Circulating Tumor Cells: A Window to Understand Cancer Metastasis, Monitor and Fight Against Cancers

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Abstract: Metastases are the major culprits behind most cancer-related death and the central challenge to the eradication of a malignancy. Circulating tumor cells (CTCs) have the potential to help us understand how metastases form, to be utilized for cancer diagnosis and treatment selection and even to be targeted for cancer treatment. Many advances have been made regarding the isolation of these rare cells. However, several challenges and limitations in CTC analysis still exist. Multiple color immunofluorescence, genetic analysis (e.g. Fluorescence *in situ* Hybridization, microarray and next generation sequencing) and CTC culture will be effective tools to study CTCs and provide information on metastatic mechanism and clinical implication. In this review, we discuss the importance of CTC study in understanding cancer metastasis and their potential clinical application as biomarkers to predict cancer progression and treatment response, as well as the current situation for CTC isolation and analysis.

Keywords: Circulating tumor cells, metastases, genetic alterations, biomarkers, prognosis, treatment response.

INTRODUCTION

An estimated 3.45 million new cases of cancer and 1.75 million deaths from cancer were reported in Europe in 2012 [1]. The top 3 cancer sites with the high incidence were cancers of the female breast (464,000 cases), followed by colorectal (447,000) and prostate (417,000). Life expectations for localized and metastatic disease differ significantly. Taking prostate cancer (PCa) as an example, the report from America showed that the average 5-year survival rate for patients with localized and metastatic prostate cancers is 100% and 28%, respectively [2]. Metastases are the greatest challenge for doctors to eradicate malignancy. Therefore, a deeper understanding of the metastatic process and the preventing its occurrence is critical to improve the survival of cancer patients and consequently a key factor to fight against malignant tumors.

Circulating tumor cells (CTCs), which are traveling in the vasculature on the way to distant metastatic sites [3], have the potential to help us understand how metastases form and hold key information for cancer diagnosis and treatment and even become a treatment target [3, 4]. This article will review the importance of CTC studies in understanding cancer metastases, their potential clinical applications as biomarkers for cancer progression and predicting treatment response and the current situation regarding CTC isolation and analysis.

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THE IMPORTANCE OF CTC ANALYSIS

1. Understanding Metastatic Mechanisms

Billions of tumor cells constitute a typical cancerous tissue, among which, some cells may acquire the ability to separate from the main tumor mass and migrate through surrounding tissue barrier, eventually get into the circulation of the bloodstream. During successful dissemination, these deciduous cells invade the surrounding tissues of the primary tumor, its blood and lymphatic vessels, translocate to distant tissues or lymph nodes, leave the vessels, adapt to the new microenvironment, and eventually seed, proliferate, and colonize to form detectable metastases. As blood is the route that disseminated cancer cells can travel to a distant organ site to establish metastatic cancers, those cells in the circulation are closely associated with cancer metastasis and of high interest. These so-called CTCs in cancer patients were first detected in 1869 by an Australian physician named Thomas Ashworth [5]. However, the potential value of CTCs were only realized by researchers until 1990s when primary tumor cells were found lodged in a cancer patient's bone marrow who had no evidence of metastasis at that moment and those cells were so-called disseminated tumor cells (DTCs) [6]. CTCs are part of DTCs and they are highly correlated, although DTCs in bone marrow were more frequently positive than in blood [7,8]. This might be explained by the fact that blood analyses allow only a 'snapshot' of tumor cell dissemination whereas bone marrow is a homing organ for DTC. However, obtaining tissue from bone marrow is challenging especially if frequent real time monitoring

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is required. Even if such material can be obtained, whether a biopsy from a single site is representative of the majority of metastatic lesions is questionable because cases of marked heterogeneity have already been reported [9]. CTCs in bloodstream then attracted more and more attentions from researchers.

CTCs were reported to be rare cells, constituting as few as 1 cell per 1×10^9 normal blood cells in patients with metastatic cancer [10]. Other major types of blood cells to be considered may comprise leukocytes (approximately 7 million/mL of blood) and red blood cells (approximately 5 billion/mL of blood). On the other hand, as Butler and Gullino stated in 1975, entry of tumor cells into the bloodstream is a frequent event [11]. he observation from xenografted models [12,13,14] showed that millions of tumor cells were continuously shed into circulation, but only a small proportion might survive to reach a distant organ and stay in a dormant status or to form metastases. All the other cells underwent apoptosis. The various fates are shown in Figure **1**; 1. anoikis, disintegration by immune-editing, or transition to a dormant state while remaining in the circulation [15]; 2. cell death following extravasation [16]; 3. forming occult micrometastases and becoming dormant for a long period of time (5-25 years) without subsequent aggressive proliferation (possibly resulting from deficient angiogenesis) [17]; 4. developing a malignant macrometastasis. In fact, only a small fraction (~0.1%) may remain alive in the circulation after 24h, among which even fewer cells (<0.01%) are progenitors of a metastatic mass. The majority undergo anoikis and are generally lysed within a few hours after invasion [18]. More knowledge of how those CTCs survive will help a better understanding of cancer metastasis.

