

REVIEW ARTICLE

Circulating Tumor Cells: Detection Methods and Potential Clinical Application in Breast Cancer

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Circulating tumor cells (CTCs) are defined as tumor cells circulating in the peripheral blood of patients. CTCs have long been regarded as an attractive research topic. Because of recent technological advances, it is now possible to detect CTCs in the bloodstream. Interestingly, CTCs are present in both of patients with metastatic disease and early stage localized disease in patients with breast cancer. An assay detecting CTCs seems to have significant future potential

value in the clinical management of breast cancer as a prognostic marker, monitoring treatment response and selecting target therapy. This review addresses the technical overview of detection methods, possible clinical application and future direction of CTCs research.

Key Words: Breast neoplasms, Circulating neoplastic cells, Clinical application, Detection

INTRODUCTION

Circulating tumor cells (CTCs) are defined as tumor cells circulating in the peripheral blood of patients, shed from either the primary tumor or a metastatic site. The presence of CTCs in the peripheral blood of cancer patients was recognized more than a century ago.⁽¹⁾

In patients with breast cancer, metastasis is the main reason for cancer mortality. Although the survival rate has increased, thanks to early detection and improved adjuvant therapy, the occurrence of distant metastases remains high. Distant relapse after definitive local treatment indicates that there might be undetected spread of the tumor at the time of primary local treatment. It has been shown that cells can be shed from the tumor at all stages of the disease, and these cells may remain in the patient's circulation for lengthy periods after initial treatment of the primary tumor.^(2,3) For this reasons, researchers have been interested in CTCs because these

cells could represent an important link to the process of metastasis.

In addition, CTCs may become a valuable tool to refine prognosis. Several clinical trials have established the correlation between the presence of CTCs at diagnosis and decreased progression free survival and overall survival in patients with breast cancer.^(4,5) Furthermore, a potential role of CTCs for the prediction of responsiveness of systemic chemotherapy in adjuvant setting has been rapidly expanding. Finally, CTCs are a potential source of biological information that can be used to predict responsiveness to various targeted agents. In this way, a paradigm shift may be introduced in the treatment strategy, from the present one that is based only on primary tumor characteristics to the future one that considers molecular characterization of CTCs as well. Here we will discuss the technical aspects of different detection methods and possible clinical application of CTCs in patients with breast cancer.

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STRATEGY FOR CTCs DETECTION

In the past few years, much work has been carried out to detect and quantify cancer cells in peripheral blood.

The key question in the evaluation of CTCs is a choice of neoplastic markers with unique properties in cancer cells but not in normal tissue. One of the most important challenges is the sensitivity of assays primarily because of the fact that CTCs are rare events with their numbers as low as one CTC in 10^6 – 10^7 leukocytes,⁽⁶⁾ and their fragility in the blood.⁽⁷⁾ The specificity is also a main issue because of heterogeneity of tumors in biologic marker expression and physical properties such as size and density. To date, detection methods of CTCs can be divided into nucleic-acid based techniques and a whole cell separation (cytometric) technique.

Nucleic acid based techniques

Genetic alterations in plasma DNA such as mutations in proto-oncogenes, tumor suppressor genes, or aberrantly methylated DNA can be surrogate markers as at least some of the DNA could have derived from the primary tumor. For example, greater amounts of amplified MYC-N DNA were detected in patients with neuroblastoma compared with healthy individuals.⁽⁸⁾ However, the detection of tumor-associated DNA in the plasma could indicate the presence only of circulating nucleic acids, not necessarily CTCs. It is not possible to determine whether the DNA came from CTCs or if it is simply being shed as free DNA from primary tumors. In addition, DNA has a much longer plasma half-life, the presence of circulating free DNA may reflect merely the presence of nucleic acids, not tumor cells. A further difficulty is that because changes such as DNA methylation occur early in tumorigenesis, such abnormalities might be detected in DNA shed from dysplastic lesions in patients who do not have a neoplastic lesion.⁽⁹⁾ Although DNA-based CTCs detection is promising, there are many challenges to be overcome before it can be used in clinical practice.

