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Chemokines in triple-negative breast cancer heterogeneity: New challenges for clinical implications

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A B S T R A C T

Tumor heterogeneity is a hallmark of cancer and one of the primary causes of resistance to therapies. Triple-negative breast cancer (TNBC), which accounts for 15–20% of all breast cancers and is the most aggressive subtype, is very diverse, connected to metastatic potential and response to therapy. It is a very diverse disease at the molecular, pathologic, and clinical levels. TNBC is substantially more likely to recur and has a worse overall survival rate following diagnosis than other breast cancer subtypes. Chemokines, low molecular weight proteins that stimulate chemotaxis, have been shown to control the cues responsible for TNBC heterogeneity. In this review, we have focused on tumor heterogeneity and the role of chemokines in modulating tumor heterogeneity, since this is the most critical issue in treating TNBC. Additionally, we examined numerous cues mediated by chemokine networks that contribute to the heterogeneity of TNBC. Recent developments in our knowledge of the chemokine networks that regulate TNBC heterogeneity may pave the way for developing effective therapeutic modalities for effective treatment of TNBC.

1. Introduction

While the death rate from breast cancer (BC) has fallen significantly during the last two decades, recent cancer data indicates that the disease remains a significant global public health burden [1,2]. Despite advances in early diagnosis and therapies, BC continues to be the most significant cause of tumor-related death [2]. Breast cancer and distant metastases are heterogeneous, a key reason for therapeutic failures [3,4]. The heterogeneity might be due to the occurrence of driver mutations and alterations in cancer genes, prompting clonal evolution and dissemination of polyclones to metastatic niches [5]. Combinatorial assessment of the histopathology of the primary tumor and the expression pattern of hormone receptors (estrogen and/or progesterone receptors; ER/PR) and epidermal growth factor receptor 2 (HER2/Neu), as well as additional genomic and transcriptomic profiling, enabled for the identification of subtypes of breast cancer and paved the way for the development of targeted therapies [6]. Based on gene expression, BC has been categorized into four subtypes: luminal A, luminal B, HER2-enriched, and TNBC. TNBC is a highly aggressive subtype that accounts for approximately 15–20% of all diagnosed cases, with limited access to targeted therapy owing to the lack of hormonal receptors (ER and PR) and HER2 amplification [7,8]. TNBC is a highly heterogeneous BC subtype, classified into six stable subtypes based on gene expression profiling, namely basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM) subtype, and luminal androgen subtype (LAR) [8,9]. TNBC is a substantial clinical challenge due to its poor prognosis, high recurrence, and, most importantly, high incidence of metastatic disease [8]. The breast tumor heterogeneity maintained by different molecular cues in tumor cells by tumor microenvironment (TME), promote aggressive tumor phenotype

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and therapeutic resistance [10]. The TME influences the epigenetic and gene expression patterns of TNBC cells and promotes their heterogeneity via secreted substances and physical interactions [10].

Chemokines are small proteins with a low molecular weight that drive leukocyte chemotaxis in persistent inflammatory or pathogenic cues [11]. Numerous studies have established that chemokines and associated cognate receptors influence the growth and spread of tumors [12,13]. With their many functions, chemokines play a critical role in the communication between tumor cells and their surroundings [14]. Recent research revealed that the role of chemokines and their cognate receptors in metastasis is multifaceted since several chemokines regulate site-specific tumor development and metastasis, indicating tumor heterogeneity due to heterogeneous receptor expression [15,16]. The fundamental challenge in metastatic cancer treatment is the establishment of therapeutic interventions capable of tackling heterogenous tumors across metastases and within metastases. Tumor cell heterogeneity is connected with their ability to metastasize, their response to chemotherapy or targeted immunotherapy, and many other aspects [4,17]. In this review, we have emphasized tumor heterogeneity and the function of chemokines in regulating tumor heterogeneity, the most crucial problem for treating TNBC. Besides, we have discussed various cues regulated by chemokine networks contributing to TNBC heterogeneity. Recent breakthroughs in research of chemokine networks regulating TNBC heterogeneity may open the way for the development of a possible personalized therapeutic approach for metastatic cancer.

2. Breast tumor heterogeneity

Tumor heterogeneity, a defining characteristic feture of cancer, describes tumor cell diversity at genetic, morphological and phenotypic level [4,18]. This phenomenon can be characterized as inter-tumor heterogeneity (between tumors) or intra-tumor heterogeneity (within tumors) [3,18]. Tumor heterogeneity is defined by an abnormal distribution of genetically different cancer subgroups across and within disease locations (spatial heterogeneity) or by temporal alterations in the genomic makeup of cancer cells (temporal heterogeneity) [3,19]. BC is a complex heterogeneous tumor triggered by several discrete genetic alterations in mammary epithelial cells, resulting in widely disparate clinical presentations in individual patients [20,21]. The diverse population of cells enables interactions between tumor cell subpopulations and TME stromal cells via secreted substances and physical interactions that promote cancer growth [10]. Individual tumor cells exhibit various characteristics regulated by cell-intrinsic signals such as genetic, epigenetic, and proteomic alterations [20,22,23]. Tumor heterogeneity is caused by differences in genetic instability, metastatic potential, epithelial-to-mesenchymal transition (EMT), cell plasticity, and stemness [22]. Furthermore, tumor vasculature and stromal cells in the TME contribute to tumor heterogeneity through physical interactions and secreted substances. Within tumors, heterogeneity evolves, resulting in uneven response to therapy and resistance in BC patients [24–27].

2.1. Intertumor heterogeneity

2.1.1. Histopathologic heterogeneity

Breast cancer is most effectively staged using the TNM staging approach, which best reflects the intertumor heterogeneity of the disease [28]. BC is classified histopathologically according to its morphologic heterogeneity. The grade is assigned using a three-tiered system (low, moderate, and high) based on three phenotypic parameters: the mitotic rate, the percentage of the tumor organized in tubular structures and glands, and the degree of nuclear pleomorphism [29]. The grading is a highly predictive prognostic factor in clinical decision-making tools such as the NPI and Adjuvant Online [30,31]. Proteomics, genomics, and transcriptomics all have a role in differentiating BC grades [32–34]. Breast carcinomas of grade 1 and grade 3 are very certainly two distinct diseases, and molecular data shows that transition from low- to high-grade carcinoma is highly unusual [31].

2.1.2. Biomarker heterogeneity

Immunohistochemistry (IHC) is often used to examine the expression of the hormone receptors (ER, PR) and HER2 in tumors [35,36]. It has been shown that these biomarkers are both predictive and prognostic indicators, and their presence in breast carcinomas is critical for patient treatment and outcome recommendations [37,38]. The estrogen and progesterone receptors are expressed in around 80% and 60–70% of breast carcinomas, respectively [39,40]. While around 70–80% of ER-positive tumors co-express PR (ER+/PR+), some breast carcinomas are ER+ /PR- or ER-/ PR+ in rare circumstances. Hormone treatment response varies as well, with ER+ /PR+ tumors responding most robustly (about 60%) and ER+ /PR- and ER-/PR+ tumors responding less robustly [31]. In 15–20% of initial breast carcinomas, the oncoprotein HER2 is overexpressed. High (3 +) HER2 staining is usually linked with gene amplification; depending on the criterion for HER2-equivocal (2 +) staining, between 10% and 20% of HER2-equivocal breast carcinomas are HER2-amplified using in situ hybridizations (ISH) [31]. Breast carcinomas that are HER2-positive have the worst prognosis of any invasive breast cancer. Nonetheless, they have a high response rate to anti-HER2 targeted therapy, as indicated by pathologic complete response post-neoadjuvant treatment in 50–60% of patients with HER2-positive tumors [41,42].

In terms of histology, genetics, and prognosis, "triple-negative" breast carcinomas constitute a wildly diverse group. According to a recent study, nuclear androgen receptor (AR) expression is found in 12–55% of triple-negative breast cancer patients and has been linked to improved survival in other tumor subtypes [43–46]. Several more BC indicators have already been investigated for their potential diagnostic, prognostic, and therapeutic utility, such as cell proliferation markers (Ki-67, survivin, NGAL), cell invasion, and metastasis indicators (p53, MMP-9, SK1, DcR3, COX2, EZH2, microRNAs miR-105, and miR126), epithelial-mesenchymal transition (EMT) markers (WNT5A/B, Pea3), immunological function (PD-L1), treatment resistance (HER216, pSTS3, KLK) [47]. The extent to which tumor heterogeneity influences biomarker expression, as well as its clinical significance, is unknown. A systematic procedure and reliable quantitative reporting of biomarkers are required to guide therapy decisions better.

