

Advances in isolation and detection technologies and immunotherapy applications of circulating tumor cells (Review)

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Abstract. Circulating tumor cells (CTCs) are shed from the primary tumor into the peripheral bloodstream, where they play crucial roles in tumor metastasis and recurrence. As a cornerstone of liquid biopsy, CTCs hold significant potential for early tumor diagnosis, therapeutic response monitoring, and prognosis. However, the rarity and heterogeneity of CTCs pose considerable challenges for their isolation and

enrichment. Additionally, their predictive usefulness in tumor immunotherapy remains relatively limited. The present review summarizes recent advancements in CTC isolation and detection technologies, explores their clinical applications in immunotherapy, and discusses current challenges alongside potential strategies for improvement. The integration of these technologies into clinical practice could pave the way for more personalized and precise cancer treatment strategies in the future.

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Abbreviations: CTCs, circulating tumor cells; PFS, progression-free survival; OS, overall survival; FDA, U.S. Food and Drug Administration; EpCAMs, epithelial cell adhesion molecules; ISET, isolation by size of epithelial tumor cell; DEPArray, dielectrophoretic array; MAP, magnetophoretic; DEP, dielectrophoretic; MFM, multi-flow microfluidic; CKs, cytokeratins; HER2, human epidermal growth factor receptor 2; EGFR, epithelial growth factor receptor; DLD, deterministic lateral displacement; EMT, epithelial-mesenchymal transition; CNFs, cellulose nanofibers; NGS, next-generation sequencing; FISH, fluorescence *in situ* hybridization; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; NSCLC, non-small cell lung cancer; OS, overall survival; ICI, immune checkpoint inhibitor; PFS, progression-free survival; LUAD, lung adenocarcinoma; OXA, oxaliplatin; MBC, metastatic breast cancer

Key words: CTCs, isolation, detection, immunotherapy

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1. Introduction

Cancer remains one of the most serious diseases that threaten human health, with metastasis being the leading cause of cancer-related deaths worldwide and posing a major challenge in cancer treatment (1,2). Metastasis is a complex process involving multiple steps (3), including intravasation, extravasation, migration and regeneration. During metastasis, tumor cells from the primary site invade distant tissues through the bloodstream, where they form metastatic foci (4). In recent years, the prevailing view has been that metastatic disease is usually extensive and incurable. However, the advent of immunotherapy has led to significant breakthroughs in cancer treatment. When combined with surgery, radiotherapy and chemotherapy, immunotherapy can improve patient survival (5). Tissue biopsy is among the clinical methods used to guide immunotherapy (6). However, traditional tissue biopsies are not 100% accurate and have several limitations: They are invasive (7), have limited sensitivity and specificity, provide only local tissue samples, and fail to capture tumor heterogeneity (8). Circulating tumor cells (CTCs) are a

cornerstone of liquid biopsy and offer undeniable advantages that can compensate for some of the limitations of traditional tissue biopsy. They are non-invasive (9), easy to administer, and more patient friendly. Additionally, they address the issue of tumor heterogeneity, which enables more effective monitoring of tumor progression through serial testing and provides valuable insights to guide treatment decisions (10-12).

CTCs are tumor cells that are shed from primary tumors or metastatic sites and enter the peripheral bloodstream (13,14). The concept of CTCs was first introduced in 1869 by the Australian physician Thomas Ashworth, who observed cells in the blood of a patient with metastatic cancer that were similar to the primary tumor cells (15,16). However, given the technical limitations of that time period, CTCs were not further examined (17). It was not until 1976 that Nowell redefined CTCs as tumor cells originating from primary or metastatic tumors that have acquired the ability to detach from the basement membrane and invade the vasculature through tissue stroma. In 1998, Peck *et al* (18) explored the feasibility of using CTC assays for the rapid assessment of chemotherapeutic responsiveness. Their study revealed that serial assays of changes in CTC counts correlated with tumor burden and therapeutic response in patients. Furthermore, in 2004, a prospective multicenter study demonstrated that the number of CTCs in peripheral blood prior to treatment was an independent predictor of progression-free survival (PFS) and overall survival (OS) in patients with metastatic breast cancer (19,20). That same year, the U.S. Food and Drug Administration (FDA) approved the CellSearch System for the treatment of metastatic colorectal, breast and prostate cancers (21). With continued technological advancements, the CTC chip developed by Nagrath *et al* (22) in 2007 markedly improved the efficiency of CTC enrichment. This microfluidic platform enables the efficient and selective isolation of viable CTCs from peripheral whole blood under precisely controlled laminar-flow conditions. Using this system, CTCs were successfully detected in 115 of 116 peripheral blood samples (99%) obtained from patients with metastatic lung, prostate, pancreatic, breast, and colon cancers. The detected CTC counts ranged from 5-1,281 cells/ml of blood, with an approximate purity of 50% (22). However, most CTC enrichment techniques during that period were performed *in vitro*. Hartmann *et al* (23) reported that this approach is inherently limited by the minimal number of CTCs that can be captured from small-volume blood samples. Moreover, preanalytical handling procedures, including sample transport, preservation, centrifugation, and subsequent *in vitro* enrichment and isolation, frequently introduce artifactual alterations, such as cellular contamination, loss, inactivation and morphological distortion (23). To overcome these challenges, the first *in vivo* CTC detection system, known as Cell Collector, received CE certification from the European Union in 2012. The cells captured by this system require no pretreatment, such as transfection or fluorescent labeling, and can be directly identified within the immunomagnetic separation device. This feature effectively minimizes the detrimental effects associated with clinical sample collection, preprocessing, and CTC isolation on cellular integrity and overall quality (24). Compared with the Cell Collector system, the CTC enrichment platform developed in recent years by Hazra *et al* (25), which employs

cellulose derivatives, may reduce costs and thus improve affordability for a larger patient population; moreover, this method achieves an overall capture efficiency of ~40%. Despite these advancements, isolating CTCs from blood with high sensitivity and specificity remains a significant challenge (26). Most patients with cancer have extremely low concentrations of CTCs in their peripheral circulating blood (from 1-100 cells/ml) (27), and factors such as blood shear stress and immune system activity result in a short half-life for CTCs, usually from 1-2.5 h (28,29). Additionally, CTCs exhibit significant heterogeneity across individuals and tumor types, even within the same patient. With the rapid advancement of new digital technology, researchers have established a new interdisciplinary field of medical, industrial integration by combining biology, chemistry, physics, computer science and medicine. This integration has led to the development of innovative technologies for the capture, detection, and analysis of CTCs. These advances have significantly facilitated the clinical application of CTCs in cancer screening, treatment response monitoring and prognosis (28).

