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Review

Circulating Tumor Cells: Beyond Isolation and Detection

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ABSTRACT

Circulating tumor cells (CTCs) are the precursors to metastases and increased numbers of CTCs in the peripheral circulation have been shown to correlate with decreased progression-free and overall survival. Although the current clinical utility has been focused on the prognostic significance, other clinical applications are being explored, such as determining if a patient is a candidate for treatment, determining the efficacy of treatment, evaluation for resistance to therapy, prediction of metastatic site, or as an early predictor of metastases. Current methodologies are based on quantifying CTCs and include technologies based on physical, immunological, and molecular techniques. However, these have limitations, of which most of them do not have the ability to perform morphological evaluation. Using morphological evaluation, CTCs in body fluids could be used for primary diagnosis in the setting of cancer of unknown primary (CUP) or in initial or early diagnostic scenarios. Additionally, cytological specimens have been shown to be useful for ancillary testing in patients when surgical resection specimens or biopsies are not available. Evaluation of CTCs should incorporate histological, immunehistochemical, and molecular characterization to enable clinicians to obtain the comprehensive diagnostic, prognostic and therapeutic information necessary to provide appropriate personalized care to cancer patients.

Keywords
Circulating tumor cell (CTC); Circulating; Tumor cell; Cancer; Isolation; Detection; Metastasis; Prognosis.

Abbreviations
CTC: Circulating tumor cell; RT-PCR: Reverse transcriptase-polymerase chain reaction; EMT: Epithelial-mesenchymal transition; MET: Mesenchymal-epithelial transition; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; CEA: Carcinoembryonic antigen; CUP: Cancer of unknown primary; CAP: College of American Pathologists; AMP: Association for Molecular Pathology; ASCO: American Society of Clinical Oncology.

INTRODUCTION

Cancer is the second leading cause of death in the United States,[1] and most cancer-related deaths are a result of metastatic disease.[2,3] Thousands of circulating tumor cells (CTCs) are shed by tumors daily and are the means by which these cancers metastasize.[4] There has been emerging evidence that the presence of CTCs in the peripheral circulation correlates with decreased progression-free and overall survival in many cancers. Although the 5-year-survival rate for most types of metastatic cancer is fairly dismal,[5] the complexity of the metastatic process makes it difficult to completely understand their prognostic significance. Additionally, their clinical relevance is not limited to their metastatic potential and their prognostic impact, and their assessment can possibly be expanded to other applications with development of improved isolation and detection technologies.

CLINICAL RELEVANCE OF CTCS

CTCs have been shown to provide prognostic information in patients with metastatic cancer. The presence of CTCs in early breast cancer is predictive of decreased progression-free and overall survival.[6-11] This data has proven to be sufficiently compelling that the American Joint Committee on Cancer (AJCC) has incorporated the presence of CTCs into the staging for breast cancer.[12,13] CTC evaluation is a valuable tool for early screening, prognostic assess-

ment, monitoring of treatment efficacy, and monitoring of disease progression or relapse.[14-17] Furthermore, studies being conducted on other tumor types, including lung, prostate, and colorectal cancer, have yielded similar results, however, research is still ongoing to completely characterize the presence of CTCs and its relationship to disease progression.[18-22]

Although biopsy is considered the "gold standard" for cancer diagnosis and for characterization of tumors,[23] there are many advantages to using CTCs for evaluation. Often patients are unable to undergo biopsy procedures due to poor clinical status, or the tumor may be in an inaccessible location. It is also common for tumors to demonstrate significant intra-and/or intertumoral heterogeneity and frequently, primary and metastatic tumors are phenotypically and genetically discordant.[24-26] Therefore, CTCs are likely more representative of the overall tumor status and disease progression than a biopsy, particularly since tumors frequently continue to undergo genetic evolution as they progress.[24-26] The evidence also suggests that CTC enumeration alone is insufficient, and that enumeration combined with downstream analysis would be ideal to provide comprehensive prognostic and therapeutic information.[11,27]

Most studies to date have focused primarily on the prognostic impact of CTCs *via* molecular characterization or enumeration using a very limited number of tumor markers to determine cell lineage or biomarker status. However, there are many other potential clinical applications for the isolation and detection of disease beyond mere enumeration.

