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Review Article

Circulating Tumor Cells and Extracellular Nucleic Acids in Breast Cancer

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Circulating tumor cells (CTCs) are defined as tumor cells circulating in the peripheral blood of patients, shed from either the primary tumor or from its metastases. The detection of circulating tumor cells (CTCs) in the peripheral blood of breast cancer patients may account for the different steps in the biologic progression of the disease. The detection of microscopic disease in patients with breast cancer is imperative to prognosis and can predict the efficacy of targeted treatments. In general, there are two main methods for their detection. These are based on cytometric and nucleic acid manipulation. Both methods generally require an enrichment step to increase sensitivity of the assay. This step is based on either detection of specific surface markers using immuno-selection and/or on morphological features, such as cell size or density. We review the methods of detecting CTCs, their prognostic implications, and opportunities to exploit the properties of CTCs to develop personalized therapy.

Key Words: Breast cancer, Circulating tumor cells, Extracellular nucleic acids

The presence of tumor cells in the tissue and organs at various times in the natural progression of breast cancer is of prognostic significance. In breast cancer when tumor cells enter the lymphatic system, they travel to the sentinel nodes in the axilla and intercostal spaces before entering the bloodstream and subsequent progression to other organs. In addition, tumor cells can disseminate directly through the blood to distant organs. The detection and characterization of such cells would be an advantage in the identification of patients who are at risk of disease recurrence and the subsequent tailoring of individualized treatments.[1] The search for metastatic deposits in the lymph nodes of patients with newly diagnosed breast cancer remains the standard practice for breast cancer patients and a strong prognostic factor. Likewise, the presence of cytokeratin-positive (CK+) cells in the bone marrow in women with primary breast cancer is an independent prognostic indicator of death from cancer. However, the discrepancy among the results of several studies investigating the presence of epithelial cells in the bone marrow has fueled the controversy regarding the clinical value of bone marrow screening. Recent advancement in immunomagnetic separation technology, with its higher level of sensitivity and specificity, has improved the detection of CTCs compared with detecting occult CTCs by reverse transcriptase-polymerase chain reaction (RT-PCR). Each of the several methods available to detect CTCs have distinct advantages and disadvantages. We present a brief description of the most clinically relevant assays used, including their associated advantages/disadvantages.

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1. CellSearch®

The CellSearch Epithelial Cell Kit contains ferrofluid particles coated with anti-EpCAM antibodies; phycoerythrin-conjugated anti-CK antibodies recognizing CK 8, CK 18, and CK 19; anti-CD45 antibody conjugated with allophycocyanin to identify leukocytes; a nuclear dye 4′,6-diamidino-2-phenylindole (DAPI) to label the cell nuclei; and a buffer that permeabilizes the cell plasma membrane to facilitate entry of anti-CK antibodies into epithelial cells.[2] Isolated CTCs are defined as EpCAM+, CK+, DAPI+, and CD45-. In metastatic breast cancer an initial and first follow-up CTC count of ≥5 per 7.5 mL blood is predictive of shorter progression free survival and overall survival While a CTC count ≥5 per 7.5 mL blood at any time during therapy is indicative of a rapid disease progression and mortality. A further study highlights the importance of use of the CellSearch® system as an independent prognostic factor for early breast cancer relapse. While it is recognized that this method is highly sensitive, reproducible, commercially available, FDA approved, and has the capability of distinguishing between viable/non-viable cells, limitations include: usage of EpCAM to capture CTCs may miss some EpCAM- tumor cells, result in false positives (due to expression of the same antigens on non-tumor cells), the multiple enrichment/processing steps may also result in loss of CTC detection and no further processing of the CTC samples is possible.[3] And its clinical utility has been limited by its high cost. However, at present (based on the most recent publication) the American Society of Clinical Oncology (ASCO) does not recommend the use of CTC detection for diagnosis or clinical management decisions in breast cancer.[4]

2. Epithelial Immunospot (EPISPOT)

EPISPOT is an alternative antibody-based immunological approach. It detects cells that secrete proteins such as cathepsin-D, mucin-1 (MUC1), and CK19 thus one of the advantage of this approach is that an assessment of non-viable CTCs can be avoided. But limitation of this study included that isolated CTCs require 48 hours culturing before analysis—delaying analysis and as for CTC-microchip—clinical validation has yet to be achieved.[5]

