Hepatoma Research

Review

Detection of circulating tumor cells in hepatocellular carcinoma: applications in diagnosis, prognosis prediction and personalized treatment

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and is associated with poor clinical prognosis, which is mainly caused by tumor recurrence and metastasis. Circulating tumor cells (CTCs) are tumor cells shed into the bloodstream and regarded as the "seeds" of tumor recurrent or metastatic lesions. Over the past decade, the clinical value of CTC analysis has been extensively explored. CTC analysis is a representative form of liquid biopsy, offering a novel solution that can bypass the problems of invasive biopsy procedures, enabling comprehensive, non-invasive, and real-time disease monitoring. In HCC, CTC analysis has facilitated early detection and prognosis prediction, as well as treatment monitoring and therapeutic intervention guiding. In this review, we summarize available literature and provide an overview of CTC biology, detection technologies, and clinical applications in the diagnosis, prognosis prediction, and personalized treatment of HCC.

Keywords: Hepatocellular carcinoma, liquid biopsy, circulating tumor cells, biomarkers, personalized medicine

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most prevalent malignancy worldwide and is currently listed as the fourth leading cause of cancer-related death[1]. HCC is a highly aggressive neoplasm; distant organ metastasis can occur at a very early stage[2]. Thus, early detection of HCC is of great importance in the management of HCC. Surgical resection or liver transplantation remains the primary therapy for HCC patients. However, only approximately 20%-30% of patients are eligible for surgical intervention at the time of the first diagnosis, most patients have already reached an advanced cancer stage[3]. The 5-year survival rate of HCC with Barcelona Clinic Liver Cancer (BCLC) stage 0-A treated with curative therapeutic modalities is 40%-70%, but the median overall survival (OS) for patients with BCLC stage B-C is only 11-20 months[4]. Even after curative resection, the five-year cumulative recurrence rate is 50%-70%, which results in the unsatisfactory long-term outcomes of HCC patients[5,6]. Currently, early detection or monitoring HCC recurrence mainly relies on imaging examinations and serum tumor biomarkers such as alpha-fetoprotein (AFP); however, their diagnostic sensitivity is limited and often fails to foresee the tumor metastatic potential[7,8]. Therefore, there is an unmet need for reliable biomarkers for early HCC detection and tumor recurrence monitoring.

In recent years, various "liquid biopsy" techniques have emerged and shown significant promise as novel biomarkers for HCC. Liquid biopsy represents a modality of collecting bodily fluids instead of solid tissue for pathophysiological or sequencing analysis. Liquid biopsy offers a solution that can bypass the problems of invasive biopsy procedures, enabling repeated and real-time disease status monitoring[9]. Circulating tumor cells (CTCs) and circulating tumor DNA are two of the most widely studied biomarkers in liquid biopsy[10]. CTCs are the cells that derive from the primary or metastatic lesions and migrate into circulation and are regarded as the "seeds" of tumor metastasis[11]. CTCs represent a unique liquid biopsy form that is different from any of the existing cancer biomarkers, as they are a sampling of the patient's live tumor cells, carrying comprehensive biological information of the primary tumor, including genomic mutations, cancer subtypes, and drug sensitivity[12]. CTC research has flourished over the past decade, spanning fields including CTC detection, identification of prognostic significance, and evaluation for treatment response and disease surveillance[13,14].

In the context of HCC, excellent progress has been made using CTCs as blood-based biomarkers[9,14,15]. Herein, to gain comprehensive insight into the role of CTCs in HCC, this review article provides an overview of their biology, detection technologies, and clinical significance in HCC.

OVERVIEW OF CTC BIOLOGY

To date, the definition of CTC from CellSearch[TM] system is considered as the current standard: a CTC is an intact nucleated epithelial cell, expressing epithelial cell adhesion molecule (EpCAM) and/or cytokeratin 8, 18, and 19, and negative for the leukocyte-biomarker CD45[16]. EpCAM[+] CTCs were also identified as a stem cell-like subpopulation in HCC, with a highly invasive and metastatic capacity[17].

Thousands of tumor cells are shed into the bloodstream everyday[18]; however, most CTCs are eliminated in the bloodstream by shear stress, immune attack, and anoikis[11,19]. Only a small number of viable and highly metastatic subpopulations of CTCs could eventually survive and develop into metastatic lesions[20-22]. However, the biological mechanism of how CTCs survive the bloodstream and home to distant organs remains largely unknown. CTCs may undergo several adaptations to survive in a hostile environment. A recent study used *in vivo* genome-wide CRISPR to screen and identify a subset of CTCs with the deregulation of ribosomal protein expression and translation. This CTC subset was significantly associated with enhanced metastatic capabilities and poor clinical outcome[23]. Meanwhile, one of the key biological events is epithelial-to-mesenchymal transition (EMT), during which CTCs downregulate epithelial markers such as E-cadherin and gain mesenchymal markers, thus acquiring increased ability to invade the

adjacent tissues and survive the environmental stress[24-26]. Recently, Sun *et al.*[27] compared EMT-features of CTCs isolated from different vascular sites of HCC patients prior to resection, including peripheral vein, peripheral artery, hepatic veins, infrahepatic inferior vena cava, and portal vein. Single-cell transcriptional characterization demonstrated that CTCs were initially epithelial phenotype at release, but they switched to EMT-activated phenotype during hematogenous transit via Smad2- and b-catenin-related signaling pathways. They suggested such heterogeneous EMT status during the CTC transition may be the result of shear stress in circulation. Nevertheless, while the loss of E-cadherin increased invasion, it also reduced cancer cell proliferation and survival of CTCs in multiple models of breast cancer, indicating the complex phenotypic plasticity of CTCs in EMT status[28,29].