A search of the PubMed database through December 2023 using 'CTCs' as a keyword retrieved more than 33,000 studies. The number of publications per year has shown an upward trend, reaching more than 2,000 by 2020. Although there has been a slight decline since then, the annual number of relevant publications still exceeds 1,500, confirming continued interest in advancing the technology and developing new applications. These findings indicate that research on CTCs is advancing rapidly, yielding significant findings (Fig. 1). The present study provides a systematic comparison of the advantages and disadvantages of the current methods for collecting and identifying CTCs and assessing their clinical applicability in immunotherapy.

2. CTC enrichment technology

In general, technologies for identifying CTCs involve three critical steps: Capture and enrichment, detection and identification, and release for further analysis (30). As noted by Wang *et al* (31), capture and enrichment methods rely primarily on the interaction between specific materials and certain physical, chemical or biological properties of CTCs. After collection, various identification methods can be used, such as flow cytometry, fluorescence microscopy and DNA sequencing. Released CTCs are typically utilized for downstream analyses, such as genomics, transcriptomics, proteomics, and immunofluorescence assays of cultured cells (31). Enrichment methods for CTCs are based on certain biological and physical properties (32,33). Since most tumors originate from epithelial tissues, epithelial cell adhesion molecules (EpCAMs) are the most commonly used biomarkers for CTCs. Physically, CTCs can be differentiated by properties such as size, density, charge, and deformability (34-37). In recent years, detection techniques that integrate both the physical and biological properties of CTCs have emerged, offering enhanced efficiency and sensitivity (38,39) (Fig. 2).

Enrichment methods based on the physical properties of CTCs
Size-based enrichment. Studies have shown that CTCs are typically larger and firmer than hematopoietic cells are, and

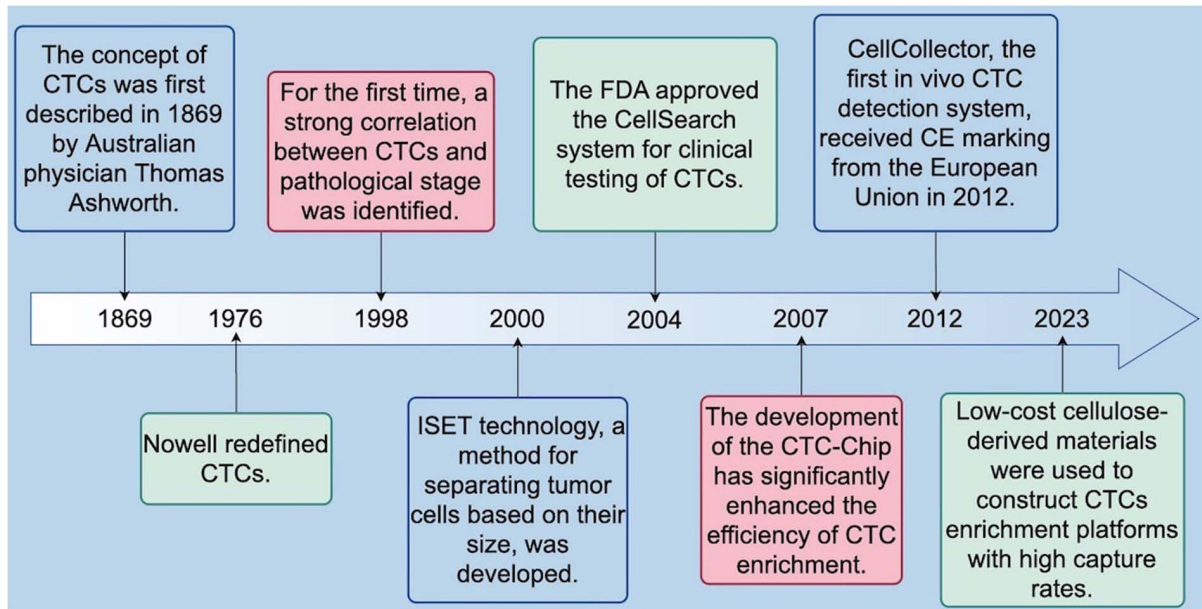


Figure 1. A brief chronological overview of the development of CTC enrichment techniques. Image was created using www.Figdraw.com. CTCs, circulating tumor cells.

