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Detection of circulating tumor cells and their implications as a novel biomarker for diagnosis, prognostication, and therapeutic monitoring in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is among the leading causes of worldwide cancer-related morbidity and mortality. Poor prognosis of HCC is mainly attributed to tumor presentation at an advanced stage when there is no effective treatment to achieve the long term survival of patients. Currently available tests such as alpha-fetoprotein (AFP) have limited accuracy as a diagnostic or prognostic biomarker for HCC. Liver biopsy provides tissue that can reveal tumor biology but it is not used routinely due to its invasiveness and risk of tumor seeding, especially in early stage patients. Liver biopsy is also limited in revealing comprehensive tumor biology due to intra-tumoral heterogeneity. There is a clear need for new biomarkers to improve HCC detection, prognostication, prediction of treatment response, and disease monitoring with treatment. Liquid

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biopsy could be an effective method of early detection and management of HCC. Circulating tumor cells (CTCs) are cancer cells in circulation derived from the original tumor or metastatic foci, and their measurement by liquid biopsy represents a great potential to facilitate the implementation of precision medicine in patients with HCC. CTCs can be detected by a simple peripheral blood draw and potentially show global features of tumor characteristics. Various CTC detection platforms utilizing immunoaffinity and biophysical properties have been developed in order to identify and capture CTCs with high efficiency. Quantitative abundance of CTCs, as well as biological characteristics and genomic heterogeneity among the CTCs, can predict disease prognosis and response to therapy in patients with HCC. This review article will discuss the currently available technologies for CTC detection and isolation, their utility in the clinical management of HCC patients, their limitations, and future directions of research.

Keywords

Hepatocellular Carcinoma; Circulating tumor cells; Biomarkers; Immunoaffinity; Epithelial-to-Mesenchymal Transition; CTC Heterogeneity

Introduction

Hepatocellular carcinoma (HCC) is an aggressive primary liver cancer that typically occurs in the setting of chronic liver disease and cirrhosis, and it is the sixth leading cause of cancer incidence and the fourth cause of cancer death globally(1). While a select group of patients with small, localized HCC may undergo curative therapies, those with large tumor burden, vascular invasion, or metastasis have a poor prognosis and are managed with systemic treatment and supportive care. There exists an unmet need for HCC biomarkers for early detection and prognostication, as well as prediction and monitoring for treatment response.

Currently, alpha-fetoprotein (AFP) is the most widely used biomarker for HCC. Biannual liver ultrasound with or without serum AFP is the main HCC screening strategy recommended by major societies(2–4). AFP is used as a prognostic and predictive biomarker in patients with HCC. Elevated levels of AFP have been associated with increased tumor size and portal vein thrombosis, as well as increased risks of liver transplant waitlist dropout and posttransplant recurrence(5, 6). Serum AFP is also a predictor of treatment response in HCC patients after liver transplant and ramucirumab treatment(7, 8). However, AFP's role as a biomarker for early detection of HCC is limited by its poor sensitivity. Alternative protein-based serum tumor markers such as AFP lectin fraction (AFP-L3) and des-y-carboxy prothrombin (DCP) have been shown to improve the diagnostic performances when used in combination with AFP(9). Glypican-3 (GPC3)(10), cytokeratin 19 (CK19)(11), golgi protein 73 (GP73)(12), midkine(13), osteopontin(14), squamous cell carcinoma antigen (SCCA) (15), and annexin A2(16) have all been shown to have diagnostic and prognostic roles in HCC as well, but they have not been adopted for widespread clinical practice.

Liver biopsy allows direct sampling of the tumor tissue and molecular characterization of the tumor. However, it is an invasive test with a risk of bleeding and concern for possible tumor seeding. Moreover, as HCCs exhibit significant inter- or intra-tumoral heterogeneity from genetic aberrations, transcriptional and epigenetic dysregulation, a single biopsy specimen

containing a small amount of tumor tissue may not be representative of the whole HCC tumor(17).

Over recent years, a variety of “liquid biopsy” techniques have shown significant promise as novel biomarkers for HCC. In liquid biopsy, samples of body fluids are collected to obtain vital pieces of phenotypic, genetic, and transcriptomic information about the primary tumor(18). The primary forms of liquid biopsy include circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), microRNA (miRNA), and extracellular vesicles (EVs). First described in 1869, CTCs are malignant cells derived from either the primary tumor or metastases that migrate into the systemic circulation(19). CTCs represent a unique biomarker different from any of the existing cancer biomarkers as they represent a sampling of the patient’s live tumor cells (20). Analysis of CTCs can help guide treatment plans by identifying specific mutations in target genes and predicting response or resistance to specific treatments. This review article will discuss the CTCs and provide an overview of their biology, current and emerging techniques, and various studies investigating their roles as potential diagnostic and prognostic biomarkers for HCC.

Overview of CTC biology and clinical implications

As a malignant tumor proliferates and invades into the adjacent tissue, the tumor cells secrete matrix metalloproteinase (MMP) which breaks the basement membrane, enabling the tumor cells to gain direct access to the nearby blood and lymphatic vessels (21) (Figure 1). Once in the bloodstream, the tumor cells become CTCs and can remain in circulation until they reach different tissues of the body and invade them. Most of the CTCs introduced into circulation get rapidly killed by processes such as anoikis, immune attacks, or shear stress(22). Thus, the CTCs undergo a number of adaptations in order to survive in their hostile new environment. A key process is epithelial to mesenchymal transition (EMT), where CTCs lose epithelial-type surface markers and gain mesenchymal markers, allowing them to behave like mesenchymal cells. CTCs that have undergone EMT can easily detach themselves from the primary tumor tissue and invade the capillaries and possess significantly improved ability to survive and metastasize. Also, CTCs may form aggregates with fibroblasts, leukocytes, endothelial cells or platelets to form CTC clusters, which possess a significantly higher metastatic potential and increased ability to survive compared to individual CTCs(23). It should be noted that CTCs are not identical clones of each other, but represent a heterogeneous population of cells from different tumor foci, with abilities to change their phenotypic and molecular characteristics under selective microenvironmental and therapeutic pressures(24). Studies have shown that profound heterogeneities exist between tumor cells within the primary tumors and those within sites of metastasis. Therefore, liquid biopsy using CTCs should not be regarded merely as a less invasive alternative to an actual biopsy, but a novel way to obtain a comprehensive understanding of the heterogeneous tumor cells throughout the body. Detection and isolation of CTCs can enable the implementation of precision medicine by revealing the molecular characteristics of the tumor and identify markers for targeted therapy.