CTCs can be assessed for specific biomarkers to determine if a patient is a candidate for specific types of treatment, and they can be used to monitor for efficacy of treatment, either by decreasing CTC counts or by evaluation of genetic evolution by itself or as a surrogate for resistance to therapy.[28] CTCs have also been found to have preferential sites of metastasis depending on the primary tumor type, and thus may lend itself to the prediction of metastatic locations.[29] Since CTCs have been found in the peripheral circulation of patients without clinically detectable disease,[30,31] it has been postulated that they can be used to detect early metastases.[32] Finally, since platelets mediate the survival of CTCs in the circulation, studies are being conducted to exploit this and use genetically-modified platelets that express tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to induce apoptosis of these tumor cells.[33]

ISOLATION AND DETECTION TECHNIQUES

Molecular Characterization

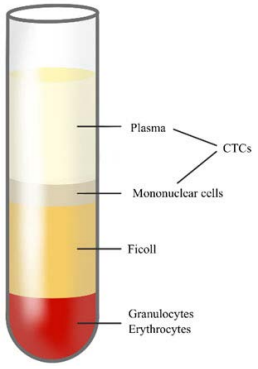
Reverse transcriptase-polymerase chain reaction (RT-PCR) is the most commonly used technique for molecular characterization and is considered highly sensitive assay, although this may vary depending on tumoral heterogeneity, contamination by genetic material from leukocytes or other cells, illegitimate transcription of cancer-associated markers in non-malignant cells, the presence of polymerase chain reaction (PCR) inhibitors, or down regulation of target genes after therapy. Also, although some tumors may have tumor-specific abnormalities, most have no tissue-specific markers. Another limitation to PCR is that it requires cell lysis which prevents CTC enumeration or other analyses.

Physical Properties

Size and deformability: Although many CTCs are typically much larger than most cells in the peripheral blood, there is significant variability in their morphologic appearance. The size of leukocytes ranges from 8-11 μm in diameter, and some CTCs can be of similar size which can make it difficult to separate them based on size alone. CTCs have decreased deformability, or a decreased ability to change their shape in relation to blood cells, however, they are more deformable than benign epithelial cells.[34,35] Therefore, it is postulated that increased deformability correlates with increased metastatic potential.[36] ISET®, Isolation-by-Size-of-Epithelial-Tumour (Rarecells, France), is a filtration device that separates CTCs in the peripheral blood based on size, which are then analyzed by standard cytomorphological microscopy.[37] The advantages to this technique are that it is not dependent on Epithelial cell adhesion molecule (EpCAM) expression or expression of other epithelial markers, however, it can have CTC loss, and it can under-represent small CTCs.

Density: Density gradient centrifugation is based on the fact that the various constituents of whole blood have different compositions and therefore, different densities and will separate into different layers when subjected to centrifugation (Figure 1). Essentially, there are three layers, the bottom layer where heavier particles are, such as red blood cells and neutrophils, the top layer consisting of plasma and platelets, and the middle layer, which is the buffy coat containing the mononuclear cells and the CTCs.[38] Although this is often referred to with the generic term "ficoll," this terminology was derived from a common product called Ficoll-Paque™ media (GE Healthcare, Chicago, IL, USA). The advantage to this is that it is inexpensive, and it does not rely on EpCAM or other epithelial markers. The disadvantages are that there may be loss of CTCs, the specimen must be processed shortly after collection, there is

Figure 1. *Density Gradient Centrifugation*



CTCs can be separated into the plasma layer instead of the mononuclear cell layer.

contamination with other blood elements, and CTCs may separate into the plasma layer.

Another centrifugation system, OncoQuick® (PA, USA) uses a porous barrier to prevent the separation medium from mixing with the specimen prior to centrifugation. During density gradient centrifugation, the CTCs and the mononuclear cells pass through the barrier, with the higher density red blood cells and granulocytes remaining below the barrier. While the recovery rate is similar to that of ficoll, the OncoQuick® system results in less contamination by blood elements and thus a higher concentration of evaluable CTCs on the slide.[39]

