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Review Article

Exploring Circulating Tumor Cells: Detection Methods and Biomarkers for Clinical Evaluation in Hepatocellular Carcinoma

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Abstract

Circulating tumor cells (CTCs), originating from primary neoplastic tissues, infiltrate blood vessels, migrate through the bloodstream, and establish secondary tumor foci. The detection of CTCs holds significant promise for early-stage identification, diagnostic precision, therapeutic monitoring, and prognostic evaluation. It offers a non-invasive approach and has broad clinical relevance in cancer management. This comprehensive review primarily focused on CTCs as biomarkers in the diagnostic, therapeutic, and prognostic surveillance of hepatocellular carcinoma, compared their correlation with key clinical parameters and the identification of gene characteristics. It also highlighted current methodologies in CTC detection. Despite approval by the U.S. Food and Drug Administration for select malignancies, the comprehensive integration of CTCs into routine clinical practice requires procedural standardization and a deeper understanding of the underlying molecular intricacies. The challenges in CTC detection, including limited quantity, technical impediments, and cellular heterogeneity, call for concerted and further investigational efforts to advance precision in cancer diagnostics and prognostication, thus realizing the objectives of precise and personalized medicine.

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Introduction

Cancer remains a leading global cause of mortality, despite advancements in early detection and treatment. The persistent rise in global cancer incidence and mortality rates underscores the urgent need for precise and advanced detection methods and appropriate biomarkers. Primary liver cancer ranks as the sixth most prevalent malignancy globally and the third leading cause of cancer-related mortality, with hepatocellular carcinoma (HCC) accounting for 75–85% of these cases.[1] Globally, the Asia-Pacific region accounts for three-quarters of HCC-related deaths.[2] The major risk factors include chronic viral hepatitis infection, alcohol-related liver disease, and metabolic-associated fatty liver disease. The high burden and mortality of HCC are exacerbated by suboptimal surveillance strategies and early diagnosis.[3]

Circulating tumor cells (CTCs), which are malignant cells shed from primary tumors into the bloodstream, have emerged as valuable non-invasive biomarkers for monitoring tumor characteristics, metastasis, and minimal residual disease. CTCs serve as indicators of tumor burden in HCC, facilitating the evaluation of treatment response and disease progression. Additionally, CTCs have the potential for early diagnosis, either in the *de novo* development or recurrence of HCC. This review systematically introduces current techniques for enriching CTCs, including methods that leverage their biological and physical properties. Furthermore, relevant studies on CTC analysis in HCC are discussed, elucidating the relationship between cell collection time, CTC types, and biochemical values to explore the utility of CTCs in HCC diagnosis, treatment, and prognosis. Finally, the review examines the current limitations of CTC detection and potential avenues for future improvements and developments. We hope this review will serve as a reference for utilizing liquid biopsy for CTC detection in HCC and provide a foundation for future CTC research.

Search strategy

A bibliometric analysis of studies related to CTCs in HCC was conducted using PubMed and VOSviewer software (version 1.6.20, Leiden University, Netherlands) for visualization. The data sources included academic publications retrieved from PubMed and Google Scholar in August 2023. Figure 1A depicts the annual publication count and growth trend of papers using the keyword "circulating tumor cells" from 1945 to 2023. The number of CTC-related publications reached 2,044 in 2020. Through co-occurrence network visualiza-

Keywords: Circulating tumor cells; CTC; Hepatocellular carcinoma; HCC; Clinical application; CTC enrichment; Biomarker; Progression; Microvascular invasion; MVI; alpha-fetoprotein; AFP.

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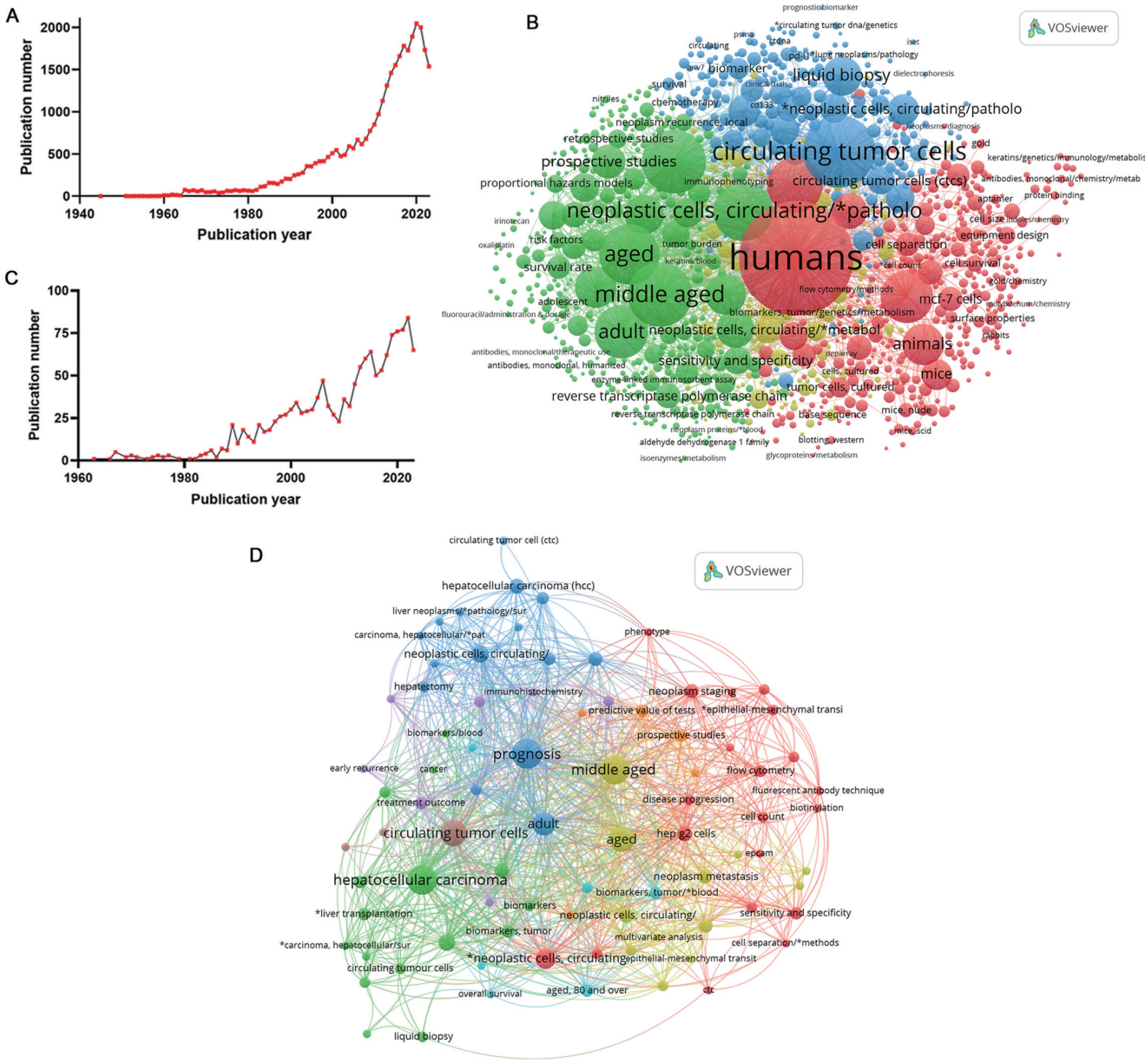

Fig. 1. Bibliometric analysis of circulating tumor cells (CTCs) and their application in hepatocellular carcinoma (HCC). (A) The number of publications on CTCs has gradually increased since 1980; (B) Keyword co-occurrence network of circulating tumor cells generated using VOSviewer software. The nodes size represents the frequency of occurrence; (C) The number of publications on CTCs and HCC in 2020 is three times that of 2000; (D) Keyword co-occurrence network of circulating tumor cells and HCC. The nodes size represents the occurrence frequency, and the lines between the nodes indicate co-occurrence. *the text represents a "truncation" or "wildcard".

tion of keywords, we identified those that appeared more than five times in PubMed core data. Out of 12,379 screened keywords, 1,061 met the threshold. The most frequent keyword was "humans," followed by "circulating tumor cells" and "middle-aged" (Fig. 1B). We further explored PubMed for the keywords "circulating tumor cells," "hepatocellular carcinoma," and "liquid biopsy," identifying a total of 84 papers published in 2022 (Fig. 1C). Similarly, we performed co-occurrence network visualization for this set of keywords. Of the 347 screened keywords, 87 met the threshold. The most frequent keyword was "prognosis," followed by "HCC" and "middle-aged," with "CTC" ranking fourth (Fig. 1D). We prioritized original research articles focusing on human clinical studies, excluding case reports, review articles, and letters to the editor. The selection process is depicted in Figure 2, and 51 relevant studies were ultimately included for analysis.