CTCs are being extensively studied in a variety of solid organ malignancies. In patients with metastatic prostate cancer, CTC enumeration has been shown to be a reliable predictor of

prognosis and treatment response(25). Detection of HER2+ CTCs in patients with breast cancer can identify candidates for targeted therapy(26). While CTCs are highly promising as a form of liquid biopsy, there are technical challenges to be overcome before there can be widespread utilization of CTCs. Most importantly, CTCs have an extremely rare frequency in the circulation and the number of CTCs tends to be proportional to tumor volume, which makes their detection in early-stage disease challenging(27). Therefore, the challenge of CTC research lies in improving the sensitivity and specificity of CTC detection to the levels necessary for accurate and comprehensive molecular characterization.

Another cornerstone of liquid biopsy, ctDNA is the fraction of cell-free DNA (cfDNA) which originates from dying tumor cells or macrophages that have phagocytized tumor cells(28). As ctDNA contains genetic mutations identical to those of their originating tumor cells, ctDNA can be used to identify heterogeneous tumor-specific mutations and epigenetic changes, and to monitor tumor dynamics in patients who are undergoing therapy. ctDNA is already used for treatment response monitoring or early detection of relapse, and also to identify specific mutations to make therapy decisions(29–31). Similar to CTCs, the clinical application of ctDNA is challenged by the technical difficulty of identifying ctDNA in the background of a significantly larger amount of cfDNA from other tissues. Also, it is unclear whether ctDNA accurately represents the genetic make-up of actively proliferating and metastasizing tumor cells, as ctDNA may disproportionately represent DNA from “weaker” tumor cells that are more prone to dying and releasing their contents into circulation(32). Compared to ctDNA, the main advantage of CTCs is that intact, viable tumor cells are being captured. As long as their cellular integrity is preserved, the captured CTCs can be used for functional assays and be cultured to evaluate drug resistance(33).

CTC Detection and Isolation

Several systems have been developed to improve the detection of CTCs, utilizing their distinct physical and molecular characteristics. The platforms can largely be broken down into three categories of immunoaffinity-based enrichment, biophysical property-based enrichment, and enrichment-free methods (Table 1).

Immunoaffinity—Immunoaffinity-based CTC enrichment techniques use antibodies against cell surface markers tethered to the device surface or a magnetic substance. Positive enrichment refers to capturing CTCs by using antibodies against specific tumor-associated antigens expressed on CTC surfaces, while negative enrichment refers to depleting the hematopoietic cells in the background by using antibodies against CD45(34). Until recent years, CTCs were defined as nucleated EpCAM+/CK+/CD45- cells. The only FDA-approved CTC diagnostic technology, the CellSearch™ system, uses an immunomagnetic separation system of ferrofluid beads coated with antibody to EpCAM (anti-EpCAM)(35). Microfluidic devices such as the CTC-Chip™ are composed of antibody-coated microposts with a geometric arrangement to optimize cell attachment(36). The CTC-iChip™ combines microfluidic and immunomagnetic technology and has demonstrated a greater sensitivity of CTC detection compared to CellSearch™(37). In parallel, Wang et al. pioneered a unique concept of “NanoVelcro™”, which utilizes antibody-coated nanostructured substrates with

integrated microfluidic chaotic mixers to facilitate CTC-substrate contact to enhance CTC capture(38).

Epithelial markers such as EpCAM are often down-regulated or lost during the epithelial to mesenchymal transition (EMT)(39). These CTCs with EMT phenotype have highly metastatic properties and can escape positive enrichment systems that target the epithelial markers such as EpCAM and CK. Given such limitations of relying on epithelial markers, positive enrichment strategies targeting stem cell markers (e.g., CD133), mesenchymal markers (e.g., vimentin), and cancer-specific antigens (e.g., HER2, PSMA) have also been developed(40).

However, even antibody cocktails targeting a wide variety of antigens may not account for the heterogeneity of CTC antigens. Negative enrichment strategies address this by targeting and removing background hematopoietic cells. There are commercialized platforms dedicated to negative enrichment, and some of the positive enrichment technologies such as magnetic activated cell sorting (MACS) and CTC-iChip™ can also be used for negative enrichment by replacing anti-EpCAM with anti-CD45(34). While they allow for higher sensitivity compared to positive enrichment technologies, negative enrichment technologies alone typically have a much lower purity(41).

Biophysical Properties—Compared to blood cells, CTCs are distinguished by their large size, mechanical plasticity, and dielectric mobility properties. This enables them to be isolated using techniques such as membrane filtration, density gradient stratification, inertial focusing, and dielectric mobility. These so-called “label-free” methods are increasingly popular, as they avoid the challenges of targeting numerous specific antigens. Also, they make downstream processing easier as the isolated CTCs are not tagged with antibodies(34).

Microfiltration utilizes the larger, more rigid phenotype observed by the CTCs. Isolation by Size of Epithelial Tumor Cells (ISET™)(42) and ScreenCell™ (43) use track-etched membranes, while CellSieve™(44) and Flexible Micro Spring Array (FMSA) ™(45) use photolithography to construct membranes that minimize captured cell damage. The Cluster-Chip™ is a unique 3D microfiltration system, specifically designed to capture CTC clusters(46). The main advantage of microfiltration is its ability to rapidly process blood for CTC enrichment. However, microfiltration systems are subject to clogging, and size overlap between leukocytes and CTCs makes it challenging to achieve high purity(34).

