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Clinical Applications of and Challenges in Single-Cell Analysis of Circulating Tumor Cells

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Technological advancements in next-generation sequencing are continually changing the landscape of genomic, transcriptomic, and epigenetic research at the single-cell level. These technologies have been used to detect and analyze circulating tumor cells (CTCs) at the molecular level and provide a new approach for the management of cancer patients. A series of unanticipated discoveries, including the heterogeneity of cancer cell populations, new driver mutations responsible for the resistance of tumors to chemotherapy, and the mechanism of tumor metastasis, have been made using single CTC sequencing. CTC detection has been used in cancer diagnosis and monitoring and in determining the prognosis of cancer patients. Traditional treatment for cancer patients is universal and does not consider genetic variations among patients, but in the era of precision medicine, giving the right drug to the right patient at the right time is the core philosophy. In this study, we review the fundamental principles of CTC isolation and single-cell sequencing and discuss recent progress in their application in both basic research and clinical fields and describe the current challenges.

Keywords: precision medicine, circulating tumor cells (CTCs), capturing, single-cell sequencing

Introduction

Liquid biopsy is a new technology that analyzes non-solid biological tissues, such as blood, and it is less invasive and more sensitive than traditional biopsies. Liquid biopsies can be used for a broad range of samples, such as circulating tumor cells (CTCs), circulating cell-free DNA (cfDNA), cell-free microRNA, exosomes, and blood platelets, and are widely used in exploring the molecular landscapes of tumors. Compared with tissue-based profiles, liquid biopsy can provide time sequenced picture rather than a snapshot of tumor heterogeneity for the samples that can be obtained repeatedly. For such reasons, liquid biopsy is suitable for longitudinal surveillance of patients and evaluation of prognosis to guide patient care and be integrated into clinical practice (Siravegna *et al.*, 2017). As a new type of liquid biopsy, CTCs are a population of cells shed from a primary or metastatic tumor into the peripheral blood and lymph (Pantel *et al.*, 2009; Yu *et al.*, 2011). Analysis of CTCs through next-generation sequencing (NGS) and whole genome amplification (WGA) can reveal the mechanism of tumor metastasis, intra-tumor heterogeneity, and genetic alterations. In recent years, CTCs have exhibited great potential for noninvasive diagnosis and real-time monitoring of cancer.

Single-cell sequencing enables researchers to recognize a problem from a new perspective and provides a new method to identify etiology at the genomic level. Analysis of CTCs using single-cell sequencing can help to reveal the underlying mechanisms of tumorigenesis and metastasis and to identify gene mutations that potentially contribute to tumor metastasis or drug resistance. Through identifying specific mutations and markers in CTCs, individualized therapy may be formulated, thus improving understanding of the molecular mechanisms of tumor metastasis, relapse, and chemotherapy resistance.

CTCs and Tumor Metastasis

CTCs are a population of cells that are shed from primary or metastatic tumor deposits and migrate into peripheral blood. CTCs are related to tumor metastasis (Pantel *et al.*, 2009; Yu *et al.*, 2011), which is considered to be the major cause of cancer-associated mortality (Fig. 1). CTCs are

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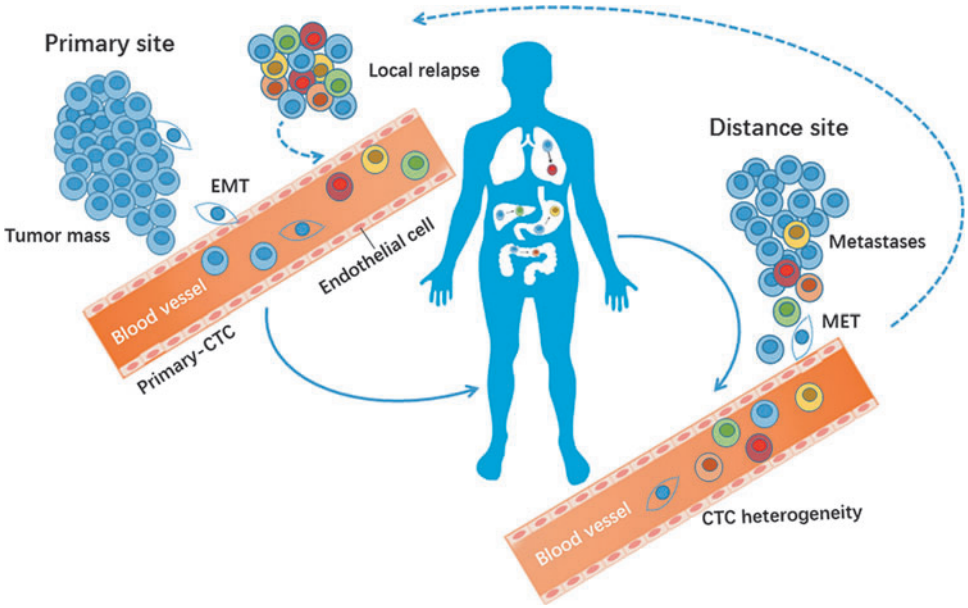

