POTENTIAL APPLICATION OF CIRCULATING TUMOR CELL MARKERS TO EVALUATE RESPONSE TO CHEMOTHERAPY IN BREAST CANCER PATIENTS: A NARRATIVE REVIEW

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Abstract

Breast cancer treatment has shifted from conventional therapy to personalized medicine based on heterogeneous and dynamic cancer biology characteristics. Cancer cells can actively spread from the primary tumor into the peripheral circulation as circulating tumor cells (CTC) to initiate cancer progression. The liquid biopsy technique, which includes repeated assessment of the number and molecular profile of CTCs, can be used to detect changes in CTC biological behavior, determine chemotherapy resistance, and predict recurrence at the molecular level. Only 3% of the CTC population, in the form of proliferating CTCs, survive and continue the metastatic process as a result of the immune system, biophysical factors, and chemotherapy. These viable CTCs can be identified through 7-Amino Actinomycin D labels, which bind to DNA in the nucleus. These CTCs have the property of cancer stemness and plasticity through the presentation of epithelial and mesenchymal markers due to the epithelial-mesenchymal transition (EMT) process, which enables CTCs to escape the immune system by expressing PD-L1 as immune checkpoint surface signals. When CTCs are still in circulation, they express MUC1, which binds to endothelium and initiates the metastatic adhesion cascade process. Using a flow cytometry method based on surface and intracellular protein markers, these proliferating CTCs can be identified using a label-dependent approach. The response to chemotherapy can be assessed by analyzing information on the changes in the number and characteristics of breast cancer CTCs based on a combination of EMT, immunological checkpoints, cancer stem cells, cell viability, and endothelial adhesion marker protein.

Keywords

Circulating tumor cell, breast neoplasm, flow cytometry, induction chemotherapy

Background

Breast cancer management methods have shifted from conventional therapy to more individualized therapy, also known as personalized medicine. One of the problems in implementing personalized therapy is that many treatment strategies are still based on tissue biopsies. However, tissue biopsy is an invasive procedure and cannot provide comprehensive information about the heterogeneity of breast cancer. In addition, in some conditions, this tissue biopsy is difficult or impossible to perform due to the unavailability of tumor tissue after surgery or the recurrence in a distant organ. This restricts the ability to assess the latest tumor biology to guide adjustments in the form of applied treatment.[1, 2]

Currently, there is a growing use of preoperative induction chemotherapy in breast cancer, which is given to both advanced local stage and aggressive early-stage breast cancer. The administration of induction chemotherapy has the goal of eradicating micrometastases and obtaining a complete pathological response.[1-3] One of the limitations of the current clinical and radiological evaluation of chemotherapy resistance is its assessment of only macroscopic lesions with or without clinical symptoms. At the same time, pathological chemoresistance can only be evaluated following definitive surgery on the primary tumor. In breast cancer, changes in the biological properties of cancer cells, either naturally or due to the influence of the treatment, require a change in treatment strategy. Serial clinical examination and post-curative therapy radiological examination have limitations in detecting microscopic lesions so that it affects worse clinical outcomes because it is known that cancer progression is already in the form of macroscopic lesions.[4, 5]

Liquid Biopsy

Liquid biopsy is an examination performed on blood, urine, saliva, or other non-invasive biological samples to detect the presence of cancer cells or DNA/RNA fragments from circulating tumor cells in the bloodstream. Liquid biopsy has several advantages, including (i) allowing rapid assessment of biomarkers when tissue biopsies are difficult to perform or have high risk, (ii) providing molecular information describing tumor heterogeneity, (iii) can be repeated during treatment or during the patient's observation period to assess treatment efficacy; and (iv) can be used to detect genomic changes that occur when resistance to therapy develops.[6-8] Sampling from peripheral blood makes it easy to carry out serial examinations within a short time, allowing improved early detection of cancer progression. Liquid biopsy is also able to provide information related to cancer plasticity, which can support more personalized therapy.[8-10]

Currently, the most frequently analyzed components of liquid biopsies are circulating tumor DNA (ctDNA) and circulating tumor cell (CTC).[8] CtDNA is a subsection of total cell-free DNA (cfDNA) in patients with cancer that is released through the process of apoptosis and necrosis of cells from healthy tissues and cancer. CTCs are released into the peripheral circulation of the primary tumor as intact cells and are generally considered the first step in the metastasis process. This provides an opportunity to analyze its relationship with treatment response and cancer progression within the scope of liquid biopsies of blood specimens.[11]