3. Adna Test Breast Cancer Detect Kit™

Adna Test Breast Cancer Detect Kit is an PCR-based nucleic acid analysis.[6] It is reported to detect CTCs that has cell surface markers including MUC1, HER2, and GA73 3-2. In this system, 5 mL of blood is collected in preservative collection tubes (AdnaCollect, AdnaGen) to stabilize the RNA and then enriched by immunomagnetic separation using a ready-to-use antibody mixture comprised of EpCAM and MUC1, commercialized as Adna Test Breast Cancer Select (AdnaGen). The Dynabeads mRNA DIRECT Microkit (Invitrogen Dynal AS, Smestad, Norway) included in the Adna Test Breast Cancer Detect Kit is used to isolate mRNA. Complementary DNA synthesis is performed and used as a template in a two-step quantitative real-time PCR for selected markers, including. One of the advantages of this approach is that it capitalizes on the prevailing state-of-the-art of CTC isolation and enhances the likelihood of detecting very low numbers of CTCs or occult CTCs based on their expression of tumor-associated genes. The AdnaGen system has high analytical sensitivity (two cells in 5 mL of blood) and high specificity (>90%), which is achieved through the combination

of multimarker tumor cell enrichment and multiplex gene-expression profiling.[7] Whilst the AdnaTest approach has been described as potentially having the same limitations (as for CellSearch® and CTC-Chip) of false positives and/or false negatives due to loss of antigens on CTCs, AdnaTest has also been proposed as suitable for characterizing events from stem cells to epithelial—mesenchymal transition (EMT).

4. MILTENYI AutoMACS system

CTCs was defined as CD326-positive cells in this system. Peripheral blood mononuclear cells are incubated with magnetic beads coated with anti-CD326 (Miltenyi Biotec, Auburn, California) at 4 to 8 °C for 15 minutes prior to passage through a magnetic column to enrich for CTCs.[8] After washing, the cell pellet is suspended in buffer and loaded onto the magnetic column of an AutoMACS system (Miltenyi Biotec, Auburn, California). The CTCs can then be spun onto glass slides with a cytospin centrifuge to determine the morphology, viability, and purity of the preparation.

5. EasySep System

Another immunomagnetic cell selection system is the EasySep (Stem Cell Technologies, Vancouver, Canada), which combines the specificity of monoclonal antibodies with the simplicity of a column-free magnetic system.[9] In this procedure, the cells of interest are allowed to react with antibodies to specific cell surface antigens and are enriched by positive selection. Cells targeted for selection are cross-linked to the EasySep nanoparticles via the formation of tetrameric antibody complexes (Stem Cell Technologies) in a standard centrifuge tube, which is then placed in the EasySep magnet chamber. The hand-held magnet chamber is gently inverted to discard the cells that are not bound to the specific nanoparticle complexes adhering to the walls of the tube in the magnet chamber. To obtain a population of higher purity, the residual cells in the tube are rinsed twice and then harvested by removing the tube from the magnet. Enriched CTCs can then be used for further interrogation for the differential expression of EpCAM or other markers of interest.

6. RosetteSep

Positive selection is most effective for specimens with a generous complement of the desired cell population.[10] When a specimen such as peripheral blood contains few or inadequate numbers of the cells of interest, a negative selection approach is preferred to enrich for these cells. One negative selection technique is RosetteSep (Stem Cell Technologies), which uses a cocktail of highly purified combination of mouse and rat monoclonal antibodies. When allowed to react with peripheral whole blood, these antibodies bind in bispecific tetrameric antibody complexes directed against cell surface antigens on human hematopoietic cells (CD2, CD16, CD19, CD36, CD38, CD45, and CD66b) and glycophorin A on erythrocytes. The unwanted cells of hematopoietic origin form rosettes by cross-linking to multiple erythrocytes that can be easily removed by performing a typical Ficoll-Hypaque density gradient procedure.[11] The rosettes, free erythrocytes, and granulocytes form a pellet, and the unlabeled, desired CTCs are collected from the interface between the plasma and the buoyant density medium.