In addition, CTCs may aggregate to form CTC clusters [also referred to as CTC microemboli (CTM)] and travel together in circulation, with or without fibroblasts, leukocytes, endothelial cells, or platelets, which possess significantly higher invasiveness and increased survival ability compared to individual CTCs[30]. Notably, clusters of circulating tumor cells (CTCs) possessed an up to 50 times greater metastatic potential compared with single CTCs[31]. CTC clusters have been identified in many cancer types including HCC; these clinical studies confirmed that CTC clusters are a much stronger prognostic factor for cancer metastasis than single CTCs[27,32-34]. A recent study comprehensively profiled the DNA methylation landscape of single CTCs and CTC clusters from breast cancer patients and mouse models, and the results revealed that binding sites for stemness- and proliferation-associated transcription factors were specifically hypomethylated in CTC clusters, thus promoting the stemness and metastasis of CTC clusters[35]. Szczerba *et al.*[36] identified a specific of CTC-neutrophil cluster in circulation and further confirmed this interaction drove cell cycle progression within the bloodstream and expanded the metastatic potential of CTCs. Targeting this interaction may be rational in treating cancer metastasis. Additionally, the formation of CTC clusters can induce a hypoxic environment that drives hypoxia-inducible factor 1-alpha-mediated mitophagy, clearing damaged mitochondria, and limiting reactive oxygen species. Such a metabolic switch may support the survival and metastatic spread of CTCs in circulation[37]. Although the specific mechanism driving CTC cluster formation remains unclear, it is currently considered that CTC clusters arise from oligoclonal tumor cell groupings from the primary tumor or intra-vascular aggregation of single CTCs in circulation[31,38]. A recent study has proposed that CTC cluster formation was a dynamic event rather than grouped migration derived from the primary tumor by their multi-vascular sampling, in which CTM displayed an "aggregated-apart-aggregated" pattern during the circulatory pathway. They hypothesized that single CTCs might aggregate spontaneously in blood vessels against the unfavorable microenvironment in the bloodstream[27]. Characterization of the mechanism that determines the cluster formation may identify viable therapeutic targets for inhibiting metastasis.

CTC DETECTION TECHNOLOGY

CTCs are extremely rare and surrounded by numerous blood cells in the bloodstream, thus CTCs are generally required to be firstly enriched from blood samples[39]. Reliable CTC enrichment technologies are essential to the downstream comprehensive CTC analysis. Many different technologies have been developed for CTC detection in the past years, and they can be classified into two categories based on whether they make use of the physical or biological properties of the target cells [Table 1][40]. However, most of these technologies for CTC capture and downstream analyses are designed for scientific research, requiring multiple batch-process steps and having relatively limited throughput. The price of CTC analysis is still on the high side (it generally costs hundreds of dollars per patient). A standardized and easy-to-use platform that facilities integrated CTC analysis is still needed.

Immunoaffinity-based method

The immunoaffinity-based method for CTC detection relies on the tumor-specific antibodies against cell surface markers, including EpCAM, human epidermal growth factor receptor (EGFR)2, prostate-specific

Table 1. Overview of CTC detection technology

Platform	Enrichment method	Phenotypic Markers	Study cohort	Blood volume	CTC positive rate	Ref.
Immunoaffinity-based method						
CellSearch platform	Immunomagnetic	EpCAM, CK8/18/19	964 metastatic carcinomas, 199 NLD, and 145 HD	7.5 mL	36.0% (≥ 2 CTCs)	[16]
Flow cytometric analysis	Immunomagnetic	CD90	34 HCC, 19 LC, and 19 HD	10 mL	91.2%	[44]
Flow cytometric analysis	Flow cytometry	ICAM-1	60 HCC	NA	50.0%	[46]
ASGPR sorting	Immunomagnetic	ASGPR, Hep Par 1	85 HCC, 37 NLD, 20 HD, and 14 patients with other advanced cancers	5 mL	81.0%	[67]
ASGPR sorting	Density gradient centrifugation and Immunomagnetic	ASGPR, pan-cytokeratin, CPS1	27 HCC patients	5 mL	89.0%	[68]
Glypican-3 sorting	Density gradient centrifugation and Immunomagnetic	Glypican-3	85 HCC patients	8 mL	38.8% (≥ 5 CTCs)	[69]
NanoVelcro CTC assay	Microfluidic device	ASGPR, glypican-3, EpCAM, vimentin	61 HCC, 11 NLD, and 8 HD	4 mL	97.0%	[70]
Biophysical properties-based method						
qRT-PCR-based platform	Density gradient centrifugation and Ficoll-Paque	AFP (mRNA)	44 HCC, and 7 HD	5 mL	72.7%	[39]
qRT-PCR-based platform	Density gradient centrifugation and Ficoll-Paque	Cytokeratin 20 (mRNA)	65 patients with colorectal cancer	10 mL	41.4%	[50]
qRT-PCR-based platform	Density gradient centrifugation and OncoQuick	Cytokeratin 20 (mRNA)	37 patients with gastrointestinal tumors	10 mL	30.0%	[51]
ISET platform	Microfiltration	AFP (mRNA)	37 HCC	15 mL	42.9%	[54]
ISET platform	Microfiltration	AFP	44 patients with primary liver cancer, 30 patients with chronic active hepatitis, 39 LC, and 38 HD	6 mL	52.3%	[55]
ScreenCell platform	Microfiltration	Cytokeratins	NA	NA	NA	[56]
CTC-Chip	Microfluidic device	EpCAM	116 patients with metastatic cancer	1 mL	99.0%	[58]
HB-Chip	Microfluidic device	EpCAM	15 patients with metastatic cancer	1 mL	93.0%	[59]
CTC-iChip	Microfluidic device	Cytokeratins	NA	8 mL	97.0%	[60]
Cluster-Chip	Microfluidic device	Cytokeratins, Ki67	27 patients with breast cancer, 20 patients with melanoma, and 13 patients with prostate cancer	1 mL	30%-40%	[61]
qRT-PCR-based platform	Density gradient centrifugation and RosetteSep Human CD45 Depletion Cocktail	EpCAM (mRNA)	299 HCC, and 120 HD	5 mL	41.2%	[71]
digital PCR-based platform	Microfluidic device and immunomagnetic	10 liver-specific transcripts (mRNA)	16 HCC, and 31 NLD	4 mL	56.0%	[72]
CanPatrol platform	Microfiltration	EpCAM, CK8/18/19, vimentin, twist (mRNA)	33 HCC, and 10 HD	5 mL	NA	[73]
Labyrinth microfluidic device	Microfluidic device	Glypican-3, Glutamine Synthetase, HepPar-1, CD44	42 HCC	10 mL	88.1%	[74]

