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Cancer stem cell-mediated drug resistance: A comprehensive gene expression profile analysis in breast cancer

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ABSTRACT

Breast cancer is the most frequently diagnosed malignancy in women and a major public health concern. In the current report, differential expression of the breast cancer resistance promoting genes with a focus on breast cancer stem cell related elements as well as the correlation of their mRNAs with various clinicopathologic characteristics, including molecular subtypes, tumor grade/stage, and methylation status, have been investigated using METABRIC and TCGA datasets. To achieve this goal, we downloaded gene expression data of breast cancer patients from TCGA and METABRIC. Then, statistical analyses were used to assess the correlation between the expression levels of stem cell related drug resistant genes and methylation status, tumor grades, various molecular subtypes, and some cancer hallmark gene sets such as immune evasion, metastasis, and angiogenesis. According to the results of this study, a number of stem cell related drug resistant genes are deregulated in breast cancer patients. Furthermore, we observe negative correlations between methylation of resistance genes and mRNA expression. There is a significant difference in the expression of resistance-promoting genes between different molecular subtypes. As mRNA expression and DNA methylation are clearly related, DNA methylation might be a mechanism that regulates these genes in breast cancer cells. As indicated by the differential expression of resistance-promoting genes among various breast cancer molecular subtypes, these genes may function differently in different subtypes of breast cancer. In conclusion, significant deregulation of resistance-promoting factors indicates that these genes may play a significant role in the development of breast cancer.

1. Introduction

The global cancer burden is projected to reach 18,989,634 new cases and 10,052,507 deaths in 2020 [1]. Of all new cases and deaths related to breast cancer, 11.7% are new cases and 6.9% are new deaths. [2]. On the basis of communications and interactions occurring within tumor microenvironments, as well as mutations occurring at genetic and epigenetic levels, cancer progresses. [3,4]. Females aged 20–50 years are most likely to develop breast cancer (BC), which is the second leading cause of cancer death in women [5]. As the result of new lifestyles, industrialization, pollution, etc., BC is not limited to women and may affect males as well (1% of all cases). [6]. Early diagnosis, appropriate management, and timely treatment can reduce BC mortality rates to an appropriate level [7]. There is, however, considerable heterogeneity and complexity in BC, characterized by a wide range of phenotypic, morphologic, and clinical characteristics, which complicates the treatment process [8]. In accordance with the origin of the cancer, BC can be classified into various types, thereby influencing treatment

