



Current biological implications and clinical relevance of metastatic circulating tumor cells

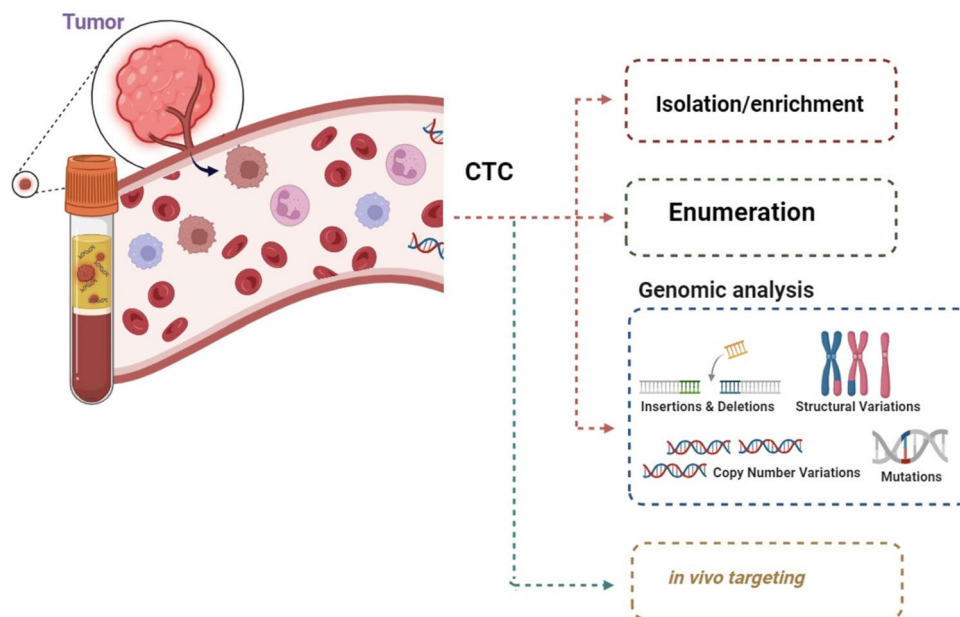
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Abstract

Metastatic disease and cancer recurrence are the primary causes of cancer-related deaths. Circulating tumor cells (CTCs) and disseminated tumor cells (DTCs) are the driving forces behind the spread of cancer cells. The emergence and development of liquid biopsy using rare CTCs as a minimally invasive strategy for early-stage tumor detection and improved tumor management is a promising advancement in recent years. However, before blood sample analysis and clinical translation, precise isolation of CTCs from patients' blood based on their biophysical properties, followed by molecular identification of CTCs using single-cell multi-omics technologies is necessary to understand tumor heterogeneity and provide effective diagnosis and monitoring of cancer progression. Additionally, understanding the origin, morphological variation, and interaction between CTCs and the primary and metastatic tumor niche, as well as and regulatory immune cells, will offer new insights into the development of CTC-based advanced tumor targeting in the future clinical trials.

Graphical Abstract



Keywords Cancer metastasis · Circulating tumor cells · Disseminated tumor cells · Liquid biopsy · Targeted therapy

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Background

In 2024, it is projected to be 2,001,140 new cases of cancer and 611,720 deaths from cancer in the USA. Incidence rates for cancers of the breast, pancreas, and uterus increased by 0.6–1% per year between 2015 and 2019. For cancers of the prostate, liver, kidney, human papillomavirus-associated oral cancers, and melanoma, the rate increased by 2–3% per year [1]. Despite advances in cancer treatment, metastasis is still the number one cause of death from cancer around the world [2]. Metastasis is the primary factor causing mortality in colorectal cancer (CRC) [3] and breast cancer patients [4]. A significant barrier to improving outcomes in patient with advanced solid tumor is the limited ability of current clinical diagnostic tools to predict metastasis and detect minimal residual disease (MRD) [5]. This is often the case due to inadequate diagnostic cutoffs, sampling biases caused by tumor tissue or biomarker heterogeneity and limited sampling of metastases [6]. Moving forward, oncologists have introduced liquid biopsy using blood samples from tumors as an alternative, real-time, and minimally invasive method for early detection, prognosis, and prediction of response to anticancer drugs [7]. Tumor-derived blood samples contain analytes such as circulating tumor cells (CTCs), cell-free DNA (cfDNA), cell-free RNA (cfRNA), and exosomes that provide a promising tool for tailoring the management of each patient with advanced cancers [8, 9].

Naturally, invasive primary tumors and metastatic tumors shed CTCs into the bloodstream to seed distant secondary sites and induce metastases. Therefore, isolating CTCs from blood samples using technological tools and analyzing them according to their established cutoff number and molecular profile could be interesting biomarkers for monitoring cancer patients [10]. In addition, targeting CTCs *in vivo* provides more opportunities to expand their clinical utility in the field of cancer treatment [11].

However, realizing the full potentials of CTCs and applying them in clinical settings is met with technological and biological challenges that must be addressed. Addressing these challenges and promoting translational applications demands a deep understating of the origin and formation of CTC populations, their interactions with tumor vasculature, stromal and immune cells in the bloodstream, as well as their presence in the niche and tumor microenvironment as disseminated tumor cells (DTCs). This review discusses these topics.

