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REVIEW

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Liquid biopsy derived circulating tumor cells and circulating tumor DNA as novel biomarkers in hepatocellular carcinoma

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ABSTRACT

Introduction: The diagnosis of hepatocellular carcinoma (HCC) is made at a relatively advanced stage resulting in poor prognosis. Alpha-fetoprotein and liver ultrasound have limited accuracy as biomarkers in HCC. Liver biopsy provides information on tumor biology; however, it is invasive and holds high threat of tumor seeding. Thus, more accurate and less invasive approaches are needed

Areas Covered: Highly sensitive liquid biopsy assays have made possible the detection and analysis of cells or organelles such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and tumor-derived exosomes. Here, we focus on CTCs and ctDNA components of liquid biopsy and their clinical application as diagnostic, prognostic, and predictive biomarkers in HCC. Unlike tissue biopsy, liquid biopsy involves attaining a sample at several time frames in an easy and a non-invasive manner. They have been efficacious in detecting and classifying cancer, in predicting treatment response, in monitoring disease relapse and in identifying mechanisms of resistance to targeted therapies.

Expert Opinion: Although interesting and highly promising, liquid biopsy techniques still have many obstacles to overcome before their wide spread clinical application sees the light. It is expected that these techniques will be incorporated into traditional methodologies for better diagnostic, predictive and prognostic results.

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1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide, but has a high death rate ranking it third among cancers in mortality. Risk factors for HCC are infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) [1], alcohol abuse [2], aflatoxicosis [3], and nonalcoholic fatty liver disease/nonalcoholic steatohepatitis [4]. Since few clinical characteristics exist for early diagnosis, HCC is mainly diagnosed at an advanced stage. The prognosis of patients with different HCC stages varies greatly. For instance, early stage (I) patients have a significantly better 5-year survival of 59% compared to 29% in patients with advanced stage (III) HCC [5]. Unfortunately a big portion of HCC patients are diagnosed at an advanced stage resulting in less than 40% of them being fit for surgery [6]. HCC is the consequence of deregulation of tumor suppressors and proto-oncogenes caused by the build-up of mutations in the genome of normal cells. While proto-oncogenes induce cell division and proliferation, tumor suppressors negatively regulate cell proliferation and result in apoptosis [7]. Thus, the detection of genetic and epigenetic modifications resulting in pathogenesis could be used for HCC therapy management, prediction and prognosis [8].

To molecularly characterize HCC tumors and recognize potential therapeutic targets, material directly taken from the tumor has to be investigated. Alpha-fetoprotein (AFP) with a sensitivity of 25% to 65% and liver ultrasound with a sensitivity of 60% are currently being used for the diagnosis of HCC but with limited accuracy [9]. Another method is liver biopsy which provides information on tumor biology; however, it is not routinely applied because it is invasive and holds a high threat of tumor seeding especially in patients with early stage disease. Unlike tissue biopsy, liquid biopsy involves attaining a sample in an easy and non-invasive way at several time frames of a patient's illness. Highly sensitive liquid biopsy assays have made possible the detection and analysis of cells or organelles such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and tumor-derived exosomes [10]. These get released from the main tumor and enter into the circulation. They are considered as 'seeds and soil' of discrete tumors [11]. Liquid biopsy is useful in detecting and classifying cancer, in predicting treatment response, in monitoring disease recurrence and in identifying mechanisms of resistance to targeted therapies. Here, we focus on CTCs and ctDNA components of liquid biopsy and their clinical applications as diagnostic, prognostic, and predictive biomarkers in HCC.

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Article highlights

- A substantial number of HCC patients are diagnosed at a late stage resulting in less than 40% of HCC patients being fit for surgery
- Traditionally used biomarkers such as alpha-fetoprotein and liver ultrasound have limited accuracy while liver biopsy is invasive
- Liquid biopsy techniques including ctDNA and CTCs have potential to be diagnostic, prognostic and predictive markers in HCC

2. CTCs

2.1. CTC biology

When a tumor invades adjacent tissues, the tumor cell releases matrix metalloproteinase, a substance which breaks down basement membrane thus easing the movement of tumor cells into the blood and the lymphatic circulation [12]. CTCs detach from tumor cells and circulate in the blood where they reside and survive into distal tissues. The majority of CTCs that enter into the blood are killed by anoikis, immune attacks or shear stress. However, some CTCs possess properties which allow them to avoid immune attacks, induce gene alterations and modulate signal transduction [13]. An important process allowing the survival of CTCs in circulation is termed epithelial mesenchymal transition (EMT). In this process, CTCs lose their epithelial markers and take on mesenchymal markers which makes them act similar to mesenchymal cells (Figure 1). CTCs that have undertaken EMT have the ability to release themselves from the primary tumor and also possess enhanced survival and metastatic abilities. Moreover, CTCs can form aggregates with fibroblasts, leukocytes, endothelial cells or platelets the end result of which are CTC clusters. These clusters have better survival and metastatic potential compared to individual CTCs [11]. CTCs are heterogeneous in nature and this characteristic allows them to modify their phenotypic and molecular characteristics depending on the surrounding micro-environment and the ongoing therapeutic strategies [14]. Thus, individual CTCs and CTC clusters that survive in the circulation continue on to metastasize (Figure 1). Several different enrichment methods have been developed in order to capture CTCs and CTC clusters in the circulation which are detailed below.