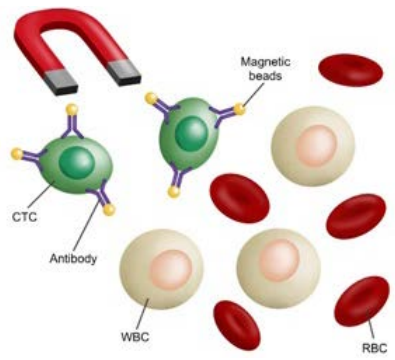
Electrical properties: Changes in the cellular content, particularly proteins, nucleic acids, and peptides, alters the dielectric properties of CTCs from that of leukocytes or benign epithelial cells. This is exploited by the DEPArray™ (Silicon Biosystems, Italy), which is an automated microfluidic system that includes an automated instrument, a disposable microfluidic cartridge, and proprietary analysis software. It has the advantage of being automated, not being dependent on expression of EpCAM or other epithelial markers, and the cells are viable and available for downstream analysis.[40] However, there is some CTC loss, and the system is expensive.

Immunoaffinity-based methods: This will not be an exhaustive discussion of enrichment methods, but a few are listed in Table 1. Immunoaffinity-based methods of CTC detection are based on marker expression and use labelled antibodies to isolate or sort the CTCs. These methods use positive or negative enrichment in which the tumor cells are enriched, or the non-tumor cells are depleted from the specimen, respectively. Often these methods result in non-viable cells that cannot be used for downstream analysis, although some microfluidic technologies have overcome this limitation. Most of the immunoaffinity-based methods use a combination of positive and negative enrichment, but EasySep™ (Chennai, India) is an immunomagnetic CTC separation kit based on depletion of leukocytes by CD45 (Figure 2). The advantage to this method is that it is easy to use and offers batch separation, however, there may be CTC loss and contamination with other blood elements.

Table 1. *Examples of CTC Enrichment Methods*

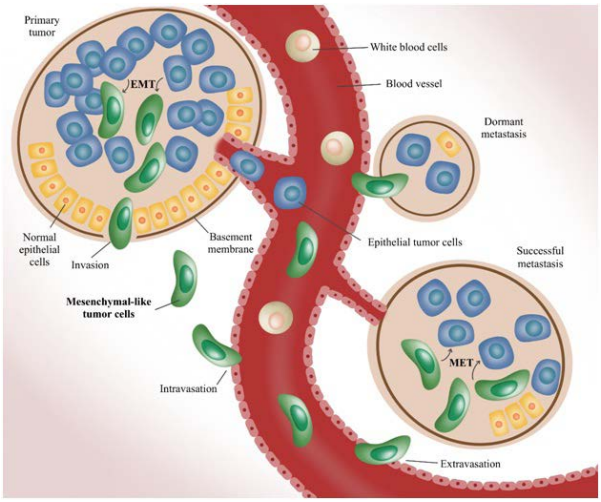
Method	Principle	Advantages	Pitfalls	References
ISET®	Filtration	Inexpensive, fast, easy, captures aggregates, not dependent on surface markers	May under-represent smaller CTCs, possible CTC loss, need to pretreat specimens, clogged membranes	14,37,77-79
DEPArray™	Dielectrophoresis	Automated, cells are available for downstream analysis	Possible CTC loss, fluorescent imaging without morphological assessment	14,78,80,81
Ficoll	Density gradient centrifugation	Inexpensive, fast, easy, not dependent on surface markers	CTCs can be in the plasma layer, low purity	4,14,82,83
OncoQuick®	Density gradient centrifugation and size	Relatively inexpensive, fast, easy, not dependent on surface markers	CTCs can be in the plasma layer, low purity	4,14,82,83
CELLSEARCH®	Immunoaffinity, positive and negative enrichment	FDA-approved, automated	Relies on EpCAM, does not detect EpCAM-negative cells, fluorescent imaging without morphological assessment, expensive	4,8,30,43,78,80,84
EasySep™	Immunoaffinity, negative enrichment	Easy to use, batch separation	Possible CTC loss, low purity	85-87

Figure 2. *Immunoaffinity-Based Method*



Immunoaffinity-based method for CTC isolation and detection using immunomagnetic beads.

Figure 3. *Epithelial-Mesenchymal Transition and Mesenchymal-Epithelial Transition in the Metastatic Process*



Epithelial cells gain mesenchymal characteristics enabling them to invade, intravate into the circulation, where they may undergo apoptosis or remain dormant for varying periods of time. They are capable of extravasating into a target tissue, regaining epithelial characteristics and establishing a metastatic lesion.