The technique of CTC isolation

The discovery of CTCs in blood has led to the development of various techniques for CTC isolation. These techniques can be broadly categorized into biological property-based separation and physical property-based separation (Fig. 3). Biological property-based separation primarily relies on the high expression of cell surface antigens on CTCs. This method involves the use of antibodies that bind to cell surface an-

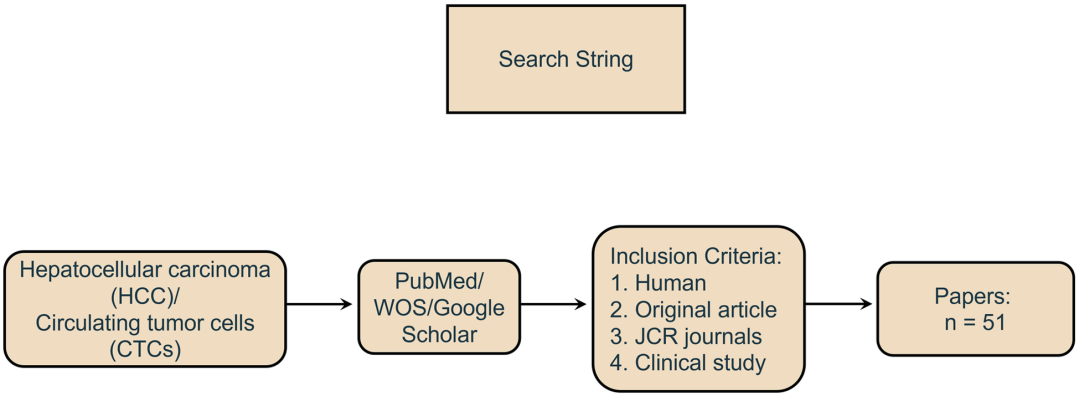

Fig. 2. Flowchart of the literature search.

tigens on CTCs, followed by their separation using magnetic beads or flow cytometry/EuroFlow system. On the other hand, physical property-based separation methods include density gradient separation, size-based membrane filtration, microfluidic devices, and electrostatic separation. These techniques rely on the size, density, or charge characteristics of cells for CTC isolation. Each of these methods will be discussed in detail below.

The biological separation of CTCs

Cell phenotype-based differences are one of the essential methods for CTC collection. These techniques predominantly employ a positive enrichment strategy for CTCs based on surface markers such as EpCAM, CK8/18/19, mesenchymal markers (e.g., Vimentin, N-cadherin, Fibroblast Activation Protein), stem cell markers (e.g., OCT4, SOX2, CD133, NANOG), and cancer-specific antigens (e.g., HER2, PSMA, MUC1).[4] By utilizing antibodies with immunoreactivity to cell surface markers, immobilized on the device surface, these antibodies bind to the corresponding CTC surface markers, enabling the isolation of CTCs. Typically, during CTC collection, a negative enrichment method is first employed using CD45 antibodies to exclude white blood cells. Subsequently, positive enrichment is performed using antibodies against CTC surface markers to collect CTCs. The primary reagents currently in use include CellSearch, Canpatrol, MagSweeper,

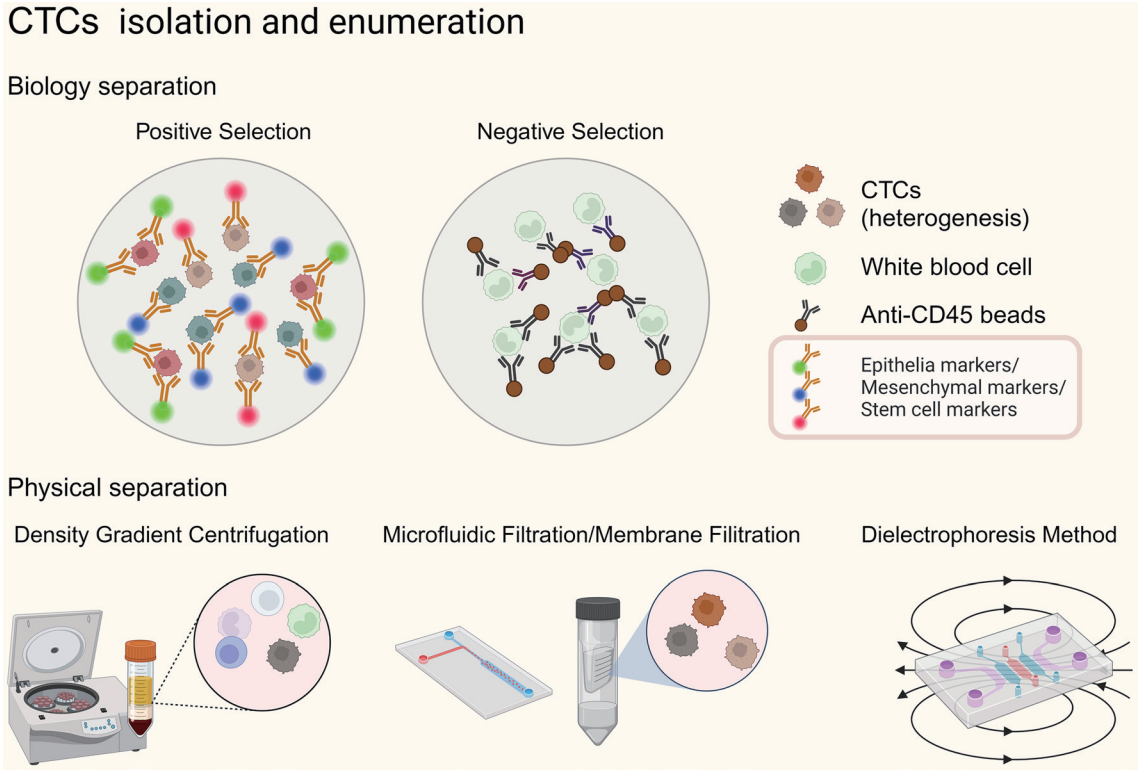

Fig. 3. Separation methods for circulating tumor cells (CTCs). The separation methods for CTCs can be categorized into biological and physical techniques. Biological separation methods, such as antibody-based approaches, further distinguish between positive and negative selection. In contrast, physical separation methods utilize factors such as density, size, and charge, including gradient centrifugation, microfluidics, membrane filtration, and electrophoresis.

and NanoVelcro. Among them, the CellSearch system (Menarini Silicon Biosystems Inc.) is the U.S. Food and Drug Administration (FDA)-approved CTC diagnostic technology.[5] However, the high heterogeneity of CTC surface antigens may not be fully addressed, even by a mixture of antibodies targeting various antigens. Additionally, CTCs with an epithelial-to-mesenchymal transition (EMT) phenotype exhibit highly migratory characteristics, often downregulating or losing surface antigens during EMT, resulting in the inability to enrich CTCs with lower surface marker expression.[6] Consequently, researchers are exploring CTCs with highly sensitive and specific tumor markers or integrating different biological and physical techniques for CTC isolation to achieve better results.

The physical separation of CTCs

The enrichment methods for the physical separation of CTCs are based on distinguishing the characteristics of CTCs from blood cells in terms of size, density, deformability, and electrical properties.[7–11] Isolating CTCs through physical characteristics reduces the dependence on cell surface-specific antigens and simplifies the subsequent experimental processing since CTCs are not labeled with antibodies. However, in the case of hematologic tumors, where tumor cells originate from blood cells, separating CTCs based on physical properties becomes more complex. Currently, physical property-based separation of CTCs can be achieved through several methods, including density gradient centrifugation, membrane filtration, microfluidic filtration, inertial focusing, and dielectrophoresis.

Density gradient centrifugation

Density gradient centrifugation primarily relies on the differences in density between CTCs and white blood cells, which is achieved through centrifugation stratification. Ficoll-Paque (GE Healthcare Life Sciences), originally designed for separating peripheral blood mononuclear cells, can also be utilized to detect CTCs in the blood of cancer patients.[8] An improved method, RosetteSep™ Immunodensity Cell Separation (STEMCELL Technologies, Inc.), employs an antibody cocktail to bind unwanted cells, thereby altering their density, and then utilizes Ficoll-Paque for CTC separation.[9] This method employs negative selection/depletion with antibodies to efficiently collect CTCs in large quantities. OncoQuick (Greiner Bio-One), on the other hand, utilizes centrifugation and filtration to separate and purify cancer cells from peripheral blood.[10] While density gradient centrifugation is cost-effective and widely used for CTC separation, variations in centrifuge rotor speeds can sometimes lead to cell damage or ineffective CTC separation. In cases where CTCs have a similar density to blood cells, the centrifugation effect may not be optimal. Therefore, it is advisable to consider alternative enrichment strategies before resorting to this method.

Membrane filtration and microfluidic filtration

Membrane filtration and microfluidic filtration are both cell selection methods based on cell volume size. The primary distinction is that membrane filtration only allows cells smaller than a fixed pore size to pass through, while retaining cells larger than the pore size.[11] Conversely, microfluidic technology utilizes channels with varying pore sizes and is often combined with inertial focusing.[7] In this approach, cells behave like pinballs, flowing into different regions based on their size and utilizing inertial effects to aid in CTC separation. However, microfluidic filtration systems are prone to clogging, and when there is a similarity in volume between white blood cells and CTCs, it can lead to misjudgment. Therefore, this method is used less frequently for separating CTCs in cases of malignant blood tumors. Current examples of this approach include CellSieve (Creatv MicroTech, Inc., Rockville, MD), Cluster-Chip (a unique 3D microfiltration system designed specifically for capturing CTC clusters by Massachusetts General Hospital), ISET (Isolation by Size of Epithelial Tumor Cells; Berlex Laboratories, Inc., Montville, NJ), and ScreenCell (Sarcelles, France).

