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REVIEW

Development and Clinical Prospects of Techniques to Separate Circulating Tumor Cells from Peripheral Blood

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Abstract: Detection of circulating tumor cells (CTC) is an important liquid biopsy technique that has advanced considerably in recent years. To further advance the development of technology for curing cancer, several CTC technologies have been proposed by various research groups. Despite their potential role in early cancer diagnosis and prognosis, CTC methods are currently used for research purposes only, and very few methods have been accepted for clinical applications because of difficulties, including CTC heterogeneity, CTC separation from the blood, and a lack of thorough clinical validation. Although current CTC technologies have not been truly implemented, they possess high potential as future clinical diagnostic techniques for individualized cancer. Here, we review current developments in CTC separation technology. We also explore new CTC detection methods based on telomerase and nanomaterials, such as in vivo flow cytometry. In addition, we discuss the difficulties that must be overcome before CTC can be applied in clinical settings.

Keywords: circulating tumor cells, cancer, liquid biopsy, tumor metastasis, cancer diagnosis

Introduction

Circulating tumor cells (CTC) enter peripheral blood circulation either spontaneously or during cancer treatment. Due to high activity and metastasis potential, CTC are sometimes considered as the primary mechanism of tumor metastasis.[1] Thus, they are important marker of tumor screening, diagnosis, prognosis, and efficacy evaluation.[2–6] Therefore, imaging CTC should improve the accuracy of lung cancer screening and early detection. CTC are extremely rare, with only one per 106–107 peripheral blood leukocytes from cancer patients.[7] Because of their rarity, research on CTC imaging did not emerge until the development of the cell search system, a Food and Drug Administration (FDA)-approved medical device for CTC selection and counting. However, challenges remain in terms of formulating a uniform standard for CTC separation (Table 1).[8,9]

The heterogeneity of CTC is the primary obstacle in their detection. These cells differ in size, shape, and immunotyping across tissue types. For example, during epithelial-to-stromal transformation (EMT), both epithelial cell adhesion molecule (EpCAM) and cytokeratin (CK) are downregulated resulting in failure of conventional EpCAM-based capturing techniques in detecting CTC subpopulations with more mesenchyme-like phenotype.[10,11] In addition, due to multi-step cell preparation processes, circulating tumor cells may be destroyed and fragmented, resulting in inaccurate test results.[7]

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Table 1 Circulating Tumor Cells Were Selected Based on Surface Analysis

Isolation Method	CellSearch	EasySep	MACS	IMS	STMBs	HTMSU	CTC-Chip	GEDI Chip
CTC enrichment	EpCAM	CD45	EpCAM	EpCAM	EpCAM, HER2, EGFR	EpCAM	Anti-EpCAM functionalized microposts and chip structure	Immunocapture and microfluidics
CTC detection	Immunofluorescence analysis for CK, CD45, and DAPI	Immunofluorescence analysis for CK, CD45, and DAPI	Immunofluorescence analysis for CK, CD45, and DAPI	Immunofluorescence analysis for CK, CD45, and DAPI	Immunofluorescence analysis for CK, CD45, and DAPI	Immunofluorescence analysis for CK, CD45, and DAPI	Immunocytochemical analysis or RT-PCR	Immunofluorescence analysis for PSMA, CD45, DAPI
Viability	na	92.59%	Viable	Mostly viable	85%	80%	Viable	Viable
Recovery	42%, 85%	na			79±10%	96%	> 60%	
Purity	0.1%, 1.4%	42%			84±3%	100%	52–67%	68%
Throughput	9 mL/h	1–4 mL/h			na	1–2 mL/h	1–2 mL/h	
Sample volume	9 mL	0.5–2 mL			1 mL	1 mL	2.7 mL	1 mL
Key features	FDA approved for advanced breast, prostate, and colorectal cancers; ferrofluid nanoparticles;	Easy-to-use batch separation; high background	Pos/neg enrichment; high surface area to volume; difficult to use with whole blood	Low background leukocytes (not tested on clinical samples)	Uses antibodies simultaneously; low sample volume	Single-step separation; low volumes of blood; conductivity-based enumeration	Microvortex increases the efficiency; various CTC-specific antigens can be used	Functional assays in situ; size and collision inclination dependency
References	Talasaz AH77	Nolan J75	Miltenyi S40	Xiong K43	Lu N-N25	Adams AA52, Dharmasiri U53, Dharmasiri U78	Nagrath S61	Gleghorn JP59