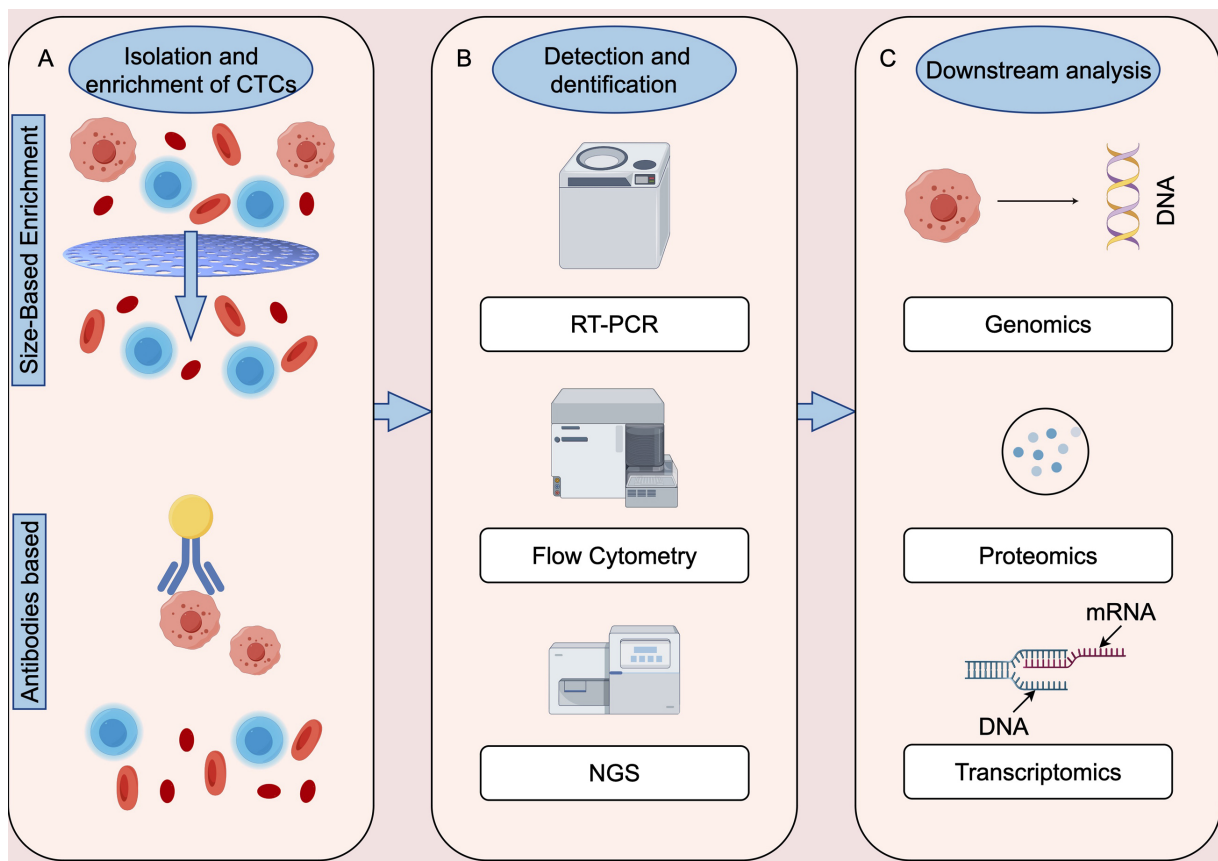


Figure 2. Brief process of CTC technology. (A) Separation techniques: These can be divided into three categories: Based on physical properties, based on biological properties, and combining physical properties and biological properties. (B) Detection techniques: RT-PCR, flow cytometry and NGS. (C) Downstream analyses: Genomics, proteomics and transcriptomics. Image was created using www.Figdraw.com. CTCs, circulating tumor cells; RT-PCR, reverse transcription-polymerase chain reaction; NGS, next-generation sequencing.

numerous CTC enrichment techniques have been developed on the basis of these physical properties (40). For example, the epithelial tumor cell size separation (ISET) method

primarily uses a two-dimensional membrane microfilter and is the first assay to allow the direct filtration of peripheral blood. In this system, blood samples are diluted 1:10 and

passed through a polycarbonate track-etch-type membrane with 8- μm pores (41,42). CTCs, which are generally larger than peripheral blood leukocytes, are retained, while the other cells pass through. However, this method is not very efficient. A similar system, the ScreenCell, enriches CTCs by processing blood samples through a microporous membrane filter with 8 μm pore size. Unlike ISET, ScreenCell system can consistently process large numbers of blood samples with high throughput (43). In addition, the ScreenCell system offers other advantages, including the use of cellular and molecular technologies for identifying CTCs and analyzing their underlying genetic abnormalities, which facilitate the easy analysis of tumor cells extracted onto the filter (44). Furthermore, the cells isolated from blood maintain a high level of activity and integrity, allowing for *in vitro* tissue culture. To minimize cell damage and preserve cell viability, a flexible miniature spring array device ensures the harvesting of CTCs with greater viability (45). This device uses a pressure-regulated system to capture CTCs with an efficiency of up to 90%. However, experiments have shown that the device tends to yield variable CTC counts across different samples, making it less reliable (46). There are also 3D membrane microfilters, such as the Parsortix system (47), Resettable Cell Trap (RCT) (48) and FaCTChecker and Cluster-Chip, which allow for the separation of individual CTCs and/or CTC clusters, depending on the size and/or variability of the CTCs (38). Unlike the FaCTChecker microfilter, the Parsortix system employs a horizontal configuration rather than a vertical one. This microdevice features a stepped architecture with channel widths progressively narrowing to $\leq 10 \mu\text{m}$. CTCs larger than the channel width become trapped in the gaps, while smaller cells pass through. After CTC capture, reverse flow releases the captured CTCs for collection and subsequent molecular characterization. The RCT employs a strategy similar to that of Parsortix; however, rather than reducing the channel height, the device uses pneumatically controlled microvalves to modulate the channel aperture and capture CTCs within pockets that are taller than the main channel. When the microvalves are relaxed, the aperture widens, enabling the release of the captured CTCs (44). Both 2D and 3D membrane microfilters have distinct characteristics in the separation and enrichment of CTCs, with the choice depending primarily on the enrichment principle of the specific method. With respect to microfiltration techniques, the cross-sectional area of 2D surfaces is more critical, whereas volumetric measurements are more relevant for 3D shapes in flow separation. However, since the size of CTCs may vary in size during aggregation, apoptosis, and different stages of the cell cycle and can partially overlap with the size of leukocytes, false-positive results may occur during the enrichment process (38). Although size-based enrichment of CTCs is a simple and label-free technique for high-throughput assays, the capture rates and specificity are influenced by the size heterogeneity of CTCs, necessitating combination with other assays to improve accuracy.

Density-based enrichment. In addition to size-based enrichment methods, density-based enrichment is a workable strategy with distinct advantages. One commonly used density gradient centrifugation technique involves the use of a sucrose polymer with a high synthetic molecular weight (Ficoll-Paque; GE Healthcare) as the primary density gradient

medium (49). The high centrifugal force applied in this method creates a density gradient and the size and density of the cells determines their sedimentation rate. The cell loading and harvesting procedures must be determined empirically as they are significantly influenced by the Ficoll-Paque, the rotor, and the type of centrifuge tube used. Excessive cell loss and low separation efficiency for CTCs isolated by Ficoll-based density gradient centrifugation have been attributed to density-related toxicity (38,50). The OncoQuick gradient system addresses these problems by minimizing cell loss and contamination, thereby achieving higher CTC enrichment rates than the traditional Ficoll-Paque technology does (38,51).