CTC: circulating tumor cells; EpCAM: epithelial cell adhesion molecule; CK: cytokeratin; NLD: non-malignant liver disease; HD: healthy donor; HCC: hepatocellular carcinoma; LC: liver cirrhosis; ASGPR: asialoglycoprotein receptor; CPS1: carbamoyl phosphate synthetase 1; qRT-PCR: quantitative reverse transcription polymerase chain reaction; AFP: alpha-fetoprotein

antigen, and so on[41]. EpCAM is the most frequently used antigen in CTC recognition, as the only FDA-approved semi-automated CTC detection device, CellSearch[TM] system, is based on the expression of surface EpCAM. CellSearch[TM] system utilizes anti-EpCAM-coated magnetic beads for CTC sorting in 7.5 mL of blood, and the extracted CTCs are then fixed, stained by antibodies against EpCAM and cytokeratin, and counted. EpCAM and cytokeratin also have been regarded as a clinical standard in CTC labeling among other markers[16,42]. CellSearch[TM] system can retain morphological and immunological characters of isolated cells, thus allowing the following fluorescence-based assays. However, CellSearch[TM] system is incompatible with direct downstream single-cell molecular analysis since these cells have been fixed, which limits their clinical utility in CTC-based comprehensive analysis[43]. Fluorescence-activated cell sorting is another widely used CTC detecting method that combines conventional flow cytometry technique and immunoaffinity sorting, while this strategy is limited by the makers' selection[44-46].

Biophysical properties-based method

The biophysical property-based enrichment utilizes various physical properties including density, size, shape, inertia, and electrical property of CTCs to distinguish them from other blood cells[47]. These so-called "label-free" methods are gaining increasing attention, as they avoid cell loss when choosing specific antigens targeting CTCs. In addition, unlabeled CTCs are generally compatible with a variety of downstream analyses[48].

Among these strategies, density-based gradient centrifugation is the most commonly used method, which utilizes the differences in specific densities CTCs to separate the target tumor cells and blood cells[49]; this is also a common pre-processing step integrated with many methods for CTC detection. Ficoll-Paque[TM] is a cell separation medium used for the isolation of CTCs in patients with various types of cancer, including liver cancer and colorectal cancer[39,50]. Oncoquick[TM] has made improvements in the centrifuge tubes that combines filtration and centrifugation and has superior CTC recovery rate and less blood cell contamination[51]. RossetteSep[TM] CTC enrichment cocktail is as an example of label-free CTC enrichment, where a mixture of antibodies is used to target and cross-link unwanted blood cells to form immunorosettes, and then density gradient centrifugation is performed to deplete the unwanted cells[52,53].

Another common method that utilizes the biophysical properties of CTC is size-based filtration. The diameter of tumor cells is generally larger than blood cells, and CTCs would be retained in the filter, while smaller blood cells pass through. Isolation by size of epithelial tumor cells (ISET) was developed as a microfilter to isolate tumor cells by a polycarbonate membrane with calibrated pores[54]. ISET[TM] system can visualize and count CTCs and CTC clusters in blood samples obtained from HCC patients[55]. More recently, ScreenCell[TM] device was developed as an advanced microfilter to isolate viable CTCs with a high recovery rate. Immunocytochemistry assays for CTCs can be performed directly on the filter[56]. To conclude, the filtration method is one of the simplest and most widely studied methods for capturing CTCs. However, this method is limited by its low specificity that the products may be contaminated by other cells owing to natural variation in size of leukocytes, and small CTCs may be lost during the filtration[34].