Circulating Tumor Cell (CTC)

Circulating tumor cells, or CTCs, are tumor cells that are actively intravased or passively released from primary tumors or metastatic lesions into the bloodstream and contain a population of cells that have the potential to initiate recurrence and distant metastasis.[12] Sequential changes in the number and molecular profile of CTCs, which can be assessed from surface and intracellular marker proteins, can be used as clues to changes in the biological behavior of CTCs that are initially dormant to more aggressive, determination of treatment resistance and recurrence at the molecular level.[13] CTC examinations after definitive therapy to detect minimal residual disease can detect recurrence at the molecular level earlier than recurrence from radiological examinations in 72% of cases, with an average of 2–9 months. This allows enough time to treat patients when the tumor burden and heterogeneity are at their lowest. Changes in treatment strategies based on dynamic information at the molecular level allow risk stratification to direct a more personalized approach to breast cancer treatment.[2]

CTC has advantages in providing molecular information from cancer cells through examination at the level of proteins, RNA, and DNA. Analysis of intact tumor cells rather than some CtDNA fragments confirmed that the information obtained came from viable tumor cells contributing to tumor progression. Another advantage of using CTC in liquid biopsy is the ease of studying the mechanism of CTC in avoiding the immune system, which generally occurs through interactions in cell surface proteins, such as MHC, and immune checkpoint PDL-1. This information, along with information obtained from genomic analysis (DNA mutations), can add to our knowledge of the biological mechanisms of resistance to immunotherapy and other systemic therapies.[13, 14]

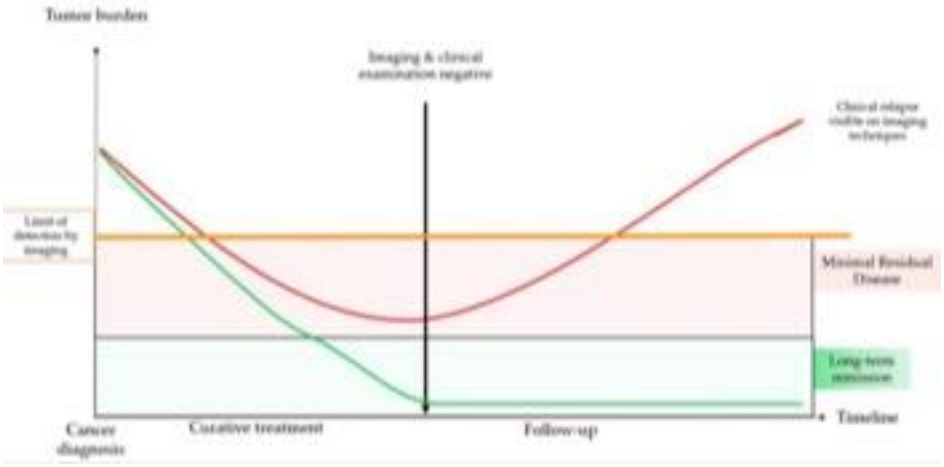

Figure 1. Schematic curve of minimal residual disease in cancer[7]

CTCs in the peripheral circulation can be detected as single cells or clusters consisting of two or more CTCs and platelets, also known as circulating tumor embolism. CTC clusters are formed from groups of oligoclonal tumor cells with intercellular adhesion linked by the cell junction component of placoglobin and many express proteins that play a role in cell adhesion, including tight junctions and desmosomes. Much clinical evidence is increasingly supportive of the character of cluster CTC, which is different from that of solitary CTC. Cluster CTCs have a higher metabolic capacity and durability in circulation than solitary CTCs, with the ability to seed metastatic cells about 23–50 times compared to solitary CTCs. In vitro analysis obtained a dominance of CTCs in solitary form of 97.4% compared to cluster CTCs of 2.6%.[15]

CTC Detection Methods

CTCs can be isolated from other cells in the peripheral circulation based on specific markers of those tumor cells. CTCs are in very low concentrations, about 1 CTC per 1 million white blood cells, even in metastatic breast cancer. Therefore, an enrichment process is needed to increase its concentration before the detection process.[6, 11] This enrichment process can increase the concentration of CTC into several log units to make it easier to detect.[11]