Density-based gradient centrifugation utilizes the differences in specific gravities of leukocytes and CTCs(47). Though not initially developed for CTC isolation, Ficoll-Paque™ has been able to detect CTCs in patients with various cancers(48). OncoQuick™ combines centrifugation and filtration and has superior CTC capture ratio compared to Ficoll-Paque™ (49). Finally, the RosetteSep™ CTC Enrichment Cocktail integrates immunoaffinity with density centrifugation, using antibody complexes targeting an extensive mixture of antigens(50). Centrifugation is widely used for CTC isolation due to its reliability and inexpensiveness, but it is best used as an initial step prior to additional enrichment using other strategies(34).

Inertial focusing utilizes the differential inertial effect on different sizes of cells to help with the isolation of CTCs. It is applicable to CTCs larger than background hematologic cells. This method has been combined into many microfluidic devices(51). By establishing a model accounting for wall interaction force, shear gradient lift force and secondary-flow drag force as well as other effects including particle properties (i.e., cells in CTC isolation devices), rotation, interparticle spacing, and fluid properties, researchers have recently had significant improvements predicting the motion of cells according to inertial focusing in microfluidic devices.(52) These devices have shown promising results with a high sensitivity of CTC detection and viability of retrieved CTCs(53).

Another novel technology, dielectrophoresis separates cells using distinct electrical fingerprints between different cell types(54). The dielectric characteristics of cancer cells are significantly different from blood cells. By applying an alternating electrical field, cells are electrically polarized, and CTCs can be isolated through differential electric forces. Becker et al. has demonstrated differential dielectric parameters in breast cancer cell lines, lymphocytes and erythrocytes. The dielectric differences between cancer and blood cells may result from the different morphologic features of the cell membrane including microvilli, membrane folds and blebbing.(54) DEPArray™ can trap single cells in dielectrophoresis cages and has been used to recover single CTCs for highly specific genetic analyses(55).

Enrichment-Free Methods—All enrichment technologies require verification of the captured cells, which can be significantly time-consuming. Advancements in the field of high-speed, fluorescence imaging have led to the development of imaging platforms that enable a direct identification of CTCs from blood samples without the enrichment step(56). Imaging flow cytometry distinguishes CTCs from leukocytes using parameters such as higher karyoplasmic ratio and size(57, 58). Another unique, in-vivo direct imaging method to detect CTCs is photoacoustic flow cytometry (PAFC), which enables real-time detection of CTCs in veins using a laser-based technology(59). Lastly, there are functional assays to detect CTC which exploit aspects of liver cellular activity such as secretion of tumor-associated proteins and preferential adhesion of CTCs to a specialized matrix(60).

CTCs as a Biomarker in HCC

EpCAM-based CTC detection in HCC—The commonly used, immunoaffinity-based CTC enrichment techniques such as CellSearch™ have been used to detect and capture EpCAM+ CTCs in patients with HCC (Table 2). In 2013, Sun et al. used CellSearch™ to detect EpCAM+ CTCs in HCC patients undergoing tumor resection. The authors preoperatively detected EpCAM+ CTCs in 67% of HCC patients. A preoperative CTC count of two or greater was shown to be a predictor of tumor recurrence after surgery(61). Additional studies using CellSearch™ demonstrated that the presence of EpCAM+ CTCs in HCC patients was associated with vascular invasion(62, 63), significantly elevated AFP(63), more advanced BCLC stage(62), disease progression(64), higher recurrence rate(65), and shorter overall survival(62–66). In 2014, Guo et al. established an optimized platform based on negative enrichment and qRT-PCR for the detection of EpCAM+ CTCs and exhibited 76.7% consistency with the CellSearch™ system(67). In 2016, Wang et al. used CTC-

BioTChip to detect EpCAM+ CTCs in 60% of 42 HCC patients and found significant correlations between both the positive rate and number of CTCs with TNM staging(68). In 2016, Zhou et al. studied the prognostic value of EpCAM+ CTCs and regulatory T cells (Treg) in HCC patients and found that elevated EpCAM+ CTC and Treg levels were associated with early recurrence and poor clinical outcome(69). Unfortunately, EpCAM-based CTC enrichment platforms are limited by the loss of epithelial markers in CTCs undergoing EMT. Furthermore, a study showed that only a small proportion (30–40%) of HCC cells express EpCAM(70).

Combined Markers based CTCs detection in HCC—To overcome the low sensitivity of EpCAM-based CTC detection platforms in HCC, alternative markers have been investigated (Table 2). For example, asialoglycoprotein receptor (ASGPR), a transmembrane protein exclusively expressed on hepatocyte surfaces, has been used in a variety of enrichment methods for CTC detection in HCC patients(71–78). In 2011, Xu et al. performed immunomagnetic separation using HCC cells bound by biotinylated asialofetuin, a ligand of ASGPR, and was able to detect CTCs in 81% of HCC patients(71). Both the presence of CTCs and the quantity of CTCs significantly correlated with tumor extent, portal vein tumor thrombus, and differentiation status(71). In 2014, the same group used a synthetic anti-ASGPR antibody instead of ASGPR ligand with successful CTC detection in 89% of HCC patients(73). Another study achieved an even higher CTC detection rate of 91% in HCC patients by using a mixture of antibodies against ASGPR and CPS1, combined with negative enrichment(75). In 2016, Zhang et al. used a CTC-Chip with antibodies to ASGPR, P-CK and CPS1 and isolated CTCs from 100% of 36 HCC patients(76). Recently, Court et al. used the NanoVelcro CTC Assay with a multimarker panel of EpCAM, ASGPR, and GPC3, and captured CTCs in 97% of patients with HCC(78).

