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Sensitive and specific detection of circulating tumor cells promotes precision medicine for cancer

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Abstract

Circulating tumor cells (CTCs) have the potential to provide genetic information for heterogeneous tumors, which may be useful for monitoring disease progression and developing personalized therapies. However, the isolation of CTCs for molecular analysis is challenging due to their extreme rarity and phenotypic heterogeneity, which hinders the transformation of CTCs into traditional clinical applications. In order to achieve clinically significant CTC detection, devices utilizing novel microfluidics and nanotechnology have been developed to achieve high sensitivity and specificity capture of CTCs. In this review, we discuss these newly developed devices for CTC capture and molecular characterization for early diagnosis and determining ideal treatment regimen to better manage these cancers clinically. In addition, the potential prognostic values of CTCs as treatment guidelines and that ultimately contribute to realize personalized treatment are also discussed.

Keywords: Circulating tumor cells, sensitivity, treatment, precision medicine

INTRODUCTION

The main cause of cancer-related mortality is cancer metastasis. During this greatly complicated and multi-stage disseminative process, tumor cells (the seeds) detach from primary roots, shed into blood and lymph circulation, undergo the immune attack and shear stress, travel to preferable metastasis soil, and eventually seed and proliferate to develop metastases. On their way to the potential organs, these circulating tumor cells (CTCs) undergo epithelial-mesenchymal transition (EMT)[1], thereby resulting in enhanced motility and migratory ability that facilitates vasculature invasion. Upon reaching a suitable niche, the CTCs undergo mesenchymal-epithelial transition (MET), subsequently reacquiring the stem cell properties and reactivating proliferative capability to colonize at metastatic sites[2]. In order to prevent and surveil the development of metastasis disease, especially metastasic carcinoma, the detection and characterization of CTCs are of great interest to scientists. CTCs were first detected in cancer patient in 1869 by Australian physician named Thomas Ashworth. In the past couple of decades, numerous studies have suggested that the presence of CTCs in the blood of cancer patients has the clinical potential as a noninvasive diagnosis marker and a prognosis indicator known as a "liquid biopsy" to replace traditional invasive biopsy, whilst also facilitating technical advances for detection of CTCs.

CTC analysis has a variety of clinical applications, including real-time non-invasive monitoring of CTCs as biomarkers for new cancer therapies as well as identifying new potential therapeutic targets that directly inhibit cancer metastasis. Although the potential applications of CTC analysis appear to be very promising, due to the rarity (one CTC per billion hematologic cells) and heterogeneity (e.g., differences in morphology and gene expression) of CTCs in the blood of cancer patients, there are few commercially available techniques for clinical use. High sensitivity and specificity of CTC detection methods thus have a great impact on improving patient outcomes. Therefore, currently available technologies for CTC detection have become increasingly more sensitive and reliable, with the goal of early cancer detection and thus successful cancer treatment. An important new direction in this field is the development of devices and materials that provide information beyond CTC enumeration. Integrated devices allow for the separation of heterogeneous CTCs to facilitate a more in-depth characterization of these cells (e.g., phenotypic and molecular profiling) to develop a personalized treatment plan. Nanomaterials and microfluidic-based nanotechnologies may be the most promising strategies for implementing ideal CTC capture devices to replace traditional tools, primarily relying on their small size, high throughput capacity and large surface-to-volume ratio to solve the problem of CTC heterogeneity[3]. In this review, we will provide an overview of current CTC isolation strategies and molecular characterization with brief insights into the potential clinical implications of CTC capture and characterization.

SENSITIVE CTC ISOLATION METHODS

CTCs may have the potential to predict the disease progression in patients with early-stage or advanced cancer, even before the formation of primary tumor. However, the extreme rarity of CTCs in blood poses a challenge for detecting CTCs from blood; for example, one study indicated that only 1.43% of 350 metastasis cancer patients had ≥ 500 CTCs/7.5 mL blood[4]. Inability to draw large volume of blood from patients highlights the need for improved CTC isolation methods to achieve sensitive and specific CTC detection in small sample volumes. CTC isolation methods have been developed based on either biological (surface antigen, cytoplasmic protein, invasion capacity, *et al.*) or physical (size, density, deformability and charge, *et al.*) properties of tumor cells. We discuss the most popular technologies and latest advances in the following sections [Figure 1].

Detection of CTCs based on their biological properties

Immunomagnetic beads-based isolation

The most widely used enrichment method is a positive selection method based on the epithelial cell adhesion molecule (EpCAM) antibodies[5-7]. So far, CellSearch System (Menarini Silicon Biosystems, Italy) is the first and also the only one being up to the standard of US Food and Drug Administration (FDA),

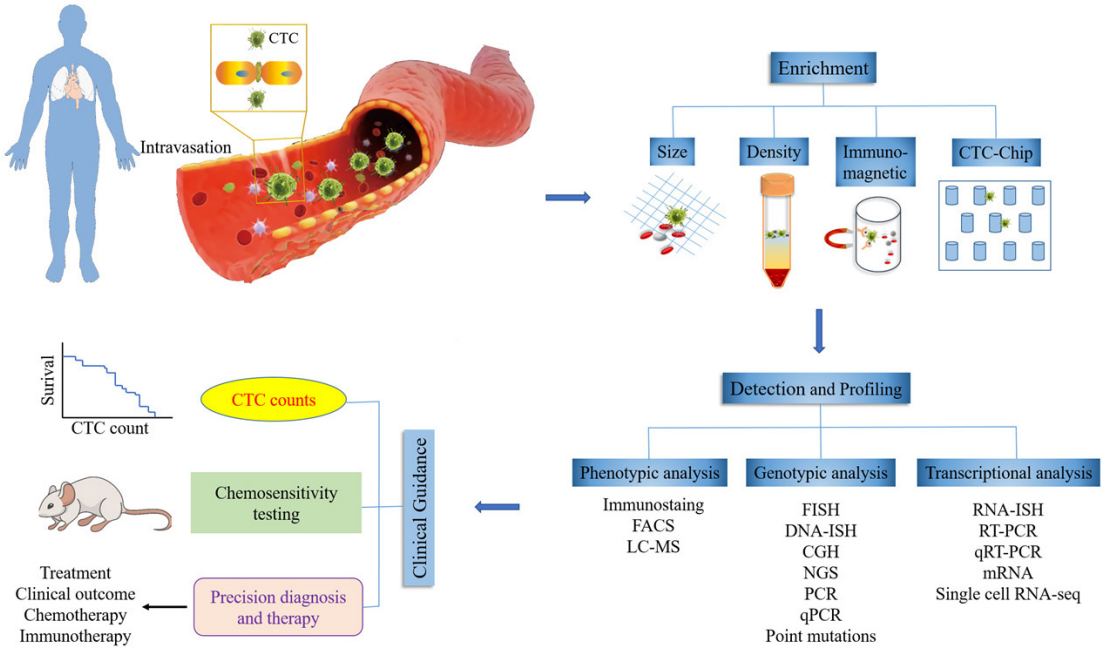

Figure 1. Illustration of current applications of CTC technologies. The CTCs exit the primary tumor and intravasate into the bloodstream. CTCs are enriched through various CTC isolation technologies such as size, density, immunomagnetic and CTC-Chip. Detection methods are utilized to detect CTCs based on phenotypic, genotypic and transcriptional analysis. The clinical applications of isolated and detected CTCs. The CTC count is associated with the potential of patient's survival. CTCs can be good chemotherapy monitoring markers for predicting drug sensitivity/resistance in preclinical and clinical settings. CTC: circulating tumor cells; FACS: fluorescence activated cell sorting; LC-MS: liquid chromatograph-mass spectrometry; FISH: fluorescence in situ hybridization; CGH: comparative genomic hybridization; NGS: next-generation sequencing; PCR: polymerase chain reaction; qRT-PCR: quantitative reverse transcription-polymerase chain reaction

