1. Background of the Study
1.1. Introduction

Breast cancer is a leading cause of death in women throughout the world [Torre LA et al, 2015; Spitale A et al, 2009]. In the year 2012, in USA, 232,714 women were diagnosed with breast cancer and out of them, 43,909 were died due to it [Breastcancerindia.net. N.p., 2017]. So, approximately, 1 of every 5 or 6 breast cancer-diagnosed women is dying in USA. In China, 187,213 women were newly detected and 47,984 were died of breast cancer in 2012. So roughly, in China, 1 of every 4 breast cancer-diagnosed women is dying. In India, 144,937 women were newly detected and 70,218 were died of breast cancer in 2012. Therefore, in India, for every 2 women newly diagnosed with breast cancer, one lady is dying of it [Fig. 1.1]. Recent statistics have indicated that among the total patients diagnosed with cancer in India, 27.0 % suffer from breast cancer [Breastcancerindia.net. N.p., 2017] [Fig. 1.2].

![Fig. 1.1. Comparison of breast cancer incidence and mortality percentages between India, USA and China in 2012.](image)

It is a complex disease comprising of distinct pathological features and clinical implications [Spitale A et al, 2009; Tang P et al, 2008; Desmedt C et al, 2009; Iwamoto T and Pusztai L, 2010; Reis-Filho JS et al, 2010; Sotiriou C and Pusztai L, 2009; Weigelt B et al, 2010]. It is evidenced that breast cancers with dissimilar clinicopathological features exhibit distinct behaviors leading to different treatment responses [Blows FM et al, 2010]. Conventionally,
patient prognosis and management is done by classical immunohistochemistry (IHC) markers such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2) together with traditional clinicopathological variables such as tumor size, tumor differentiation grade and lymph node metastasis (LNM) [Vallejos CS et al, 2010; Cheang MC et al, 2009].

![Percentage analysis of occurrence of different cancer types in India in 2012.](image)

**Fig. 1.2.** Percentage analysis of occurrence of different cancer types in India in 2012. The figure shows that breast cancer is the top most cancer type among the patients diagnosed with cancer in India, accounting for approximately 27.0 % of total cancer occurrence in the year 2012.

Gene expression analysis has shown that response of tumor cells to treatment is determined by inherent molecular individuality that can be probed using molecular methods [Iwamoto T and Pusztai L, 2010; Reis-Filho JS et al, 2010; Sotiriou C and Pusztai L, 2009; Weigelt B et al, 2010]. This has led to a new model on how breast cancer patients are stratified and treated. Further, it provides a superior precision and reproducibility of disease prognosis and therapeutic decision making [Pusztai L et al, 2008]. Novel molecules with promising roles are gaining their importance with increasing understanding of the breast cancer tumorigenesis process, further contributing to our understanding of breast cancer sub typing and also helped in interpreting breast cancer heterogeneity.
Molecular types of breast tumor

Breast tumors are primarily categorized into three main groups which are luminal, Her2 enriched and triple negative phenotypic tumors (TNP) or the basal subtype [Brenton JD et al, 2005]. Brief description of each of these three subgroups is discussed below:

1.2.1. Luminal tumors

The luminal type tumors express hormone receptors i.e., ER and PR with expression profiles suggestive of the luminal epithelial component of the breast, including the expression of luminal cytokeratins 8/18, ER activation genes such as Liv1 and Cyclin D1 [Perou CM et al, 2000; Sotiriou C et al, 2003]. Two distinct subtypes exist within luminal-like tumors, i.e., luminal A and luminal B.

Both luminal A and luminal B are ER positive-PR positive (ER+ PR+) [Vallejos CS et al, 2010]. Luminal A tumors are Her2 negative (Her2-) whereas, parts of luminal B tumors are Her2 positive (Her2+) [Vallejos CS et al, 2010]. Luminal tumors are the most common subtypes among breast cancer. In luminal A tumors, ER-related genes have higher expression and proliferative genes have lower expression than comparatively higher grade luminal B tumors [Sørlie T et al, 2001; Sørlie T et al, 2003]. Majority of breast cancers contain luminal A tumors. Usually, both luminal subtypes have a good prognosis. Further, luminal B tumors have a significantly poorer prognosis than luminal A subtype [Sørlie T et al, 2003]. Use of hormone therapy responds well in luminal tumors rather than conventional chemotherapy [Brenton JD et al, 2005]. However, the recurrence score is relatively higher in luminal B
tumors when compared to luminal A tumors [Paik S et al, 2004]. Thus, luminal A tumors could be effectively treated with endocrine therapy, while more proliferative luminal B tumors could be treated more effectively by combined treatment of chemotherapy and hormonal therapy. Approaches such as anti-angiogenic strategies are also helpful for luminal tumors management [Brenton JD et al, 2005].

1.2.2. Her2 enriched tumors

Typically Her2 enriched tumors are ER negative, PR negative, Her2 positive (ER-PR-Her2+) [Vallejos CS et al, 2010]. Though, not all clinically Her2 positive tumors show changes at the transcriptional level. The Her2 enriched tumors are characterized by overexpressing other genes in the Her2 amplicon such as GRB7 and PGAP3 [Perou CM et al, 2000; Dai X et al, 2014] and 40%-80% of these tumors harbor p53 mutation. Her2 overexpression tumors are more likely to be of grade 3. Despite the fact that Her2 overexpression in breast tumors predict poor prognosis, they are susceptible to neoadjuvant chemotherapies with anthracycline [Brenton JD et al, 2005].

The poorer prognosis of this subtype appear to get from a higher probability of early recurrence among those without complete destruction of tumor cells, and furthermore, are suggested to get the most advantage from improvements in chemotherapy [Brenton JD et al, 2005]. AntiHer2 monoclonal antibody, trastuzumab, is used to efficiently treat Her2 enriched cancers. However, not all Her2 enriched tumors respond to trastuzumab and trastuzumab resistance is found to be associated with loss of PTEN [Nagata Y et al, 2004] and upregulation of CXCR4 [Brenton JD et al, 2005].

1.2.3. Basal tumors

Basal tumors are likely to be of grade 3 tumors [Sørlie T et al, 2001; O’Brien KM et al, 2010]. This subtype is also known as triple negative breast cancer (TNBC), composed of ER-PR-Her2- tumors with expression profiles similar to that of the basal epithelial cells of other body parts and normal breast myoepithelial cells [Perou CM et al, 2000]. Expression profiles of basal tumors include very low or no expression of hormone receptors and Her2, and high expression of basal markers such as keratins 5, 6, 14, 17, epidermal growth factor receptor (EGFR) and proliferation related genes [Perou CM et al, 2000; Sotiriou C et al, 2003]. Low BRCA1 expression [Abd El-Rehim DM et al, 2005] and p53 mutation [Sørlie T et al, 2001; O’Brien KM et al, 2010] are observed in tumors expressing basal cytokeratins.
Among the triple negative breast cancer cases, 60%-90% are basal tumors [Fan C et al, 2006; Swenson RR et al, 2009]. These tumors are specifically compelling in light of the fact that they follow violent clinical course and at present do not have any type of standard systemic treatment. In contrast to other subtypes, these tumors are associated with more youthful patient age and higher risk of relapse [Ho-Yen C et al, 2012]. Generally, the size of basal tumors is comparatively larger than the other subtypes [Rakha EA et al, 2006] and tends to show faster growth [Ho-Yen C et al, 2012]. As most of the basal tumors are triple negative in nature, they are amenable only to chemotherapies. These aggressive tumors are sensitive to conventional chemotherapies such as anthracycline and taxane [Brenton JD et al, 2005]. Besides these conventional therapies, novel targets for basal tumors have been suggested by many studies. Reports have suggested the role of EGFR in basal tumors [Nielsen TO et al, 2004]. The ‘wound response’ signature genes associated with angiogenesis and matrix remodeling, has been shown to be linked with basal tumors, signifying other potential therapeutic targets for basal breast cancers [Chang HY et al, 2005].