2.2. CTC detection methods

CTCs are of great potential as they can be obtained at multiple times during the course of illness; however, some technical challenges exist which are related to their frequency in the blood. The amount of CTCs available in circulation is proportional to the volume of the tumor from which they are released and thus their detection at an early stage is still a major concern [15]. Several platforms were created to enhance the recognition and isolation of CTCs, relying on either biological capture methods, physical separation techniques, nanovectors, or microfluidic chip (Figure 2).

One commonly used biological capture method that deploys the immune magnetic bead capture system is CellSearch [16] which is centered on the immune interaction between epithelial cell adhesion molecule (EpCAM) expressed on CTCs and antibodies to EpCAM immobilized on magnetic particles. Another method is NanoVelcro [17] which recognizes several cell surface markers, including EpCAM,

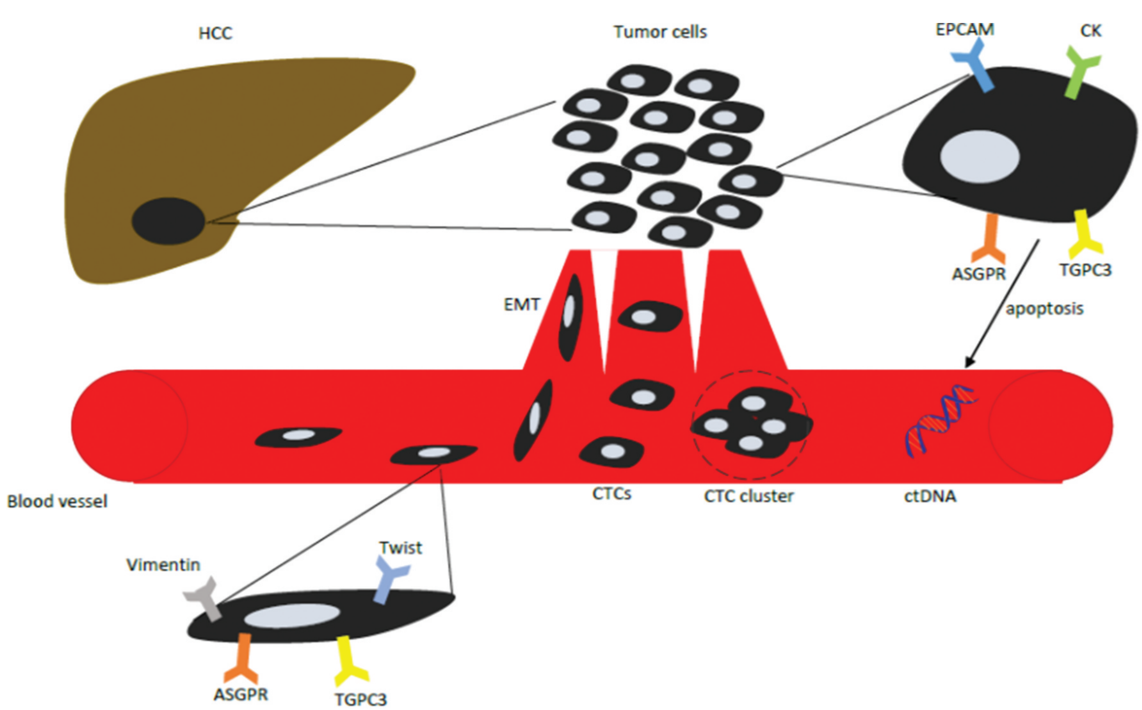

Figure 1. Circulating tumor cells and ctDNA in HCC. HCC tumor cells express epithelial markers such as EpCAM and CK, and hepatocyte markers such as asialoglycoprotein receptor (ASGPR) and Glypican-3 (GPC3). Tumor cell enter the circulation either as single CTC cells maintaining their epithelial characteristics, or undergo EMT where they express mesenchymal markers like vimentin and twist, or form CTC clusters. ctDNA is obtained after tumor cells undergo apoptosis and becomes available in the circulation.