The detection of CTC has three main steps: 1) blood sample preparation for tumor cell separation, 2) antibody staining or DNA probe detection, and 3) imaging of cells. Improved cell separation technology allows us to obtain complete CTC for biological characterization and functional analysis (Figure 1). In this review, we focus on the development of CTC separation technique, particularly the advantages and disadvantages of various separation methods. We then summarize the areas in which separation technique still requires improvement and the current limitations of its clinical application. Finally, we examine the potential future directions of CTC use in clinical settings.

Cell Separation Methods

Surface Antigen-Based Separation

Circulating tumor cells are recognized by their round or oval morphology, along with the presence of surface antigens epithelial cell adhesion molecule (EpCAM) and cytokeratin (CK), as well as the absence of CD45.[12] Separation methods using surface antigens can be divided into positive and negative selection. The most representative positive-selection method is the cell search system. Negative selection generally involves removing unnecessary cells to indirectly capture CTC. For example, all CD45+ peripheral blood cells can be excluded to leave only the CD45-CTC. However, this method usually leads to low purity.[13–16] Another technique called as EasySep (developed by Stemcell) is an immunomagnetic method that separates CTC through negative enrichment using a mixture of CD45-containing antibodies and differently sized magnetic beads. With an average recovery of 42.23%, EasySep can produce living cells without markers and also be used for subsequent downstream analysis.

The prognosis of patients with breast, prostate, or colorectal cancers is related to CTC count.[17–19] After revealing this potential, the FDA approved the cell search system as the only technology for clinical CTC detection. This immunocytochemistry method is based on magnetic immunity and staining. First, EpCAM antibody beads are used to enrich CTC, which are then extracted from blood under a strong magnetic field. Next, tumor cells are fixed and identified through fluorescent-stained keratin. A semi-automatic four-color fluorescence microscope is used to detect stained cells, and those with the tumor cytological characteristics are identified as CTC.[20] However, a related method is superior to simply using EpCAM antigen to enrich CTC[21] at least in case of lung cancer. Because

