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Review

Breast Cancer Metastasis

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Abstract. *Breast cancer metastasis accounts for the majority of deaths from breast cancer. Detection of breast cancer metastasis at the earliest stage is important for the management and prediction of breast cancer progression. Emerging techniques using the analysis of circulating tumor cells show promising results in predicting and identifying the early stages of breast cancer metastasis in patients. Additionally, a deeper understanding of the metastatic cascade in breast cancer will be critical for developing therapeutic interventions to combat breast cancer metastasis. In this review, the current and novel methods for detection of breast cancer metastasis, as well as the mechanisms involved in metastasis and the treatment of breast cancer metastasis, are discussed.*

Breast cancer is the most common type of cancer and the primary cause of cancer mortality in women (1). The majority of deaths from breast cancer are not due to the primary tumor itself, but are the result of metastasis to other organs in the body (2).

Detection of Breast Cancer Metastasis

Currently, detection of breast cancer metastasis relies on clinical manifestations of the spread to distant organs, biopsies of affected organs, radiological evaluations, imaging methods and serum tumor markers (3, 4).

According to the American Society of Clinical Oncology (ANCO) guidelines on breast cancer follow-up and management, symptoms of breast cancer recurrence include presence of new breast lumps, pain in the bone, chest or abdomen, dyspnea and constant headaches (5). In addition, ASCO also recommends mammography for the early detection of relapse in breast cancer (5). Nicolini *et al*. (6) emphasized that the inclusion of serum tumor markers is an important factor in the postoperative monitoring of breast cancer patients (7, 8). Another suggestion is to have intensive postoperative follow-up which includes consultations every 4-6 months, physical examination and evaluation of serum carcinoembryonic antigen (CEA), tissue polypeptide antigen (TPA) and breast cancer-associated antigen 115 D8/DF3 (CA15.3), at each visit. Additionally, imaging methods such as bone scintigraphy, liver echography and chest X-ray are to be performed bianually. Computed-tomography and magnetic resonance imaging should be performed if suspicion arises from the earlier mentioned methods (6).

Although mammographic screening has reduced the mortality rate associated with metastasis as a result of early diagnosis (2), the methods described above are frequently inept at detecting metastasis at the earliest stage and at accurately predicting the clinical outcome of the disease (3, 4). An emerging method to detect metastasis is the analysis of circulating tumor cells (CTCs), which has shown promise in filling the gaps left by other diagnostic methods.

CTCs are tumor cells originating from primary sites or metastases that circulate in the patients' bloodstream and are very rarely found in healthy individuals (9, 10) (Figure 1). CTCs are recognized as playing important roles in the metastasis of carcinomas (11, 12) and their analysis enables the prediction of metastatic relapse and progression (11). Generally, CTCs are firstly isolated and enriched through either morphological or immunological techniques (4). Morphological-based isolation separates CTCs according to size discrepancies, using isolation by size of epithelial tumor cells (ISET) or according to density, using density-gradient separation (4). Immunological techniques, which are the most widely used methods, employ immunomagnetic isolation (4). This method uses either epithelial cell-specific markers which are generally expressed in all tumor cells, or tumor markers expressed by specific types of cancer (13). After isolation, the source and genetic make-up of CTCs are