The presence of CTCs can be indirectly estimated by the detection of tumor-associated mRNA using RT-PCR.⁽¹⁰⁾ As RNA disappears quickly from the blood after cell death, the detection of RNA likely indicates the presence of a whole tumor cell.⁽¹¹⁾ Cytokeratin 19 seemed to be a good surrogate marker of circulating epithelial tumor cells.⁽¹²⁾

Although RT-PCR has high sensitivity, the specificity is generally low, resulting in higher false positive rates due to contamination and expression of markers in normal cells.^(13–15) Quantitative RT-PCR with multiple specific markers might resolve the issue of the specificity, but the target mRNA transcript needs to be carefully selected.^(9,16) Other limitations of RT-PCR based assays include inability for further evaluation of morphological analysis and molecular profiling assays of CTCs.⁽¹⁷⁾

Cytometric methods

Cytometric approaches isolate individual CTCs, allowing further morphological and molecular characterization of the cells, including steroid receptor or HER2 status. Enrichment of epithelial tumor cells is generally needed to increase the sensitivity to an acceptable level because CTCs are rare event. In recent years, there have been many efforts to devise automated technologies.

CellSearch

A CellSearch System (Veridex, Warren, USA) is an automated device using anti-epithelial cell adhesion molecule (EpCAM) antibody linked to small magnetic beads as an enrichment method.⁽¹⁸⁾ After immunomagnetic enrichment, the captured cells are fluorescently stained with monoclonal antibodies directed against pan-cytokeratin and CD-45, a leukocyte marker. CD-45 is used to exclude nonspecifically stained leukocytes. CTCs are defined as cytokeratin positive and CD-45 negative. This highly automated system has low inter-sample variability and high reproducibility.^(7,19) However, the sensitivity of this technique is relatively low, which currently limits the clinical use of this system.

CTC-chip

A CTC-chip is a microfluidic device which consists of 78,000 microposts coated with anti-EpCAM antibodies. Sample preparation and manipulation are a critical step in the process of detecting CTCs. To avoid losing CTCs, a simpler procedure should be preferred. To improve the CTCs capture sensitivity, the CTC-chip system passes

whole blood through the coated microposts without any additional preparation step. The captured CTCs on the chip are identified by fluorescence microscopy after staining with an anti-cytokeratin epithelial marker with an anti-CD-45 antibody for negative selection.(20) This single-step system can efficiently detect CTCs as it does not require steps for centrifugation or washing.

AdnaTest

AdnaTest BreastCancer™ (AdnaGen, Langenhagen, Germany) enables the immunomagnetic enrichment of tumor cells via a mixture of beads coated with epithelial and tumor-associated antigens.(21,22) After immunomagnetic enrichment, this system extracts mRNA for a subsequent qRT-PCR procedure for the detection of amount of epithelial cell associated mRNAs such as MUC1, HER2, and the surface glycoprotein. The AdnaTest system combines traditional antibody techniques with RT-PCR and shows a higher sensitive compared to the Cell-Search system.

CAM method for invasive CTCs

Ficoll density gradient centrifugation is used to obtain the mono-nucleated cells (MNCs) from whole blood, and MNCs is subsequently seeded and cultured with Cancer Cell Culture (CCC) media for 12 hr (Vita-Assay™; Vitatex Inc., Stony Brook, USA). Non-adherent cells are washed away, and remaining cells are stained for CTCs using fluorescein-conjugates anti EpCAM antibody with anti-CD45 antibody. An advantage of the CAM assay is that it detects only viable CTCs with invasive phenotype.(23)

MAINTRAC® analysis

The positive rate of CTCs varies between zero and 100% of patients with operable and metastatic disease.(9) Recently, Pachmann et al.(24) improved the method to avoid cell loss resulting from enrichment procedures and centrifugation, and were able to detect an even higher number of epithelial cells. MAINTRAC® analysis identifies CTCs by the use of an automated laser scanning cytometer after incubating the cells with fluorescent-labeled anti-EpCAM and anti-CD 45 antibodies. CTCs are defined

as EpCAM positive and CD-45 negative. MAINTRAC® analysis is one of the most sensitive assays with more than 90% positive rate of CTCs in patients with early breast cancer. However, the question then arises regarding whether these epithelial cells which are detectable in such high numbers are indeed tumor cells, and the methodology of this analysis is still controversial.(25)

Membrane microfilter assay

A basic strategy of “micro filter” for CTCs detection is using a size difference between CTCs and normal blood cells. This method enriches relatively larger CTCs in blood cell using the filter for microscopic identification.(26) After separation, RT-PCR is performed on the microfilter. This system is relatively easy, inexpensive and highly sensitive for CTCs enrichment. It is one of the promising alternative technologies for the detection of CTCs.