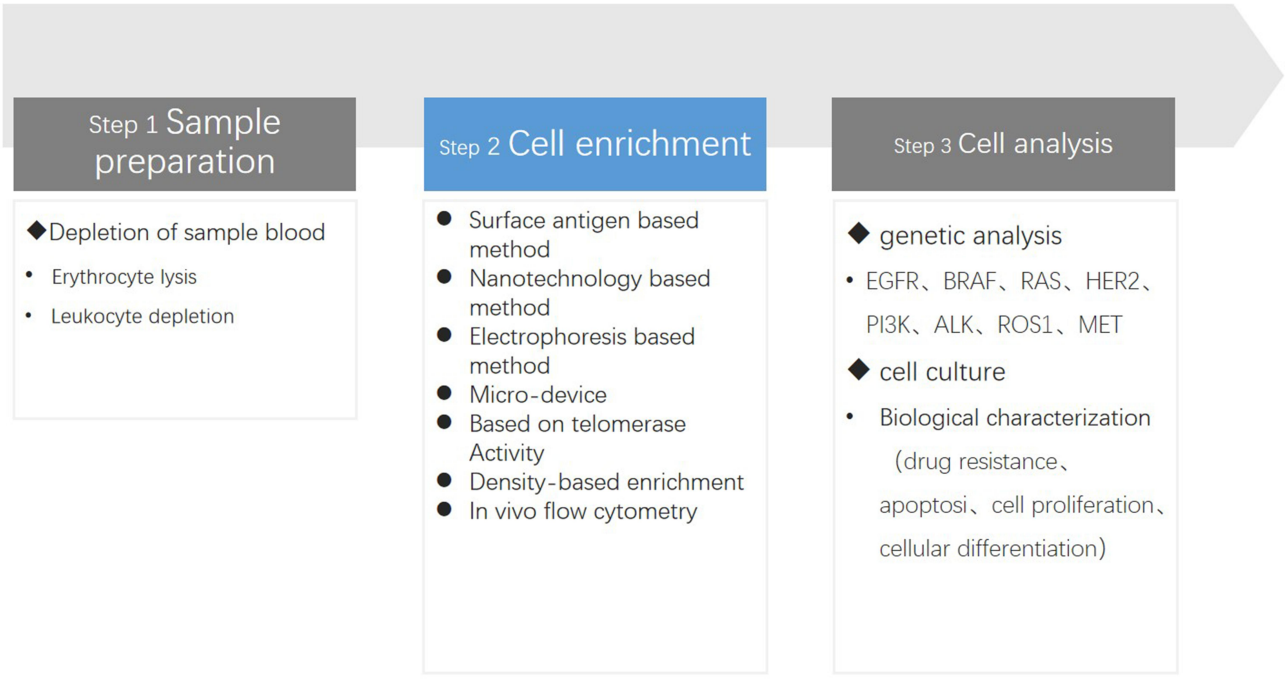

Figure 1 Graphic illustration of three major steps in a CTC assay. Step 1. Sample preparation: in order to isolate and detect CTCs at a high frequency, the blood sample should be pretreated to remove the erythrocytes and/or leukocytes as much as possible to provide a low interference background. Step 2. Cell enrichment: CTC can be detected by multiple methods, depending on the different theories, they are divided into seven groups: surface antigen based, physical property based, nanotechnology based, electrophoresis based, micro-devices, telomerase, Density-based and in vivo flow cytometry. Step3. Cell analysis: At present, the research on the biological characteristics and functional analysis of circulating tumor cells mainly involves two aspects: genome/transcriptome analysis and in vitro culture. We can be understood through genetic analysis to guide clinical treatment, and by vitro culture can further understand the biological characteristics of CTC.

EpCAM is expressed mostly in lung cancers that originate from epithelial cells, anti-EpCAM antibodies can be used in CTC, and profile kits to detect CTC in peripheral blood. However, the use of CellSearch device for CTC detection was discontinued a long time ago owing to severe problems in clinical application.

Another surface antigen useful for CTC analysis is melanoma cell adhesion molecule (MCAM). When used in combination with EpCAM as a cell-search enrichment marker, CTC detection rate is improved from 18% (EpCAM only) to 25%.[22] However, we require more data to conclude whether changes to MCAM+ CTC during localized or metastatic cancer treatment are related to clinical outcomes.

A recently developed method uses *MET* gene to detect amplified tumor cells in a rapid, noninvasive, sensitive, and specific way.[23] *c-MET* is a protein product encoded by the *MET* proto-oncogene, which is a hepatocyte growth factor receptor with tyrosine kinase activity and is associated with a variety of oncogene products and regulatory proteins. The sensitivity of this method to the cells highly expressing *CMET* was 40–80%, and specificity to *CMET*-negative cells was 100%. Given that *CMET*+ CTC and *MET* amplification are highly correlated, the former is a potential biomarker for predicting individualized treatment for patients with cancers that highly express *CMET* (eg, gastric, colorectal, and renal cancers). Technologies using other surface markers (eg, *EGFR, HER2, and MUC1*) have also been developed, along with antibodies that target stem cells and stromal markers.[24] In the integrated immunomagnetic separation system, chemical coupling is used to prepare Strep-tag-II-derived immunoglobulin G (IgG). This is then reversibly loaded on Strep-tag-combined magnetic beads (stmbs) to fix antibodies, including anti-EpCAM, anti-*HER2*, and anti-*EGFR*. Using different antibodies that can capture more CTC subgroups simultaneously, the technique results in high capture efficiency (79%) for IgG stmbs.[25]