which consists of the CellTracks Autoprep and the CellSearch Epithelial Cell kit, integrating EpCAM based immunomagnetically enrichment, 4',2-diamidino-2-phenylindole (DAPI) based cell nuclei staining, CD45-Allophycocyan specified leukocyte negative selection and cytokeratin 8,18,19-Phycoerythrin specified epithelial cells positive selection into an objective indicator (EpCAM+, DAPI+, CD45-, cytokeratin+) of CTC counts. In 2004, it was cleared for monitoring the outcome of therapies and optimizing clinical decision for breast cancer; later, it was also cleared for use in prostate and colorectal cancers. Through the CellSearch, which has become the benchmark for all other CTCs isolation methods, CTC counts have been associated with prognosis for progression-free survival (PFS) and overall survival (OS) in these three kinds of metastatic cancer[8-10].

Although clinical correlations have been identified, methods for large scale isolation of CTCs from peripheral blood are lacking, therefore, efforts have been focused on improving the isolation sensitivity and efficiency. Talasaz *et al.*[11], reported a magnetic sweeping device (MagSweeper, Stanford University, Stanford) consisting of a nonadherent plastic sheath covered magnetic rod with anti-EpCAM antibody functionalized beads, allowing for a ~60% capture efficiency to target cells and a purity of 100% for HLA-A2 cells. Ephesia technology integrated anti-EpCAM functionalized self-assembled magnetic beads with microfluidics, demonstrating a capture efficiency > 94%[12]. Similar immunomagnetic platforms [Figure 2A] also included the Magnetic Sifter with magnetic pores incorporated into a microfluidic chip[13]. Moreover, compared with CellSearch system, Adna Test (Qiagen, Hannover), a highly specific immunomagnetic cell-isolation system, with its improved antibody cocktails provided an effective approach to increase the efficiency of CTCs capture and complements the CellSearch for detection of CTCs[14]. Mayo *et al.*[15] utilized MACS cell separation platform (Miltenyi Biotec) based on a mixture of cytokeratin (CK) coated magnetic beads to isolate CTCs in lung cancer patients.