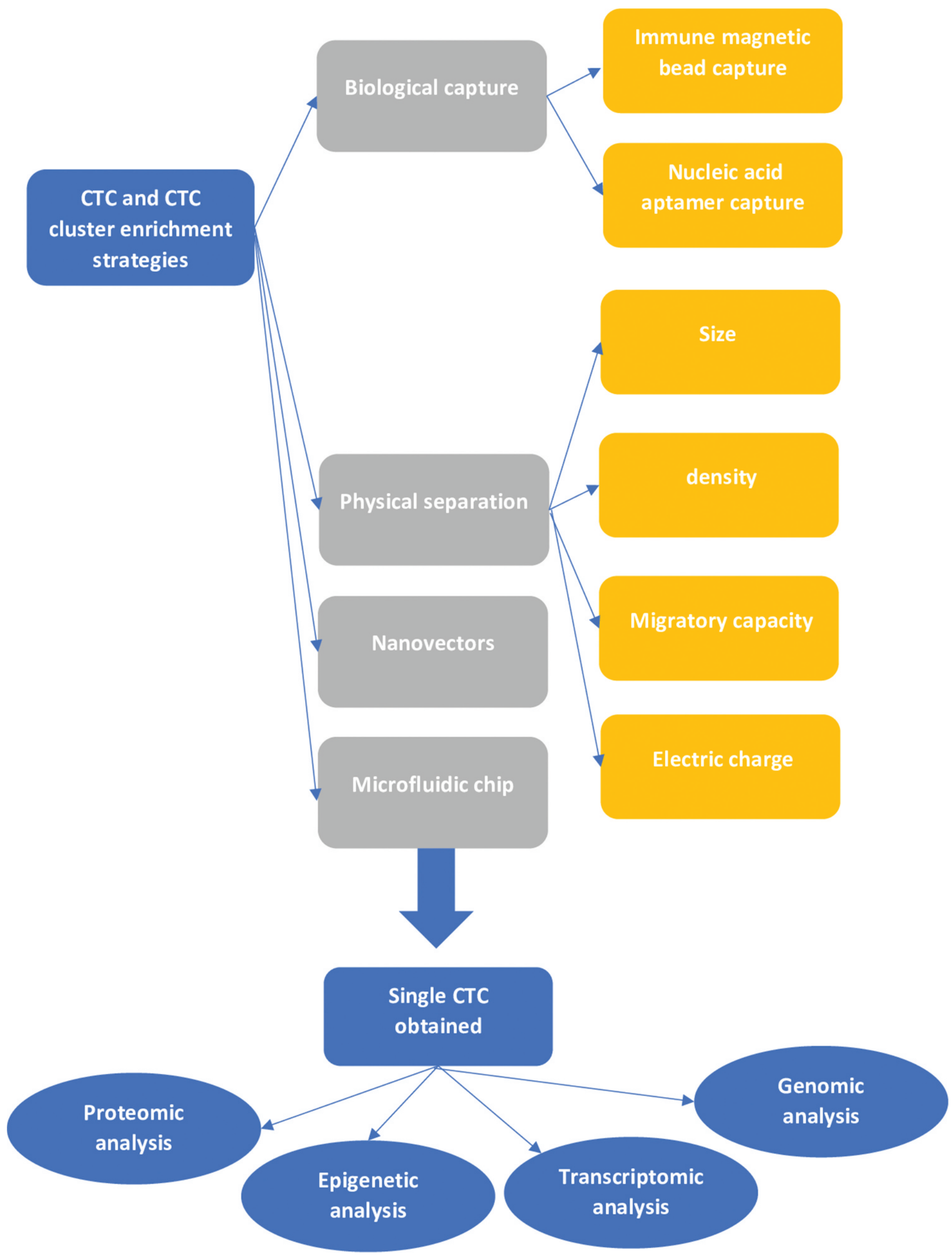

Figure 2. Circulating tumor cell (CTC) capture methods involving physcial and biological methods as well as microfluids and nanovectors. Isolation of single circulating tumor cell allows for single-cell sequencing technology of CTCs.

asialoglycoprotein receptor (ASGPR) and Glypican-3 (GPC3). The NanoVelcro system successfully identified HCC-CTCs in 97% of patients. Nucleic acid aptamer capture technique was used by Wang et al. where they made use of sLex-AP to adhere onto Hydroxyapatite/chitosan nanofilm for effective capture of CTCs in 59.5% of patients [18].

Physical methods that rely on size of CTCs usually involve the microfiltration technique. Platforms using this method include ISET [19], ScreenCell [20] and CellSieve [21]. Although, microfiltration is a rapid process for CTC capture, this method is hindered by clogging and size overlap between CTCs and leukocytes [22]. Other physical methods relying on density of CTCs usually involve centrifugation. Ficoll-Paque [23] deploys this technique while OncoQuick [24] uses both centrifugation and microfiltration to accurately capture CTCs. Dielectrophoresis (DEP) is also a physical method that involves separating cells by using discrete electrical fingerprints for diverse cell types [25]. The DEPArray [26] uses this technique to isolate single CTC cells in DEP cages for use in genetic analyses.

Two platforms that combined both biological and physical methods included the RosettSep [27] which employs both immunoaffinity and density centrifugation, using antibody complexes targeting several antigens and the CanPatrol system [28] which uses microfiltration followed by RNA in situ hybridization assay, to differentiate between epithelial and mesenchymal markers of CTCs.

Emergent microfluidic CTC isolation methods successfully deplete leukocytes without influencing tumor cells thereby preserving high-quality RNA-content [11,29,30]. The CTC-chip [31] is a silicon chip on which microposts are engraved with a geometric pattern to augment CTC capture. CTC-iChip [32], on the other hand, associated hydrodynamic separation based on size with inertial focusing of nucleated cells to deplete leukocytes. These methods allow for the use of RNA-based digital PCR (dPCR) methodologies to mark molecular signatures of cancer cells by providing detailed information on CTCs within blood samples. Kalinich et al. tested the practicability of RNA-derived digital scoring of CTC-enriched cell populations by first isolating CTCs using CTC-iChip microfluidic device followed by amplification using dPCR. Their results revealed that this technique represents a sensitive and specific CTC readout, which allows for the non-invasive screening for HCC especially in viral hepatitis and cirrhosis [33]. The CTC Cluster-Chip is a unique microfluidic chip which is used to differentiate between CTC clusters from single CTCs [34].

Nanovector methods use a magnetically assisted surface-enhanced Raman scattering (SERS) biosensor composed of anti-ASGPR antibody-Fe_3O_4@Ag magnetic nanoparticles and anti-GPC3 antibody-Au@Ag@DTNB nanorods. Based on this dual-selectivity, a limit of detection of 1 cell/mL for HCC CTC in human peripheral blood samples with a linear relationship from 1 to 100 cells/mL can be achieved [35].