CTC biology and tumor metastases

Although each invasive tumor releases thousands of CTCs daily into the bloodstream; however, most of them attach and die due to both mechanical evidences (e.g., shear forces and oxidative stress) and biological reactions (e.g., immune

system responses). Therefore, CTCs have a short half-life in bloodstream and need to employ survival mechanisms, such as activating immune evader pathways, more importantly, forming cluster phenotypes (groups of > 2 CTCs). Clustering protects CTCs against attacks from antitumor immune cells, facilitates their rolling in blood vessels, and even allows them to use platelets or neutrophils as escorts to seed and colonize distant organs, eventually contributing to metastasis [12, 13] (Fig. 1). Previous studies have shown that potent CTC cluster may originate from (i) assembling in the primary tumor due to the density of the extracellular matrix (ECM) before intravasation and through the upregulation of mesenchymal traits, as confirmed in epithelial-to-mesenchymal transition (EMT) necessary for tumor metastasis [14], (ii) overexpression of cell surface adhesion molecules and induction of adherent junctions by plakoglobin and keratin 14 [15, 16], (iii) remodeling of ECM or tumor architecture to clear a path, collective invasion provided by the upregulation of stemness factors (OCT4, NANOG, SOX2) [17], and (iii) the formation of CTC clusters in bloodstream through the coupling of CTC-expressing heparanase-mediated expression of intercellular adhesion molecule-1 (ICAM-1) as cell–cell adhesion molecules in the focal adhesion kinase (FAK)-Src-paxillin-dependent pathway [18].

In comparison to single CTCs, CTC clusters are experiencing hypoxia [19], demonstrating hybrid epithelial-mesenchymal features [20], and expressing more adhesion-dependent survival signals that inhibit the anoikis. They also overexpress transforming growth factor- β (TGF- β), increasing their ability to survive. Their presence in the bloodstream is correlated with elevated circulating levels and is associated with worse prognosis [21, 22]. Endothelial cells express N-cadherin or galectin-3, which interact with multiple areas of CTC clusters, facilitating their extravasation [23]. CTC clusters appear to have a lower turnover rate in the bloodstream compared to single cells, which may enable more efficient metastasis extravasation [24]. Platelets associated with clusters can secrete TGF β that subsequently induces EMT and facilitates the extravasation of CTC clusters [25]. In addition, these platelets release granules containing ATP, which act as potent disruptors of endothelial junctions and may therefore facilitate CTC intravasation and extravasation [26]. CTCs overexpress galectin-4, which binds to red blood cells (RBCs) to form CTC-RBC clusters that enable CTCs to roll steadily along the vessel wall at low flow rates, increasing their adhesion affinity to the endothelial wall and facilitating extravasation [27, 28].

In addition, the expression of cancer stem cell (CSC) markers such as BMI1, CD44, CD133, ALDH1, DLG7, and tendency to cluster (spheroid formation in CSCs) in invasive CTCs indicate the possibility that they originate from CSCs, which are known as metastasis-initiating cells (MICs) [29–31]. Like CSCs, stem cell-like CTCs demonstrate EMT,

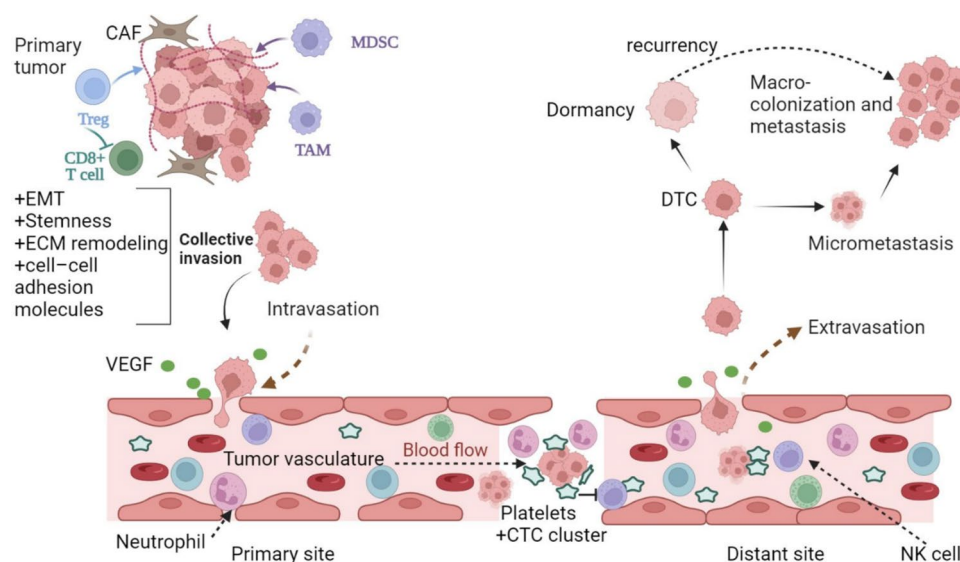


Fig. 1 Models of CTC invasion, cluster formation, intravasation, and induction of tumor metastasis are crucial in understanding cancer progression. The immune suppressive tumor microenvironment, along with stromal cells such as CAFs at the primary site, play significant role in promoting tumorigenesis. In tumors prone to metastasis, EMT allows single CTCs to detach from the primary tumor lesion. This is followed by the upregulation of stemness factors (OCT4, NANOG, SOX2), ECM remodeling, and upregulation of cell–cell interaction mediators, all of which support collective invasion and migration of tumor cells. After intravasation, platelets and neutrophils