Alternatively, microfluidic techniques are now increasingly being exploited in CTC isolation, which allows for precise control of fluids in a small volume and rapid sample processing at relatively low cost and high sensitivity[57]. Microfluidic platforms enable on-chip CTC isolation, identification, and even culturing. The first microfluidic device named "CTC-chip" was developed to capture rare CTCs in 2007, which successfully identified CTCs in the peripheral blood of patients with metastatic lung, prostate, pancreatic, breast, and colon cancer[58]. In 2010, an improved herringbone-chip was developed. The herringbone-shaped grooves of this chip can generate a microvortex when blood is pumped, which enhances the contact between the chip surface and tumor cell. Its clinical utility was demonstrated in specimens from patients with prostate cancer[59]. Another novel CTC-iChip platform utilizes the distinct differences between cancer

cells and blood cells in size and deformability, reaching a 97% yield of rare tumor cells[60]. The Cluster-Chip is a unique 3D microfiltration system, designed specifically to capture CTC clusters[61]. One obvious advantage of microfluidic chip is that it can isolate CTCs from whole blood in a high-throughput fashion without complicated initial preparation step, thus decreasing the possibility of destruction and loss of CTCs[62].

Technology developed for detecting CTC in HCC

CTCs in patients with HCC are a highly heterogeneous population; currently, there are no widely accepted antigens specifically targeting HCC CTCs[27,63]. Although CellSearch[TM] system is commonly used in CTC detection, it is reported that the EpCAM-based CTC-sorting strategy could only identify approximately 10%-35% of the total amount of tumor cells in blood due to the EMT process and heterogeneous CTC molecular phenotypes[64,65]. Thus, a clinically relevant subset of CTCs may be missed by singular epithelial markers-based sorting strategies[29,66]. Some research groups have broadened target epitopes to include alternative candidates specific to hepatocytes. For example, Xu *et al.*[67] developed a novel CTC enumeration system for HCC by taking advantage of a "biotin and anti-biotin antibody" combination, in which circulating HCC cells were bound by biotinylated asialofetuin, an asialoglycoprotein receptor (ASGPR) ligand and subsequently magnetically labeled by anti-biotin antibody-coated magnetic beads, followed by magnetic separation. This system was able to detect CTCs in 69 out of 85 (81%) HCC patients. The same group used an anti-ASGPR antibody instead of ASGPR ligand with successful CTC detection in 89% of HCC patients[68]. Glypican-3 (GPC3) is an oncofetal heparan sulfate proteoglycan that is currently used as a pathologic biomarker for HCC diagnosis. It can also be used for the detection of HCC-specific CTCs[69]. Recently, the novel NanoVelcro CTC assay uses an antibody cocktail targeting the cell-surface markers ASGPR, GPC3, and EpCAM to detect CTCs. It has been showed that multi-marker capture detected greater numbers of CTCs than any individual antibody alone in both cell line and HCC patient samples[70].

"Label-free" strategies have also exhibited great utilities in HCC CTC enrichment. RosetteSep[TM] and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were combined for the optimized CTC detection in HCC patients, yielding a 41.2% positive rate of EpCAM[mRNA+] CTCs[71]. Kalinich *et al.*[72] integrated a microfluidic chip device and RNA-based digital PCR to detect molecular signatures derived from HCC CTCs. Using the identified 10 liver-specific transcripts, 9 out of 16 (56%) untreated HCC cases had detectable CTCs. The CanPatrol[TM] CTC analysis platform used a two-step technique including microfiltration and subsequent RNA *in situ* hybridization (ISH) assay, to characterize epithelial (EpCAM and cytokeratin 8/9/19) and mesenchymal (Vimentin and Twist) markers of CTCs from patients with HCC[73]. Wan *et al.*[74] used a labyrinth microfluidic device to detect CTCs in patients with HCC. They showed that 71.4% of the HCC patients had CTCs positive for cancer stem cell marker CD44, while 55% of the patients had the presence of CTM, which was correlated with advanced HCC stage.

EARLY DETECTION AND PREDICTION OF TUMOR PROGRESSION IN HCC

Research focusing on clinical implications of CTCs in HCC patients has flourished over the past decade. CTCs have shown great potential for the early diagnosis and prognostication of HCC patients[14]. The clinical applications of CTC detection in patients with HCC are summarized in Table 2.

Currently, EpCAM[+] CTCs have been extensively investigated in HCC, as immunoaffinity-based CTC enrichment techniques such as CellSearch[TM] have been widely used to capture EpCAM[+] CTCs. In 2013, Sun *et al.*[17] used CellSearch[TM] to detect EpCAM[+] CTCs in HCC patients undergoing tumor resection. They found that 66.7% of HCC patients had detectable EpCAM[+] CTCs preoperatively; moreover, EpCAM[+] CTCs ≥ 2 per 7.5 mL of blood was the strongest predictor of HCC recurrence. The prognostic value of CTCs was retained in patient subgroups with minor recurrence risk by traditional evaluation. They also found that CTC numbers were significantly correlated to the systemic immune-inflammation index