2.1.3. Genetic heterogeneity

Based on gene expression analyses, BC has been classified into four main intrinsic molecular subtypes: luminal A, luminal B, HER2-enriched, and basal-like [48]. ER-positive breast carcinomas viz, luminal A and luminal B subtypes have better survival than the HER2-enriched and basal-like subtypes. Both luminal subtypes express ER; however, luminal B tumors express more proliferation-associated genes and have a worse prognosis than luminal A tumors [49]. The HER2-enriched subtype is defined by increased HER2 and proliferative gene expression in ER-/PR-/HER2 + and ER+ /PR+ /HER2 + tumors. In most cases (70%), the basal-like subtype is triple-negative and enriched for genes expressed in basal epithelial cells [48]. Other subtypes include claudin-deficient tumors with stem-like characteristics [50] and AR-positive molecular apocrine malignancies [51]. A meta-analysis of transcriptomics demonstrated a correlation between the prediction value of different signatures and proliferation-associated genes [52]. While gene expression patterns can be used to predict chemotherapeutic response and recurrence risk [53], breast cancers clinical and molecular heterogeneity makes categorization difficult. Patients with the same molecular subtype of breast cancer who get the same therapy may experience varying clinical results and develop resistance to the treatment [54]. Breast carcinomas with somatic mutations in TP53, PIK3CA, or GATA3 are common ($>$ 10%) [55]. Several more molecular subgroups were identified, including a molecular classification of breast cancer patients based on integrated genomic and transcriptome analyses, which identified eleven unique breast cancer subtypes with

variable clinical outcomes [22,56].

IHC stains have been assessed as prospective alternatives to molecular assays for the indirect assessment of molecular subtypes that can be employed in the majority of laboratories because of the high cost, time, and technical competence necessary for molecular tests. The IHC staining panel for ER, PR, HER2, Ki-67, epidermal growth factor receptor (EGFR), and cytokeratin 5/6 (CK5/6) can reliably identify the following breast cancer molecular subtypes: (1) Luminal A (ER+/PR/HER2-/Ki-67); (2) Luminal B (ER+/PR/HER2/Ki-67 +; with Ki-67 positivity); and (3) Luminal/HER2 + [57,58]. It is well established that not all TNBC tumors are basal-like and ER-positive luminal tumors also exhibit a wide range of heterogeneity, indicating that genetic heterogeneity in breast cancer is likely to be substantially more complex than our current understanding and warrants immediate investigation.

2.2. Intratumor heterogeneity

2.2.1. Histopathologic heterogeneity

Intratumor heterogeneity can be defined as the diversity of tumors in diverse areas (spatial heterogeneity) or the evolution of tumors through time (temporal heterogeneity) [59]. Spatial heterogeneity within a single tumor is readily apparent, but it can also be found between the primary breast cancer and synchronous lymph node metastases, or even between synchronous metastases from different locations. Breast carcinomas with a truly mixed morphology consist of two morphologically distinct components (e.g., IDC and mucinous carcinoma). On the other hand, some tumors have ambiguous morphologic characteristics (e.g., IDC with lobular features) or discrete differentiation foci (e.g., IDC with localized squamous/basaloid or spindle cell differentiation). Individual tumors with physically diverse sites might be clonal, with each cancer exhibiting its unique set of genetic abnormalities [60–62]. Temporal variability is seen in the growth of an invasive tumor over time or in response to therapy [63,64], the formation of asynchronous metastatic disease [65,66], and the shift from in situ to invasive carcinoma [67,68].

While current BC therapies are guided by the histologic, immunohistochemical, and molecular characteristics of the primary tumor, changes in metastases morphologic and immunohistochemical features may influence treatment success [59,69]. The percentage of ER discrepancy varies between 16% and 33.6%, the rate of PR discrepancy varies between 32% and 40%, and the rate of HER2 contrast varies between 10% and 15.7% [70–72]. Exposure to therapy as well as lack of treatment can result in biomarker expression differences between primary and metastatic malignancies [73–75]. Significant changes have been observed in genomic diversity [65,76], single nucleotide or copy number variants [66,69,77,78], as well as chromosomal rearrangements and insertions/deletions [78]. Due to a scarcity of evidence that modulating therapy based on biomarker status enhances clinical outcomes, current practice guidelines propose biopsy and retesting for biomarkers on accessible metastases only when clinically indicated [79].

2.2.2. Biomarker heterogeneity

The expression of biomarkers within a tumor may vary significantly, resulting in challenging interpretation and different outcomes in small biopsies. It has long been established that there might be variations in ER/PR labeling within the same tumor [80,81]. Individual tumors vary in their percentage of ER/PR-expressing tumor cells, and expression levels are strongly correlated with response to endocrine treatment [40,82]. Even tumors with low ER/PR levels (1%) can react, demonstrating the ASCO/CAP recommendations acceptance of a 1% limit for ER/PR positive malignancies [83]. However, because this technique ignores intratumor heterogeneity, labeling tumors with different patterns of ER-cells as ER+ has limited therapeutic benefit [84].

Furthermore, the staining and amplification of HER2 might differ significantly, thereby impacting disease-free survival [81,85–87]. The incidence of discrepancy in HER2 IHC findings varies from 1 to > 50%; however, the frequency of heterogeneity in gene amplification ranges from 5% to 30% [85,88–90]. IHC labeling of HER2-positive malignancies displays total circumferential membrane staining in 10–100% of tumor cells (3 + staining). Certain malignancies show partial and/or indecisive circumferential membrane staining in more than 10% of cells or total, robust circumferential membrane staining in 10% of cells (2 + staining) by IHC but do not demonstrate gene amplification by ISH [35]. Protein overexpression without gene amplification has been documented in rare situations, as has gene amplification lacking protein upregulation. While the ASCO/CAP recommendations recognize uneven amplification and urge disclosure from many sites, identifying gene amplification in a single location is adequate to label cancer as HER2-enriched [90]. This technique optimizes individual selection for treatment modality while oblivious to the clinical implications of intratumor heterogeneity [84].

Other indicators with variable expression include EGFR [91], p53 [81,91], c-myc [88], proliferation markers such as Ki-67 [81,91,92], cyclin-D1 [88], and PCNA [88]. Ki-67 is a non-histone nuclear protein expressed throughout the cell cycle except G0 and has been reported to be prognostic and predictive in both estrogen receptor-positive and estrogen receptor-negative breast tumors [93,94]. On the contrary, Ki-67 expression is much greater around the periphery of the tumor, with varying staining throughout the tumor in the form of hot patches [95]. Additionally, heterogeneity in Ki-67 expression within tumors has been demonstrated in breast carcinomas of diverse histologic subtypes and grades [92]. Numerous grading systems have been proposed for Ki-67 staining, including assessing hot regions, calculating the average score while including hot patches, and even removing them completely [95].

In comparison to original tumors, lymph node metastases display Ki-67 uniformly. Additionally, metastatic tumor cells are proliferative and associated with high Ki-67 levels in initial tumors with the most abundant expression hot spots. This might be explained by temporal variation in the initial tumor growth fraction, facilitating metastatic dissemination via clonal proliferation [96]. Although it is unknown whether intratumor heterogeneity is an actual physiological phenomenon or a technological artifact caused by insufficient fixation and processing [81,97], extensive sample collection and IHC or other staining procedures with sufficient negative and positive controls are frequently recommended.

2.2.3. Circulating tumor cells (CTCs)

During malignancy, circulating tumor cells separate from the primary tumor and spread [98]. In numerous trials [99–107], CTC count was demonstrated to be an independent predictor of poor survival, resistance to treatment, and early recurrence. However, the practical use of CTC-based assays as "liquid biopsies" is complicated by the high molecular and functional heterogeneity of CTCs [108–110], which includes protein (HER2, ER, Ki-67) and gene (PIK3CA) variability [37,111–113], as well as EMT markers [114,115]. Tumor cells lose epithelial characteristics such as cell polarity, cell-to-cell adhesion, and epithelial marker expression during the EMT process, which is thought to occur before developing lymphovascular invasion and metastasis, and acquire mesenchymal characteristics such as motility and invasiveness [114,115]. Although it is thought that CTC heterogeneity contributes to therapeutic resistance, the ASCO guidelines do not propose adjusting therapy only based on CTC counts when assessing treatment response due to a lack of clinical data [36,59].