Epithelial markers, such as CK 8, 18, 19, and EpCAM, are the commonly used biomarkers for CTC identification, although they could identify only tissue origin and not the biological behavior (benign vs malignant). It is known that tumor cells could decrease or lose epithelial marker expression during metastasis/dissemination, causing significant heterogeneity, however, researchers have not been able to identify a universal tumor antigen which can trap all potential CTC that fail to express epithelial markers.[7] This limitation invalidates the CTC immunomagnetic enrichment and staining procedures, reducing the application of cell search systems in several cancers, including lung, gastric, and liver.[11,25,26] In addition, only the screening and isolation tables would be available for any cancers that affect the epithelial-to-stromal transformation, because surface antigens such as EpCAM, keratin, MCAM, and c-Met may cease being expressed during this process. However, negative selection enrichment has the disadvantage of potential contamination by CD45 + cells that are not completely separated, lowering the sample specificity and purity. However, further cleaning would cause cell destruction and fragmentation, thereby increasing the cost while reducing the efficiency.

Telomerase-Based Detection

Recent research has identified a special reverse transcriptase in tumor cell nuclei that increases telomere length repeatedly. This characteristic suggests that the reverse transcriptase, or the telomerase, may be a potential biomarker and prognostic indicator of tumors. Telomerase activity is reactivated in almost all the tumor cells, but never in normal cells.[27] Therefore, telomerase activity should be a viable option for detecting CTC in cancers without EpCAM expression, including prostate, bladder, colon, breast, and brain cancers.[28] For example, while most of the available methods cannot detect CTC in prostate cancer, analysis of telomerase activity successfully achieved high detection rate (75%) and specificity (100%).[29] Telomerase activity has also been successfully used to identify 100%, 84%, 73%, and 90% of patients with stage IV ovarian cancer,[30] stage IV breast cancer,[31] stage IIIB/IV NSCLC (no small cell lung cancer),[32] and metastatic bladder cancer,[31] respectively. However, the telomere-based method has a major limitation in that the peripheral blood cells must be split, precluding any other further analysis.[33]

Electrophoresis-Based Separation

The electrophoretic method involves using dielectrophoresis (DEP) field flow to isolate CTC from normal peripheral blood cells through their morphological and biophysical differences (eg, membrane capacitance, shape, size, and conductivity).[34] Previous studies have shown that differences in conductivity between normal blood cells and CTC depend on the effective surface area of the cell membrane, which in turn influences the membrane capacitance.[35] Isolating cells with different dielectric phenotypes using DEP clarifies the range in conductivity between cancer cells and normal blood cells (Table 2).[36] Another study proposed the use of staggered electrode

Table 2 Circulating Tumor Cells Were Selected Based on DEP

Isolation Method	eDEP	TWDEP	cDEP	iDEP	Optical DEP	Two Steps Separation Methods
CTC enrichment	Dielectrophoresis field-flow assist principles		Dielectrophoretic field	Dielectrophoretic field	Dielectrophoretic field	Dielectrophoretic field and microfluidic
CTC detection	Immunofluorescence analysis for ASPL-TFE3 type I fusion protein and vimentin					Immunofluorescence analysis, PCR and sequencing methods
Viability	0.9	Viable	Viable	Viable	Viable	Viable
Recovery	0.85		0.8	0.75	0.71	na
Purity	0.9		Low	Low	0.92	High
Throughput	5mL/2h		2.2 mL/h	na	High	na
Sample volume			na	na	na	na
Key features	Separated by electrophoresis; demonstrated by fluorescence in situ hybridization		The absence of contact between the electrodes and the media	Single-cell trapping; separation based on the cell size and electrophoresis; a high-throughput method	Size-based separation; higher resolution of the cell separation	Requires CD45 depletion and manual staining; Different antigens can be used for immunofluorescence analysis
References	Chan,[79] Vykoukal[80], Huang,[81] Moon,[82] Cheng[83]	Cen[84]	Zhao[85]	Kang[86], Bhattacharya[87]	Chuang,[88] Wu[89], Qin[90]	Huang[91], Huang C[92]

structure to capture CTC, resulting in 92% efficiency in isolating human lung cancer cell line NCI-H1975 at high flux (flow rate of 6 mL/h).[37] A study isolating CTC using side electrophoresis processed 5 mL of samples continuously within 2 h, with 85% recovery rate and >90% CTC purity.[38] Finally, two-dimensional electrophoresis successfully isolated human breast cancer cells and colorectal cancer cells.