Charge-based enrichment. The dielectrophoretic array (DEPArray) system, developed in 2013, is a semiautomated platform capable of selecting and isolating individual CTCs and small populations of cells (52). The primary advantage of the DEPArray system lies in its ability to identify multiparameter immunofluorescence staining characteristics of individual cells before isolation. Additionally, it can be integrated into a continuous, semiautomated workflow platform, such as with the CellSearch System. However, the system's advanced capabilities for imaging and isolating individual CTCs have significant drawbacks, including high costs and an average cell loss of $\sim 40\%$ during sample preparation and separation (52). To address these challenges and achieve higher separation resolution, researchers developed an integrated dielectrophoretic (DEP) and magnetophoretic (MAP) microfluidic chip in 2022. This integrated MAP-DEP approach enables the separation of various types of CTCs on the basis of their size and dielectric properties (53).

Inertia-based enrichment. In 2019, an investigator introduced a novel multifold microfluidic (MFM) system designed for the high-purity separation of CTCs. By utilizing the inherent differences in the inertial migration of cells flowing within microchannels, the MFM system demonstrated a sensitivity that achieved over 87% purity in label-free separations. However, the system has serious limitations. Its sensitivity and recovery rates are compromised when CTCs exhibit significant size overlap with other cell types. Additionally, compared with other label-free separation systems, the device operates at a relatively low flow rate. To address these limitations, a new class of collectors based on similar principles, known as inertial microfluidic labyrinth devices, was developed (54). These devices incorporate 12 distinct arrays of microfilters and combine inertial focusing with Dean flow dynamics. This approach achieves size-based cell separation by continuously focusing all cells while selectively isolating CTCs from smaller blood cells at the device's outlet. Current CTC and CTC cluster separation techniques often rely on biomarker-dependent antibody-based capture. However, compared with individual CTCs, CTC clusters pose unique challenges: They have a lower surface-area-to-volume ratio (55) and often exhibit mixed epithelial-mesenchymal phenotypes, resulting in reduced antibody binding regions (56). Consequently, these methods may result in lower enrichment rates for CTCs and CTC clusters with stem-like or mesenchymal characteristics. By contrast, physical property-based separation methods, such as inertial microfluidic labyrinth devices, avoid the biases inherent in molecular marker-dependent systems. These advanced devices offer a more effective solution for separating CTCs and CTC

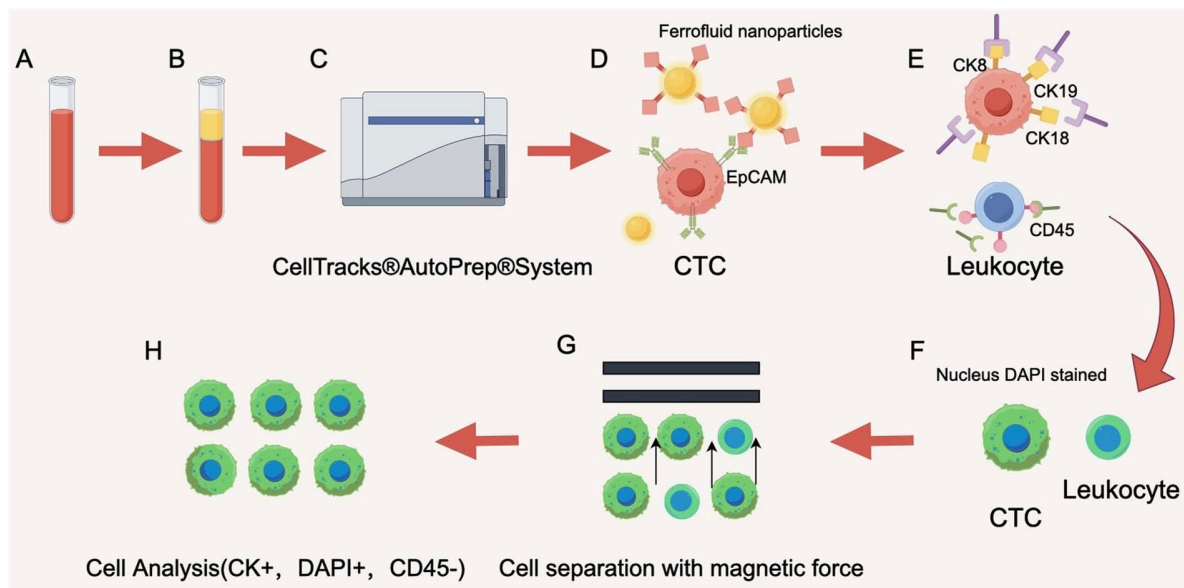


Figure 3. Schematic diagram of the Cell Search system for detecting CTCs. (A) Blood is drawn into a Cell Save protection tube containing EDTA and cytoprotectant. (B) A 7.5-ml aliquot of blood is transferred to a separate tube for centrifugation. (C) The samples are processed using the CELLTRACKS@AUTOPREP@ system, during which plasma is aspirated and the cells are resuspended in a buffer solution. (D) Magnetic nanoparticles coupled with EpCAM antibodies are added to bind EpCAM-positive cells, enriching for epithelial-derived CTCs. The bead-bound cells are then separated from other cells via magnetic force. (E) The CTCs are stained with antibodies targeting CK8, CK18 and CK19. CD45-positive cells (leukocytes) are excluded from the analysis. (F) The nuclei are stained with DAPI for identification. (G) Magnetic separation is performed to isolate EpCAM-positive cells bound to the magnetic beads. (H) Cells that are CK-positive, DAPI-positive and CD45-negative are identified as CTCs and selected for further analysis. Image was created using www.Figdraw.com. CTCs, circulating tumor cells; EpCAMs, epithelial cell adhesion molecules.

clusters, including those with diverse phenotypes, because they rely on size-based rather than molecular-based characteristics (54).

In summary, CTC enrichment methods based on physical properties are label-free and relatively simple to perform. However, they often suffer from limited enrichment purity and a risk of cell loss. As a result, they are best suited for preliminary enrichment, and their combination with immunoaffinity methods may offer a more effective strategy to increase detection sensitivity and specificity.

Enrichment methods based on the biological characteristics of CTCs. CTCs are typically enriched through positive selection using antibodies targeting tumor-associated antigens. These antigens include epithelial cell adhesion molecule (EpCAM), mesenchymal markers, cytokeratins (CKs), mucin-1, human epidermal growth factor receptor 2 (HER2) and epithelial growth factor receptor (EGFR), among others (57,58). Alternatively, CTCs can be enriched through negative selection by removing unwanted leukocytes using antibodies against markers such as CD45; however, the EpCAM-dependent positive selection technique is the most widely used (59,60).