2.3. CTC single cell sequencing

With single-cell sequencing technology, CTCs are handled and examined as single cells followed by single-cell sequencing which involves genomic, transcriptomic, proteomic, and epigenetic analyses that can reveal the genetic heterogeneity of different cancer cells, distinguish each cell subgroup in the tumor microenvironment and disclose the clonal characteristics of cancer cells (Figure 2). Genomic analysis involves the analysis of gene mutations related to the tumor. Amplification of whole-genome nucleotides is required before sequencing since there are only two copies of DNA molecule in each cell. A new method termed multiple annealing and looping-based amplification cycles (MALBAC) has the unique properties of quasi-linear amplification and has been shown to reduce amplification bias and increase genome coverage [36]. The data obtained on microsatellite instability, single nucleotide and copy-number variations are helpful in companion diagnostics [37].

Transcriptomic analysis involves studying the complete set of RNA transcripts to provide data on tumor heterogeneity, immune evasion, seeding and metastasis as well as resistance to therapy. D'Avola et al. demonstrated CTC heterogeneity and detected known oncogenic drivers such as Oct4 [38]. Losic et al. performed scRNA-seq from seven tumoral regions from two patients with HCC. Profound differences in transcription factor signaling among the tested tumoral regions of the two patients were found. Also, significant heterogeneity in the activation status of transcription factors across distant regions existed within the same tumor [39]. Sun et al. revealed heterogeneity of EMT status among the diverse vascular compartments and demonstrated that CTCs exhibited epithelial characteristics when they first detach from the tumor and then transformed to EMT phenotype via Smad2 and β-catenin signaling [40]. Also, the same group recently reported spatial heterogeneity and an immune-escape mechanism of CTC through the overexpression of chemokine CCL5 which is regulated by p-38-MAX signaling that recruits regulatory T cells to facilitate immune escape and metastatic seeding of CTCs [41]. Another recent study also reported on potential immune evasion mechanisms in recurrent tumor cells that dampen dendritic cells antigen presentation and recruit CD8 + T cells [42]. Ho et al. found that tumor-associated macrophages suppress tumor cell infiltration and TIGIT-NECTIN2 interaction regulated the immunosuppressive environment in HBV-associated HCC [43]. Transcriptomic analysis can also dissect signaling pathways associated with resistance to chemotherapy [29,44].

In the epigenetic analysis of single-cell CTCs, more information regarding tumor heterogeneity is obtained including DNA methylation, hydroxymethylation, histone modification, regulation of non-coding RNA, chromatin configuration and chromatin binding structural and regulatory proteins. In a study by Ogunwobi et al., the authors revealed that through hematogenous dissemination, CTCs exhibit EMT under the effect of increased amount of hepatocyte growth factor and the amplification of mesenchymal–epithelial transition factor (c-MET) via promoter demethylation at 6 CpG sites [45]. Although, much on the epigenetic characteristics of CTCs is yet to be explored, the present data highlights the potential of epigenetic analysis of CTCs to identify molecular aspects of HCC metastasis. Single-cell proteomic analysis recognizes heterogeneity of apparently similar tumor cells by studying structure of proteins as well as their localizations, functional status and interactions. Recently, Wang et al. developed a microchip-assisted single-cell proteomic method for profiling surface proteins of CTCs based on antibody and cellular DNA barcoding strategies [46]. Implementation of this method facilitated the phenotypic and functional analysis of single CTCs.

2.4. CTC clinical application in HCC

Many experiments have confirmed that the detection of CTCs contributed to the diagnosis, prognostic assessment and the prediction of tumor response to therapy in patients with HCC.

2.4.1. Diagnostic role of CTCs

Guo et al. detected EpCAM+ CTCs using reverse-transcription PCR with a 76.7% consistency with CellSearch although requiring smaller amounts of blood [47]. And in 2018, using a qRT-PCR platform which makes use of the multimarkers EpCAM, CD90, CD133, and CK19, the same group identified CTCs with stem-like phenotypes. In a multicenter cohort involving 1006 patients, utilizing the aforementioned panel resulted in a sensitivity of more than 70.0% and a specificity of more than 90.0%. This panel outperformed AFP and identified HCC patients with early-stage disease. Moreover, it allowed for the distinction between patients with HCC and other forms of liver disease [48]. Since CTCs undergo EMT, CellSearch may fail to detect CTCs undergoing EMT and therefore several other groups utilized the CanPatrol platform to analyze CTCs. For instance, Liu et al. retrospectively enrolled 33 HCC patients and 10 healthy controls. The authors detected CTCs in all HCC patients and none in healthy controls. The average number of CTCs was 17.84 (2–81), while the mean number of epithelial,

epithelial-mesenchymal-mixed and mesenchymal CTCs were 0.81 (4.54%), 13.81 (77.41%) and 3.21 (17.99%), respectively. The authors also found that the number of CTCs exhibiting epithelial characteristics correlated with tumor size, while CTCs exhibiting EMT were related to tumor number and CTCs exhibiting mesenchymal characteristics were associated with metastasis [28]. These data were further confirmed by Chen et al. where they reported that the number of CTCs and epithelial/mesenchymal characteristics influenced clinical disease stage as well as metastatic potential [49]. Cheng et al. studied a cohort of 170 patients composed of HCC patients (n = 113) and non-malignant liver disease patients (NLD) (n = 57). Comparing the two groups, the authors determined epithelial CTCs (median:0vs0), mixed CTCs (median:2vs0), mesenchymal CTCs (median:0vs0), and total CTCs (total CTCs of all phenotypes) (median:4vs1) in the HCC and NLD groups. Total CTCs ≥3 indicated positive and had the highest sensitivity and specificity (sensitivity: 61.95%, specificity: 89.47%). Moreover, total CTC count represented a higher diagnostic value compared to AFP and a combination of CTCs and AFP was associated with the highest diagnosis [50]. Future approaches to improve the diagnostic capability of CTCs ought to involve multiple biomarkers which could overcome the decreased sensitivity and specificity of conventional single biomarker diagnosis.