2.2.4. Genetic heterogeneity

BC is highly heterogeneous intratumorally in terms of chromosomal and genomic abnormalities, affecting many processes and activities, including signaling pathways, antitumor immunity, cell senescence, migration and metastasis, angiogenesis, and therapeutic response [3,22,116,117]. Different cell clones may cluster in separate tumor regions or disseminate and mix within the same area [118]. The complexity of intratumor genetic heterogeneity is best illustrated by a study of 100

tumors in which driver mutations in > 40 cancer genes were observed, including AKT2, ARID1B, CASP8, CDKN1B, MAP3K1, MAP3K13, NCOR1, SMARCD1, and TBX3, as well as 73 combinations of mutant genes [119]. Intratumor genetic heterogeneity can be analyzed using bulk sequencing and single-cell or single-molecule sequencing [18]. On the contrary, bulk tumor sequencing cannot identify the cellular origin of genetic alterations, the location of the tumor, or the degree of heterogeneity, and single-cell sequencing cannot offer information about the remaining cell population, limiting its practical value in clinical practice [84]. An autopsy study compared molecular alterations in multiple synchronous breast carcinoma metastases and documented tumor cell molecular evolution and clone selection in response to targeted therapy, highlighting the difficulties associated with targeted treatment due to metastatic tumor molecular heterogeneity [120].

2.2.5. Non-genetic (Epigenetic) heterogeneity

Epigenetic heterogeneity refers to variations in gene expression that do not result from changes in DNA sequence [84,121,122]. Epigenetic suppression of tumor suppressor genes such as p16INK4A, RASSF1A, and ER/PR/HER2 can be altered by histone modification or DNA methylation [3]. Transient phenotypic variations of cells can also occur due to stochastic biochemical processes occurring inside cells [122], involving changes in chromatin states or messenger RNAs [123]. Non-genetic heterogeneity affects the destiny of cells and their differential responsiveness. Additionally, non-genetic stem cell and stromal cell heterogeneity are critical for cancer development, particularly in BC, however, non-genetic heterogeneity is clinically insignificant.

3. Chemokines in TNBC

Chemokines are a broad class of low-molecular-mass cytokines (8–14 kDa) and regulate immune cell trafficking, modulate stromal composition and inflammation [11]. They are classified according to the position of crucial amino acids (Cys): CC, CXC, CX3C, and XC [124]. Most chemokines communicate via receptors linked to G-proteins, and 25 distinct (GPCRs) have been identified that bind chemokine and control multiple aspects of cell function [11]. Additionally, the chemokine family has been separated into two functional categories: homeostatic chemokines and inflammatory chemokines [124,125]. While inflammatory chemokines are released in response to infection or cell damage, homeostatic chemokines are required for the immune system to operate normally [14,125]. The significance of chemokines in breast cancer growth, angiogenesis, immune suppression, and site-directed metastasis has been extensively established during the previous decade [12]. Consequently, we analyzed the expression pattern of chemokines in BC utilizing the TCGA BRCA dataset on the Gepia2 online database (http://gepia2.cancer-pku.cn/#index), a comprehensive online web resource for analyzing gene expression levels [126]. The study revealed that chemokines were dramatically dysregulated in breast cancer. Ten chemokines were found to be highly deregulated with a log2FC > ± 2 when compared to normal tissues. These chemokines include CCL14, CCL21, CCL28, CX3CL1, CXCL12, CXCL2, CXCL11, CXCL13, CXCL9, and CXCL10 Table 1.

The following sections will shed light on the multifaceted role of chemokines in breast tumorigenicity and clinical challenges in targeting chemokine networks.

3.1. Chemokines in TNBC heterogeneity

Recent research has proven that chemokines are crucial in developing and maintaining tumor heterogeneity, notably in breast malignancies [4127]. It has been revealed that stromal and immunological cells in the TME control breast tumor heterogeneity via secretion of chemokines such as CCL2, CCL5, CXCL8, and CXCL1 [13,15,128]. Additionally, gradient exposure to the factors released by TME results in heterogenous production of chemokines and their corresponding

Table 1
Chemokine expression in Breast cancer patients and healthy controls.

S.No	Chemokines	Tumor (Median)	Normal (Median)	Log2 (Fold Change)	Adj.p
1.	CCL14	14.79	285.569	-4.182	1.32E-137
2.	CXCL2	1.02	20.77	-3.43	4.62E-198
3.	CCL21	8.34	72.69	-2.98	4.83E-62
4.	CX3CL1	14.13	108.45	-2.855	7.86E-114
5.	CCL28	5.92	41.091	-2.605	4.78E-68
6.	CXCL12	55.521	247.811	-2.138	7.23E-100
7.	CXCL14	65.009	210.123	-1.677	1.21E-16
8.	CCL23	0.35	2.68	-1.447	1.44E-103
9.	CXCL1	0.54	2.43	-1.155	1.43E-28
10.	CCL15-CCL14	0.18	1.59	-1.134	2.12E-46
11.	CXCL3	0.250	1.740	-1.132	2.45e-109
12.	CCL13	0.99	2.64	-0.871	1.54E-27
13.	CCL16	0.03	0.6	-0.635	3.95E-134
14.	CCL24	0.05	0.41	-0.425	8.16E-39
15.	CXCL7	0	0.24	-0.31	1.57E-31
16.	CXCL6	0.07	0.32	-0.303	5.26E-18
17.	CCL2	38.03	46.96	-0.297	9.43E-10
18.	CXCL4	0	0.14	-0.189	1.40E-34
19.	CXCL5	0.06	0.2	-0.179	8.69E-08
20.	CCL25	0.1	0.04	0.081	1.06E-04
21.	CCL7	0.17	0	0.227	6.01E-13
22.	CCL20	0.69	0.25	0.435	3.12E-12
23.	CXCL17	4.11	2.24	0.657	4.72E-04
24.	CXCL16	49.731	30.439	0.69	1.39E-06
25.	CCL17	1.26	0.3	0.798	6.53E-22
26.	CCL22	2.02	0.72	0.812	3.45E-11
27.	CCL11	1.15	0.04	1.048	1.02E-71
28.	CCL3L3	3.08	0.85	1.141	8.36E-13
29.	CCL5	25.751	9.1	1.405	7.37E-16
30.	CXCL8	25.324	9.7	1.435	7.12E-16
31.	CCL4L2	8.08	1.44	1.896	5.49E-23
32.	CXCL11	5.15	0.27	2.276	1.53E-65
33.	CXCL13	6.93	0.35	2.554	6.88E-25
34.	CXCL9	16.63	1.58	2.773	7.17E-54
35.	CXCL10	25.419	1.53	3.384	2.08E-93

receptors, resulting in primary tumor heterogeneity [129,130]. Chemokines have been involved in breast tumor site-directed metastasis, resulting in the heterogeneous spread of breast tumor cells [23,131]. Norton et al. reported two different subsets of TNBC cells with variable CXCR3, CCR5, and CXCR1 receptor expression, along with distinct metastatic potential and chemokine receptor heterogeneity, in orthotopic breast cancer models [132]. CCL5, a CCR5 ligand, is overexpressed in breast tumors, and the CCL5/CCR5 axis is highly active in TNBC cells [133]. Notably, only a fraction of cells express CCR5 and respond to CCL5, and these cells are more aggressive than cells with low CCR5 expression [133]. Song et al. showed the key role of CCL2 chemokine in shaping the breast TME resulting in a distinct response to therapeutics [134]. A recent study demonstrated that CXCL8 promotes the mesenchymal phenotype of TNBC cells and neutralization of CXCL8 with antibodies reduced the mesenchymalization [135]. The expression pattern of chemokines in different breast cancer subtypes was studied using the UCSC online database (https://xenabrowser.net/) [136]. We categorized the TCGA BRCA dataset on PAM50 mRNA expression defining breast cancer subtypes. Among the BC subtypes, basal breast tumors, followed by HER2 enriched, showed high expression of chemokines compared to luminal A or B subtypes. The heterogeneous expression of chemokines amongst breast cancer subtypes indicates the importance of intertumor heterogeneity in breast cancer and correlates with tumor grades (Fig. 1). Among the screened chemokines, several chemokines showed heterogeneous expression in BC subtypes, viz CXCL2, CXCL3, CXCL1, CXCL6, CXCL5, CX3CL1, CCL13, CCL18 were highly upregulated in basal breast tumors, while as highly downregulated in luminal A and luminal B breast tumors. In addition, CXCL12 was found highly downregulated in basal tumors, in contrast, overexpressed in luminal A tumors. The CXCL12-CXCR4 axis has been highly implicated in BC metastasis and attributed to the tissue-specific metastasis in basal tumors in particular brain metastases [137]. The heterogenous expression of chemokines between the low grade (luminal) and high grade (basal) breast tumors indicates key role of chemokines in breast tumorigenicity.