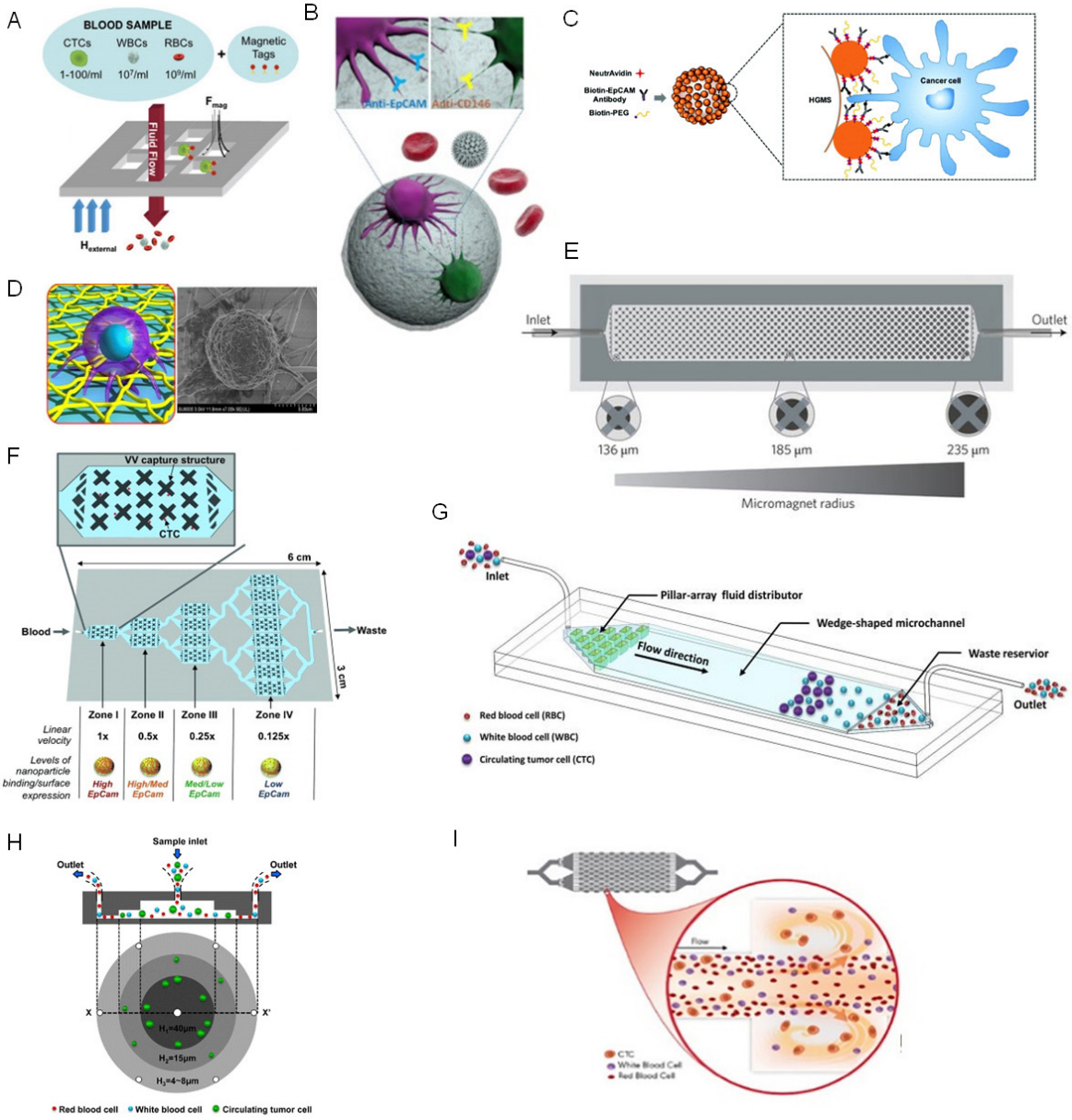

Figure 2. Strategies for CTC enrichment. A: A magnetic sifter device for CTC isolation when a magnetic field is applied. Magnetically labeled CTCs are captured at the edges of the pores, while unlabeled cells pass through the pores under fluid flow; B: dual antibodies (anti-EpCAM and anti-CD146) and biodegradable gelatin nanoparticle-coated microbeads for the capture of mesenchymal CTCs; C: a microfluidic device embedded a wedge-shaped microchamber for cell separation based on multiple biophysical properties; D: a 3D bionic cytosensor with PLGA nanofibers for CTC capture; E: vortex technology exploited for CTC isolating; F: a multizone velocity valley device for isolating heterogeneous CTCs in four different regions of varying linear velocities; G: the hollow glass microspheres with nanotopographical structures (NSHGMS) for excellent CTC isolation; H: a microfluidic device embedded a pyramid-shaped microchamber for size-based CTC separation; I: a MagRC approach for separating and in-line profiling of heterogeneous CTCs. A: Copyright Royal Society of Chemistry, 2013. Reproduced with permission from reference[13]; B: Copyright Ivyspring International Publisher, 2013. Reproduced with permission from reference[27]; C: Copyright Royal Society of Chemistry, 2018. Reproduced with permission from reference[42]; D: Copyright BioMed Central, 2018. Reproduced with permission from reference[43]; E: Copyright Nature, 2017. Reproduced with permission from reference[45]; F: Copyright Wiley, 2015. Reproduced with permission from reference[46]; G: Copyright Institute Of Electrical And Electronics Engineers, 2018. Reproduced with permission from reference[52]; H: Copyright Springer, 2018. Reproduced with permission from reference[53]; I: Copyright Wiley, 2018. Reproduced with permission from reference[54]

However, a number of studies have shown that the levels of CTCs estimated by EpCAM-based methods including CellSearch, is uncorrelated with prognosis in patients with some types of carcinomas. Most of the evidence attributes this inconsistency to the large degree of heterogeneity in CTCs. Specifically, CTCs might undergo full (or partial) EMT during dissemination, resulting in several phenotypes including epithelial, mesenchymal or hybrid (epithelial/mesenchymal) CTCs. These subpopulations of cells may insufficiently bind to antibodies, thereby evading detection[16,17]. Therefore, a lack of sensitive and specific biomarkers still hinders the isolation and detection of CTCs. Recent studies provide some probabilities. Here are some examples of successful markers. Glycan sialyl-Tn (STn) is often associated with cancer metastasis and expressed in metastatic colorectal and bladder tumors. Neves *et al.*[18] fabricated a STn affinity-based microfluidic device for specifically isolating STn+ CTCs, following an enzyme-based method to recover viable CTCs for downstream analyses. It showed greatly higher isolation efficiency from the blood of patients with advanced bladder and colorectal cancers. Plastin3 (PLS3) is expressed in metastatic cancer cells but absent in normal cells[19]. Similarly, telomerase which is expressed at high levels in almost all the cancer cells, but not in normal cells, plays an important role in cancer immortality by replenishing chromosome ends[20]. Green fluorescent protein (GFP) fused adenoviral was employed as a probe to target telomerase in cancer cells, and this strategy was applied to detect and isolate CTCs in Non-Small Cell Lung Cancer (NSCLC) to evaluate response to radiation therapy and to potentially detect recurrence and progression of disease. Oncofetal chondroitin sulfate (ofCS) is expressed in both epithelial and mesenchymal tumor cells, as well as the cells that have undergone EMT, suggesting that it may be an ideal candidate for isolating and analyzing CTCs[21,22]. Agerbæk *et al.*[23] employed recombinant VAR2CA (rVAR2) to efficiently target ofCS expressed CTCs from patients with hepatic, prostate, pancreatic or lung cancer, allowing for isolation of a larger and more diverse population of CTCs compared to anti-EpCAM-antibody approaches. More recently, Ding *et al.*[24] detected Folate receptor (FR) positive CTCs in peripheral blood from 200 patients with lung adenocarcinoma, and further determined that FR+ CTC number could be used for screening solitary pulmonary nodules (SPNs) in patients and diagnosing early-stage lung cancer with sensitivity of 70.2% and specificity of 79.3%. Meanwhile, more specific biomarker for specific subgroup of CTCs is of interest. Cyclooxygenase-2 (COX-2) has been implicated in transforming growth factor-β (TGF-β1) mediated EMT progress[25] and has a higher level of expression in subpopulations of mesenchymal CTCs correlated with distant metastases[26]. These results suggested FR might be a novel biomarker for isolation and therapy targets. A subpopulation of tumor cells can express cluster of differentiation 146 (CD146) during EMT process, during which EpCAM expression is reduced. Therefore, Huang *et al.*[27] [Figure 2B] designed dual antibodies (anti-EpCAM and anti-CD146) and biodegradable gelatin nanoparticle-coated microbeads to improve the capture of mesenchymal CTCs, achieving high efficiency (> 80%) and high cell viability (92.5%).

All aforementioned *ex vivo* detection systems require substantial quantities of blood. The GILUPI CellCollector (NANOMEDIZIN), approved by Conformite Europeenne in 2012, is another commercial EpCAM positive based selection device and is the first developed *in vivo* CTC isolation system to overcome the limitations of blood sample volume[28-30].

Except those EpCAM-based positive selection, CD45 negative selection is applied to deplete the CD45+ cells, mostly using RosetteSep system (Stem Cell Technology, Vancouver), and to analyze the EpCAM-negative CTCs in combination with EPISPOT (Epithelial Immunospot assay, France). Ramirez *et al.*[31] first evaluated the EPISPOT assay on a large cohort of metastatic breast cancer patients with a positive rate of 59% compared with the 48% positive rate using CellSearch, demonstrating its clinical prognostic relevance.

Microfluidic and nanotechnology-based CTC devices

Microfluidic devices enable efficient processing of complex blood samples with minimal damage to target cells. Owing to the synergistic benefits of the microfluidic devices and immunomagnetic separation, microchip-based immunomagnetic technologies are also commonly used for CTC detection. The most representative

microfluidic device based on anti-EpCAM for CTCs isolation is a microscope slide sized CTC-Chip with a mass of geometrically distributed microposts coated with anti-EpCAM[32]. A 98% viability of captured CTCs was reported with minimal preprocessing and low flow stress[33]. Ozkumur *et al.*[34] developed an automated platform, termed "CTC-iChip", combining the strengths of microfluidics and the benefits of magnetic-based cell isolation for single-cell separation. This CTC-iChip was able to detect the *EML4-ALK* gene fusion in lung cancer, suggesting that it could be a promising tool for clinical diagnosis. Then Stott *et al.*[35] developed a microvortex-generating herringbone (HB)-Chip for effective capture of CTCs. The micromixer device was fabricated to enhance the cell-surface interaction. Subsequently, improvement was achieved by employing nanostructured substrates and chaotic micromixers, increasing the recovery rate up to 95%[36].