2.4.2. Prognostic role of CTCs

By deploying the CellSearch platform, EPCAM+ CTCS were associated with vascular invasion [51,52], increased AFP levels [52], advanced Barcelona Clinic Liver Cancer (BCLC) stage [51], increased disease progression [40,53], increased recurrence rate [40,54–56] and decreased overall survival [51,54]. However, the CellSearch platform cannot detect CTC which are undergoing EMT and thus only around 30–40% of HCCs are EpCAM positive [57].

By deploying the qRT-PCR method, Guo et al. showed that decreased CTC levels prior to treatment were correlated with improved prognosis following surgery, transcatheter arterial chemoembolization (TACE) or radiotherapy [47]. Moreover, Zhou et al. found that elevated CTC/Treg levels were associated with increased risk of recurrence after surgery compared to preoperative low CTC/Treg levels [58].

Other studies exploring the clinical relevance of CTCs on prognosis in HCC relied on metastasis-associated CTC phenotypes. First, pertaining to stem-like phenotypes, Liu et al. showed that in 60 HCC patients, elevated CD45-intercellular cell adhesion molecule-1 (ICAM-1) positive CTCs were associated with decreased disease-free and overall survival [59]. As for Fan et al., the authors showed that circulating cancer stem cells (CD45− CD90+ CD44+) were associated with HCC recurrence after hepatectomy. Patients with more than 0.01% circulating cancer stem cells had a significantly lower 2-year recurrence-free survival rate and overall survival rate than patients with ≤0.01% circulating cancer stem cells [60]. Second, in relation to EMT phenotypes, mixed EMT status CTCs or mesenchymal-like CTCs were reported to have a worse clinical outcome due to advanced BCLC stages [49,61], earlier tumor relapse [28,62–64], higher metastatic potential [28,49,61,63,64] and elevated serum AFP levels [49,61]. However, these results were inconclusive as two other studies failed to report the same [65,66]. Such conflicting results may be attributed to the retrospective study nature, difference in CTC capture platform being used, small cohort size, short follow-up time, and data from a single study center. As for CTC clusters, although these are rare in circulation, reports have shown that they are 23–50 times more metastatic than single CTCs [11,67]. Sun et al. demonstrated that circulating tumor microemboli were identified in tumor efferent vessels in around 50% of patients revealing that these initiate from oligoclonal tumor cell groupings. Of note, around 15 patients showed circulating tumor microemboli in hepatic veins but not in peripheral artery, whereas 5 patients showed that these were circulating in peripheral vein thereby suggesting that CTCs might be capable of aggregating in the blood [40].

2.4.3. Predictive role of CTCs

Although surgery is still the gold standard treatment for HCC, CTCs could be manipulated to aid in assessing the effectiveness of surgical techniques and in monitoring tumor progression. Recently, Qi et al. studied a cohort of HCC patients treated with anatomical or non-anatomical resection. The group reported that anatomical resection may increase survival, only in patients with a decreased CTC count and negative mesenchymal- and epithelial mesenchymal-CTC phenotypes. Thus, analyzing CTCs prior to surgery can be utilized to better direct the type of resection strategy [68]. Several clinical studies reported that CTC frequency decreased following tumor resection and that an elevated CTC count following surgery is correlated with poor prognosis [47,48,54,56,69]. Using 3 CTC markers including MAGE3, survivin, and CEA, Shi et al. evaluated the effectiveness of cryosurgery in unresectable HCC patients and showed that these markers have the capacity to predict treatment response [70]. Moreover, Jin et al. reported that positive AFP mRNA of CTCs can be a crucial predictor for HCC metastasis pre- and post-hepatectomy [71].

Moreover, identifying CTCs that show specific tumor markers and drug targets aid in predicting response to therapy. Guo et al. reported that HCC patients having low CTC counts were more likely to benefit from treatment whereas patients showing elevated CTC counts were more likely to progress after treatment [47]. Wu et al. explored the predictive importance of serum dickkopf-1 (DKK1) and CTCs following TACE. Responders to TACE treatment had decreased levels of DKK1 and CTCs whereas non-responders had elevated levels [72]. Three days following percutaneous radiofrequency ablation, CTC counts and CTCs expressing mesenchymal characteristics were elevated but no correlation was found between changes in CTC levels and the radiofrequency ablation factors [73]. Recently, Rau et al. reported the results of a longitudinal analysis of 17 patients with locally advanced or metastatic HCC. A change in CTC count was related to response to treatment and was specifically useful in patients who had low levels of serum AFP [74]. Yan et al. reported that the number of CTCs was associated with disease progression and treatment response. Sorafenib was able to significantly decrease the number of CTCs

[75]. Moreover, Li et al. showed that CTCs could be manipulated instead of tumor tissue for description of pERK/pAkt expression, and that HCC patients with pERK+/pAkt- CTCs were more responsive to treatment with sorafenib [76]. Use of EGFR inhibitor during TACE treatment had the capacity to reduce CTC numbers [77]. Also, the presence of PD-L1 positive CTCs differentiated between early-stage disease and advanced/metastatic disease. Patients demonstrating response to anti-PDL1 had PD-L1 positive CTCs suggesting the possibility that PD-L1 positive CTCs might be predictive of response to immunotherapy [78]. Thus, by monitoring changes in CTCs and CTC quantity, drug resistance could be discovered in a timely manner and modifications to therapeutic plans could be implemented.