Dielectrophoresis method

The dielectrophoresis method involves the movement of particles induced by the asymmetrical displacement of an electric field.[12] Because different cells possess distinct dielectric properties, the non-uniform electric field leads to an uneven distribution of cell charges. Even in a neutral electrostatic environment, cell surfaces can attract both positive and negative ions from the solution due to this non-uniformity. Consequently, differences in cell charge polarization speed and solution charge can be harnessed by modifying the electric field to achieve cell separation. Currently, various methods employ dielectrophoresis, including bioelectric chips, DEP-based instruments, MOFF-DEP, 3D-asymmetric microelectrodes, and ApoStream, among others.[13]

The role and impact of CTCs in HCC

CTC tests could be implemented during the pre-, intra-, and post-treatment phases in HCC patients. By employing non-invasive liquid biopsy methods to collect peripheral blood, the detection of CTCs could potentially provide an alternative assessment of HCC prognosis (Fig. 4).[14] Current studies on CTCs in HCC have demonstrated that the quantity of CTCs, the composition of different CTC types, and their molecular biological characteristics are associated with traditional biochemical analyses and tumor features (Table 1).[4,15–23] Concurrently, these characteristics correlate with prognosis factors such as treatment efficacy and recurrence.[4,15–17,24–43] Table 2 summarizes recent research on CTCs in the context of HCC.[4,15–64]

CTCs or mesenchymal CTCs are associated with tumor size and grading in HCC

The analysis of CTCs in HCC patients reveals that advanced-stage HCC patients have higher CTC counts compared to early-stage patients, suggesting a direct correlation between CTC quantity and cancer severity. Additionally, research by Takahashi *et al*. highlights that the incidence of CTCs in HCC patients surpasses that in individuals with liver cirrhosis, with 14% of cirrhotic patients demonstrating CTCs compared to 38.9% in HCC patients.[44] Various studies have also shown a significant correlation between different cancer stages and either the Barcelona Clinic Liver Cancer (BCLC) or the American Joint Committee on Cancer tumor staging systems.[24–30,33,45] Interestingly, a negative correlation exists between CTC count and cell differentiation grade (Edmondson grading).[33] In addition to the association between CTCs and cancer staging, the number of CTCs correlates with tumor size, suggesting that larger tumors tend to release more CTCs into the bloodstream.[42,46,47] Larger tumors and more severe portal vein thrombosis are associated with higher CTC counts in HCC patients. Furthermore, the median survival of HCC patients without detectable CTCs is approximately 4.5 times longer than that of patients with detectable CTCs.[42] Notably, CTCs are prevalent in 76.9% of metastatic liver cancer patients, with metastatic HCC exhibiting significantly higher

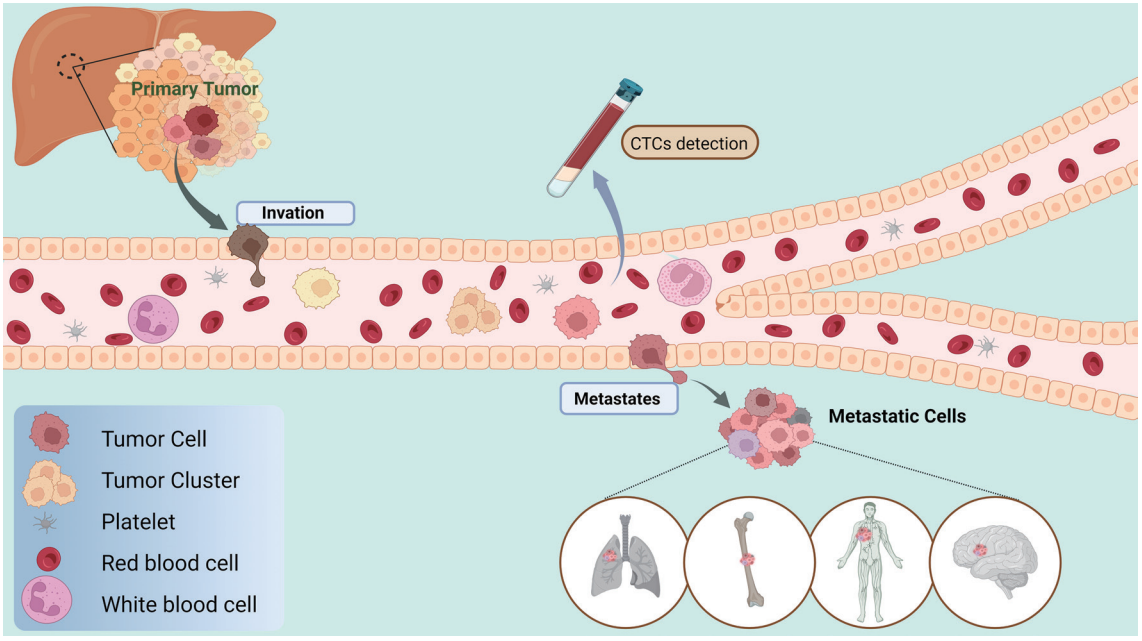

Fig. 4. The process of circulating tumor cells (CTCs) formation, invasion, and migration. CTCs detach from the primary tumor (liver), infiltrate blood vessels, and form either individual CTCs or CTC clusters. Subsequently, these cells egress from the bloodstream, traverse blood vessels, undergo metastasis, and give rise to metastatic cells in secondary tumors (lung, bone, lymph nodes, and brain). Blood samples from these vessels enable the detection of CTCs or CTC clusters.

CTC counts compared to localized HCC. Regarding patient survival rates, those with CTC counts of fewer than five cells per 8 mL of blood show significantly extended mean survival exceeding 36 months, while those with five or more CTCs have significantly reduced median survival of approximately 4.6 months.[25] Furthermore, the presence of positive CTCs (CTC ≥ 10) significantly diminishes the overall survival rate of HCC patients, particularly in those with localized HCC.[65]

Recent studies demonstrate that M-CTCs, a subtype of CTC with metastatic capabilities, can predict the severity of HCC.[18,19,24,29,31,48,49] Nearly half of HCC patients have at least one detectable M-CTC per 5 mL of blood, and the analysis of M-CTCs is significantly correlated with tumors measuring 5 cm or more.[31] These findings collectively indicate that a higher peripheral CTC or M-CTC count is associated with tumor size, tumor grading, and metastasis. Moreover, significant associations between the presence of CTCs and increased tumor invasiveness, as well as poorer survival in HCC patients, suggest the potential utility of CTCs as biomarkers for evaluating the malignancy of hepatocellular carcinoma.

The impact of surgical procedures on CTCs and postoperative outcomes in HCC

Blood samples collected during surgery provide a comparative analysis of CTC counts before, during, and after surgical procedures, offering insights into the prognostic implications of surgery. For example, preoperative CTC analysis has demonstrated a significant difference in disease-free survival between HCC patients with fewer than one interstitial CTC (<1) and those with one or more (≥1) prior to surgery, with survival periods of 13.3 months and 5.0 months, respectively.[33] Interestingly, intraoperative manipulations do not increase CTC counts, whereas surgical tumor resection results in a decrease. Elevated postoperative CTC levels (≥5) indicate a heightened risk of early recurrence.[33] In 2020, Zhou *et al*. conducted a study analyzing CTCs in peripheral blood collected during surgery and found no significant increase compared to preoperative levels.[34] Furthermore, CTC counts significantly decreased postoperatively in patients without recurrence, a trend not observed in those with early recurrence.[34] It suggests that the surgical process itself does not substantially increase CTC numbers, and persistently high postoperative levels may indicate an increased risk of early recurrence. Additionally, Li *et al*. compared CTC counts between open surgery and laparoscopic surgery, finding a similar reduction in postoperative circulating cancer stem cells in both procedures.[50] Although the laparoscopic approach resulted in less pronounced increases in inflammatory factors such as IL-6 and IL-8 postoperatively—likely due to smaller incisions—this did not significantly affect CTC dynamics. Similarly, CTCs serve as biomarkers for assessing postoperative recurrence rates and disease progression in HCC. Analysis of CTCs following treatment reveals variations in CTC numbers based on different surgical approaches or liver transplant procedures. For instance, patients who received ablation therapies exhibited a significant reduction in CTC proportions.[48,51]

CTC detection after liver transplantation may serve as a predictor for post-transplantation prognosis and liver graft status in patients undergoing liver transplantation. Following liver transplantation, there is also a decrease in postoperative CTC numbers.[47,52] However, persistently high or increasing postoperative CTC levels can lead to higher recurrence rates and lower survival rates for HCC patients.[20,21,34,52–55] In a cohort of 50 liver transplant recipients, those with detectable CTCs after liver transplantation had a one-year disease-free survival rate two-thirds lower than patients without CTCs.[47] Conversely, a study comparing CTC differences between liver resection and transplantation found that one month postoperatively, the proportion of CTC clusters was higher in liver resection than in liver transplantation, impacting survival rates and suggesting that liver resection may not completely eliminate CTCs.[56] The recurrence rate increased over time in CTC-positive patients and was significantly higher than in

Table 1. Summary of the most cited articles

Author(s)	Article title	Journal	Quartile	citations(n)	Year
Wu, S; *et al*.[4]	Classification of Circulating Tumor Cells by Epithelial-Mesenchymal Transition Markers	PLOS ONE	Q1	241	2015
Qi, L. N; *et al*.[19]	Circulating Tumor Cells Undergoing EMT Provide a Metric for Diagnosis and Prognosis of Patients with Hepatocellular Carcinoma	CANCER RESEARCH	Q1	210	2018
Vona, G; *et al*.[16]	Impact of cytomorphological detection of circulating tumor cells in patients with liver cancer	HEPATOLOGY	Q1	199	2004
Xu, W; *et al*.[15]	Isolation of Circulating Tumor Cells in Patients with Hepatocellular Carcinoma Using a Novel Cell Separation Strategy	CLINICAL CANCER RESEARCH	Q1	144	2011
Kalinich, M; *et al*.[17]	An RNA-based signature enables high specificity detection of circulating tumor cells in hepatocellular carcinoma	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA (PNAS)	Q1	134	2017
Li, Y. M; *et al*.[18]	Epithelial-mesenchymal transition markers expressed in circulating tumor cells in hepatocellular carcinoma patients with different stages of disease	CELL DEATH & DISEASE	Q1	130	2013
Sun, Y. F; *et al*.[23]	Circulating Tumor Cells from Different Vascular Sites Exhibit Spatial Heterogeneity in Epithelial and Mesenchymal Composition and Distinct Clinical Significance in Hepatocellular Carcinoma	CLINICAL CANCER RESEARCH	Q1	121	2018
Guo, W; *et al*.[20]	Circulating Tumor Cells with Stem-Like Phenotypes for Diagnosis, Prognosis, and Therapeutic Response Evaluation in Hepatocellular Carcinoma	CLINICAL CANCER RESEARCH	Q1	109	2018
Kelley, R. K; *et al*.[22]	Circulating tumor cells in hepatocellular carcinoma: a pilot study of detection, enumeration, and next-generation sequencing in cases and controls	BMC CANCER	Q2	103	2015
Guo, W; *et al*.[21]	Clinical Significance of EpCAM mRNA-Positive Circulating Tumor Cells in Hepatocellular Carcinoma by an Optimized Negative Enrichment and qRT-PCR-Based Platform	CLINICAL CANCER RESEARCH	Q1	99	2014