In circulation, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-AKT pathways activation can inhibit anoikis of tumor cells [19]. Besides self-anoikis, the tumor cells in the circulation have to resist the turbulence or shear forces of blood flow and immune defenses to survive [20]. For example, some cancer cells [21] have been shown to alter the intracellular processing of presented antigens and generate altered peptide ligands (APLs), so that tumor recognition by antigen-specific cytotoxic T-lymphocytes (CTLs) will be interfere with and immune tolerance will be induced. Notably, CD47, a molecule that helps tumor cells evade macrophage phagocytosis [22], as well as 'don't

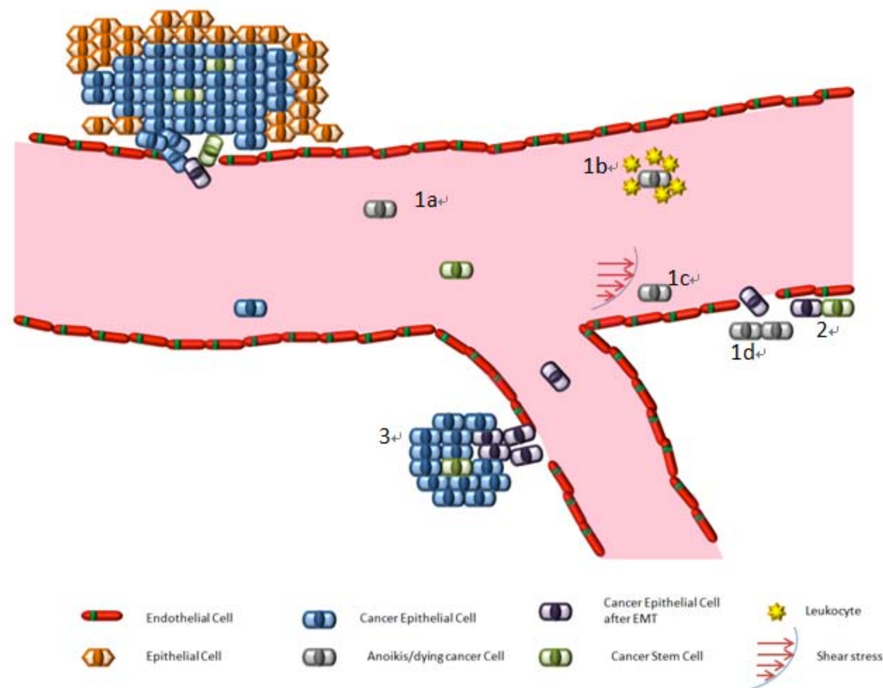

Figure 1: Various fates for CTCs.

1. Anoikis or dying cells: 1a. self anoikis of sloughed cells; 1b, cells attacked by leukocytes resulting disintegration; 1c, dying by shear stress from flow fluid; 1d, dying after extravasation.
2. Form occult micrometastases and become dormant.
3. Form macrometastases.

eat me' signal [23], was found on the surface of various human solid tumor cells, including breast, ovarian, colon, bladder, brain, liver and prostate cancer samples. Blockade of these pathways may offer an attractive target for controlling cancer metastasis. The ability of cancer cells to adhere and aggregate with platelets, which helps protect them from the turbulence and shear stresses of the circulation and facilitate arrest in the microvasculature, is another interest to researchers. Activation of protease-activated receptor 1 (PAR1), which was found on tumor cells, has been shown to increase cell adhesion to platelets and be associated with increased bone metastases [24,25]. Additionally, treatment with antiplatelet antibodies or heparin, which prevents platelet aggregation, has been demonstrated to decrease the incidence of lung metastases by inhibiting platelet-tumor cell interaction [26,27]. Moreover, the presence of circulating fibroblast-like cells in the blood was reported to be able to help distinguish men with and without metastatic cancer [28].

In contrast to our natural view that tumor dissemination occurs only at late stages, CTCs are also reported to be detected in patients with early stage cancers [7,29-32]. One or more CTCs were reported to be detected in 100% of 7 patients with early-stage PCa [29], 19.6% of 692 node-negative [33] and 24% of stage 1-3 breast cancer patients [34]. Whether this early release happens in all types of tumors and what the association is between these early CTCs and ultimate metastasis remain unclear. Cancers recurring after a successful surgical primary tumor resection indicates that metastases without local recurrence may originate from CTCs that are already present [35]. Yet it is not known which specific subpopulation of the heterogeneous CTC pool found in patients after surgery initiates metastases. Long-term prospective follow-up studies in patients should be carefully designed to evaluate the clinical relevance of early and late disseminated cells. A subpopulation of CTCs found in the blood of patients has been hypothesized to initiate metastasis, named as metastasis-initiating cells (MICs) [36], such as $EpCAM^+CD44^+CD47^+MET^+$ CTCs, whose abundance correlates with lower overall survival and increased number of metastatic sites compared to all $EpCAM^+$ cells. Another observation against our long-held view of tumor recurrence is the self-seeding hypothesis, proposing circulating and metastatic tumor cells can return to orthotopic sites. This finding demonstrated that bone marrow or blood, the reservoir for DTCs, would be a promising drug target to help controlling metastatic and local relapse. This hypothesis is supported by some clinical observations, including a reduction in the number of DTCs using bone-targeting drugs (such as bisphosphonates) [37], which has been linked to fewer local relapses [38], and one recent phase 3 study, showing that Radium-223 dichloride (radium-223) can significantly improve overall survival for men with castration-resistant prostate cancer (CRPC) and bone metastases, when used to selectively target bone metastasis [39]. However, firmer proof is still required, for which detected CTCs may provide insight.