Label-Free (Biophysical and Enrichment-Free) Methods of CTC detection in HCC—"Label-free" methods have also been investigated for CTC detection in HCC patients (Table 2). In 2000, Vona et al. used the ISET™, a 2-D microfiltration system, to detect CTCs in HCC patients undergoing liver resection, and was able to capture tumor microemboli during surgery(42). Vona et al. also found that the number of CTCs detected by ISET™ correlated with the presence of a diffuse tumor, portal tumor thrombosis, more advanced liver disease, as well as shorter survival(79). A direct comparison of CellSearch™ and ISET™ in their ability to detect CTCs in HCC patients revealed a significantly superior performance by ISET™ (ISET™: 100%, CellSearch™: 28%)(80). However, one disadvantage of the ISET™is the difficulty of releasing CTCs for downstream genetic analysis(27).

In 2016, Liu et al. used imaging flow cytometry (IFC) to detect CTCs from blood samples of HCC patients, utilizing a higher karyoplasmic ratio (HKR) seen with CTCs. When compared to the traditional CTC detection method based on CD45- EpCAM+ cells, IFC using HKR cells showed significantly greater AUROC of 0.82 vs. 0.73(57). Ogle et al. studied IFC focusing on cell size and compared its effectiveness to a multi-marker panel consisting of CK, EpCAM, AFP, GPC-3, and DNA-K. IFC with the inclusion of size criteria, combined with the depletion of CD45 cells, led to the capture of all positive

biomarker CTCs, as well as an additional 28% of CTCs which did not express any of the biomarkers tested(58). The advantages of IFC are its ease and cost-effectiveness without requiring antibodies or complex handling of blood samples. However, its specificity could be limited by the presence of immune cells which also have large size and high karyoplasmic ratios(57).

Biological Characterization and Heterogeneity of CTCs—Multi-marker combination panels and downstream molecular analysis can reveal biological characteristics and heterogeneities of HCC CTCs (Table 2). Identification of CTCs expressing specific tumor markers and drug targets allows providers to predict response to therapy. Shi et al. used three CTC markers MAGE3, survivin, and CEA for evaluating the efficacy of cryosurgery on unresectable HCC, and found that they were reliable in predicting treatment response(81). In 2016, Li et al. used the expression status of pERK and pAkt to detect CTCs in 93% of patients with advanced HCC undergoing sorafenib treatment(82). Among different patterns of pERK/pAkt expression, presence of pERK+/pAkt- CTCs was significantly associated with longer progression free survival and higher rate of response to sorafenib(82). Winograd et al. evaluated for CTCs expressing programmed death-ligand 1 (PD-L1) in HCC patients, and found that presence of PD-L1+ CTCs discriminated HCC patients with early stage and advanced/metastatic disease. Of six patients receiving anti-PD1 therapy, all three patients demonstrating response had PD-L1+ CTCs, compared to only one of three non-responders, suggesting the possibility that PD-L1+ CTCs might be predictive of treatment response to immunotherapy(83). In 2017, Kalinich M et al. also devised a unique strategy of using CTC-iChip™ to isolate CTCs, then applying RNA-based digital PCR to detect a panel of liver-specific transcripts which were combined into a single metric CTC score. The CTC score was highly correlated with BCLC staging and declined in patients receiving therapy(84).

Expression of EMT markers such as twist and vimentin in CTCs of HCC patients have been studied as markers for predicting recurrence and prognosis. The majority of these studies used the CanPatrol™ CTC analysis platform, a two-step technique including microfiltration and subsequent characterization of CTCs using a variety of epithelial (EpCAM, CK8/9/19) and mesenchymal markers (Vimentin and Twist)(85). Using this method, Chen et al. detected CTCs in 95% of 195 HCC patients(86). The proportion of hybrid and mesenchymal CTCs was associated with increased age, BCLC stages, metastasis, and AFP levels(86). Wang et al. studied 62 HCC patients undergoing surgical resection and found that patients with a higher number of mesenchymal CTCs had increased risk of recurrence and shortened disease-free survival(85). Other studies using CanPatrol™ showed a consistent association between mesenchymal CTCs and poor clinical outcomes(87–91).

An emerging body of literature suggests profound heterogeneity among the HCC CTCs. D'Avola et al. developed a novel analytical technique that combines IFC and single-cell mRNA sequencing (92). Genome-wide expression profiling of CTCs using this approach demonstrated significant transcriptome heterogeneity, even amongst CTCs from the same HCC patient(92). Such marked heterogeneity among CTCs was also reported by Sun et al., who found significant heterogeneity in the EMT status of CTCs across different vascular compartments, with predominantly epithelial phenotype at release but switching to EMT

phenotype during transit(93). A recent study demonstrated even more heterogeneity among CTCs, both in size and karyotype. Wang et al. used subtraction enrichment and immunostaining-fluorescence in situ hybridization (SE-iFISH), which employs *in situ* characterization of phenotypes and karyotypes to examine both chromosomal aneuploidy and tumor marker expressions. Using this novel strategy, the authors discovered the existence of small HCC CTCs smaller than WBCs, with a very high prevalence of chromosome 8 aneuploidy. The postsurgical quantity of triploid or multiploid CTCs significantly correlated to poor prognosis(94).

Conclusion and Future Directions

Decades of research have led to significant advancements in the clinical utility of CTCs in patients with HCC (Figure 1). As a form of liquid biopsy, CTCs hold great potential to facilitate the implementation of precision medicine in patients with HCC. CTCs are already FDA-approved for disease monitoring and prognostication in patients with metastatic breast and colorectal cancer, and there is an ongoing NIH-sponsored clinical trial (NCT02973204) to investigate CTCs and ctDNA as clinical support tools in HCC. However, significant challenges remain before CTCs can be adopted for widespread clinical use in HCC. In addition to the technical challengs with CTC detection and isolation, there are numerous CTC detection methods, each with its own protocols for sample preparation, enrichment, and analysis. Most studies are small, single-center, case-control studies with widely varying patient demographics such as ethnicity, etiology of liver disease, and stage of HCC, which makes validation studies very difficult. Therefore, there needs to be a standardized assay protocol with high sensitivity and specificity that can capture the full spectrum of CTCs. This can potentially be achieved by multicenter, prospective studies with a larger sample size using a uniform CTC detection platform, which can provide effective validation of the findings(95).