7. OncoQuick

OncoQuick (Arcisen Bio-One GmbH, Frickerhausen,

Germany) is size or density-based approaches for CTC analysis. This filtering (through 8 μm-pore) approach is based on size differential between larger epithelial cells/CTCs and smaller leucocytes, followed by fixing and staining of the retained larger cells. OncoQuick (Arcisen Bio-One GmbH, Frickerhausen, Germany), is composed of a centrifugation tube preloaded with a liquid density separation medium and a porous barrier membrane; optimized for the enrichment of CTCs, it is currently available and has been used successfully. Cell preparations enriched for CTCs by the initial enrichment methods can be subjected to immunophenotyping for definitive identification of CTCs. Evaluation of the immunostained specimens and detection of CTCs are possible using different platforms, including conventional fluorescent microscopy, flow cytometry, laser scanning cytometry, and automated digital imaging. An automated scanning method using a laser scanning cytometer that combines the speed of flow cytometry with the ability to analyze every positive event for its morphologic features has been used successfully to analyze CTCs.[12] Other imaging systems such as fiberoptic array scanning technology, which applies laser printing techniques to detect rare cells, have increased speed of detection compared with other automated digital microscopy systems with equivalent sensitivity and superior specificity. Limitations of this approach, which deem it to be of limited sensitivity and specificity, include loss of small CTCs and retention of large leucocytes/clumps of leucocytes.[13]

CIRCULATING NUCLEIC ACIDS AND CANCER

Circulating nucleic acids are defined as extracellular nucleic acids (DNA or RNA) found circulating in the blood throughout the body. Such extracellular nucleic acids are thought to be released from normal cells, primary malignant cells or metastatic cancer cells.[14] The presence of circulating nucleic acids in plasma and serum has implications for minimally invasive diagnostic and predictive applications in benign and malignant conditions. Stroun et al.[15] first suggested that malignant tumors may be a source of exDNA, but it was only with the detection and verification of tumor-derived mutations of K-ras in plasma from pancreatic adenocarcinoma patients and point mutations of N-ras in plasma of patients with myelodysplastic syndrome and acute myelogenous leukemia that an association between tumor and exDNA was convincing. However the origin of extracellular nucleic acids is indeterminate which can be found as free nucleic acids, bound to a cell's surface via proteins with specialized nucleic acid binding properties, complexed with proteolipids and within apoptotic bodies. Studies have shown that they (DNA and RNA) can be released spontaneously from cells. However, the exact mechanism of release from normal cells and tumor cells remains to be fully elucidated.

1. Cytokeratin 19

Cytokeratin (CK) form the cytoskeleton of epithelial cells, and their main function is to maintain the epithelial cell integrity. Cytokeratin (CK) constitute the largest intermediate filament protein subgroup and represent a multigene family with more than 20 different types of polypeptides that are divided into acidic type I (CK9-CK20) and basic type II (CK1-CK8) keratins. The most commonly used broad spectrum anti-CK antibodies, A45-B/B3 and AE1/AE3 (hereafter referred to as "A45" and "AE"), have

been shown to be specific for DTC detection as shown by analysis of large cohorts of noncancer control patients.[16] Epithelial cells are targets of caspase-mediated proteolysis during apoptosis.[17] CK19 is cleaved by caspase 3 and soluble fragments (CYFRA 21-1), released into extracellular space, have been detected in sera of cancer patients and reportedly do not correlate with that of full-length CK19 mRNA. Silva et al.[18] identified CK19 mRNA in plasma of breast cancer patients (49%) in comparison to healthy controls (20%). As the recurrence rate in stage I and stage II breast cancer is between 10-20% and 40-65%, respectively, it was proposed that the high proportion of patients in the study with early stage cancer (stage II)and showing positivity for CK19 mRNA expression, might be an indicator of patient clinical outcome and recurrence.