Genetic characterization of CTCs may fill a gap in our understanding of the relationship between primary and metastatic tumors. Copy number profiles in primary tumors have been reported to be highly similar to the metastases, indicating the metastatic cells emerge as a main advanced expansion, but not from a completely different subpopulation [40]. Although the similarity of genetic alterations between primary and metastatic tumor exists, two fundamental concepts of systemic cancer metastasis through CTCs/DTCs have been suggested [41]. The first is linear progression, in which DTCs from primary cancer are thought to expand and grow to a metastasis after variable time of adaptation to the ectopic microenvironment. In contrast, the second is parallel progression, suggesting that genetically less advanced cancer cells disseminate in an early stage and progress at the ectopic site. Adaptation to a distant site here mainly refers to a process of mutation, selection and inheritance [42]. The main findings [43-46] regarding the genetic disparity between primary tumors and DTCs, focused mainly on prostate and breast cancer, including: 1) DTCs from bone marrow generally display fewer genetic abnormalities than matched primary tumors. 2) DTCs displaying typical changes, such as chromosomal gains and losses for the respective type of tumor, are mostly undetectable until when manifest metastasis is diagnosed rather than at the time of primary surgery. These observations, supporting the parallel progression model, suggest that metastatic cells evolve independently from primary tumors, which are incompatible with the linear progression. More evidences are required to make a conclusion which model represents the real status better, or a combination of them.

Biomarkers based on the gene sets and genomic profile of CTC subsets also have the potential to predict homing and colonization to specific distant metastatic sites or even reveal sites of primary tumor origin [3].

Furthermore, in breast [47] and prostate [48,49] cancers, systemic drug may impose various selective pressures to CTCs, probably influencing the evolutionary history of these latent seeds of metastasis which tend to make residual tumor cells more aggressive and resistant, acquiring the characteristics of stem cells.

2. Clinical Implications as a Biomarker

Apart from the role of CTCs in understanding the mechanism of metastasis, they are also promising to be developed into novel surrogate biomarkers. Biomarkers, including those for disease staging, prognosis, therapeutic response/efficacy prediction, and resistance monitoring are invaluable for patient management [50]. Accurate and repeated biopsy of tumor tissues is crucial for our improved understanding and monitoring of changes in tumor cell populations during disease progression and in response to therapies. However, repeat biopsies of overt metastases require difficult invasive procedures that are not easily acceptable and impractical in the clinic. CTCs, also called liquid biopsies, have the potential to be used as a real-time, minimal-invasive monitor of cancer progression and to measure the effectiveness of therapies in anti-metastasis, as they only require a minimum invasive blood sampling. The potential of using CTCs as biomarkers has been explored in several types of human cancers, including breast cancer [51,52,23,53-56], prostate cancer [50,57-61], lung cancer [62-64], colorectal cancer [65-67], pancreatic cancer [68], gastric cancer [69], and hepatocellular cancer [70].

The progression of cancers diagnosed with similar clinical and pathological characteristics by existing methods often distinct in disease progression and response to therapies, which makes molecular diagnosis essential. Detection and characterization of CTCs pose the potential to establish the risk for metastatic relapse, to stratify patients to different adjuvant therapy, to identify various therapeutic targets, and to monitor systemic anticancer therapies, showing its potential as the next-generation diagnostic and evaluation tool [6,18]. For example, analysis of biomarker status in CTCs collected prior to treatment could potentially be used to select an appropriate targeted therapy, while appearance of resistance markers can be detected before disease progression by repeated longitudinal sampling during treatment and potentially guide switching to a more appropriate therapy. Recent reports have shown that CTCs are currently included as biomarkers in >400 clinical trials [71] to assess the survival.

2.1. Biomarkers in Tumor Staging

As CTCs are a source of metastatic cells, they should be included in the staging algorithms for malignant tumors, which are currently focused on the primary tumor and node/bone/visceral metastasis. Adding CTC as a staging parameter can provide additional tumor information. Whichever isolation method was used, most results showed that baseline CTC numbers among different cancer types [34,60,72,73] were not correlated with tumor size detected by imaging analysis, serum markers, such as prostate specific antigen (PSA) for prostate cancer, pathological axillary lymph node status, or index for tumor differentiation, either from the preoperative biopsy or from the more complete pathological analysis of the resected tumor specimen.

The proliferative or quiescent status of tumor deposits representative by CTCs provides another informative support for cancer staging. A significant variation in the proliferative index (from 1 to 81%) by Ki67 staining of CTCs found among patients at different stages of disease has been described [73]. Patients with metastatic prostate cancer, which was highly responsive to androgen withdrawal, had a low Ki67-postive fraction, whereas those with progressive castration-resistant disease had a considerably higher proliferative index.

CTC enumeration is not included in current guidelines [74] by the American Society of Clinical Oncology for non-metastatic breast cancer patients, however, is provided as an option for those with metastatic breast cancer, indicating the increasing recognition of the importance of information that CTCs provide in tumor staging.

2.2. Biomarkers in Disease Prognosis

When doctors discuss the option of surgery with patients, they always introduce an unknown and unpredictable recurrent rate after the excision of primary lesion. For those inoperable ones, usually there is only a general survival rate provided, rather than a specific individual one. Could this status be changed or at least improved?

Numerous prospective studies on patients with different metastatic cancers have indicated that a higher number of CTCs at the baseline or any time during the therapy is associated with a shorter survival

time [60,75-80]. De Bono *et al.* [75] demonstrated that the enumeration of CTCs predicted overall survival better than change of PSA levels in the CRPC patient population. One important but still controversial issue is whether the presence of a single CTC may be clinically relevant and what CTC count thresholds should be used to establish survival analysis. Generally, a cut-off of five CTCs per 7.5 mL whole blood has been adopted as a positive result in breast cancer by most researchers, based on the early study by Cristofanilli and colleagues [54,81]. However, the ability to detect CTCs in early stage patients is an unneglectable issue, and that's why most researches were focused on the application in metastatic patients. One recent study [34] showed the prediction value for early recurrence and overall survival in non-metastatic breast cancer patients by the presence of one or more CTCs.