The most frequently used marker for CTC isolation is EpCAM which is not expressed on all CTCs due to a phenomenon called epithelial-mesenchymal transition (EMT), which is part of the complex metastatic cascade that confers the ability for tumor cells to metastasize to distant sites (Figure 3). Although the process by which CTCs are formed and the mechanisms involved in the development of metastases are not the focus of this review, a brief overview provides a basic understanding of this limitation. It involves phenotypic alterations that are significant when considering the development of methodologies to isolate and detect CTCs. In order for CTCs to develop the capacity to metastasize, they require the ability to alter their phenotype from epithelial to mesenchymal through EMT.[4,14-17] This involves the selective loss of epithelial adhesion molecules, such as E-cadherin and integrins, that allow the tumor cells to detach from the adjacent cells, digest the extacellular matrix, change their shape and deformability, and migrate to and enter the blood circulation, a process called intravasation. Although thousands of CTCs can be shed by tumors daily,[41] most do not survive in the circulation due to hemodynamic forces, physical damage from interaction from other blood elements, destruction by immune cells, or apoptosis.[4,14-17] Of the rare cells that do survive, some may become dormant for prolonged periods of time and may not lead to metastasis, or a metastasis may arise years after the CTCs entered the circulation.[4,14-17] Once in the circulation, CTCs are often coated by activated platelets which promote the survivability of these cells by preventing NK cell destruction.[42] At some point, the CTCs can extravasate into a target organ or tissue, undergo mesenchymal-epithelial transition (MET) whereby they regain their epithelial characteristics and their ability to proliferate, creating a metastatic tumor.[4,14-17] This concept of EMT and MET partially contributes to the phenotypic heterogeneity seen in tumors and in CTCs in particular.

The CELLSEARCH® system (Veridex, Warren, NJ, USA) is the only Food and Drug Administration (FDA)-approved detection system for the enumeration of CTCs in breast cancer patients. It is an immunoaffinity-based system which uses immunomagnetic enrichment to detect cells labeled with antibodies to EpCAM, CD45, and DAPI conjugated to fluorochromes analyzed with a cytometry-based automated instrument. The cells are visualized using a fluorescent imaging system that images the DAPI-stained nuclei for adjunctive nuclear evaluation to confirm that the DAPI-positive, EpCAM-positive, CD45-negative cells have nuclei with the size and shape consistent with carcinoma cells. The sensitivity of this method for detecting EpCAM-positive CTCs is ≥85%.[9,30,43,44] A CTC count of ≥5 per 7.5 mL of blood is associated with significantly shorter progression-free and overall survival.[6-11,44] A meta-analysis evaluating the literature published from 1990 to 2012 confirmed this and also showed that it was not influenced by the detection method or the time point at which the CTCs were assessed.[45] Further, a study to analyze pooled individual patient data showed that the prognostic value is superior to that of serum markers (CEA and CA15.3), and that it is unrelated the tumor histologic type, the number of metastases, or the type of treatment.[46]

A prospective multicenter study comparing the outcomes of patients with metastatic breast, colorectal, and prostate cancer confirmed that the presence of CTCs was a strong predictor of poor overall survival.[47] In a separate study involving a small number of samples, CELLSEARCH® detected varying percentages of CTCs in the peripheral blood of patients with a variety of metastatic tumor types, including 64% in colon cancer, 33% in gastric cancer, 66% in rectal cancer, 60% in ovarian cancer, and 20% in prostate cancer.[48] This illustrates one major limitation of EpCAM-based assays in that not all epithelial CTCs express EpCAM or other epithelial markers. The success of the CELLSEARCH® system is variable depending on the type of cancer and the stage of disease. Some other studies, particularly those for other tumor types, have used other tumor markers with some success.[49-54] However, the challenge of detecting CTCs is in large part due to their phenotypic heterogeneity.[55] Therefore, a combination of isolation techniques involving selection based on physical properties along with positive and negative enrichment, with immunological-based methods would yield the most sensitive and specific results.

