Circulating Tumor Cells (CTCs)

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Circulating tumor cells (CTC) have been recognized as the cause of distant metastasis. Their unique role as metastatic seeds renders them a potential marker in the circulation for early cancer prognosis, as well as monitoring therapeutic response. This review summarizes existing CTC isolation technologies, advances in downstream analysis of CTC and their potential applications in precision medicine.

circulating tumor cells | therapeutic response | single-cell analysis | circulating tumor microemboli | ex vivo CTC culture | liquid biopsy

1. Introduction

Cancer metastasis, the process of tumor cells spreading from a primary tumor to distant organs, is the primary cause of cancer morbidity and mortality, which is responsible for about 90% of cancer-related deaths Circulating tumor cells (CTCs) detach from the primary tumor and invade surrounding tissue and travel to different sites through circulatory systems. However, it is difficult to identify CTCs from the blood of a patient with non-hematological cancers due to their extreme rarity, with numbers ranging from one to a hundred cells in a 7.5 mL tube of blood drawn [1][2]. Many studies have shown the potential of CTCs as a valuable prognostic and predictive biomarker in cancer management, helping to monitor the efficacy of therapies and detect early development of metastases via their downstream functional and molecular analysis [3][4].

2. CTC Isolation and Detection Techniques

In the past decade, we have witnessed remarkable improvements in the efficiency and accuracy of isolation of CTCs from peripheral blood samples. Several CTC isolation technologies are now commercially available and can overcome previous limitations, such as the rarity of CTCs in blood samples and the heterogeneity of CTCs. We summarize current techniques categorized by their use of CTCs' physical properties (size, shape, density, and flexibility), biological properties based on tumor-specific markers expressed in the membrane of CTCs, and a combination of physical and biological properties using microfluidic devices.

The differences in density of the diverse blood components have led researchers to use the density centrifugation method (Ficoll-Paque®) (CYTIVA, Marlborough, MA, USA) to separate peripheral blood mononuclear cells and CTCs from whole blood [5][6]. The OncoQuick® system (Greiner Bio-One International GmbH, Kremsmunster, Austria) combines the density gradient centrifugation system and a porous barrier that increases the depletion of

mononuclear cells [7]. Recently, an integrated platform was developed (RareCyte Inc, Seattle, WA, USA) for enrichment, detection, and single-cell analysis of CTCs in a single workflow, through combining the AccuCyte CTC system for CTC collection based on density, together with CyteFinder (RareCyte Inc, Seattle, WA, USA), a scanning digital microscope with a six-channel fluorescence detection system, and CytePicker for individual CTC isolation [8][9]. These separation technologies are all based on the density of CTCs.

Tumor cells in circulation are larger than the vast majority of normal blood cells. Perhaps the most efficient isolation and enrichment technology for CTC is sized based separation, and one of the most effective ways to exploit size differences is via sophisticated microfiltration systems. An example of this is the faCTChecker system (Circulogix, Inc, Florida)[22,23]. The main system captures fixed cells ready for analysis. Moreover, the faCTChecker is capable of live CTC capture by simply changing the geometry of the pores on membrane filters, which reduces flow resistance to prevent damage of captured viable cells [10]. Releasing captured cells from the faCTChecker is also achieved by coating temperature-responsive polymers on filters, which enables the detachment of cells as the temperature increases [11]. Staining of the captured cells can be performed directly in the filtration cassette. Additionally, the system enables the capture of viable CTCs and later release of the captured cells from the filtration cassette for further downstream analysis, such as single-cell analysis and ex vivo CTC culture.

The VTX-1 Liquid Biopsy System (Vortex Biosciences, Pleasanton, CA, USA) uses a microfluidic approach that does not use any antibodies or microfilters to capture cells; instead, it uses two types of forces: a shear-gradient lift force and a wall-effect lift force that directs particles away from the wall of the microfluidic channels where the blood is injected. The separation is based on the heterogeneity of the cells in structure and conductivity and on an electric field exerting a positive force (pDEP) and negative force (nDEP) The separation is performed via migration mechanism, using pDEP to attract CTCs and nDEP to repel leukocytes; the ApoStream® technology (Precision for Medicine, Bethesda, MD, USA) leverages differences in the dielectric properties of the cell, capturing cancer cells with positive DEP [12]. (Menarini Silicon Biosystems, Bologna, Italy) separates single cells by retention mechanism and the use of nDEP forces to trap CTCs in deep cages by the gentle dye electrophoresis force [13].