Positive enrichment. Currently, several methods are popular for the positive enrichment of CTCs. The CellSearch system, which uses an EpCAM-based immunoassay, is the first and only FDA-approved CTC assay (61) for colorectal, breast, prostate and other tumors (62). The CellSearch system's principle involves the use of ferromagnetic fluid nanoparticle-labeled antibodies that specifically bind to EpCAM, which is highly expressed on the surface of a wide range of tumor cells. CTCs are then separated from other blood cells by means of a magnet. The detailed collection

process is illustrated in Fig. 3. However, EpCAM-antibody interactions can cause CTCs to adhere to the device's surface, making their release difficult. To address the shortcomings of the CellSearch system, researchers developed MagSweeper, an immunomagnetic cell separator that enriches CTCs while eliminating nonmagnetic particle-bound cells (63).

Advances in microfluidic chip technology and nanomaterials have further improved the sensitivity, specificity, and cost efficiency of CTC detection (32). A key feature of microfluidic chips is their flexible combination and large-scale integration of multiple functional units on a miniature platform. These chips represent an interdisciplinary achievement combining physics, chemistry, material science, medical science, mechanics, optics, mechanical engineering and bioengineering. Microfluidic devices utilizing antigen-antibody interactions can be categorized into two types: Microcolumns (requiring chemical modification of the monoclonal antibody coating) and devices using antibody-coated surfaces (38). The CTC microarray isolates target cells by enabling the interaction of CTCs with an array of 78,000 microcolumns coated with EpCAM antibodies under precisely controlled laminar flow conditions. This method does not require pretreatment of the samples and preserves cellular activity (22).

The size-determined immunocapture chip, a deterministic lateral displacement (DLD) microarray, captures CTCs with high sensitivity, efficiency and purity. This chip features microcolumns that are surface modified with EpCAM antibodies. Relatively large CTCs are deflected laterally as they collide with microposts, prolonging their flow time, while smaller blood cells advance with the laminar flow (64) and selectively increase the probability of interaction with the microposts. This method achieves capture efficiencies exceeding 92%, with a

purity of 82% (65,66). Another approach employs microfluidic encapsulated microarrays with a staggered herringbone design on channel ceilings, increasing cell-surface contact points. This design significantly enhances capture efficiency while preserving cellular activity, enabling downstream analyses such as cell culture and genomic studies. However, CTCs undergoing epithelial-mesenchymal transition (EMT) exhibit increased invasive and metastatic potential (67). During the EMT, the expression of tumor markers on the cell surface is downregulated (68,69), posing significant challenges for devices that rely exclusively on anti-EpCAM antibodies for CTC capture (70). A synergistic, dual-antibody chip based on the DLD principle partially addresses this challenge. In this approach, anti-ASGPR and anti-EpCAM antibodies are simultaneously modified in parallel on a microfluidic synergistic chip, with a capture efficiency of more than 87% per channel (71).

Over time, advancements in research have transitioned CTC capture technology from first-generation chips, such as the CTC-Chip, to second-generation chips, such as the HB-Chip, GO-Chip and GEM-Chip. Currently, a third-generation microfluidic chip, called the CTC-iChip, has emerged as a recognized innovation in the field (72-74). Compared with first-generation CTC microarrays, second-generation microarray technology, specifically surface capture devices, is simpler to fabricate and more efficient at capturing CTCs. However, these devices have serious limitations, as the captured CTCs are immobilized on the device surface, making their recovery for downstream genomic analyses challenging. While trypsin digestion can be used to release the captured cells, this process may remove some receptor proteins from the surface of the CTCs and interfere with subsequent analyses.

Rostami *et al* (75) proposed overcoming this limitation by developing a novel microfluidic chip incorporating smaller magnetic pores and utilizing aptamers as an alternative to conventional antibody-based CTC capture. Aptamers provide several advantages, including high binding affinity, low cost, facile chemical modification, and straightforward release mechanisms (75). To increase the capture rate of specific CTCs, researchers have developed novel capture platforms with specially designed surfaces conjugated with engineered capture molecules. A representative example is the TDN-nanogold click chip developed by Sun *et al* (76), which features a gold-modified surface functionalized with DNA nanostructures. This advanced design, combined with a microfluidic chamber, enhances specific CTC capture. Traditional methods often have difficulty capturing CTCs that have undergone EMT because of the consequent reduction in the expression of common capture targets such as EpCAM. However, the TDN-Nanogold Click Chip uses click chemistry (77) to effectively and irreversibly capture CTCs with a capture rate as high as $91.3 \pm 6.7\%$ (76).

Third-generation microfluidic chip technology, such as the inertial focusing-enhanced microfluidic system (CTC-iChip), enables the efficient negative depletion of anuclear RBCs and platelets. This is achieved through the hydrodynamic separation of nucleated leukocytes and CTCs in plasma, followed by inertial focusing into a single stream. Magnetic bead-labeled leukocytes are then efficiently removed by magnetophoresis, resulting in a solution enriched with CTCs with both epithelial

and mesenchymal features (78). However, this system has notable drawbacks, including high initial cost a long setup time, limited ability to analyze single-cell molecules, reliance on multiple manually attached chips, and challenges in clinical implementation (32).