provide cluster survival against shear stress and antitumor immune cells such as T and NK cells. Following CTC extravasation and dissemination to distant organs, they may enter a dormant phase for an unknown period or induce micro and macro colonization, ultimately leading to tumor metastasis. *Abbreviations:* CAF, cancer-associated fibroblasts; CTC, circulating tumor cell; DTCs, disseminated tumor cells; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; VEGF, vascular endothelial growth factor; TAM, tumor-associated macrophages; Treg, regulatory T cell

self-renewal, and more aggressive properties, supporting their survival and resistance to anticancer therapies, ultimately contributing to the formation of distant metastases [32]. Interestingly, in distant organs, the colonized CTCs, known as disseminated tumor cells (DTCs), exhibit dormancy and recurrence, consistent with potentials of CSCs [33]. Overall, EMT and clustering provide a survival advantage for a small fraction of CTCs, protecting them from cell detachment-induced apoptosis and shear forces, thereby promoting of metastasis. In addition, clustering formation protects CTCs from immune assault, as discussed in the following sections.

CTC survival-promoting interactions

During EMT and the formation of invadopodia, at the molecular level, the hypoxic solid tumor environment results in the expression of hypoxia-inducible factor 1 α (HIF1 α), which subsequently induces the expression of L1 cell adhesion molecule (L1CAM) [34] and C-X-C chemokine receptor 4 (CXCR4) [35]. Both L1CAM and CXCR4 facilitating the binding of CTCs to endothelium and their intravasation [34]. Before invasion and intravasation, CTCs also bind to perivascular macrophages, which further increase

endothelial permeability through the production of vascular endothelial growth factor A (VEGFA) [36]. Stromal cancer-associated fibroblasts (CAFs) interact with invasive tumor cells at the primary site to reduce shear stress and augment collective invasion, while at the secondary site, they provide TME to CTC seeding and colonization [37]. Indeed, CAF-CTC heterotypic clusters indicate higher CTC metastatic potential in vivo [38].

Once CTCs enter the bloodstream, they can overexpress antiapoptotic proteins such as BCL-2 and programmed cell death protein 1 (PD1) ligand 1 (PD-L1) or CD47, which protect them against cell death induced by apoptosis and immune cells [39, 40]. In addition, CTCs can be masked by platelets expressing MHC I or neutrophils, which provide CTCs with survival advantages by inhibiting immune clearance by natural killer (NK) cells and CD8 T cells, respectively [41, 42]. Similar to neutrophils, myeloid-derived suppressor cells (MDSCs) and macrophages can provide survival advantages for CTC clusters and protecting them from immune surveillance [43] [44] (Fig. 1).

In addition to tumor-supportive immune or stromal cells, recent research has demonstrated that CTC- or tumor-derived extracellular vesicles (EVs) support tumor metastasis by shedding CTCs, protecting them in circulation, and promoting their localization in distant sites to induce

metastasis [45]. EVs carry TGF- β 1 as an EMT promoting factor, or miRNAs that regulate key genes in the EMT/Wnt pathways, such as miR-19b-3p, and miR-10527-5p, and ECM remodeling-related MMP enzymes, all of which facilitate CTC shedding from the primary tumor [46–48]. In addition, EVs carry tumor vascular permeable factors that compromise endothelial junction integrity and facilitate the processes of CTC intravasation and extravasation. In the circulation, tumor-derived EVs contain CD63, PD-L1, and tissue factor (TF), which activate platelets to protect CTCs from shear stress and immune surveillance mediated by T cells, NK cells, and B cells [49, 50].

In distant organs or secondary sites, CTC clusters trap in small capillaries. They express ligands and receptors to interact with tumor-associated endothelial cells (TECs) in the vascular wall, mimicking the diapedesis of leukocytes for extravasation [51]. TEC-derived VEGF and other inflammatory mediators further facilitate vasculature permeability and CTC extravasation [52]. Platelets and neutrophils also support extravasation by creating a pro-inflammatory condition and trapping CTCs in the TEC wall, inducing vascular leakiness [12, 53].

After exiting the bloodstream, CTC metastatic colonization depends on the condition of the TME. Physical and biological factors determine whether metastasis is induced or if dormancy is promoted in CTC for an unknown period of time. The heterogeneity of CTCs in the terms of genetic, phenotypic, metabolic, and other factors ensures their survival throughout the metastatic process. For example, enhanced mitophagy and downregulation of surface biomarkers (e.g., HER2) protect CTCs from ROS-mediated oxidative stress and immune cells identification, respectively [54, 55]. Overall, the phenotypic plasticity and heterogeneity of CTCs impact their ability to acquire adaptive phenotypes for metastasis.