The geographical and temporal heterogeneity of secreted factors results in the formation of distinct cancer cell subpopulations within the tumor and metastatic environment, which has been reported by several groups [138,139]. Because of the varying geographic and temporal distribution of CXCL8 expression at the tumors periphery and core, subpopulations with a wide range of angiogenesis, invasiveness, and metastatic potential have been identified [139–141]. Aside from that, TAMs, which constitute a large proportion of the TNBC TME population, secrete CXCL8 and CXCL1 and a range of other pro-tumor factors. TAM-produced CXCL1 promotes the development of tumor spheroids and the differentiation of CSC subpopulations in human TNBC cells [142,143]. According to the existing studies on chemokines in TNBC, chemokines seem to have an evident influence on TNBC cells and diverse stromal factors in primary tumors and metastases via modulating the heterogeneous subpopulations of cancer cells in primary tumors and metastases [15]. Additionally, chemokine-mediated cellular processes such as survival, proliferation, senescence, angiogenesis, EMT, and stemness confer phenotypic alterations in TNBC cells and their milieu, regulating TNBC heterogeneity Fig. 2 [15]. While these findings provide insight into the role of chemokines in breast tumor heterogeneity, the molecular mechanisms underlying chemokine-mediated inter-and intra-tumor heterogeneity in TNBC merits immediate attention.

3.1.1. Chemokines in TNBC survival, proliferation and senescence

Chemokines and their receptors regulate cell survival, proliferation, and cellular senescence in TNBC. When activated by their ligands, they mediate these processes through different signaling pathways. Chemokines like CXCL1, CXCL2, CXCL3, and CXCL12 are involved in the growth and development of many cancers and inhibition of chemokine receptors like CXCR2 and CXCR4 in several cancers, halts their proliferation and can lead to apoptosis. These ligands and their receptors show differential expression in the breast. Mesenchymal stem cells increase cell proliferation in heterogeneous TNBC by upregulating the expression of CXCL8, CCL2, and CCL5 [144,145]. In aggressive forms of breast cancers, CCL5 is highly expressed and can be detected in the serum of high-grade tumor patients [147,148]. The chemokines increase cell proliferation and metastasis by enhancing the expression of cyclin D1 and c-Myc via the mTOR signaling pathway [149]. In breast cancers,

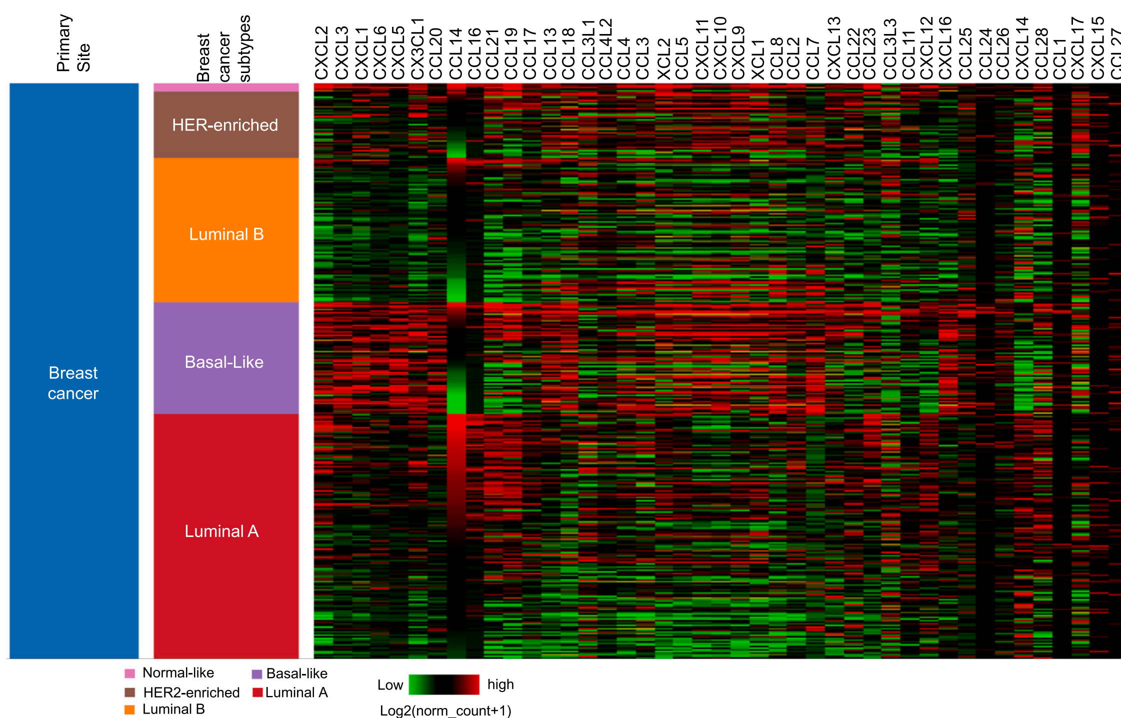

Fig. 1. Expression pattern of chemokines in breast cancer subtypes. The expression pattern of chemokines in breast cancer subtypes is classified based on PAM50 mRNA levels. The expression analysis showed high heterogeneity in the expression profile of chemokines across breast tumors.

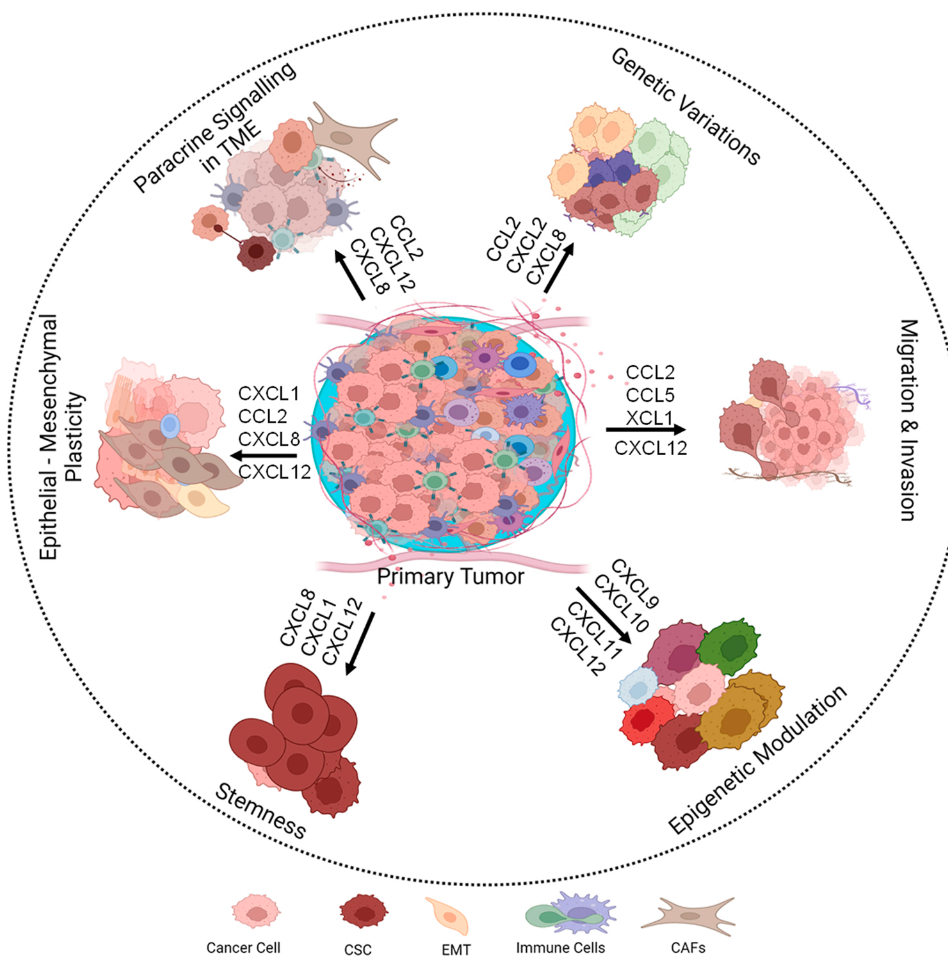

Fig. 2. Chemokines in breast tumor heterogeneity. Individual tumor cells at a tumor site have different capacities regulated by intrinsic and extrinsic factors. Chemokines regulate several aspects of tumor biology and promote the heterogeneous nature of tumors due to their gradient levels, temporal/spatial positioning at the tumor site, and the expression of cognate receptors on cancer cells. Activating cognate receptors by chemokines on tumor cells promotes metastatic phenotypes such as cancer cell migration and invasion, epithelial-to-mesenchymal transition (EMT), cell plasticity, and stemness. Moreover, through cell-cell interactions and paracrine signalling, extrinsic factors in the tumor microenvironment (TME), such as the tumor vasculature and resident and invading immune cells, also contribute to intratumoral heterogeneity. Intratumoral heterogeneity develops through time and space, changes with treatment response and the development of resistance, and differs across individuals.