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decisions. Most commonly, ductal carcinomas arise from milk ducts and can spread to lymph nodes, lungs, skin, bone, liver, and brain as well as other parts of the body. It is also possible to develop lobular carcinoma in milk-producing lobules, which is an invasive tumor. Inflammatory BC has the poorest prognosis and results from inflamed lymphatic vessels which are blocked with cancerous cells. As well as comedocarcinoma and medullary carcinoma, and colloidal carcinoma, there are other forms of BC [9]. Local or regional treatment is effective for BCs without distant metastases, but metastases adversely affect the ability to cure the disease. [10]. Similarly, this type of cancer is classified into three main categories and is treated differently based on immunohistochemical characteristics (IHC) and hormone receptor status (HR status): 1) hormone receptor-positive BCs which are positive for both estrogen receptor (ER+) and progesterone receptor (PR+) and include 85% of all BCs. These cancers can further be divided into luminal A, ER+ and/or PR+ and human epidermal growth factor receptor 2 negative (HER2-) and luminal B, ER+ and/or ER+ and/or PR+ and HER2 + (or HER2- with high Ki6). Hormone-positive BCs can be cured with endocrine hormone therapies like selective ER antagonists such as tamoxifen, ER expression modulators such as fulvestrant and aromatase inhibitors such as letrozole (Femara); 2) HER2 positive BCs, which are more aggressive, and fast-growing as a result of receiving more growth factors. This subtype has a poor prognosis and includes 20% of all BCs. Treatment strategies comprise anti-HER2 drugs such as trastuzumab (Herceptin) and tyrosine kinase receptor inhibitor such as lapatinib (Tykerb); 3) Triple-negative BC (TNBC) also known as basal-like subtype, which is ER, PR and HER2 negative and include 15% of all BCs. In light of the lack of targeting therapeutics and the higher likelihood of recurrence, TNBC is thought to have the worst prognosis, and surgery, chemotherapy, and radiotherapy are the current treatment recommendations. [8]. A majority of BCs are treated through surgery. In addition, chemotherapy, radiation therapy and hormone therapy are the adopted strategies for BC treatment in which chemotherapy is accepted as a traditional method and is used to shrink the tumor before surgery or prevent remissions and relapses after surgery [11]. In spite of all the advances in BC treatment strategies and anticancer agents, drug resistance still remains one of the major causes of failure in all BC types and is a major impediment to effective cancer treatment. In many cases, the drug response for the same anticancer agent differs from person to person as a result of drug resistance mechanisms [12]. There are intrinsic factors which can result in the emergence of drug resistance (de novo resistance), such as specific cell membrane transporter proteins that pump the drug out of the tumor cells or altered expression and function of the drug targets. This results in the cancerous cells showing a poor response to the anticancer drugs when they are exposed for the first time. Alternatively, acquired drug resistance occurs when a favorable initial response is followed by poor results following prolonged exposure to anticancer agents, resulting in relapse of the disease. [13]. The development of acquired drug resistance can result in cross-resistance with anti-proliferative drugs, which are key to the treatment of BC [14]. The mechanism responsible for this type of resistance is believed to be genetic alterations in DNA sequence or epigenetic changes such as methylation or expression of the underlying gene [13]. Due to the heterogeneity and complexity of BC [15], it has also been documented that BC stem cells (BCSCs) are responsible for cancer drug resistance and metastasis [16]. Since the cellular heterogeneity within breast tumors and resistance are the major threats in the clinical setting, studying the differential expression pattern of the drug resistance factors is crucial to find biomarkers and therapeutic targets for BC management and treatment. Cancer stem cells (CSCs) possess characteristics associated with normal stem cells, such as the ability to self-renew and the ability to differentiate into other types of cells [17]. A significant role is played by CSCs in the initiation, maintenance, progression, chemoresistance, tumor recurrence and metastasis, and the poor prognosis associated with cancer. Furthermore, CSCs are significant hurdles to successful BC treatment. The understanding of how these cells contribute to drug resistance in BC will support the development of novel therapies that target their elimination [18,19].

2. Methods

2.1. Human clinical data analyses

The gene expression data with their clinical information in patients with BC were extracted from two publicly available BC datasets, the TCGA (The Cancer Genome Atlas BC) [20] and METABRIC (Molecular Taxonomy of BC International Consortium) [21] available at the TCGA data portal, and cBioPortal for Cancer Genomics (http://www.cbioportal.org). In the case of METABRIC, the RNASeq data performed on breast Invasive Carcinoma samples are provided with clinical information (2509 samples). In the case of TCGA, gene expression data is reported as in RSEM normalized count (log intensity levels) for 1108 Breast Invasive Carcinoma samples. According to the PAM50 classification, METABRIC BC dataset was divided into 5 subtypes, including the Basal (n = 199), HER2 + (n = 220), Lum A (n = 679), Lum B (n = 461) and Normal-like (n = 140) subtypes. Clinical data for each TCGA sample is downloaded directly from the TCGA Data Portal. ER, PR, and HER2 status are assessed using the consensus of clinical tests ER Status By IHC, PR status by IHC, and IHC-HER2, respectively.

2.2. Correlation analyses

The metastasis (GILDEA_METASTASIS) [22], angiogenesis (HALLMARK_ANGIOGENESIS), and immune evasion (LIN_TUMOR_ESCAPE_FROM_IMMUNE_ATTACK) [23] gene signatures were obtained from The Molecular Signatures Database hallmark gene sets (MsigDB, https://www.gsea-msigdb.org/gsea/msigdb). Pearson's correlation analysis was used for the analysis of the correlation between the methylation degree and gene expression. The data were downloaded from the TCGA database. The RNA-seq data and methylation data for level 3 were downloaded from TCGA, and the selected samples were all patient tissue samples.