2. Human telomerase reverse transcriptase

Telomerase is a multi-component ribonucleoprotein located within the nucleus. It is an RNA-dependant DNA polymerase, the function of which is to synthesise the repetitive nucleotide sequence (TTAGGG in humans) forming the telomeres at the end of chromosomes.[19] Without telomerase activity each round of nuclear division results in shortening of the telomeres and reaching a critical length seems to trigger off cellular apoptosis. Continued telomerase activity appears to stabilize telomeres and thus leads to cellular immortality. Telomerase is active in 80-90% of malignant tissues and many immortal cell lines. It is composed of two subunits: an RNA component (TERC) and a telomerase reverse transcriptase (TERT). TERT is generally not expressed in most differentiated somatic cells; therefore, telomerase activity is absent. TERT is the major determinant for activation of telomerase. Chen et al.[20] identified hTERC and hTERT mRNA in primary tumors and sera of breast cancer patients presenting with invasive lobular carcinoma and invasive ductal carcinoma. Of the specimens analyzed, hTERT was identified in 25% of sera and 94% of primary tumors, while hTERC was identified in 28% of sera and 94% of primary tumors. hTERC or hTERT was not detected in sera from control subjects or patients presenting with benign disease. A more recent study identified hTERT mRNA-positive CTCs. Shen et al. 2009 reported hTERT expression in 59.6% of patients presenting with breast cancer in comparison to healthy volunteers (0%) and benign controls (0%). Importantly, detection of hTERT mRNA significantly correlated with the TNM (internationally used tumor, node, metastases staging system) stage and lymph node metastasis, with hTERT present in 24.2, 67.9, 80.0, and 100% of patients with a TNM stage of I, II, III, and IV, respectively. The authors conclude that the use of peripheral blood would be advantageous in clinical monitoring of early stage hematogenous spreading that may further develop into breast cancer metastasis or recurrence.

3. Human mammaglobin

Mammaglobin (MGB) is a breast tissue-specific member of secretoglobin subfamily C. Mammaglobin expression is elevated in breast cancer tissue relative to non-malignant breast tissue. While little is known about the potential function of mammaglobin in breast carcinoma, several studies have emphasized mammaglobin's potential as a marker for breast cancer. Human mammaglobin (MGB) belongs to the uteroglobin/Clara cell protein family of small epithelial secretory proteins and was discovered by Watson and Fleming in their isolation of sequence

fragments that were abundantly expressed in breast tumors relative to normal breast tissue. Two isoforms of MGB have been reported. These are mammaglobin 1 (also referred to as MGB1, mammaglobin A, SCG2A2) and mammaglobin 2 (MGB2, mammaglobin B, SCGB2A1).[21] Most research on the use of MGB as a potential diagnostic biomarker in cancer has focused on MGB1, although there are reports of high levels of MBG2 mRNA and protein being associated with well (Grade 1) and moderately (Grade 2) differentiated endometrioid endometrial tumors.[22] Initially, Watson and Fleming detected MGB1 expression in several breast carcinoma cell lines but not in primary breast stromal cells nor in immortalized luminal ductal breast cell lines. They concluded that MGB1 expression is mammary-specific, as MGB1 was expressed exclusively in mammary epithelial cells. It was since reported, however, that MGB1 is expressed at the mRNA and protein level in tumors of the endometrium, ovary, and cervix . A more recent study reported high MGB1 levels in primary breast cancers to be indicative of a less aggressive tumor phenotype and correlating with the expression of ER and PR, low Ki-67 labeling and absence of nodal invasion. Mikhitarian et al.[23] used a panel of biomarkers (CK19, MUC1, MGB1, EpCAM, HER-2, prolactin inducible protein) in their investigation of a possible correlation between mRNA expression in peripheral blood CTCs, bone marrow DTCs and clinicopathological indicators (tumor type, TNM, ER/PR status, HER-2 status, age, and race). Their main finding was the lack of correlation between biomarker positivity in CTCs and tumor type and/or biomarker status of axillary lymph nodes. However, a significant association was reported between biomarker positivity in CTCs and tumor grade (grade II-III versus grade I), with this association holding true for expression of MGB1 mRNA alone. As this was an interim report of an ongoing study, the clinical significance in terms of overall survival and disease-free survival remain to be seen.