deregulated MAPK and AKT signaling pathways have increased cell survival and proliferation [150,151]. Overexpression of tumor-suppressing chemokine receptor, XCR1, inhibits cell survival and cell proliferation in the cancerous breast cell by decreasing the activation of p38 and JNK; simultaneously, the expression of p53 protein is enhanced [152]. In TNBC, the Ras/MAPK signalling pathway mediates increased cell proliferation via CXCR2 dependent process. It has also been reported that CXCR2 is involved in metastasis and increases chemoresistance in BC cells by upregulating the COX2 and downregulating AKT1, E-cadherin, and β-catenin [153]. CCR2, which is upregulated in BC, increases cell survival via ERK1/2 signalling pathways by increasing the activation of Smad3 protein, and interference with Smad3 gene suppresses CCR2 mediated cell survival in mammary carcinoma [154]. Mutations in oncogenes/tumor suppressor genes also deregulate the expression of many chemokines. Protein tyrosine kinase, Syk, a tumor suppressor candidate in breast cancer, suppresses cell proliferation by reducing the expression of growth-related oncogene (GRO) chemokine [155]. Chemokine receptor CXCR4, which is upregulated in BC and involved in cell proliferation and metastasis, is regulated by tumor suppressor gene p53. Mutation or deletion of p53 in breast cancer cells increases the expression of CXCR4 [156]. Mutation in the Ras gene increases the expression of CXCL8, whereas inactivation of retinoblastoma and PTEN increases the expression of CCL2. The chemokine/chemokine receptor interaction pathway showed the most significant difference in gene expression after tumor suppressor PBRM1 knockdown. In knockdown cells, protein levels of IL6ST and CCL2 increased while levels of IL-8, IL-6, and CXCL2 decreased [157]. Chemokines also have pro-apoptotic functions in some cancers. For example, CCR1 and CCR5 inhibits cell proliferation in hepatocellular carcinoma and breast cancer cells, respectively. It has been found that CCR5 mediates its effect through p53 protein.

Chemokines and their receptors also arrest cell growth and lead to cellular senescence. It is worth noting that senescent cells secrete many proteins, including extracellular proteases and matrix components, growth factors, cytokines, and chemokines, to arrest cell growth. It is an essential process by which aberrant cells delay tumorigenesis. Cellular events such as DNA damage, increased ROS production, and replicative irregularity can lead to cell senescence [158–160]. CXCL8 and IL-6 promote cellular senescence in human fibroblasts via cell membrane-bound receptor IL-1α [161] CXCR2 (IL8RB) also cause fibroblast cell senescence. Senescent cells secrete many chemokines that increase the production of CXCR2 via NF-kβ and C/EBPβ transcription factors. CXCR2 has been shown to mediate replicative and oncogene-induced senescence in a DNA damage response in a p53-dependent manner. Furthermore, it has been found that CXCR2 is transactivated by p53 and mediate cellular senescence through p38. Ectopic expression of CXCR2, on the other hand, causes premature senescence through a p53-independent mechanism [162].

3.1.2. Chemokines in TNBC angiogenesis

Chemokines and their receptors have been reported to regulate angiogenesis in several malignancies. While several chemokines are pro-angiogenic, some inhibit the formation of new blood vessels. To avoid hypoxic conditions, tumors cells secrete angiogenic factors that promote the growth of new capillaries. CXC chemokines like CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8 have glutamic acid leucine, and arginine (ELR) motif at N-terminus which promotes angiogenesis via chemokine receptor CXCR2 [163]. CXCL12, which also belongs to

CXC chemokines and lacks the ELR motif at the N terminus has also been reported to promote formation of new capillaries [164]. Angiogenesis is an essential process for the survival and proliferation of cancerous cells and contributes to tumor heterogeneity. CXCL8 induces proliferation of endothelial cells by increasing the expression of MMP-2 and MMP-9 via CXCR1-and CXCR2 chemokine receptors. Some CC chemokines like CCL1, CCL2, CCL11, CCL15, and CCL16 also function as angiogenic factors and promote neovascularization [165–169]. Recent studies indicate that these chemokines directly bind the endothelial cells and promote angiogenesis by increasing nitric oxide production, leading to increased cell proliferation and migration of endothelial cells [165,170]. CCL2 also upregulates MMP14 and induces the endothelialization of endothelial progenitor cells. CCL5 was found to promote angiogenesis by increasing the expression of MMP-9. CX3CL1, a member of the CX3C family, regulates angiogenesis in BC via immunosuppressive factor TAM and acts on endothelial cells to increase their proliferation and migration [171–175].

Some chemokines have been reported to inhibit the formation of new blood vessels and suppress the growth of tumors. When activated by ligands, angiostatic chemokines inhibit endothelial cell migration and proliferation. Some members of the CXC family of chemokines that are angiostatic and communicate via CXCR3 include CXCL4, CXCL9, CXCL10, CXCL11, and CXCL14 [176]. CXCL4, the first identified chemokine which inhibits angiogenesis, supresses the binding of the VEGF and FGF-2 to their receptors [177,178]. Chemokines CXCL-9, CXCL − 10, and CXCL − 11 mediate their angiostatic function through CXCR3 receptor.

3.1.3. Chemokines in TNBC EMT

The epithelial to mesenchymal transition (EMT) is a well-characterized developmental pathway. Cancer cells have been demonstrated to hijack embryonic development systems such as EMT to boost their dynamic state, which confers various benefits during successful metastasis. Chemokines have a well-established role in site-specific metastasis. Their modulation of behavior, morphology, migration/invasion, and gene regulation has been reported in various malignancies, including TNBC. It is strongly associated with an acquiring of a more aggressive phenotype [13,23,180].

In the investigation of chemokine activities, it has been found that chemokines can directly promote mesenchymal properties in tumor cells, such as increased vimentin expression and specific transcriptional repressors (such as twist, snail, slug, and ZEB) as well as decreased E-cadherin expression in tumor cells [16]. Higher degrees of EMT has been found associated with enhanced tumor cell migration and invasion, partly because mesenchymal features often facilitate motility, frequently via chemotactic gradient-mediated mechanisms (Fig. 3) [181,182]. For instance, a recent study found that CXCL1 produced by TAMs increased EMT distinctive features in luminal-A and TNBC breast cancer cells via an NF-kB-mediated mechanism that activated SOX4 [183]. Additionally, TNBC cells exhibit high amounts of CXCL1, which has been shown to mediate EMT by upregulating the expression of mesenchymal markers such as N-cadherin, Snail, Slug, Twist, and Vimentin and modifying the motility and invasiveness of TNBC cells [184]. EMT and twist expression, as well as enhanced tumor cell invasion, can be induced by CCL2 through CCR2 and CCL5, respectively, in BC cells [185–188]. Furthermore, recent research clarified the involvement of CCL5 in the TNBC EMT phenomenon. The scientists discovered that PAI-1 (Plasminogen activator inhibitor-1) secreted by TNBC cells in response to TGF-β therapy stimulates CCL5 secretion in endothelial cells, which subsequently in paracrine manner acts on TNBC cells to improve their motility, invasion, and metastasis. PAI-1—the main inhibitor of plasminogen activators—was previously reported for promoting tumor growth and angiogenesis, and increased PAI-1 expression has been linked with a poor prognosis in breast cancer patients [133,189].