![Intrinsic Subtypes](image)

**Fig. 1.3** Patient outcome based on breast tumor intrinsic subtypes and molecular subtypes.

All basic breast tumor intrinsic subtypes, their intrinsic nomenclature, featured IHC status as well as the association with clinical variables, i.e., tumor grade and patient outcome, are summarized in Table 1.1 and represented in Fig. 1.3.
Breast cancer related death is increasing throughout the world [Torre LA et al, 2015] including India [Sen U et al, 2002; Breastcancerindia.net. N.p., 2017]. As the cancer progresses, it starts spreading to secondary sites through a complex process termed as metastasis and metastasis has been established as a primary causal factor for decreasing overall survival among breast cancer patients [Jemal A et al, 2011]. Cross-talk between infiltrated immune cells and cancer cells determines metastasis [Cimino-Mathews A et al, 2015]. Heterotypic interactions between tumor cells and their microenvironment are mediated by chemokines [Zlotnik A et al, 2012]. Chemokines are small molecular-weight chemotactic cytokines, grouped into four subfamilies: CC, CXC, CX3C and C [Balkwill F, 2004]. Trafficking of immune cells into the primary tumor is mediated by secretion of chemokines from the tumor microenvironment (TME) [Kulbe H et al, 2004].

Inherent metastasis ability of breast cancer cells enhances mortality risk among the patients. The present clinical diagnostic and prognostic techniques/procedures are certainly insufficient to predict future metastasis of an early stage breast cancer patient.

1.3. Metastasis

New prognostic markers are critically expected to distinguish patients who are at high change of developing metastases, which may empower oncologists to start fitting treatment techniques to individual patients. Gene-expression profiling of primary breast tumors might be one way to identify the patients who are most likely to develop metastatic cancer and might also help to identify new therapeutic targets.

Enhancing our comprehension of the molecular mechanisms of the metastatic procedure may likewise improve clinical supervision of the disease. It has been widely accepted that, uncommon subpopulation of cells inside the primary tumor get favorable hereditary changes, which enable these cells to metastasize and shape new solid tumors at far off destinations [Fidler IJ and Kripke M, 1977; van ’t Veer LJ et al, 2002; van de Vijver MJ et al, 2002; Ramaswamy S et al, 2003]. The deoxyribonucleic acid (DNA)-microarray studies reported that metastatic primary breast tumors could be recognized by their gene-expression profile from those that stayed restricted and the metastatic capacity of ‘poor-prognosis’ breast tumors might be acquired by mutations at much prior phases of tumorigenesis than was expected [Bernards R and Weinberg RA, 2002].
Background of the study

1.3.1. Established prognostic markers of breast cancer metastasis

Metastasis to lymph nodes, increasing tumor size and loss of histopathological differentiation grade increases the risk of distant metastasis [Page DL, 1991; Koscielny S et al, 1984; Rosen PP et al, 1989; Carter CL et al, 1989; Elston CW and Ellis IO, 1991]. These are the conventional breast cancer prognostic markers. Interestingly, approximately 30% of women with LNM do not show metastasis even after 10 years of local therapy. Conversely, about one-third patients without any lymph node-dissemination show distant metastasis [Rosen PP et al, 1989; Hellman S, 1994]. Limited markers can forecast the secondary site of metastasis. Literature suggests ER+ breast tumors have a preference to metastasize to bone [James JJ et al, 2003] and lobular carcinomas have a predilection to recur in the gastrointestinal tract and ovaries [Borst MJ and Ingold JA, 1993; Arpino G et al, 2004].

Established prognostic markers are only able to assertively recognize the likely outcome of approximately 30% of patients. However, among the rest 70% patients, a considerable number develop metastatic breast cancer [McGuire WL and Clark GM, 1992]. Therefore, novel prognostic markers are desired to aid in indentifying high- and low-risk group of patients and treat them accordingly.

Considerable hard works have been done to discover other predictive markers that typify breast cancer patients who are at the uppermost possibility of metastasis progression. To fulfill the criteria to become an independent prognostic marker, retrospective studies in significantly large number of samples with an extensive follow-up and subsequent statistical analysis should be done in combination with conventional markers. Consequently, the results must be validated through independent research of different groups and should be confirmed by prospective study with the investigated marker [Hayes DF et al, 1996; Ransohoff DF, 2005].

Literature suggests a large number of putative molecular prognostic markers. However, very few of them have fulfilled the above mentioned requirements. Initial information showed that most of these markers are dependent of the conventional markers and usefulness is limited to only some sub-groups [McGuire WL and Clark GM, 1992].

A list of commonly used breast cancer prognostic markers is given in Table 1.2 [Page DL, 1991; Koscielny S et al, 1984; Rosen PP et al, 1989; Carter CL et al, 1989; Elston CW and Ellis IO, 1991; Pinder SE et al, 1994; de Mascarel I et al, 1998; Foekens JA et al, 2000;
Background of the study


Table 1.2. Breast cancer metastasis prognostic markers [Adopted from Weigelt B et al, 2005].

<table>
<thead>
<tr>
<th>Marker</th>
<th>Use in clinic</th>
<th>Metastatic determinants</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size</td>
<td>Established</td>
<td>Tumors &lt; 2 cm in diameter have a low threat of metastasis; tumors of 2–5 cm have higher metastasis chances; tumors &gt; 5 cm have a very high chance of metastasis</td>
<td>Independent prognostic marker</td>
</tr>
<tr>
<td>Axillary lymph- node status</td>
<td>Established</td>
<td>The risk of metastasis is low if LNM is negative; For LNM positive cases, the threat of metastasis is high. LNM &gt; 4 is associated with very high chances of metastasis</td>
<td>Related to tumor size</td>
</tr>
<tr>
<td>Histological grade</td>
<td>Established</td>
<td>Increasing Grade of tumors is associated with increasing risk of metastasis</td>
<td>Related to tumor size</td>
</tr>
<tr>
<td>Angioinvasion</td>
<td>Established in patients with lymph node-negative tumors</td>
<td>Infiltration of tumor cells in &gt; 3 blood vessels is coupled with metastasis</td>
<td>In patients with LNM negative tumors</td>
</tr>
<tr>
<td>uPA / PAI1 protein level</td>
<td>Newly established marker</td>
<td>High protein levels of urokinase plasminogen activator (uPA) and (Plasminogen activator inhibitor-1) PAI1 are associated with increased risk of metastasis</td>
<td>Independent prognostic marker</td>
</tr>
<tr>
<td>Steroid-receptor expression</td>
<td>Established for adjuvant therapy decision</td>
<td>Steroid-receptor levels are inversely proportional with metastasis</td>
<td>Short-term predictor of metastasis risk (5 years); related to histological grade</td>
</tr>
<tr>
<td>Her2 gene amplification and protein expression</td>
<td>Established for adjuvant therapy decision</td>
<td>Her2 (also known as ERBB2) amplification/overexpression is associated with metastasis</td>
<td>In patients with LNM positive tumors</td>
</tr>
<tr>
<td>Gene-expression profiling</td>
<td>Currently being tested</td>
<td>A set of ‘good signature’ genes is linked with low metastasis risk whereas, set of ‘poor signature’ genes is related with high metastasis risk</td>
<td>Tested in patients with LNM negative tumors</td>
</tr>
</tbody>
</table>
1.3.2. Mechanisms of Breast Cancer Metastasis