To reduce the cost of capturing CTCs, a recent study proposed the use of EpCAM-resistant nanofiber cellulose scaffolds with immobilized Fe_3O_4 NPs for harvesting CTCs (25). Cellulose nanostructures can be categorized into two main types: Cellulose nanocrystals and cellulose nanofibers (CNFs). These nanomaterials offer several advantages, including relatively high aspect ratios, large specific surface areas, biodegradability, environmental sustainability, biocompatibility and non-toxicity (25). Compared with fibrous scaffolds, rigid nanocrystal scaffolds interact more efficiently with CTCs (25); however, CNFs have a higher aspect ratio, which increases the probability of intermolecular interactions. Kumar *et al* (79) developed a CNF-based microfluidic chip with a capture efficiency of 79-98%. However, its average flow rate of $5 \mu\text{l}/\text{min}$ poses a significant limitation for processing large volumes of blood, as it reduces the point of contact necessary for effective CTC capture (79). By contrast, a microchip developed by Cui *et al* (80) and integrated with ZnO nanowires achieved a high CTC capture efficiency of $91.1 \pm 5.5\%$ while maintaining excellent cell viability (96%). Notably, the platform also enabled damage-free release of captured cells. This advancement in microarray technology facilitates single-cell analysis and holds considerable potential for advancing personalized medicine, particularly in the development of therapeutics targeting cancer stem cells (80). Researchers have also recently developed a nanoplatform called NICHE for determining the relationships between host genes and the microenvironment in living cells (81). This innovative platform combines microfluidics and nanofluidics to efficiently capture CTCs from blood samples. The platform then integrates stable multichannel fluorescent probes into live CTCs, enabling rapid *in situ* quantification of target gene expression. The NICHE microfluidic device is unique in that it allows simultaneous gene expression and phenotypic analyses on individual cells *in situ*. It also has the potential to generate predictive indices for screening patients who are likely to benefit from ICIs.

Negative enrichment. The decreased expression of EpCAM on the surface of cells undergoing EMT results in a reduced capture rate when an EpCAM-dependent positive enrichment method is used (38). To overcome these shortcomings, label-free and negative enrichment methods have been developed. Similar to the previously mentioned CTC-iChip, a 3D microfluidic device has been developed and fabricated for the negative enrichment of CTCs from whole blood samples (82). The device captures unlabeled leukocytes on its inner surface using antibodies targeting leukocyte-specific membrane antigens and separates red blood cells and platelets from CTCs according to their small size. This method is compatible with subsequent molecular, immunocytochemical and functional analyses.

In conclusion, positive enrichment methods offer superior specificity and purity, making them effective for enriching CTCs with known markers. However, their reliance on specific markers may cause them to miss certain CTC subtypes. Conversely, negative enrichment methods can capture a more

diverse range of CTCs but generally result in lower enrichment purity and a risk of cell loss. In simple terms, positive enrichment methods are best suited for applications requiring high sensitivity and specificity, whereas negative enrichment methods are more appropriate for capturing a heterogeneous CTC population, particularly when markers are unknown or inconsistently expressed. A combined approach may provide a more thorough CTC detection strategy, despite being more complex to implement. In addition to the above-described platforms, numerous potential CTC enrichment methods have been developed in recent years (Table SI).

3. CTC detection methods

Although there are numerous complex methods for enriching CTCs, the detection procedure is still relatively straightforward. The current identification methods include immunofluorescence, reverse transcription-polymerase chain reaction (RT-PCR) and next-generation sequencing (NGS).

Immunofluorescence detection. This technique involves labeling enriched cells after fixation using fluorescent markers such as anti-human pancytokeratin (CK), anti-mouse CD45, DAPI, and a cocktail of antibodies targeting cell surface proteins such as EpCAM, EGFR and HER2. CTCs are identified as DAPI⁺/CK⁺/CD45⁻ cells (83). The primary advantage of this method is its convenience in observing the phenotype and morphology of cells under a fluorescence microscope (84). However, the expression of protein markers often lacks stability and cannot be used to distinguish between viable and non-viable cells (83,85).

RT-PCR. RT-PCR can be used to detect target cells by reverse transcription, in which the enzyme reverse transcriptase converts RNA into complementary DNA using a specific primer (86). While this method allows for the genetic identification of target cells, it cannot distinguish non-viable cells from viable cells, limiting its application in analyses requiring live cells, such as assessments of cellular deformability and drug responses.

EPISPOT. This method enables the detection of specific proteins secreted by CTCs. It involves culturing enriched CTCs and tagging secreted proteins with fluorescently labeled antibodies. Each immunofluorescent spot represents a living cell, offering high specificity and stability (87). However, this technique requires sufficient accumulation of secreted proteins to form immune spots, which can be time-consuming.

Fluorescence in situ hybridization (FISH). FISH can be used to detect CTCs by labeling abnormal chromosomal numbers or structures specific to tumor cells. This genetic method offers high sensitivity and specificity for identifying CTCs but it can suffer interference from abnormal chromosomes in the patient's blood and the limited scope of chromosomal probes, which can detect only a limited number of specific markers (54,88).

NGS. NGS provides insights into genetic structures and expression states at the single-cell level, allowing for the genotyping

of CTCs and the detection of tumor-specific mutations (89). While this method offers unparalleled precision, highly effective upstream enrichment techniques and stringent QC are needed to ensure the purity and abundance of CTCs (90) (Table SII).

4. Clinical applications of CTCs in immunotherapy

Immunotherapy involves activating immune cells for the recognition and destruction of cancer cells (91). Common immune checkpoints include cytotoxic T-lymphocyte-associated antigen 4, programmed cell death protein 1 (PD-1), programmed cell death ligand 1 (PD-L1), lymphocyte activation gene 3, B7-H3 and T-cell immunoglobulin and mucin-domain containing-3. Among these, PD-1 and PD-L1 are key molecules in the immune system, and play crucial roles in the immune escape of tumor cells (92). PD-1 is an immunosuppressive molecule that is primarily expressed on activated T and B cells, whereas PD-L1 is predominantly upregulated in various tumor cells (93). When PD-L1 binds to PD-1 on T cells, it promotes tumor immune escape. PD-1 inhibitors work by blocking the interaction between PD-1 and PD-L1 (94), thereby restoring the antitumor activity of immune cells, enhancing the immune response, reducing tumor cell proliferation and metastasis, and increasing patient survival rates (95,96). Numerous studies have demonstrated the important clinical value of CTCs in immunotherapy.