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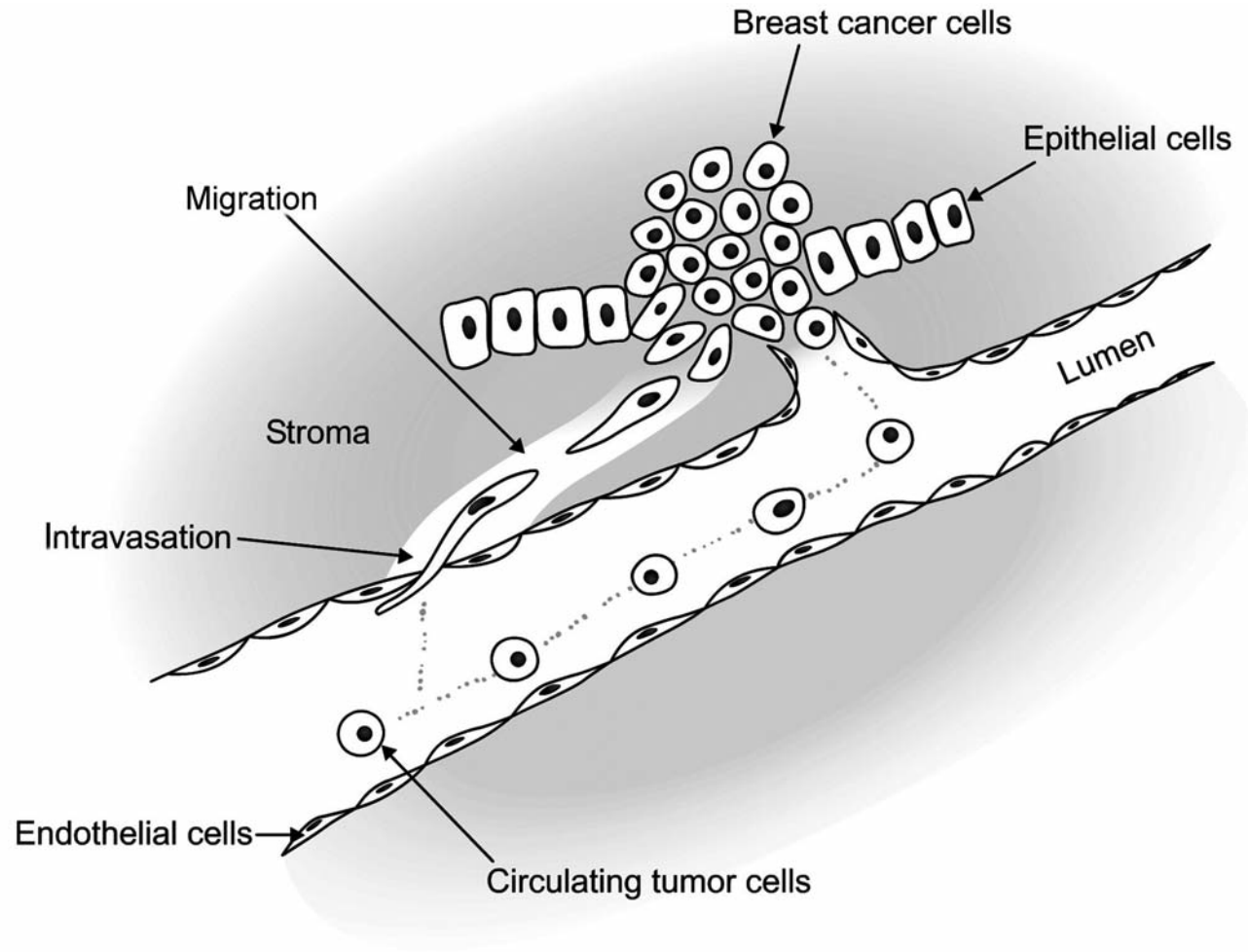

Figure 1. *Circulating tumor cells in the blood stream.*

characterized using nucleic acid-based methods, such as quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR), or cytometric-based methods, such as flow cytometry and enzyme-linked immunospot assay technology (4).

Clinical applications of CTC analysis have shown promising results. Indeed, Goodman *et al*. (14) proposed that the number of CTCs could indicate ongoing metastasis. There is accumulating evidence that CTCs are correlated with clinical outcome and survival in patients with cancer (4). A study on patients with metastatic breast cancer before treatment showed that patients with more than five CTCs in 7.5 ml of blood are predisposed for shorter progression-free and overall survival (15). Pierga *et al*. (16) demonstrated that the presence of one CTC in 7.5 ml blood after neoadjuvant chemotherapy, could be predictive of metastatic relapse. However, although promising results have been recorded, an expansion in the number of methods to detect CTCs calls for a need to standardize the techniques available, in order to ensure increased efficacy and quality (17).