Fiberoptic array scanning technology (FAST) and Epithelial immunospot (EPISPOT)

FAST system detects immunofluorescently labeled CTCs with an automated digital microscopy imaging system. This method is in an early stage of development and does not require cell enrichment.

The EPISPOT assay identifies viable tumor cells by detecting secreted proteins such as MUC1 and CK19 after 48-hr cell culture. Dead CTCs that cannot secrete epithelial-associated proteins are not identified in this system.(27)

From the technical point of view, CTCs research has been limited because of low sensitivity, specificity, and reproducibility of the detection methods. Due to the high variability of results in CTCs detection and the discrepancy of the results, a clinical use of CTCs has not yet been established, and standardized methods are urgently required.

POTENTIAL CLINICAL APPLICATION OF CTCs

There are several potential clinical applications of CTCs research in various clinical settings. At this point, several studies have suggested a prognostic and predictive role

for CTCs in metastatic and adjuvant/neoadjuvant setting.

Prognostic marker

To improve stratification of breast cancer patient, many issues remain unresolved, and new predictive and prognostic factors are needed in clinical practice. About 30% of node negative patients will present distant metastases, whereas 40% of the patients with involved axillary lymph nodes will survive for 10 yr without clinical recurrence. (28) The initial CTCs status in the patient's peripheral blood might be an important marker for the estimation of prognosis, and a negative prognostic impact associated with the detection of CTCs during the course of breast cancer is generally accepted. By stratifying the prognosis using CTCs, systemic treatment could be better targeted to patients who are most likely to relapse, thereby avoiding unnecessary toxic therapy for lowest risk patients.

CTCs detected by RT-PCR for CK19 mRNA have an independent prognostic value as a marker for poor clinical outcome in patients with breast cancers. (28-30) Pachmann et al. (31) reported a correlation between distant relapse and an increase in the number of CTCs. The presence of CTCs 3-4 weeks after surgery and before the initiation of chemotherapy was associated with shorter disease free survival and overall survival in patients with node negative breast cancer. (28)

In metastatic setting, Cristofanilli et al. (18) demonstrated that CTCs are highly prognostic. More than five CTCs per 7.5 mL of whole blood were identified as the threshold. Elevated CTCs at baseline predicted extremely short median progression free survival and overall survival. Even more interesting was that patients whose CTCs level was high at baseline and then was reduced to below five CTCs/7.5 mL of blood at the first follow up had similar progression free and overall survival compared to the group that had low levels of CTCs at baseline. (4, 18, 32) This result suggested that CTCs provided independent prognostic information beyond tumor burden. Furthermore, assessment of CTCs was suggested to be a superior surrogate endpoint than current radiology imaging studies for predicting overall survival in patients with metastatic breast cancer. (33) Other researchers

also found that a detectable level of CTCs appeared to correlate with the worse clinical course in patients with metastatic disease. (20, 34)

However, although a potential role of CTCs as an independent prognostic factor is generally accepted, the future success of CTCs test competing with conventional prognostic markers or growing data of multigene assay might be controversial.

Predictive marker—monitoring of responsiveness for treatment

Longitudinal monitoring of the CTCs status could be an effective strategy for measuring the therapy efficacy and resistance and monitoring treatment response. Early indicators of response or resistance to treatment are an important issue in clinical practice. In addition to being a sign of tumor cell dissemination, increases and decreases in their number may also serve as a surrogate marker for response to therapy. (35) Furthermore, monitoring CTCs in blood would be an ideal strategy because its non-invasive sampling procedure allows repeated analyses.