Q, Quartile.

patients without postoperative CTCs.[53] Additionally, another study indicated that the postoperative CTC count significantly decreased in the non-early and non-recurrence groups.[34] A recent study demonstrated that a higher postoperative CTC count was associated with an increased risk of extrahepatic metastasis and lower overall survival.[54]

It is essential to scrutinize the composition and proportion of different CTC subtypes, which may impact postoperative recurrence. Wang *et al*. identified that M-CTCs within the CTC population are associated with postoperative disease-free survival and can act as independent risk factors for early recurrence.[49] The quantity of CTCs and the proportion of M-CTCs in postoperative HCC patients have implications for early recurrence, multi-site extrahepatic recurrence, and lung metastasis. An increase in both the number and proportion of postoperative CTCs and M-CTCs is also linked to early recurrence.[19]

CTCs as standalone biomarkers or in conjunction with other biochemical tests in HCC

In the field of HCC prognosis, the integration of CTCs, alpha-fetoprotein (AFP), and microvascular invasion (MVI) has emerged as a critical determinant. This combination provides valuable insights for devising more effective treatment strategies. AFP, a widely recognized tumor marker particularly for HCC detection and monitoring, demonstrates superior prognostic accuracy when combined with CTC counts or clusters, as opposed to AFP alone.[25,29] Furthermore, the combination of CTCs with AFP performs better than AFP alone in predicting the prognosis of HCC patients, including disease-free sur-

Table 2. The application of circulating tumor cells (CTCs) in the detection techniques and outcomes of hepatocellular carcinoma (HCC)

Patients	Collected time points	Methods	Cell types	Results	Limitations	References
127 HCC cases, 21 NMLD patients, and 42 health control	Preoperative peripheral blood	7.5 mL blood stained with immune antibody	Epithelial CTC: PanCK; M-CTC: vimentin, and epithelial–mesenchymal CTC: PanCK, and vimentin	Total CTC number and M-CTC percent were positively correlated with the tumor characteristics, thrombosis, MVI, AJCC stage, BCLC stage, poor RFS rate, and high recurrence risk.	The study focused on EMT-associated CTC subtypes, potentially overlooking CTC heterogeneity. With a limited sample size (n = 127), the conclusions required further validation in larger, independent studies.	24
105 HCC cases	Before radical surgery	5 mL blood, CanPatrol, and ISH	Epithelial CTCs (EpCAM and CK8/18/19) and M-CTCs (Vimentin and Twist)	M-CTC positivity was significantly higher with AFP, tumor size, multiple tumors, poorly differentiated tumors, incomplete tumor capsule, BCLC stage B or C, MVI, PVTT, and Ki67.	This single-center retrospective study, with a sample size of 105, indicated a risk of selection bias and limited generalizability to other patient populations. The criteria for Ki67 expression grouping should be further validated in additional studies.	31
127 HCC patients	Before surgery	5mL peripheral blood samples with the CanPatrolTM system and expression levels of target genes in CTCs were assessed using the RNA-ISH method.	Leukocyte biomarker (CD45), epithelial biomarkers (Epithelial cell adhesion molecule, EpCAM; CK8/18/19), and mesenchymal biomarkers (vimentin and Twist)	1. CTCs served as independent factors associated with the early recurrence in HCC. 2. Survival curve analysis indicated that patients with CTCs experience a shorter recurrence-free survival period. 3. The combination of CTCs and platelet count represented a more robust prognostic marker than their individual use.	This single-center retrospective study with 127 samples needed validation through multi-center prospective studies. CTC detection via filtration may yield false negatives due to tumor cell size variability. As most HCC patients also had HBV, further research is needed to confirm applicability in HCC patients with other liver diseases.	32
56 HCC patients under liver transplantation	Before the operation and seven to ten days after the operation	5mL peripheral blood samples with CanPatrol CTC-enrichment technique platform and CTCs detected for ISH and fluorescence staining	Epithelial biomarkers (EpCAM and CK8/18/19) and two interstitial biomarkers (Vimentin and Twist)	The postoperative recurrence rates at one, two, and three years for interstitial CTC-positive groups were 21.7%, 37.5%, and 55.5%, respectively, compared to a consistent rate of 10.8% in the CTC-negative group. The recurrence rates at one, two, and three years for the group with increasing interstitial CTCs were 25.2%, 36.9%, and 66.9%, respectively, while they were 12.6%, 24.4%, and 24.4% in the group with decreasing or unchanged CTC.	These changes in total CTC count before and after surgery did not reliably assess liver transplant prognosis in HCC patients, potentially due to differences in inclusion criteria and post-operative immunosuppression protocols. Furthermore, the non-blinded, single-center design and small sample size (n = 56) may introduce bias.	53

(continued)

Table 2. *(continued)*

Patients	Collected time points	Methods	Cell types	Results	Limitations	References
73 HCC patients	N.D.	8 mL blood was detected by immunoaffinity-based method	Epithelial cell adhesion molecule (EpCAM, CD326) and MUC1	1. The detection rate of CTCs increased with advancing stages of BCLC. 2. Patients with lower CTC counts exhibited significantly longer overall survival. 3. The combination of CTCs and AFP yielded a higher HR compared to the individual impact of CTCs or AFP alone.	A single-center, prospective cohort study (n = 73) quantified only epithelial CTCs, without mesenchymal or hybrid epithelial-mesenchymal CTCs. Additionally, CTCs were measured at a single time point; serial measurements would offer more information for better outcome prediction.	25
40 HCC patients	N.D.	4 mL blood	1. NanoVelcro CTC Assay with round/ovoid cells, $DAPI^+/CD45^-/CK^+$, with sizes > 6 μm. 2. The NanoString nCounter platform for quantification of selected mRNA transcripts	The HCC-CTC Risk Score panel, which included ten prognostic genes (DDR1, EHHADH, AR, LUM, HSD17B6, PMEPA1, TSKU, NECAB2, LAD1, and SLC27A5), has been validated as an independent predictor of survival.	Patients were recruited from a single institution (n = 40), with a limited representation of different stages, tumors, and treatment characteristics. The analysis of gene expression in CTCs was a proof-of-concept study; further genomic refinement could enhance the prognostic power and utility of the relevant genes.	64
193 HCC patients	Pre- and post-operative	5 mL peripheral blood	ChimeraX ® -i120 CTC detection platform with epithelial cell (EpCAM), (Campos-Murguia, #225), CK19, and single-cell whole genome sequencing.	1. HCC patients experienced a reduction in the burden of CTCs following liver transplantation. 2. CTCs served as post-transplantation biomarkers in HCC patients, aiding in the assessment of recurrence risk.	The small cohort size (n = 193), short follow-up, single-center design, and disease heterogeneity between groups reduced the predictive value of preoperative CTC counts for tumor recurrence. Additionally, CTCs were only measured at two post-transplant time points, insufficiently assessing the predictive value of continuous CTC monitoring for recurrence.	52

(continued)

Table 2. *(continued)*

Patients	Collected time points	Methods	Cell types	Results	Limitations	References
160 HCC patients	Before surgery	15 mL of peripheral blood sample	CanPatrol™ CTC enrichment technology with RNA-ISH, Cancer stem cells marker (Nanog), epithelial marker (CK8, 18 and 19 and EpCAM), and mesenchymal marker (Twist and Vimentin)	1. The quantity of CTC expressing RNA for both EpCAM and Nanog was closely associated with postoperative recurrence of HCC. 2. Patients with CTCs showing higher expression of Nanog exhibited a higher recurrence rate. 3. CTC expressing Nanog was predominantly categorized into the mixed CTC and M-CTC subtypes. 4. The number of CTCs and the expression of Nanog were significantly correlated with BCLC stage, vascular invasion, tumor size, and HBV.	A single-center, prospective study (n = 160) collected CTCs only preoperatively, lacking post-surgery follow-up. Consequently, Nanog gene expression could only be correlated with preoperative CTC subtypes.	46
7 healthy donors, 14 liver cirrhosis patients, and 31 HCC patients	ND	3 mL peripheral blood samples	MCA system for size-based isolation of CTCs and immunofluorescence staining with positive for CK and DAPI	The positivity rate of CTCs in HCC was significantly higher than that in LC, leading to a reduction in the cumulative survival rate of HCC patients, particularly those with localized HCC. The quantity of CTCs in metastatic HCC was significantly higher than in localized HCC. The expression of AFP, GPC3, EpCAM, and ALB genes was detected in isolated CTC, with the detection rate of ALB mRNA significantly higher in the metastatic group compared to the localized group.	This study focused on MCA analysis of a small number of clinical samples. Analyzing more samples would have better evaluated the system's applicability to CTC counting and tumor characteristics. Additionally, the MCA system's reliance on specific antibodies limited its broader use.	44

(continued)