1.3.2.1. Metastatic cascade

Metastasis includes a sequence of steps (Fig. 1.4). Breakdown of either of these steps will seize the development of metastasis [Poste G and Fidler IJ, 1980]. Metastasis begins with the nearby intrusion of encompassing host tissue by primary tumor cells and proceeds until invasion and intravasation into blood or lymphatic vessels [Hunter KW et al, 2008; Talmadge JE and Fidler IJ, 2010]. Subsequently, tumor cells are then distributed to distant body parts via the blood stream or the lymphatic vessels. Accordingly, the tumor cells experience cell cycle arrest and adhere to capillary beds in the secondary organ. This is followed by extravasation into the parenchyma and increased angiogenesis within the organ [Hunter KW et al, 2008]. During the process of metastasis, tumor cells survive by concurrent escape from host’s immune system and apoptotic signals [Hunter KW et al, 2008; Fidler IJ et al, 1978]. Successful completion and repetition of these steps develop secondary metastases or ‘metastasis of metastases’ [Poste G and Fidler IJ, 1980; Talmadge JE and Fidler IJ, 2010].

1.3.2.2. Invasion

Invasion of tumor cells into the neighboring host tissue marks the start of metastasis. Initially inter-cellular adhesion and cell adhesion to the extracellular matrix (ECM) is altered. Literature suggests that change in expression of cadherin family proteins plays major role in breast cancer metastasis [Li DM and Feng YM et al, 2011] and downregulation of E-cadherin was shown to be a determinant in the outgrowth of metastatic breast cancer cells [Wendt MK et al, 2011] which further reflects breast cancer poor prognosis [Gould Rothberg BE and Bracken MB, 2006; Kowalski PJ et al, 2003]. Loss of function mutation in E-cadherin is observed in lobular breast carcinoma [Berx G et al, 1995].

Conversely, N-Cadherin is associated with epithelial-to-mesenchymal transition (EMT) [Kotb AM et al, 2011]. There is mounting evidence that EMT is associated with cancer development [Thiery JP, 2002; Guarino M et al, 2007]. EMT promotes tumor progression by inducing invasion and upregulating ECM-degrading proteases [Bonnomet A et al, 2010; Ota I et al, 2009]. Substitution of E-cadherin expression with N-cadherin induces fibrosis and cysts in mammary glands and ultimately led to malignant breast tumor in mice [Kotb AM et al, 2011]. Moreover, during stromal invasion, overexpression of N-cadherin and downregulation of E-cadherin is commonly observed in majority of epithelial cancers [Yilmaz M and...
Elevated N-cadherin expression favors the adhesion of tumor cells to stromal cells and consequently, the invasion of tumor cells into the stroma whereas, decreased E-cadherin expression is associated with adhesion of epithelial breast cancer cells with other epithelial cells [Cavallaro U and Christofori G, 2004]. Integrins mediate the adhesion of tumor cells to the ECM [Mego M et al, 2010]. Fibronectin, laminin, collagen, fibrinogen and vitronectin are commonly found integrins on ECM components [Li DM and Feng YM et al, 2011]. Prior to invasion, matrix metalloproteinases (MMPs) and the uPA system degrade ECM [Danø K et al, 2005; Egeblad M and Werb Z, 2002]. It has been reported that uPA may be used as prognostic marker for predicting the chance of breast cancer distant metastases [Harbeck N et al, 2004]. Small-interfering RNA (siRNA)-mediated inhibition of uPA reduces invasion and MMP9 expression [Huang HY et al, 2010].

Fig. 1.4. Schematic representation of the mechanism of metastasis.

MMPs function through proteolysis of ECM [Kelly T et al, 1998]. Integrins regulate the activity of ECM-degrading MMPs [Li DM and Feng YM et al, 2011]. It has been reported
Background of the study

that, both integrins \( \alpha_5\beta_1 \) and \( \alpha_3\beta_1 \) upregulate MMP9 [Mitchell K et al., 2010; Rolli M et al., 2003]. In addition, heparanase also aids in the ECM-degradation by breaking down heparan sulfate proteoglycan [Götte M and Yip GW, 2006], and thus, the expression of heparanase correspondingly increase the metastatic potential in breast cancer [Maxhimer JB et al., 2002]. Elevated expression of heparanase is positively associated with increased cell proliferation, survival, and stromal infiltration of breast cancer cells \textit{in vitro} and \textit{in vivo} [Cohen I et al., 2006].

1.3.2.3. Migration and motility

Tumor cells should move from the confined primary site to attain an invasive phenotype. Migration of tumor cells may be solitary or in a coordinated fashion depending on how the cancer cells are differentiated [McSherry EA et al., 2007]. Tumor cells of intermediate or highly differentiated lobular carcinomas migrate coordinately [Bell CD and Waizbard E, 1986]. Conversely, in poorly differentiated tumors, tumor cells carry out single-cell migration because of insufficient intercellular adhesions and lack of intercellular junctions [McSherry EA et al., 2007]. During coordinated migration, cells at the leading edge reorient collagen fibers to form microstacks using membrane type 1 (MT1) MMP [Friedl P and Wolf K, 2008; Wolf K et al., 2007]. Conversely, singular movement occurs in two ways, either by mesenchymal movement or amoeboid movement. Mesenchymal movement is protease-dependent and amoeboid movement is protease-independent [McSherry EA et al., 2007].