Mechanisms of Breast Cancer Metastasis

Metastatic cascade. The process of metastasis comprises of a series of sequential steps (Figure 2). Failure to complete any of these steps will arrest the process (18). Metastasis starts with the local invasion of surrounding host tissue by cells originating from the primary tumor and continues until the tumor cells invade and intravasate into blood or lymphatic vessels (19, 20). The tumor cells are disseminated *via* the blood stream or the lymphatic vessels to distant organs. Consequently, the tumor cells undergo cell cycle arrest and adhere to capillary beds within the target organ, before extravasating into the organ parenchyma, proliferating and promoting angiogenesis within the organ (19). While undergoing these steps, the tumor cells must simultaneously

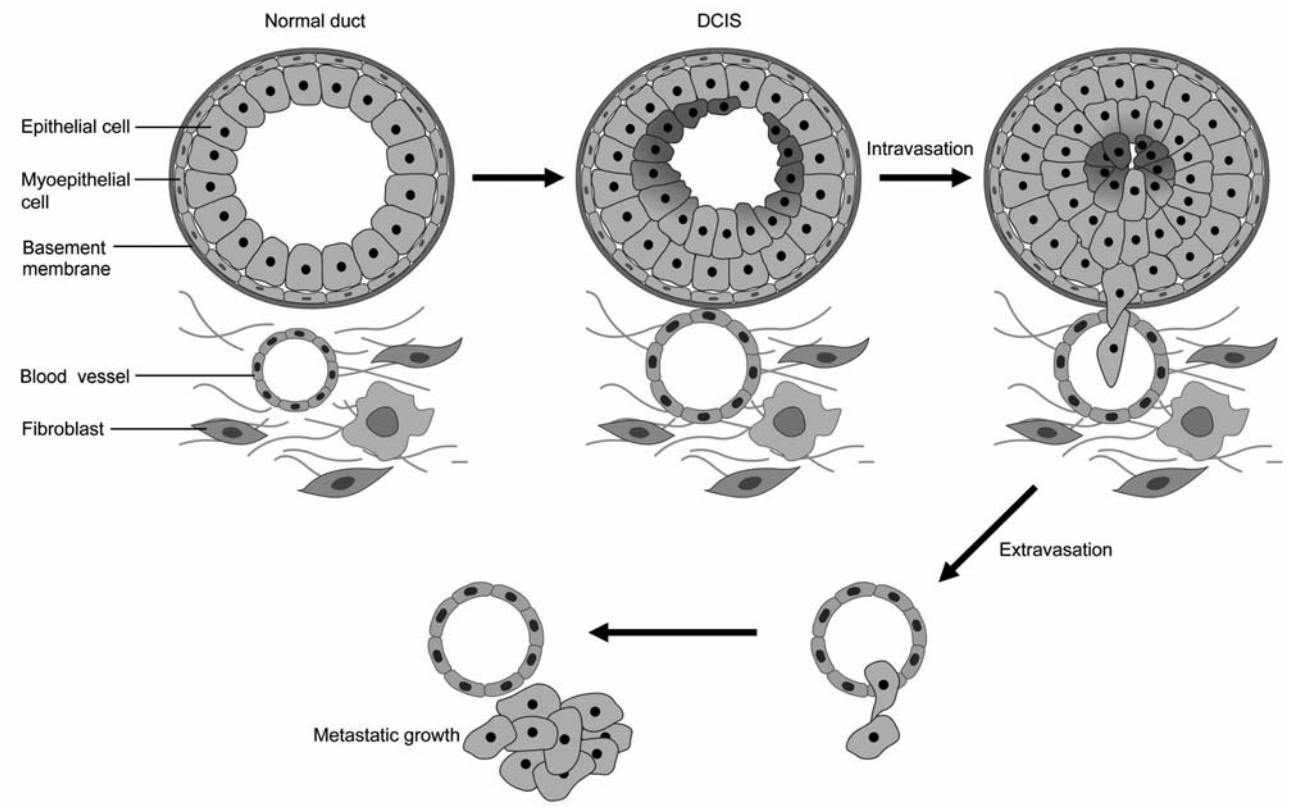

Figure 2. *Schematic showing the metastasis cascade of breast cancer.*

evade the host's immune response and apoptotic signals in order to survive (19, 21). If the tumor cells succeed in completing these steps, the process can be repeated to produce secondary metastases or 'metastasis of metastases' (18, 20).

