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Circulating tumor cells: a step toward precision medicine in hepatocellular carcinoma

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Abstract

Serum alpha-fetoprotein and radiologic imaging are the most commonly used tests for early diagnosis and dynamic monitoring of treatment response in hepatocellular carcinoma (HCC). However, the accuracy of these tests is limited, and they may not reflect the underlying biology of the tumor. Thus, developing highly accurate novel HCC biomarkers reflecting tumor biology is a clinically unmet need. Circulating tumor cells (CTCs) have long been proposed as a noninvasive biomarker in clinical oncology. Most CTC assays utilize immunoaffinity-based, size-based, and/or enrichment-free mechanisms followed by immunocytochemical staining to characterize CTCs. The prognostic value of HCC CTC enumeration has been extensively validated. Subsets of CTCs expressing mesenchymal markers are also reported to have clinical significance. In addition, researchers have been devoting their efforts to molecular characterizations of CTCs (e.g., genetics and transcriptomics) as molecular profiling can offer a more accurate readout and

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provide biological insights. As new molecular profiling techniques, such as digital polymerase chain reaction, are developed to detect minimal amounts of DNA/RNA, several research groups have established HCC CTC digital scoring systems to quantify clinically relevant gene panels. Given the versatility of CTCs to provide intact molecular and functional data that reflects the underlying tumor, CTCs have great potential as a noninvasive biomarker in HCC. Large-scale, prospective studies for HCC CTCs with a standardized protocol are necessary for successful clinical translation.

Introduction

Hepatocellular carcinoma (HCC) comprises 75%-85% of primary liver cancer cases[1] and accounts for the fourth most common cancer-related deaths worldwide.[2] In recent decades, advances in systemic therapy with both targeted therapies[3] and immune checkpoint inhibitors[4] have significantly impacted the treatment paradigm and prognosis of patients with advanced HCC. However, biomarkers for predicting or monitoring treatment response are still unavailable. As such, early diagnosis is essential to improve clinical outcomes since early-stage HCC patients can undergo curative-intent surgical or locoregional treatment.[5, 6]

Serum alpha-fetoprotein (AFP) is the most common blood-based biomarker for prognosis and monitoring treatment response in HCC. Combined with serum AFP levels, radiologic studies, including ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI), are the primary strategies for surveillance and monitoring treatment response in the current paradigm.[5] However, serum AFP levels and the imaging studies are limited by their poor sensitivity and high financial cost, respectively. Thus, reliable biomarkers are still an unmet need for early detection and dynamic monitoring of treatment response for HCC.

In the past decades, researchers have extensively developed liquid biopsies such as circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and extracellular vesicles (EVs) as promising and noninvasive biomarkers in clinical oncology.[7] Among these modalities, CTCs represent a sampling of live tumor cells that contain intact molecular or even functional information from primary and metastatic malignancies. CTC enumeration offers a systemic and reproducible assessment of tumor burden. Its prognostic and predictive value has been widely validated in various cancers. However, identifying CTCs in blood samples is technically challenging because of their rarity (around 1-100 CTCs in millions of white blood cells [WBCs]). The CellSearch assay is the only Food and Drug Administration (FDA)-cleared platform in metastatic breast, colorectal, and prostate cancer for CTC enumeration.[8] Although many efforts have been dedicated to developing new CTC technologies and improving CTC enrichment, unfortunately, there is currently no FDA-approved CTC platform for HCC.

Beyond simply enumerating CTCs, researchers have been keen to develop methodologies to allow for downstream characterization of the genetics and transcriptomics of CTCs[9] to further delineate their underlying biology as CTCs play a crucial role in cancer metastases. For example, ALK and ROS1 gene rearrangements were detected in CTCs purified from ALK/ROS1 positive non-small cell lung cancer patients.[10] The androgen receptor splice

variant 7 (AR-V7) RNA expression in prostate cancer CTCs was validated to have values in predicting hormonal therapy response in a multicenter, prospective-blinded clinical trial, and the results were consistent between two CTC platforms of different enrichment mechanisms.[11] Some researchers might argue that a single-gene expression is biased and insufficient for prognostication, so several studies have established CTC-specific gene signatures to provide comprehensive biological insights.[12–15] In this review article, we will briefly introduce the existing CTC technologies and focus on discussing clinically relevant studies with an adequate sample size in HCC.

Overview of CTC assays and their mechanisms

The CTC detection and isolation methodologies can be roughly categorized into immunoaffinity-based, biophysics-based, and enrichment-free methods (Figure 1).[6] Immunoaffinity is the most classic CTC enrichment technique that utilizes antibodies to target proteins with differential expression on different cells. Red blood cells (RBCs) are usually depleted first by either lysis buffer or centrifugation, resulting in remaining plasma and peripheral blood mononuclear cells (PBMCs). Then, a negative or positive enrichment strategy can be applied. For the negative enrichment strategy, most CTC assays deplete cells with CD45 expression, which is a classic WBC marker. Kalinich *et al.* depleted cells expressing three immune cell markers (CD45, CD16, and CD66b) to yield purer CTCs.[12] The major advantage of the negative enrichment method is less manipulation of CTCs, which theoretically gives higher viability, greater recovery rate, and less interference on CTCs. The positive enrichment strategy usually targets epithelial cell adhesion molecule (EpCAM) to capture CTCs of epithelial origin (i.e., carcinoma). However, different carcinomas have differential expressions of epithelial markers (e.g., EpCAM),[16] and loss of epithelial markers typically represents epithelial-mesenchymal transition (EMT), which is a characteristic of tumor aggressiveness and progression.[17] Thus, some CTC assays use combined markers to enrich HCC CTCs.[18] The positive selection would consume much fewer antibodies because of fewer target cells (i.e., CTCs) and yield higher purity than the negative selection. Most immunoaffinity-based platforms to conduct negative/positive enrichment can be subcategorized into magnetic-based and microfluidic-based devices. For example, the only FDA-approved CellSearch assay uses magnetic beads to isolate anti-EpCAM tagged cells.[8] The microfluidic-based devices usually rely on nanosubstrates characterized by highly increased contact area.[16] Dong *et al.* had a comprehensive review on currently existing nanosubstrates for the enrichment of circulating rare cells.[19] Of note, the CTC-iChip reported by Kalinich *et al.* combined magnetics and microfluidics to enrich CTCs.[12]