The EMT process plays a significant role in the mesenchymal movement of single migratory cells. During EMT, cells go through phenotypic changes from an epithelial to a mesenchymal one [Yilmaz M and Christofori G, 2010]. The process starts with the breakdown of intercellular adhesion after the downregulation of epithelial markers, like E-cadherin, and upregulation of mesenchymal markers, like vimentin. The major regulatory molecules of EMT include transcriptional repressors of E-cadherin such as ZEB1, ZEB2, Twist, Snail, Slug etc. The main signaling pathways which influence the process of EMT are transforming growth factor beta (TGF-\( \beta \))-signaling, Wnt-signaling, PI3K/Akt signaling [Batlle E et al., 2000; Comijn J et al., 2001; Eger A et al., 2005; Hajra KM et al., 2002; Yang J et al., 2004; Larue L and Bellacosa A, 2005; Blick T et al., 2008]. Following the loss of adhesion, cellular polarity changes from apical-basal to front-rear. This commences cellular migration through cytoskeleton remodeling by changing cortical actin and actin stress fibers. Finally, activation of MMPs and change in matrix adhesion takes place [Iwatsuki M et al., 2010]. EMT-
experienced cells have an extended fibroblast-like shape and they follow facilitated migration through ECM-channels produced by MMPs [Friedl P and Wolf K, 2010]. Conversely, amoeboid movement through pores in the matrix depends mostly on shape deformations and structural changes in the ECM [Provenzano PP et al, 2008; Mierke CT et al, 2008; Rösel D et al, 2008; Wyckoff JB et al, 2006], rather than MMP-mediated degradation of the matrix [Yilmaz M and Christofori G, 2010; McSherry EA et al, 2007]. Active myosin/actin contractions and signaling pathways such as RhoA/Rho kinase generate the mechanical force used [Yilmaz M and Christofori G, 2010; Friedl P and Wolf K, 2010]. That tumor cells primarily employ mesenchymal motility [McSherry EA et al, 2007], however, in specific situations, modifications in the molecular pathways deciding either mode could bring about a switch in the migration mode, in either ways named mesenchymal-to-amoeboid transition (MAT), or amoeboid-to-mesenchymal transition (AMT) [Panková K et al, 2010]. Inhibition of pro-amoeboid pathways such as PI3K-signaling and cell division control protein 42 homolog (CDC42)-mediated signaling cause AMT. Similarly, inhibition of ‘mesenchymal movement-promoters’ such as ras-related C3 botulinum toxin substrate (Rac) and SMAD-specific E3 ubiquitin protein ligase 1 (Smurf1) cause MAT [Panková K et al, 2010]. At the tumor ECM boundary, the spatial arrangement of encompassing collagen filaments additionally assumes a part in deciding the mode utilized by migrating cells [Provenzano PP et al, 2008].

Stromal cells play significant role in favor of migration of tumor cells. Inside the breast tumor, the predominant stromal cells are fibroblasts, and are called as carcinoma-associated fibroblasts (CAFs) [Mego M et al, 2010; Micke P and Ostman A, 2005]. CAFs-conditioned medium facilitates breast cancer cell migration and invasion in vitro [Heylen N et al, 1998] and nude mice injected with a combination of human CAFs and MCF7-ras human breast cancer cell line demonstrate significantly increased breast tumor growth and angiogenesis when compared to normal human fibroblasts-injected mice [Orimo A et al, 2005].

1.4. Tumor microenvironment

Our knowledge about the TME is increasing for the last few years. It is a very complex system consisting of different cell types [Quail DF and Joyce JA, 2013; Place AE et al, 2011; Albini A and Sporn MB, 2007]. Among the many cells, the most important cells are of immune system e.g. neutrophils, T lymphocytes, B lymphocytes, natural killer (NK) cells, macrophages, dendritic cells (DCs) etc [Balkwill F and Mantovani A, 2001, DeNardo DG et
al, 2011]. Besides immune cells, other important cell types which regulate tumor progression include endothelial cells and fibroblasts [Albini A and Sporn MB, 2007; Kalluri R and Zeisberg M, 2006]. As a whole, to understand the complete process of carcinogenesis and ensuing metastasis, a comprehensive understanding of the TME is as important as of the tumor epithelial cells [Farber E and Rubin H, 1991; Clark WH Jr, 1995; Sporn MB, 1996]. To combat cancer in the future, we need to consider the disease as a complex tissue response rather, individual cell type and therefore, the TME turn out to be an fundamental and indispensable part of the disease.

Interaction involving cancer cells and their microenvironment is vital for tumor growth as well as normal tissue homeostasis. Precisely, the cross-talk between tumor cells and associated stroma persuade the initiation and development of cancer [Howlett A and Bissell MJ, 1993; Joyce JA and Pollard JW, 2009] Earlier it was considered that cancer is a heterogeneous disease comprised of aberrant mutations in tumor cells but now it is clear that tumors are also different by their hetero-cellular complexity and their activation states [Hanahan D and Coussens LM, 2012; Hanahan D and Weinberg RA, 2011]. The TME repeatedly alters during the period of cancer progression. This emphasizes that TME regulates metastasis through a dynamic process and therefore, tumor cells themselves develop the fate of the disease.

1.4.1. Leukocyte infiltration in the TME

Tumor infiltrating leukocyte (TIL)-trafficking in breast TME is currently under intense investigation as its mechanism remains largely unknown [Balkwill F and Mantovani A, 2001; DeNardo DG et al, 2011]. Some breast tumors have substantial leukocytic infiltration and their interaction with breast tumor cells appears to be associated with disease progression (Fig. 1.5), however, the mechanisms are poorly understood.

The most diverse cell populations within the breast tumor are the immune cells [Coussens LM and Werb Z, 2002; McDaniel SM et al, 2006]. Physiologic wound healing necessitates balanced immune responses favoring re-epithelialization and new tissue formation [Coussens LM and Werb Z, 2002; de Visser KE et al, 2006]. In focal myoepithelial cell layer-disrupted ductal carcinoma in situ (DCIS), an elevated percentage of TILs are present which is suggestive of their involvement in invasive progression [Man YG and Sang QX, 2004].
Multiple reports have shown that tumor-associated macrophages (TAMs) facilitate tumor invasion and favor inter-cellular paracrine signaling between tumor cells [DeNardo DG et al, 2009; Schedin P et al, 2007]. Macrophage deficiency does not affect tumor initiation but reduces the rate of malignant progression [Lin EY et al, 2001]. MCF7-injected xenograft in colony stimulating factor 1 (CSF-1)-silenced mice have shown significantly decreased macrophage infiltration, MMPs production, vascular endothelial growth factor (VEGF)-A secretion, and endothelial cell proliferation [Aharinejad S et al, 2004]. Functional studies in mice have indicated the potential role of TAMs in breast cancer progression and their association with poor outcome. In addition to macrophages, other immune cells have also been implicated in breast cancer development. Massive infiltration of CD8+ cytotoxic T-cells (Tc cells) is positively associated with good prognosis, patient survival [Mahmoud SM et al, 2011; Liu S et al, 2014] and response to therapy [Seo AN et al, 2013] whereas, role of CD4+ regulatory T-cell (Treg) infiltration is still debated [Bates GJ et al, 2006; Nnadi D et al, 2014; West NR et al, 2013]. Among the other CD4+ T-cell subpopulations, type 1 helper T (Th1) cells are associated with favorable clinical outcomes [Gu-Trantien C et al, 2013], whereas...
type 2 helper T (Th2) cells have been reported to be associated with protumor anti-inflammatory response [Teschendorff AE et al, 2010]. T helper 17 (Th17) cells produce pro-inflammatory cytokine interleukin (IL) 17, but their effect depends on the associated cytokine milieu [Qi W et al, 2013]. Another CD4+ subset, T-follicular helper (Tfh) cells, has been found to be positively associated with patient outcome [Gu-Trantien C et al, 2013]. Role of infiltrating B-cells is controversial [Mahmoud SM et al, 2012a]. Infiltrated leukocytes are sometimes suppressed by tumor cells, may be through direct suppression of antitumor immune cells or recruitment and reactivation of immunosuppressive subsets [Dushyanthen S et al, 2015]. Tumor cells expressing PD-L1, interacts with PD-1+ CD8+ T cells and induces an unresponsive state or apoptosis, which leads to inactivation or exhaustion of TILs [Dushyanthen S et al, 2015]. This process invites a decreased antitumor immunity [Pardoll DM, 2012]. The type of lymphocyte infiltration and differentiation depends on the chemokines and cytokines secreted from cancer cells and/or tumor stroma [Thelen M and Stein JV, 2008; Masopust D and Schenkel JM, 2013]. Naïve T-cells are differentiated to Th1 by IL2; Th2 by IL4; Th17 by IL1β/TGF-β, IL6, IL23; Treg cells by TGF-β [Hsieh CS et al, 1993; Swain SL et al, 1990; Nurieva R et al, 2007; Zhou L et al, 2007; Mangan PR et al, 2006; Schramm C et al, 2004]