CTC clinical applications

CTC enrichment

In comparison to invasive tumor tissue biopsy, utilizing liquid biopsy for phenotypic and molecular analysis of CTCs with metastatic potential may serve as an ideal source of biomarkers for real-time clinical applications. However, before deciding, CTC analysis requires pure capturing these cells from the blood samples using innovative technologies and then assessing viable CTCs at molecular and functional levels. Capturing methods are classified based on CTC biophysical and biological markers (Fig. 2a). CTCs that overexpress specific antigens (Table 1) enable positive immunomagnetic selection and isolation of CTCs from blood samples that have been pretreated with a hemolysis solvent.

Immune capture of CTCs is performed using anti-CTC antigen antibodies that are immobilized on magnetic beads using magnetic-activated cell separation (MACS) technology, magnetic platforms, and nanowires for in vivo capturing of CTCs after intravascular establishment. EpCAM, EGFR, pan-CK, and CD45 are examples of CTC antigens that are utilized for immune capture of CTCs with acceptable sensitivity and specificity. The FDA-approved CellSearch system and AdnaTest CTC Select use EpCAM-based immunomagnetic capture of CTCs.

CTCs can also be enriched based on their physical criteria such as size (filter-based devices), charge (capture surfaces), density (density gradient centrifugation), or elasticity (microfluidic systems) (Fig. 2a). The pure selection and enrichment of CTCs is achieved through the combination of antigen-dependent and independent technologies [70]. In addition, in vivo capturing of CTCs using nanowires (e.g., anti-EpCAM antibodies decorated on CellCollector, Germany) reduces manipulation, contamination, and stress associated with in vitro isolation of CTCs. More importantly, CellCollector addresses the low number of CTCs in blood samples that require a high volume of blood samples, which is limited in some patients. Similar to CellCollector, cytopheresis is another strategy that combines antigen-dependent selection and provides the *ex vivo* enrichment of low CTCs from the whole blood volume of patients. The conventional method of isolation and identification of CTCs is summarized in Table 2.

CTC analysis and tumor detection and prognosis

Downstream analysis of CTCs includes direct drug phenotyping, the creation of CTC-derived xenograft models, and multi-omics interrogations at the single-cell level (e.g., epigenomic, proteomic, genomic, and transcriptomic) [9] (Fig. 2b and c). Epigenetic comparison of CTCs and CTC clusters at the single-cell level revealed that the hypomethylation profile of CTC clusters on the binding sites for OCT4, SOX2, NANOG, and SIN3A correlates with stemness, metastatic spreading, and poor prognosis in breast cancer [17]. Therefore, remodeling these sites using Na⁺/K⁺ ATPase inhibitors resulted in the dissociation of CTC clusters and suppression of metastasis, introducing potential therapeutic targets on a genome-wide scale. Genomic profiling using single-cell genome sequencing can indicate high-throughput intra-tumor heterogeneity, providing new biological insights into tumor formation [85]. In addition, mass spectrometry and microfluidic chambers are utilized for the analysis of proteins secreted by single CTCs and CTC clusters, indicating the potential for providing functional CTC profiling in cancerous samples [86, 87].

Detection of CTCs in peripheral blood has been studied extensively in major solid tumors such as breast, prostate,