Invasion. Metastasis begins with the invasion of tumor cells into the surrounding host tissue. The invasive tumor cells must first alter cell-to-cell adhesion and cell adhesion to the extracellular matrix (ECM). The cadherin family has been documented to play a large role in mediating cell-to-cell adhesion and plays predominant roles in breast cancer metastasis (22). E-Cadherin maintains cell-cell junctions, while the down-regulation of E-cadherin was shown to be a determinant in the outgrowth of metastatic breast cancer cells (23). The down-regulation of E-cadherin has been reported to reflect progression and metastasis in breast cancer associated with poor prognosis (24, 25). Mutations in E-cadherin which lead to its functional loss were discovered in lobular breast carcinoma (26). N-Cadherin is closely associated with mesenchymal cells and related to epithelial-to-mesenchymal transition (EMT) during the gastrulation stage (27). There is increasing evidence that EMT is associated with cancer progression (28, 29). EMT plays a major role in tumor progression by assisting invasion and intravasation into the bloodstream and inducing proteases involved in the degradation of the ECM (30, 31). Kotb *et al*. (27) showed that the expression of N-cadherin in place of E-cadherin caused the formation of fibrosis and cysts in mammary glands and eventually led to malignant breast tumor in mice. In addition, as reported by Yilmaz *et al*. (32), down-regulation of E-cadherin and up-regulation of N-cadherin were frequently observed in cancer cells of most epithelial cancers during stromal invasion. Down-regulation of E-cadherin is believed to result in the loss of adhesion between epithelial breast cancer cells and other epithelial cells, while increase in N-cadherin, and possibly other mesenchymal cadherins, permits the adhesion of tumor cells to stromal cells and subsequently, the invasion of tumor cells into the stroma (33).

The adherence of tumor cells to the ECM is mediated through integrins (34). Integrins are transmembrane receptors found on ECM components such as fibronectin, laminin, collagen, fibrinogen and vitronectin (22). Invasion is preceded by degradation of the ECM to enable the penetration of tissue boundaries. The degradation of ECM is

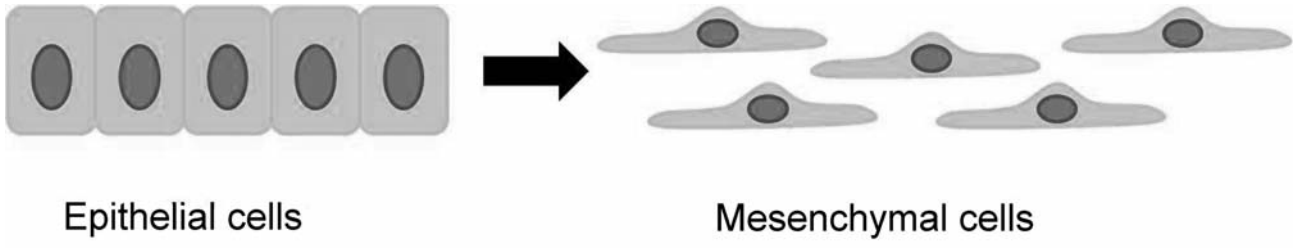

Figure 3. *Epithelial–to-mesenchymal transition (EMT). The epithelial cells undergo phenotypic changes to take on mesenchymal-like characteristics.*

carried out mainly through metalloproteinases (MMPs) and the urokinase plasminogen activator (uPA) system (35, 36). In breast cancer patients, uPA showed prognostic importance in predicting the risk of distant metastases (37). This result also encompasses patients with good prognosis at diagnosis (38). Huang *et al.* (39) showed that the inhibition of uPA *via* small-interfering RNA (siRNA) restricted invasion and reduced the expression of MMP9. MMPs mediate the proteolysis of ECM at the invadopodial front of invasive breast cancer cell lines (40). Integrins are also known to participate in the modulation of tumor motility by participating in the activity of ECM-degrading enzymes such as the MMPs (22). For instance, integrins α5β1 and α3β1 were both reported to up-regulate MMP9 (41, 42).

Additionally, heparanase, a β-glucorinidase, also aids in the degradation of the ECM by breaking down heparan sulfate proteoglycan (43), a proteoglycan existing either in the ECM or the cell surface which is important in the assembly and integrity of the ECM (44) and for mediating cell matrix adhesion and growth factor receptor interactions (45). Heparan sulfate acts as a reservoir for heparin-binding growth factors and angiogenic factors (43). By degrading heparan sulfate, heparanase helps in the release of these substances which promote tumor growth, invasion and angiogenesis (46). Indeed, the expression of heparanase correlates with metastatic potential in breast cancer (47) and an increase in heparan sulfate proteoglycans such as glypican-1 and syndecan-1 has been observed in advanced stages of breast cancer (48). In addition, Cohen *et al.* (49) reported that the overexpression of heparanase in MCF7 breast cancer cells increased cell proliferation and survival, as well as stromal infiltration, *in vitro* and *in vivo*.