1.4.2. The microenvironment of metastases

It has been largely studied that the cross-talk between tumor epithelial cells and stromal cells regulates the metastatic microenvironment within the breast tumor, during breast cancer progression. Chemokines and cytokines secreted by the secondary site and/or the primary tumor create a favorable environment which determines metastasis.

In MDA-MB-231-induced xenograft mice model, primary tumors promote recruitment and growth of bone marrow-derived cells to secondary sites in favor of metastasis via expression of osteopontin which further demonstrates the systemic influence of primary tumor [McAllister SS et al, 2008]. However, the mechanism is still poorly understood. The macrophages (F4/80+ CSF-1R+CD11b+Gr1-CX3CR1high CCR2high VEGFR1high) recruited at metastatic breast cancer cells at secondary sites are different from typical macrophages and it has also been observed that primary tumor-induced macrophages secrete MMP9 and VEGF and thereby stimulate metastasis of tail-vain injected tumor cells [Qian B et al, 2009; Hiratsuka S et al, 2002].
In human breast cancer cells, the receptor activator of nuclear factor-κB (RANK) is greatly expressed and associated with metastasis, however, its’ source is still not clearly understood. In a murine model, it has been found that RANK ligand (RANKL) expression is coupled with increased metastasis to lungs [Tan W et al, 2011].

Genetic alterations and epigenetic modifications are responsible for the superior survivability of tumor-associated stromal cells during prolonged-passage in cell culture [Orimo A et al, 2005; Qiu W et al, 2008]. Considerable changes in methylation pattern in different cell types within DCIS, infiltrating ductal carcinoma (IDC) and healthy breast tissues were observed and reported [Hu M et al, 2005]. This supports the hypothesis of epigenetic regulation of TME during breast cancer progression.

1.4.3. Involvement of TAMs in breast cancer metastasis

Macrophages are immune cells differentiated from monocytes of circulation or from embryonic precursors [Murray PJ et al, 2014]. In response to different environmental stimuli, marcophages perform unique functions depending upon different polarization and activation [Mantovani A and Sica A, 2010]. Macrophages are polarized into either classically activated M1 type or alternatively activated M2 type cells and includes a number of transitional categories [Mosser DM and Edwards JP, 2008; Ambarus CA et al, 2012]. Additionally, human M2 macrophages can be divided in three groups: M2a, M2b and M2c. M2c most immunosuppressive macrophage type (Fig. 1.6).

TAMs are a vital part of the TME. About 5–40 % of the primary breast tumor mass consists of TAMs [Mahmoud SM et al, 2012].They can be either of the two extremes of M1 and M2-like macrophages. M1 macrophages induce anti-tumor responses whereas, M2 macrophages are associated with pro-tumor response. However, within the breast tumor, TAMs are primarily of M2 type [Gabrilovich DI et al, 2012; Noy R and Pollard JW, 2014; De Palma M and Lewis CE, 2013; Mantovani A and Allavena P, 2015]. A decrease in the M1/M2 ratio in the primary tumor is coupled with poor prognosis of breast cancer [Bingle L et al, 2002].

Literature suggests that TAMs play significant roles in different steps of metastasis viz. invasion, intravasation, extravasation, angiogenesis, ECM remodeling and homing to the secondary sites [Valastyan S and Weinberg RA, 2011; Condeelis J and Pollard JW, 2006].
M1 macrophages secrete pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-6 and induce activation of the anti-tumor adaptive immune system. Conversely, M2 macrophages secrete TGF-β, IL23, IL10, VEGF etc. that induce pro-tumor anti-inflammatory responses and also favor angiogenesis and stromal breakdown (Fig. 1.6) [Mantovani A et al, 2002; Santoni M et al, 2013; Allavena P et al, 2008; Sica A et al, 2008; Mantovani A et al, 2005; Eriksson F et al, 2009]. In many cancer, intra-tumor trafficking of macrophage is correlated with poor prognosis, including breast cancer [Qian BZ and Pollard JW, 2010; Zhang QW et al, 2012].

Macrophage polarization is similar and related to Th1-Th2 polarization. In a Th1-secreted cytokine milieu monocytes differentiate into classical M1 macrophages. On the contrary, Th2-secreted cytokines favor M2-polarization [Van Ginderachter JA et al, 2006]. Polarized M1 and M2 cells possibly correspond to the extremes of macrophage phenotypes [Sica A et al, 2008]. New classification method explains macrophage populations in terms of cytokine production and function they exert. It includes classically activated macrophages that produce high levels of proinflammatory cytokines, woundhealing macrophages differentiate in IL-4 environment and rebuild ECM and regulatory macrophages that produce high level of IL-10 [Mosser DM and Edwards JP, 2008]. TAMs of breast cancer share features of both wound-
healing and regulatory macrophages. Within the primary breast tumor the percentage of myeloid-derived suppressor cells (MDSCs) increases proportionally with increase in percentage of M2-polarized macrophages, which in turn inhibits Tc cell responses [Sinha P et al, 2005; Sinha P et al, 2007].