For the sake of increasing the sensitivity of capturing exceedingly rare CTCs, many efforts have been made to fabricate nanostructures into the microfluidics to increase the interaction between ligands and cells; such devices include electropolymerized polymer nanodots[37], electrospun TiO2 nanofiber[38], and silicon nanowires[39-41]. More recently, Dong *et al.*[42] [Figure 2C] utilized a nanotopographical surface (^{NS}HGMS), based on the CTC isolation technology of anti-EpCAM antibody modified Self-floating hollow glass microspheres (HGMS), to achieve excellent capture performance (93.6% ± 4.9% efficiency and 30 cells/mL detection limit in 20 min). A preferable method was based on a combination of advantages of different approaches. Wu *et al.*[43] [Figure 2D] tactfully fabricated a 3D bionic cytosensor with electrospun polymers (PLGA) nanofibers crosswise stacked on Ni micropillars for better CTC filopodia climbing, subsequently coupled with immuno-selection by anti-EpCAM quantum dots, demonstrating a sensitive detection range and limit of 10^1-10^5 cells/mL and 8 cells/mL, respectively, as well as a recovery range of 93.5%-105%.

However, fabricating these nanoscale substrates is time-consuming. Sheng *et al.*[44] developed a microfluidic device combined with DNA aptamer modified gold nanoparticles (AuNPs) to enhance the capture performance without elaborate establishment for nanostructure. When compared with aptamer on the surface alone, the binding efficiency of AuNPs-aptamer showed a 39-fold increase and the capture efficiency rose from 49% to 92%. In order to profile the dynamic phenotypes of rare CTCs, Poudineh *et al.*[45] [Figure 2E] fabricated a magnetic nanoparticles-enabled ranking cytometry (MagRC) approach to separate and in-line profile heterogeneous CTCs based on the longitudinal profile of magnetic field gradients. They demonstrated that this device was capable of profiling CTCs with higher sensitivity at a single-cell resolution in unprocessed blood from cancer patients compared to other previously developed magnetic sorting techniques. Similarly, an immunomagnetic nanoparticle-mediated binning and profiling approach [Figure 2F] was developed to separate CTCs with different phenotypes based on the differential expression of surface markers[46]. The CTC subpopulations could be spatially sorted in different compartments of a fluidic chip, providing a powerful means to sort heterogeneous CTCs and investigate EMT in patient CTCs.

Detection of CTCs based on their physical properties

CTCs undergo cellular processes (EMT, MET, *et al.*) during dissemination, resulting in a number of phenotypes. Thus, it is important to determine which CTC fractions possess greater metastatic potential and/or stronger resistance to immune surveillance and medical treatment. In this case, CTCs would have better prognostic and therapeutic values. The aforementioned methods depend on specific markers of interest for isolation; however, subpopulations of CTCs lacking the markers may be unintentionally overlooked. Therefore, additional methods that could serve as complements to protein markers are urgently needed.

Size-based CTC isolation

Alternative methods that isolate CTCs dependent on physical properties have been developed to replace or complement the antibody-based isolation methods. Most of the CTCs are believed to be larger than normal blood cells (leukocytes, erythrocytes). And pores with ≈ 8 μm in diameter have been shown to be appropriate for CTC detainment.

Thanks to various advantages, such as retention of cell morphology, antigen independence, and high sensitivity and specificity, membranous filter devices, for example, isolation by size of epithelial tumor cells (ISET) (Rarecells Diagnostics, Paris, France), have caught more attention in CTC researches. In a comparative study, Bai *et al.*[47] estimated the clinical effect of CTCs by using CellSearch system and ISET devices among patients with renal cell carcinoma (RCC), discovering that ISET was more appropriate for RCC patients. CTCBIOPSY (Wuhan YZY Medical Science and Technology Co., Ltd., China) is a commercial one-step ISET device which could complete automatic detection and identification within 10 min[48]. The Parsortix technology (ANGLE plc) incorporated a microscope slide sized cassette for CTC separation based on cell size and compressibility[49]. Owing to the excellent capture performance and the advantage of easy retrieval of viable CTCs for downstream analysis, the FDA clearance process of this device for diagnose is underway. The ClearCell FX system (Clearbridge BioMedics, Singapore), one of the first automated cell separation and retrieval systems, is a new label-free and size-based technology with extremely high recovery rates by dean flow fractionation[50]. These devices, together with other similar size exclusion platforms, such as ScreenCell (ScreenCell, France) based on microporous membrane filter[51], CellSieveTM (Creatv Microtech), and MetaCell (Ostrava, Czech Republic), constitute the next generation label-free CTCs enrichment technologies, demonstrating CTC isolation and detection with high efficiency, purity and viability.

Moreover, size difference can be combined with other physical features to improve capture yield. For example, a recent wedge-shaped microfluidic device [Figure 2G] based on the difference in size, as well as rigidity and nuclear/cytoplasmic ratio between CTCs and normal blood cells, was fabricated to enhance CTC isolation, exhibiting excellent capture performance with ≥ 85% capture efficiency[52]. Similarly, benefiting from those multiple biophysical properties, Liu *et al.*[53] [Figure 2H] developed a pyramid-shaped microchamber to achieve a more than 85% capture efficiency and a 93% recovery yield. In addition, vortex technology has been exploited and validated for isolating CTCs based on differences in size, shape and deformability by inertial microfluidics and laminar micro-vortices. VTX-1 liquid biopsy system [Figure 2I] was developed for fully automated isolation and enumeration of CTCs with either high recovery mode or high purity mode in the vortex microfluidic chip[54].

Density-based CTC isolation

The density of nucleated CTCs lies between plasma and red blood cells, and within the scope of white blood cells. Quantitative buffy coat analysis by centrifuging for separation was established by Stephen C. Wardlaw in 1983[55]. AccuCyte separation based on this principle is the first step of the commercial RareCyte Platform (RareCyte, Inc. Seattle)[56], coupled with fluorescence analyzing (CyteFinder system) and picking (CytePiker) to count and retrieve cells for downstream single-cell characterization, overcoming the limitation of capture methods which are dependent on sizes that might miss the small sized-CTCs and immunomarkers that might not be expressed on some subpopulations. Some commercial density gradient solutions, such as Ficoll-Paque (GE Healthcare) and Percoll (GE Healthcare), provide simple-to-use and inexpensive methods for separating CTCs in the mononucleocyte layer from granulocytes and erythrocytes. OncoQuick (Greiner Bio-One/ Hexal Gentech, Germany) consists of a sterile tube with a porous barrier inserted above separation medium, allowing the simple, rapid and highly efficient enrichment of CTCs through density-based centrifugation and size-based separation.

Dielectrophoresis based CTC isolation

The overlap of size or density between CTCs and normal cells may affect the efficiency of these size-/density-based approaches. Electrical properties of CTCs have been applied to discriminate them from other normal cells using dielectrophoresis (DEP). Based on conventional DEP devices, microchips are used to manipulate electric fields to achieve higher capture efficiency and recovery rate. Nguyen *et al.*[57] fabricated a microchip to guide target lung CTCs to sensing electrodes by DEP and hydrodynamic forces, achieving a LOD of 3 cells and an efficiency over 90% at 50 kHz electric field intensity within 10 minutes. The commercial

DEPArray TM cartridge (Menarini Silicon Biosystems, Italy) that contains an array of electrodes embedded with detection sensors is based on the same principle for isolating target cells for subsequent analysis.