Table 2. *(continued)*

Patients	Collected time points	Methods	Cell types	Results	Limitations	References
204 HCC patients	Before surgery	7.5 mL peripheral blood	CellSearch™ System with CK positive for CTC detection. CTC clusters were detected as an aggregation of CTCs containing two or more distinct nuclei and with contiguous cytoplasmic membranes	1. In preoperative samples, 37.3% of patients were detected with CTCs, and 9.3% with CTC clusters. The survival period of patients with CTCs ≥ 2 was significantly shorter than that of patients with CTC < 2. 2. Patients with CTC clusters had a significantly poorer prognosis, and their survival time was also significantly shorter than those without CTC clusters. The presence of CTC clusters in HCC was associated with the activation of the Wnt/β-catenin signaling pathway.	The low detection rate and quantity of CTC clusters and CTCs in this study may have been attributed to the use of the CellSearch system, potentially leading to an underestimation of cluster numbers. Most CTCs within clusters were EpCAM-positive without EMT characteristics, which differed from other studies. This study focused only on the tumor cell component within CTC clusters, without analyzing the non-tumor cell elements.	63
41 HCC patients (31 patients underwent liver transplantation/10 patients underwent surgical resection)	Before surgery, at post-operative day 5 and at day 30	7 mL peripheral blood samples	The Isoflux® system (Fluxion Biosciences)	In HCC patients undergoing LR, CTC clusters were found more frequently 30 days postoperatively compared to patients undergoing LT. This difference arose from the incomplete clearance of CTCs and negatively impacted survival.	The prospective cohort study had a limited sample size due to slow accrual, extended monitoring, and increased CTC analysis costs. Additionally, the study only examined CTC clearance kinetics within the first month post-surgery, without long-term insights into CTC-immune system interactions.	56
17 HCC patients (10 patients underwent microwave ablation and 7 patients underwent conventional transarterial chemoembolization)	Before and after radiological interventions	10 mL peripheral blood samples	Flow cytometry with ASGPR, CD146 and CD274 (PD-L1)	The rate of CTCs in HCC patients was significantly decreased after MWA treatment.	The prospective single-center study had a small patient cohort, and the heterogeneity and rarity of CTC antigens made detection and analysis challenging.	51
179 HCC patients	ND	5 mL peripheral blood samples	The CanPatrol CTC enrichment technique with RNA-ISH with epithelial cell markers (EpCAM and CK8/18/19), and mesenchymal cell markers (vimentin and Twist). Survivin expression in CTCs was assessed using the RNA-ISH method.	The counts of CTCs and survivin-positive CTCs were significantly higher in the HCC patients and associated with tumor stage and differentiation degree.	The single-center case study had limited follow-up, preventing analysis of the relationship between survivin-positive CTC counts and overall survival.	26

(continued)

Table 2. *(continued)*

Patients	Collected time points	Methods	Cell types	Results	Limitations	References
214 HCC patients	Before surgery	7.5 mL peripheral blood samples	CanPatrol™ CTC analysis technology, epithelial cells were labeled with EpCAM and CK8/18/19, and M-cells were labeled with vimentin/twist. Epithelial CTC, M-CTC, and mixed CTC subtypes can form CTC-WBC clusters with WBCs.	Both CTC clusters and the total number of CTCs associated with tumor size and number, portal vein tumor thrombus, BCLC stage, and AFP level. CTC clusters in HCC patients were an independent prognostic indicator of DFS and OS.	The single-center retrospective cohort study cannot be generalized to non-Asian populations. It primarily focused on resectable HCC patients, lacking data on advanced-stage liver cancer cases.	27
136 HCC patients	Before resection	5 mL peripheral blood samples	The CanPatrol system with RNA-ISH EpCAM, CK8/18/19 (epithelial biomarkers), and vimentin and twist (mesenchymal biomarkers).	In patients with a lower quantity of CTCs and a negative mesenchymal and epithelial/M-CTC phenotype, tumor-free survival rates were significantly higher. Higher pre-resection CTC counts and positive mesenchymal and epithelial/M-CTC phenotypes were significantly associated with extrahepatic and multi-intrahepatic recurrence.	This single-center retrospective study used the CanPatrol system, which only collected CTCs larger than 8 μm, potentially missing smaller CTCs.	38
344 HCC patients	Preoperative	7.5 mL peripheral blood samples	CellSearch system with positive for CK8/18/19 and/or EpCAM	Patients with CTCs undergoing TACE demonstrated significantly enhanced clinical outcomes and a substantial reduction in early recurrence.	This single-center retrospective study did not evaluate the value of post-operative CTCs in guiding adjuvant TACE, and PSM analysis did not account for confounding factors or bias. Additionally, the CellSearch system did not fully assess CTC status.	39
50 HCC patients	Before liver transplantation	3.2 mL peripheral blood	imFISH, which combined the FISH probes with chromosome 8 (orange) centromere probes CTC cells positive with $CEP8^{+}/DAPI^{+}/CD45^{-}$	The CTC number was correlated with tumor size, AFP level, tumor grade, and Recurrence. CTC-negative patients had higher one-year disease-free survival rates.	This single-center prospective cohort study collected only preoperative CTCs, limiting the ability to assess the relationship between post-operative CTCs and early recurrence after LT. Extending the follow-up to three to five years would be more meaningful.	47
217 HCC patients	ND	7.5 mL peripheral blood	Ficoll solution was incubated with fluorescent antibodies including CTC positive (EpCAM; panCK19), and isolated by flow cytometry.	USP1 was frequently upregulated in CTCs and correlated with metastasis and reduced overall survival rate in HCC patients.	This single-center case study did not investigate real-time changes in patient CTCs. While it aimed to show that immune attacks limit CTC survival, the evidence was insufficient to establish the association between USP1 and immune evasion.	60

(continued)

Table 2. *(continued)*

Patients	Collected time points	Methods	Cell types	Results	Limitations	References
99 HCC patients	Pre-operatively	5 mL peripheral blood samples	CanPatrolTM with epithelial biomarker probes (EpCAM and CK8/18/19), interstitial biomarker probes (vimentin and twist)	Positive CTCs exhibited positive correlations with BCLC staging, tumor diameter and quantity, capsule integrity, MVI, portal vein thrombosis, AFP, and hepatitis B DNA in HCC. Higher expression of CXCR4 was more commonly observed in mixed CTCs than mesenchymal CTCs.	This single-center prospective analysis did not fully establish the link between CTCs and AFP. Only 22 patients provided survival-related follow-up data, and overall survival was not included.	33
137 HCC patients	Before surgery, during surgery (30 m after tumor removal) and one week, one/two/three/six months, and one year after surgery	5 mL blood samples	Isolation by Size of Epithelial Tumor Cells	1. Preoperative CTC count was an independent predictor of MVI. 2. CTC count had better predictive value than AFP and tumor diameter. 3. The number of CTCs in the non-early recurrence group decreased significantly after surgery 4. Patients with CTC count ≥ 5 had worse long-term outcomes.	This single-center case study had a short follow-up period. The ISET method may have caused non-CTC to clog filter pores and lead to background contamination, resulting in failed CTC isolation in some samples.	34
87 HCC patients (49 early-stage, 22 locally advanced, and 16 metastatic), 7 cirrhosis patients, and 8 healthy controls	ND	4 mL blood samples	CTCs were separated by gradient centrifugation with Ficoll-Paque solution then the NanoVelcro Chip defined CK^+ CTCs were defined as round/ovoid cells ($DAPI^+/CK^+/PD\text{-}L1^-/CD45^-$) and $PD\text{-}L1^+$ CTC are the subpopulation of HCC CK^+ CTC defined as round/ovoid events ($DAPI^+/CK^+/PD\text{-}L1^+/CD45^-$)	$PD\text{-}L1^+$ CTCs were predominantly found at advanced stage and had a significantly worse OS in HCC patients.	This single-center case study required additional testing for EMT markers beyond EpCAM to identify CTC surface antigens. The association between $PD\text{-}L1^+$ CTCs and immunotherapy needed further validation.	61
197 HCC patients (retrospective training cohort (144 patients) or a prospective validation cohort (53 patients))	Before surgery and one month after surgery	7.5 mL peripheral blood	CELLSEARCH system	The presence of EHM was significantly correlated with a higher postoperative CTC burden. Patients with a postoperative CTC count ≥ 3 faced an elevated risk of EHM and a shorter median overall survival.	This single-center case study collected only $EpCAM^+$ CTCs, missing other heterogeneous CTCs, which reduced the sensitivity and specificity of CTC detection.	54

(continued)

Table 2. *(continued)*

Patients	Collected time points	Methods	Cell types	Results	Limitations	References
309 HCC patients	Before surgery	7.5 mL peripheral blood	CellSearch and positive CTC was defined as CTC ≥ 1	CTC-positive HCC patients exhibited higher MVI and greater FMT. Within the CTC-positive group, a surgical margin > 1 cm independently provided protection against early recurrence and was associated with a lower early recurrence rate.	The single-center retrospective study could not eliminate potential selection bias or confounding factors. Most enrolled patients were infected with HBV, and applicability to HCC from other causes needs further validation. Additionally, only $EpCAM^+$ CTCs were collected, missing other heterogeneous CTCs.	35
85 HCC patients	Before surgery	8 mL peripheral blood	Immuno-magnetic positive enrichment (GPC3) coupled with flow cytometry (CK7/8)	Patients had higher GPC3-positive CTC counts with a higher incidence of mPVI, a lower disease-free survival, and a lower overall survival.	This single-center case study did not analyze liver transplantation or adjuvant therapy. Additionally, flow cytometry for CTCs may have included non-specific events.	62
105 HCC patients and 132 control	Before and after surgery	5 mL peripheral blood	A tapered slit filter platform based on the cell size and morphology with positive for 4′,6-diamidino-2-phenylindole, and CK	After surgery, HCC patients exhibiting an increase in CTCs were significantly associated with higher recurrence rates. CTC counts were considered as an independent predictive factor for predicting PFS. Patients with low alpha-fetoprotein levels and cirrhosis, along with positive CTCs, were correlated with lower survival rates and higher recurrence rates.	This single-center prospective cohort study might not isolate smaller or highly deformable CTCs through the TSF platform, limiting the sensitivity and specificity.	55
42 HCC patients and 5 control	ND	10 mL peripheral blood	Labyrinth microfluidic device	1. CTC positive rate was elevated in advanced HCC stages. 2. In 71.4% of HCC patients, the cancer stem cell marker CD44 was exhibited in CTCs. 3. CTM was present in 55% of HCC patients and was associated with advanced HCC stages.	This single-center case study required further research to determine the association between tumor invasion and $CD44^+$ CTCs or CTMs.	28
176 HCC patients	Before chemotherapy or radiotherapy	5 mL of peripheral blood	CanPatrol with multiplex RNA with ISH; epithelial (CK8/18/19, and EpCAM) and mesenchymal (Vimentin and Twist)	1. All types of CTCs were more numerous in HCC patients. 2. BCLC stage B-C had more M-CTCs than BCLC stage 0-A.	Due to the small subgroup size in this single-center prospective cohort study, the association between total CTC count and other factors could not be effectively analyzed.	29