Table 2. Applications of CTC detection in HCC

Year	Techniques/ Platform	Model	Objective	Main findings	Quality of the evidence (GRADE)	Ref.
2011	Flow cytometric analysis	82 HCC patients	Prognostic significance of stem cell-like CTCs in HCC undergoing liver resection	Circulating cancer stem cells > 0.01% predicted post-hepatectomy HCC recurrence with high accuracy	●●○○ Low	[86]
2012	*In vivo* flow cytometry	HCC orthotopic metastatic tumor model (mouse)	Monitor CTC dynamics *in vivo*	The number of CTCs and early metastases rates decreased significantly after the resection of primary tumor	●○○○ Very Low	[99]
2013	CellSearch	123 HCC patients	Prognostic significance of CTCs in HCC undergoing liver resection	A preoperative EpCAM CTCs ≥ 2/7.5 mL of blood was an independent prognostic factor for tumor recurrence; such prognostic value retained in patient subgroups; a significant decrease of CTC-positive rates and numbers was observed 1 month after resection	●●●○ Moderate	[17]
2013	CellSearch	59 HCC patients	Prognostic significance of CTCs in HCC undergoing liver resection	Presence of EpCAM-positive CTC was associated with intermediate or advanced HCC stage and worse OS	●●○○ Low	[77]
2013	Flow cytometric analysis	60 HCC patients	Prognostic significance of stem cell-like CTCs in HCC undergoing liver resection	Increased numbers of ICAM-1(+) cells in blood samples of HCC patients correlated with worse clinical outcomes	●●○○ Low	[46]
2013	ASGPR sorting	60 HCC patients	Relationship between the EMT status of CTCs and HCC metastasis and prognosis after surgical resection	Twist and Vimentin expression levels in CTCs could serve as promising biomarkers for evaluating metastasis and prognosis in HCC	●●○○ Low	[87]
2014	qRT-PCR-based platform	299 HCC patients	Diagnostic value of CTC; Clinical significance of CTCs in patients treated with surgical resection, TACE and radiotherapy	Negative enrichment and qRT-PCR-based platform can effectively detect CTCs; the platform might be clinically useful in auxiliary diagnosis, treatment response assessment, and early decision making of antitumor strategies for HCC	●●●○ Moderate	[71]
2015	CellSearch	20 HCC patients	Prognostic significance of CTCs and CTC-based DNA signature	CTC detection was associated with elevated AFP, vascular invasion, and poor prognostic factors; sequencing of CTC DNA identified known HCC mutations	●○○○ Very Low	[78]
2015	*In vivo* flow cytometry	HCC orthotopic metastatic tumor model (mouse)	Monitor CTC dynamics following sorafenib treatment *in vivo*	CTCs can be a biomarker in predicting disease progression and monitoring therapeutic efficacy in HCC	●○○○ Very Low	[107]
2016	qRT-PCR-based platform	49 HCC patients	Prognostic significance of CTCs and T regulatory cell in the prediction of postoperative recurrence	Combination of EpCAM CTC and T regulatory/CD4(+) cell may be a novel prognostic predictor for HCC patients	●○○○ Very Low	[83]
2016	CanPatrol platform	33 HCC patients	Diagnostic value of CTCs of EMT phenotypes in HCC; relationship between the EMT process of CTCs and HCC	Epithelial-mesenchymal-mixed CTCs might be a vital factor for intrahepatic metastasis, and mesenchymal CTCs predicated extrahepatic metastasis in HCC	●○○○ Very Low	[73]
2016	qRT-PCR-based platform	72 HCC patients	Dynamic monitoring of CTC counts after surgical resection	Increased AFP mRNA(+) CTCs can be a predictor for HCC metastasis before and after hepatectomy	●●○○ Low	[101]
2016	Density gradient centrifugation	59 HCC patients	Determining pERK and pAkt expressions in CTCs isolated from HCC patients	pERK+/pAkt- CTCs were the most sensitive to sorafenib and an independent predictive factor of PFS in HCC patients treated with sorafenib	●●○○ Low	[109]
2016	Microfluidic device and ASGPR sorting	36 HCC patients	Isolation of viable CTCs of HCC for their culture and drug sensitivity assays	The device can accurately enumerate CTCs and release viable CTCs for in vitro culture and further functional assays	●○○○ Very Low	[110]
2017	CellSearch	61 HCC patients	Prognostic significance of CTCs in HCC undergoing liver resection	Detection of CTC prior to curative-intended liver resection disclosed an elevated risk of HCC recurrence	●●○○ Low	[80]
2017	CanPatrol platform	195 HCC patients	EMT phenotypes of CTCs in the early diagnosis of HCC metastasis and progression after surgical resection	CTCs count and EMT classification are correlated with clinical stages and metastasis of HCC	●●○○ Low	[88]