The magnetic-activated cell sorting system (MACS) is a method of separation of different cell populations depending on their surface antigens or cluster of differentiation (CD) molecules [14]. The CTCs are captured and isolated by immunolabeling with the superparamagnetic particles conjugated with antibodies for the enrichment and further characterization by immunocytologic, molecular, and cytogenetic assays [15][16]. (Illumina Inc, San Diego, CA, USA), a robotic liquid biopsy device that isolates and purifies live CTCs using the EpCAM protein as a biomarker, excluding cells that are not bound to magnetic particles [17]. The workflow assures high purity, efficiency, and viability for subsequent gene expression studies.

Microfluidic-based enrichment technologies have been explored for CTC detection methods. The first microfluidic device or affinity-based microchip system is the CTC-chip, which consisted of an arrangement of microposts where the CTCs are captured as the whole blood is pushed over the surface of the chip. The platform captures and analyzes the target cells by using antibody-based separation, with antibodies such as anti-EpCAM [18]. The second-generation platform called CTC-iChip is also a system composed of two modules: the first uses continuous

deterministic lateral displacement for the size-based separation of RBCs, platelets, and other blood components from the white blood cells (WBC) and CTCs; the second uses inertial focusing to align the larger cells and facilitate the subsequent separation by magnetophoresis, acquiring the cell of interest and depleting the white blood cells and contaminants [19].

The geometrically enhanced differential immunocapture (GEDI) chip is a technique to isolate CTCs using size-dependent collision frequency that maximizes the CTC–wall interactions while minimizing the interactions of other blood cells and antibody-coated 3D posts. against tumor-associated antigens from cancer cells of both epithelial and mesenchymal phenotype, as well as cancer stem cells [20]. This system is based on immunomagnetic separation using anti-EpCAM magnetic beads after the selection of cells through the leukapheresis technique from the buffy coat sample [21]. The system is composed of a three-layer sheath flow for a positive selection of the CTC population from whole blood using antibody beads anti-EpCAM, anti-MelCAM (melanoma cell adhesion molecule), anti-HER2, anti-MUC-1, and anti-TROP2 or TACSTD2

The affinity-based microchip system, which consists of an arrangement of microposts, is a more complex microfluidic device system when compared to the surface-based microfluidic technology. An example of this technology is the microvortex-generating herringbone-chip (HB-chip), which applies a passive mixing of blood cells through the generation of microvortices to increase the interaction between the CTCs and the antibody-coated chip surface [22]. Sheng and collaborators developed a new form of the HB-chip, the geometrically enhanced mixing (GEM) chip, that improved the previous system by increasing the groove width for high efficiency and high-purity tumor cell capture [23]. It is assembled using functionalized graphene oxide nanosheets on a patterned gold surface that allows CTC capture with high sensitivity at a low concentration of target cells [24].

The NanoVelcro CTC chip (CytoLumina Technologies Corp., Los Angeles, CA, USA) is composed of an overlaid polydimethylsiloxane (PDMS)-based chaotic mixer, anti-EpCAM antibody-coated silicon nanowires (SiNW), and a multilayer chip holder that assemble the functional components to immobilize CTCs. The system engenders vertical flows of the blood sample with a considerable speed that enhances the contacts between CTCs and the capture substrate, preserving cell morphology [25][26]. CD45, a pan-leukocyte surface marker. Some of the commercially available options are The EasySep™ Human CD45 Depletion Kit (STEMCELL Technologies, Vancouver, Canada) [27] and the quadrupole magnetic separator (QMS), composed of an immunomagnetic sorting system that uses an automated cell counter, filtration, and visual counting or a cytospin for cell analysis [28].

Expanding the variety of detection methods and the including markers that help to characterize cell populations at different phenotypic stages is crucial for improving cancer cell detection. The low abundance in blood and limited blood volume accessible from a cancer patient may represent a significant constraint on the detection of a particular cell population, such as the CTCs and their clusters. For example, with the ability to report a protein expression profile for CTCs as a function of surface marker expression, magnetic ranking cytometry (MagRC) has achieved a high level of sensitivity and resolution. In addition, a new strategy for detection of CTC and single-CTC proteomics was described in estrogen receptor-positive (ER+) breast cancer patients using the microfluidic single-cell resolution Western blot method for a panel of protein expression [29].