(continued)

Table 2. *(continued)*

Patients	Collected time points	Methods	Cell types	Results	Limitations	References
126 HCC patients	Before and after cancer treatments	7.5 mL peripheral blood	Immuno-magnetic beads and Immunostaining-fluorescence ISH (EpCAM, CK18, PD-L1, and Vimentin)	CTC count was higher in HCC stages III and IV than in stages I and II.	The single-center case study lacked follow-up and prognostic assessment of patients. Performing multiploidy analysis on different chromosomes in CTCs could help clarify the relationship between multiploidy and cancer stages.	30
62 HCC patients	Postoperative	5 mL of peripheral blood	CanPatrol with multiplex RNA-ISH; epithelial (CK8/18/19; EpCAM) and mesenchymal (Vimentin and Twist).	M-CTCs were associated with shortened postoperative disease-free survival and acted as independent risk factors for early recurrence.	The single-center prospective cohort study should further examine the impact of different surgical methods on CTC phenotypes and counts. The extended follow-up aimed to clarify the link between CTC subtypes and patient survival.	49
325 HCC patients, 201 chronic hepatitis B infection and liver cirrhosis patients, 100 benign hepatic lesion patients, and health 260.	Before initial diagnosis or one week after tumor resection	5 mL of peripheral blood	RosetteSep Human CD45 Depletion Cocktail and mRNA expression levels of 10 target genes (EpCAM, CD133, CD90, CK19, ABCG2, CD24, CD44, ICAM1, Nestin, and β-actin)	1. A multiple-marker CTC detection panel distinguished early and AFP-negative HCC from CHB, LC, and BHL. 2. CTC load decreased after tumor resection, and patients with a high CTC load were associated with tumor recurrence postoperatively.	The multi-center prospective cohort study included HCC patients with cirrhosis or HBV. Validation in other regions and HCC types was needed.	20
43 HCC patients with HCC	Before and after percutaneous radiofrequency ablation	5 mL of peripheral blood	CanPatrol with ISH; epithelial (EpCAM and CK8/18/19); mesenchymal (vimentin and twist)	The levels of M-CTCs increased and were associated with a decrease in lymphocyte count following hepatic tumor PRFA.	The single-center prospective cohort study used RNA-ISH for CTC detection, differing from antigen-antibody methods. The correlation between CTC levels and immune cell subsets ($CD3^+8^+$ T cells and NK cells) was not well established.	48
139 HCC patients and 23 control	Before and after operation	7.5 mL peripheral blood	CellSearch with EpCAM and CK	1. Postoperative CTC counts increased, associated with the macroscopic tumor thrombus status, and were correlated with worse disease-free and overall survival rates. 2. Patients with preoperative high CTC counts had poor prognoses.	The single-center prospective cohort study only detected the EpCAM CTCs.	37

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Table 2. *(continued)*

Patients	Collected time points	Methods	Cell types	Results	Limitations	References
47 HCC patients	After liver transplantation	5mL of blood samples	CanPatrol with ISH; epithelial (EpCAM and CK8/18/19); mesenchymal (vimentin and twist)	The levels of epithelial and interstitial CTCs increased following LTx and continued to rise over the follow-up period.	The single-center case study, limited by sample size and the collection of post-transplant samples only for some patients, could not fully compare the association between CTCs and HCC recurrence after liver transplant, nor the differences in CTCs before and after transplant.	57
112 HCC patients	Before and after resection	5 mL peripheral blood	CanPatrol with RNA-ISH, epithelial (EpCAM, CK8/18/19), and mesenchymal (vimentin and Twist)	1. CTC count ≥ 16 and M-CTC percentage ≥ 2% were associated with early recurrence, multi-site intrahepatic recurrence, and lung metastasis before resection surgery. 2. Increased CTC count and M-CTC percentage were associated with recurrence after surgery. 3. The overexpression of BCAT1 in CTCs may trigger the EMT process, inducing CTC release.	The single-center prospective cohort study using the CanPatrol system may miss smaller CTCs and only examined the relationship between BCAT1 expression and CTCs.	19
73 HCC patients	Before resection	7.5 mL blood	CellSearch EpCAM-positive (panCK8/18/19) and Microfluidic qRT-PCR	1. The total CTC count from hepatic veins was correlated with the initiation of EMT in HCC. 2. The burden of CTCs and circulating tumor microemboli from HV could predict intrahepatic recurrence and postoperative lung metastasis. 3. CTCs were primarily epithelial upon release but switched to an EMT phenotype during dissemination through the bloodstream, involving associated Smad2 and β-catenin protein signaling pathways.	The single-center prospective cohort study limited the prognostic significance of CTCs in different vascular regions. The molecular mechanisms behind CTC spatial heterogeneity needed further investigation.	23

(continued)

Table 2. *(continued)*

Patients	Collected time points	Methods	Cell types	Results	Limitations	References
61 HCC patients, 11 had cirrhosis without HCC, adenoma, focal NMLD, and eight healthy controls	Before and after surgery	4 mL blood	NanoVelcro CTC Assay with ASGPR, GPC3, EpCAM, and Vimentin. HCC CTCs were defined as round/ovoid cells, $DAPI^+/CD45^-/CK^+$, with a size ≥ 6 μm. EMT phenotype, VIM-positive CTCs are the subpopulation of HCC CTCs defined as round/ovoid events, $DAPI^+/CD45^-/CK^+/VIM^+$, with size ≥ 6 μm	VIM-positive CTCs not only predicted OS of HCC patients but also accurately distinguished patients in the early stage, LT eligible, from those in the locally advanced/metastatic stage, LT ineligible. Furthermore, VIM-positive CTCs indicated a faster recurrence trend after surgical or local treatment of potentially curable early-stage HCC.	The single-center prospective cohort study used EpCAM, ASGPR, and GPC-3 to isolate CTCs but lacked EMT markers. Additionally, it analyzed vimentin($^+$)-CTCs without considering other CTC markers.	59
42 HCC patients	Before and after surgery	5 mL peripheral blood	CanPatrol with RNA-ISH	1. CTCs were associated with the Edmondson stage in HBV-related HCC before surgery. 2. Postoperative CTC counts and the change in CTC counts before and after surgery were associated with PFS. 3. The postoperative CTC count was associated with TP53 expression.	This single-center prospective cohort study only assessed total CTC counts, lacking further analysis of CTC phenotypes and genotypes.	45
63 HCC patients, 31 chronic liver disease patients, and 26 health control	Before or after surgery	5–15 mL of blood	Microfluidic CTC-iChip	CTCs were significantly detected in 56% of untreated HCC patients compared to 3% of nonmalignant liver disease patients, indicating a risk for HCC.	The single-center case study required establishing screening criteria and validating the sensitivity and specificity of RNA-based CTC detection.	17
57 HCC patients	Before surgical treatment	7.5 mL blood	CellSearch with EpCAM and CK8/18/19	CTC-positive patients had a significantly higher risk of recurrence with a HR of 2.3, and a shorter RFS.	This single-center prospective study did not examine EMT markers in CTCs, which may have reduced the number of detected CTCs and limited the ability to link CTCs to HCC recurrence.	40
123 patients (HCC = 52) and 12 normal controls.	ND	5mL blood samples	Flow cytometry analysis with the EpCAM.	High karyoplasmic ratios were more prevalent in HCC patients with MVI than in both HCC and non-cancer patients.	This single-center study used flow cytometry to identify high karyoplasmic ratio cells as CTCs without validation standards, risking misidentification. Additional markers are needed for verification.	36

(continued)