3. ctDNA

3.1. Biology of ctDNA

Fragments of cell free DNA (cfDNA) are released in detectable amounts by cancer cells into the circulation and other biofluids. cfDNA has the potential to act as a surrogate marker for multiple indications in cancer, including diagnosis, prognosis, and monitoring. However, harnessing the full potential of cfDNA requires the optimization and standardization of preanalytical steps, refinement of current analytical strategies, and a better understanding of its origin, physical properties, and dynamics in circulation [79]. ctDNA is the portion of cfDNA circulating in body fluids and originating from dying tumor cells or macrophages that have phagocytized tumor cells [80] (Figure 1). ctDNA contains information pertaining to genetic and epigenetic alterations that are relevant to cancer development, progression, and resistance to therapy. These alterations include loss of heterozygosity and mutations of tumor suppressor genes (such as *TP53*) and oncogenes (such as *KRAS* and *BRAF*). Additional genetic alterations that are detectable on ctDNA and used as biomarkers in cancer include the integrity of non-coding genomic DNA repeat sequences (such as *ALU* and *LINE1*) [81]. Epigenetic alterations in ctDNA bear cancer-specific histone modifications [82], nucleosome positioning [83,84], fragmentation patterns [85–87], fragment end-point motifs [88], single nucleotide mutations [89], copy number aberrations [90], and DNA methylation [91] or 5-hydroxymethylcytosines [92]. DNA methylation is the most studied epigenetic modification that happens when a methyl group is added to the carbon-5 position of a cytosine through DNA methyltransferases, occurring predominantly in CpG dinucleotides [93].

Both quantitative alterations referring to the quantity of ctDNA as well as qualitative alterations of ctDNA referring to monitoring tumor specific genetic aberrations are primarily detected in HCC patients. ctDNA has several advantages. Collection of fluid samples is easier and more convenient than tissue samples. Also, ctDNA allows the collection of samples at several time frames during the course of illness which provides insights into the kinetic mutations arising during therapy. However, similar to CTCs, the clinical applications of ctDNA are challenging. First, early detection of HCC using ctDNA is not feasible since early stage (asymptomatic tumors) are not likely to release enough ctDNA to be detectable in a typical blood draw of 10 mL [94]. Also, mutations in cfDNA can also be found in healthy individuals and are very difficult to distinguish from those associated with cancer [95]. Second, the implementation of cfDNA in a clinical setting remains limited because of the lack of standardization of cfDNA analysis [96]. In particular, several pre-analytical steps could influence the outcome of ctDNA measurement such as choice of matrix, sample collection and processing procedures, storage conditions, thawing protocols, DNA isolation method, and storage conditions of isolated circulating DNA [97]. Third, the influence of lifestyle and biological factors on the amount of circulating DNA present in blood is another issue. ctDNA may inaccurately represent DNA from weaker tumor cells which are more likely to die and release their contents in circulation than tumor cells which are actively proliferating and metastasizing [95]. For instance, given the large increase in circulating DNA induced by acute exercise, it is recommend to control for physical activity prior to blood collection when total circulating DNA levels are of interest [98]. Finally, several issues related to standardization and technical validation in clinical trials must be addressed prior to its routine clinical application in HCC [99]. Some of these challenges may be overcome through sampling of larger volume, further improvement of analytical sensitivity, detection of multiple alterations and a better understanding of cfDNA and ctDNA [100].

3.2. ctDNA detection methods

Depending on the purpose for testing ctDNA, the detection methods may differ. They could be divided into targeted techniques to detect some known mutations using PCR (such as real-time PCR, droplet digital PCR (ddPCR), beads, emulsion, amplification and magnetics (BEAMing), amplification-refractory mutation system (ARMS)-PCR), or non-targeted techniques that categorize millions of DNA fragments (such as Sanger sequencing and next-generation sequencing (NGS)).

Real-time PCR utilizes known point mutations and is intended to detect targeted single nucleotide variations in ctDNA [101–103]. Although this method is easy to use and is cost-efficient, it has the disadvantage of low sensitivity and low ability to investigate a large number of targeted loci. dPCR allows the identification of rare target-mutated genes by compartmentalization and amplification. This method screens for pre-defined known point mutations. ddPCR performs PCR based on water/oil emulsion droplet technology by employing millions of monodisperse droplets which can perform many reactions at the same time [104,105]. However, some setbacks remain in its clinical feasibility which include the difficulty in making droplets, the high cost and the time consumption. BEAMing is a highly sensitive digital PCR method that uses emulsion PCR in addition to flow cytometry to differentiate and quantitate somatic DNA mutations as rare as 0.01% [106].

NGS is based on the analysis of several millions of short DNA sequences in parallel followed by either sequence alignment to a reference genome or de novo sequence assembly. This method is used to detect the order of nucleotides in DNA or RNA with high through-put, selectivity and specificity of up to 96% [107]. However, besides being expensive, around 2–3 weeks are needed to obtain sequence information which limits the delivery of early diagnosis. NGS-based technologies can be divided into targeted amplification sequencing (TAS) and targeted capture sequencing (TCS). TAS involves the use of dozens to hundreds of pairs of PCR primers for the target gene for several rounds of PCR amplification and enrichment. A demonstrative method is tagged-amplicon deep sequencing (TAM-Seq) that utilizes known point mutations; however, it is limited to a small number of targets [108]. TCS comprises the use of a probe for capturing the targeted gene by using hybrid method of enrichment. A representative of TCS method is cancer personalized profiling by deep sequencing (CAPP-Seq) that utilizes known point mutations in addition to copy number variations and rearrangements [107].