Biophysics-based assays utilize differential biophysical properties, e.g., size and density, to isolate CTCs from background WBCs. CTCs are usually larger and more rigid than WBCs, so microfiltration can be rationally applied to enrich CTCs. For example, many research groups used the CanPatrol assay[20] to isolate HCC CTCs. Other less common biophysics-based platforms include employing differential inertial focusing effect[21] and dielectrophoresis effect[22] to enrich CTCs. The isolated cells are generally subject to immunocytochemical (ICC) staining of cytokeratin (CK) and CD45 and nuclear staining (DAPI or Hoest) to avoid false positivity. CTCs are identified as nuclear stain+/CK+/

CD45−. Finally, enrichment-free platforms such as flow cytometry ensure minimal manipulation of cells to identify CTCs quickly. In 2013, Liu *et al.* used flow cytometry to identify CD45-/ICAM-1+ cells, of which high frequency was significantly associated with worse survival and aggressive clinical behaviors (Table 1).[23] Other researchers have proposed that counting higher nucleus to cytoplasm (N/C) ratio cells was better than staining CD45 and epithelial markers.[24] However, the purity by enrichment-free platforms is much less than that by immunoaffinity- or biophysics-based platforms. For example, CTC size could be diminished after neuroendocrine differentiation and EMT.[25, 26]

CTC enumeration by protein expression or fluorescence *in situ* hybridization (FISH)

Enumeration of CTCs can assess the systemic tumor burden in cancer patients. Representative HCC CTC enumeration studies with clinical applications and significance are summarized in Table 1. Many research groups have broadly applied magnetic separation to enumerate HCC CTCs. For example, Xu *et al.* exploited the AutoMACS Pro Separator and found CTC presence associated with aggressive clinical features.[27] Li *et al.* reported that CTCs with twist and vimentin expression (both are mesenchymal markers) enriched by the MiniMACS Separator were associated with poor clinical behaviors.[28] The clinical significance of subsets of CTCs beyond total CTCs has been reported more recently. In 2016, Li *et al.* demonstrated that pERK+/pAkt− CTCs were associated with worse progression-free survival and the total CTC count in patients with pERK+/pAkt- CTCs decreased drastically after sorafenib treatment.[29]

Multiple studies leveraged the CellSearch system in HCC CTC studies. Remarkably, Sun *et al.* included a prospective cohort of 123 HCC patients undergoing curative resection and found the CTC count was an independent prognostic factor.[30] Additional studies using the CellSearch assay also reported similar results.[31–37] Among them, Morris *et al.* demonstrated that the ISET (isolation by size of epithelial tumor cells) platform had a CTC detection rate of 100%, while the CellSearch had that of 28%.[32] Sun *et al.* collected blood samples from various sites, including peripheral vein, peripheral artery, hepatic vein, infrahepatic inferior vena cava, and portal vein. They demonstrated the spatial heterogeneity of CTCs.[34]

As mentioned in the study by Morris *et al.*,[32] the HCC CTC detection rate of the CellSearch assay was relatively low, and researchers criticized that the EpCAM-based CellSearch assay was unsuitable for the HCC CTC studies because HCC cells often undergo EMT.[18] Thus, microfluidic devices were developed to achieve high CTC capture efficiency because of their maximized contact area.[19] Zhang *et al.* used the CTC-chip coated with anti-ASGPR to capture CTCs, with reported 100% of CTC detection rates and a mean of 14 CTCs/2mL blood.[38] Court *et al.* utilized the nanosilicon-structured NanoVelcro chip with combined capture antibodies targeting EpCAM, ASGPR, and GPC3. They found that vimentin positive CTCs were associated with poorer survival and were more frequently observed in advanced HCC.[18] Winograd *et al.* also showed that PD-L1 positive CTCs captured by the NanoVelcro chip were significantly more frequent in advanced HCC. The PD-L1 positive CTCs might show values in predicting treatment response to immuno-oncologics.[39]

Sized-based devices were used to isolate CTCs from background WBCs in earlier studies. In 2004, Vona *et al.* used the ISET platform to capture HCC CTCs by microfiltration. CTCs identified by their larger size, N/C ratio, and nuclear/chromatin shape were associated with overall survival, tumor diffusion, and portal tumor thrombosis.[40] Wu *et al.* optimized the size-based CanPatrol assay to enrich CTCs combined with downstream RNA *in situ* hybridization (RNA-ISH) to identify epithelial markers (EpCAM and CK), mesenchymal markers (vimentin and twist), and a WBC marker (CD45). CTCs were categorized into epithelial, mixed (or hybrid), and mesenchymal CTCs.[20] Using the CanPatrol assay, many studies showed that total CTCs and mesenchymal CTCs were associated with aggressive clinical features and survival,[41–47] though Chen *et al.* did not have a consistent result in 2019.[48] In addition, Lei *et al.* identified nanog expression of CTCs by the CanPatrol assay and revealed that nanog positive CTCs were associated with postoperative recurrence.[49]