2018	CellSearch	73 HCC patients	Spatial heterogeneity of phenotypic and molecular characteristics of CTCs	Multi-vascular measurement of CTCs facilitates precise prediction of postoperative relapse or metastasis pattern; profound spatial heterogeneity in cellular distribution and biological features of CTCs during circulation	●●●○ Moderate	[27]
2018	CellSearch	139 HCC patients	Prognostic significance of CTCs and effect of liver resection on CTCs	Both CTC detection incidence and mean CTC counts increased postoperatively; increased postoperative CTC numbers were associated with a worse prognosis	●●○○ Low	[79]
2018	CellSearch	97 HCC patients	Prognostic significance of CTCs in predicting survival outcomes of patients with unresectable HCC treated with TACE	High EpCAM-positive CTC count predicts poor survival of patients with unresectable HCC treated with chemoembolization	●●○○ Low	[81]
2018	qRT-PCR-based platform	445 HCC patients	Clinical value of CTCs with stem-like phenotypes for diagnosis, prognosis, and surveillance in HBV-related HCC	CTC panel may be a useful tool in HCC diagnosis, risk prediction, and treatment response monitoring	●●●○ Moderate	[63]
2018	CanPatrol platform	165 HCC patients	Prognostic significance of EMT phenotypes of CTCs after surgical resection	Presence of mesenchymal CTCs predicted the shortest relapse-free survival	●●●○ Moderate	[89]
2018	CanPatrol platform	80 HCC patients	Relationship between expression of Twist in CTCs and HCC clinical parameters	Twist$^+$ CTCs were closely correlated with the rate of metastasis or recurrence and the mortality rate in HCC	●●○○ Low	[90]
2018	CanPatrol platform	42 HCC patients	Prognostic significance of the change of CTC numbers in tumor recurrence and metastasis after surgical resection	Unfavorable changes after surgery in CTC counts may be independent prognostic indicators for PFS in patients with HBV-related HCC	●●○○ Low	[91]
2018	CanPatrol platform	62 HCC patients	Relationship between postoperative circulating tumor cells subtypes and HCC recurrence	HCC patients with positive postoperative peripheral mesenchymal CTCs had a higher risk of early recurrence	●○○○ Very Low	[93]
2018	CanPatrol platform	112 HCC patients	Prognostic significance of EMT phenotypes of CTCs after surgical resection	CTCs were highly correlated with HCC characteristics, representing a novel marker for early diagnosis and early recurrence prediction	●●●○ Moderate	[94]
2018	CanPatrol platform	47 HCC patients	Prognostic significance of EMT phenotypes of CTCs in HCC patients treated with liver transplantation	CTC levels and subtypes were not predictive of HCC recurrence following liver transplantation	●○○○ Very Low	[96]
2018	iFISH platform	30 HCC patients	Prognostic significance of CTCs in HCC patients treated with liver transplantation	iFISH-CTC $\geq$ 5 may be a good prognostic indicator for patients with HCC undergoing liver transplantation	●○○○ Very Low	[97]
2018	Flow cytometric analysis	43 patients with liver malignant tumor	Effect of percutaneous radiofrequency ablation on CTCs	Liver tumor ablation might increase the level of mesenchymal phenotype CTCs	●○○○ Very Low	[103]
2018	Flow cytometry	HCC orthotopic metastatic tumor model (mouse)	Monitor CTC dynamics following transcatheter arterial embolization	EGFR inhibitor application may reduce circulating cancer cells during transcatheter arterial embolization and improve the therapeutic outcomes for advanced HCC	●○○○ Very Low	[108]
2019	CanPatrol platform	113 HCC patients	Diagnostic value of different EMT phenotypes of CTC in HCC	Total CTCs were more effective than AFP in the diagnosis of HCC; combined use of total CTCs and AFP can enhance the sensitivity of HCC diagnosis	●●○○ Low	[92]
2019	CanPatrol platform	256 HCC patients	Prognostic significance of EMT phenotypes of CTCs after surgical resection	CTC count and EMT classification were not correlated with clinical stages or predictive of HCC recurrence	●○○○ Very Low	[95]
2019	Tapered slit filter platform	105 HCC patients	Monitor CTC before and after surgery and its association with clinical outcomes in early-stage HCC	Count of ΔCTC is predictive of recurrence in patients with early HCC undergoing surgery	●●○○ Low	[100]

2019	FISH analysis	155 HCC patients	Value of serum dickkopf-1 and CTCs in predicting the efficacy and prognosis of TACE treatment in HCC	CTCs levels can predict the efficacy and prognosis of TACE treatment in patients with HCC	●○○○ Very Low	[102]
2020	CanPatrol platform	136 HCC patients	Relationship between CTC status and outcomes of different surgical methods in HCC	Anatomic resection might improve the survival of HCC patients, but only those with low CTC count and negative mesenchymal and epithelial/mesenchymal-CTC phenotypes	●●○○ Low	[98]
2020	PowerMag negative selection system	30 HCC patients	Longitudinal monitoring the treatment response of patients with locally advanced HCC using CTCs	Sequential CTC enumeration during treatment can benefit the management of patients with locally advanced or metastatic HCC, in particular for the AFP-low cases	●○○○ Very Low	[104]

CTC: circulating tumor cells; HCC: hepatocellular carcinoma; EpCAM: epithelial cell adhesion molecule; OS: overall survival; ICAM-1: intercellular cell adhesion molecule-1; EMT: epithelial-to-mesenchymal transition; ASGPR: asialoglycoprotein receptor; TACE: transcatheter arterial chemoembolization; qRT-PCR: quantitative reverse transcription polymerase chain reaction; AFP: alpha-fetoprotein; pERK: phosphorylated ERK; pAkt: phosphorylated Akt; PFS: progression-free survival; HBV: hepatitis B virus; FISH: fluorescence *in situ* hybridization; EGFR: epidermal growth factor receptor

(SII), a novel index based on peripheral lymphocyte, neutrophil, and platelet counts. HCC patients who experienced unfavorable internal inflammatory alterations after radical hepatic resection suffered an earlier recurrence and distant metastasis. Thus, they proposed that the dissemination and colonization of CTCs may be influenced by host inflammatory and immune response status[75,76]. In 2018, Sun *et al.*[27] showed that CTC and circulating tumor cluster burden in hepatic veins and peripheral circulation prognosticated postoperative lung metastasis and intrahepatic recurrence in HCC patients by multi-vascular sites sampling, respectively. This study provided new insight that multi-vascular measurement of CTCs could facilitate precise prediction of postoperative relapse or metastasis patterns in HCC. Other studies using CellSearch[TM] also demonstrated that patients who had EpCAM[+] CTCs were associated with vascular invasion[77,78], significantly elevated AFP[78], more advanced BCLC stage[77], higher recurrence rate[79,80], and shorter OS[77,79]. High EpCAM-positive CTC count also predicted poor survival of patients with unresectable HCC treated with transcatheter arterial chemoembolization (TACE)[81].