Table 2. *(continued)*

Patients	Collected time points	Methods	Cell types	Results	Limitations	References
49 HCC patients	Before operation	6 mL blood	EpCAM $^{mRNA+}$ CTCs	1. The counts of EpCAM $^{mRNA+}$ CTCs and Treg/CD4^{+} cells showed a significant correlation with postoperative HCC recurrence. 2. High CTC/Treg level indicated a higher risk of postoperative HCC recurrence, significantly increasing the one-year recurrence rate.	The single-center prospective cohort study included only HBV-induced and early-stage HCC patients. The relationship between CTCs and Treg cells required further analysis.	41
72 patients	After hepatectomy at zero, three, six, nine and twelve months	3 mL blood samples	Anti-EpCAM nanoparticals with magnet	1. The positive expression of AFP mRNA in CTCs could serve as a predictor for metastasis both before and after hepatectomy. 2. The release of AFP expression by HCC into circulation was inevitably a primary source of HCC metastasis.	This single-center cohort study used only EpCAM to detect CTCs, while AFP nested RT-PCR limited technical issues, causing discrepancies.	58
69 patient and 31 control samples (15 healthy volunteers and 16 patients with cirrhosis without cancer)	ND	12 mL blood samples	Immunofluorescence of panCK4/5/6/8/10/13/18, EpCAM, AFP, GPC3, and DNA-PK	1. CTC number associated with tumor size and PVT. 2. HCC patients with fewer than one CTC had a median survival period exceeding 34 months, compared to patients with more than one CTC, whose median survival was only 7.5 months.	The single-center cohort study showed a weak association between peripheral neutrophil count and portal vein invasion.	42
20 HCC and 10 NMLD patients	Prior biopsy or resection	7.5 mL blood samples	CellSearch by immunomagnetic EpCAM enrichment and fluorescence-activated cell sorting	1. The CTC counts were associated with AFP levels and vascular invasion. 2. The frequency of low-variant DNA was higher in CTCs.	This single-center case-control study showed a limited match between CTCs, FFPE tumors, and PBMC DNA, with CTCs having lower WGA coverage than FFPE.	22
40 liver cancer patients and 27 healthy volunteers	Before surgery or treatment	5 mL blood samples	CanPatrol CTC enrichment technique with RNA-ISH	1. Three subtypes of CTCs were identified using EMT markers, including epithelial CTCs, epithelial/M-CTCs, and M-CTCs. It was observed that M-CTCs were more prevalent in patients with cancer metastasis.	The single-center case-control study isolated CTCs using filtration and RNA-ISH, a more complex technique compared to immunostaining, with non-standardized probes.	4

(continued)

Table 2. *(continued)*

Patients	Collected time points	Methods	Cell types	Results	Limitations	References
26 HCC patients	Preoperative, postoperative, and 24 h after surgery	10 mL blood samples	Quantitative flow cytometry with $CD45^{-}$/$CD44^{+}$/$CD90^{+}$ cells	HCC patients undergoing laparoscopic surgery exhibited fewer CTCs and lower secretion of IL-6 and IL-8 compared to those undergoing traditional open surgery.	This single-center prospective cohort study found that the accuracy of CTC detection needed improvement. Patient variability also affected CTC detection, and further analysis was required to explore the relationship between cytokines and CTCs.	50
299 HCC patients (Resection, n = 157; TACE, n = 76; radiotherapy, n = 66)	Pretreatment and post-treatment	7.5 mL of blood	Negative enrichment and qRT-PCR-based CTC detection platform and CellSearch system with $EpCAM^{+}$ CTC	The preoperative levels of CTCs could predict the prognosis of patients with HCC undergoing liver resection, TACE, and radiotherapy. Moreover, an increase in CTC levels after treatment indicated disease progression.	The single-center prospective cohort study had a short follow-up period, and qRT-PCR for EpCAM was prone to leukocyte contamination, leading to a higher false positive rate.	21
11 HCC patients	During treatment	20 mL of blood	CTC type detection by immunofluorescence staining with epithelial panCK and mesenchymal markers (vimentin, N-cadherin).	Different CTC subtypes could be identified in the peripheral blood of HCC patients. Changes in the ratio of epithelial to M-CTCs were associated with a longer median time to progression.	The single-center cohort study observed that CD45-positive cell depletion during negative enrichment led to CTC loss. Additionally, immunomagnetic methods showed lower recovery rates compared to density gradient centrifugation.	43
60 HCC patients, 10 patients with benign liver diseases (including patients with cirrhosis, chronic hepatitis B, hepatic hemangioma, and liver cysts), 10 healthy volunteers and 10 patients with miscellaneous advanced cancers other than HCC	ND	10 mL of blood	ASGPRs with immunofluorescence staining	In HCC tumors, the presence of positive CTCs was associated with the expression levels of E-cadherin, vimentin, and twist. The expression of E-cadherin indicated a significant role for EMT in facilitating the blood-borne dissemination of primary HCC cells. Moreover, the expression levels of twist and vimentin in CTCs could serve as biomarkers for evaluating metastasis and prognosis in HCC patients.	The single-center study used ASGPR, a receptor expressed only in HCC, to detect EMT CTCs.	18

(continued)

Table 2. *(continued)*

Patients	Collected time points	Methods	Cell types	Results	Limitations	References
85 HCC patients, 37 patients with benign liver diseases, 20 healthy volunteers, and 14 patients with other cancers.	ND	5 mL blood	HCC cells were bound by biotinylated asialofetuin and magnetically labeled	CTCs were identified in 81% of HCC patients. The positivity rate and quantity of CTCs were significantly correlated with tumor size, portal vein tumor thrombosis, differentiation status, as well as TNM classification and Milan criteria-based disease staging. Additionally, HER-2 gene amplification and TP53 gene deletion were detected in CTCs.	The single-center cohort study used ASGPR and Hep Par 1 techniques for CTC detection, which were applicable only to HCC, while HER-2 amplification in CTCs required further investigation.	15
44 HCC patients, 30 patients with chronic active hepatitis, 39 with liver cirrhosis, and 38 healthy individuals	ND	6 mL peripheral blood	The ISET method with filtration and β-Catenin mutations assay	CTCs were associated with tumor diffusion, portal vein tumor thrombosis, and shorter overall survival.	The single-center cohort study using the ISET method for detecting CTCs may have missed those with diameters less than 25 μm	16

AFP, alpha-fetoprotein; AJCC, the American Joint Committee on Cancer; AR, androgen receptor; ASGPR, Asialoglycoprotein receptor; BCLC, Barcelona Clinic Liver Cancer; BHL, benign hepatic lesion; CHB, chronic hepatitis B infection; CK, cytokeratin; CTM, circulating tumor microemboli; DDR1, discoidin domain receptor tyrosine kinase 1; EHHADH, enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase; EHM, extrahepatic metastases; EMT, epithelial-mesenchymal transition; GPC3, glypican-3; HCC, hepatocellular carcinoma; HR, hazard ratio; HSD17B6, hydroxysteroid 17-beta dehydrogenase 6; LAD1, ladinin 1; LC, liver cirrhosis; LTx, liver transplantation; M-CTC, mesenchymal CTC; MVI, microvascular invasion; NECAB2, N-terminal EF-hand calcium-binding protein 2; NMLD, nonmalignant liver disease; OS, overall survival; PMEPA1, prostate transmembrane protein, androgen-induced 1; PVT, portal vein thrombosis; PVTT, portal vein tumor thrombosis; SLC27A5, solute carrier family 27 member 5; TACE, transcatheter arterial chemoembolization; TSKU, tsukushi, small leucine-rich proteoglycan; USP1, Ubiquitin-specific protease 1; VIM, vimentin; MCA, microcavity array; ALB, albumin; ISH, *in situ* hybridization; ISET, isolation by size of epithelial tumor cells.

vival and overall survival.[22,27,33,47] The presence of M-CTCs correlates significantly with AFP levels of ≥400 ng/mL.[31] Moreover, Guo *et al*. utilized a qPCR panel consisting of nine putative cancer stem cell biomarkers (EpCAM, CD133, CD90, CK19, ABCG2, CD44, ICAM1, CD24, and Nestin) to detect CTCs. This panel effectively identified early-stage and AFP-negative HCC, demonstrating high sensitivity and specificity, making it a promising tool for early prediction and treatment monitoring.[20]

Post-surgical monitoring of HCC patients has revealed a substantial correlation between CTCs and M-CTCs with early recurrence and a decline in recurrence-free survival, establishing both as independent factors. Monitoring their quantity has proven more effective than AFP monitoring.[24] Testing for AFP mRNA in CTCs pre- and post-hepatectomy revealed that a significant proportion of patients expressing AFP mRNA developed metastasis. Subsequent examinations confirmed the persistence of AFP mRNA expression in these patients' CTCs.[58] This finding implies that AFP mRNA positivity in CTCs serves as a predictive factor for pre- and postoperative metastasis in HCC. In HCC patients undergoing liver transplantation, CTC testing was also correlated with AFP mRNA levels, suggesting its role in evaluating HCC recurrence post-transplantation.[47]

MVI, characterized by the infiltration of cancer cells into the hepatic microvasculature, is typically associated with metastasis and is a crucial factor in assessing prognosis and treatment strategies for liver cancer. Studies have shown a close relationship between the quantity of CTCs or M-CTCs and MVI, indicating that as CTC counts increase, so does the severity of MVI. Surgical margins greater than 1 cm are recommended for CTC-positive patients to ensure disease eradication and preserve liver function.[35] Preoperative CTC counts exceeding one exhibit the most significant predictive capacity for the presence of MVI. Furthermore, patients with preoperative CTC counts exceeding one had surgical margins of >1 cm, which protected against early recurrence, showing lower early recurrence rates than those with surgical margins of ≤1 cm.[35] CTC counts or M-CTCs in HCC patients are closely associated with the presence of MVI,[24,31,34–36] and may serve as independent predictors for MVI in HCC. Moreover, tumor thrombosis is a severe complication with grave outcomes in HCC patients, and research has indicated an association between CTCs and tumor thrombosis.[15,16,24,27,33,37] In summary, the combination of CTCs, AFP, and MVI offers more accurate prognostication for HCC patients, facilitating the development of more effective treatment strategies.