For example, an increase by 10-fold or more at the end of therapy by MAINTRAC[®] analysis is a strong predictor for relapse. (36) Although patients could be monitored for therapy response by longitudinal CTCs assay, the ASCO group concluded that the measurement of CTCs was not to be used to influence treatment decisions in patients with breast cancer in the 2007 update of their recommendations. (37)

The Southwest Oncology Group and the Breast Cancer Intergroup of North America are now conducting a prospective trial in which patients with metastatic breast cancer who have a positive CTC count using the CellSearch system after 1 cycle of first-line chemotherapy will be randomly assigned to a group that will continue with the current therapy or to a group that will switch the current therapy to a different chemotherapeutic agent (Southwest Oncology Group protocol S0500). This prospective trial could solve the uncertainty in the possibility of treatment monitoring by CTCs assay.

CTCs in neoadjuvant setting

The courses of neoadjuvant chemotherapy could influence the number of CTCs. In one neoadjuvant trial, the reduction in the number of CTCs with chemotherapy strongly predicted for the final tumor size, suggesting that CTCs could potentially be used to monitor the response to primary chemotherapy. (38) The correlation between the reduction in CTCs and the final reduction in tumor size indicates that monitoring of CTCs could predict tumor responsiveness of particular therapy and avoid unnecessary toxicity during neoadjuvant therapy by discontinuing an ineffective therapy.

However, the role of CTCs in neoadjuvant setting is unclear. For clinical use of CTCs in neoadjuvant setting, we need long term follow up data about the correlation between a change in the number of CTCs and patients prognosis.

FUTURE DIRECTION

Technical challenge in CTCs assay

Recently, many investigators have reported CTCs detection methods in breast cancer patients, including one that uses different volumes of peripheral blood and others that use different enrichment procedures (density centrifugation, filtration, immunomagnetic beads), different methods (RT-PCR, immunocytochemistry, immunofluorescence) or epithelial tumor markers (cytokeratins, mucins, mammaglobin, etc.) Thus, it is extremely difficult to compare one study with others.

In CTCs research, the main challenge lies in development of an optimized and standard assay platform with a reliable sensitivity, specificity and reproducibility. Once the optimum assays are developed, techniques validated in the laboratory should be taken into clinical setting in the context of meticulously designed trials.

Molecular profiling of CTCs– target treatment selection

Ascertainment of the phenotype of CTCs also has a potential role in tailored treatment. CTCs can exhibit different characteristics compared to the primary tumors,

and molecular characterization of CTCs might contribute to improving more individualized therapies. (5,39)

Many investigators are developing methods to evaluate biologically important markers in CTCs. Treatment-related markers such as estrogen receptor (ER) and HER2 can also be detected in CTCs, and these phenotypic markers are now being investigated as possible biological indicators of target modulation. For example, HER2 status of CTCs might be different from that of the corresponding primary tumors. (40–42) The fact that trastuzumab is known to have a substantial positive impact on the survival of patients with HER2 positive disease, monitoring of CTCs for HER2 could help identify patients who would not otherwise receive trastuzumab due to a negative HER2 status on the primary tumor to benefit from this therapy. In terms of ER status, there has been very limited data about comparison of the expression between primary tumor and CTCs, which might be useful in predicting the benefit of endocrine therapy. (43) In addition, gene-expression profiling of CTCs might be feasible in decision making of tailored individual treatment strategies in the near future.

Circulating cancer stem cells

Finding tumor cells in peripheral blood raises questions about the possibility of the existence of subset of cells sharing many characteristics with cancer stem cells (CSCs). According to the CSC hypothesis, at least some of the CTCs should have properties of metastatic potential. (44) The majority of CTCs lack the ability to metastasize. It appears that the potential to grow into metastases may be restricted to a small fraction of stem cells. (45) Being a heterogeneous population, some of these cells exhibit a more aggressive behavior by generating early metastases, and CTC subpopulations may be identified and used as predictive tools to measure patient response to targeted agents. (46)

CONCLUSION

In conclusion, current prognostic and predictive factors are inefficient and the information regarding CTCs could

provide an additional clue. Although CTCs research in breast cancer has rapidly expanded, the clinical relevance of CTCs has not been firmly established. Further optimization and standardization of CTCs detection techniques could lead to the inclusion of CTCs detection in daily clinical practice. Molecular characterization of CTCs might open the new horizon in improving tailored treatment. In the future, identify circulating cancer stem cell may clarify the metastatic cascade and may help develop new targets in cancer treatment.

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