The positive feedback loop between PAI-1 and CCL5 increased MMP9/10, N-cadherin, Twist, and Snail expression while decreasing E-cadherin expression. TNBC cells' migratory and invasive capacity is reduced when they are treated with a neutralizing CCL5Ab or PAI-039 [133]. Simultaneously, multiple investigations have shown the

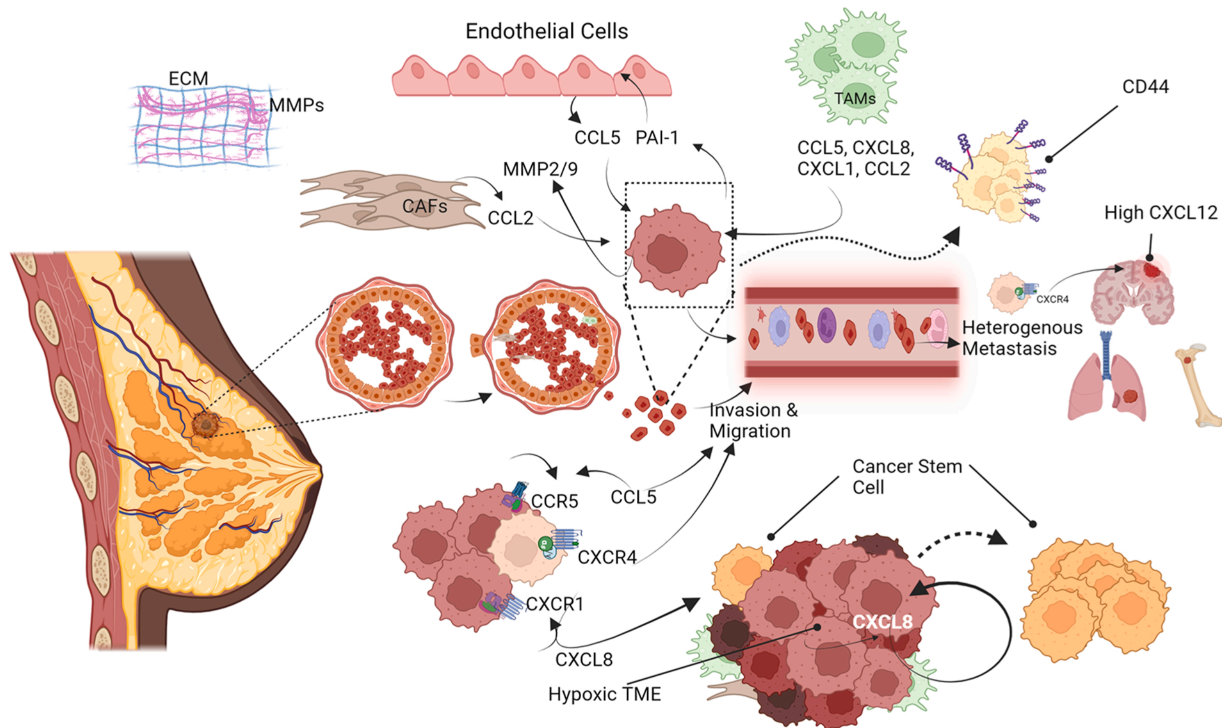

Fig. 3. Chemokines promote epithelial-mesenchymal plasticity and stem cell-like characteristics in TNBC cells. Chemokines such as CXCL1, CXCL8, CXCL12, CCL5, and CCL2 promote epithelial to mesenchymal transition and invasive phenotype of breast tumor cells by upregulation of MMPs, and mesenchymal phenotype promoting genes and downregulation of epithelial genes. CXCL8 in the TME regulates breast cancer stemness, contributing to tumor heterogeneity.

involvement of CXCL8 in TNBC EMT and metastasis. Recent research established that overexpression of Brachyury results in increased production of various chemokines, including CXCL8, CCL5, and CXCL1, and that blocking the CXCL8/CXCR1 axis inhibits EMT [190]. Although it is widely established that TNBC has a more excellent glycolytic capability and is linked with an inflammatory milieu, the regulatory mechanisms and metabolic interactions between the tumor and the tumor microenvironment (TME) remain largely unknown. Recent studies reported that GLUT3 controls CXCL8 production and is required for EMT in TNBC cells and increases their invasiveness and distant metastases. CXCL8 secretion further contributes to the activation of inflammatory TAMs by GLUT3 overexpressing TNBC cells, which further promotes GLUT3 expression and motility of TNBC cells. The findings indicate that aerobic glycolysis not only increases tumor cell aggressiveness but also begins a positive regulatory loop via CXCL8 that promotes tumor growth by modifying the inflammatory TME [191]. Neutralization of CXCL8 with a monoclonal antibody (HuMax-IL8) reverted mesenchymalization in claudin-deficient TNBC models and dramatically reduced the recruitment of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) to the tumor site, an effect that was substantiated when used in combination with docetaxel [135]. Additionally, HuMax-IL8 increased the vulnerability of claudin-deficient breast cancer cells to immune-mediated lysis in vitro employing natural killer (NK) and antigen-specific T cells. These findings demonstrate the numerous ways in which neutralizing this single chemokine reverses mesenchymalization, reduces MDSC recruitment to the tumor site, and aids in immune-mediated killing, establishing a rationale for using HuMax-IL8 in combination with chemotherapy or immune-based therapies to treat TNBC [135].

One of the most extensively studied chemokine pathways in TNBC is the CXCL12-CXCR4 axis, and its role in site-directed metastasis in TNBC is well known [137]. CXCL12-CXCR4 axis has been implicated in the EMT phenomenon [192–194]. Mechanistic investigations revealed that overexpression of CXCL12 in breast tumor cells resulted in a decrease in E-cadherin expression via activation of the NF-kB pathway and upregulation of β-catenin expression [192,194]. Also, we previously reported that the CXCR7-CXCL12 axis enhances the EMT phenomenon in breast tumor cells by activating proinflammatory STAT3 signaling and angiogenic markers and modulation of TME [195]. Simultaneously, recent research indicated that TNF stimulation promotes CCL5, CCR2, and CXCR2 expression, increasing neutrophil recruitment into the TME. CXCR2 + neutrophils were shown to promote the expression of genes associated with metastasis in the TME, including CXCR4 and MMP-2 [196]. CXCL12 has also been shown to decrease the expression of Kiss1, a critical gene related to metastasis inhibition. Kiss1 expression correlates negatively with matrix metalloproteinase-9 (MMP-9) and CXCL8 levels in human breast cancer specimens—genes associated with metastatic invasion [197]. Another study found that dipeptidyl peptidase (DPP)− 4, a membrane glycoprotein, promotes CXCL12 cleavage and inhibits CXCL12-induced EMT in breast cancer [198]. DPP-4 has been shown to affect multiple biological processes, such as cell differentiation, adhesion, immunomodulation, and apoptosis and plays a key role in cancer progression [199]. DPP-4 inhibition was reported to activate the CXCL12/CXCR4 and mTOR signaling pathways, resulting in EMT and migration in TNBC and non-TNBC/ breast cancer cells, encouraging metastasis. Additionally, DPP-4 deficiency increases metastasis in vivo [198].

A recent study performed serial and site-directed mutation analyses on the promoter regions of CXCL9, CXCL10, and CXCL11 and found that NF-kB binding areas were responsible for TNF-induced promoter activation of CXCR3 ligand chemokines produced from BM-MSCs. Additionally, it was shown that all three CXCR3 ligands improved the migratory and invasive motility of CXCR3-expressing MDA-MB-231 breast cancer cells. CXCL10 treatment of MDA-MB-231 cells activated small GTPases belonging to the Rho family, such as RhoA and Cdc42. The invasive capacity of MDA-MB-231 cells promoted by CXCL9, CXCL10, or CXCL11 was entirely reduced in the presence of a neutralizing anti-CXCR3 antibody in the culture medium. Additionally, CXCL9, CXCL10, and CXCL11 increased the expression of MMP-9 in MDA-MB-231 cells, but not MMP-2. These findings imply that BM-MSCs increase breast cancer cell motility via TNF-induced actin rearrangement mediated by CXCR3 ligands in the tumor microenvironment [200].