The two primary types of CTC selection methods are the independent label, which is based on negative selection or certain biophysical features, and the dependent label, which is based on positive enrichment involving proteins on the surface of cancer cells.[6] Label-dependent methods are more specific but can give false-negative results if antigen expression is lost in some subpopulations of CTCs and less viable cancer cells after isolation. The label-independent method is not based on a specific phenotype of CTC and is less specific. However, it is relatively better at preserving the viability of cancer cells for

subsequent molecular analysis.[6] One of the main problems in CTC detection methods based on the biophysical character of cell size is the possibility of contamination by other cells, such as white blood cells that are relatively the same size as the lower limit of CTC size.[16]

Flow Cytometry examination allows the assessment of cell characteristics based on surface and intracellular antigens of cancer cells using a variety of monoclonal antibodies conjugated with fluorochrome dyes. CTC examination using several cell marker proteins can improve the diagnostic value of CTC detection and help comprehensively detect heterogeneous CTC populations. These cell marker proteins are expressed as CTC responses and mechanisms to survive in systemic circulation.

Relationship between CTC and Breast Cancer Progression

CTC analysis can allow studying cancer progression through molecular metastatic cascade processes. This metastatic cascade begins with a complex event when cancer cells break away from the primary tumor, invade/migrate to surrounding local tissues, perform intravasation into the circulation as CTC, defend themselves in circulation, and extravasation to the target organ and colonize as metastatic lesions.[15] CTCs in the systemic circulation will be exposed to biochemical (cytokines, immune cells) and biophysical (shear stress) influences that make most CTCs experience apoptosis. Only a small percentage of the CTC population (3%), in the form of proliferation CTC, can survive and continue the metastasis process.[17]

The metastasis process begins with transforming cancer cells from initially non-motile epithelial cells with structured polarization, under the influence of various cytokines from the stroma that reactivate the epithelial-mesenchymal Transition (EMT) process, to cells with a phenotype that is easy to migrate. The EMT process is a dynamic process when cancer cells lose part or all of their epithelial character and gain a mesenchymal phenotype.[15, 18] These cells lose adhesion to surrounding cells partially or entirely and have a higher ability to migrate intravascularly into the circulation as CTCs. Many in vitro and in vivo studies have shown the activation of the EMT process in metastatic cancer cells to overcome various obstacles that can arise in the new microenvironment. CTC has been considered a direct mediator of the metastatic process, and the presence of CTC in various solid tumors is associated with a poor prognosis.[15]

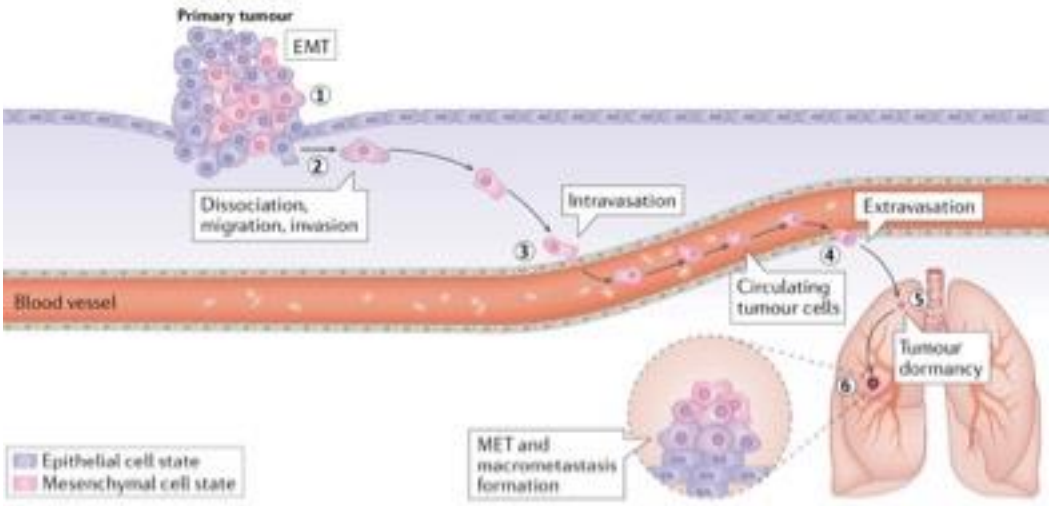

Figure 2. Stages of the metastatic process mediated by the EMT process.[19] (1) Tumor cells detached from the primary tumor; (2) migration penetrates the basal membrane and extracellular matrix; (3) intravasation into systemic circulation as CTC; (4) extravasation into the target organ; (5) some tumor cells can survive and become dormant; (6) tumor cells undergo the MET process forming macrometastatic lesions. CTC: Circulating tumor cells; EMT: Epithelial-mesenchymal transition.