Migration and motility. In order to achieve an invasive phenotype, tumor cells need to migrate from the confined primary site. Tumor cells are able to migrate either singly or coordinately (50). Tumor cells are inclined to migrate coordinately from intermediate or highly differentiated lobular carcinomas of the breast (51). It is suggested that coordinated cell migration may switch to single-cell migration, mainly in poorly differentiated tumors due to structural and functional abnormalities of the intercellular adhesion proteins (50). Tumor cells that migrate collectively need the presence of intercellular junctions. As a result, after invasion and intravasation, they commonly circulate as emboli in the blood or lymphatic vessels (52, 53). Cells at the leading edge of the migrating tumor will create tube-like microtracks by cleaving and orienting collagen fibers using the membrane type 1 (MT1) MMP for the ensuing collective mass migration of tumor cells through the ECM (54, 55). On the other hand, single tumor cells migrate in two ways, mainly by protease-dependent mesenchymal movement or the protease-independent amoeboid movement (50).

The EMT is a critical pathway in the mesenchymal movement of single migratory cells. Here, the cells will undergo changes from an epithelial phenotype to a mesenchymal-like phenotype (32) (Figure 3).

EMT starts with the disintegration of cell-cell adhesion by losing epithelial markers, such as E-cadherin, and expressing mesenchymal markers, such as vimentin. Accordingly, the expression of transcriptional repressors of E-cadherin including zinc finger E-box-binding homeobox 1 (ZEB1), zinc finger E-box-binding homeobox 2 (ZEB2), twist-related protein (Twist), zinc finger protein, Snail and Slug, involved in signaling pathways such as transforming growth factor- β (TGF-β), the wingless-type MMTV integration site family (WNT) cascade and the phosphatidylinositol 3' kinase-serine/threonine kinase (PI3K/AKT) axis linked to the EMT programs, are associated with poor prognosis in breast carcinoma (56-62). Following the loss of cell adhesion, cell polarity is altered from apical-basal polarity to front-rear polarity to initiate cell migration through changes in cortical actin and actin stress fibers that induce cytoskeleton remodeling. And lastly, proteolytic enzymes such as MMPs are activated and cell matrix adhesion is changed (63). Thus, cells which have undergone EMT have an elongated fibroblast-like shape and their movement is facilitated by channels which are produced in the ECM by matrix-degrading enzymes, such as MMPs (54). In contrast, cells with amoeboid movement are round cells and resemble to

primodial unicellular organisms (32, 50). Similarly to those organisms, they push and squeeze through pores in the matrix by relying mostly on shape deformations and structural changes in the ECM (64-67) rather than actual degradation of the matrix (32, 50). These cells are loosely attached to the ECM, lose cell polarity and move through the paths of least resistance (34). The mechanical force used is generated by active myosin/actin contractions and cortical actin *via* signaling pathways such as RhoA/Rho kinase (ROCK) (32, 54).

It is postulated that tumor cells predominantly utilize mesenchymal motility (50). However, under certain circumstances, alterations in the molecular pathways determining either mode could cause a switch in the migration mode, either from mesenchymal to amoeboid movement, named mesenchymal-to-amoeboid transition (MAT), or *vice-versa*, the amoeboid-to-mesenchymal transition (AMT) (68). At the molecular level, the inhibition of pathways related to Rho/ROCK, such as PI3K and cell division control protein 42 homolog (CDC42)-mediated signaling, which are pro-amoeboid, caused AMT, while molecules such as ras-related C3 botulinum toxin substrate (Rac) and SMAD-specific E3 ubiquitin protein ligase 1 (Smurf1) encourage mesenchymal movement and their inhibition caused MAT (68). Events such as inhibition of pericellular proteolysis (69) or high Rho/ROCK levels (70) also caused MAT. The spatial arrangement of surrounding collagen fibers at the tumor ECM boundary also plays a role in determining the mode used by migrating cells (64). When collagen fibers were pre-aligned perpendicularly to the tumor ECM boundary, amoeboid movements of MDA-MB-231 mesenchymal cells were not associated with the Rho/ROCK pathway. Conversely, activation of the Rho/ROCK pathway was observed in these cells when collagen fibers were not pre-aligned to tumor ECM boundary (64).