CTC molecular information

The promotion and clinical translation of existing CTC enumeration assays have been challenging because of technical limitations, including the complicated CTC capture, imaging, and counting process and the lack of throughput. Furthermore, CTC enumeration provides limited insights into tumor biology. Therefore, researchers have been moving efforts toward molecular characterizations of CTCs.[13] Because of existing background WBCs, a principle of detecting CTC expression of target genes is to select genes with high expression in tumors and minimal expression in WBCs. The representative studies investigating HCC CTC molecular information are summarized in Table 2. In 2001, Wong *et al.* used Ficoll-Paque for centrifugation and isolation of all PBMCs. The resulting PBMCs were subject to albumin and AFP RNA quantification by reverse transcription-polymerase chain reaction (RT-PCR). The AFP RNA expression and serum AFP levels could predict HCC metastasis and recurrence.[58] Although the AFP RNA expression of a single CTC is much higher than that of a WBC, the amount of WBCs is significantly greater than CTCs, so it is not suitable to neglect background AFP noise directly without enrichment of CTCs.

Guo *et al.* demonstrated that the EpCAM RNA expression of CTCs enriched by MACS was associated with prognosis and reflective of treatment response.[59] Zhou *et al.* showed that patients with higher CTC EpCAM RNA expression had significantly greater postoperative HCC recurrence rates.[60] Basically, the quantification of EpCAM expression is equivalent to quantifying EpCAM+ cells. Shi *et al.* included three markers (i.e., MAGE-3, survivin, and CEA) with biological implications and found that their expression in CTCs was decreased after cryosurgery.[53] Guo *et al.* developed a four-gene CTC panel consisting of EpCAM, CD133, CD90, and CK19. Higher expression of the 4-gene panel was associated with earlier recurrence.[61]

In 2017, Kalinich *et al.* published an HCC CTC digital scoring system that integrated CTC-iChip for CTC enrichment and downstream droplet digital PCR (dPCR) for highly sensitive RNA quantification. The introduction of the droplet dPCR specifically addressed the challenge of quantifying small amounts of DNA/RNA (as sensitive as one copy per cell) in the high background by using water-oil emulsion technology. They used dPCR to quantify the RNA expression of a 10-gene panel comprising AFP, AHSG, ALB, APOH, FABP1,

FGB, FGG, GPC3, RBP4, and TF. The 10-gene expression of CTCs had values in screening HCC in high-risk cirrhotic patients.[12] Sun *et al.* translated the same 10-gene panel into an HCC EV purification system and found that the 10-gene expression of HCC EVs was also valuable for early detecting HCC in at-risk cirrhotic patients.[62]

The NanoVelcro assay[10, 63], another microfluidic-based device, was also used to obtain molecular messages from HCC CTCs. Court *et al.* incorporated laser microdissection technology to isolate single CTCs for profiling somatic copy number alterations (SCNAs). There was an 80% concordance of SCNA profiling between HCC CTCs and primary tumors.[64] Sun *et al.* displayed that the CTCs purified by the latest NanoVelcro assay, i.e., Click Chip, possessed highly concordant expression of 64 HCC tissue-derived genes from The Cancer Genome Atlas (TCGA).[14] Lee *et al.* established a 10-gene HCC CTC Risk Score panel (DDR1, EHHADH, AR, LUM, HSD17B6, PMEPA1, TSKU, NECAB2, LAD1, and SLC27A5) quantified by the NanoString nCounter platform[65] and the risk score was significantly associated with overall survival.[15]

Besides serving as a biomarker assay, many researchers also utilized CTC platforms to explore cancer biology. For example, Sun *et al.* conducted single-cell RNA sequencing of HCC CTCs collected from various human body sites. The results explored the immune invasion of CTCs and the role of EMT in the hematogenous dissemination of CTCs.[34, 66] Hu *et al.* applied 3-dimensional culture of collected CTCs from HCC patients to investigate the biological behavior of early recurrence and metastasis.[67]

Conclusion

The prognostic value of HCC CTC enumeration has been extensively validated in multiple studies across different CTC platforms. However, accessing molecular information in a noninvasive manner was eagerly needed. Since Kalinich *et al.* introduced digital reports combining multiple genes,[12] the digital scoring of a multiple-gene panel offers a more objective and reproducible means with more comprehensive biological understandings for clinical applications of liquid biopsies. Nevertheless, there remain challenges to the widespread use of HCC CTC platforms in clinical settings. In addition, large-scale prospective studies of HCC CTC platforms are still lacking. As new therapies have been rapidly developed in clinical oncology, biomarkers for the selection of treatment and the prediction of response are becoming more urgent. CTCs hold great potential to serve this purpose since CTCs contain intact molecular and even functional information despite the shortcomings mentioned above. Therefore, a standardized protocol for establishing CTC assays is necessary to promote their clinical applications in advance.