MOLECULAR CHARACTERIZATION OF CTCS

Not all the CTCs are detectable and not all detected CTCs have the potential for metastases, indicating that CTC enumeration alone may not be an effective marker of progressive disease. A commonly used chemotherapy agent, isosfamide, was reported to decrease the number of lung cancer nodules but also increase CTC frequency in a pre-clinical model of osteosarcoma[58]. Although a large number of clinical trials have suggested that CTC presence is associated with poor survival in patients with some metastatic cancers[59]. Their characterization, including phenotyping and genotyping, could lead to a better understanding of heterogeneity of metastatic tumor and further facilitate the management of patients for individualized treatment.

Protein analysis of CTCs

Most CTC detection assays are compatible with identification systems for numeration and follow-up characterization. The most common procedure consists of morphological analysis (size, shape, nuclear cytoplasmic ratio, cell shrinkage), immunohistochemical analysis[4] [Figure 3A], and fluorescence immunocytochemistry (ICC). There are various markers that are useful for ICC analysis. For instance, DAPI (nuclear counterstaining), pan-keratin (positive marker), and CD45 (negative marker) are applied in the CellSearch system. Fluorescence channels and specific antibodies are now accessible for users to define the detection of more established markers, for example, epidermal growth factor receptor (EGFR)[60], androgen receptor (AR)[61], folate receptor (FR)[62], and several EMT-related markers (such as vimentin) etc., which are not only the identification markers but also therapy-associated targets. Furthermore, the ELISPOT assay combines cell culture and an immunospot test to quantitatively and qualitatively detect single viable cells expressing-cancer associated marker proteins.

One of the more reliable analytical approaches is protein profiling of captured CTCs, which can shed light on the roles of CTCs in tumor metastases and disease progression. At present, proteomic analyses mostly rely on mass spectrometry and the sensitivity of this method is improved with appropriate sample preparation and targeted cell enrichment. High-resolution porous layer open tube based liquid chromatograph-mass spectrometry (PLOT-LC-MS) lead to identification of approximately 4000 proteins of 100-200 MCF-7 cells with zeptomole detection sensitivity. Recently, Zhu *et al.*[63] incorporated the nanodroplet processing platform (nanoPOTS) with ultrasensitive LC-MS, allowing identification of 1500-3000 proteins from 10-140 cells. He subsequently combined CD45 negative selection and laser capture microdissection-based purification with nanoPOTS-LC-MS for studying protein expression of rare or single CTCs, identifying 164 and 607 protein groups of 1 and 5 spiked LNCaP cells, respectively[64].

Because of these analytical advances, a wealth of information regarding disease metastasis and progression is being discovered. For example, a recent report about profiling of single live CTC protease activity demonstrated the increased expression of matrix metalloproteases (MMPs) secreted by CTCs relative to normal cells is capable of triggering proteolytic processes that assist in invasion and immune evasion[65].

Gene analysis of CTCs

Fluorescence *in situ* hybridization (FISH) technology is highly commercial and easily accessible for analysis of genetic alterations (deletion, insertion, translocation and rearrangement) at the chromosomal level, and is widely used in CTC identification and characterization. For example, RNA-ISH has been utilized for detection of onco-miRNA (such as miRNA-21)[66], while DNA-ISH has been used for quanitifying copy number of cancer-related genes (such as HER2/*neu* gene)[13]. Based on an optimized FISH method, Frithiof *et al.*[67] [Figure 3B] performed a CellSearch-based CTC separation assay to quantitatively measure HER-2