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List of Abbreviations

AFP	alpha-fetoprotein
AUROC	area under the receiver operating characteristic curve
BCLC	Barcelona Clinic Liver Cancer
CTC	circulating tumor cell
DEP	dielectrophoresis
EMT	epithelial to mesenchymal transition
FDA	Food and Drug Administration
FMSA	flexible micro spring array

HCC	hepatocellular carcinoma
HCV	hepatitis c virus
HKR	higher karyoplasmic ratio
IFC	imaging flow cytometry
ISET	isolation by size of epithelial tumor cells
NAFLD	non-alcoholic fatty liver disease
NPV	negative predictive value
PACF	photoacoustic flow cytometry
PPV	positive predictive value
RT-PCR	reverse transcriptase polymerase chain reaction
scRNA-seq	single-cell mRNA sequencing
SE-iFISH	subtraction enrichment and immunostaining-fluorescence in situ hybridization
TNM	tumor, node, and metastases
Treg	regulatory T cells

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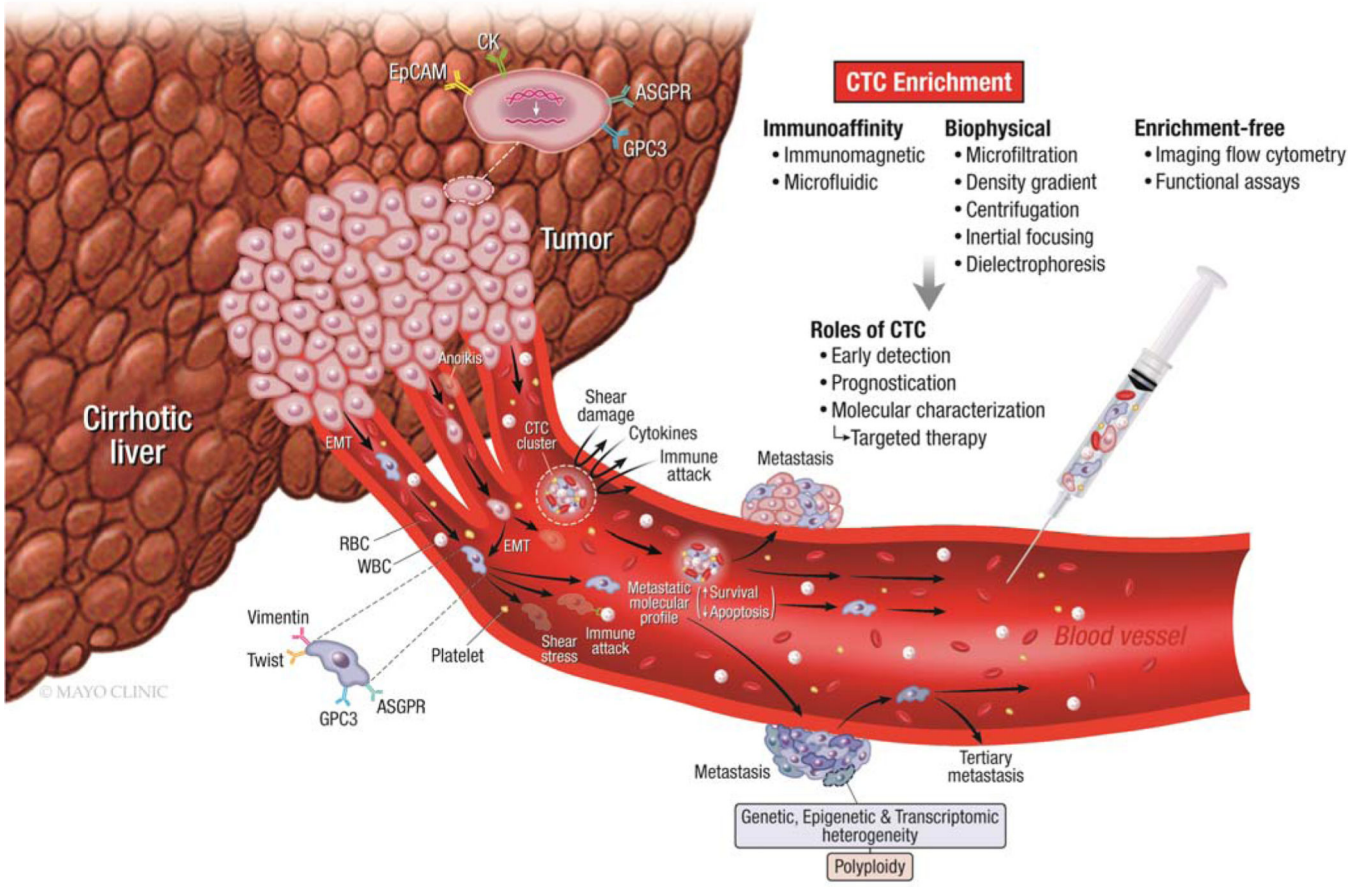

Figure 1.

Circulating Tumor Cells in Hepatocellular Carcinoma

HCC exhibits epithelial characteristics with surface expression of epithelial markers such as EpCAM and CK, as well as hepatocyte-specific markers such as ASGPR and GPC3. HCC cells enter the bloodstream to become CTCs in different ways - (1) maintaining the epithelial characteristic, (2) undergoing EMT with the expression of mesenchymal markers such as Twist and Vimentin, and (3) as CTC clusters consisting of multiple tumor cells as well as RBCs, platelets, stromal cells and fibroblasts. Upon entering the circulation, most CTCs get destroyed by anoikis, shear stress or immune attack. Some epithelial CTCs undergo EMT in transit. Eventually, CTCs that survive have acquired a more metastatic molecular profile with increased survival and decreased apoptosis. CTC clusters are protected from the processes that would kill isolated CTCs. Both the individual CTCs and CTC clusters that survive go on to metastasize and develop highly heterogeneous genetic, epigenetic and transcriptomic heterogeneity as well as polyploidy. The CTCs and CTC clusters can be collected and isolated from the peripheral blood via a variety of different enrichment techniques and can help with early detection, prognostication, and molecular characterization of HCC.