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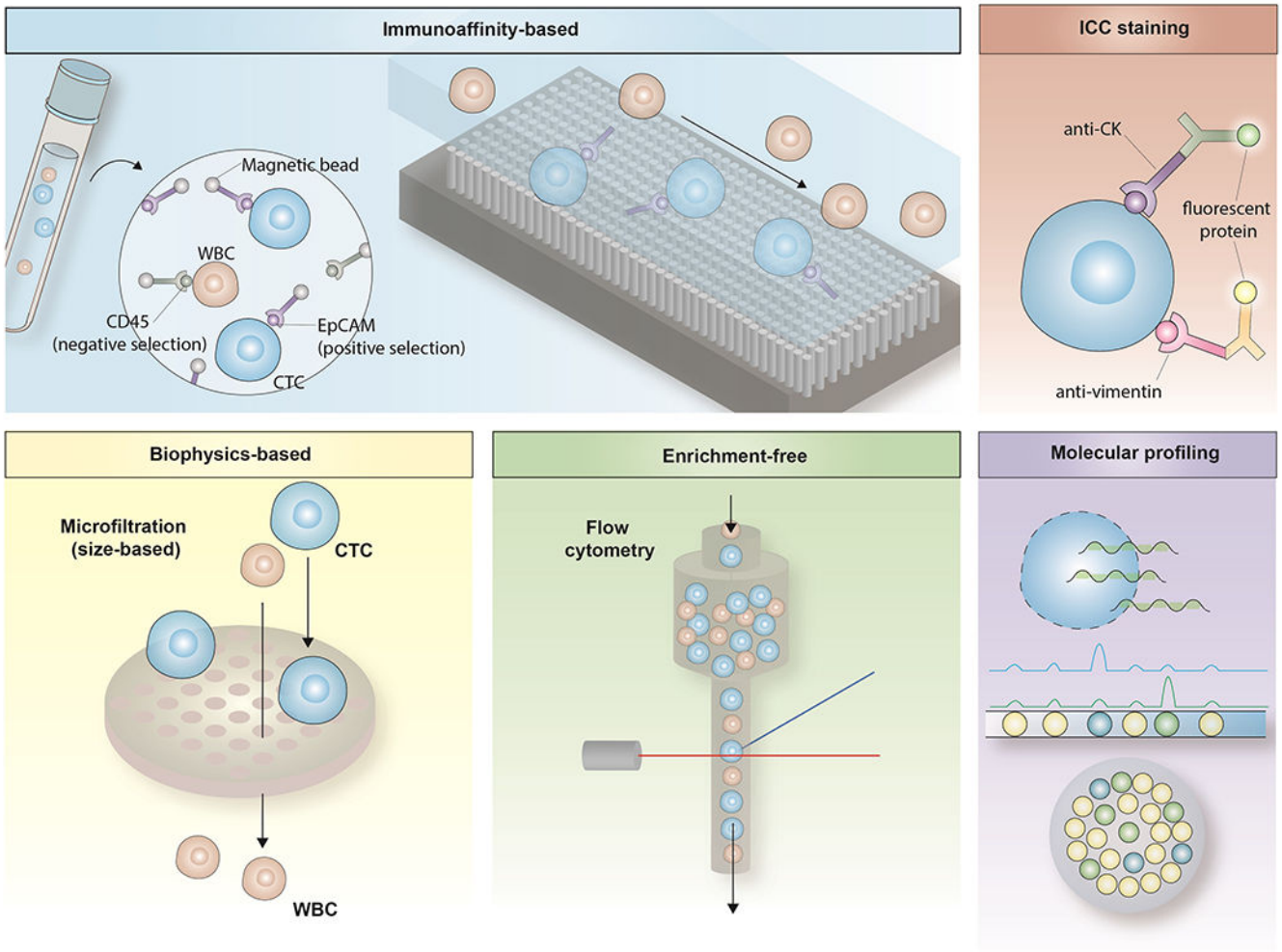

Figure 1. CTC isolation methods and downstream characterizations.

Most CTC assays can be categorized into immunoaffinity-based, biophysics-based (e.g., microfiltration), and enrichment-free methods. The immunoaffinity-based platform is the most common and can be subcategorized into magnetic-based and microfluidic-based (e.g., nanosubstrates in the figure) devices. Besides, a negative selection strategy (depleting WBCs) or a positive selection strategy (capturing CTCs) is conducted for enriching CTCs. The resulting CTCs are generally subject to immunocytochemical (ICC) staining or molecular profiling (e.g., droplet digital PCR in the figure).

Table 1.

CTC enumeration for clinical outcomes in HCC.