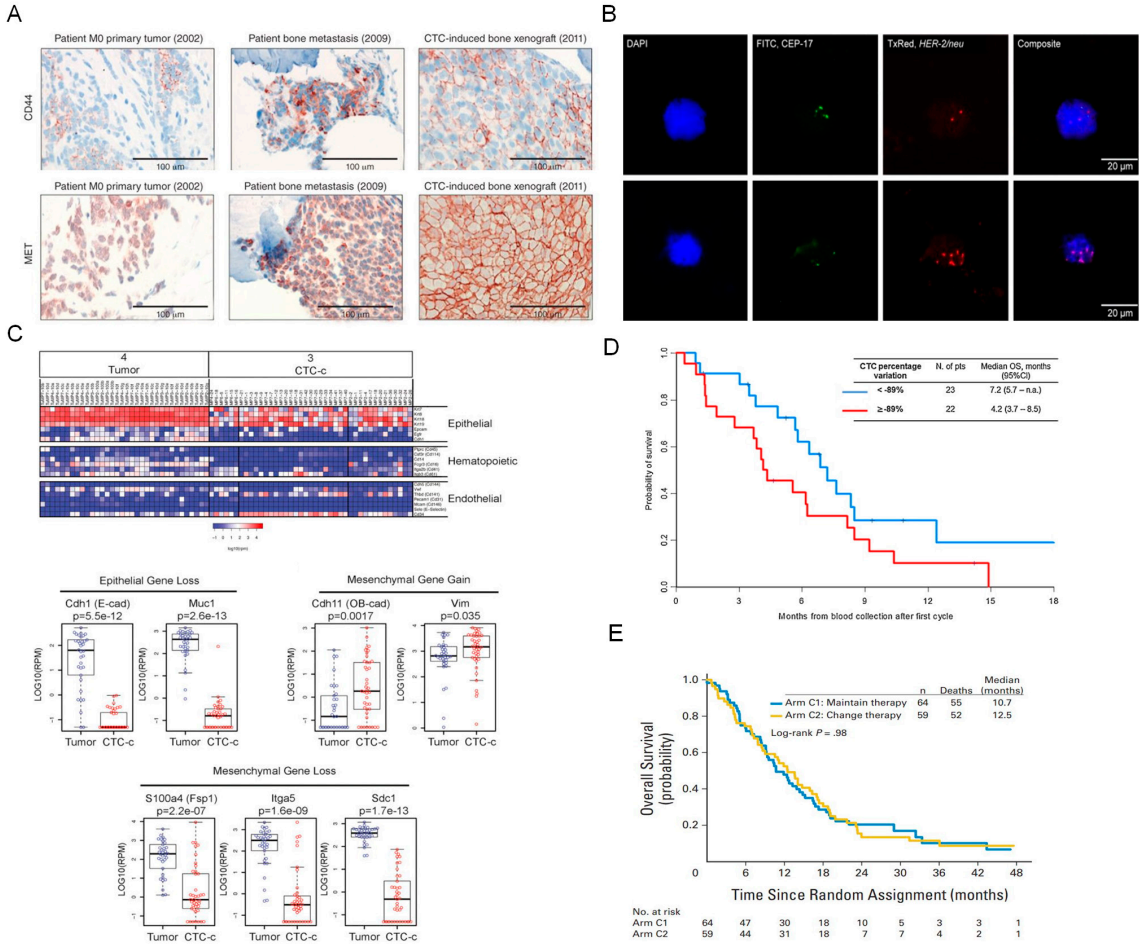

Figure 3. Molecular profiling and clinical application of CTCs. A: Immunohistochemical analysis of the expression of CD44 and MET in primary tumor (M0, nonmetastatic stage), bone metastasis and CTC-induced bone xenograft after transplantation of sorted CTCs; B: FISH images of a patient with metastatic breast cancer have no detectable HER-2 amplification (top panel), and the lower panel is HER-2 amplified CTC; C: the expression heatmap of epithelial, hematopoietic, and endothelial markers in primary tumors and classical epithelial CTCs (CTC-c). Epithelial and mesenchymal genes differentially expressed in CTCs vs. tumors; D: Kaplan-Meier curves of overall survival according to CTC change after one cycle of chemotherapy; E: overall survival (OS) and PFS in patients with metastatic breast cancer for whom therapy failed to reduce CTCs at first follow-up (approximately 21 days after first dose of chemotherapy), or randomly assigned to maintain the original chemotherapy (arm C1) or to switch to an alternative chemotherapy (arm C2). A: Copyright Nature Publishing Group, 2013. Reproduced with permission from reference[4]; B: Copyright Dove Medical Press, 2016. Reproduced with permission from reference[67]; C: Copyright Cell Press, 2014. Reproduced with permission from reference[75]; D: Copyright Elsevier, 2014. Reproduced with permission from reference[79]; E: Copyright American Society of Clinical, 2014. Reproduced with permission from reference[80]

amplification in breast cancer CTCs. They validated that FISH was superior to protein evaluation of *HER-2* status in predicting breast cancer patients' response to *HER-2* targeted immunotherapy and found that one in six patients underwent CTC *HER-2* amplification during the treatment of metastatic disease.

Hybridization analyses, much like ICC analyses, are limited by the availability of both antibodies and microscope filters. Multiple polymerase chain reaction (PCR) targeting associated RNA and DNA for detecting assorted genetic mutations may overcome these limitations. Real-time PCR (RT-PCR) can be used to determine the differences of gene expression between CTCs and normal cells. And quantitative reverse transcription PCR (qRT-PCR) takes advantage of a reverse transcriptase reaction to convert RNA into cDNA before regular RT-PCR. Global or specific gene expression profiling of CTCs has been generated by real-time reverse transcription-PCR analysis, providing insights about mechanism of cancer and development of novel diagnostic biomarkers and therapeutic targets[68]. These technologies following CTC isolation has been used for identification of various gene markers, such as ALDH1 (stem cell marker), phosphoinositide kinase-3 (PI3Kα), TWIST1, TP53, and Akt2

(stem cell markers)[69]. Mostert *et al.*[70] performed a CTC isolation assay and mRNA expression profiling using the CellSearch technique in 142 metastatic colorectal cancer (mCRC) patients. They measured 95 mRNAs by RT-qPCR and found that 34 CTC-specific mRNAs were higher in patients with ≥ 3 CTCs compared with healthy donors. This CTC-specific gene panel for mCRC patients, such as *KRT19*, *KRT20* and *AGR2*, may aid in characterizing how CTCs with different expression profiles contribute to malignancy, thereby furthering the realization of individualized cancer treatment. Campton *et al.*[71] developed a comprehensive and sensitive platform, named as AccuCyte®-CyteFinder® system, for identification and characterization of individual CTCs. Using the whole genome amplification (WGA) product, they confirmed that the TP53 gene, which is known to contain the R175H mutation in SKBR3, enables personalized, molecularly-guided cancer treatment.

Ampli1 TM (Menarini Silicon Biosystem), a product developed for single-cell WGA, can be used to amplify DNA for downstream genotyping analysis, including comparative genomic hybridization (CGH) and next-generation sequencing (NGS). Upon its development in 1992, CGH technology opened a new avenue in genomic investigation and, more particularly, in cancer gene analysis. In the past, CGH was applied for analysis of tumor tissues, but many studies have suggested that data from primary tumors alone is insufficient. Array CGH, which exploits ordered arrays of genomic DNA sequences, is widely used for analysis of CTCs, including identification of genomic alterations which include insertion/deletion, single-nucleotide variations, copy number variations (CNVs)[72]; identification of candidate oncogenes or tumor suppressors; identification of novel biomarkers involved in metastasis, cancer progression and therapy response; and identification of subgroups of CTCs[73]. High-throughput NGS is another strong technology to analyze heterogeneity of CTCs and reveal the mechanisms of metastasis, which might be the Achilles' heel in disease progression. Bertucci *et al.*[74] utilized aCGH and NGS to compare DNA copy number and mutational profiles of 365 cancer-related genes between primary tumors and metastases and discovered a degree of divergence for actionable driver genes that might be extremely relevant with cancer metastasis. Profiting from whole genome amplification technology, the limited amount of single-cell genomic DNA sample can be amplified indistinguishable for sequencing.

Single-cell RNA sequencing was used to detect the heterogeneity of CTCs, unveiling the mechanism of drug resistance of androgen receptor (AR) inhibitors in prostate cancer[61]. The technique was also used to identify conduct a transcriptomic analysis in pancreatic CTCs, finding increased expression of stromal-derived extracellular matrix (ECM) proteins, which facilitate cell migration and invasiveness, in CTCs from mice and humans with pancreatic cancer[75] [Figure 3C].

Single-cell exome sequencing of isolated CTCs from cancer patient, revealed insertion/deletion and single-nucleotide variation in CTCs after whole genome amplification. The results showed cancer-type specific CNVs that are reproducible within cells of the same patient, or even between patients with the same type of cancer[76].

CLINICAL IMPLICATIONS

Prognostic and diagnostic value of CTCs

Several studies have highlighted the correlation between CTC burden and treatment effect, indicating the prognostic value for patients receiving chemotherapy or surgery and the potential of surveillance of disease recurrence or metastasis [Table 1]. Several years after chemotherapy, patients with high-risk breast cancer with elevated CTC counts in their peripheral blood were reported to have worst survival prospect[77]. Similarly, patients with colorectal cancer with elevated CTC counts were more likely to have recurrence after 3 years of curative resection, showing the relation between post-operative CTCs and poor prognosis[78]. The prognostic value of CTCs was demonstrated by Nicola *et al.*[79] [Figure 3D] in 60 patients with extensive SCLC. After assessment with the CellSearch system, the group isolated and analyzed CTCs in 90% (54/60)

of patients at baseline and demonstrated that CTC count was significantly associated with the number of organs involved. A reduction in CTC count of more than 89% after chemotherapy significantly improved the prognosis accuracy and was associated with a better outcome. They concluded that only the change of CTC count after the first chemotherapy cycle provided clinically relevant information. However, there were still some clinical trials (such as: gov NCT00382018) that failed to observe improved PFS or OS for cancer patients with decreased CTCs after therapies[80] [Figure 3E]. Compared to either CTCs levels or cancer specific antigen levels alone, the combination of both two biomarkers provided a notably better predictive indicator for patients with advanced cervical cancer[81].