Predictive role of CTCs in tumor immunotherapy. Numerous studies have shown the usefulness of CTCs in clinical prognosis; however, their role in predicting the response to tumor immunotherapy has been relatively unexplored. Research has demonstrated that the presence of CTCs serves as an independent prognostic indicator of shorter overall survival (OS) in patients with various cancers, including non-small cell lung cancer (NSCLC) (97). Lin *et al* (98) reported a significant reduction in the number of CTCs in the peripheral blood of patients with stage IV NSCLC who were treated with NK cell immunotherapy on Days 7 and 30 of treatment. These findings suggest that CTCs may serve as valuable biomarkers for evaluating treatment efficacy (98). A different study revealed that the presence of CTCs was a predictor of poor durable response rates following immune checkpoint inhibitor (ICI) therapy. A durable response is defined as stable disease or a partial or complete response without disease progression at six months (99). The prognostic and predictive value of CTCs was further demonstrated in a phase 1b study using lysosomal virus immunotherapy, where a reduction in CTC counts was observed in baseline-positive patients who responded effectively to treatment. Moreover, a study by Castello *et al* (100) revealed that the mean CTC count was significantly lower in patients with NSCLC treated with ICIs than in untreated patients. The results of the aforementioned study also revealed that combining the mean CTC count with the median metabolic tumor volume was associated with PFS and OS after 8 weeks of ICI treatment (100). Importantly, CTC counts were identified as independent predictors of both PFS and OS (101,102). Gu *et al* (103) developed a risk model to predict prognosis on the basis of aberrantly methylated DNA in CTCs from patients with lung adenocarcinoma (LUAD).

In the aforementioned study, patients were categorized into high-risk and low-risk groups on the basis of their median risk score. High-risk patients were associated with poor prognosis, whereas low-risk patients demonstrated hyperimmunocompetence, which correlated with favorable prognosis. Univariate and multivariate Cox regression analyses confirmed that the risk score was an independent prognostic factor for survival in patients with LUAD (103).

Antitumor immunotherapeutic strategies have emerged as promising approaches for the treatment of various cancers. The expression level of PD-L1 is a crucial indicator of the immune status of patients with cancer and a key marker for predicting the efficacy of immunotherapy (104). Studies have suggested that PD-L1 expression serves as a predictive biomarker for immunotherapy response in certain solid tumors, including NSCLC, melanoma and renal cell carcinoma (95,105,106). For example, in a study of immunotherapy for NSCLC, patients with PD-L1-negative CTCs at baseline, as well as at 3 and 6 months of nivolumab treatment, achieved favorable clinical benefits at the 6-month mark. By contrast, tumor progression occurred in patients with PD-L1-positive CTCs. These findings suggest that PD-L1 expression on CTCs could serve as a predictive marker for the immunotherapy response (107). Dall'Olio *et al* (108) reported that pretreatment with PD-L1-positive CTCs was associated with improved survival, whereas post-treatment with PD-L1-positive CTCs was correlated with worse survival in patients with advanced NSCLC receiving PD-L1/PD-1 inhibitors as second- or third-line therapy (108). In a phase 1 trial of the PD-1 inhibitor IBI308 for the treatment of gastrointestinal tumors, the presence of CTCs with high PD-L1 expression was associated with significantly better disease control rates. The abundance of PD-L1-positive CTCs at baseline was also found to predict PFS, suggesting that monitoring CTC dynamics could provide an early indication of therapeutic response.

The authors' hypothesis that CTCs can serve as predictive biomarkers of tumor immunotherapy efficacy was also supported by a study on metastatic genitourinary tumors. PD-L1-positive CTCs, elevated CTC counts, and specific CTC morphological subtypes were associated with shorter OS from immunotherapy for metastatic genitourinary cancers (109). Combining an assessment of CTC cluster abundance with single CTC counts significantly improved prognostic accuracy (110). Thus, CTCs not only are useful for assessing the prognosis of patients with cancer, but they also serve as important predictive biomarkers for monitoring the efficacy of immunotherapy and developing robust personalized treatment strategies. Most studies have focused on NSCLC, colorectal cancer, breast cancer, prostate cancer and melanoma; the predictive value of CTCs in other cancer types requires further exploration.

Immunotherapeutic strategies against CTCs. Tumor metastasis and recurrence remain the primary causes of cancer-related death and present difficult treatment challenges. The development and successful application of ICIs represent major breakthroughs in tumor therapy in recent years. While it is not yet clear whether CTCs are more susceptible to interventions targeting PD-L1 than cells in solid tumors are, activating the immune system by blocking PD-L1 expression

on CTCs and reducing their numbers in peripheral circulation could decrease the likelihood of malignant tumor recurrence and metastasis, representing a promising new therapeutic approach (8,111). In addition to PD-L1, CD47 is recognized as a crucial immune checkpoint that is highly expressed on tumor cells. CD47 interacts with signal-regulated protein alpha on macrophages, transmitting inhibitory signals that suppress phagocytosis (112). Thus, PD-L1 acts as the key 'don't find me' signal for the adaptive immune system, whereas CD47 functions as the 'don't eat me' signal for the innate immune system and regulates the adaptive immune response (113,114). Targeting both PD-L1 and CD47 simultaneously with specific antibodies has been shown to be more effective at suppressing lung metastases than targeting PD-L1 or CD47 alone (114) (Fig. 4).

CTCs often express multiple immune checkpoints, including PD-L1 and CD47 (115). Lian *et al* (115) demonstrated a significant reduction in tumor growth and metastasis by using antibodies specific to PD-L1 and CD47 expressed on the surface of CTCs. Additionally, Yu *et al* (116) reported on O-TPNVs, a type of nanovesicle created by fusing TIGIT-expressing cell membranes with platelet-derived membranes (TPNVs) and encapsulating oxaliplatin (OXA). The platelet-derived membrane components enable O-TPNVs to specifically target postsurgical wounds and interact with CTCs. OXA not only directly eliminates residual tumor cells and CTCs, but it also induces immunogenic cell death and activates the immune system. Furthermore, O-TPNVs exhibit synergistic chemotherapeutic and immunotherapeutic effects, effectively preventing the recurrence and metastasis of triple-negative breast cancer (4T1) after surgery (117). In addition, clinical trials are actively exploring the mechanisms involved in blocking tumor immune escape through ICIs. For example, Jacot *et al* (118) evaluated the clinical and pathological relevance, as well as the prognostic value, of PD-L1-positive CTCs in a cohort of 72 patients with metastatic breast cancer (MBC) as part of a prospective clinical trial. PD-L1-positive CTCs were associated with survival outcomes in patients with MBC (Clinical Trial Registry: NCT02866149) (118). In conclusion, immunotherapeutic targeting of CTCs is likely a therapeutic strategy for preventing tumor metastasis and recurrence.