Study	Patients	CTC assay	Enrichment method	CTC identification	Main results
Vona *et al.*,[40] 2004	44 HCC, 30 CH, 39 cirrhosis, 38 HVs	ISET	Filtration	Diameter > 25 μm, high/NC ratio, irregular chromatin or nuclear shape	23 of 44 (52%) liver cancer patients had detectable. CTC(+) patients had more tumor portal tumor thrombosis (P= .006). The CTC positivity (P= .01) and count (P= .02), and circulating tumor microemboli were significantly associated with overall survival.
Xu *et al.*,[27] 2011	85 HCC	AutoMACS Pro Separator	Magnetic separation (anti-asialofetuin)	DAPI(+), HepPar-1(+), CD45(−), morphology of malignant cells	69 of 86 (81%) HCC patients had CTCs. The presence and number of CTCs were significantly associated with tumor size, portal vein thrombosis, Edmondson–Steiner grade, TNM stages, and Milan criteria.
Li *et al.*,[28] 2013	60 HCC	MiniMACS Separator	Magnetic separation (anti-ASGPR)	DAPI(+), hepatocyte-specific antigen(+), CD45(−)	46 of 60 (77%) HCC patients had detectable CTCs. 39 (85%) and 37 (80%) of the 46 patients had twist(+) and vimentin(+) CTCs, which were significantly associated with portal vein tumor thrombus. Coexpression of twist and vimentin was found in 32 of the 46 (70%) patients and was associated with TNM stages and tumor size.
Liu *et al.*,[23] 2013	60 HCC	Flow cytometry	No enrichment	The median frequency of CD45(−) ICAM-1(+) cells (0.157%) was used as a cutoff.	High CD45(−) ICAM-1(+) cell frequency patients had worse disease-free survival (P< .0001) and overall survival (P= .013).
Sun *et al.*,[30] 2013	123 HCC undergoing curative resection	CellSearch	Magnetic separation (anti-EpCAM)	CK(+), DAPI(+), CD45(−)	CTCs were detected in 67% of patients. 51 patients had ≥ 2 CTCs/7.5mL blood preoperatively and had earlier tumor recurrence than those with < 2 CTCs (P< .0001). Decrease of CTC(+) rates (67% to 28%, P< .05) and CTC counts (2.60 ± 0.43 to 1.00 ± 0.36, P< .05) were found 1 month postoperatively.
Schulze *et al.*,[31] 2013	59 HCC, 19 non-HCC controls	CellSearch	Magnetic separation (anti-EpCAM)	EpCAM(+), DAPI(+), CD45(−)	18 of 59 (31%) HCC patients and 1 of 19 (5%) non-HCC controls had detectable CTCs. CTC(+) patients had worse overall survival compared to CTC(−) patients (460 vs. 790 days, P= .017). CTC status was also associated with BCLC stages, vascular invasion, and AFP levels.
Morris et al.,[32] 2014	54 HCC	CellSearch (50 patients) and ISET (19 patients)	Magnetic separation (CellSearch) and filtration (ISET)	As CellSearch and ISET above	14 of 50 (28%) patients had detectable CTCs by CellSearch and 19 of 19 (100%) by ISET. GPC3(+) CTCs by ISET were 100% concordant with GPC3(+) in the original tumor (N=5). CTC numbers had a positive trend with Child-Pugh scores and TNM stages but did not reach clinical significance.
Li *et al.*,[50] 2014	27 HCC, 61 others (see main results)	AutoMACS Pro Separator	Magnetic separation (anti-ASGPR)	DAPI(+), CK(+), CPS1(+)	89% of HCC patients had detectable CTCs and other patients (12 other cancers, 13 cirrhosis, 5 HBV, 3 acute HAV, 2 HCV, 11 benign liver tumors, 15 HVs) did not have detectable CTCs.
Liu *et al.*,[51] 2015	32 HCC, 77 others (see main results)	Ficoll-Paque	Magnetic separation (excludes CD45+ cells)	DAPI(+), CD45(−), ASGPR(+), CPS1(+)	29 of 32 (91%) HCC patients had detectable CTCs and others (17 other cancers, 12 benign liver tumors, 3 acute HAV, 6 HBV, 4 HCV, 15 cirrhosis, and 20 HVs) did not.
Zhang *et al.*,[38] 2016	36 advanced HCC, 14 non-HCC	CTC-chip	Microfluidic chip (anti-ASGPR)	DAPI(+), CD45(−), CK/CPS1(+)	All the HCC patients had detectable CTCs with an average of 14 ± 10 per 2mL whole blood. Captured CTCs could be released in a 3D cell culture assay for drug sensitivity tests.
Ogle *et al.*,[52] 2016	69 HCC, 31 non-HCC controls	BigEasy magnet	Magnetic separation	DAPI(+), CD45(−), morphology, size, antigen expression	45 of 69 (65%) HCC patients had detectable CTCs. CTCs positive for CK and EpCAM were in 29% and 18% of patients, respectively. The CTC count was