As in staging, mere enumeration gradually is insufficient to meet the clinical need. The deeper understanding of CTCs is required and the importance of molecular profiling of CTCs has been well recognized. For example, patients having CTCs with a PSA median expression >50% demonstrated a significantly better survival than those with <50% expression [82], which is consistent with the finding [83] that PSA$^{-/low}$ cells have properties characteristic of cancer stem cells.

2.3. Biomarkers in Prediction for Treatment Response

Nowadays, the efficiency of existing treatment against cancer, whether it be surgery, chemotherapy, targeted therapy, immunotherapy, or even gene therapy, is mainly hampered by the inter-individual heterogeneity of the cancer [74]. During the last decade, cancer therapy has undergone a remarkable change. While cytotoxic therapies, based on the hypothesis that cancer cells are characterized by rapidly growing cells, target mostly on tumor cells undergoing mitosis, more recent drugs try to interfere specifically with pathways that are genetically altered in tumor cells, in which cancer cells are addicted. Normal cells do not display those changes and will be spared [84]. Therefore, the specific genomic alteration pattern for metastatic in an individual is crucial for the understanding and predicting therapeutic success [40]. A key to successful development of gene/pathway targeted therapy is the ability to pre-select patients who harbor the particular pathway of interest through diagnosis at the molecular level [85,86]. Molecular characterization of CTCs, which can be performed frequently and in real time, should provide such essential information on therapeutic targets, with the result of a more tailored personalized therapy.

Danila *et al.* [87] investigated the role of *TMPRSS2-ERG* status in CTCs as a predictive marker for sensitivity in CRPC patients treated with Abiraterone acetate post-chemotherapy. Although the fusion characterized by reverse transcription polymerase chain reaction (RT-PCR) did not predict for response to Abiraterone treatment, it demonstrated the role of CTCs as surrogate tissue that can be obtained in a routine practice setting. A recent study [88] reported the predictive value of the detection of AR-V7 in CTCs from CRPC patients in the treatment response for enzalutamide and abiraterone. In breast cancer, human epidermal growth factor receptor 2 (*HER2*) amplification is a requirement to administer anti-HER2 directed drugs such as trastuzumab (Herceptin®) or lapatinib [89,90,91], which is currently mainly analyzed by primary tumor tissue only. However, several researchers [51,52,92] reported HER2 positive CTCs for those who previously had HER2 negative primary tumors, suggesting that additional patients might exist who would benefit from this treatment. Furthermore, molecular activation of the epithelial growth factor receptor (EGFR) pathway as well as the absence of *KRAS*, *BRAF* or *PI3K* mutations was reported to be as prerequisite for anti-EGFR antibody response [93,94]. A remarkable *KRAS* mutation heterogeneity was reported to occur in the analysis of individual CTCs [95], which could be used to explain for the failure of drug-mediated EGFR inhibition in some patients, or on the other hand to help stratify patients who would benefit from this treatment.

2.4. Biomarkers in Monitoring Treatment Response

A marker to monitor the response during treatment is critical. Reduction in the number of CTCs (or high-risk CTCs) may be one of such biomarkers if it can be proved to reliably correlate with disease response to treatment. It will be a crucial development for drugs used in long-term prophylactic or adjuvant settings, because the assessment can be facilitated in a reliable way without waiting years for survival analysis and the optimization of drug schedule can be speeded up as well.

Taking PCa as an example, PSA, the most used biomarker for PCa worldwide at present and as well the most frequently altered biomarker in the disease, may not reflect the status of the disease accurately as

anticipated. Up to 20% of CRPC patients, who eventually respond to a systemic cytotoxic therapy proven to prolong life, have an initial PSA increase before the decline and the decline even may not occur for up to 12 weeks or not occur at all. Meanwhile, in a study of patients with CRPC, post-treatment CTC numbers were a stronger prognostic factor for survival than a 50% decline in PSA (receiver operating characteristic area under the curve: 0.87 vs 0.62), and the authors suggested that CTC numbers measured at 4 or 8 weeks can even discriminate between favorable or unfavorable outcomes with therapy [60]. Decrease of number of CTCs after chemotherapy for CRPC patients was also observed correlated with better survival in a Phase I study [96]. In metastatic colorectal cancer patients, a conversion of baseline unfavorable CTC number to a favorable one at 3-5 weeks after treatment was reported to be correlated with significant longer survival compared to those without this change [77]. Similar results were also reported in breast cancer [97], indicating elevated CTCs after therapy is an accurate indication of subsequent rapid disease progression. Moreover, CTC detection was also reported to be a superior surrogate than standard imaging procedures for therapy response [72].