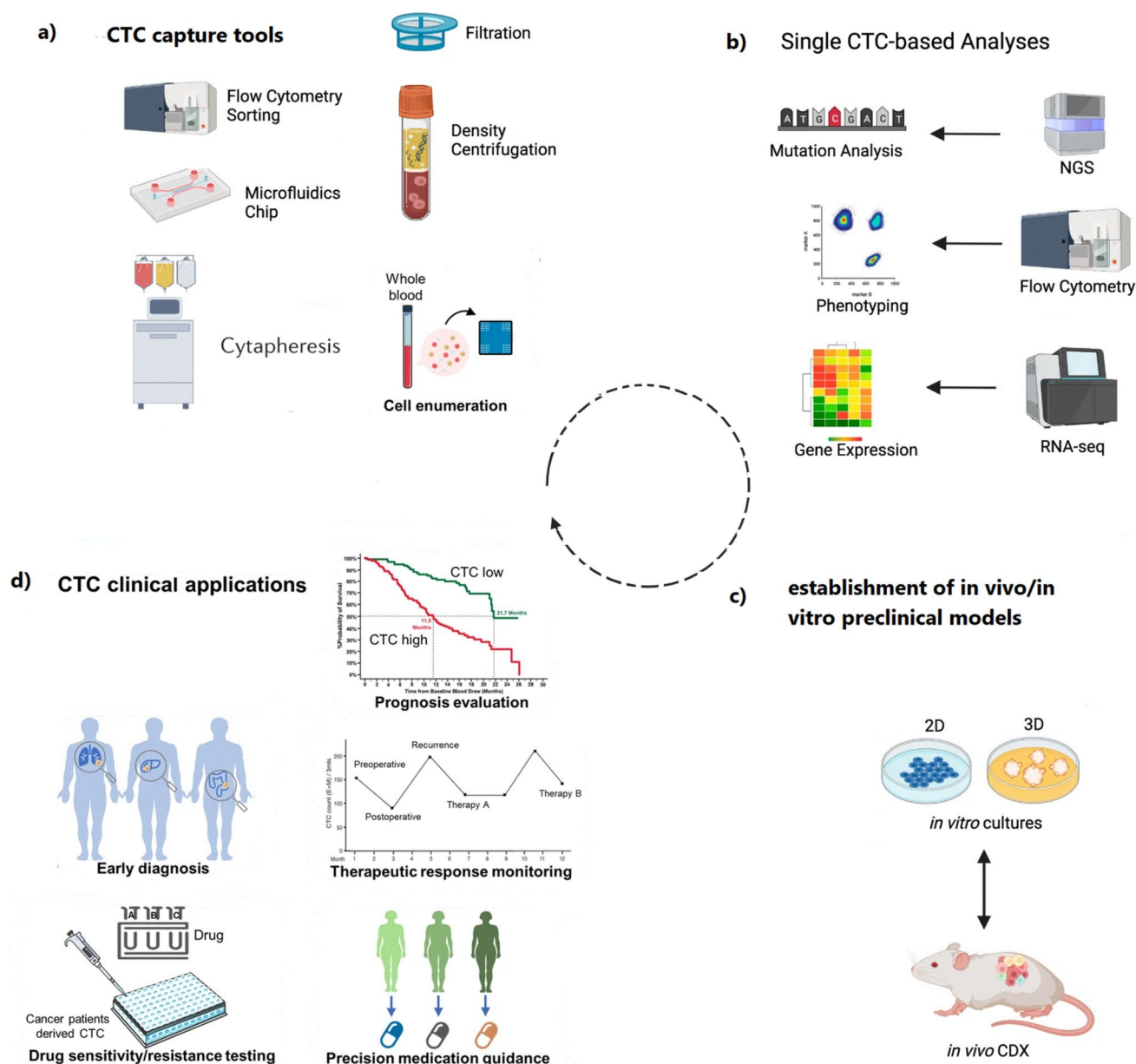


Fig. 2 **a** Viable circulating tumor cells (CTCs) are isolated or enriched from peripheral whole blood using techniques such as FACS, MACS, filtration, density gradient centrifugation, and microfluidic devices. **b** These purified CTCs can then be characterized using single-cell analysis technologies (single-cell genomic/transcriptomic/proteomic analysis) or **c** used for establishment of in vitro 2D

culture (low adherent) or 3D culture (tumoroids) and in vivo CDX. **d** Examples of clinical applications of CTCs. *Abbreviations:* CDX, CTC-derived xenografts; FACS, fluorescence-activated cell sorting; MACS, magnetic-activated cell sorting. Figure 2 is redesigned from references [88] and [89]

colorectal, and small cell and non-small-cell lung cancers. These studies have focused on the prognostic value and the ability to predict disease-free and overall survival [9]. Elevated CTC counts (five or more CTCs per 7.5 mL of blood) or changes in CTC numbers in response to therapy have been associated with worse prognosis and can help predict progression-free survival (PFS), disease-free survival (DFS), and overall survival (OS) [90].

In addition, the profile of CTC biomarkers presents the benefit of therapy choice. The randomized phase III DETECT III trial (NCT01619111) compared lapatinib in combination with standard therapy versus standard therapy alone in patients with initially HER2-negative metastatic breast cancer (MBC) and HER2-positive CTCs. The results indicated that patients with HER2-negative MBC

Table 1 Molecular markers of CTCs in human solid tumors

Type of cancers	Specific marker(s)	EMT marker	Epithelial markers	Stemness marker	Ref
Breast cancer	HER2, ER, AR	Vimentin, Twist, N-cadherin, β -catenin	EpCAM/CK8,18,19 and E-cadherin	CD44, CD24 ⁺ , and ALDH1	[56, 57]
Bladder cancer	CK, EGFR, MUC7	–	EpCAM/CK8,18,19	CD44v6	[58–60]
Colorectal cancer	PI3K α , CEA, PRL3	Vimentin, Twist, SNAI1, Plastin3	EpCAM/CK8,18,19	Wnt, CD44	[22, 61]
Gastric cancer	MT1-MMP	N-Cadherin	EpCAM/CK19/CK20	CD24, CD44v6	[62, 63]
Hepatocellular carcinoma	GPC-3, ASGPR	Vimentin, Twist	EpCAM/CK8,18,19	NANOG, CD44, and CD133	[64, 65]
Pancreatic cancer	GAS2L1, CA19.9	Vimentin, Twist, KLF8	EpCAM/CK8,18,19	CD44	[66, 67]
Small-cell lung cancer	DLL3	Vimentin	EpCAM/CK8,18,19	CD44v6 + / CD31-	[68, 69]

ASGPR, asialoglycoprotein receptor; CEA, carcinoembryonic antigen; DLL3, delta-like ligand-3; GPC-3, glypican-3; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor-2; PRL3, Phosphatase of Regenerating Liver 3; MT1-MMP, membrane type1-matrix metalloproteinase

and HER2-positive CTCs benefit from additional HER2-targeted therapy with lapatinib [91].

