-Pugh score, BCLC stage, AFP level, tumor size, and vascular invasion. Nevertheless, Guo *et al.* showed that in EpCAM-positive HCC patients, low CTC levels correlate with treatment success and patients showed decreased CTC count after treatment with tumor resection, TACE and radiotherapy (79). On the contrary, patients with increased CTC levels showed disease progression and extrahepatic metastasis after curative surgical resection.

Rau *et al.* investigated the CTC count in HCC patients during longitudinal follow-up before and at different time points during systemic/targeted therapy. They showed that changes in the CTC count correlate with the patients' treatment response in most cases and were particularly useful for monitoring HCC with low AFP levels (<100 ng/ml) (80). In patients with AFP<100 ng/ml who were originally treated with sorafenib alone, or who were afterwards enrolled in clinical trials with systemic therapy by ramucirumab and intravenous chemotherapy (IV-CT) or intra-arterial chemotherapy (IA-CT), CTC counts decreased and were consistent with the disease status. In patients with AFP >100 ng/ml who have a trend towards decreasing AFP levels, CTC counts remained low during the follow-up period and consistent with the clinical status of stable disease (SD) or partial response (PR). In patients with stable or increased AFP levels after treatment, the CTC amount increased as the disease progressed.

Genomic and Transcriptomic Profiling of Exosomal Non-coding RNAs

Extracellular vesicle biogenesis and regulation. Extracellular vesicles (EVs) are cell-derived membranous particles that are released from cells into the extracellular space. EVs are found in various body fluids and serve for intercellular communication by delivering their cargo molecules to other cells (81). EVs can be categorized into three types depending on the mode of biogenesis, which is an evolutionarily conserved process (82). The first type is exosomes (of about 30 to 100 nm in size) that are first formed by budding into the endosomes to create the multivesicular bodies (MVBs), which either fuse with the lysosomes or with the cell membrane (Figure 1). The second type is microvesicles (MVs) or ectosomes of about 100 nm to 1 µm in size; they arise by direct budding and shedding from the cell membrane. The third type is apoptotic bodies (APBs) of about 1-5 µm in size; these arise from cells by blebbing during apoptosis (83).

Electron microscopy data demonstrate that exosomes are bilipid membranous vesicles that possess a nearly spherical

shape and a concave cavity structure containing electron-dense and coated vesicles (84). Exosomes have irregular and diverse external morphologies, indicating the possible existence of subpopulations with specific functions. The exosomal content is modulated by changes in the intra- and extracellular microenvironment; therefore, the exosomes can serve as homeostasis sensors (85). Exosome ingredients include mRNAs, micro-RNAs (miRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), proteins, lipids, and various metabolites. Exosomes can be internalized by neighboring or distant cells to regulate multiple target genes in the recipient cells and have been implicated in cell-to-cell communication, cell-microenvironment interaction and mediating cellular signaling and metabolism (86).

The content of exosomes derived from tumor cells can induce oncogenic reprogramming of target cells, which can enhance cell proliferation, tumor growth and metastasis (87). The characteristics and dynamic changes of the cargo molecules of exosomes directly reflect those in their parental tumor cells. This creates a basis for their usage in cancer diagnosis and prognosis, as well as prediction of individual responsiveness to anti-cancer therapy (88). To further facilitate investigations on exosomal markers including oncogenes and tumor suppressors, the manually curated and publicly available databases ExoCarta (<http://www.exocarta.org>) and Vesiclepedia (<http://www.microvesicles.org>) have been developed (89, 90).

Exosomal miRNAs and their target genes. miRNAs are small, 18- to 25-nucleotide (nt) endogenous cell-free, non-coding RNAs with the capability of post-transcriptional gene silencing (91). Exosomal miRNAs can be either downregulated or upregulated in HCC depending on the roles of their target genes in cancer growth. Therefore, some of them can function as oncogenes, whereas others can serve as tumor suppressors. For example, high-throughput sequencing of miRNAs and mRNA within the exosomes derived from Hep3B cells with EMT activation revealed 119 upregulated and 186 downregulated miRNAs along with 156 upregulated and 166 downregulated mRNAs. The most differentially expressed miRNA was miR-374a-5p that targets *GADD45A* encoding the growth arrest and DNA-damage-inducible protein, GADD45-α (92).