Study	Patients	CTC assay	Enrichment method	CTC identification	Main results
			(excludes CD45+ cells)	(EpCAM, CK, AFP, GPC3, DNA-PK)	associated with tumor size and portal vein thrombosis. Patients with ≥ 2 CTCs had poorer overall survival than those with < 2 CTCs (7.5 vs. > 34 months, P< .001).
Shi *et al.*,[53] 2016	47 HCC undergoing cryosurgery	MACS	Magnetic separation (anti-EpCAM)	DAPI(+), CD45(−), EpCAM(+), CK(+)	CTC count was assessed on preoperative day 1, postoperative day 7, and postoperative day 30. The number was 17.70 ± 5.725, 14.64 ± 6.761, and 10.28±5.598, respectively. The CTC number decreased over time.
Li *et al.*,[29] 2016	109 HCC (including 59 patients receiving sorafenib)	Ficoll-Paque	Magnetic separation (excludes CD45+ cells)	DAPI(+), CK(+), pERK(+/−), pAkt(+/−)	90% of patients had a concordant phenotypic classification of tissues with that of CTCs. CTC count declined sharply in patients with pERK(+)/pAkt(−) CTCs after 2 weeks of sorafenib treatment (P< .01). Patients with pERK(+)/pAkt(−) CTCs had worse progression-free survival (HR= 9.39, P< .01).
Liu *et al.*,[24] 2016	52 HCC, 5 CCA, 12 liver diseases, 12 HVs	Flow cytometry	No enrichment	DAPI(+), N/C ratio, CD45, EpCAM	56.2 ± 23.8 high N/C ratio cells/100,000 cells were detected in cancer patients, while non-cancer patients had 7.6 ± 2.2/100,000. Patients with microvascular invasion had more high N/C ratio cells than those without.
Wang *et al.*,[54] 2016	42 HCC	CTC-^{BioT}Chip	HA/CTS nanofilm coating aptamer for carbohydrate sialyl Lewis X	DAPI(+), CK(+), CD45(−)	25 of 42 (60%) HCC patients had detectable CTCs with an average of 2 ± 2 CTCs/2mL whole blood. The CTC positive rate and the CTC count were associated with tumor size, portal vein tumor thrombus, and TNM stages.
Chen *et al.*,[41] 2017	195 HCC	CanPatrol	Filtration	mRNA *in situ* hybridization (EpCAM, CK8/18/19, vimentin, twist, CD45)	95% of patients had detectable CTCs. The CTC count was significantly associated with BCLC stages, metastasis, and serum AFP levels. The fraction of hybrid and mesenchymal CTCs was associated with ages, BCLC stages, metastasis, and AFP levels. Recurrent HCC patients also had more total CTCs, hybrid CTCs, and mesenchymal CTCs.
von Felden *et al.*,[33] 2017	61 HCC underdoing resection	CellSearch	Magnetic separation (anti-EpCAM)	DAPI(+), CK(+), CD45(−)	CTC(+) patients had significantly higher HCC recurrence rates (HR= 2.3, P= .027) and worse recurrence-free survival (5 vs. 12 months, P= .039). The predictive power of the CTC positivity was independent of complete resection.
Sun et al.,[34] 2018	73 localized HCC undergoing resection	CellSearch	Magnetic separation (anti-EpCAM)	DAPI(+), CK(+), CD45(−)	The CTC positivity was highest in hepatic vein and lowest in infrahepatic inferior vena cava. CTCs and circulating tumor microemboli in hepatic veins and peripheral circulation were associated with postoperative metastasis and intrahepatic recurrence, respectively.
Yin *et al.*,[42] 2018	80 HCC undergoing resection or TACE, 10 HVs	CanPatrol	Filtration	mRNA *in situ* hybridization (EpCAM, CK8/18/19, twist, CD45)	54 of 80 (68%) patients had twist(+) CTCs. The twist positive rates were associated with portal vein tumor thrombus, TNM stages, serum AFP levels, cirrhosis, tumor number, tumor size, and microvascular invasion. CTC positive rates of twist were associated with metastasis, recurrence, and mortality rates.
Yu *et al.*,[35] 2018	139 HCC, 23 benign liver tumors	CellSearch	Magnetic separation (anti-EpCAM)	DAPI(+), CK(+), CD45(−)	Blood was drawn on preoperative day 1 and postoperative day 3. Patients with elevated postoperative CTC counts (from < 2 to ≥ 2) had worse disease-free survival (P< .0001) and overall survival (P= .0005) compared to patients with persistent CTC < 2.
Qi *et al.*,[45] 2018	112 HCC undergoing R0 resection, 12 HBV, 20 HVs	CanPatrol	Filtration	mRNA *in situ* hybridization (EpCAM, CK8/18/19, vimentin, twist, CD45)	101 of 112 (90%) HCC patients had detectable CTCs. 2 of 12 HBV patients were CTC(+) and later were found to have small HCCs within 5 months. Preoperative CTC ≥ 16 and mesenchymal CTC ratio ≥ 2% were associated with earlier recurrence, multi-intrahepatic recurrence, and lung metastasis.
Ye *et al.*,[43] 2018	42 HBV-related HCC	CanPatrol	Filtration	p21, NM23, CD34, TP53, VEGF	The preoperative CTC count was significantly associated with Edmondson stages. The postoperative CTC count and pre/postoperative CTC number change were associated with progression-free survival.