Taken together, these results showed that DEP is capable of screening normal blood cells, tumor cells, malignant cell clusters, and malignant cell groups with different heterogeneity in peripheral blood. However, DEP also has obvious disadvantages. Processing time is too long, resulting in low efficiency. In addition, inappropriate cell concentration in samples easily causes contamination of CTC from normal cells, reducing purity. Finally, the specific buffer used by DEP has a strong correlation with cell survival rate, suggesting a strong confounding factor that would influence interpretation of results.

Nanotechnology-Based Separation

Nanoparticles are similar in size to cell membrane pores. Additionally, covering nanoparticles with CTC-specific antibody can increase the surface area for CTC binding. These characteristics suggest that techniques using nanoparticles can promote cellular interaction, enhance cell adhesion, and isolate CTC, greatly improving efficiency, purity, sensitivity, and repeatability in malignant cell capture. In the past few decades, nanotechnology research has greatly advanced, resulting in tools such as the multifunctional immunomagnetic nano carrier platform (Table 3). However, nanotechnology methods also suffer from high contamination from normal blood cells. To address this issue of high contamination, Wang et al[39] developed a CTC capture platform that combines silicon dioxide nanoparticles (SINP) with antibodies. The platform has anti-Epcam-coated SINP substrate and a PDMS (polydimethylsiloxane) chip to increase cellular surface area. The PDMS chip also has a serpentine mixed channel that promotes CTC and lining.

Table 3 Circulating Tumor Cells Were Selected Based on Chip

Isolation Method	GO Chip	SiNP Platform	NP-HBCTC-Chip
CTC enrichment	EpCAM	EpCAM	EpCAM, HER2, EGFR, or cocktail
CTC detection	Immunofluorescence analysis for CK, CD45, and DAPI	Immunofluorescence analysis for CK, CD45, and DAPI	Immunofluorescence analysis for CK, CD45, and DAPI
Viability	0.92		87–93%
Recovery	91–95%	0.95	91–92%
Purity	High		
Throughput	1–3 mL/h	1 mL/h	1 mL/h
Sample volume	1 mL	1 mL	3.5 mL
Key features	Graphene oxide nanosheets; easy fabrication; high purity	Antibody-coated silicone nanopillars for capture enhancement; 1.5–3.0-psi pressure	Antibody-coated gold nanoparticles for capture enhancement
References	Yoon[9]	Wang[39]	Park[50]

Another enrichment technology based on immunomagnetic nanoparticles is the magnetic cell separation system (MACS).[7,40] This method involves high-gradient magnetic separation to capture CTC that are labeled using magnetic nanoparticles coupled with EpCAM antibodies. Although there are studies using MACS to capture CTC from peripheral blood of metastatic cancer patients,[41,42] the technique is more suitable for tissue samples than for whole blood samples.[43] Biomimetic immunomagnetics (IMS) were introduced recently to address the problem of poor MACS detection in whole blood. This technology formed magnetic corpuscles through camouflage magnetic nanoclusters with white blood cell membrane fragments. As a result, adsorption of non-specific white blood cells in peripheral blood samples was inhibited, reducing the contamination of CTC by background cells. Although this equipment has not been tested in a clinical setting, experiments revealed a near-complete lack of background cells, as a major advantage.[43,44]