The ability of exosomes to transfer miRNAs to cancer cells has been shown under both in vitro and in vivo conditions using normal hepatic stellate cell (HSC)-derived exosomes, which can be loaded by miR-335-5p that is taken up by HCC cells. These exosomes then supply miR-335-5p to the recipient HCC cells to induce the inhibition of cancer cell proliferation and invasion (93). Transferring HCC cell-derived exosomes that are enriched in miR-155 into new target cells, where miR-155 directly binds to 3'-untranslated regions (3'-UTR) of the PTEN gene can stimulate cancer cell

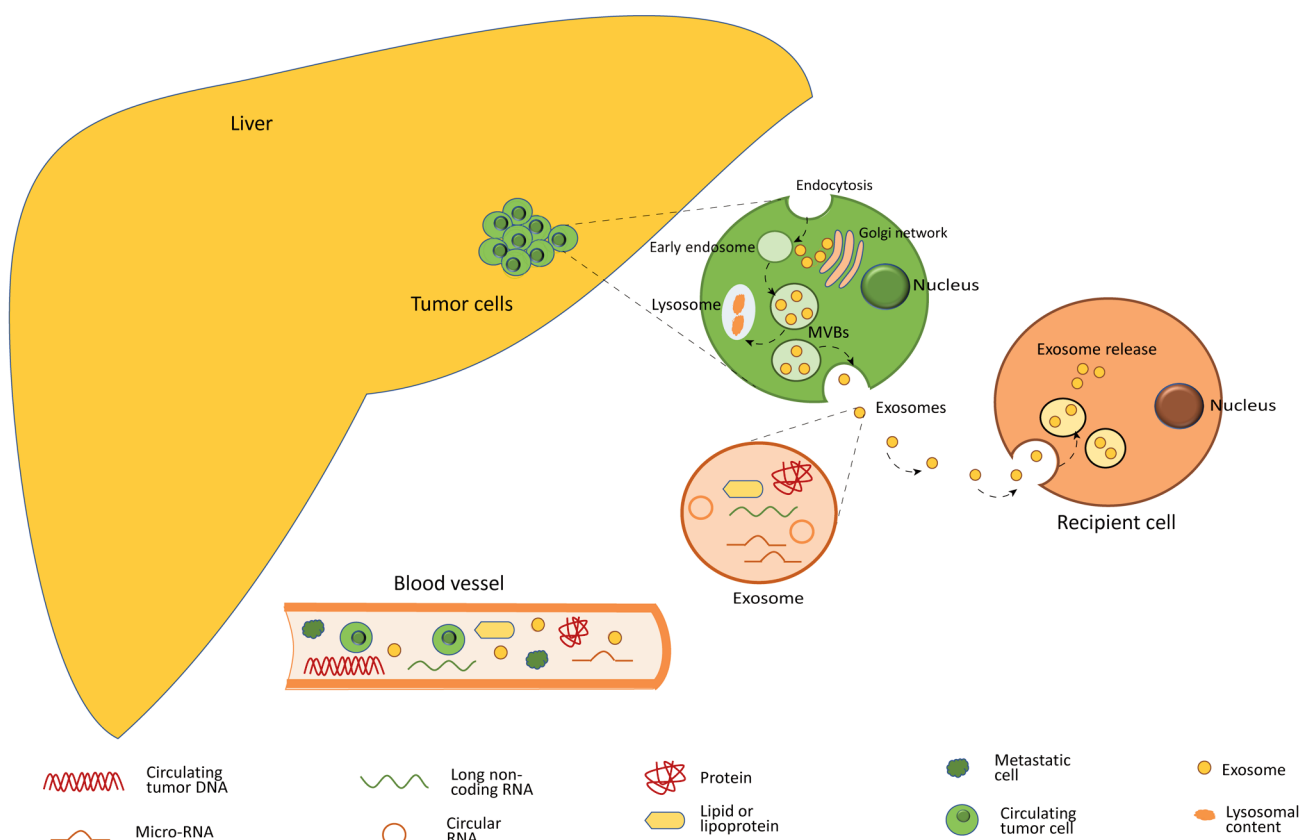


Figure 1. Components of liquid biopsy and biogenesis of exosomes from liver tumor cells. Circulating tumor DNA, circulating tumor cells and exosomes carry many phenotypic and genotypic characteristics of the tumor cells. Exosomes derived from tumor cells deliver their cargo molecules including micro-RNAs, long non-coding RNAs and circular RNAs to recipient cells. Biogenesis of exosomes proceeds through budding into early endosomes to give rise to late endosomes or the multivesicular bodies (MVBs), which further fuse either with the lysosomes or with the cell membrane.

proliferation (94). Exosomal miR-21 secreted from HCC cells can also directly target the PTEN gene leading to activation of phosphoinositide-dependent kinase 1 (PDK1)/Akt signaling in normal HSCs. This converts HSCs to cancer-associated fibroblasts (CAFs) that further promote the secretion of angiogenic cytokines including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor- β (TGF- β) and matrix metalloproteinases 2 and 9 (MMP2 and MMP9, respectively) followed by cancer progression (95).