There is also compelling evidence that stromal cells aid migration of tumor cells. The majority of stromal cells within breast cancer are fibroblasts and are usually referred to as carcinoma-associated fibroblasts (CAFs) (34, 71). Conditioned medium collected from CAFs was found to promote cell motility and invasion in breast cancer *in vitro* (72). Moreover, immunodeficient nude mice when injected with both human CAFs and MCF7-ras human breast cancer cell lines, also exhibited enhanced breast tumor growth and angiogenesis compared to mice injected with normal human fibroblasts (73).

Tumor microenvironment. In the 1980s, Stephen Paget proposed the 'seed and soil' theory for metastasis whereby the 'seed' (tumor cells) is postulated to only grow when it finds a suitable 'soil' (environment) (74). This theory is being revisited, as increasing evidence points to the tumor microenvironment as a critical factor in metastasis.

The microenvironment of metastatic tumor cells is critical for tumor cell proliferation. A suitable microenvironment is a requirement for and equally important in establishing tumor growth and malignant progression (75). Many different specialized cells, including fibroblasts, immune cells, endothelial cells and mural cells of the blood and lymph vessels, together with the ECM make up the microenvironment which influences tumor progression (76-78). Malignant cells constantly interact with cells of the microenvironment at both the primary and metastatic sites (79-84). These interactions pave the way for the progression of '*in situ*' breast cancer to metastatic breast cancer (85). For example, the recruitment of macrophages by non-invasive breast tumor cells induced angiogenesis and promoted malignant transformation (86). Tissue-associated macrophages, which are capable of influencing tumor invasion, angiogenesis, immune evasion and migratory behavior (87-90), were found to form interactive niches with breast cancer cells and endothelial cells, thus promoting intravasation and metastatic spread (91). In the bone, it is known that interactions between tumor cells and the stromal components, such as osteoclasts and osteoblasts, influence the growth and dormancy of the tumor cells; hence, success of the outgrowth of metastatic cells into bone, heavily depends on the bone stroma (92, 93).

It is also postulated that tumor cells themselves might secrete substances to prime the 'soil' prior to metastasis to establish a 'pre-metastatic niche' supporting future metastatic sites (75). Hiratsuka *et al*. (94) showed that signals from primary tumor which induced MMP9 expression in lung endothelial cells and macrophages prior to metastasis, promoted preferential invasion of tumor cells to the lungs. In addition, vascular endothelial growth factor receptor 1 (VEGFR-1)-positive hematopoietic progenitor cell clusters were observed in pre-metastatic lymph nodes of patients with breast cancer before the arrival of tumor cells, suggesting the formation of a pre-metastatic niche (75). Indeed, breast cancer has been observed to preferentially metastasize to the bone and lungs and less frequently to other organs such as the liver and brain (95). Gene expression signatures accounting for the preferential metastasis of breast cancer cells to the bone marrow and lung have been identified, providing evidence that metastasis exhibits tissue tropism (96, 97). Interestingly, evidence also suggests the involvement of chemokines in the homing of tumor cells to target organs. Breast cancer tissue highly expresses the chemokine receptor, chemokine (C-X-C motif) receptor 4 (CXCR4) while its ligand, chemokine (C-X-C motif) ligand 12 (CXCL12), is predominantly expressed in lymph nodes, lung, liver and bone marrow but weakly expressed in small intestine, kidney, brain, skin and skeletal muscle (98). Organs with higher expression of CXCL12 are associated with being common sites of metastatic breast cancer (99). Furthermore, Muller *et al*. (98) demonstrated that the CXCR4-CXCL12

interaction encouraged migration of breast cancer cells to the common sites of breast cancer metastasis.

Another important aspect in metastasis is the establishment of tumor vasculature. Angiogenesis plays a significant role in generating metastasis and subsequent metastasis growth (100). It is a critical microenvironmental adaptation for tumors and is regarded as a hallmark of cancer (101). In tumorigenesis, the balance between pro-angiogenic and anti-angiogenic factors is disrupted with a slant towards the pro-angiogenic side (102-104). Genetic mutations, mechanical stresses, inflammatory processes, tumor expression of angiogenic proteins and, predominantly, hypoxia are believed to cause the 'angiogenic switch' (105, 106). Unlike under normal physiological conditions, the tumor vasculature is distorted and is structurally, functionally and genetically different from that of normal vasculature. The abnormal blood vessels are insufficient to supply oxygen to the tumor, which causes tumor hypoxia (107). This, in turn, encourages tumor cells to produce more pro-angiogenic factors, resulting in an increase in abnormal vasculature. Hence, this vicious cycle continues. In order to escape the severely hypoxic microenvironment induced by this cycle, invasive and metastatic programs are turned on (108).