The use of immunomagnetic nanocarriers can achieve high capture rates in cancers lacking EpCAM expression.[45] Moreover, the different nano-materials available for linings (eg, quartz,[46] polymers,[47] and gold[48]) allow flexibility that improve CTC capture efficiency of EpCAM-expressing cancers and detection efficiency of low EpCAM-expressing malignant tumors. The recently developed nanoparticle HBCTC chip can be coated with surface markers including EpCAM, HER2, and EGFR, or a mixture of the three, achieving >90% capture efficiency for low EpCAM-expressing cells (such as the MDA-MB -231 line). The thiol exchange reaction then releases captured cells from nanoparticles, allowing for subsequent CTC molecular analyses, such as next-generation RNA sequencing and cell culture.[49,50]

Methods that improve nanomaterial adhesion to cancer cells over normal cells include a nano-surface subjected to reactive ion etching.[51] The nanotube array has also been developed as a sensitive biodetector for CTC detection. Compared with an anti-EpCAM-modified planar substrate, the anti-EpCAM-modified conducting polymer nanotube has a CTC capture rate of 70% and a cell survival rate of 97%.

Although nanotechnology has significantly improved capture efficiency, little data are available on how nanostructured substrates influence the interaction between tumor cells and normal peripheral blood cells. Moreover, the purity of CTC extracted via nanomaterials still needs improvement.

Micro-Device-Based Separation

Micro-devices are any tools created through microprocessing technology and chemical synthesis for specific molecular biological test functions in a very small area. Numerous studies show that micro-devices have short processing time, simple operation, and high separation efficiency.[7] Currently available micro-devices include the microsingular chip,[52,53] microfilter,[54–56] micro EDAR (sensitive decision aliquot ranking) cytometer,[57] and microGEDI (geometrically enhanced differential

Table 4 Circulating Tumor Cells Were Selected Based on Micro-Devices

	Micropost Chip	**Microfilter**	**Microsinusoidal Chip**	**MagSweeper**	**Micropillar Chip**	**Microcrescent Chip**	**Microwall Chip**	**MicroGEDI Chip**	**Microvortex Chip**	**Nanopillar Chip**
CTC enrichment	Antibody based	Cell size	Antibody/aptamer based	Antibody based	Antibody based	Cell size and deformability	Cell size and deformability	PSMA/HER2 (+ size selection)	Antibody based	Antibody based
CTC detection		0.85	Isolation method	0.94	0.71	0.8		0.92	0.95	
Viability	~99%		na		na	na	na	na		na
Recovery	> 60%	86–90%	90–97%	> 50%			na	80–100%		> 95%
Purity	9%, 50%	na	1	51–100%	na	83–89%	na	62–74%	0.14	0.14
Throughput	> 387~609	> 142	> 260	> 120	> 480	> 575	> 450	> 550	> 300~420	> 550
Sample volume	2.7 mL	7.5 mL	1 mL	9 mL	1 mL	1–3 mL	1 mL	1 mL	4 mL	1 mL
References	Stott[49], Nagrath[61]	Zheng, Lin, Zheng[54–56]	Adams,[52] Dharmasiri[53]	Nagrath[61], Talasaz, Ameri[4,9]	Helzer[9]	Tan[6,9], Tan[7]	Mohamed[9]	Kirby[58] Gleghorn[59]	Stott[49]	Wang[39]

immunocapture) chip (Table 4).[58,59] Among these, the high-throughput microsampling unit is a microfluidic device that uses surface-immobilized monoclonal antibodies to separate CTC from the blood. The device releases unlabeled live CTC via trypsin.[52] Next, the CTC chip promotes antibody attachment through geometrical arrangement of 78,000 anti-EpCAM antibody microcolumns with the surface area of ~970 mm^2 [60] and fluid flow rate.[61] Gedi combines positive enrichment (using antibody-coated microcolumn) with hydrodynamic chromatography to maximize the streamline distortion of its geometry, reducing non-specific leukocyte adhesion and capturing high-purity CTC.[59] In comparison to the detection sensitivity of the cell search system (96%), microfilter,[55] microGEDI chip,[58] and microedar cell analyzer[57] have sensitivities of 75%, 94%, and 100%, respectively, whereas these four methods have specificities of 16%, 22%, 0%, and 0%.