2.3. Methylation analyses

DNA methylation (Illumina Infinium HumanMethylation450) datasets were extracted from UCSC Cancer Browser (https://www.cancer.gov/tcga), along with the clinical-pathological phenotypes. Methylation (HM450) beta-values for genes in 885 cases (Breast Invasive Carcinoma (TCGA, Firehose Legacy)) were used. Pearson's correlation analysis was used for the analysis of the correlation between the methylation degree and gene expression.

2.4. Statistical analyses

Data were analyzed by an unpaired t-test between cancer and normal groups for every single gene and one-way ANOVA followed by t-test. Statistically significant values of *$p < 0.05$, * *$p < 0.01$, * **$p < 0.001$ and * ** *$p < 0.0001$ were determined.

3. Results

3.1. Deregulation of drug resistance genes in breast tumors compared to normal samples

The differential expressions of drug resistance genes have been assessed at mRNA level in healthy and BC tissues. There was a significant rise in transcript levels of EIF2AK3, ANTXR1, EPCAM, B4GALNT1, PGP, CHEK2 and CHEK1 in BC tissues than in normal samples ($p < 0.0001$, $p < 0.0001$, $p = 0.0007$, $p = 0.0017$, $p = 0.0008$, $p < 0.0001$, $p < 0.0001$, respectively) (Fig. 1). CXCL8 and ABCC1 exhibited a trend of slight up-regulation in breast tumors in comparison with healthy individuals, although it was not statistically significant. On the other hand, the

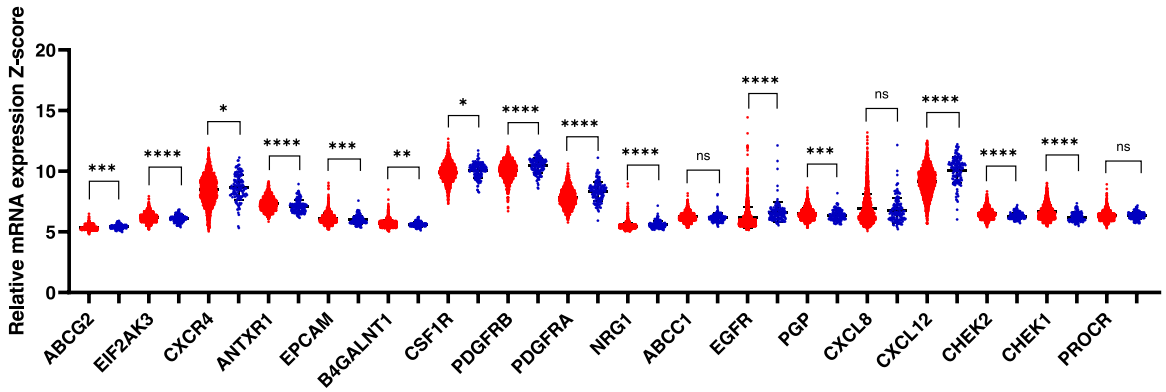

Fig. 1. Differential gene expression pattern of stem-cell related drug resistance factors in breast cancer and normal tissues. RNA-Seq (mRNA expression) data for breast cancer (red color) and normal (blue color) tissues in METABRIC cohort. Normal tissues (n = 148) and cancer tissues (n = 1826) of primary breast tumors have been used. Data were analyzed by an un-paired t-test between cancer and normal groups for every single gene. Statistically significant values of *p < 0.05, * *p < 0.01, * **p < 0.001 and * ** *p < 0.0001 were determined.

expression levels of ABCG2, CXCR4, CSF1R, PDGFRB, PDGFRA, NRG1, EGFR, and CXCL12 were significantly downregulated in BC tumors compared to control tissues from healthy individuals (p = 0.0004, p = 0.0311, p = 0.0352, p < 0.0001, p < 0.0001, p < 0.0001, p < 0.0001, p < 0.0001). Moreover, though not statistically significant, the mRNA level of PROCR was slightly lower in BC samples as compared to healthy normal tissues (Fig. 1).