The gene expression in CTCs and interaction with immune cells

In the ongoing quest to understand HCC, recent studies have illuminated the role of CTCs and their genetic makeup. Despite their limited abundance in the bloodstream, CTCs have been the subject of extensive investigation, particularly regarding the expression of specific genes within them. Findings from these studies suggest that gene expression in CTCs is associated with various clinical parameters of HCC, including prognosis, recurrence, tumor stage, size, and MVI. Notably, genes such as *Nanog*, *vimentin*, *Survivin*, *CD44*, *al-*

Table 3. The markers of CTCs and the clinical impacts

Genes	Cells	The clinical impacts
PanCK, EpCAM (CD326), E-cadherin, CK8/18/19, mucin 1	E-CTC biomarker	The common markers of CTCs and the quantity of CTCs were positively correlated with poor prognosis in HCC.
VIM, Twist, CD274 (PD-L1), N-cadherin	M-CTC marker	The process of CTCs undergoing EMT not only leads to poor prognosis in HCC but also increases the likelihood of HCC recurrence.
CD133, CD90, ABCG2, CD44, ICAM1, CD24, and Nestin	cancer stem cell biomarkers	The CTC detection panel effectively detected early-stage and AFP-negative HCC
Nanog	cancer stem cell biomarkers	Higher Nanog levels were associated with a higher recurrence rate
BCAT1, Smad2 and β-catenin	Metastasis biomarker	Triggered the EMT process, inducing CTC release
DDR1, EHHADH, AR, LUM, HSD17B6, PMEPA1, TSKU, NECAB2, LAD1, and SLC27A5	prognostic genes	The genes expressed in CTCs reduced the survival rate of patients.
GPC3	CTCs	A risk factor of poor prognosis with low OS, and lower disease-free survival.

CK, cytokeratin; CTC, circulating tumor cells; DDR1, discoidin domain receptor tyrosine kinase 1; EHHADH, enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase; EMT, epithelial-mesenchymal transition; GPC3, glypican-3; HCC, Hepatocellular carcinoma; HSD17B6, hydroxysteroid 17-beta dehydrogenase 6; LAD1, ladinin 1; M-CTC, mesenchymal CTC; NECAB2, N-terminal EF-hand calcium-binding protein 2; OS, overall survival; PMEPA1, prostate transmembrane protein, androgen-induced 1; SLC27A5, solute carrier family 27 member 5; TSKU, tsukushi, small leucine-rich proteoglycan; VIM, vimentin.

bumin, *USP1*, *CXCR4*, *BCAT1*, *PD-L1*, and *GPC3* have been detected in CTCs. For instance, *Nanog*, a specific marker for cancer stem cells, is associated with a higher recurrence rate in HCC patients when highly expressed in CTCs. Similarly, the presence of vimentin-positive CTCs indicates a faster recurrence trend in early-stage HCC patients post-treatment.[59] Furthermore, the expression of *Nanog* and *Survivin* correlates with BCLC stage, vascular invasion, and tumor size.[26,46] Another cancer stem cell marker, *CD44*, is found to be abundantly present in CTCs from HCC patients.[28] A*lbumin* expression in CTCs has been detected more frequently in patients with metastatic HCC compared to those with localized HCC.[44] Notably, *USP1* expression is often upregulated in CTCs from metastatic HCC patients, correlating with a reduced survival rate.[60] The expression of *CXCR4*, commonly found in mixed CTCs or mesenchymal CTCs, signifies the initiation of EMT and the onset of metastasis.[33] *BCAT1*, which is highly expressed in CTCs, is considered a driver of EMT, promoting the release of CTCs into the bloodstream.[19] *PD-L1* expression in CTCs, associated with advanced stages of HCC and poorer overall survival, further underscores the prognostic value of these cells.[61] *GPC3,* another gene associated with low overall survival, is expressed in CTCs and correlates with a higher probability of microvascular invasion and lower disease-free survival.[62] Studies employing RNA sequencing and tissue microarray analysis to investigate the positive expression of CTC clusters in HCC demonstrated a significant upregulation of Wnt/β-catenin signaling proteins within the CTC clusters.[23,63] Furthermore, there was a significant gradient in the number and size of CTCs between tumor efferent vessels and post-pulmonary peripheral vessels, with CTCs spreading in an aggregated-singular-aggregated pattern. Single-cell qPCR analysis revealed that the Smad2 and β-catenin signaling pathways activate the EMT process in CTCs.[23] A proposed HCC-CTC Risk Score Panel by Lee *et al*. comprises 10 prognostic genes (*DDR1*, *EHHADH*, *AR*, *LUM*, *HSD17B6*, *PMEPA1*, *TSKU*, *NECAB2*, *LAD1*, and *SLC27A5*). This panel leverages the high expression of these genes in HCC and their low expression in white blood cells, serving as an independent predictor of survival. It offers a non-invasive approach for real-time disease analysis and dynamic prognosis assessment for HCC.[64] The relationship between CTC markers and HCC is summarized in Table 3.

In addition to gene expression in CTCs, the association between CTCs and immune cells can provide insights into the progression of HCC. Luo *et al*. conducted an in-depth analysis of CTCs in HCC patients, particularly their interactions with immune cells in the peripheral blood.[27] This investigation was prompted by the potential role of immune cells in promoting the proliferation and dissemination of CTCs. The presence of CTC-immune cell clusters in peripheral blood may signify a less favorable prognosis. There was a significant association between the presence of CTC-immune cell clusters and various clinical parameters, including tumor size, tumor number, portal vein tumor thrombosis, BCLC staging, AFP levels, and total CTC count. In summary, the combination of CTCs, their gene expression, and their interaction with immune cells offers a more comprehensive understanding of HCC, facilitating the development of more effective treatment strategies.

The enhanced accuracy in predicting HCC prognosis can be attributed to the notion that the presence of CTCs in the blood may reflect immune system dysfunction, indicating an inability to effectively clear CTCs. Furthermore, functional changes in CTCs, such as undergoing EMT after entering the bloodstream, increase their invasiveness, leading to extrahepatic metastasis or MVI in patients. While studies emphasize the superiority of CTC quantity in diagnosis and prognosis prediction, controversies remain regarding the required blood volume for CTC detection, the choice of detection techniques, and the establishment of diagnostic thresholds for CTC counts with clinical significance. This highlights the need for standardized guidelines to obtain more definitive and targeted results.

Limitations and challenges

Certain limitations and challenges remain in the routine application of CTC detection for HCC.

Limited clinical application

Currently, the CellSearch system is the only CTC detection technology approved by the U.S. FDA, primarily for breast, colorectal, and prostate cancers. CTC detection has not yet

been officially validated or standardized for HCC; therefore, further multicenter, large-scale studies are necessary in this area.

Low quantity and high variability

CTCs are shed from primary HCC tumors into the bloodstream. However, the low number and high heterogeneity of CTCs make detection a challenging task. Improving enrichment efficiency, as well as enhancing cell identification and isolation techniques, is crucial for increasing the accuracy and sensitivity of CTC detection.

Challenges with CTC subtypes

In HCC patients, both heterogeneous epithelial tumor cells and mesenchymal-like cells can be found. Additionally, CTCs may exhibit different subtypes, varying in cell surface antigens and size. Consequently, detecting and capturing these specific CTC subtypes presents further challenges, complicating the detection of particular CTCs.

Future directions

Regarding the future directions of CTC research, several aspects can be considered:

Single-cell analysis techniques

Current cell analysis methods have increasingly shifted toward single-cell analysis techniques. For instance, single-cell sequencing technologies can be employed to understand the characteristics of CTCs, including gene mutations and expression profiles. Mass cytometry or Cytometry Time-Of-Flight can analyze protein expression at the single-cell level. These single-cell analysis techniques can facilitate personalized treatment and cancer progression monitoring.

Nanotechnology

The utilization of nanotechnology to enhance the isolation and separation efficiency of CTCs is a promising avenue. Nanotechnology can provide higher sensitivity while reducing the risk of false positives or false negatives. Using nanoscale isolation devices or filters, such as nanostructures, offers advantages due to their high specificity, increased surface area, and varying filter sizes. These technologies can improve isolation efficiency, eliminate the influence of normal cells, and achieve effective CTC separation.

Artificial intelligence and machine learning

Machine learning and artificial intelligence can be used to train recognition algorithms for the automated identification and separation of CTCs, along with subsequent analysis of CTC data. These algorithms can be based on CTC characteristics such as shape, size, surface antigens, and other features, enabling automated collection, separation, and analysis. This approach aids in identifying tumor characteristics and predicting disease progression, thereby enhancing the efficiency and precision of CTC detection.

In summary, while there are current technical challenges and limitations that need to be addressed, continuous development of new CTC detection methods and technologies is essential for improving accuracy and clinical applicability.

Conclusions

In this review, we provide an overview of sampling methods and detection technologies for CTCs, focusing on the latest research developments in the field of HCC. We elaborate on various detection methods and discuss the clinical applications of CTCs in predicting, diagnosing, and prognosticating these malignancies, comparing them with current diagnostic approaches. Exploring the potential of CTCs as biomarkers offers significant opportunities for future applications in liquid biopsy for HCC.CTCs have gained FDA approval for monitoring and predicting diseases in cancer types such as breast, colorectal, and prostate cancers. However, significant challenges remain in HCC. Beyond the technical challenges associated with CTC detection and isolation, there exists a multitude of CTC detection methods, each with distinct sample preparation, enrichment, and analysis protocols, making validation studies exceptionally challenging. Therefore, establishing a standardized detection protocol with high sensitivity and specificity, capable of capturing the entire spectrum of CTCs, is essential.

While CTCs have opened avenues for valuable clinical applications, a comprehensive understanding of the molecular mechanisms remains incomplete. Future research efforts are anticipated to delve deeper into the processes and mechanisms of CTC formation and the transition of epithelial tumor cells to mesenchymal cells. These endeavors will pave the way for more targeted and clinically relevant applications in cancer management.

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Conflict of interest

JFH has been an Editorial Board Member of *Journal of Clinical and Translational Hepatology* since 2022. The other authors have no conflict of interests related to this publication.

Author contributions

Conceptualization (CMH, JFH), supervision (YCL, JFH), writing – original draft (CMH), writing – review & editing (YCL, JFH), and visualization (CMH, YCL, JFH). All authors have made significant contributions to this study and have approved the final manuscript.

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