Micro-devices benefit CTC biological characterization as they maintain high efficiency and purity, while they also ensure CTC integrity and activity. We note that micro-devices using surface antigen recognition have higher purity than the cell search system, but require more processing time. However, neither method can recognize CTC without related surface antigens, meaning that they will lose key malignant cells. Additionally, size-based CTC-screening micro-devices have faster processing speed than cell search systems, but insufficient purity. Though including the deformability parameters in size-based screening can further improve the purity, this increase is limited.

Microfluidics have also been combined with aptamers, single-stranded RNA, DNA, or peptide molecules with high specificity and affinity for specific target molecules to improve the purity. But these technologies have significant limitations. Screening requires a median of ~10 h. This longer processing time may affect the CTC survival, as well as the stability of cellular immunophenotype and genotyping. Thus, micro-devices may not be conducive for further CTC research.

Separation Based on Differences in Cell Density

Separating cells based on buoyancy is one of the oldest isolation methods. Also called as density gradient centrifugation or equal density gradient centrifugation.[7] Buoyancy-based methods have the advantages of low cost, simple to operate, and independent of specific antigen expression. Whole blood can be divided into three layers: plasma layer, denuclearized cell layer, and CTC layer.[62] However, these methods have low efficiency and purity of this method is low due to a large number of hematopoietic monocytes. The development of Oncoquick with a porous membrane[63] improved recovery rate to 87%.[64] CTC purity still remains very low using buoyancy-based techniques, barely reaching to 1%, even with the most advanced versions. A new centrifugal microfluidics platform was developed to address such problems. First, CTC were bound to anti-EpCAM-labeled beads to distinguish them from normal blood cells. Next, CTC were precipitated and separated under the density gradient medium, thereby improving the separation purity. Despite an improvement through the use of EpCAM, the lack of a standardized CTC density range may still result in CTC being lost during extraction.[66]

Another buoyancy-based device is the Accucycle system. Through a unique separation tube, blood is separated into three layers hematocrit, plasma, white blood cells, and platelets. The CTC are processed with row classification in the Cytefinder, an automatic scanning digital microscope and image analysis system. The average recovery rate of tumor cells is 90% using this method. Clinical trials reveal that after CTC enrichment, Accucycle can be used for genome analysis of a single CTC.[64]

The main advantage of buoyancy-based CTC separation is simplicity, resulting in viable cells that can be used for subsequent downstream experiments. These methods are also cost-effective and do not require much enrichment time. However, buoyancy-based methods are highly prone to contamination by other peripheral blood cells. In addition, the size and density range of different CTC subtypes are unknown, meaning that some CTC may not be detected.

Separation Based on Cell Size

Cell-size-based methods mainly involve microfiltration and therefore do not depend on surface antigen expression.[67] ISET is the most suitable representative of these techniques.[68] In ISET, pre-fixed peripheral blood is filtered using an 8 μm pore membrane. Next, CTC are immunostained for cell counting and morphological analysis. Enrichment of CTC was identified using ISET, mixed with epithelial and stromal phenotypes in the peripheral blood of NSCLC patients for the first time.[69] This outcome further demonstrated the significance of EMT and the mechanism of metastasis. Another study using ISET detected CTC in 31

patients with uveal melanoma by ISET, along with a single CTC or CTC cell cluster in 17 others. These results confirmed that CTC predicts poor prognosis in patients with uveal melanoma. Although not all the CTC are larger than normal blood cells, ISET has a >90% capture rate. Another major advantage is that ISET can separate epithelial cells without destroying cell morphology.[68] However, it and the other cell-size-based methods may miss the smaller tumor cells. More research using different cell lines and tumor types are required to determine the application scope and size threshold of ISET. In addition, the technique's specificity and purity must be increased.