2.5. Relationship with Cell-Free DNA

Briefly mentioned here is cell-free DNA (cfDNA) in the circulation, which was first described by Mandel and Metais in 1948 [98] and applied into clinical arena by recent development of digital analysis of DNA sequences. Several groups reported that mutations present in cfDNA were highly concordant with those present in the matched tumor [99-102]. Furthermore, enumeration of cfDNA amounts can be utilized to accurately track dynamic changes in tumor burden [100, 101]. More refined studies have expanded this work to detect structural genomic alterations such as rearrangements [103] and amplifications [104] in cfDNA and have shown the emergence of acquired resistance mutations in patients treated with targeted chemotherapy [99,102]. CfDNA is also thought to represent a pool of tumor genomes derived from multiple independent lesions within the body [104]. However, partly released from dying tumor cells, cfDNA is questioned by its representative for the accurate and synchronous information on resistant clones [105]. In contrast, CTCs represent intact viable tumor cells that can be analyzed for various biological molecules of interest (such as DNA, RNA and proteins) [106,107] and, most importantly, allow subpopulation study by different biomarkers to identify stem cells. Genomic analysis of these stem cells then may reveal therapeutic targets and drug resistance mechanisms that are highly relevant to pausing or even reversing cancer progression. CTC and cfDNA analyses may reveal complementary information [105].

CTC ISOLATION TECHNOLOGIES

The rarity of CTCs and the complexity of the background that CTCs survive, along with the limitation of current technologies, all hinder the study of CTCs. One thing for sure is that, to fully characterize CTCs, reasonable efficiency of CTC isolation is required. Only in the past decades, researchers managed to capture CTCs with certain degree of efficiency using the emerging technologies. Multiple approaches have been employed to detect CTCs, ranging from standard flow cytometry [108,109], fast scanning cytometry [110], size based systems [111-114], technology based on the application of immune-magnetic beads [58,115, 116] to newly developed isolation chips [29,117-119]. One or more unique properties of CTCs, that distinguish them from surrounding non-tumor cells, are utilized in CTC enrichment and isolation technologies, such as physical properties (size, density, electrical charges and deformability) and biological properties (such as surface protein expression and expression of specific genes).

Flow cytometry was tested for CTC detection long ago [109]. Owning the ability to simultaneously perform multiple parameters (e.g. cell size, DNA content, expression of cell markers, and cell viability, etc) on a cell-by-cell basis, it has a high specificity in detecting CTCs. However, its sensitivity was scrutinized. Another major limitation to this approach was that no additional tests could be performed once cells were assessed by antibody accessible biomarkers.

Currently, biological property based CTC isolation is a mainstream of methodology, which is mainly focused on epithelial cell adhesion molecule (EpCAM) [120], also known as CD326. Initially discovered in human colon carcinoma, CD326 is a transmembrane glycoprotein that is highly expressed in rapidly proliferating carcinomas [121]. To note, EpCAM is also expressed on normal epithelial cells due to its mark for cells of epithelial origin. However, the expression levels in normal cells are generally much lower than those in malignant cells [121] and distribution is more homogenous on the cancer cell surface [120,122-124]. EpCAM based CTC enrichment and isolation uses immune-magnetic beads coated with anti-EpCAM

antibody to enrich EpCAM-expressing epithelial cells. Captured cells can be further immuno-stained to distinguish CTCs. In 1999, criteria were published by the European ISHAGE Working Group on Standardization of Tumor Cell Detection. CTCs were defined as objectives with cell morphology and clearly enlarged nucleus as well as particular immune-positive and immune-negative staining using specific antibodies. Cells that do not fit in all these criteria may be 'false positive' hematopoietic cells, skin squamous epithelial cells or artifacts [125].

The magnetic-activated cell sorting system (MACS™ [Miltenyi Biotec GmbH, Bergisch Gladbach, Germany]) [126] was designed and applied to enrich disseminated epithelial tumor cells from peripheral blood using anti-EpCAM antibody conjugated superparamagnetic microbeads. Such harvested cells have been used to detect chromosomal rearrangements affecting the *ERG* gene in PCa patients by Fluorescence *in situ* Hybridization (FISH) analysis [127]. The only Food and Drug Administration approved device for CTC detection, the CellSearch® platform, is a system designed for semiautomated immunomagnetic enrichment based on the same principle. The CellSearch system defines a CTC as a cell ≥4 μ m and with the presence of cytokeratin (CK) staining and the absence of the leukocyte marker CD45 and DAPI positive. Data on its application on breast cancer control group showed 0.1±0.2 CTCs/7.5mL blood in healthy women and 0.1±0.9 CTCs/7.5mL blood in patients with benign breast disease and none of them had more than 2 such cells per 7.5mL blood. In early 2004, CellSearch® reported the relationship between the number of detected CTCs and patient prognosis [54]. Later, they further demonstrated its specificity, only 1 of the 344 (0.3%) healthy and non-malignant disease subjects had ≥2 CTCs, and sensitivity, 57% (107 of 188) of metastatic PCa patients were detected to have ≥2 CTCs [128]. Despite the prognosis value validated by multiple studies, the limits to this technique are emerging. It requires fixation of cells prior to isolation, which would limit further analysis. The process is semi-automated and very time consuming. Sample purity is not satisfied, as there are about 1000-3000 cells captured per sample dependent on donors, resulting in a purity of 0.1% or less with 10 CTCs according to the total number of captured cells [129]. Several other emerging systems, such as the MagSweeper™ system [115], IsoFlux system [116] and Microvortex chip [117] are based on similar principles with improved technologies, demonstrating both a relatively higher sensitivity and purity. For example, IsoFlux [116], using immune-magnetic capture plus flow control to enhance CTC isolation, has been reported of a higher recovery rate compared with CellSearch® (95% vs. 36% in PCa patients) and a median purity of 1.4% [116].