A recent study examined the effects of XCL1 on migration and intracellular signaling in TNBC cells. It revealed that XCL1 promoted the expression of N-cadherin and nuclear localization of β-catenin in a concentration-dependent manner. Additionally, it was reported that XCL1 promotes HIF-1α accumulation and ERK1/2 activation in breast cancer cells. These results suggest that XCL1-induced SK-BR-3 cell migration is mediated by activating the ERK/HIF-1/EMT pathway [201]. Simultaneously, another research established that TECK promotes EMT in TNBC cells by activating the Akt pathway and that this promotion may be inhibited with the Akt phosphorylation inhibitor LY294002 [202]. Chemokines play an important role in the EMT phenomenon, and these findings suggest that controlling chemokine-signaling cascades reduces tumor phenotypes, viz EMP induced tumor heterogeneity.

3.1.4. Chemokines in TNBC stemness

Breast cancer stemness and EMT are two inextricably related phenomena that may explain tumor cell phenotypic plasticity that is not genetic [203]. Al et al. extracted BC stem cells (BCSCs) effectively from cancer tissues in 2003, and they have subsequently been identified as critical contributors to metastasis, treatment resistance, and tumor relapse in BC patients [204]. Due to their capacity for self-renewal and differentiation, breast cancer stem cells (BCSCs) are resistant to the effects of chemoradiotherapy and endocrine therapy. This enables BCSCs to commence and sustain tumor development, invasion, metastasis, resistance to chemotherapy, and recurrence [205]. BCSCs are typically identified by the CD44 + /CD24- low phenotype and/or by the presence of the ALDH1 enzyme, as indicated by an increased proportion of an ALDEFLOUR+ cell population; frequently, an increased extent/size of tumor spheroids (mammospheres) is also considered a possible marker for CSC enrichment [205–207].

Accumulating data indicates that chemokine ligands and their corresponding receptors, as well as cellular motility, govern breast cancer stemness, contributing to the tumor heterogeneity Fig. 3 [23,208]. Several in vitro and in vivo investigations established that, in addition to EMT, the CXCL8/CXCR1 axis directly enhances breast cancer stemness (revived in [209]). Charafe-Jauffre et al. revealed that CXCL8 was required to maintain competent cancer stem cells with metastatic potential and improved the ALDEFLOUR+ population and spheroid formation via activation of Akt signaling. Besides, inhibiting the CXCL8/CXCR1 axis decreased the ALDEFLOUR+ tumor cell population and metastasis, confirming CXCL8s involvement in sustaining BC stemness [210,211]. Later, elegant research established that when tumor cells are exposed to chemotherapeutic agents (paclitaxel and gemcitabine), they produce CXCL8 via activation of HIF signaling, which is elevated in hypoxic TME, and enhance BC stemness. Additionally, the study discovered that CXCL8 neutralizing antibodies inhibited tumor cells capacity to produce stem cell-like properties (tumor spheroids and ALDH expression) in response to treatment exposure [212]. Additionally, Deyong Jia et al. revealed an autocrine inflammatory forward-feedback loop that activates Wnt/β-catenin and NF-κB signaling, enhancing breast cancer stemness and therapeutic resistance following chemotherapy withdrawal. The scientists further reported that the autocrine forward-feedback loop and CSC enrichment might be successfully prevented using a CXCL8-neutralizing antibody or reparixin-mediated inhibition of the CXCL8 receptors CXCR1/2 [213]. Additionally, a parallel investigation indicated that decreasing CXCR1/CXCL8 in conjunction with paclitaxel increased paclitaxel's inhibitory action by reducing stemness induction and brain metastases [214]. Simultaneously with these findings, Hartman et al. investigated

the genomic profile of TNBC patients across numerous databases and discovered a group of inflammation-related genes that were differently expressed in TNBC cells [215]. Among these, the proinflammatory mediators IL6, CXCL8, and CXCL1 were requisite for TNBC cells to proliferate anchorage independently but had a negligible effect on non-TNBC counterparts. All twelve TNBC cell lines tested had significantly more significant amounts of mRNA transcripts and chemokine/cytokine release into the medium than the six ER-positive/luminal-like breast cancer cell lines via activation of nuclear factor-kappa B (NF-kB) signalling [215]. Inhibiting the IL6/IL8 autocrine loop, researchers significantly reduced TNBC cell survival and anti-apoptotic potential and successfully sensitized TNBC cells to chemotherapy [215]. Similarly, CXCL1 produced from TAMs increased the CD44 + /CD24 - subpopulation and tumor spheroid development in human TNBC cells. (78).

CXCL12-CXCR4 axis, as mentioned earlier, is one of the most well-defined chemokine axis in cancer biology [137]. Recent research has established that CXCL12-CXCR4 plays a critical role in establishing and sustaining breast cancer stemness [131,137]. For instance, over-expression of CXCL12 has been shown to increase the CD44 + /CD24- and ALDH+ cell populations and the transcription of cancer stems cell markers such as Sox2, Oct4, and Nanog [192]. Also, CXCR4 + BC cells exhibit a high capacity for mammosphere formation than CXCR4- cells. [216]. Similarly, CXCR7/ACKR3 was discovered to be critical in fostering the CSC subpopulation, as down-regulation of CXCR7/ACKR3 reduced CD44 + /CD24- & ALDH+ cell population, as well as reduced expression of Oct4 and Nanog [217]. CCL2 and CCL5, two proinflammatory CC chemokines, were also reported to enhance the formation of BCSCs. The paracrine release of CCL2 by CAFs triggered by tumor-stroma cross-talk enhanced NOTCH1 expression and CSC characteristics in breast cancer cells [218]. Put all together; these findings offer insight into the role of chemokines in increasing breast cancer stemness and establishing a unique phenotype of tumor cells that is associated with increased survival and resistance to therapy.

4. Challenges for clinical implications

Presently, there are no clinically approved anti-metastatic therapeutic strategies, and the cancer research community is focused on developing effective methods for treating and preventing metastatic tumor spread [15]. Recent research unravels the layers of a complex interaction network between tumor cells and the host cell, providing the necessary information for efficient metastasis suppression [10,54,208]. As described above, the chemokine networks are central to the development of heterogeneous cell populations, enhancing metastatic potential, tissue tropism, and breast cancer stemness, all phenomena leading to metastatic disease and poor prognosis of breast cancer patients.

Several chemokine receptor inhibitors are evaluated in preclinical studies and clinical trials to treat different primary tumors and metastasis [15]. In preclinical settings, chemokine receptor inhibitors showed promising results in reducing metastatic burden when combined with chemotherapy or immune checkpoint therapy. Independent laboratories have found evidence for a CXCL8-CXCR1/2 axis in CSCs, indicating a possible therapeutic target. Preclinical findings reveal that BCSCs multiply in vitro in response to exogenous CXCL8. However, a small molecule antagonist of CXCR1/2 (reparixin) or a monoclonal antibody blocking CXCR1 (but not CXCR2) was able to deplete CSCs in vitro [211, 219]. In human breast cancer cell lines or xenografts from breast cancer patients orthotopically implanted in mice, the combination of weekly docetaxel and reparixin for four weeks was more successful in reducing tumor growth than either therapy alone [211]. CXCL8 binding with CXCR1 on the surface of CSC protects CSCs against apoptotic signals prompted by FASL.

On the other hand, when reparixin inhibits CXCR1 signaling in CSC, these cells undergo FASL-mediated death. Independent laboratories later provided evidence to support this notion. As Ginestier and coworkers first showed, tumor cells subjected to taxane in vitro express CXCL8 [220]. Additionally, tumor cells from immunocompromised mice following two doses of paclitaxel demonstrated a significant and dose-dependent increase in mammosphere formation efficiency as compared to untreated animals [220]. Clinical trials examining the efficacy of this axis in targeting CSC has been done in breast cancer, where the most preclinical data are available [209]. Although reparixin proved to be well-tolerated, such trials had various difficulties assessing its effectiveness, including the deficient number of CSCs in primary operable breast cancer. To overcome this constraint, circulating markers for monitoring the action of anti-CSC drugs were investigated; however, these assays were insufficient [209]. Prospects for CSC targeting medicines include the development of accurate assays to quantify stem cell quantity and/or activity in serial biopsies from accessible tumors (e.g., window-of-opportunity studies) and the use of alternative endpoints in metastatic clinical trials. Concerning CXCR1/2 inhibition, enrollment has concluded in a randomized, placebo-controlled clinical study (NCT02370238) comparing weekly paclitaxel with and without reparixin as the first-line treatment of metastatic TNBC. Identification of clinical (e.g., disease sensitivity to chemotherapy) and/or cellular/molecular biomarkers of patients is most likely to benefit from treatment and represents a future research direction. At the same time, analysis of data generated in metastatic patients may fuel the development of this strategy in the (neo)adjuvant setting [209].