CTC Protein Marker Expression on Therapy Resistance and Cancer Progression

Phenotypic analysis of CTCs can provide information about subpopulations of CTCs that have a more aggressive character, which can help assess the risk of recurrence, stratify high-risk patients, and for monitoring. In addition, selecting therapies that target CTC populations with high metastatic potential can provide hope for better clinical outcomes. CTC shows heterogeneous population phenotypes that have different clinical and biological characteristics.

Various expressions of cell-marker proteins in CTCs appear in response to various stresses within the systemic circulation, interactions with the immune system, and responses to systemic therapies administered. These CTC marker proteins comprise EMT markers, immune checkpoints, cancer stem cells, cell viability, and endothelial adhesion. The advantage of using CTC based on the combination of various cell marker proteins is that it will add to the diagnostic value of CTC detection. The information obtained is sourced from intact tumor cells, viable with a higher potential for survival and metastasis than other CTC populations. [15, 20]

1. CTC Epithelial and Mesenchymal Markers

Many studies show that CTCs that display epithelial and mesenchymal phenotypes simultaneously, which are in partial EMT status, have a higher potential for metastasis. CTCs in partial EMT status have more plastic CTC properties; these phenotypes display separate epithelial and mesenchymal characteristics, such as increased resistance to anoikis processes in mesenchymal phenotypes and high proliferation rate characters in

epithelial phenotypes. Changes in the CTC phenotype during therapy administration can be used as a dynamic observation method of therapy response.[21] Patients with CTC expressing EMT markers have a worse prognosis than epithelial CTC phenotypes. The transitional form between the epithelial and mesenchyme phenotype and the reversal mesenchyme-epithelial transition (MET) process is considered a hallmark of cellular plasticity that allows cancer cells to efficiently metastasize.[15, 22]

Some epithelial markers often used for CTC detection are cytokeratin (CK 8,18,19) and EpCAM (epithelial cell adhesion molecule). EpCAM is an epithelial biomarker used in the CellSearch system and is the only CTC method approved by the FDA.[11, 23, 24] It has a sensitivity of approximately 65% for detecting CTC (>1 cell per 7.5 ml of blood) in metastatic breast cancer patients.[25] EpCAM expression decreased during cancer dissemination due to the EMT process followed by an increase in the expression of mesenchymal markers.[26]

Vimentin and Pi3K are the most commonly used biomarkers for detecting CTC with mesenchymal profiles.[24] The CTC detection method with vimentin enrichment and negative selection with CD45 can detect cancer progression more accurately than Cellsearch, which mainly detects epithelial CTC. The concordance degree of both methods was 66.67% for patients with stable cancer conditions but decreased to 45% in cases with advanced cancer, indicating the role of EMT in breast cancer progression. Reliable assessment of CTC requires a method capable of detecting epithelial and mesenchymal components simultaneously.[24, 27]

2. CTC Stem Cell Markers

One of the causes of cancer cell chemoresistance is the presence of intratumor heterogeneity, with the primary role of the CTC population displaying cancer stem cell markers through its ability to self-regenerate, high plasticity, drug transporter expression, and higher survival in circulation.[28] Some biomarkers that indicate the properties of cancer stem cells in solid tumors are ALDH1, CD133, CD90, α6-integrin, or a combination of CD44/CD24 status. Several studies have shown that cluster CTCs have properties similar to cancer stem cells accompanied by increased expression of CD44 cell surface antigens. Cluster CTCs may provide appropriate protection and microenvironment for cancer stem cells during their journey to the target organ. This is in line with the discovery of increased expression of genes involved in the preparation of the cancer microenvironment. Conventional systemic therapy is generally less effective in radiating cancer cells that have entered the cancer stem cell condition through this EMT process, resulting in recurrence after treatment.[15]