Xue *et al.* showed that 8 serum-derived exosomal miRNAs including miR-122, miR-125b, miR-145, miR-192, miR-194, miR-29a, miR-17-5p, and miR-106a demonstrate statistically significant differences between HCC and normal serum samples (96). The authors found a correlation between patients' survival and exosomal levels of miR-106a that can function through mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways. The exosomal miR-638, miR-663a, miR-3648 and miR-4258 downregulated

the expression of endothelial cell markers, E-cadherin, and zonula occludens-1 (ZO-1) (97).

Exosomal miR-93 is upregulated in HCC to promote cancer cell proliferation and invasion by directly targeting *TP53INP1*, *TIMP2*, and *CDKN1A* genes (98). miR-1290 is also overexpressed in HCC patient serum-derived exosomes and its delivery to human endothelial cells enhances their angiogenic ability. Targeting of *SMEK1* by miR-1290 in endothelial cells diminishes the inhibition of VEGFR2 phosphorylation by *SMEK1* to promote angiogenesis and tumor growth (99). Additionally, the higher serum exosomal miR-638 expression associates with tumor recurrence and correlates with tumor size, vascular infiltration and TNM stage in HCC patients (100).

Under *in vivo* conditions, mice treated with exosomes derived from the highly intrahepatic metastatic cell line HuH-7M exhibited increased tumorigenesis and liver metastases. Yang *et al.* reported that miR-92a-3p is an abundant miRNA in exosomes from highly-metastatic HCC

cells and is enriched in the plasma of HCC patient-derived xenograft mice with high metastatic potential (101). The authors also showed that the E2F1 transcription factors and c-Myc proto-oncogene upregulate exosomal miR-92a-3p by directly binding to *miR17HG*. In turn, miR-92a-3p promotes EMT in recipient cancer cells *via* targeting *PTEN* and activating Akt/Snail signaling. Additionally, hypoxic conditions induce increased exosomal production by HCC cells to enhance EMT and cancer cell proliferation, migration, and invasion. Under hypoxic conditions, miR-1273f presents at higher levels and targets the *LHX6* gene, promoting Wnt/ β -catenin signaling (102). The acidic microenvironment triggers the activation of hypoxia-inducible factors-1 α and -2 α (HIF-1 α and HIF-2 α , respectively) and stimulates exosomal miR-21 and miR-10b expression, significantly promoting HCC cell proliferation, migration and invasion both *in vivo* and *in vitro* (103).

Roles of exosomal miRNAs in drug sensitivity. The exosomal miR-32-5p from the multidrug-resistant cell line Bel/5-FU has been shown to significantly increase in HCC to target the *PTEN* gene and activate the PI3K/Akt pathway; this induces multidrug resistance by promoting angiogenesis and activating EMT (104). In contrast, miR-744 is downregulated in HCC tissues, cell lines and exosomes; this promotes HepG2 cell proliferation and inhibits the chemosensitivity of HepG2 cells to sorafenib *via* targeting the *PAX2* gene (105). However, several studies have reported on the ability of exosomal miRNAs to sensitize cancer cells to chemotherapy and systemic/targeted therapy. A recent study showed that treatment of HepG2 and Hep3B cells with human cerebral endothelial cell-derived exosomes carrying elevated miR-214 (hCEC-Exo-214) in combination with oxaliplatin or sorafenib, significantly reduces cancer cell viability and invasion, compared to monotherapy with either drug (106). This is achieved *via* targeting P-glycoprotein (P-gp) and splicing factor 3B subunit 3 (SF3B3) in HCC cells.