Paired CTCs are correlated strongly with origin tumor cells, thus characterization of this subset of genes of CTCs might help to predict primary tumor origin. And CTCs detached from different parts within same tumor or even from different tumors are originally heterogeneous in nature. Gene expression profiling of CTCs from patients with different metastatic cancers showed the different but unique gene expression patterns of those cancer types, providing novel noninvasive diagnostic tools[68] and essential information for personalized treatment.

On the other hand, CTCs undergoing certain transitions, such as EMT and MET, might generate new genetic alterations which are absent in the primary tumor, but related with potential distant tumor. Characterization of these subgroups of CTCs might assist in localizing specific distant metastatic sites. Additionally, some subgroup of CTCs may harbor changes that are undetectable in the tumor of origin, but are related to drug-resistance and management of treatment. Revealing the qualitative and quantitative divergence between the CTCs and the primary tumor or within CTCs by genotyping and phenotypic analysis is crucial for future studies of individualized medicine in metastatic disease. However, some changes present in CTCs homogenously take place within primary tumor, suggesting CTCs, to some extent, might be a noninvasive and real-time indicator for following cancer progression and monitoring therapeutic response.

CTCs predict therapy outcome and provide personalized therapeutic targets

CTC enumeration can be a good marker for predicting drug sensitivity/resistance in preclinical and clinical settings. CTC quantity reasonably correlates well with clinical and instrumental tumor response. To investigate the clinical significance of CTCs in predicting the tumor response to chemotherapy, Wu *et al.*[82] detected CTCs at baseline and during chemotherapy in 453 eligible lung cancer patients, indicating that disease control rate (DCR) of CTC-negative patients was significantly higher than that of CTC-positive patients; more importantly, patients also showed higher OS. The CTC status has been reported to be related to prognosis and is altered in response to chemotherapy in many other tumor types, such as colorectal cancer[10], breast cancer[83], osteosarcoma[58]. Smerage *et al.*[80] confirmed the prognostic significance of CTCs in patients with metastatic breast cancer, demonstrating that an increased number of CTCs was associated with poor prognosis. Early conversion to alternate cytotoxic therapies was ineffective in prolonging OS in patients with increased CTCs after receiving 21 days of first-line chemotherapy. For this population, a more effective treatment than standard chemotherapy is needed.

CTC characterization can generate predictions of drug potency and therapeutic efficacy before or during treatment according to analysis of targeted protein expression and signaling pathway activity. The development of immune check point inhibitors for cancer therapy, for example, anti-PD-L1, have achieved much success due to increased efficacy and decreased toxicity[84]. However, PD-L1 expression detection for prediction of therapeutic response using tumor biopsies prior to treatment is invasive and insufficiently precise to guide treatment planning, resulting in some cancer patients being treated with an expensive but ineffective and toxic therapy. Instead, serial monitoring of patients treated with immune checkpoint inhibitors showed that a decrease of CTC score correlated with improved PFS and OS, and further demonstrated that RNA-based CTCs score during the immunotherapy has the potential to be a predictive biomarker for immunotherapeutic outcome[85].

Table 1. Presence of CTCs and clinical outcome in cancer patients

Tumor type	Technique	Patient number	CTC cut-off	Patient Ratio	Baseline				Treatment	Post/During Therapy				Ref.
					PFS		OS			PFS		OS		
					HR (95%CI)	*P* value	HR (95%CI)	*P* value		HR (95%CI)	*P* value	HR (95%CI)	*P* value	
IBC (stage III)	CellSearch	63	≥1 CTC/ 7.5 mL	27%	NR	NR	NR	NR	CT	4.22 (1.67-10.67)	log-rank *P* = 0.005	1.53 (0.41-5.79)	log-rank *P* = 0.54	[99]
Breast Cancer	CellSearch	1,574	≥ 2 CTC/ 7.5 mL	12.6%	3.73 (2.82-4.90)	< 0.001	3.93 (2.81-5.45)	< 0.001	Neoadjuvant CT	NR	NR	NR	NR	[83]
MBC	CellSearch	1,944	≥ 5 CTC/ 7.5 mL	46.9%	1.92 (1.73-2.14)	< 0.0001	2.78 (2.42-3.19)	< 0.0001	NR	NR	NR	NR	NR	[100]
MBC	CellSearch	595	≥ 5 CTC/ 7.5 mL	53.6%	NR	NR	NR	NR	First line CT	1.94 (1.52-2.47)	< 0.001	2.13 (1.63-2.79)	< 0.001	[80]
CRC (stage IV)	qRT-PCR	50	NR/7.5 mL	26%	3.39 (1.67-6.83)	0.001	2.96 (1.14-6.81)	0.003	CT	2.49 (1.12-5.11)	0.013	3.58 (1.59-8.07)	0.002	[101]
mCRC	Immuno-fluorescence	75	≥ 2 CTC/ 7.5 mL	28%	0.82 (0.38-1.78)	0.61	1.97 (0.89-4.37)	0.09	CT	0.92 (0.30-2.78)	0.88	3.55 (1.1-11.5)	0.03	[102]
NSCLC (stage III/IV)	CellSearch	59	≥ 2 CTC/ 7.5 mL	32%	3.93 (1.38-11.15)	0.01	5.32 (1.62-17.50)	0.006	CT	NR	NR	3.07 (1.33-7.05)	0.008	[103]
NSCLC (stage IIIB/IV)	CellSearch	43	≥ 5 CTC/ 7.5 mL	23.2%	2.4 (1-5.7)	0.16	3.1 (1.2-8.2)	0.016	First-line CT	4.3 (1.2-14.4)	0.016	2.9 (0.7-11.4)	0.11	[104]
SCLC	CellSearch	83	≥ 5 CTC/ 7.5 mL	60%	2.4 (1.4-4.1)	0.001	3.4 (1.8-6.3)	< 0.001	CT	1.9 (1-3.5)	0.05	NR	NR	[105]
SCLC	CellSearch	56	≥ 5 CTC/ 7.5 mL	50%	0.3 (0.2-0.6)	< 0.001	0.3 (0.2-0.6)	0.001	CT	0.7 (0.3-1.7)	0.486	0.4 (0.2-0.9)	0.034	[106]
AGC	CellSearch	106	≥ 3 CTC/ 7.5 mL	41%	NR	NR	NR	NR	CT	2.152 (1.11-4.16)	0.022	3.463 (1.82-6.58)	< 0.001	[107]
AGC	FACS-ICC	24	> 2 CTC/ 8 mL	67%	2.5 (0.76-8.27)	0.133	2.7 (0.82-8.89)	0.101	drug exposure	3.9 (1.13-12.7)	0.031	4.8 (1.37-16.8)	0.014	[108]
GGEA	CellSearch	106	≥ 2 CTC/ 7.5 mL	46%	1.6 (1.0-2.4)	NR	1.8 (1.2-2.9)	NR	no treatment	4.8 (2.4-9.7)	NR	3.1 (1.5-6.3)	NR	[109]
GGEA	qRT-PCR	62	all marker (+) *vs.* others	69.4%	4.96 (2.2-11.2)	0.000	4.1 (1.7-9.5)	0.001	CT	3.84 (1.6-9.3)	0.003	6.5 (2.0-21.3)	0.002	[110]
GEC	IsoFlux platform	43	> 17 CTC/ 7.5 mL	46.5%	NR	NR	4.4 (1.7-11.7)	0.0013	NR	NR	NR	NR	NR	[111]
EC	CellSearch	22	> 2 CTC/ 7.5mL	37.5%	1.06	0.91	0.52	0.23	NR	NR	NR	NR	NR	[112]
BC	CellSearch	1,087	> 1 CTC/ 7.5mL	20.6%	(pre+/post+) 3.73 (1.96-7.12)	Log-rank *P* < 0.001	(pre+/post+) 10.57 (3.92-28.56)	Log-rank *P* < 0.001	Drug and adjuvant CT	(pre-/post+) 2.49 (1.51-4.10)	Log-rank *P* < 0.00	(pre-/post+) 7.38 (3.19-17.08)	Log-rank *P* < 0.001	[77]
CRC (stage I-III)	ISETdevice-CTCBIOPSY	138	> 1 CTC/ 2.5mL	79%	2.17 (0.75-6.31)	0.153	NR	NR	surgery	2.82 (1.39-5.75)	0.004	NR	NR	[78]
Cervical cancer	NEim-FISH	99	3 CTCs/ 3.2 mL	45.5%	NR	NR	NR	NR	radiotherapy	2.425 (1.131-4.477)	0.005	NR	NR	[81]
Lung cancer	CellSearch/ CD45-FISH	453	56%	NR	3.16 (2.23-4.48)	< 0.001	3.43 (2.21-5.33)	< 0.001	CT	3.78 (2.33-6.13)	< 0.001	3.16 (2.23-4.48)	< 0.001	[82]
MM	Digital CTC Assay	49	48	NR	0.97 (0.47-2.3)	0.95	0.46 (0.14-1.52)	0.20	ICIT	0.17 (0.05-0.62)	0.008	0.12 (0.02-0.91)	0.04	[85]