4. Cyclin D1

Cyclin D1 is a cell cycle regulator. It is induced by a variety of factors, such as epithelial growth factor, hormones and oncogenic signals and its over-expression is one of the most commonly observed alterations in cancer.[24] In breast cancers, cyclin D1 is over-expressed by 30-50% of tumors and is documented as being predictive of poor clinical outcome. García et al.[25] investigated whether plasma mRNA from breast cancer patients is related to disease-free survival and overall survival. Presence of cyclin D1 plasma mRNA was significantly associated with non-responsive patients following treatment after relapse. Additionally the authors reported a trend towards a significant relation between reduced overall survival rates and cyclin D1 presence. No association was detected between cyclin D1 plasma mRNA presence and disease-free survival.

5. HER-2

HER2 is a 185 kDa protein composed of three domain; a cytoplasmic domain, a transmembrane domain and an Extra-Cellular Domain (ECD). The ECD of HER2 can be cleaved from the surface of breast cancer cells by matrix metalloproteases and released into the serum, where it is detectable using an enzyme-linked immunosorbent assay (ELISA).[26] The currently approved cutoff for an elevated serum HER2 is greater than 15 ng/mL, which results in a positive test in approximately 5% of healthy controls. The

extracellular domain of HER-2 can be proteolytically cleaved from the cell membrane and this domain can be measured in peripheral blood. While the presence and thus relevance of HER-2 mRNA in serum or plasma has yet to be investigated, detection of HER-2 mRNA-positive CTCs in blood specimens from patients with operable breast cancer have been reported as prognostic of unfavorable outcome, in terms of both relapse-free survival and overall survival.

MICRORNA-21 (MIR-21) IN BREAST CANCER

MicroRNAs (miRNAs), small non-coding endogenous RNA gene products consisting of 18 to 25 nucleotides, were first discovered in 1993. The first report of circulating microRNAs (miRNAs) associated with diffuse large B-cell lymphoma, circulating miRNAs have been reported in studies of a range of other cancer types. After a multistage process commencing in the nucleus, pri-mRNAs are trimmed into pre-mRNAs and are exported to the cytoplasm. Following cleavage by the endoribonuclease dicer, pre-miRNAs are converted into mature miRNAs that are incorporated into the RNA-induced silencing complex (miRISC) and target messenger RNA (mRNA), resulting in cleavage or translational repression. By targeting mRNAs, miRNAs play critical roles in cell proliferation, differentiation, and apoptosis and, moreover, can act as tumor suppressors or oncogenes. Studies of circulating miRNAs associated with breast cancer have been limited to date. Iorio et al.[27] first reported 29 miRNAs associated with breast cancer and many more have since been discovered. Among these miRNAs, miR-21 is known to be overexpressed in breast cancer. Studies have demonstrated that miR-21 functions as an oncogene by targeting tumor suppressor genes including tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4), and phosphatase and tensin homolog (PTEN), leading to cell proliferation and inhibition of apoptosis and regulating cancer invasion and metastasis in breast cancer.[28] Limitations of blood-based biomarkers and future directions Although research into potential cancer biomarkers has been ongoing for many years, the actual number of markers used in clinical settings remains limited. The main reason being the discovery and development of useful biomarkers pose many challenges, with several factors contributing to the slow rate of biomarker development.

So far, there are no proven advantages of one molecule type over another with regards to their potential as biomarkers. Although, recently CTCs, results emerging from exploratory studies suggest that RNAs—which are proving to be reproducibly detected and can be amplified using a multiplex approach (thus minimizing the amount of specimen required for analysis) will form members of useful breast cancer biomarker panels. To date, what could possibly be best described as proof-of-concept studies have been reported on RNAs (mRNAs and miRNAs) as biomarkers. CTC detection may allow a more rational selection of treatments for patients with newly recurrent disease, and this approach could maximize the chance of a particular combination or single new drug showing clinical benefit and, eventually, prolonging survival. It is possible that CTC detection could be used in the design of efficacy trials of different therapeutic approaches. The efficacy of these treatments could be more easily assessed if patients were stratified by their prognosis, leading to

more tailored treatment strategies. We believe that the challenge for the next generation of clinical trials and the responsibility for both clinical investigators and the pharmaceutical industry will be to incorporate these concepts into the process of drug development.

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