Recent advances in nanomaterials and nanotechnology offer high sensitivity, specificity, and multiplexed measurement capacity in CTC isolation and detection [30][31][32]. For example, the gold nanoparticles (AuNPs) have a strong binding capacity and the ability to be easily synthesized, prompting their use as an option for detection and capture of CTCs. They will serve as a good platform for assembling aptamers for high efficiency cell capture [33]. Other 3D nanosurfaces used as ultrasensitive platforms in CTC detection are silicon nanowires [34], graphene oxide [24], carbon nanotubes [35], and polymer nanofibers [36].

In addition to in vitro isolation of CTCs, several techniques and devices have been developed and show the potential for in vivo applications (e.g., in vivo CTC collection and direct detection). [37] developed a wearable in vivo indwelling intravascular aphaeretic CTC isolation device to continuously collect CTCs directly from a peripheral vein, showing its capability to screen 1–2% of the entire blood over 2 h in canine models. Nolan et al. demonstrated that GFP+ CTCs can be directly detected by using in vivo flow cytometry (IVFC) in animal models [38]. Moreover, the recent development of Cytophone technology (University of Arkansas for Medical Science, AR, USA) shows direct detection of CTCs in patients with melanoma, based on the detection of melanin-bearing CTCs by using an in vivo photoacoustic flow cytometry [39].

3. Downstream Analysis of Circulating Tumor Cells

CTC counts have shown to have a promising prognostic value in monitoring cancer progression and therapeutic response for advanced metastatic patients. Moreover, CTCs are extremely rare in early-stage cancer patients and in advanced metastatic patients. Other factors can also affect the baseline CTC count of cancer patients. Inflammatory breast cancer patients and small-cell lung cancer (SCLCL) patients with chronic obstructive pulmonary disease (COPD), for example, may have a greater chance of releasing tumor cells into the bloodstream than other types of cancer patients due to high vascularity and increased microvessel density [40][41].

Currently, CTC research is extending beyond enumeration for more accurate cancer diagnostics. Immense efforts have been devoted to a genotypic and phenotypic analysis of CTCs, such as molecular characterization, ex vivo expansion, and investigation of crosstalk between CTCs and their associated cells (e.g., stromal cells and immune cells). The information obtained from these studies provides unprecedented insights into the metastatic process and allows us to explore prognostic biomarkers and therapeutic targets for cancer management (**Figure 1**).

Identifying molecular defects associated with cancer progression and drug resistance is essential for the improvement of cancer diagnostics and therapeutics. Although molecular interrogation of CTCs holds great promise as a noninvasive “liquid biopsy” for real-time monitoring of the change of cancer drivers, it may also introduce a strong bias in the molecular analysis due to the extremely rare events of CTCs in the impure samples, even after an enrichment process. As a result of recent advances in technology (e.g., next-generation sequencing and single-cell isolation), researchers are now able to perform in-depth molecular characterization of CTCs at the single-cell level, which provides more clinically useful information when studying CTC heterogeneity and improving early patient stratification [42]. Additionally, the comparison of the copy number alternations (CNA) between single

primary tumor cells and circulating tumor cells reveals the focal CNAs affecting the MYC gene and the PTEN gene, which are the drivers for cancer metastasis [43].

In comparison with genomic analysis, transcriptome analysis provides more detailed information that allows us to precisely identify cancer drivers for diagnosis and therapeutic targets [44]. Although CTC enrichment steps may cause an effect on gene expression, researchers are still able to identify key genes by analyzing their RNA contents from different types of cancer patients, determining which expression is associated with cancer progression and treatment response [45][46][47][48][49][50]. Similar work has been performed in pancreatic cancers, showing that expression of Wnt2 regulates a metastasis-associated survival signal pathway that increases metastatic propensity. Several commercial devices enable automatic isolation and analysis of individual CTCs in a single workflow, such as the Polaris system (Fluidigm), Chromium (10X Genomics), and DEPArray system (Siliconbiosystems) [51][52].

In addition to the investigation of DNA and RNA biomaterials, researchers are also investigating the important roles that microRNAs and epigenetic modification play in mediating gene expression (e.g., oncogenes and tumor suppressor genes). While the limitation impacts single-cell proteomics, mass cytometry has enabled the measurement of up to 40 different target proteins in single CTCs. Mass cytometry uses antibodies conjugated with heavy metal isotopes to label cellular proteins, which allows for precise quantification of target proteins at the single-cell level [53]. Moreover, another technique has been developed for profiling protein expression in single CTCs using microfluidic Western blotting [29].