IBC: inflammatory breast cancer; MBC: metastatic breast cancer; CRC: colorectal cancer, NSCLC: non-small-cell lung cancer, SCLC: small-cell lung cancer; GC: gastric cancer; GGEA: gastric and gastroesophageal adenocarcinomas; GEC: gastroesophageal cancer; EC: esophagogastric cancer; MM: metastatic melanoma; HR: hazard ratios; CI: confidence interval; PFS: progression-free survival; OS: overall survival; CT: chemotherapy; NR: not reported; ICIT: immune checkpoint inhibition therapy

Beyond enumeration alone, CTCs could provide crucial information of tumor malignancy via molecular characterization, leading to better treatment monitoring and molecular-/cancer cell-targeted therapies. The genotypic changes in CTCs provided the best suitable targeted therapy and enabled assessment treatment regimen efficacy over time. Epidermal growth factor receptor (EGFR) on the CTC surface has been verified as extremely significant in the process of tumor growth and progression. EGFR inhibitors (HER2 inhibitors, tyrosine kinase inhibitors, TKI and monoclonal antibodies) have been licensed for treatment of cancers caused by EGFR up-regulation, such as NSCL, breast, renal cell, squamous cell, colon and pancreatic cancers. However, in some cases, EGFR-targeted inhibitors are not effective due to the emergence of drug-resistance mutations. Mutation screening analysis of EGFR in CTCs may provide an explanation for drug-resistance mechanism and also reveal possibilities for diagnostic and therapeutic interventions[60]. Inhibitors of other therapeutic molecular targets including mTOR, such as temsirolimus, and phosphoinositide 3-kinase (PI3K), such as ZSTK474, LY294002, have shown to have anti-proliferation in clinical trials[86,87]. Most cell populations of the immune system play an important role in survival and seeding, or even enhancing the growth of tumorigenic subpopulations of CTCs. The molecular characterization of CTCs might assist in unveiling intercellular interaction mechanisms and providing potential therapeutic targets. For instance, the extracellular surface interacting protein, PD-L1, is one such target that is currently generating much interest[84]. In consideration of costs and toxicity of anti-PD-L1 therapy, predictive biomarkers able to distinguish responders from non-responders are in urgent demand. Real-time CTC analysis provides significant information on drug resistance[88]. In addition, CTCs are now regarded as a new cellular therapeutic target. Photodynamic therapy was used to selectively kill GFP-expressing CTCs by energy transfer between expressed GFP and pre-accumulated rose bengal (RB) in cells, demonstrating that clearance of CTCs could reduce metastasis and extend survival[89].

CONCLUSION AND OUTLOOK

The potential clinical value of CTCs has been established. Advances in CTC isolation and molecular characterization offer the possibility for early detection and diagnosis, improve the satisfaction of therapies, as well as expand our knowledge about underlying mechanisms of cancer dissemination and progression.

Although the tremendous technical advances in CTC isolation and detection make it possible to analyze extremely rare CTCs, there are still many hurdles. First, a criterion to standardize different kinds of detection assays is urgently needed in clinical applications. Secondly, while the emergence of new predictive biomarkers leads to clearer recognition about tumor metastases and disease progression, novel targets for prognosis and treatment need to be further validated and standardized. The next frontier of CTCs detection lies in thorough characterization, which might rely on developing single-cell multi-omic technologies, including genomics, proteomics, transcriptomics etc.. Finally, research findings provide arguments in favor of the hypothesis that only a subpopulation (metastasis-initiating cells, MICs) of CTCs in patient blood is responsible for initiating carcinoma metastasis. A majority of cancer cells may never develop into metastatic phase, but instead maintain a dormant state or die from the anoikis, immune attacks and physical shear stress in the vasculature. However, our understanding about the requirements for CTCs being activated from latency into overt metastases is far from complete. Apart from CTCs, other noninvasive "liquid biopsies" might provide more supplementary information, including some cell-free components such as circulating tumor DNA (ctDNA)[90], microRNAs (miRNA), exosomes, as well as long-coding RNA (lncRNA)[91-94]. Recently, due to the success in immunotherapy of cancers, immune checkpoint blocker programmed death-ligand 1 (PD-L1), whose expression in CTCs correlate with tumor status[95], has gained interest as a potential independent prognostic marker for PFS and OS[96], extending the spectrum of noninvasive liquid biopsies[97]. And, CTC detection may also have the potential to monitor the efficacy of anti-PD-L1 therapy[98]. Comprehensive and systemic liquid biopsy analyses may contribute to thorough understanding of metastatic malignancy and better management of cancer patients.

DECLARATIONS

Authors' contributions

Huang QQ and Chen XX contributed equally to this work.

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

Not applicable.

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