The most widely used method to detect ctDNA methylation is mehtylation-specific (MS-PCR). This method is based on treating DNA with bisulfite to chemically modify the non-methylated cytosine and convert them to uracil [109]. The methylation profile of ctDNA can then be further investigated using downstream applications such as PCR or NGS [110].

3.3. ctDNA clinical application in HCC

3.3.1. Diagnostic role of ctDNA

DNA methylation pathway can lead to changes in chromatin structure, DNA conformation, DNA stability and DNA and protein interactions, thereby controlling gene expression. ctDNA positivity has surpassed positive AFP values in terms of diagnosis in HCC [111,112]. Its effectiveness in early diagnosis has surpassed conventional diagnostic approaches by a period of 4 years [113]. Moreover, ctDNA methylation analysis was capable of differentiating early stage HCC disease [92]. These results suggest the practicability of ctDNA as an early biomarker for HCC diagnosis.

Several groups reported on alteration in DNA-methylation at specific genes such as p15 [114], p16 [115], APC [116], SPINT2 [117], SFRP1 [118], p16INK4a [119], TFPI2 [120], GSTP1 [121] and Ras association domain family protein 1A (RASSF1A) [122]. These alterations were mainly correlated with progressive HCC disease. Concurrent methylation at p15 and p16 was seen in 48% of tumors and p15/p16 methylation was found in the plasma of 92% of HCC patients [114]. Methylation in RASSF1A on the other hand was found in 90% of HCC patients. Further utilization of serum methylated levels can identify HCC patients from controls with an overall predictive accuracy of 77.5% [123].

Several studies used combination marker panel. For instance, Xu et al. utilized a combination marker panel composed of 10 methylation genes and reported a sensitivity and specificity of 85.7% and 94.3% in the training cohort and a sensitivity and specificity of 83.3% and 90.5% in the validation cohort [91]. Cai et al. used a panel composed of 32 genes and reported better performance differentiating early-stage disease or small tumors (≤2 cm) from non-HCC compared to AFP [92].

3.3.2. Prognostic role of ctDNA

Telomerase reverse transcriptase (TERT) mutation is a very common reported genetic alteration in HCC. Plasma TERT mutation was detected in 44% of HCC patients and in none of non-HCC cirrhotic patients; and was associated with increased mortality [124]. In another study, TERT mutations were reported in 47.7% of plasma cfDNAs and were also correlated with poor prognosis [125]. Variations in TP53 and CTNNB1, were also reported to have a poor prognosis in HCC patients [126,127]. Human mutL homolog 1 (MLH1) is a tumor suppressor gene involved in DNA mismatch repair and defects influence genomic instability and cancer developments. A single nucleotide variant chr3:37025749 T > A, in addition to increased ctDNA level, were predictors of poor survival in HCC patients [128]. Methylation of insulin-like growth factor-binding protein 7 (IGFBP7) is important in HCC development. In a study of 155 HCC patients undergoing hepatectomy, IGFBP7 promoter methylation correlated with overall survival and early tumor recurrence, suggesting its feasibility as a prognostic marker in HCC after hepatectomy [129].

3.3.3. Predictive role of ctDNA

Data on the predictive response of ctDNA to various treatment strategies in HCC has been studied including response to targeted therapy and surgery (Table 1). Although immunotherapy with checkpoint inhibitors has become a standard of care in patients with advanced HCC, some challenge still exists because mutational DNA molecules in HCC have not been defined and certain abnormalities which are present in HCC are also common in benign liver disease.

No identical genomic profile in HCC patients has been detected which suggests that studying the mutational genomic setting of a patient can enable personalized treatment [130,131]. The ctDNA gene sequence is subject to change with changes in treatment strategies. For instance, in the study by Alunni-Fabbroni et al., in the case of HNF1A, 50% of patients showed the variant already before starting sorafenib. At 8 weeks, only one patient was still positive, suggesting a good therapy response. However, at first follow-up after using sorafenib, the variant showed a mutation allele frequency of 82%. This finding suggests a clonal expansion of cells that no longer responded to sorafenib treatment [132]. Moreover, ctDNA may also be used as a biomarker for drug resistance specifically to sorafenib which constitutes the upfront treatment of HCC patients with advanced disease [133].

Surgical resection is main stay treatment for early stage HCC patients. Nevertheless, HCC patients recur after surgical intervention with a recurrence rate of >60% after 5 years. Thus, specific aberrations in ctDNA have been utilized as biomarkers for post-operative recurrence in HCC patients [112,134]. ctDNA is able to detect recurrence beforehand and can monitor longitudinal changes of different mutants and response to treatment [135].

4. Conclusions

Liquid biopsy is a novel method that is non-invasive and is capable of monitoring tumor progression and recurrence as well as therapeutic response in real time. CTCs and ctDNA hold great prospect to smooth the execution of precision medicine in patients with HCC. CTCs measured using CellSearch system are already FDA-approved in metastatic breast cancer and colorectal cancer for prognosis and

Table 1. Predictive role of ctDNA in HCC patients receiving targeted therapy or surgery.