Additionally, exosomes from adipose tissue-derived mesenchymal stem cells (AMSCs) can effectively mediate miR-199a delivery to HCC cells. This sensitizes HCC cells to doxorubicin by targeting the *MTOR* gene and subsequently inhibiting the mTOR pathway (107). Exosomal miR-451a is down-regulated in HCC, while its overexpression induces apoptosis of HCC and endothelial cells *via* targeting the *LPIN1* gene, which encodes lipin-1 phosphatidase phosphatase (108). The miR-451a from human umbilical cord mesenchymal stem cells-derived exosomes targets *ADAM10* to suppress paclitaxel resistance, cell cycle transition, proliferation, migration, and invasion, and to promote HCC cell apoptosis (109) (Table II).

Exosomal lncRNA-miRNA-mRNA axes. lncRNAs are involved in the regulation of cell functioning due to their

Table II. *Exosomal micro-RNAs and their target genes implicated in HCC.*

miRNA types	Target genes and/or signaling pathways	References
miR-374a-5p	<i>GADD45A</i>	(92)
miR-155	<i>PTEN</i>	(94)
miR-21	<i>PTEN</i> and PDK1/Akt signaling	(95)
miR-106a	<i>MAPK/JNK</i>	(97)
miR-93	<i>TP53INP1</i> , <i>TIMP2</i> and <i>CDKN1A</i>	(98)
miR-1290	<i>SMEK1</i> and VEGFR2 pathway	(99)
miR-92a-3p	<i>PTEN</i> and Akt/Snail signaling	(101)
miR-1273f	<i>LHX6</i> and Wnt/ β -catenin signaling	(102)
miR-32-5p	<i>PTEN</i> gene and PI3K/Akt pathway	(104)
miR-744	<i>PAX2</i>	(105)
miR-199a	<i>MTOR</i> and mTOR signaling	(107)
miR-451a	<i>LPIN1</i>	(108)
miR-451a	<i>ADAM10</i>	(109)

interaction with miRNAs to sponge them from their target mRNA; therefore, they can act as competitive endogenous RNAs (ceRNAs) (110). For example, lncRNA H19 promotes proliferation and migration but inhibits apoptosis of HCC cells *via* sponging miR-520a-3p, thereby, upregulating the *LIMK1* gene that encodes the LIM domain kinase 1 protein (111). Additionally, exosomal lncRNA ASMTL-AS1 can promote HCC malignancy *via* sponging miR-342-3p and transactivating c-Myc to promote oncogenic MAPK family Nemo-like kinase (NLK) expression and yes-associated protein 1 (YAP) activation (112).

In contrast, the transfer of exosomal lncRNA SENP3-EIF4A1 secreted by normal cells to HCC cells stimulates apoptosis and decreases the invasive, migratory and malignant properties of HCC cells (113). The inhibition of tumor growth under *in vivo* conditions was achieved through modulating the expression of the *ZFP36* gene encoding zink-finger protein 36 homolog (*ZFP36*) *via* sponging miR-9-5p. A panel of differentially expressed serum exosomal lncRNAs including lnc-FAM72D-3, lnc-GPR89B-15, lnc-ZEB2-19 and lnc-EPC1-4 has been identified in HCC (114). Among them, the expression of lnc-GPR89B-15 and lnc-EPC1-4 correlated with that of AFP, while lnc-FAM72D-3 and lnc-EPC1-4 functioned as an oncogene and tumor suppressor, respectively.

Additionally, exosome-mediated transfer of lncRNAs such as linc-VLDLR and linc-ROR to cancer cells can be involved in tumor cell drug resistance. Exosomal linc-ROR acts *via* sponging miR-876-5p causing the release of forkhead box protein M1 (FOXO1) and decreasing cancer cell sensitivity to sorafenib (115). The exposure of HCC cells to diverse anti-cancer agents including sorafenib, camptothecin, and doxorubicin increased the expression of linc-VLDLR in HCC cells and HCC-derived extracellular vesicles. This caused

Table III. Regulatory axes with the involvement of exosomal long non-coding RNAs/circular RNAs implicated in HCC.

LncRNA/circRNA types	miRNA targets	Target genes and/or signaling pathways	References
lncRNA H19	miR-520a-3p	<i>LIMK1</i>	(111)
lncRNA ASMTL-AS1	miR-342-3p	c-Myc-MAPK signaling	(112)
lncRNA SENP3-EIF4A1	miR-9-5P	<i>ZFP36</i>	(113)
lncRNA linc-ROR	miR-876-5p	<i>FOXM1</i>	(115)
circPTGR1	miR449a	MET pathway	(120)
circ-0051443	miR-331-3p	<i>BAK1</i>	(122)
circUHRF1	miR-449c-5p	<i>TIM-3</i>	(123)

increased linc-VLDLR expression in recipient cells and reduced chemotherapy-induced cell death (116).