In vivo Flow Cytometry

In vivo flow cytometry (IVFC) allows the quantitative analysis of circulating cells. The technique involves laser scanning of surface blood vessels to detect cells via various visualization methods, including fluorescence excitation and emission, photoacoustic effects, and photothermal effects.[70] The biggest advantage of IVFC is that the blood collection and treatment are not required.[71,72] At present, fluorescent and photoacoustic IVFC are the most widely used.[73] Fluorescent IVFC is approximately 1.8 times more sensitive than conventional whole blood flow cytometry.[72] This technique helps to identify the effects of other treatments, through fluorescence IVFC, as in case where sorafenib was revealed to reduce CTC count and lung metastasis in patients with advanced liver cancer.[74] Another study has used IVFC to observe the interactions between circulating breast cancer cells and dendritic cells. Mixed fluorescence and photoacoustic IFVC was also used successfully to detect GFP + breast cancer cells[75] and to identify apoptotic CTC in vivo. After androgen deprivation therapy, patients with prostate cancer were monitored using IVFC, revealing that the therapy reduced CTC count in peripheral blood. One use of fluorescent IVFC in an orthotopic liver cancer transplantation model and subcutaneous prostate cancer model demonstrated that the number of CTC clusters increased with primary tumor development. Thus, IVFC allowed researchers to confirm the key role of CTC clusters in tumor metastasis.

Nevertheless, IVFC has a clear disadvantage: its detection speed is 1 μL/min,[76] while ~5 L/min blood passes through human blood vessels. Therefore, IVFC is too slow for clinical needs. However, as seen in the examples provided, this technique is particularly useful for CTC detection and counting, which should be valuable for clinical monitoring and prognosis evaluation. The fact that CTC in circulating blood are not purified means that IVFC is not conducive to the detection of CTC molecular typing and the study of biological characteristics.

Conclusions

The detection and isolation of CTC have progressed in recent years. Advancements have been mainly driven by interdisciplinary research involving cancer biology, oncology, cell physics, materials science, chemistry, nanotechnology, and bioengineering. The diversification of CTC detection methods greatly improved the efficiency and reduced the cost. Unfortunately, every available detection method has major flaws. The cell search system involving surface antigen screening and enrichment possess the ease of automation and high specificity. Yet its clinical application has been hindered by low sensitivity and inability to detect all cancer types. In addition, most current CTC isolation methods require multi-step cell preparation, leading to CTC loss or damage, thus adversely affecting detection efficiency, accuracy, and sensitivity. The difficulty of extracting complete and viable CTC means further analysis is frequently impossible, thereby limiting the utility of these techniques for clinical decision-making.

The availability of high-throughput DNA sequencing means the whole genome and transcriptome of a single CTC can be obtained. These data allow increased attention to CTC biological characteristics, along with more exploration of genetic informatics and phenotypic functions (eg, through genome/transcriptome analysis and in vitro culture). These experiments require high molecular integrity and cell survival rate of isolated CTC, highlighting the importance of improving CTC separation technology. Therefore, future research on CTC detection should first focus on acquiring basic knowledge on CTC, specifically understanding the phenotypic changes accompanying the epithelial-stromal transformation. Insight into this process will help researchers improve the CTC separation efficiency, survival rate, and integrity while developing new methods. Additionally, new capture mechanisms should be explored to reduce blood cell contamination while improving survival and molecular integrity of purified CTC. Finally, antigen markers should be combined to increase cell-capture specificity and solve the issue of CTC heterogeneity. We also require in-depth research to address the experimental protocols, such as determination of detection timing and cut-off values, as well as standardization of specific techniques. Finally, we recommend

investigations to clarify the consistency of CTC biological characteristics.

Although there are problems to be solved, CTC analysis is a simple and feasible liquid biopsy technology that has gained serious attention and has achieved major success. With further development, CTC-based diagnosis should have considerable value in individualized treatment of patients with cancer.

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Disclosure

Xinhua Xu reports a licensed patent (ZL201920055267.9): A special petri dish for magnetic trap. The authors report no other potential conflicts of interest in this work.

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