Researchers have widely used established cancer cell lines for studies of cancer pathological mechanisms and anticancer drug testing. Despite their significant role in cancer research, cancer cell lines do not completely mimic the original solid tumors [54]. Instead, the primary culture of patient-derived cancer cells can be a good model for identifying cancer markers and predicting the drug response of individual patients' tumors. Based on the mechanism of the metastatic cascade, CTCs offer valuable information about metastatic development over primary tumor cells obtained from a piece of tumor tissue.

Although the establishment of cell lines from CTCs has been challenging due to the rarity of CTCs in blood, significant advances in viable CTC enrichment techniques and culture systems have been making it possible. These generated CTC cell lines (6 out of 36 patients) were maintained for more than six months under hypoxic conditions with a combination of cocktail of growth factors and low attachment plates. They also performed drug sensitivity testing for these CTC cell lines, which revealed potential new therapeutic targets. , captured CTCs from patients with lung cancer using a microfluidic device, and these captured CTCs were cultured in situ along with tumor-associated fibroblasts and extracellular matrix [55], showing a model of the tumor microenvironment and subsequent ex vivo expansion of CTCs.

Cancer drug screening in patient-derived tumor cells holds great promise for translational and personalized medicine. In organoid culture systems, single cancer cells are automatically grown and differentiated into original tissue architecture embedded in a 3D extracellular matrix (laminin and collagen) along with stem cell niche factors.

established organoid cultures from metastatic biopsies and CTCs from patients with metastatic prostate cancer [56]. These patient-derived organoid lines harbor genetic defects similar to parental prostate cancer, which can be used as genetically manipulatable models for drug response.

Circulating tumor microemboli (CTM), so-called CTC clusters, the heterogeneous multicellular clumps that are released from the primary tumor, have been shown to have higher metastatic potential than single CTCs in animal and clinical studies [57][58]. Other than tumor cells, the overall CTM is composed of different types of cells, such as stromal cells and immune cells. Growing evidence has shown that CTCs may use platelets as a protective shield to escape from host immune surveillance and as facilitators to enhance the attachment to endothelial cells at the metastatic site [59][60][61]. The same group also developed genetic engineering platelets to neutralize circulating tumor cells [62].

The tumor microenvironment is a complex ecological system where tumor cells, stromal cells, infiltrating immune cells, and extracellular matrix, as well as the blood and lymphatic vascular networks, together orchestrate cancer metastasis. In the primary tumor, tumor-associated macrophages (TAMs) have been well studied as critical metastatic effectors and demonstrated to stimulate angiogenesis and promote tumor cell proliferation, invasion, and intravasation [63]. Interestingly, recent discoveries show that CTCs can fuse with macrophages, and these fusion cells play an important role in tumor heterogeneity and chemoresistance [64]. In addition to circulating macrophages, myeloid-derived suppressor cells (MDSCs) have been shown to facilitate cancer metastasis by shielding CTCs from immune surveillance [65].

Tumor-associated stromal cells can also significantly contribute to the tumor microenvironment, acting as feeder cells to support tumor growth and development [66]. During intravasation, cancer-associated fibroblasts (CAFs) have been shown to degrade extracellular matrix, creating tunnels that allow tumor cells to pass through and lead to blood vessels. Yadav et al. showed that Bcl-2-overexpressed circulating endothelial cells were released from primary tumors and co-migrated with CTCs to distal sites in an animal model. The interaction between the two types of cells enhanced anoikis resistance via the activation of the Src/FAK pathway [67].

4. Current Challenges and Future Direction for Clinical Utility

Based on the current standard procedure of CTC tests, one of the challenges comes from CTC sampling where 7.5 mL of blood sample is withdrawn from venous blood vessels. Another concern of CTC sampling is whether a greater number of CTCs can be detected in arterial blood or venous blood. [68] provided evidence that CTCs were detected in all patients when arterial blood was analyzed, whereas only half of the patients showed positive for CTCs by using venous blood. These findings suggest that arterial blood may be a better source of CTCs than venous blood.

However, downregulation of epithelial markers on CTCs, such as EpCAM, is associated with epithelial–mesenchymal transition (EMT), a biologic progress where epithelial cells acquire a mesenchymal phenotype and thus become more aggressive and invasive. As a result, the system underestimates the number of CTCs and only

detects them in about 60% of metastatic breast cancer patients. The researchers have used cell surface vimentin, a well-known EMT marker, to isolate CTCs and showed that EMT CTCs correlate with disease progression or relapse of cancer patients [69][70]. Notably, given that EMT-related biomarkers are also expressed on the surface of certain types of normal cells (e.g., fibroblasts, monocytes, and macrophages), it is imperative to identify specific and broad markers for isolation and characterization of CTCs.

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