3.2. Differential expression of drug resistance genes based on breast cancer grades

In this study, mRNA expressions of all drug resistance genes in various grades of BC (grade I vs. II, and grade I vs. III) were compared (Fig. 2). Higher mRNA expression of ABCG2 was identified in grade I compared to grades II and III. (p = 0.0094, and p < 0.0001, respectively). Similarly, we did detect significantly higher mRNA levels of PDGFRA, CXCL12 as well as PROCR in the first grade of breast tumors in comparison with second-and third-grade tumors (PDGFRA (p = 0.0040, p = 0.0077), CXCL12 (p = 0.0002, p < 0.0001), PROCR (p = 0.0438, p < 0.0001)). There was also a significant rise in mRNA expression of PDGFRB in grade I compared to grade III (p < 0.0001). In contrast, transcript levels of CXCR4, ANTXR1, EPCAM, B4GALNT1, EGFR, and CXCL8 were significantly higher in grade III vs. grade I (p < 0.0001, p = 0.0011, p = 0.0003, p = 0.0063, p < 0.0001, p < 0.0001). Additionally, ABCC1, CHEK1, and CHEK2 showed upregulation in grades II and III BC samples in comparison to grade I tumors (ABCC1 (p = 0.0039, p < 0.0001), CHEK1 (p < 0.0001, p < 0.0001), CHEK2 (p = 0.0004, p < 0.0001). However, no significant differences were observed in EIF2AK3, CSF1R, NRG1, and PGP mRNA expression among various BC grades (Fig. 2).

3.3. Differential expression of Hippo pathway genes in breast cancer molecular subtypes

As part of the study, we compared the levels of mRNA expression of drug resistance genes in various molecular subtypes of BC. The 10 comparisons performed here are as follows: Basal/Claudin-low vs. HER2, Basal/Claudin-low vs. Lum(inal) A, Basal/Claudin-low vs. Lum (inal) B, Basal/Claudin-low vs. Normal, HER2 vs. Lum A, HER2 vs. Lum B, HER2 vs. Normal, Lum A vs. Lum B, Lum A vs. Normal, and Lum B vs. Normal. Based on our analyses, the highest ABCG2 mRNA levels were detected in Normal subtypes, with HER2 subtype showing the lowest rate. ABCG2 expression was significantly higher in Basal/Claudin-low vs. HER2 (p = 0.0011) while ABCG2 mRNA showed upregulation in Lum A and Normal subtypes compared to Basal/Claudin-low (p < 0.0001, p < 0.0001) (Fig. 3). HER2 subtype expresses a significantly lower level of ABCG2 compared to Lum A, Lum B, and Normal subtypes (p < 0.0001, p = 0.0004, p < 0.0001, respectively). Both Lum A, and Normal subtypes showed substantially higher ABCG2 mRNA than the Lum B subtype (p < 0.0001, p < 0.0001).

CXCR4 mRNA expression was significantly higher in Basal/Claudin-low subtype compared to all other subtypes including HER 2 (p < 0.0001), Lum A (p < 0.0001), and Lum B (p < 0.0001) and Normal (p = 0.0004). An increased level of CXCR4 transcript was also observed in the Normal BC subtype vs. HER2, Lum A, and Lum B (p = 0.0060, p < 0.0001, p = 0.0005, respectively). No other significant alteration of CXCR4 level was identified among the other subtypes (Fig. 3).

In contrast to CXCR4 expression pattern in BC subtypes, Basal/Claudin-low subtype expresses the lowest level of EIF2AK3 mRNA among subtypes, followed by Normal subtype. mRNA expression of EIF2AK3 was significantly lower in Basal/Claudin-low subtype vs. HER2, Lum A, Lum B subtype (p = 0.0005, p = 0.0016, p < 0.0001). However, the difference in EIF2AK3 mRNA levels between Normal and Basal/Claudin-low subtypes was not statistically significant. Additionally, mRNA levels of EIF2AK3 were significantly reduced in Normal subtype of BC in comparison with HER2 (p = 0.0002), Lum A (p = 0.0002), Lum B subtypes (p < 0.0001). Intensified level of EIF2AK3 was identified in Lum B when compared to Lum A (p < 0.0001) as well.