Exosomal circRNA-miRNA-mRNA axes. One more type of ncRNAs is circRNAs that are well-expressed at low but stable levels in different species and exhibit cell-type, tissue-type and developmental-stage specificity and regulatory potency (117). Due to these features, exosomal circRNAs have a biomarker potential for early and minimally invasive cancer diagnosis and prognosis (118, 119).

Indeed, three isoforms of circPTGR1 are upregulated in serum exosomes from HCC patients and associate with the disease clinical stage and prognosis. The higher metastatic LM3 HCC cells have been shown to confer this potential on those with lower (97L) or no metastatic (HepG2) potential *via* exosomes containing circPTGR1 (120). This resulted in increased migratory and invasive capabilities of cancer cells *via* targeting miR449a and the hepatocyte growth factor receptor (HGFR or c-Met)-mediated pathway. Additionally, circRNA-100338 is highly expressed in both highly metastatic HCC cells and their secreted exosomes, suggesting that the metastatic ability of HCC cells could be enhanced by transferring exosomal circRNA-100338 to recipient cells (121).

In contrast, circRNA circ-0051443 is transmitted to HCC cells *via* exosomes to suppress their malignant behavior by promoting cell apoptosis and causing cell cycle arrest. Additionally, exosomal circ-0051443 decreases the weight and volume of the xenograft tumors in nude mice *via* *BAK1* upregulation by targeting miR-331-3p (122). The higher level of circUHRF1 expression in human HCC tissues as compared to adjacent nontumor tissues associates with natural killer (NK) cell dysfunction and poor clinical prognosis. In the plasma of HCC patients, exosomal circUHRF1 inhibits NK cell-derived interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) secretion (123). Moreover, circUHRF1 targets miR-449c-5p to upregulate the expression of *TIM-3* and may drive resistance to immunotherapy by immune checkpoint blockade with the use of anti-programmed cell death 1 (anti-PD1) mAb in HCC patients. Data on lncRNA-miRNA-mRNA and circRNA-

miRNA-mRNA regulatory axes implicated in HCC are summarized in Table III.

Conclusion

In recent years, enormous progress has been achieved in non-invasive, liquid biopsy technologies, and cancer cell whole-genome analysis, which, if combined, can greatly enhance cancer early detection and monitoring. Genomic, epigenomic, and transcriptomic profiling of liquid biopsy components, ctDNA, CTCs, and exosomal ncRNAs (miRNAs, lncRNAs, and circRNAs) enables the identification of individual variabilities in gene expression and mutational patterns. This provides personalized patient follow-up and tailored treatment strategies.

Whereas ctDNA genomic and epigenomic profiling demonstrates the most significant potential in HCC diagnosis and prognosis and in predicting treatment response, there is only limited success in CTC and exosome phenotyping and genotyping so far. CTCs have many characteristics of the tumor tissue from which they originate and, therefore, their EMT phenotype allows assessment of cancer aggressiveness. However, there are still many challenges in CTC isolation and enumeration using intracellular and cell-surface antigens. Moreover, there are discrepancies in CTC number cut-offs and the unit volume of blood samples used by different groups; this makes it difficult to compare data from different studies to assess the CTC prognostic and predictive values. Additionally, there are only limited data on mutational profiles of CTCs in HCC patients.

EVs can give information about the properties of cancer tissue since their cargo molecules directly reflect characteristics and dynamic changes in their parental tumor cells. However, unlike non-exosomal circulating miRNAs, lncRNAs, and circRNAs, gene expression and mutational profiles of target genes of exosomal miRNAs and exosomal lncRNA-miRNA-mRNA and circRNA-miRNA-mRNA regulatory axes are poorly studied. More comprehensive investigations and more data in this field are required to assess their utility in the management of HCC patients.

Conflicts of Interest

The Authors declare no competing interests.

Authors' Contributions

Nurbubu T. Moldogazieva performed the literature search, wrote the draft and revised the manuscript. Sergey P. Zavadskiy contributed to writing the draft and figure preparation. Alexander A. Terentiev provided conceptualization and critically revised the manuscript. All Authors have read and agreed with the published version of the manuscript.

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