Better than single CTCs counting, evaluation of CTC clusters can improve early cancer detection [92], monitoring of response [93], prognostic value [94], detection of MRD that determined by the clearance of CTC clusters after standard therapy [94, 95], and prediction of tumor relapse in late stages of disease [96]. These factors are all helpful in guiding therapeutic decisions. Comprehensive analysis of the size or quantity of CTC clusters and associated peripheral blood cells (PBCs) could provide valuable insights into a biomarker-driven strategy for further management after the completion of standard therapy. This analysis could also aid in the risk stratification of patients with unresectable late-stage metastatic tumors [95, 97]. Beyond CTC enumeration, the integration of single-cell multi-omics approaches provides characterization of CTCs at the single-cell level. This approach also sheds light on their role in inter-/intra-tumor heterogeneity, mechanisms of therapeutic resistance, and the discovery of rare CTCs that drive tumor progression and metastasis (Fig. 2b).

However, CTCs are relatively rare in most cancer patients. Functional characterization of rare CTCs requires in vitro or ex vivo expansion, which can be achieved through low-adherent 2D culture or CTCs cultured in ultralow attachment plates to support 3D culture. Alternatively, CTCs can be seeded in a 96-well plate under hypoxic conditions to support long-term CTC culture [98]. Once CTCs reach 90% confluence, they can be collected for molecular characterization [99], used to create lysate in DC vaccines [100], or utilized for developing CTC-derived xenografts (CDX) to better understand cancer biology and identify potential prognostic markers and therapeutic targets [101] (Fig. 2c). These approaches pave the way for personalization of therapeutic option as discussed in previous reviews [9, 102, 103]) (Fig. 2d).

Target CTCs in vivo

Targeting the primary lesions but not the metastatic tumor, and ignoring the CTCs or DTCs, has resulted in the failure of current therapies such as surgery or systemic chemoradiotherapy for advanced human cancers [104]. Therefore, targeting CTCs before dissemination may interrupt the metastatic progression. Nevertheless, this strategy should be implemented at all stages of metastasis, from CTC shedding, cluster formation, CTCs in the bloodstream, and DTCs in secondary tumors at distant sites (Fig. 3).

As mentioned above, hypoxia supports collective invasion of tumor cells and intravasation of CTC clusters. Targeting hypoxia or inducing tumor vascular normalization by proangiogenic agents can lead to reduced CTC cluster formation, shedding rate, and suppression of metastasis in animal models [19]. Integrin, cell surface glycoproteins [105], and heparanase (HPSE)-induced hemitropic cluster formation (HPSE upregulates cell–cell contact mediators such as ICAM-1) [18], and targeting these mediators has resulted in clustering suppression and decreased CTC induced tumor metastasis. In addition, disrupting heterotypic cell–cell interactions that occur in the bloodstream and provide the clusters survival, such as CTC-platelet [106] or CTC-neutrophil clusters [12], could reduce the metastatic potential in ovarian and breast cancers (Fig. 3b).

Regarding surface biomarker targeting, CTCs that overexpress antigens and immune checkpoint inhibitors (ICIs) can be targeted using anti-EpCAM, HER2 PD1, PD-L1, and CTLA4 antibodies. These antibodies mediate T cell killing as a single-agent therapy, or can be improved by dual targeting CTC antigen and ICIs. Dual targeting of EpCAM or HER2 with neutralizing antibodies in combination with ICIs has been shown to eradicate CTCs in models of human colorectal cancer [107] and breast cancer [108] (Fig. 3c). Inspired by the physical interaction between platelets and