3. CTC Immune Check Point Markers

CTCs that enter the peripheral circulation can be susceptible to elimination by the immune system. However, CTCs can utilize immune system components such as leukocytes and other circulomes such as platelets, endothelial cells, and cancer-associated

fibroblasts to increase survival and metastasis capacity. CTC can inhibit the binding process between MHC I in CTC and T cell receptors and induce a decrease in the regulation of MHC class II and genes that activate T cells. In addition, CTC has a good capacity to avoid the immune system involving many mechanisms, including interaction with platelets that envelop the CTC cluster, expression of the PD-L1 protein that functions as an immune checkpoint inhibitor, CD47 expression in CTCs that bind to proteins α in macrophages and dendritic cells, inhibition of phagocytosis process, and changes in FAS/FASL protein expression that play a role in the apoptosis process.[13] The interaction of PD-1 and PD-L1 on the surface of cancer cells will reduce the infiltration of CD4+ and CD8+ cells and the production of cytokines that play a role in the immunosuppression process. PD-L1 relays intracellular signals that induce the proliferation and survival of cancer cells and protection against interferon-mediated pro-apoptosis stimulus. There is clinical evidence of cross-communication between molecules involved in EMT and PD-L1.[13]

4. CTC Endothelial adhesion Markers

Mucin 1 (MUC1) is a transmembrane protein generally found in epithelial cells in the mucous membrane and provides lubrication and protection. The extracellular domain of MUC1 (MUC1-ECD) can act as a ligand for adhesion receptors in the stroma and endothelium. In contrast, the cytoplasmic domain (MUC1-CD) is associated with various interactions that result in increased migration and invasion.[29-31]

When the CTC is already in circulation to continue the extravasation process, the CTC must have an initial interaction with the vascular endothelium, which will then facilitate the tethering and rolling process. The tethering and rolling phenomena of CTCs under the influence of shear stress on the blood vessel walls are mediated by adhesion molecules from the selectin family expressed in the endothelium and their ligands in the CTC. MUC1 is a ligand of E-selectin to initiate the splicing and turnover of CTCs in the endothelium. After being in a stable turnover phase, MUC1 can bind to ICAM-1 present in the endothelium to stabilize the CTC adhesion condition. The synergistic effect between MUC1, E-selectin, and ICAM1 has an important role in the metastatic process of breast cancer through systemic circulation. The entire stage of events, from the initial interaction to the adhesion of CTCs to the endothelium, is called the metastatic adhesion cascade.[30, 32]

5. CTC Apoptotic Markers

Apoptosis is the primary mechanism of cancer cell death at chemotherapy administration. Only CTCs that remain viable can survive in circulation and continue the metastasis process. CTCs that are still viable and survive the metastatic cascade process generally play a role in the occurrence of chemoresistance and have more aggressive biological characteristics. Determining a viable CTC population in breast cancer patients can help determine prognostics and select more individualized therapies. CTC apoptosis in circulation is associated with therapy response, disease remission, and a better prognosis.

In addition, detecting CTCs that have undergone apoptosis helps remove these cells from the analysis process. CTCs that have experienced death can cause artifacts due to binding to non-specific antibodies that give false negative results of fluorosin capture.[17]

The detection of CTC apoptosis is quite complex because the half-life of CTC apoptosis in circulation is quite short (10 minutes), so the timing of the analysis is very important in assessing the effect of therapy.[33] Flow cytometry can be used as a fast and reliable detection technique to assess viable CTCs based on the principle of label-dependent method by using specific antibodies that bind to cell surface proteins or DNA. Phosphatidyl serine is a phospholipid component of the cell membrane located in the inner layer on the cytoplasmic side. When a cell undergoes apoptosis, serine phosphatidyl will be exposed to the outer layer of the plasma membrane. Macrophages will recognize phosphatidylserine on the cell surface and subsequently perform phagocytosis. Annexin V has a strong affinity for phosphatidylserine; thus, it can be a marker of cells in the early phase of apoptosis.[34] 7-Amino Actinomycin D (7-AAD) staining is a fluorescent-labeled agent that can bind to DNA in the cell nucleus at the position between cytosine and guanine bases and can be used as a marker of cells that have reached an advanced stage of apoptosis. A positive result indicates a cell that is not viable, as the new 7-AAD can bind to DNA if the apoptosis cell has lost membrane integrity.[17, 35]

Conclusion

Sequential changes in CTC values of breast cancer based on a combination of EMT, immune checkpoint, cancer stem cells, cell viability, and endothelial adhesion markers might be used to predict chemotherapy resistance.

Conflict of Interests

The author declared no conflicts of interest.

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