**Table 1.3.** Comparison of the features and functions of M1 and M2 macrophages.

<table>
<thead>
<tr>
<th>Functions</th>
<th>M1 macrophage</th>
<th>M2 macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activate adaptive immune response through antigen presentation on upregulated MHC class II molecules</td>
<td>Downregulate adaptive immune response through downregulation of MHC class II molecules</td>
<td></td>
</tr>
<tr>
<td>Produce reactive oxygen intermediates and nitric oxide for microbial killing</td>
<td>Increase tumor invasion through production of EGF</td>
<td></td>
</tr>
<tr>
<td>Produce soluble VEGF receptor</td>
<td>Produce MMPs</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Features</th>
<th>M1 macrophage</th>
<th>M2 macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decreased IL10</td>
<td></td>
<td>Elevated IL10</td>
</tr>
<tr>
<td>Elevated IL23</td>
<td></td>
<td>Elevated TGF-β</td>
</tr>
<tr>
<td>Elevated TNF</td>
<td></td>
<td>Elevated IL17</td>
</tr>
<tr>
<td>Elevated IL12</td>
<td></td>
<td>Decreased IL12</td>
</tr>
<tr>
<td>Decreased IL4</td>
<td></td>
<td>Activated Stat3</td>
</tr>
<tr>
<td>Elevated 6</td>
<td></td>
<td>Elevated CD206, CD163</td>
</tr>
</tbody>
</table>

M1 and M2 macrophages can be differentiated and identified from a heterogeneous cell population by their characteristic surface markers (Fig. 1.6). M2 macrophages exclusively express CD163 and CD206 and using these markers M2 macrophages can be quantitatively measured [Shabo I et al, 2008; Satoh T et al, 2010; Zaynagetdinov R et al, 2011]. On the other hand, M1 macrophages have elevated class-II-major histocompatibility complex (MHC) expression [Ma J et al, 2010; Lugo-Villarino G et al, 2011]. Table 1.3 includes major functions and characteristics of M1 and M2 macrophages [Mukhtar RA et al, 2011].

**1.5. Chemokines and breast cancer**

Chemokines are chemotactic cytokines of very small molecular weight, participate in the critical steps of tumor growth, cancer progression and metastases [Raman D et al, 2011; Sarvaiya PJ et al, 2013]. Chemokines function primarily in regulating immune response and inflammation by controlling leukocyte trafficking into the primary tumor. The binding of the chemokines to their specific transmembrane G-protein-coupled receptors (GPCRs) induces a
conformational change followed by activation of signaling pathways [Raman D et al, 2011; Zlotnik A and Yoshie O, 2000; Murphy PM et al, 2000]. Chemokines and their receptors are subgrouped into CXC, CC, CX3C and C, according to the pattern of their N-terminal cysteine residues [Figure 1.7]. ‘X’ symbolizes non-cysteine amino acids and ‘C’ stands for the cysteine [Zlotnik A and Yoshie O, 2000; Murphy PM et al, 2000]. There are almost 50 different human chemokine ligands and 20 receptors identified [Table 1.4, Fig.1.8]. Functional characterization divides chemokines in major two categories: homeostatic chemokines and inflammatory chemokines [Fig. 1.8], Homeostatic chemokines have constitutive expression whereas, expression of inflammatory chemokines is induced by inflammation [Zlotnik A and Yoshie O, 2000; Murphy PM et al, 2000].

Table 1.4. List of chemokine receptors and their cognate ligands.

<table>
<thead>
<tr>
<th>Chemokine receptors</th>
<th>Specific chemokine ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1</td>
<td>CCL3, CCL4, CCL5, CCL7, CCL14, CCL15, CCL16, CCL23</td>
</tr>
<tr>
<td>CCR2</td>
<td>CCL2, CCL3, CCL7, CCL8, CCL12, CCL13</td>
</tr>
<tr>
<td>CCR3</td>
<td>CCL5, CCL7, CCL11, CCL13, CCL15, CCL24, CCL26, CCL28</td>
</tr>
<tr>
<td>CCR4</td>
<td>CCL2, CCL3, CCL5, CCL17, CCL22</td>
</tr>
<tr>
<td>CCR5</td>
<td>CCL3, CCL4, CCL5, CCL8</td>
</tr>
<tr>
<td>CCR6</td>
<td>CCL20</td>
</tr>
<tr>
<td>CCR7</td>
<td>CCL19, CCL21</td>
</tr>
<tr>
<td>CCR8</td>
<td>CCL1, CCL4, CCL17</td>
</tr>
<tr>
<td>CCR9</td>
<td>CCL25</td>
</tr>
<tr>
<td>CCR10</td>
<td>CCL27, CCL28</td>
</tr>
<tr>
<td>CCR11</td>
<td>CCL2, CCL7, CCL8, CCL12, CCL13, CCL19, CCL21, CCL25</td>
</tr>
<tr>
<td>CXCR1</td>
<td>CXCL6, CXCL8</td>
</tr>
<tr>
<td>CXCR2</td>
<td>CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8</td>
</tr>
<tr>
<td>CXCR3</td>
<td>CXCL4, CXCL9, CXCL10, CXCL11</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXCL12</td>
</tr>
<tr>
<td>CXCR5</td>
<td>CXCL13</td>
</tr>
<tr>
<td>CXCR6</td>
<td>CXCL16</td>
</tr>
<tr>
<td>CXCR7</td>
<td>CXCL11, CXCL12</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>CXC3CL1</td>
</tr>
</tbody>
</table>
Some set of chemokines are secreted at low levels by the normal breast epithelial cells. Interestingly, human milk contains CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8 [Maheshwari A et al, 2003; Basolo F et al, 1993]. Additionally, CXCL8 is also detected in the culture medium of primary culture of normal breast epithelial cells [Basolo F et al, 1993]. Chemokines are found at differential levels in cancer tissues and normal tissues and sometimes, a number of them are significantly secreted from tumor but missing in normal breast e.g., CXCL1, CXCL2, CXCL5, CXCL6, CXCL8, CXCL20, CX3CL1, CCL2 and CCL7 [Porter DA et al, 2001].

CXCL8 exerts significant function in multiple cancer types [Xie K, 2001]. In breast cancer, CXCL8 is overexpressed in the primary tumor tissues relative to healthy tissues [Greene GF et al, 1997; Chavey C et al, 2007; Bièche I et al, 2007] and in the peripheral blood [Kozlowski L et al, 2003; Benoy IH et al, 2004]. CCL5 is primarily expressed in breast tumor epithelial cells and hardly found in normal breast tissues [Luboshits G et al, 1999; Yaal-Hahoshen N et al, 2006]. Moreover, CCL5 levels in peripheral blood and metastatic sites is also elevated and positively correlated with grade of breast tumors [Niwa Y et al,
Background of the study

Not only chemokine ligands, but some chemokine receptors are also differentially expressed during breast cancers. For example, receptor of CXCL12 i.e., CXCR4 and the common receptor of CCL19/CCL21, CCR7 are overexpressed during breast cancer [Müller A et al, 2001; Scotton CJ et al, 2001].

![Diagram of chemokine ligands and their receptors](image)

**Fig. 1.8.** Chemokine ligands and their receptors. (a) List of chemokine ligand-receptor pairs grouped into inflammatory and homeostatic chemokines. (b) Chemokine mediated cellular trafficking, network and cross-talk during breast cancer. TILs produce an array of additional chemokines that can amplify or control immune-mediated effects.

CCL21 is largely expressed in lymph nodes, suggestive of breast tumor cell migration to the lymph node [Müller A et al, 2001]. Most importantly, CXCL12 is expressed highly in lymph nodes, bone marrow, liver and lung. This indicates a higher probability of migration and
metastasis of CXCR4-positive breast cancer cells to the lymph nodes and distant secondary sites [Zlotnik A et al., 2006; Müller A et al., 2001; Zlotnik A, 2006; Zlotnik A, 2006a]. Expression of a chemokine receptor does not necessarily mean that the cell will become responsive to the specific ligand chemokine of the receptor. In fact, only in metastatic breast cancer cell lines, CXCL12-stimulation is capable to turn on intracellular signaling though most of the breast cancer cell lines have a substantial CXCR4 protein level [Holland JD et al., 2006].