Table 2 Overall methods for isolating CTCs

Isolation principle	Example technology	CTC/cancer type	Advantages	Disadvantages	Ref
<i>Size-based methods</i>					
Membrane microfilters	ISET	Non-small cell lung, colorectal cancers, melanoma	+ High sensitivity + Isolation of CTC clusters	-High loss of small cells	[71]
FAST	Colorectal, breast, stomach, lung cancers	KRAS mutation analysis	+ High sensitivity	-High loss of small cells	[72]
Microfluidic technologies	ieSCI-chip	Breast cancer	+ High capture efficiency + Fast isolation and processing	-Limited sample volume -Slow flow rate	[73]
Microfluidic technologies	Labyrinth	Non-small cell lung, liver cancers	+ Isolation of clusters + Depletion of more than 95% of leukocytes + High cell viability	-High loss of small cells	[74]
<i>Immuno-affinity strategy (cell biological properties)</i>					
Negative selection	RosetteSep	Liver, breast cancers	+ Isolation of CTC clusters + Fast isolation (40 min) + CTC marker-free isolation	-High number of untargeted cells	[75]
Positive selection	CELLSEARCH® (Janssen Diagnostics)	Metastatic breast cancer, Colorectal, Prostate	+ FDA-approved + marker's dependence + Most clinically validated capture technique + high Specificity	-Low purity of captured CTC -low Sensitivity -cannot Identify EMT	[76]
Positive selection	MACS system (Miltenyi Biotec)	Non-small-cell lung cancer (NSCLC) Breast (HER2 +)	+ Identifies EpCAM negative CTCs + Can combined with leukocytes depletion	-Expensive -Low cell viability -Dependence on expressed proteins -Hard to automate	[77]
<i>Functional Assays</i>					
Secretory Protein	EPISPOT	Breast cancer	+ High sensitivity / specificity + Independent Ag phenotype + Allows CTC quantifying	-Just allows CTC detection based on protein secretion	[78]
Adhesive CTC	Vita-Assay (Vitatex)	Prostate	+ Allows detection of invasive n CTC	-Low purity	[79]
<i>Dielectrophoresis</i>					
Dielectrophoretic cage	DEPArray™ (Silicon Biosystems)	Breast	Isolation of single CTCs for downstream gene analysis	-High loss of small cells	[80]
DEP-FFF	ApoStream® (ApoCell)	Breast	+ Independent of EpCAM + useful for viability analysis and culture	Processes more than 10 mL/h	[81]
<i>Combined methods</i>					
Positive selection and microfluidic approaches	RBC-chip	Colorectal cancer	+ High sensitivity and specificity + High viability	-Isolation of only EpCAM-positive CTCs	[82]
Positive selection and microfluidic approaches	Herringbone-Chip	Lung cancer	+ One step method + High capture efficiency + Isolation of clusters	-Isolation of only EpCAM- and EGFR-positive CTCs	[83]
Non-microfluidic approaches and cell biological properties	AccuCyte-RareCyte/PIC & RUN	Prostate, breast, lung cancers	+ High viability + High capture efficiency + provide CTC culturing	-The presence of false-positive CTCs	[84]

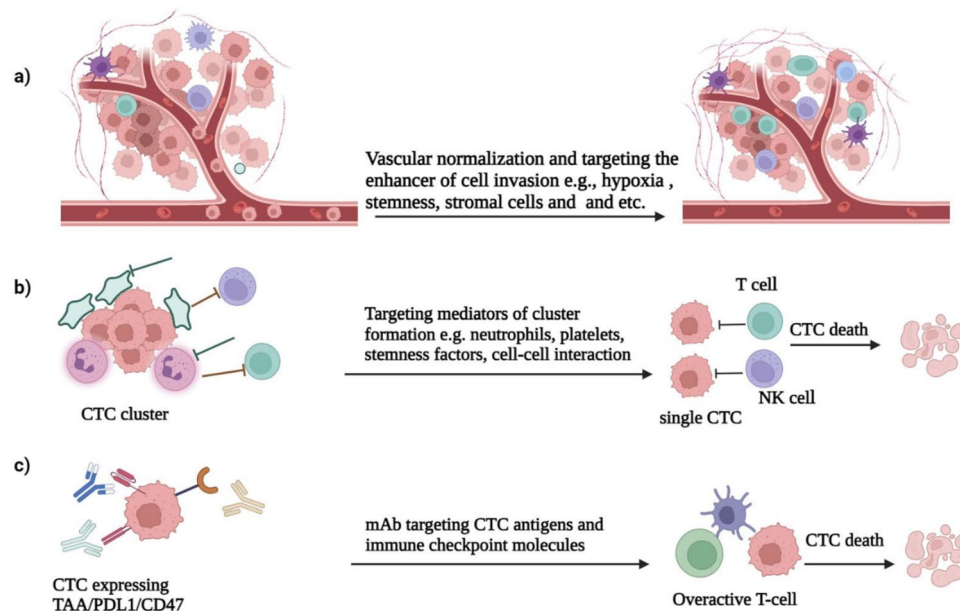


Fig. 3 The approaches for targeting CTCs in vivo. **a** Inhibition of cancer cell invasion and intravasation can be achieved through vascular normalization and targeting of hypoxia, stemness factors, and tumor-supportive immune cells or stromal cells in the solid tumor microenvironment. **b** Blocking cellular interactions with platelets or neutrophils that support cluster formation and survival can lead to the dissociation of CTC clusters, producing immune-prone single CTCs

for eradication in the systemic circulation. **c** Targeting CTC survival can be down using small molecules or monoclonal antibodies (mAb) targeting tumor-associated antigens (TAAs) such as EpCAM, HER2, PSA, and immune checkpoint molecules (PD1, PD-L1, CTLA4, CD47) on CTCs or T cells. This reprograms of antitumor immune responses and promotes immune clearance of CTCs in both the bloodstream and their niches in distant sites

CTCs that help them evade immune elimination, Li et al. genetically engineered platelets to express tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). This induced apoptosis in metastatic tumor cells in the bloodstream and reduced systemic prostate cancer metastases [109].