In addition, hypoxic conditions allow factors such as hypoxia inducible factor-1 (HIF-1) to trigger the production of angiogenic proteins (100, 109, 110). Among them, vascular endothelial growth factor (VEGF) and its receptors (VEGFR) have been extensively studied (111). VEGF belongs to a family of growth factors which includes VEGF-A, -B, -C, -D and -E and placental growth factor (112, 113). Generally, VEGF stimulates vasculogenesis and angiogenesis and its functions are mediated through different VEGFRs (100). VEGF stimulated the proliferation, invasion and migration of endothelial cells and enhanced microvascular permeability (114-116). In solid tumors, the expression of VEGF denotes poor prognosis and a tendency for metastasis (111, 117).

Treatment of Breast Cancer Patients with Metastatic Disease

Although advances in the treatment for metastatic breast cancer have significantly improved the survival of patients (118), metastatic breast cancer is still considered an incurable disease (6, 119). In general, the treatment for breast cancer metastasis can be divided into standard chemotherapy and targeted therapy.

Cytotoxic drugs used in standard chemotherapy for metastatic breast cancer include anthracyclines, taxanes and 5-fluorouracil as first, second and third lines of therapy, respectively (6). However, anthracycline use has been associated with cardiac dysfunction (120). Newer cytotoxic chemotherapeutic agents that have been developed are epothilones and ixabepilone (121). Both these agents exhibited increased efficacy in patients with metastatic breast cancer who had prior treatment with anthracyclines and taxanes (119).

Targeted therapies include hormone therapy, immunological therapy and antiangiogenic therapy. Hormone therapy either blocks estrogen receptor (ER) or reduces estrogen by inhibiting the enzyme aromatase. Aromatase converts adrenal androgen to endogenous estrogen, and in post-menopausal women, this conversion is the sole source of endogenous estrogen (6). Tamoxifen is an agent that blocks the ER and when used as an initial hormone therapy in post-menopausal women with metastatic disease, it results in tumor regression (122). Examples of aromase inhibitors are letrozole, anastrozole and exemestane. Interestingly, letrozole and anastrozole were shown to have better therapeutic index as first-line therapy of post-menopausal patients with metastatic disease, compared to tamoxifen (123, 124).

Trastuzumab is a monoclonal antibody that selectively binds to the extracellular domain of human epidermal growth factor receptor 2 (HER-2) and blocks the proliferation of tumors that overexpress HER-2 (6, 125). This antibody is regularly used with combination chemotherapy for both adjuvant treatment of breast cancer and metastatic breast cancer (126). The addition of trastuzumab to chemotherapy in the treatment of metastatic breast cancer was reported to improve overall survival rate, response rate and time-to-progression (127). The newer generation of HER-2-targeting antibodies, such as trastuzumab-MCC-DM1 and pertuzumab, have shown promising results in the treatment of metastatic breast cancer (119).

As mentioned earlier, angiogenesis is considered a hallmark of the malignant process and antiangiogenic therapy focuses on inhibiting new blood vessel growth (6). Bevacizumab is a humanized monoclonal antibody derived from murine VEGF, targeting all human VEGF-A isoforms but not other members of the VEGF family (128). It inhibits endothelial proliferation and starves tumor cells of vascular supply (129). The combination of bevacizumab with other chemotherapeutic agents has led to increased progression-free survival duration (119). However, this therapy also poses significant risks to patients with breast cancer such as severely high blood pressure, bleeding and hemorrhage, and even heart failure (119).

Conclusion

Breast cancer metastasis is a complex process determined by many factors and pathways. New and effective ways to detect and predict breast cancer metastasis at the earliest stage possible are important for the management of this disease. In addition, the unraveling of the mechanisms behind breast cancer metastasis could give rise to novel therapeutic approaches to combat this disease.

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