1.5.1. Function of chemokines in breast tumor growth

In addition to build pro-tumor environment, chemokines induce growth and proliferation of tumor cells. Among many, CCL2 or monocyte chemoattractant protein-1 (MCP-1) is an important chemokine involved in tumor growth and progression. It controls the trafficking of monocytes, memory T lymphocytes, and NK cells [Deshmane SL et al., 2009]. CCL2 is highly secreted by cells of the TME and resulted in increased angiogenesis and trafficking of leukocytes [Soria G and Ben-Baruch A, 2008; Ben-Baruch A, 2006]. CCL2 expression increases the percentage of TAMs, followed by increased tumor growth. Furthermore, elevated amount of CCL2 is secreted by CAFs upon their co-culture with cancer cells [Tsuyada A et al., 2012]. The promotion of chemo/radio-resistant cells with CSC phenotype facilitates to uphold the tumor heterogeneity and ensuing metastasis. Another chemokine associated with breast cancer development is CCL20. It mainly attracts lymphocytes and DCs [Palacios-Areola MI et al., 2014]. It has been reported that elevated CCL20 is coupled with higher cyclin E and lower p27, which are the signatures of cellular proliferation [Marsigliante S et al., 2013]. CXCL8 is also found to promote cell division and knockdown of CXCL8 upregulates p27 and downregulates cyclinD1, resulted in G1-S arrested cell division and delayed cellular proliferation [Shao N et al., 2013]. Importantly, it has been reported that CXCL8 increase the activity and renewal of breast CSCs by direct binding to CXCR1 on their surface [Singh JK et al., 2013]. Besides from its well-known involvement in metastasis, CXCR4 expression is found to be associated with increased tumor growth [Rhodes LV et al., 2011]. On the contrary, breast tumor tissues express significantly decreased CXCL14 as compared to normal breast tissues. Moreover, CXCL14 inhibits breast tumor growth and metastasis both in vitro and in vivo [Gu XL et al., 2012].
1.5.2. Chemokines and TME

The complexity of the TME is achieved through trafficking of non-cancer cells into and from the tumor. Particularly macrophage-recruitment is utmost important. Infiltrated macrophages acquire a protumor M2 phenotype induced by the surroundings cytokine and growth factors. The resulting TAMs help ECM remodeling and cancer cell migration.

Chemokines regulate infiltration of leukocytes into the TME. The two chemokines that are mostly studied in breast cancer in recent years are CCL2 and CCL5. CCL2 attract monocytes and macrophages and CCL5 recruits T cells and eosinophils [Rot A and von Andrian UH, 2004; Soria G and Ben-Baruch A, 2008]. Both CCL2 and CCL5 are found at very low levels at normal breast and show similar patterns of elevated expression in tumor tissues, reactive lymph nodes, fibroblasts and secondary sites [Soria G and Ben-Baruch A, 2008, Ben-Baruch A, 2006]. Trafficking of DCs is regulated by CCL19, CCL20, and CCL21 [Ben-Baruch A, 2006]. Role of DCs in breast cancer is still debated though, elevated expression of CCL19, CCL20, CCL21 is positively associated with DCs infiltration [Ben-Baruch A, 2006].

Regarding T cell and NK cell trafficking, migration of activated T cells and NK cells are regulated by CXCL9 and CXCL10 [Neville LF et al, 1997; Farber JM, 1997]. Moreover, it has been reported that CXCL12 favor cytotoxic activities of CD8+ Tc cells [Williams SA et al, 2010]. Receptor of CX3CL1 i.e., CX3CR1 is expressed on the surface of monocytes, NK cells, and T cells and literature suggests that CX3CL1 support proliferation and migration of these cells [White GE and Greaves DR, 2012]. Correspondingly, CX3CL1 expression is positively associated with stromal Tc cells, NK cells and intratumoral DCs [Park MH et al, 2012] and therefore, elevated CX3CL1 level predict better prognosis for breast cancer patients.

CCL22 attracts mainly CCR4+ Th2 cells and also some monocytes, DCs and NK cells. CCL22 promote Treg infiltration in multiple cancer types [Mizukami Y et al, 2008; Maruyama T et al, 2010; Curiel TJ et al, 2004]. Correspondingly, in breast tumors that are short of CCL22 expression does not show considerable Treg accumulation [Faget J et al, 2011].
1.5.3. Chemokines and angiogenesis during breast cancer

Angiogenesis is a typical feature of cancer and marks the increased nutrient and oxygen requirement of the growing tumor cells [Hanahan D and Weinberg RA, 2011]. Angiogenic CXC chemokines CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8 mediate their activity through CXCR2 and function alone or with other angiogenic factors like VEGF [Strieter RM et al, 2006]. CXCL8 is a strong angiogenic inducer [Ben-Baruch A, 2006; Strieter RM et al, 2006] and it has been reported that estrogen upregulates CXCL8 expression during breast cancer [Haim K et al, 2011]. CXCL12 has angiogenic activity and found to be involved in neovascularization, endothelial cell migration and enhancing VEGF release [Ben-Baruch A, 2006]. On the other hand, angiostatic CXC chemokines CXCL4, CXCL9, CXCL10, CXCL11, and CXCL14 signals through CXCR3 [Sarvaiya PJ et al, 2013]. CXCL4, CXCL9, and CXCL10 inhibit proliferation and migration of microvascular endothelial cells [Strieter RM et al, 2006].

CCL2 and CCL5 induce neovascularization within the TME. CCL2 supports angiogenesis by directly acting on endothelial cells and CCL2 expression is correlated with VEGF, CXCL8 level [Ueno T et al, 2000]. TAMs, accumulated within the TME through CCL2-mediated chemoattraction, produce other angiogenic factors [Soria G and Ben-Baruch A]. Within the tumors, new lymphatic vessel formation, known as lymphangiogenesis, occurs simultaneously with formation of new blood vessels. Lymphangiogenesis contribute to lymphatic metastasis [Zhuo W et al, 2012] and CXCL12 induces lymphangiogenesis in vivo in cancer tissues [Zhuo W et al, 2012].

1.5.4. Chemokines in breast cancer metastasis

The most widely studied chemokine axis in metastasis is the CXCL12-CXCR4 chemokine axis. Low-CXCR4-expressing MCF7 cells remain unsuccessful to metastasize whereas CXCR4-high MDA-MB-231 cells develop distant metastases when injected into mice [Dewan MZ et al, 2006]. Likewise, CCL21 triggers migration and invasion of CCR7+ breast cancer cells, particularly to lymph nodes, where CCL21 expression is high [Müller A et al, 2001]. Significantly elevated CXCR4 level in malignant breast tumor tissues [Müller A et al, 2001] promote chemotaxis of cancer cells toward secondary sites such as lung, bone, liver, and lymph nodes where considerable CXCL12 secretion is observed [Mukherjee D and Zhao J, 2013; Kucia M et al, 2005]. It has been reported that expression of CXCR4 is relatively
higher and more frequent in TNBC samples than that of non-TNBCs and is coupled with a superior rate of metastasis [Chen HW et al, 2013].