HER2 subtype exhibited the highest level of ANTRX1 expression in comparison with Basal/Claudin-low, Lum A, Lum B, and Normal (p < 0.0001 for all comparisons). Moreover, a statistically significant rise in ANTRX1 mRNA expression was observed in Basal/Claudin-low as compared to that seen in Lum A (p = 0.0002), Lum B (p = 0.0022), and Normal (p < 0.0001) subtypes. Both Lum A and Lum B subtypes expressed a higher level of ANTRX1 mRNA compared to the Normal subtype (p = 0.0095, p = 0.0122, respectively), while the difference between the mRNA expression of ANTRX1 in Lum A and Lum B was not statistically significant.

The highest level of EPCAM mRNA expression was detected in Lum B subtype (Lum B vs. Basal/Claudin-low: p = 0.0156, Lum B vs Lum A: p < 0.0001, Lum B vs. Normal: p < 0.0001), with Normal subtype showing the lowest expression of EPCAM transcript (Normal vs. Basal/Claudin-low: p = 0.0017, Normal vs HER2: p < 0.0001). Furthermore, we observed a significant upregulation of EPCAM mRNA in HER2 vs. Lum A subtype (p < 0.0001) and in Basal/claudin-low vs. Lum A (p < 0.0001).

A significant increase in B4GALNT1 mRNA expression was identified in Basal/Claudin-low subtype compared to all other subtypes including HER2 (p = 0135), Lum A (p < 0.0001), Lum B (p = 0.0096), and Normal (p < 0.0001) subtypes. There was also a significant rise in B4GALNT1 expression in HER2 subtype compared to Normal breast