In addition to monoclonal antibodies targeting surface tumor antigens, CTCs could also be targeted by engineered chimeric antigen receptor (CAR)-T cells. This was most recently indicated by EpCAM-specific CAR-T cells, which were successful in eliminating CTCs that have intravasated into circulation in a surgery-induced tumor metastasis model in immunocompetent mice [110]. Granzyme B (GrB)-based CAR-T cells were also developed to target membrane-bound HSP70 (mHSP70), effectively inhibiting cancer metastasis in spontaneous metastasis models without causing any apparent toxic effects [111]. CAR-T cells targeting other tumor antigens, such as HER2, PSCA, CEA, TAG-72, and CBT-511, have been developed and are currently being evaluated in preclinical and clinical trials for solid tumors [112, 113]. The promising anti-tumor activity of these tumor antigen-specific CAR-T cells in preclinical investigations led to the study of the therapeutic effect of EpCAM CAR-T cells in clinical trials (NCT02915445) involving 12 patients with EpCAM-positive epithelial tumors. The result showed that two

patients had a partial response, three patients had more than 23 months of progression-free survival (PFS), and one patient experienced a 2-year PFS with detectable EpCAM CAR-T cells 200 days after infusion [114].

However, the administered antitumor agents cannot distinguish disease-relevant CTCs from disease-irrelevant CTCs in the bloodstream, which may be better targeted toward colonized tumor cells at metastatic sites. Various biophysical, biochemical, and immune barriers limit the delivery and therapeutic effects of chemotherapies in the mechanoenvironment of cancer metastasis [115]. Using cell-based vectors (e.g., MSCs, mesenchymal stem cells) with the potential for homing to cancer metastases is a novel platform for both tumor diagnostics and therapies [116]. CTC offer a super benefit with self-homing or self-seeding properties. CTCs armed with both a reporter gene and a cytotoxic prodrug gene therapy, converting nontoxic 5'-fluorocytosine (5FC) into the cytotoxic compound 5'-fluorouridine monophosphate, have been used as a theranostic platform in metastatic disease. This approach resulted in tumor lysis and a reduction of tumor burden in a mouse model, as demonstrated by bioluminescence imaging [117]. Therefore, distinguishing metastasis-relevant CTCs, understanding CTC heterogeneity, and determining the molecular interactions between immune or stromal cells, neutrophils, and CTCs, expands the metastatic potential of CTCs. This provides a

rationale for targeting invasive single CTCs or CTC clusters in the treatment of human cancers.

Limitations of the routine analysis of CTCs and prospective approaches

Interestingly, the circadian rhythm may have an effect on CTC release and metastatic dissemination in breast cancer. For example, during sleep or rest, melatonin (and other unknown mechanisms) may increase the CTC count in the bloodstream [118], highlighting the importance of the time of sampling for analyzing CTC. This can influence the prognostic and predictive value, as well as decision-making in the clinic.

Beyond enumeration, the rhythmicity of CTC release into the bloodstream can guide time-controlled clinical trials of chemotherapy by utilizing available therapeutic agents to achieve maximum effectiveness during peaks of CTC generation. Efforts have been made to address the low number of CTCs in blood samples, with cytopheresis and nanowire technologies explored for ex vivo and in vivo capturing of CTCs from large blood volumes, respectively. However, the implementation of these approaches in routine clinical practice may be limited due to their invasive nature and the poor vascular health of heavily treated cancer patients [119, 120].

Since tumor-draining vessels contain higher number of CTCs compared to peripheral locations, it is important to develop attractive liquid biopsy approaches that have access to these vasculatures, especially in patients with early-stage cancer who have undergone surgery [121]. In addition to enumeration, during the capturing process, some CTCs may become loose or deformed, necessitating rapid identification and automated downstream analysis of CTCs [70].

Upon metastasis, the EMT process results in the downregulation of EpCAM and upregulation of mesenchymal markers in CTCs, limiting the application of EpCAM-based CTC enrichment [122]. Therefore, there is a need to develop EMT-independent methods capturing CTCs. In addition, issues such as clogging formation and the requirement of large blood samples have restricted the isolation of CTCs using size/filter-dependent systems (e.g., ISET, MetaCell system, ScreenCell Cyto, and CellSieve) and have also affected their viability in density-based centrifugation.

CTCs are informative sources of biomarkers, even for personalized medicine (Fig. 2). However, presenting precise functional assays requires improvement of optimized culture methods and efficient CTC-derived animal models [70]. In addition, metastasis-targeting agents are used in the advanced stages of tumors, while CTC shedding, dissemination, and metastasis may have already occurred even in the early stages and during tumor formation [123]. The timing and mechanism of CTCs and their clusters entering the

bloodstream, their organotropism, as well as dormancy or induction of micro/macrometastasis remain elusive, and this aspect warrants further investigation.

Conclusion

Analysis of liquid biopsy-associated CTC analytes at the single-cell level has the potential to revolutionize cancer management in the future. However, to broaden its application in the clinic and across the cancer spectrum, innovative study designs will likely be required to address unanswered questions about CTC biology and enumeration validity in patients with advanced cancers. New bioengineering techniques, such as implantable, rapid, automated, and noninvasive microchips, are being developed to enable the simultaneous capture and high-throughput analysis of CTCs using multi-omics technologies. However, not all CTCs may induce metastasis, so it is necessary to distinguish the most metastasis-prone CTC subpopulations. In addition, before designing relevant clinical trials, further research into culture conditions and animal models is needed to maximize the effectiveness of interventions and optimize standards of care.

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of interests The authors declare no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate Not applicable.

Consent to publish Not applicable.

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