Abbreviations: CTC, circulating tumor cells; EMT, epithelial to mesenchymal transition; HCC, hepatocellular carcinoma

Table 1.

CTC Detection Technologies

	Subcategory	Key Features	Capture yield*	Ref.
Immunoaffinity				
CellSearch	Immunomagnetic	Ferrofluid beads functionalized with anti-EpCAM. The only FDA-approved platform for CTC enumeration in metastatic prostate, breast and colorectal cancer.	≥85%	35
MACS	Immunomagnetic	MNPs conjugated to various antibodies. Large surface area to volume ratio. Can be used for both positive/negative enrichment.	40–90%	34
SERS	Immunomagnetic	MNPs used as the CTC enrich platform and the SERS signal amplification substrate. Dual-selectivity using anti-ASGPR nanoparticles and anti-GPC3 nanorods for HCC CTC isolation.	> 90%	77
SE-iFISH	Immunomagnetic, *in situ* karyotyping	Combines subtraction enrichment followed by *in situ* phenotypic and karyotypic characterization, especially useful for identifying chromosome aneuploidy.	70–87%	94
CTC-Chip	Microfluidic	Microposts ($^{\mu p}$CTC-Chip) with geometric arrangement to generate laminar flow to optimize cell attachment. Herringbone-shaped grooves (^{HB}CTC-Chip) and microvortices to increase cell contact toward antibody-coated surfaces. High purity, process whole blood.	> 60%	36
CTC-iChip **	Microfluidic, Immunomagnetic, Inertial Focusing	Sequential steps of micropillar array, inertial focusing and magnetophoresis (isolation of nucleated cells including CTCs and WBCs using deterministic lateral displacement, alignment of nucleated cells in a microfluific channel and collection of magnetically tagged cells). Combining strengths of microfluidics and magnetic-based cell sorting.	97%	37
NanoVelcro **	Microfluidic	Anti-EpCAM/anti-ASGPR/anti-GPC-3 coated nanosubstrates, with integrated microfluidic chaotic mixers to facilitate CTC-substrate contact to achieve enhanced HCC CTC capture. Vimentin(+) CTCs identified as a poor prognostic subset in HCC.	80–94%	38
Biophysical property				
ISET	Microfiltration	Size-based isolation of CTCs that are usually larger than hematologic cells. Track-etched membranes with nano to micron-sized pores in thin polycarbonate films.	N/A	42
ScreenCell	Microfiltration	Size-based isolation of CTCs that are usually larger than hematologic cells. Track-etched membranes with nano to micron-sized pores in thin polycarbonate films.	74–91%	43
CellSieve	Microfiltration	Precision pores arranged in arrayed patterns to enable CTC capture under low-pressure state and preserve intracellular architecture.	83–91%	44
FMSA	Microfiltration	Flexible polymer micro springs minimize cell damage. Allows rapid enrichment directly from whole blood.	90%	45
CanPatrol	Microfiltration, Immunomagnetic	Microfiltration followed by detection of EMT markers using RNA *in situ* hybridization.	80–89%	85
Ficoll-Paque	DGC	Inexpensive, easy-to-use in combination with other techniques. The ratio of CTCs to PBMCs remain unchanged.	84%	48
OncoQuick	DGC Microfiltration	Porous membrane above separation media for additional separation by filtration. Superior CTC capture ratio compared to Ficoll-Paque.	87%	49
RosetteSep CTC Enrichment Cocktail	DGC Immunomagnetic	Antibody complexes targeting an extensive mixture of CTC antigens. Can be used with centrifugation platforms such as Ficoll-Paque to enhance capture efficiency.	36–60%	50
DEPArray	Dielectrophoresis	Traps single cells in dielectrophoresis cages generated via an array of electrodes. Can recover single CTCs.	N/A	55

	Subcategory	Key Features	Capture yield*	Ref.
Enrichment-free				
ImageStream	IFC	Utilizes size and karyoplasmic ratios to detect CTCs, without requiring antibodies or complex handling of blood samples.	N/A	57
PAFC	*In vivo* direct imaging	Absorbtion of laser by nanoparticles which are tagged on targeted cells via antibodies. Enables real-time detection of CTCs in veins using a laser-based technology.	N/A	59
EPISPOT	Functional assay	Detects viable CTCs at the single-cell level by identifying proteins secreted/released/shed (antibody-based) from single epithelial cancer cells. Theoretically can be combined to any CTC enrichment step with live tumor cells.	N/A	60

CTC, circulating tumor cells; DEP, dielectrophoresis; DGC, density gradient centrifugation; IFC, imaging flow cytometry; MNP, magnetic nanoparticle; PAFC, photoacoustic flow cytometry; PBMC, peripheral blood mononuclear cell; SE-iFISH, subtraction enrichment and immunostaining-fluorescence in situ hybridization; SERS, surface-enhanced Raman scattering

*From cell line spiking study (the capture yield may vary in different cancer types and different generations of the technology)

**In each processed patient sample, background 500 and 200–1,000 WBCs were non-specifically captured in the CTC-iChip and NanoVelcro platform, respectively. Other platforms did not report a reliable purity.

Table 2.