Metastasis to bone is favored by the chemokines secreted by osteoblasts and bone marrow endothelial cells. Differentiated osteoblasts secrete CCL2 and attract cancer cells with CCR2 [Molloy AP et al, 2009]. CX3CL1 is expressed by human bone marrow endothelial cells and thereby, attracts breast tumor cells with CX3CR1 [Jamieson-Gladney WL et al, 2011].

Most interestingly, binding of CXCL12 with CXCR7 has different impact compared to the usual binding of CXCL12 with CXCR4 [Hernandez L et al, 2011]. CXCR12-CXCR4 interaction induces chemotaxis and invasion of breast cancer cells, whereas, CXCL12-CXCR7 interaction decreases invasion and metastasis rather, increases primary tumor growth and angiogenesis [Hernandez L et al, 2011]. Some chemokines and receptors, such as CCL2, CCL5, CCL20, CXCL12 and CXCR7 positively regulate expression of MMPs [Soria G and Ben-Baruch A, Ben-Baruch A, 2006; Marsigliante S et al, 2013; Hernandez L et al, 2011]. CCL18 increases the invasiveness of tumor cells and it has been found that within the TME, CCL18 is secreted sometimes by TAMs [Yu M et al, 2010].

1.5.5. CXCL13 and cancer

CXCL13 (BLC or BCA1) is an important chemokine, classically known to be involved in trafficking of B lymphocytes [Gunn MD et al, 1998; Legler DF et al, 1998]. B cells express CXCR5, the receptor of CXCL13 and thereby attracted towards the site of CXCL13 expression [Jenh CH et al, 2001]. Recent understanding of CXCL13-CXCR5 signaling in the regulation of cancer progression including breast cancer has made this signaling utmost important [Panse J et al, 2008; Meijer J et al, 2006; Bürkle A et al, 2007; Airoldi I et al, 2008; El Haibi CP et al, 2010; Zeng J et al, 2013].

It has been reported that binding of CXCL13 with CXCR5 induces intracellular signaling involving PI3Kp110, Src and FAK (Figure 1.9) [El Haibi CP et al, 2010]. CXCL13 signaling could promote invasion, migration and expression of MMPs during prostate cancer progression [El Haibi CP et al, 2010]. Interestingly, literature has suggested that CXCL13 is overexpressed in primary breast tumor tissues [Panse J et al, 2008] Moreover, serum level of CXCL13 is significantly higher in patients diagnosed with metastatic breast cancer [Panse J et al, 2008]. Additionally, it has also been reported that transcript levels of CXCL13 and
CXCR5 is relatively higher in LNM positive tumors and of higher stage [Razmkhah M et al, 2012].

**Fig. 1.9.** Schematic representation of CXCR5-CXCL13 intracellular signaling pathways.

Apart from B lymphocytes, some other immune cells express CXCR5 on their surface. Therefore, secretion of CXCL13 from tumor tissues could promote infiltration of those CXCR5+ cells into the TME. Approximately 20-25 % of central memory T cells (Tcm) in the peripheral blood express CXCR5 [Schaerli P et al, 2000; Kim CH et al, 2001]. Elevated CXCR5 expression is also a key feature of CD4+ Tfh cells. Tfh cells are mainly confined in B cell follicles and germinal centers of secondary lymphoid organs [Vinuesa CG et al, 2005; King C, 2009; Yu D et al, 2009]. Tfh cells also express high levels of CXCL13 [Vinuesa CG et al]. The constitutive CXCR5 expression by Tfh and a part of Tcm cells imply their role in formation of germinal centers [Rivino L et al, 2004]. However, the ultimate function of these cells is still not clearly understood. It has been reported that infiltration of Tfh cells into the breast tumor is associated with good prognosis and survival of breast cancer patients [Gu-Trantien C et al, 2013]. It helps in the formation of tertiary lymphoid structures which could induce effective anti-tumor response [Gu-Trantien C et al, 2013].
1.6. Statement of the problem and scope

Breast cancer is a massive threat towards womanhood worldwide and more than 30% of breast cancer deaths occur due to metastasis and recurrence. Inherent ability of breast cancer cells towards metastasis enhances mortality risk among patients. Metastasis is the result of several sequential steps and represents a highly organized, non-random and organ-selective process.

Although a number of molecules have been implicated in the metastasis of breast cancer, the precise mechanisms determining the directional migration and invasion of tumor cells into specific organs remain to be established. Currently, we have only few routine diagnostic markers such as ER/PR and Her2/Neu, which are being evaluated to choose chemotherapeutic drugs. Apart from this, some specific biomarkers are available with significant prognosis value for prediction of disease progression or tumor fate.

It is evident from research outcomes that metastases-associated deaths are predominant in breast cancer. Recent developments on early diagnosis using mammographic screening and the implementation of adjuvant therapies may have reduced breast cancer associated deaths in decent numbers, although new markers for prognosis are of utmost importance for patients with higher risk of developing metastases or recurrence. Targeting either specific immunomodulators and/or intervening molecular mechanisms is thought to be a potential therapeutic option. As a part of immune response, both pro-tumor and anti-tumor immune cell subsets infiltrate to the lymphoid organ or tumor microenvironment through disease stages. Thus, the degree of infiltration and recruitment of different immune cell subsets as well as the in between cross-talks become the primary determining factor for tumor progression.

Chemokines are the master regulator of the immune cell trafficking and can be targeted to reduce the intra-tumoral regulatory T cells (Treg) for anti-tumor immunity. Henceforth, we proposed the study for a comprehensive prediction of the tumor fate as well as to explore possible markers for breast cancer prognosis and future chemotherapy with more precision.
1.7. Objectives of the study

1.7.1. Objectives of Chapter-I

CXCL13-CXCR5 co-expression regulates epithelial to mesenchymal transition of breast cancer cells during lymph node metastasis

a. Study the relevance of expressions of CXCL13 and its receptor CXCR5 in breast cancer patients from eastern India
b. Investigate the potential of CXCL13-CXCR5 signaling in EMT and migration of breast cancer cells
c. To decipher the intra cellular CXCL13-CXCR5 signaling pathway

1.7.2. Objectives of Chapter-II

Regulation of cxcl13 and cxcr5 gene transcription during breast cancer

a. To investigate the potential of RelA and Nrf2 to regulate cxcl13 transcription
b. Regulation of cxcr5 transcription beyond RelA-mediated positive expression

1.7.3. Objectives of Chapter-III

CCL17 and CCL22 determine Th2-dependent M2-macrophage polarization and associated CXCL13 secretion within breast tumor microenvironment

a. To identify the particular cell types that are the major source of intra-tumoral CXCL13 within the primary breast tumor
b. How the trafficking of CXCL13-secreting cells are being regulated by intra-tumoral chemokines and/or cytokines