5. Conclusions and perspectives

CTCs were first discovered more than a century ago, and extensive studies have since demonstrated their importance in clinical applications. CTCs offer the potential to detect relevant target lesions at an early stage, even before imaging can reveal a tumor. They can also be used in combination with other tumor markers to guide therapy, monitor patients' postoperative treatment, and predict prognosis. Despite the promising results from studies of these novel applications, significant challenges remain before CTCs can be fully integrated into clinical practice.

The rarity and heterogeneity of CTCs present major obstacles. While recent advances in CTC enrichment and detection technology have been achieved, differences in equipment and detection methods can lead to differences in the interpretation of results. Currently, standardized, robust CTC detection procedures are lacking. Moreover, most existing

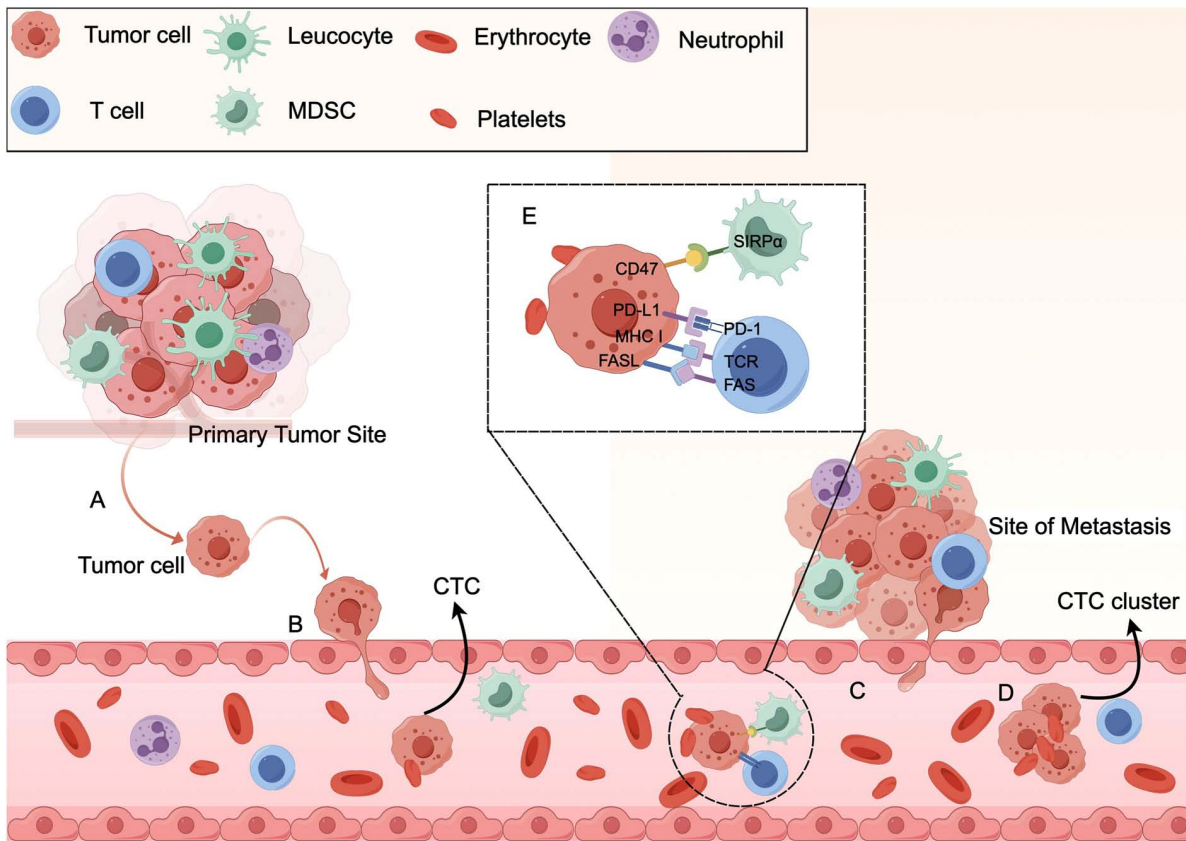


Figure 4. Mechanisms underlying CTC immune evasion. (A) Tumor cells are shed from the primary focus. (B) Dislodged tumor cells traverse the mesenchymal connective tissue, penetrate the blood vessel walls and enter the circulation as CTCs. (C) Tumor cells that enter the bloodstream remain in circulation temporarily before extravasating (exiting the circulatory system) to attach to other organs or tissues. These cells then begin to proliferate, eventually forming new metastases. (D) In addition to individual CTCs, clusters of CTCs exhibit significantly higher metastatic potential. (E) Immune escape mechanisms: These include modifications of MHC-I expression; ‘don't eat me’ signaling via CD47, upregulation of PD-L1 expression, and the expression of FasL, which induces apoptosis in Fas-positive effector immune cells. Image was created using www.Figdraw.com. CTCs, circulating tumor cells; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; MHC, major histocompatibility complex; TCR, T cell receptor; MDSC, myeloid-derived suppressor cell.

CTC enrichment and detection methods are expensive, limiting their widespread use in clinical settings. To address this issue, numerous researchers are now focusing on utilizing nanoscale materials to develop more cost-effective platforms for CTC separation and enrichment. While laboratory testing of these approaches has shown excellent results, large-scale experiments are needed to validate their feasibility for clinical applications.

In summary, CTCs not only are useful for assessing the prognosis of patients with cancer in clinical applications, but they also serve as important predictive biomarkers for monitoring the effectiveness of immunotherapy and personalized treatment strategies. However, current research on CTCs has focused primarily on a limited number of cancer types, and their predictive value in other cancers requires further investigation. Immunotherapy is a prominent research focus for the treatment of malignant tumors, but no large studies have been conducted that definitively show the efficacy of using CTCs as targets for tumor immunotherapy. Using immunotherapy to target CTCs has the potential to reduce the risk of metastasis and recurrence in malignant tumors, particularly in postoperative patients, offering a greater hope for permanent remission.

In conclusion, advancements in CTC isolation and detection technologies, along with their gradual integration into

clinical applications, hold great potential as key components of precision individualized medicine in the future.

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Availability of data and materials

Not applicable.

Authors' contributions

PZ and QY conducted data mining, writing, draft, and figure preparation. YH and YF conceptualized, designed and supervised the study, and revised the manuscript. LZ and KX

reviewed and edited the manuscript. All authors read and approved the final version of the manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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