PCR-based methods can detect tumor-specific mRNA in blood cells with high sensitivity and specificity. Yao *et al.*[82] reported the combination of positive/negative cell sorting methods and RT-PCR could effectively detect AFP mRNA-positive CTCs in HCC patients. Guo *et al.*[71] established an optimized platform based on negative enrichment and qRT-PCR for the detection of EpCAM mRNA-positive CTCs in HCC. Using their platform, they reported good sensitivity and specificity of PCR-based CTC detection in a cohort of 299 HCC patients. The novel platform exhibited 76.6% consistency with the CellSearch[TM] system while required a reduced blood volume (5 mL). They also reported that low pretreatment CTC levels were significantly correlated to a better prognosis after curative resection, TACE, or radiotherapy for patients with HCC. Using the same platform, Zhou *et al.*[83] discovered that patients with high CTC/Treg levels exhibited higher recurrence rates than those with low CTC/Treg counterparts (66.7% *vs.* 10.3%, $P < 0.001$) by combining the measurement of EpCAM[mRNA+] CTCs and CD4^{+} CD25^{+} Foxp3^{+} Treg cells. Currently, AFP examination remains the most extensively used screening method to indicate early HCC. However, about 30%-40% of HCC patients are AFP negative and the specificity of AFP may be flawed by false-positive results[84,85]. CTC enumeration can be useful in the early detection of HCC since tumor dissemination can occur at the early stage of tumor development[2]. In 2018, the same group developed a qRT-PCR-based multimarker (EpCAM, CD90, CD133, and CK19) diagnostic CTC panel for the identification of CTCs with stem-like phenotypes. They obtained a sensitivity of over 70.0% and specificity of over 90.0% in a well-designed multicenter cohort (n = 1,006). This panel performed equally well in detecting early-stage and AFP-negative HCC as in differentiating HCC from patients with benign liver diseases. The CTC panel outperformed AFP as a biomarker in terms of differential diagnostic capability, yielding higher area under

curve (AUC) value than AFP alone. This study demonstrated the clinical significance of using CTC panel in diagnosis and real-time risk evaluation for HCC[63].

Increasing efforts have been made to investigate the correlation between different molecular phenotypes of CTCs and corresponding clinical outcomes. The stem-like phenotype of CTC has been explored as a strong predictor of the clinical outcome of patients with HCC. For instance, circulating CD45− intercellular cell adhesion molecule-1 (+) (ICAM-1+) cells were regarded as HCC CTCs with stem cell-like properties. Liu *et al.*[46] showed that patients with a higher burden of ICAM-1+ CTCs had significantly shorter disease-free survival and OS. Fan *et al.*[86] also reported that circulating cancer stem cells (CD45− CD90+ CD44+) predicted post-hepatectomy HCC recurrence with high accuracy. Moreover, EMT subtypes of CTCs have been studied for the correlation to clinicopathological features and prognosis of HCC patients. It is reported that a presence or dominance of mesenchymal-like CTCs represented worse clinical outcomes for HCC patients due to earlier tumor relapse and metastasis[73,87]. The majority of these studies used the CanPatrol™ platform for CTC analysis[73,88-94]. In a cohort of 113 HCC patients (65% BCLC 0/A) and 57 non-malignant liver diseases patients, the system presented a higher diagnostic value (AUC = 0.774, 95%CI: 0.704-0.834) of HCC than AFP [0.669 (AUC = 0.669, 95%CI: 0.587-0.750)]. A further combination of CTCs and AFP showed the highest diagnostic capability (AUC = 0.821, 95%CI: 0.756-0.886)[92]. The proportion of mixed EMT status CTCs or mesenchymal CTCs was associated with advanced BCLC stages, higher metastatic tendency, and elevated serum levels of AFP[88,89]. Yin *et al.*[90] reported that twist expression in CTCs could serve as a biomarker for evaluating HCC metastasis and prognosis. Similarly, Wang *et al.*[93] studied 62 HCC patients undergoing surgical resection and found that mesenchymal CTC positivity was an independent risk factor for early recurrence. A similar study using CanPatrol™ platform also found that CTCs undergoing EMT were significantly associated with early recurrence, multi-intrahepatic recurrence, and lung metastasis[94]. However, a recent study reported that CTCs undergoing EMT were poorly correlated with clinical stages or predictive of recurrence of HCC using the platform[95]. Another study using this platform also failed to uncover significant associations between change in total CTCs or CTC subtypes and HCC recurrence in a cohort consisting of 47 patients who underwent liver transplantation[96]. Nevertheless, Xue *et al.*[97] utilized an iFISH® platform to detect CTCs in patients undergoing liver transplantation and found that patients with preoperative iFISH-CTCs ≥ 5 in 7.5 mL of blood had significantly shorter recurrence-free survival than those with lower CTCs. Further large, multicenter studies are still needed to confirm the association between different molecular phenotypes of CTCs and HCC prognosis.