Study	Patients	CTC assay	Enrichment method	CTC identification	Main results
Ou *et al.*,[46] 2018	165 HCC undergoing radical resection	CanPatrol	Filtration	mRNA *in situ* hybridization (EpCAM, CK8/18/19, vimentin, twist, CD45)	CTC(+) was defined as ≥ 2 CTCs/5mL whole blood. 71% of the 165 HCC patients were CTC(+). Total CTC count and mesenchymal CTCs were associated with serum AFP levels, tumor number, advanced TNM and BCLC stages, and embolus/microembolus.
Wang *et al.*,[44] 2018	62 postoperative HCC	CanPatrol	Filtration	mRNA *in situ* hybridization (EpCAM, CK8/18/19, vimentin, twist, CD45)	Total CTC count and mesenchymal CTCs were significantly more in the recurrence group than in the non-recurrence group. Mesenchymal CTC was an independent risk factor for early recurrence (HR= 3.45, P= .007).
Shen *et al.*,[36] 2018	97 unresectable HCC undergoing TACE	CellSearch	Magnetic separation (anti-EpCAM)	DAPI(+), CK(+), CD45(−)	Patients were categorized into the low (≤ 1 CTC), moderate (2-5 CTCs), and high (≥ 6 CTCs) CTC groups with clinical significance. In a multivariate Cox regression model, the CTC count was associated with overall survival (P= .049) and progression-free survival (P= .007).
Court *et al.*,[18] 2018	61 HCC, 11 nonmalignant liver diseases, 8 HVs	NanoVelcro	Microfluidic chip (anti-EpCAM, anti-ASGPR, anti-GPC3)	DAPI(+), CK(+), CD45(−), vimentin(+/−)	59 of 61 (97%) HCC patients had detectable CTCs. Vimentin(+) CTCs could differentiate early-stage HCC from locally advanced or metastatic HCC with area under curve of 0.89. Higher vimentin(+) CTC count was associated with poorer overall survival (HR= 2.21, P= .001) and postoperative HCC recurrence (HR= 3.14, P= .002).
Chen *et al.*,[48] 2019	143 HCC	CanPatrol	Filtration	mRNA *in situ* hybridization (EpCAM, CK8/18/19, vimentin, twist, CD45)	The total CTC count and EMT classification were not associated with clinical stages or HCC recurrence.
Wan *et al.*,[55] 2019	54 HCC	Labyrinth chip	Microfluidic device (isolates CTCs by size)	DAPI(+), CD45(−), HCC markers including GPC3, glutamine synthetase, HepPar-1, and CD44	88.1% of the patients had detectable CTCs. The CTC positive rate was associated with HCC stages. 71.4% of the patients showed CTCs positive for CD44, a cancer stem cell marker.
Cheng *et al.*,[47] 2019	113 HCC, 57 non-HCC liver diseases	CanPatrol	Filtration	mRNA *in situ* hybridization (EpCAM, CK8/18/19, vimentin, twist, CD45)	Combining the total CTC count and serum AFP, the area under the curve of differentiating HCC from non-HCC patients was 0.82. Late-stage HCC patients had more mesenchymal CTCs.
Zhou *et al.*,[37] 2020	309 HCC undergoing resection	CellSearch	Magnetic separation (anti-EpCAM)	DAPI(+), CK(+), CD45(−)	The CTC count was positively associated with microvascular invasion (mVI) counts (r= .655, P< .001) and the farthest mVI from the tumor (FMT) (r= .495, P< .001). CTC(+) patients had higher mVI counts (P= .032) and FMT (P= .008).
Winograd *et al.*,[39] 2020	87 HCC, 7 cirrhosis, 8 HVs	NanoVelcro	Microfluidic chip (anti-EpCAM, anti-ASGPR, anti-GPC3)	DAPI(+), CK(+), CD45(−), PD-L1(+/−)	PD-L1(+) CTCs were found in 4 of 49 (8%) early-stage, 12 of 22 (55%) locally advanced, and 15 of 16 (94%) metastatic patients. Patients with PD-L1(+) CTCs had poorer overall survival (HR= 4.0, P= .001). In 10 HCC patients receiving immune checkpoint inhibitors, all 5 responders had baseline PD-L1(+) CTCs, while 1 of 5 nonresponders had PD-L1(+) CTCs.
Rau *et al.*,[56] 2020	30 HCC	PowerMag	Magnetic separation (excludes CD45+ cells)	Hoest(+), EpCAM(+)	Patients with progressive disease had more CTCs than patients with partial response or stable disease (P= .0002). In addition, a longitudinal analysis of 17 patients showed that changes in the CTC count were associated with treatment response even in patients without elevated serum AFP levels.
Amado *et al.*,[57] 2021	41 HCC undergoing resection or transplant	IsoFlux Liquid Biopsy System	Magnetic separation (anti-EpCAM)	Hoest(+), CK(+), CD45(−)	CTC clearance within the first month was more in the liver transplant group. Baseline CTC clusters were associated with incomplete CTC clearance (54.2% vs. 11.8%, P= .005), which correlated with worse overall survival (P= .038).

Study	Patients	CTC assay	Enrichment method	CTC identification	Main results
Lei *et al.*,[49] 2021	160 HCC undergoing R0 resection	CanPatrol	Filtration	mRNA *in situ* hybridization (EpCAM, CK8/18/19, vimentin, twist, CD45, nanog)	EpCAM(+) and nanog(+) CTCs were strongly associated with postoperative recurrence. Most epithelial CTCs did not express nanog, while most mixed and mesenchymal CTCs expressed nanog (positive rate: 38.7%, 66.7%, and 88.7%, respectively).

Abbreviations: ASGPR, asialoglycoprotein receptor; BCLC, Barcelona Clinic Liver Cancer; CH, chronic hepatitis; CK, cytokeratin; CPS1, carbamoyl phosphate synthetase 1; CTC, circulating tumor cell; DNA-PK, DNA-dependent kinase; EpCAM, epithelial cell adhesion molecule; FISH, fluorescence *in situ* hybridization; GPC3, glypican 3; HAV, hepatitis A virus; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HV, health volunteer; ISET, isolation by size of epithelial tumor cells; MACS, magnetic-activated cell sorting; N/C ratio, nucleus to cytoplasm ratio; TACE, transarterial chemoembolization; TNM, tumor-node-metastasis.

Table 2.

CTC molecular information for clinical outcomes in HCC.