CYTOMORPHOLOGY

Most reports in the literature regarding tumoral heterogeneity refer to clonal genetic alterations expressed in different parts of a tumor. However, there are many different ways in which tumor heterogeneity can manifest, and these phenotypic changes can be detected or measured in a number of different ways. Tumoral heterogeneity is simply different phenotypic features seen within the same tumor, whether it involves different histological patterns, altered protein expression, or genetic mutations, that can be routinely evaluated using a combination of morphological evaluation in conjunction with ancillary studies, including immunohistochemical staining, immunofluorescence, flow cytometry, in situ hybridization, or a variety of molecular tests. Some of the biomarkers that are targeted are evaluated for diagnostic purposes, and some have prognostic or therapeutic relevance. To date, most investigations regarding CTCs have focused on their prognostic significance, however, CTCs are also present in patients with cancers of unknown primary (CUP), both in body fluids, as well as, in the peripheral circulation. In such cases, performing molecular tests to determine the clonal genetic status in the absence of a definitive diagnosis and identification of the primary tumor is insufficient.

Methodologies such as the CELLSEARCH® system or other methodologies that only evaluate the immunophenotypical or genetic characteristics of CTCs are limited by their inability to provide a true morphological evaluation of the cells, which demonstrate characteristic features that confirm malignancy.[56-58] Morphological evaluation can overcome the limitations related to the histological characteristics, such as size, shape, and nuclear and cytoplasmic features, which can be characteristic of malignant cells. This is important because of the morphological heterogeneity demonstrated by CTCs, some of which can be the size of leukocytes, and especially because benign epithelial cells have been found in the peripheral circulation of healthy subjects with inflammatory conditions or benign neoplasms.[30,59,60] It is unknown

whether or not these cells represent malignant cells in the early stages of cancer development.[32]

Although morphological evaluation may not be completely necessary in patients with a known primary tumor, this does not exclude the possibility of a new or a concurrent malignancy, and it would be essential in cases of CUP or initial or early diagnoses. In fact, malignant cells detected in effusion cytology is often the first indication of malignancy and require pathological workup for a diagnosis whichmost frequently includes immunohistochemistry and possibly flow cytometry, in situ hybridization, or other molecular testing.[61,62]

Cytology of pleural effusions and ascitic fluid is able to diagnose malignancy in approximately 70% of cases.[63-66] Often the morphological features of CTCs resemble that of the primary tumor type.[57,67] This is of particular importance considering that less than 30% of lung cancer patients are able to undergo resection and that many cases are diagnosed by cytology alone.[68-70] Although diagnostic molecular profiling can be performed on tumor cells, the specificity can be as low as 75% due to genetic heterogeneity or genetic evolution.[71] Therefore, despite the trend towards smaller specimens and the necessity for molecular testing for prognostic biomarkers, the diagnostic algorithm remains the same with histological evaluation with limited immunohistochemical stains followed by molecular testing being standard.[72] Historically, cell block specimens have been preferred over cytological smears because the original diagnostic slides can be archived, and ancillary tests can be added on after the initial diagnosis.[73] However, recent studies have shown that cytology smears have sufficient, and sometimes higher, cellularity than cell block specimens and yield results concordant with biopsies and resection specimens.[68,73-75]

In 2013, the College of American Pathologists (CAP), the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology (AMP) issued updated guidelines regarding molecular testing in lung cancer that were endorsed by the American Society of Clinical Oncology (ASCO). Direct smears were recommended as the specimen of choice for molecular testing provided that they had adequate cellularity and preservation.[76] This makes it possible to use non-cell block specimens, such as direct smears, cytospin preparations, touch preparations, and liquid based cytology for molecular testing in lung cancer. The rationale is because cell blocks are limited by variable cellularity, the inability to perform on-site adequacy assessments, and decreased nucleic acid quality due to formalin fixation.[76] This provides the opportunity to expand testing to more specimen types that may be more readily available in certain cancer types.

CONCLUSION

With the evolving understanding of the mechanisms involved in the development of metastases, CTCs have become increasingly relevant, not only regarding their prognostic significance in breast, lung, prostate, colorectal, and other cancers, but their potential to be applied to other clinical applications. The development of methodologies for isolation and detection is the first step in a pathway that incorporates the identification and histological, immunohistochemical, and molecular characterization to enable clinicians to obtain the comprehensive diagnostic, prognostic and therapeutic information necessary to provide appropriate personalized care to cancer patients.

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