TUMOR MONITORING AND GUIDING PERSONALIZED THERAPEUTIC INTERVENTION IN HCC

Surgical resection remains the most effective therapy for HCC. CTCs could serve as a complementary tool to assess the efficacy of surgical resection and monitor tumor progression[8,14]. Qi *et al.*[98] recently compared the outcomes of patients undergoing anatomical or non-anatomical resection according to the number and EMT phenotype of CTCs. They suggested that anatomic resection may improve the survival of HCC patients, for those with low CTC count, negative epithelial/mesenchymal hybrid CTCs, and mesenchymal CTCs. Thus, CTC analysis before surgery can be used to better guide the resection method for HCC. Meanwhile, the decrease of CTC count after surgical treatment often reflects therapeutic efficacy. Fan *et al.*[99] investigated the effect of liver tumor resection on CTC dynamics using *in vivo* flow cytometry (IVFC) in a green fluorescent protein-transfected HCC orthotopic metastatic mouse model. Their preliminary study found that the number of CTCs and early metastases rates decreased significantly after the resection of the primary tumor. Several clinical studies obtained similar results that CTC load decreased significantly after tumor resection, while increased CTC numbers after surgery were associated with a worse prognosis in patients with HCC[17,63,71,79,100]. Besides, Jin *et al.*[101] explored the clinical value of serial postsurgical observation (at 0, 3, 6, 9, and 12 months) of AFP mRNA level of CTCs in assessing the therapeutic effectiveness of hepatectomy.

In addition, the dynamic change of CTC counts reflected the treatment response in patients treated with locoregional therapies, including TACE, radiotherapy, and radiofrequency ablation[63,71,102,103]. Li *et al.*[103] found that the total number of CTCs and mesenchymal phenotype CTCs significantly increased three days after percutaneous radiofrequency ablation of liver tumor. However, no significant correlation was identified between changes in CTC levels and all the radiofrequency ablation factors. Recently, Rau *et al.*[104] demonstrated the clinical utilities of sequential CTC monitoring in a patient cohort (n = 17) with locally advanced or metastatic HCC accepted systemic/targeted therapy. They found that a change in the CTC count correlated with the patient treatment response in most of the cases and was particularly useful for monitoring patients without elevated serum AFP levels.

Drug therapy is an important component of the comprehensive treatment of HCC. However, only a few patients are sensitive to chemotherapy drugs. Compared with other liquid biopsy biomarkers, CTCs contain more information on the functional characteristics and biological behaviors of tumor[105,106]. Thus, CTCs can be a more relevant biomarker in guiding personalized therapeutic intervention for cancer patients. Yan *et al.*[107] monitored the effect of sorafenib on CTC count in an orthotopic HCC mouse model by IVFC. They showed that the sorafenib treatment could dramatically reduce the number of CTCs, associated with a decreased probability of lung metastasis. Zhu *et al.*[108]. showed that the application of EGFR inhibitor could reduce CTC numbers caused as a side effect of transcatheter arterial embolization. Moreover, Li *et al.*[109] presented a novel system to simultaneously detect the expressions of the phosphorylated extracellular signal-regulated kinase (pERK) and phosphorylated protein kinase B (pAkt) in CTCs. They showed that CTCs can be used in place of tumor tissue for characterization of pERK/pAkt expression, and HCC patients with pERK+/ pAkt- CTCs were more sensitive to sorafenib treatment. Another potential application of CTCs in drug therapy is to test the drug sensitivity by CTC culture. Zhang *et al.*[110] used a microfluidic chip to isolate and release viable CTCs and then performed chemotherapeutic drug assay. The number of spheroids formed by CTCs declined greatly when cultured with sorafenib or oxaliplatin. Furthermore, the novel single-cell sequencing technology makes individual CTC profiling possible, which may provide more valuable drug target information and guide individualized treatment in the future clinical practice[12,111,112].

CONCLUSION

CTC analysis is an exciting field that is gaining increasing attention thanks to the significant technological advancements in CTC isolation and detection. As an important component of "liquid biopsy", CTC analysis enables early cancer detection, prognosis prediction, therapy response monitoring, and novel therapeutic target identification in patients with HCC. However, significant challenges still exist in translating CTC analysis from bench to bedside. Most of the current studies in HCC used different technologies or platforms to detect CTCs in a relatively small, single-centered cohort with widely varying patient demographics, making it difficult to compare studies. Therefore, clinical utilities of CTC should be validated in more multicenter, large, and long-term studies using a standardized CTC assay. Moreover, CTCs hold great promise as a tool to deepen our knowledge of the complicated metastasis process. In recent years, CTCs researches have moved beyond simple CTC enumeration towards more sophisticated molecular analyses. Single-cell sequencing technology may pave the way for using CTCs to understand the underlying mechanisms of cancer metastasis and provide critical insights for new therapeutic strategies. Hopefully, routine CTCs evaluation will become a clinical reality in the near future.

DECLARATIONS

Authors' contributions
Manuscript writing, design, planning: Wang PX, Cheng JW, Yang XR
Manuscript review and editing: Yang XR
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