Type of treatment	Patients	Assay	Agents used	Main findings	Reference
Targeted therapy	14 HCC	NGS-based	Sirolimus Cabozantinib Palbocilib celecoxib	Treatment with Sirolimus and Cabozantinib were effective in PREN and MET mutations while treatment with Palbocilib and Celecoxib decreased AFP level and was beneficial in CDKN2A and CTNNB1 mutations.	[104]
	13 HCC	NGS-based	sorafenib	After sorafenib, 68% of gene variants were detected suggesting a clonal choice prompted by sorafenib.	[106]
	27 HCC	NGS-based	Sorafenib refametinib	In HCC patients with RAS mutations, ctDNA evaluated effectiveness of treatment with refametinib alon or in combination with sorafenib.	[105]
	12 HCC	NGS-based	sorafenib	DNA methylation are the drivers behind EMT-mediated resistanc eto sorafenib therapy. These changes could aid in predicting tumor response to sorafenib.	[107]
Surgical resection	34 HCC	NGS-based	Surgical resection	ctDNA identified patients at high risk of disease recurrence and metastasis.	[87]
	81 HCC	ddPCR	Hepatectomy	Patients with increased mutant allele frequency (MAF) had more incidences of microvascular invasion and recurrence. Patients with increased MAF had shorter DFS and OS.	[108]
	1 HCC	NGS-based	TACE Hepatic surgery	HCKp.V174M mutation was identified following TACE. The same mutation became undetectable after liver surgery and then increased after recurrence.	[109]

disease monitoring. Currently, ctDNA FDA approved tests in non-small cell lung cancer, colorectal cancer and breast cancer include the following five tests: FoundationOne Liquid CDx, Guardant360 CDx, cobas EGFR Mutation Test v2, Therascreen PIK3CA RGQ PCR Kit and Epi ProColon. ctDNA analysis is beginning to transition from the research setting into the clinic. Analysis of gene panels in plasma has now become available as a potential clinical tool. Large studies are under way to establish the overall performance and clinical utility of such assays when a tumor biopsy is not available for analysis [136].

An ongoing trial (NCT02973204) is investigating the role that CTCs and ctDNAs play as clinical support tools in HCC. The purpose of the study is to identify which patients need special monitoring and individualized therapy and explores the importance of these tests in supporting clinical decision-making. Moreover, the transformational idea of a multi-cancer early detection (MCED) test that has the ability to detect multiple cancers and dramatically increase the cancer detection rate (CDR) in the population is being investigated. A new generation of cfDNA-based MCED tests is being developed and will be available for clinical use in the near future [137]. Currently, a few large prospective studies, such as DETECT-A, STRIVE (ClinicalTrials.gov Identifier: NCT03085888), SUMMIT (ClinicalTrials.gov Identifier: NCT03934866), PATHFINDER (NCT04241796) and ASCEND (ClinicalTrials.gov Identifier: NCT04213326), are completed or are under way to clinically validate MCED tests.

5. Expert opinion

The identification of reliable non-invasive biomarkers that could personalize the management of HCC patients are of utmost importance. The vital aspect of managing HCC patients is detecting the disease at an early stage followed by accurate monitoring and choice of best therapeutic approach. Although data on the role of liquid biopsy in HCC is limited compared to other malignancies, the results from some studies in HCC as well as from other malignancies show very promising and motivating results. It must be noted however that the majority of studies in HCC are small, retrospective, single-center, case-control studies with broadly varying patient demographics, an issue which makes validation studies very problematic. This brings along the need to have multicenter, prospective studies with a larger sample size using a uniform CTC detection platform to provide effective validation. Even though liquid biopsy has great potential in overcoming many obstacles encountered with current diagnostic, prognostic and predictive tools used in HCC, its prevalent clinical application has not yet witnessed the light. The procedures of obtaining 'liquid', and the isolation, enrichment, or detection of CTCs and ctDNA ought to be standardized. Most of the current studies used different methods to detect CTCs or ctDNA, resulting in varied sensitivity and specificity. Thus, to have a chance to be used in clinical practice, liquid biopsy biomarkers ought to be specific to distinguish early-stage HCC from other liver diseases such as cirrhosis or chronic hepatitis where the molecular pathways leading to cancer may already be in place. Also, highly sensitive tests ought to be in place to identify HCC at an early stage even when tumor burden is low. As for ctDNA, although it has high specificity in diagnosis, a multi-marker analysis may offer a more inclusive perception into cancer specificity.

Moreover, one highly discouraging drawback is cost. Current available methodologies are time-consuming and expensive, and most have insufficient sensitivity and cannot cover the entire genomic loci. It is most likely that future directions in the field of liquid biopsy will aim at developing more advanced techniques that would be less expensive and less time consuming. It is less likely however that these techniques will become completely independent and reliable by themselves in diagnosing HCC and providing prognostic and predictive information without the aid of currently used methodologies. What is more likely is the integration of liquid biopsy techniques into the currently used tools to achieve more predictive power. For instance, surveillance using liver ultrasonography in addition to liquid biopsy techniques is one method of integration which could prove to be efficient and reliable and remains to be evaluated in ad hoc studies. By being real-time monitors of disease, liquid biopsy techniques not only have the potential to monitor disease progression but could also have interventional potential. It is likely and very encouraging to integrate the use of liquid biopsy techniques such as CTCs and ctDNA in large prospective trials and rely on these biomarkers to direct therapeutic strategies.

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