FIG. 1. Editing of the CTC pool by the microenvironment of distance metastatic sites. Tumor cells leave the primary tumor and circulate through the bloodstream. Each time the CTCs reach a new niche (distant organs, e.g., the thymus, liver, lung, or colorectal region), they undergo an organ-specific mimetism and may leave this site with a new organ-specific signature. CTC, circulating tumor cell; EMT, epithelial to mesenchymal transition; MET, mesenchymal to epithelial transition.

predictive biomarkers in clinical care, owing to their close relationship with tumor metastasis (Aceto *et al.*, 2014). Notably, CTCs have been found to appear in vasculature before detection of the primary tumor (Kalluri and Weinberg, 2009). Tumor cell metastasis is a complex process consisting of a series of stages that include the loss of adhesion proteins (e.g., E-cadherin), shedding of tumor cells, intravasation, dissemination, arrest at secondary sites, extravasation, colonization, and formation of metastatic tumors (Chaffer and Weinberg, 2011; Gradilone *et al.*, 2011; Krebs *et al.*, 2014). During this process, two crucial changes typically occur in CTCs: epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET). EMT is a highly complicated process in which cancer cells revert to a state resembling the mobile cells in the developing embryo (Hanahan and Weinberg, 2011). MET usually occurs when CTCs arrest at the primary tumor or other metastatic lesion site and become arrested in a state known as dormancy or undergo alterations to adjust to the new environment. Whereas EMT has been demonstrated in human cancer cells in the circulation, the requirement for EMT to initiate metastasis remains a debated topic (Ledford, 2011; Yu *et al.*, 2013).

CTC Detection and Isolation

In recent years, CTCs have exhibited potential for tumor diagnosis, treatment, and monitoring, and great efforts have been made to detect and separate CTCs. Along with advancements in CTC enrichment techniques, the efficacy and accuracy of CTC capture have greatly improved. CTC isolation techniques can be classified into three types: nucleic acid-based, physical property-based and surface marker-based methods (Fig. 2). Each method has specific characteristics, application range, and limitations, which are listed below.

Nucleic acid-based methods

Nucleic acid-based CTC detection identifies specific tumor DNA or mRNA (cfDNA) to confirm the presence of CTCs indirectly (Esmaeilsabzali *et al.*, 2013). Detection involves designing specific primers to combine with specific DNA or cDNA sequences that are extracted from enriched samples. These genes represent specific tumor genes that contain known mutations, translocations, and methylation patterns (Riethdorf *et al.*, 2008). The nucleic acid-based method has the highest sensitivity but lacks specificity, owing to the potential of captured noncancerous cells to generate false-positive signals, thus decreasing the overall accuracy. According to previous work, not all the detection targets are tumor specific, because the same targets can be found in blood cells (Fehm *et al.*, 2009; Punnoose *et al.*, 2010). Pantel *et al.* (2008) have reported that CK-19, a major marker for CTC detection, is also presenting immune cells.

Physical property-based methods

Physical property-based methods isolate CTCs on the basis of physical properties, including size, density, mechanical plasticity, and dielectric properties, and have the advantage of allowing CTC separation without marker-based preselection. CTCs (20–30 μm) are larger than most blood cells (8–12 μm), and thus, they can be isolated through specific porous filters that allow only blood cells to cross. This technique has been used in commercialized ISET® (Rarecells, Paris, France) and ScreenCell® systems (ScreenCell, Paris, France) for isolation of fixed and live CTCs (Desitter *et al.*, 2011). Owing to their relatively larger size and larger mass than those of blood cells, CTCs can also be separated using density gradient centrifugation. In addition to size and density disparity, differences in mechanical properties between blood cells and CTCs have been exploited to capture CTCs; specifically, blood cells have inherent deformability and are smaller, whereas CTCs are larger and stiffer (Yap and Kamm, 2005). On the basis of different cells have different conductivities and polarities, CTCs can be isolated on the basis of their unique dielectric properties when they are placed in an electric field (Kobayashi *et al.*, 2015). Nevertheless, physical property-based CTC detection is subject to the physical properties of tumor cells, and