Technologies have been recently developed to utilize microfluidic platforms, and a so-called 'CTC-chip' [29] was reported which can isolate viable CTCs from whole blood. Using EpCAM-coated microposts and controlled laminar flow conditions, it was designed to reduce the shear stress to ensure optimal cell-micropost attachment and reported to achieve a level of purity up to much higher than other technologies. More recently, a second-generation microfluidic device, so-called 'Herringbone-chip' [117], was introduced. Comparing to the CTC-chip, it is able to induce a microvortex mixing, disrupting laminar flow of blood and increase the cell-surface interaction in antibody-coated device.

Notably, the sensitivity of the CellSearch system is being challenged by the fact that CTC number in many metastatic cancer patients was less than 2 per milliliter of blood [54,60], which is recognized to be the common critical limitation for all EpCAM-based detection. CTCs express variable levels of EpCAM, resulting of down-regulation of epithelial markers and up-regulation of mesenchymal markers. This process in cancer metastasis, called epithelial-to-mesenchymal transition (EMT) [130], is reported to help cancer cells become more invasive and resistant to therapy. Therefore, CTCs may be missed by EpCAM based detection, since they are less likely to express high levels of this protein in the process of EMT. In fact, the down regulation of epithelial markers, such as EpCAM, CK and E-cadherin, and up regulation of mesenchymal markers, such as vimentin and N-cadherin, have already been reported in CTCs from cancer patients [123,131]. Even considering epithelial markers alone, the expressions of EpCAM and CK are not always correlated, that is some cells expressing high level of CK expressed low or no EpCAM, or vice versa [132].

To combat the EMT problems, various cocktails of antibodies have been used in some laboratories to capture more CTCs by combining different biomarkers representing epithelial, mesenchymal or organ specific features [119,133]. Some others [134-136] have also tested so-called negative filtration approach, which used antibodies, e.g. CD45, to identify white blood cells and remove them from a sample, thereby leaving

residual tumor cells. Although this methodology had the potential to purify CTCs independent of cell surface antigens, limited recovery rates and purity were also reported due to its low efficiency [135].

People have also attempted to utilize CTC trapping methods to develop a sized-based system, which is based on the fact that most, though not all, of the tumor cells in the circulation are larger than blood cells. Independent of cell surface antigen expression, size based methods can help acquire potential mesenchymal cells. However, the size of the captured tumor cells from non-sized-based systems was reported to range from 4 to 30μM [137]. Therefore, recovery rate from this system is an issue. Size overlapping between CTCs and WBCs (8~20 μM in diameter) will result in a loss of considerable proportion of small CTCs and retain certain leukocyte contamination [131]. The average recovery rate from one size based Vortex technology was reported to be only 20.7% [114]. Besides, some size based systems can only run small quantities of whole blood, and harvested cells were in some cases trapped on filter membrane. For example, the isolation by size of epithelial tumor cells (ISET) [111] is a direct size based method for CTC isolation, in which tumor cells were captured by filter membrane and each spot can only correspond to 1mL whole blood. However, due to the simple process, irrespective of any antibody and no interference with beads for downstream analysis, several different pores and filter-based approaches are still being developed, some of which [138,139] have shown their promise in CTC capture using spiked cell lines. The Parsortix system is a novel isolation platform based on cell size, which allows viable cells captured within a designed cassette to be harvested or cultured outside the cassette with easy access to downstream analysis. Based on the results from our laboratory, Parsortix has a recovery rate of 42% and a purity of 5.58% using 100 PC3 cells spiked in 7.5mL human blood. Immunofluorescence, FISH and PCR all well suited on harvested samples and cell culture was successfully tested using 100 PC3 cells spiked in human blood. More evaluation of its application in advanced prostate cancer patients is in process.

Although various isolation strategies are available or under development in the laboratory, in all, a standard set of parameters [140] to evaluate the performance of a typical CTC isolation system should include the following: 1) Recovery rate (or isolation sensitivity): The ratio of isolated CTCs to all CTCs present in a sample. It is also necessary to determine the smallest number of CTCs per sample that can be isolated. The ability to isolate low concentration CTCs is crucial for the early diagnosis of the primary tumor and metastasis, as the number of CTCs in the circulation is often low. 2) Purity rate (or isolation specificity): The ratio of isolated CTCs to all captured cells from a sample. Contamination by WBCs co-captured with CTCs might hamper downstream analysis, esp. genetic investigations, such as allele specific PCR [141], which often only have sensitivity down to approximately 1% mutant DNA in a background of wild-type DNA. 0.5% purity is required for next generation sequencing (NGS) with 1000X depth. Therefore, a higher degree of cell capture purity is crucial for downstream genetic studies. 3) Enrichment rate: The ratio of CTCs to blood cells before and after CTC enrichment; 4) Throughput: the speed by which the sample is processed; 5) Viability: the percentage of viable CTCs to all isolated CTCs from sample. This parameter is important for CTC isolation aiming to culture the cells; 6) Release Efficiency: the percentage of captured target cells that can be successfully removed from a device to the downstream analysis.

Clearly, each platform developed so far has its unique advantages and limitations. The selection of a system to use should be based on the downstream analysis required. Some laboratories have attempted to combine different platforms to help isolate single CTC [142, 143] which resulted in a high purity, aiming for genetic investigation, such as NGS. Further technology advance will help us acquire an improved system, aiming for an efficient capture of the entire spectrum of collected CTCs, while keeping least leukocytes captured.