Study	Patients	CTC assay	Enrichment method	CTC marker	Main results
Wong et al.,[58] 2001	84 HCC under radiotherapy, resection, or chemotherapy; 53 healthy or non-HCC controls	Ficoll-Paque	Centrifugation (isolates all PBMCs)	Albumin and AFP mRNA expression by RT-PCR	Compared to the upper limit of 53 healthy/non-HCC controls, albumin RNA expression increased 2-10 folds in 6% pretreatment and 2-2.6×10^4 folds in 32% posttreatment patients. AFP RNA expression increased 3-210 folds in 17% of pretreatment and 4-5×10^5 folds in 30% of posttreatment patients. Combined with serum AFP test, AFP mRNA quantification could predict metastasis/ recurrence in 56% of patients.
Guo *et al.*,[59] 2014	299 HCC (157 curative resection, 76 TACE, 66 radiotherapy)	MACS	Magnetic separation (excludes CD45+ cells)	EpCAM mRNA expression by RT-PCR	Pretreatment CTC EpCAM RNA expression was associated with prognosis (P< .05). Most patients had decreased posttreatment EpCAM RNA levels, reflecting treatment response.
Zhou *et al.*,[60] 2016	49 HCC undergoing curative resection, 50 HVs	RosetteSep	Centrifugation (excludes CD45+ cells)	EpCAM mRNA expression by RT-PCR	Patients with CTC EpCAM RNA expression of ≥ 2.22 had significantly higher postoperative HCC recurrence (HR=6.67, P= .003).
Shi *et al.*,[53] 2016	47 unresectable HCC undergoing cryosurgery	MACS	Magnetic separation (anti-EpCAM)	MAGE-3, survivin, CEA expression by RT-PCR	The expression of MAGE-3, survivin, and CEA (carcinoembryonic antigen) on postoperative day 30 was significantly decreased compared to postoperative day 7 (P< .01).
Kalinich et al.,[12] 2017	63 HCC, 31 chronic liver diseases, 6 liver metastasis, 38 other cancers, 26 HVs	CTC-iChip	Microfluidic chip and magnetic separation (excludes CD45+, CD16+, and CD66b+ cells)	10-gene panel by RT digital PCR (AFP, AHSG, ALB, APOH, FABP1, FGB, FGG, GPC3, RBP4, TF)	Using the 10 liver-specific transcripts, HCC-derived CTCs were present in 9 of 16 (56%) untreated HCC patients vs. 1 of 31 (3%) with non-HCC liver diseases (P< .0001). The positive CTC scores decreased in treated patients. Combining the digital scoring and serum AFP, the positive and negative predictive values for HCC screening in high-risk cirrhosis patients were 80% and 86%, respectively.
Sun et al.,[34] 2018	73 localized HCC undergoing resection	CellSearch	Magnetic separation (anti-EpCAM)	Single-cell RNA sequencing	Single-cell RNA sequencing demonstrated that CTCs were most epithelial at release and turned to EMT-activated along with the hematogenous dissemination via Smad2 and β-catenin associated pathways.
Guo *et al.*,[61] 2018	445 HCC, 201 HBV/ cirrhosis, 100 benign liver lesions, 260HVs	RosetteSep	Centrifugation (excludes CD45+ cells)	EpCAM, CD133, CD90, and CK19 mRNA expression by RT-PCR	The area under the curve of the 4-gene CTC panel was 0.93 in the validation set, which performed well to identify early-stage and AFP-negative HCC and differentiate HCC from other non-HCC liver diseases. Higher expression was associated with earlier tumor recurrence (HR= 3.13, P= .007 in the validation set).
Court *et al.*,[64] 2020	10 HCC undergoing resection	NanoVelcro	Microfluidic chip (anti-EpCAM, anti-ASGPR, anti-GPC3)	Somatic copy number alterations (SCNAs)	18 CTC samples from 10 HCC patients using a whole-genome sequencing strategy with a median of 0.88 million reads/sample demonstrated frequent SCNAs reported previously, such as 8q amplification and 17p deletion. SCNAs of CTCs showed a median of 80% concordance with those of primary tumors.
Sun *et al.*,[14] 2021	20 HCC	NanoVelcro Click Chip	Microfluidic chip (anti-EpCAM, anti-ASGPR, anti-GPC3)	64 HCC-specific genes quantified by the NanoString nCounter platform	The expression of HCC-specific genes from purified CTCs demonstrated high concordance with HCC tissue-derived gene signatures from The Cancer Genome Atlas (TCGA) database.
Lee *et al.*,[15] 2022	40 HCC	NanoVelcro	Microfluidic chip (anti-EpCAM, anti-ASGPR, anti-GPC3)	10-gene HCC-CTC Risk Score panel by NanoString nCounter platform	The HCC-CTC Risk Score panel included 10 prognostic genes (DDR1, EHHADH, AR, LUM, HSD17B6, PMEPA1, TSKU, NECAB2, LAD1, and SLC27A5). Higher risk scores were significantly associated with poorer overall

Study	Patients	CTC assay	Enrichment method	CTC marker	Main results
					survival in the TCGA HCC cohort (HR= 2.0, P< .001) and an independent cohort (N=40, HR= 5.7, P= .009).

Abbreviations: AFP, alpha-fetoprotein; CTC, circulating tumor cell; EMT, epithelial-mesenchymal transition; EpCAM, epithelial cell adhesion molecule; HCC, hepatocellular carcinoma; HR, hazard ratio; HV, healthy volunteer; N/C ratio, nucleus to cytoplasm ratio; PMBC, peripheral blood mononuclear cells; RT-PCR, reverse transcription-polymerase chain reaction; TACE, transarterial chemoembolization.