CTC Studies in HCC

Study	Patients	Method	CTC Marker	Main Findings
EpCAM-based CTC detection in HCC				
Sun YF et al[61]., 2013	123 HCC; 5 BLD; 10 HV	CellSearch	EpCAM	CTCs identified in 67% of preop pts, and 28% 1 month after resection. ≥2 CTCs/ 7.5mL predicted recurrence.
Schulze K et al[62]., 2013	59 HCC; 19 BLD	CellSearch	EpCAM	CTCs identified in 31% of HCC pts, associated with advanced stage, vascular invasion and shorter OS.
Guo W et al[67]., 2014	299 HCC; 24 BT; 25 CLD; 71 HV	RosetteSep, MACS, qRT-PCR	EpCAM	Negative enrichment and qRT-PCR-based platform had AUC 0.70, sensitivity 42.6% and specificity 96.7%. Combined with AFP level, AUC improved to 0.86 with sensitivity 73.0% and specificity 93.4%.
Kelley RK et al[63]., 2015	20 HCC; 10 BLD	Cell Search	EpCAM	CTCs detected in 40% metastatic HCC pts. ≥ 1/7.5mL associated with vascular invasion and decreased OS.
Wang S et al[68]., 2016	42 HCC	CTC-^{BioT}Chip	EpCAM	CTCs detected in 60% HCC pts. Positive rate and number of CTCs highly correlated with TNM staging.
Zhou Y et al[69]., 2016	49 HCC undergoing curative resection; 50 HV	RosetteSep qRT-PCR	EpCAM $CD4^+CD25^+Foxp3^+$	Pts with high EpCAM $mRNA^+$ CTCs and $CD4^+CD25^+Foxp3^+$ Treg cells had higher risk of postoperative recurrence (67% vs. 10%) and 1-year recurrence (50% vs. 10%).
von Felden J et al[65]., 2017	57 HCC undergoing resection	Cell Search	EpCAM	CTCs detected in 15% HCC pts. CTC positivity associated with higher recurrence and shorter median RFS.
Shen J et al[64]., 2018	89 HCC undergoing TACE	Cell Search	EpCAM	CTCs detected in 56% HCC pts. Higher number of CTCs associated with mortality and progression.
Yu JJ et al[66]., 2018	139 HCC; 23 BHT	Cell Search	EpCAM	Pts with CTC ≥2 had shorter DFS and OS compared to pts with CTC < 2.
Combined Markers-Based CTC Detection in HCC				
Xu W et al[71]., 2011	85 HCC; 37 BLD; 14 OT; 20 HV	AutoMACS	ASGPR, HER2, TP53	CTCs identified in 81% of HCC pts. Positivity and number of CTCs correlated with tumor size, portal vein tumor thrombus, differentiation status, and disease extent.
Li J et al[73]., 2014	27 HCC; 12 OT; 13 LC; 11 BLT; 7 CH; 15HV	AutoMACS	ASGPR; CPS1 P-CK	CTCs detected in 89% of HCC pts. Anti-ASGPR specific and efficient for HCC CTC enrichment. Combining anti-P-CK and anti-CPS1 was superior (96.7%) to using one antibody alone (CPS1: 62.1%, P-CK 85.2%).
Mu H et al[74]., 2014	62 HCC; 7 CH; 15 HV	MidiMACS	ASGPR, GPC3, CK	GPC3, ASGPR, and CK expression increased in HCC pts. PPV and NPV: 90% and 71% for GPC3; 93% and 75% for ASGPR; 83% and 29% for CK. GPC3 and ASGPR expression associated with decreased OS.
Liu HY et al[75]., 2015	32 HCC; 17 OT; 12 BLT; 15 LC; 10 CH; 3 AH; 20 HV	Ficoll-Paque, RosetteSep	ASGPR CPS1	CD45 depletion of leukocytes recovered more CTCs compared to $ASGPR^+$ selection. Combining anti-ASGPR and anti-CPS1 improved CTC detection vs. either antibody alone, and detected CTCs in 91% of HCC pts.
Zhang Y et al[76]., 2016	36 HCC; 14 BLD	CTC-Chip	ASGPR, P-CK, CPS1	CTCs detected in 100% of HCC pts. Captured CTCs readily released from CTC-chip and could subsequently be expanded to form a spheroid-like structure in a 3D cell culture assay.