CTC ANALYSIS

PCR methodology, which is highly sensitive in identifying the presence of specific mRNA or DNA fragments, has been used to detect genetic changes in CTCs [144], which are mixed with millions of peripheral blood mononuclear cells (PBMC) after isolation by gradient centrifugation. However, the specificity, instability and inability to directly visualize CTCs all challenged the application of this technique [145]. With the improvement of the technology to isolate CTCs, many downstream CTC analysis approaches have recently been explored. CTC enumeration has been used clinically for disease prognosis as mentioned above and molecular characterization of CTCs will ultimately accelerate the application of personalized medicine for cancer patient management.

In addition to the three-color (CK, CD45 and DAPI nuclei counterstain) identification of CTCs as used in the CellSearch system and downstream analysis of CTCs isolated by other isolation systems, a 4th fluorescence channel provides researchers the possibility to define the tumor cell with relevant markers of their own interest, such as prostate specific membrane antigen (PSMA), PSA [146], Ki67 [73], EGFR [95] and EMT markers [123] (e.g. N-cadherin, vimentin, etc.). However, such enumeration is difficult to be done by conventional fluorescence microscope, and either Ariol [132] or Confocal [58] microscope has been used for automated cell imaging capture and analysis. Using four fluorescence channels, CK can also be replaced by another marker of researchers' interest, with a result of two markers of interest, CD45 to exclude lymphocytes and DAPI for nuclear staining.

Genomic CTC analysis is a good approach to approve the malignancy of isolated tumor cells and to reveal gene mutations relevant to disease progression or therapy resistance. FISH analysis is a well-established and effective tool for monitoring locus copy number in individual cells. A study [147] performed FISH analysis on CTCs isolated from patients of different cancer type, including breast, kidney, prostate and colon. The majority of them were reported to be aneusomic and cytogenetically heterogeneous, demonstrating the malignant nature of those cells. Our team reported a high frequency of *TMPRSS2:ERG* fusion in CTCs of PCa patients by FISH in 2008 [127], which revealed the organ specific genetic alteration and malignancy of the isolated cells. Subsequently, more FISH studies of isolated CTCs have been performed [148, 149].

As PCR is a sensitive method to detect cancer specific genetic alterations, RT-PCR has been used in several recent CTC studies to detect the expression of tumor-related genes, including *MUC1* [150], *TMPRSS2-ERG* fusion gene [151], *CK19* [152, 153], *ERBB2* [51, 52, 92], EMT related markers [154], or genes associated with drug resistance, such as *PIK3CA* [155]. Same as FISH, specific primer and target gene selection is required for this analysis. PCR method, using nucleotide acid extracted from a collection of tumor cells will miss the information of intra-tumor heterogeneity. Another limitation of this approach is the difficulty in accurate quantification of CTCs due to the heterogeneous levels of gene expression. Moreover, considering the false-positive tumor-related gene expression in some normal cells [145] and limited volume of harvested samples, multiplex PCR [153,154,156] targeting a panel of tumor-associated genes have been used to assess CTCs.

With the advance of genomic analysis technologies, such as microarray analysis and NGS, all the genomic alterations in a cell can be detected in one experiment. These technologies have been commonly used for human cancer genetic studies. However, those technologies are difficult to be applied for CTC analysis, due to the purity of isolated CTCs. Although very deep NGS can be achieved nowadays, it is still too expensive and inefficient to apply NGS to CTCs without the separation of single cells. Therefore, currently microarray and NGS have been applied to study genetic alterations of CTCs by analyzing separated single CTCs, which have revealed somatic single-nucleotide variants and copy number alterations [107, 143, 157-162]. This single cell analysis has the advantage to reveal intra-tumor heterogeneity, but significantly increase the cost, as many cells, each considered as a separate sample, have to be analyzed. In addition to genomic alteration detection, Next generation RNA sequencing has also be applied to detect gene expression changes in CTCs [163-165] and pathways implicated in pancreatic [163] and prostate [164] cancer metastasis have been identified.

Successful culture of CTCs isolated from cancer patients are of high interest, although the difficulty is also foreseeable. If CTCs can be cultured, much more cellular and molecular features can be investigated and CTCs can also be used to test the tumor response to therapies. In lung cancer, CTCs has been cultured to validate their tumorigenicity using mouse model [166]. In prostate cancer, CTCs has been cultured to overcome the limit access to the rare sample, or even establish organoid lines recapitulating specific molecular diversity [167]. In breast cancer, cultured CTC lines with multiple mutations have been tested for drug sensitivity, which is promising in revealing new therapeutic targets [168]. Another recent report [36] identified MICs using a xenograft assay but *in vitro* culture was not successful. Other attempts using different isolation platforms were also recently reported [169-173].

Taken together, various strategies are available for CTC identification and characterization. A combination might be beneficial to help deeper understand the heterogeneity and role of CTCs in cancer metastasis and therapy response and/or resistance. Besides, different isolation platforms should also be taken into

consideration, depending on the specific interests, such as downstream analysis platforms and requirement of viable cells.

CURRENT LIMITATION AND FUTURE DIRECTION

CTCs in patients with several types of metastatic cancers can now be counted every few weeks to predict disease progression or gauge the effectiveness of the treatment. However, currently only the CellSearch system was approved by FDA for clinical prognosis and treatment prediction with a few advanced cancers, including colon, breast and prostate cancers [174]. However, CellSearch is not an efficient system to capture CTCs and the CTCs captured using this system is difficult for downstream biological and molecular analysis. Recently, various alternative CTC isolation and or analysis platforms have been developed, either with higher efficiency than or being complementary to CellSearch system. However, their robust application in clinical sample analysis still remains to be established.