Additionally, CCL2 downregulation via gene silencing mediated by siRNA complexed with cell-penetrating peptides proved to be more successful than standard antibody neutralization. In *vivo* CCL2 gene silencing resulted in the reduction of primary tumor development and metastasis, which was coupled with a reduction in cancer stem cell renewal and the recruitment of M2 macrophages [221]. Inadequate T cell infiltration in TNBC has impeded its susceptibility to checkpoint inhibitors (ICB), which has prompted the development of immunostimulatory techniques to augment ICB therapy. In a murine TNBC model, recent research indicated that decreasing CXCR4 enhanced anticancer efficacy and extended survival time compared to monotherapies. The study established that immune activation by liposomal administration of CXCR4 inhibitors has the potential to significantly broaden the use of ICB treatments to hitherto ICB-insensitive cancer types [222]. These preclinical studies demonstrate that specific targeting of the chemokine axis may enhance immunotherapy response in TNBC patients.

The rationale for targeting the CCR5-CCL5 axis is based on the following observations: (I) CCL5 appears to be vital for mammary tumor growth while being redundant for overall physiology and broad immune function; (ii) CCL5, knockout mice, progress and live an everyday life; their steady-state immune compartments also appear to be intact, and (iii) CCR5 delta32-homozygous human individuals are resistant to HIV infection and have no general immune deficiency [223]. Because of this, targeted suppression of CCL5, particularly in the bone marrow and the MDSC milieu of tumor-infiltrating MDSCs in TNBC patients, may have a significant therapeutic benefit without overt toxicity. Delivering therapeutic molecules to where MDSCs grow (bone marrow) and function (TME) should be the focus of future studies.

Based on preclinical evidence of reduced metastasis, blocking the chemokine axis has been investigated in metastatic breast cancer clinical trials. Balixafortide, a CXCR4 antagonist, has completed phase I studies in HER2-negative patients with severely pretreated and relapsed metastatic breast cancer combined with eribulin chemotherapy [224]. As mentioned before, multiple clinical studies are evaluating chemokine antagonists and inhibitors; hence, targeting chemokines and their receptors for metastasis treatment is not novel [14,209,223], although it is complicated for a multitude of reasons [14,225]. Firstly, chemokines/receptors are expressed by both tumor cells and various host cells. Thus, inhibiting a chemokine/receptor combination may have unintended consequences, such as affecting normal immune cells that express the same receptor. Immune cells are essential to eliminate residual cancer cells and to inhibit recurrence of the illness.

Additionally, a homeostatic dose must be administered to minimize adverse immunological responses and allergies. Second, the promiscuous nature of chemokines/receptors complicates their interactions, and inhibition or inactivation of a chemokine/receptor pair may result in compensating effects. Thirdly, inhibiting specific chemokine receptors may not be beneficial throughout the metastatic phase and may have a limited treatment scope. Likewise, the profile of chemokines varies with cancer stage, therapeutic administration, and resistance to chemotherapy, further narrowing the therapeutic window. Finally, chemokines as therapeutic agents must target cancer cell propagation and existing metastases, overcoming heterogeneity in metastasis. Apart from the difficulties inherent in targeting chemokines/receptors, addressing metastatic heterogeneity is also tricky. Ideally, we should tackle metastatic heterogeneity by addressing genetic instability as a source of heterogeneity. With the large number of genes involved and other possible heterogeneity issues that arise over a clinical course, it is more challenging than addressing cellular heterogeneity, even though targeting cellular heterogeneity has significant implications for metastasis therapy. Cells found in the primary tumor do not have to be representative of tumor cells found in metastases. The diverse range of cancer cells precludes the efficacy of a single anticancer treatment, or a single therapy, in eradicating all cancer cells present in a malignant tumor and metastases. Thus, new treatment targets or techniques should concentrate on the properties of malignant cells that enable them to metastasize, ways to restrict the number of distinct cancer cell subpopulations inside a tumor, or the ability of tumor cells to produce new variations. Notably, the initial tumor response to treatment and the response of metastatic subpopulations should be used to establish a therapeutic strategy. When anticancer drugs are combined, the kind of combination, the administration sequence, and the time gap between subsequent administrations all contribute to eliminating tumor cells distinct subpopulations.

5. Conclusions and future directions

Due to the lack of a systematic therapeutic strategy at both the early and late phases, TNBC is a challenging disease to treat. The heterogeneity found among TNBC tumors, both intra as well as inter-tumor heterogeneity, further adds to the poor prognosis. Tumor heterogeneity may be a result of both the genetic instability of tumor cells and the diverse TME compositions. As previously noted, tumors are characterized by dysregulation in the expression of chemokine/chemokine receptors at distinct stages of tumor development and metastasis. It is widely established that cancer cells acquisition of chemokine receptors increases their "specific" dissemination to tissues with high expression of cognate ligands. Apart from site-directed metastasis, chemokines have been implicated in several non-conventional facets of tumorigenesis, including proliferation, angiogenesis, stemness, and EMT. However, in light of tumor heterogeneity, the tumor-promoting conventional and non-traditional functions of chemokines in cancer should be carefully examined. The etiology and progression of tumors vary greatly across cancer types and within the same disease, as seen in BC subtypes and intra-tumor heterogeneity of TNBC tumors. Consequently, while investigating the involvement of chemokines in cancer, it is necessary to take into account the existence of facets of intertumoral and intratumoral heterogeneity. More specifically, the functions of chemokines in cancer and their significance for treatment must be considered in the context of the wider concept of "chemokine heterogeneity." In this context, tumors and metastases must be interpreted as multi-chemokine organs. The effect of chemokines on tumor growth and cancer development is largely determined by their relative levels, temporal/spatial positioning at the tumor site, and the expression of cognate receptors on cancer cells, endothelial cells, leukocytes, and stromal cells, as well as the source of chemokine at the tumor site or metastatic organ. In due course, these defining features will determine to a considerable extent which chemokine/s will govern the overall malignant environment, affecting the tumor cells or the TME.

Understanding the fascinating chemokine pathways prevalent in tumor cells and their interaction with the tumor environment paves the way for a new approach to metastasis therapy. Secondly, identifying the underlying processes by which chemokines regulate tumor phenotypes associated with metastasis can assist in discovering cellular and molecular targets for developing efficient molecular-targeted treatments. To sum up, our knowledge of chemokine functions in cancer development has advanced significantly during the past three decades. Chemokines may have both pro-tumor and tumor-suppressing effects in specific situations. Despite this fact, chemokines motility-driven and pro-tumor activity dominate the scene and lead to an increased disease course and poor prognosis. Preclinical and preliminary clinical research indicates that inhibitors of specific chemokines or their receptors may be useful as cancer therapeutics, particularly when combined with other modalities such as chemotherapy or ICBs. Chemokine biology, as now understood, implies that chemokine/receptor antagonists might be used in tandem with already utilized chemotherapeutic agents or that various pairs of chemokine/receptors could be targeted to treat metastasis. Additionally, the expression of several chemokines and their receptors is related to the survival of many cancer patients; consequently, in the future, chemokine/receptor expression may serve as a predictive biomarker. Chemokine/receptor expression may also be used to create "molecular signatures" that can be used to predict tumor aggressiveness, choose suitable therapies for cancer patients, and monitor therapeutic responses.

The ability of chemokines to regulate "immune infiltration" is another promising treatment strategy for cancer and metastasis in the future. Antitumor immune response induction in the tumor microenvironment may be accomplished by viral transmission of chemokines, nanoparticle administration, or reactivating epigenetic blocks that suppress antitumor chemokines in the tumor microenvironment, all of which are therapeutic options. Extensive investigation into the connection between metastatic heterogeneity and chemokine/receptor heterogeneity at the tumor and metastases, however, is required before chemokines and receptors may be used as therapeutic cancer targets in the clinic.

CRediT authorship contribution statement

Conceptualization, U.M. (Umar Mehraj). and, U.M. Umar Mushtaq), NAW, and M.A.M (Manzoor A Mir); Formal analysis, U.M (Umar Mehraj); Writing – original draft, U.M. (Umar Mehraj), U.M. (Umar Mushtaq), NAW, A.S., and M.A.M (Manzoor A Mir); Writing – review & editing, M.A.M. (Muzafar A. Macha), M.N.L., A.S., A.H., M.A.Z., S.M.A and N.A.W; Supervision, NAW.

Declaration of Competing Interest

The authors declare no conflict of interest in this research.

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