Study	Patients	Method	CTC Marker	Main Findings
Pang Y et al[77]., 2018	8 HCC; 5 OT; 5HV	SERS	ASGPR, GPC3	SERS nanoplatform detected CTCs in 100% of HCC pts, and enabled detection with small volume of blood.
Label-Free (Biophysical and Enrichment-Free) Methods to Detect CTCs in HCC				
Vona G et al[42]., 2000	7 HCC undergoing hepatectomy; 8 CH; 8 HV	ISET RT-PCR	AFP PSA	CTCs detected in 43% and 86% of HCC pts before and after surgery. ISET allowed subsequent analysis of cell morphology, enumeration of CTCs, and demonstration of tumor microemboli during surgery.
Vona G et al[79]., 2004	44 HCC; 30 CH; 39 LC; 38 HV	ISET	N/A	CTCs found in 52% of HCC pts, associated with portal tumor thrombosis and shorter survival.
Morris KL et al[80]., 2014	52 HCC	ISET Cell Search™	EpCAM GPC3	CellSearch detected CTCs in 28%, while ISET detected CTCs in 100% of HCC pts. Presence of GPC3-positive CTCs by ISET was 100% concordant with GPC3-positive cells in the original tumor.
Liu Z et al[57]., 2016	52 HCC; 12 HV	Imaging Flow Cytometry	Karyoplasmic Ratio	Using high karyotype ratio (HKR), imaging flow cytometry had AUROC of 0.82. HKR pts had significantly higher presence of microvascular thrombosis, as well as higher risk of recurrence and mortality.
Ogle LF et al[58]., 2016	69 HCC; 16 LC; 15 HV	Imaging Flow Cytometry	Cell Size CK, EpCAM, AFP, GPC-3, DNA-K	Size criteria + absence of CD45 led to capture of all positive biomarker CTCs, and 28% of biomarker negative CTCs. Increased CTCs associated with advanced tumor stage, portal vein thrombosis and poorer survival.
Biologic Characterization and Heterogeneity of CTCs in HCC				
Li YM et al[72]., 2013	60 HCC; 10 BLD; 10 OT; 10 HV	MiniMACS Flow Cytometry	ASGPR, Vimentin, twist, ZEB1/2, snail, slug, cadherin	CTCs detected in 77% of HCC pts. CTC positivity higher in pts with portal vein thrombus and advanced stages. EMT markers highly correlated with portal vein thrombus, TNM classification and tumor size.
Li J et al[82]., 2016	109 HCC on sorafenib treatment	Ficoll-Paque RosetteSep	pERK pAkt	pERK/pAkt expression in CTC and tissue concordant in 90%. pERK+/pAkt- CTCs associated with PFS and predicted good prognosis. In vitro, pERK+/pAkt- HCC cells showed the greatest response to sorafenib.
Shi J et al[81]., 2016	47 HCC undergoing cryoablation	MACS RT-qPCR	MAGE-3, Survivin CEA	Average CTCs decreased significantly following cryosurgery. Gene expression for tumor markers MAGE-3, survivin and CEA all significantly decreased following cryosurgery as well.
Kalinich M et al[84]., 2017	63 HCC; 31 CLD; 6 LM; 38 OT; 26 HV	CTC-iChip Digital PCR	AFP, AHSG, ALB, APOH, FABP1, FGB, FGG, GPC3, RBP4, TF	10 liver-specific transcripts identified CTCs in 56% of untreated HCC pts vs. 3% of pts with nonmalignant liver disease at risk of developing HCC. CTC positivity declined in HCC pts receiving therapy.
Chen J et al[86]., 2017	195 HCC	CanPatrol™	CK, EpCAM, Twist, Cadherin, Snail, Vimentin, AKT2	CTCs detected in 95% HCC pts, able to discriminate metastatic HCC with AUC 0.86, 86% sensitivity, 81% specificity. Mesenchymal CTCs associated with age, BCLC stages, metastasis, AFP levels, recurrence.
Court CM et al[78]., 2018	61 HCC; 11 BLD; 8 HV	NanoVelcro CTC Assay	EpCAM, ASGPR GPC3, Vimentin	CTCs detected in 97% HCC pts. Panel identified HCC with sensitivity 84.2%, specificity 88.5%, PPV 69.6%, NPV 94.7%, and AUC 0.92. Vimentin-positive CTCs associated with aggressive disease and metastasis.
Qi LN et al[87]., 2018	112 HCC treated with R0 resection; 12 HBV; 20 HV	CanPatrol™	EpCAM, CK, Vimentin, Twist	CTCs detected in 90% HCC pts. CTC count ≥16 and mesenchymal–CTC percentage ≥2% associated with recurrence and metastasis. BCAT1 gene identified as a potential trigger of EMT.

Study	Patients	Method	CTC Marker	Main Findings
Ou H et al[88]., 2018	165 HCC undergoing radical resection	CanPatrol™	EpCAM, CK, Vimentin, Twist	CTCs detected in 71% HCC pts. Increased CTCs associated with higher AFP, multiple tumors, advanced staging, and tumor embolus. Mesenchymal CTCs predicted earlier recurrence and shortest RFS.
Yin LC et al[89]., 2018	80 HCC; 10 HV	CanPatrol™	Twist	Twist+ correlated with tumor burden/aggressiveness, staging, as well as postop recurrence and mortality.
Ye X et al[90]., 2018	42 HCC	CanPatrol™	TP53	Postop CTC counts and changes in CTC counts were independent prognostic indicators for PFS.
Cheng Y et al[91]., 2018	113 HCC; 57 BLD; 6 LM	CanPatrol™	CK, EpCAM Vimentin, Twist	Total number of CTCs had AUC 0.774, which improved to 0.821 when combined with serum AFP. Mesenchymal CTCs were increased in late-stage HCC pts.
Wang Z et al[85]., 2018	62 HCC undergoing radical resection	CanPatrol™	CK, EpCAM, Vimentin, Twist	CTCs detected in 84% of HCC pts. Pts with postop recurrence had significantly higher number of CTCs, mesenchymal CTCs, and mixed CTCs. Mesenchymal CTCs associated with shortened postoperative DFS.
D'Avola D et al[92]., 2018	6 HCC; 1 HV	Single cell RNA sequencing	CK, EpCAM GPC3, ASGPR1	Single cell RNA sequencing of CTCs showed significant transcriptome heterogeneity. Non-hepatic expression of ASGPR1 was detected in a significant proportion of non-CTCs, mainly monocytes.
Sun YF et al[93]., 2018	73 HCC undergoing curative resection	Cell Search™ qRT-PCR	EpCAM, CK, Cadherin, Slug, Vimentin, Snail	EMT status of CTCs was heterogeneous across different vascular compartments (peripheral vein, peripheral artery, hepatic vein, portal vein, IVC).
Wang L et al[94]., 2018	14 HCC; 16 CCA; 4 GBC undergoing resection	SE-iFISH	C8 Aneuploidy	Among CTCs detected, 8% were EpCAM+, and 86% EpCAM$^-$. Small aneuploid HCC CTCs were discovered. Postsurgical quantity of triploid CTCs, multiploid CTCs or CTMs correlated to poor prognosis.
Winograd P et al[83]., 2018	73 HCC; 8 HV; 11 BLD	CTC-iChip	PD-L1	Presence of PD-L1+ CTCs predicted response to anti-PD1 therapy.

AH, acute hepatitis; AUC, area under the curve; BHT, benign hepatic tumor; BLD, benign liver disease; CCA, cholangiocarcinoma; CH, chronic hepatitis; CLD, chronic liver disease; CTC, circulating tumor cell; CTM, circulating tumor microemboli; DFS, disease-free survival; EMT, epithelial to mesenchymal transition; GBC, gallbladder cancer; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HD, hepatic disease without HCC; HV, healthy volunteers; IVC, inferior vena cava; LC, liver cirrhosis; LM, liver metastasis; NPV, negative predictive value; OS, overall survival; OT, other malignant tumors; PFS, progress-free survival; PPV, positive predictive value; RFS, recurrence-free survival