The current CTC isolation systems have a major limitation in CTC harvest efficiency, both in the CTC recovery rate and purity, making it insufficient to capture the full image of CTCs in a patient and complicating downstream biological and molecular analysis. Due to the heterogeneity of CTCs in many physical and biological features, most of the current CTC isolation platforms can only capture a proportion of CTCs based on certain specific properties of tumor cells. EMT-related loss of captured cells is one of the most concerned problems for the EpCAM dependent isolation technology. A mixture of antibodies to capture the cell surface expressed proteins which are specific for and cover all sub-population of CTCs may help to address this issue. However, it is unlikely that all CTCs express certain tumor specific cell membrane antigens and if they do so, it is difficult to known what they are before they were analyzed. Antibody independent approaches may also be used to tackle this problem, but no features have been found to completely distinguish cancer cells from normal cells in the circulation. Therefore, current antibody independent systems can only select a proportion of CTCs with a balance of minimum retain of leukocytes, for example the size-based system. Until a stable and reliable isolation approach is introduced, the so-called ‘liquid biopsy’ will be difficult to be applied to cancer patients of all stages to replace tissue biopsy, the current golden standard for diagnosis. On the other hand, due to the low number of detected CTCs in circulation, heterogeneity of those cells, uncertain origin from primary tumor or metastatic sites, it is quite questionable whether those detected tumor cells can represent the whole primary tumor. Moreover, as discussed before, while lineal progression model supports CTCs are representative for primary tumor even at early stage, parallel progression hypothesis proposed a more naive genetic abnormality in CTCs than that in primary tumors. So far, no report regarding the diagnosis role of CTCs in non-confirmed cancer patients has been published. It will be evaluated by extensive genetic/molecular analysis of CTCs with improved isolation technology and in comparison with the genetic/molecular alteration in tumour tissues, if CTC analysis can fully replace tissue biopsy. High purity of isolated CTCs is another critical unmet need for current CTC study. Until recently, most CTC isolation systems yield a CTC purity less than 1%, making it difficult for downstream molecular analysis, such as microarray and NGS analysis of cancer cell genomic copy number and gene expression analysis. Further improvement of the CTC isolation platforms, both in CTC recovery and purity, is urgently required.

The CTC isolation technique is the bottle-neck of CTC analysis. Once it is sorted out or drastically improved, there are certain biological issues of CTCs to be addressed and CTC analysis platforms for clinical application to be developed. Firstly, among the captured CTCs, does each of them have the clinical implication or what proportions are the true MICs or so called ‘stem cells’? The genetic makeup of CTCs from patients with metastatic disease is a source of important information we currently lack, which is probably more closely resemble that of metastatic tumors and may serve as a better surrogate than primary tumor biopsies for evaluating the genetic stages of metastasis. It will be important to assess which genetic changes in CTCs are representative for systemically spread cancer cells and characterize the role of each sub-populations of CTCs in tumor metastasis process, in particular those with stemness property and the progenitor of metastatic cancer.

The biological function and aggressive potential/features of cancer cells can be much better investigated if those cells can be cultured *in vitro* and grow *in vivo* in animal models. Low number of harvested CTCs for culture, suitable culture conditions, level of contaminated leukocytes, all make CTC culture difficult to perform. Only in the recent couple of years CTCs have been successfully cultured [166-173]. The utilization of xenograft models will be a promising

approach to understand tumor cell dissemination, but even fewer such successful studies have been reported [166] and not parallel to *in vitro* culture [36]. Those cultured CTCs have also been used to test the therapeutic response to help treatment method selection, but again only with one report [168]. Extensive studies of cultured CTCs are urgently needed to develop novel form of therapies targeting CTCs.

For clinical application using CTCs and associated molecular changes as biomarkers, the reliability, accuracy and robustness of CTC analysis methods are required. Currently, most of technologies applied for CTC analysis are solely for research purpose. Certain techniques currently used for CTC analysis, such as immuno-staining, PCR and FISH analysis are routinely used in the clinic for molecular diagnosis. Standardization of these techniques for CTCs should be performed before they are applied for clinical use. Due to the complexity of molecular changes in tumor cells and the heterogeneity between cancer cells, predicting cancer progression and therapeutic responses based on a single molecular characterization may not be sufficient and can even be erroneous. Multiple biomarker analysis is required. Of note, immune-staining and FISH analysis for the same individual tumor cell will be interesting and informative, once the issue of removal of immune-signals can be tackled. The potential clinical application of NGS on CTCs is also an attractive future diagnostic approach for personalized cancer medicine. When the effectiveness of CTC isolation can reach a high standard and the downstream analysis can be robustly and accurately performed, CTCs, may have the potential not only, as a 'liquid biopsy', to replace current invasive biopsy for cancer diagnosis and treatment stratification, but also serve as a therapy target to fight against metastasis. The self-seeding hypothesis has shown the promising prospects to select CTCs as therapy targets for effective control of disease progression to metastatic tumor [37-39]. However, there is a long way to go. We may not be able to efficiently kill CTCs, in particular MICs until we have deep understanding of the biological characteristics and genetic alterations which determines the fate of CTCs.

CONCLUSION

Analysis of CTCs in the peripheral blood is promising in assessing the prognosis and predicting therapy response for cancer patients. It will also help us understand the mechanism of tumor metastasis and consequently development novel strategies for therapeutic to control cancer metastasis. Many CTC isolation technologies have been developed and different approaches have been applied for CTC analysis in recent years. With further improvement of CTC isolation methodologies and analysis strategies, we believe that it will be routinely used as a 'liquid biopsy' in many aspects of personalized managements for cancer patients in the near future.

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