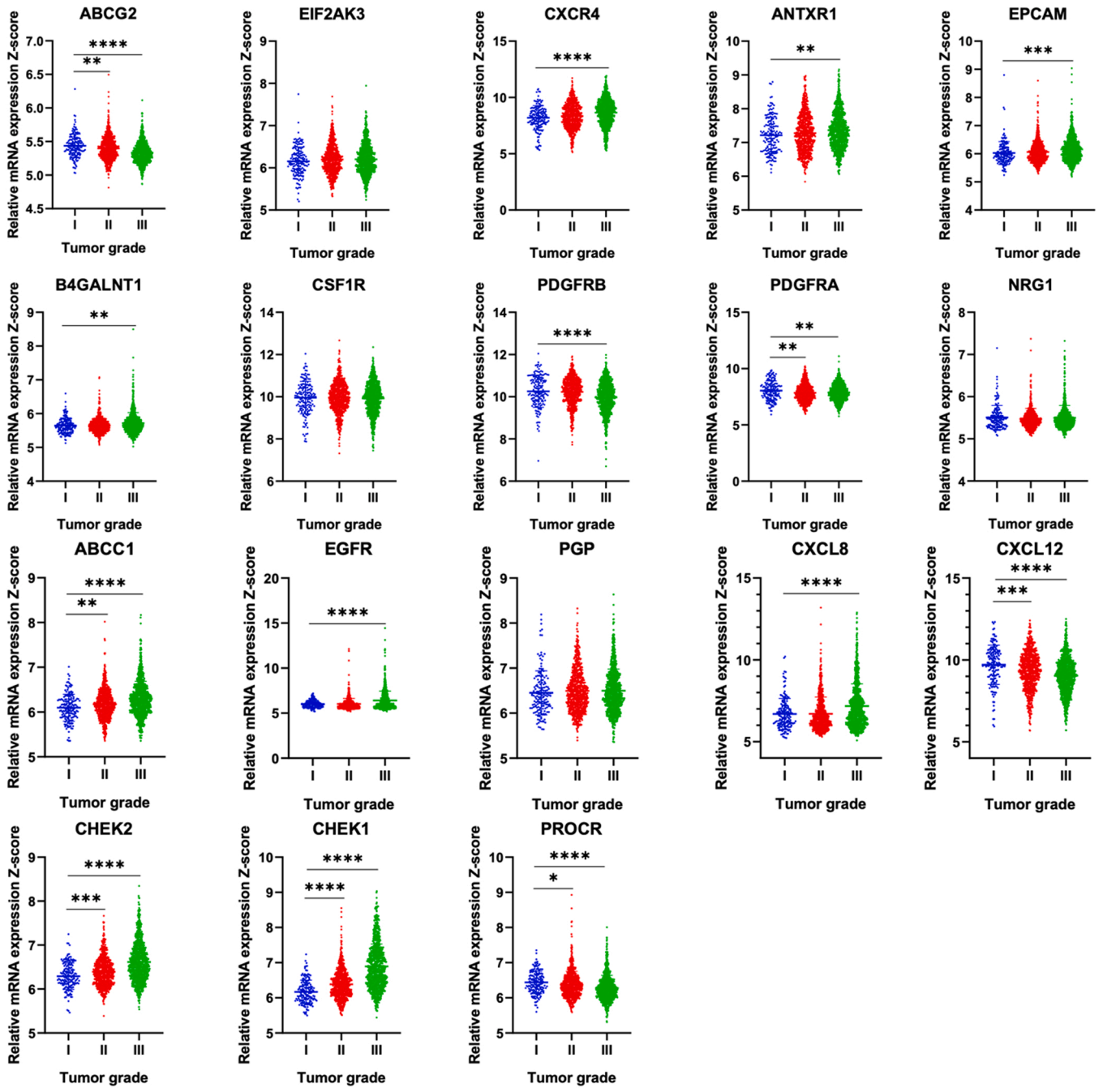

Fig. 2. Relative expression of stem-cell related drug resistance factors across different breast cancer tumor grades. Data were extracted from the METABRIC dataset and analyzed by one-way ANOVA followed by t-test. Statistically significant values of (P-value*: P-value < 0.05, **: P-value < 0.001, ***: 0.0001 < P-value = 0.0001, ****: P-value < 0.0001) were determined.

tumors subtype ($p = 0.0149$), while no significant alterations in B4GALNT1 mRNA levels were found between HER2 and Lum A/B. Additionally, Lum B expressed a higher level of B4GALNT1 mRNA in comparison with Lum A and Normal ($p = 0.0023$, 0.0019, respectively).

Our analyses demonstrated that Basal/claudin-low subtype showed the highest expression of CSF1R mRNA compared to all other subtypes ($p < 0.0001$ for all four comparisons). Also, a significant increase in CSF1R mRNA expression was observed in Normal subtype vs. Lum A ($p = 0.0141$), Lum B ($p < 0.0001$), and HER2 subtypes ($p < 0.0001$). Significant upregulation of CSF1R mRNA was also identified in Lum A compared to Lum B subtype. PDGFRB mRNA expression in the Normal subtype was substantially higher in comparison with all other four subtypes ($p < 0.0001$ for Normal vs. HER2, Basal/Claudin-low, Lum B, and $p = 0040$ for Normal vs. Lum A). However, Lum B breast tumors expressed the lowest rate of PDGFRB mRNA among various subtypes (Lum B vs. Basal/Claudin-low: $p = 0.0021$, Lum B vs. HER2: $p = 0.0030$, Lum B vs. Lum AL: $p < 0.0001$). Moreover, PDGFRB transcripts level in the Lum A subtype was higher compared to HER2 ($p < 0.0001$), and Basal/Claudin-low ($p < 0.0001$).

mRNA expression of PDGFRA was significantly increased in the Basal/Claudin-low subtype compared to the HER2, Lum A, and Lum B subtypes ($p < 0.0001$ for all). However, no significant difference in PDGFRA expression was noted between Basal/Claudin-low subtype and Normal subtype showing the highest level of PDGFRA mRNA among BC subtypes (Normal vs. HER2: $p < 0.0001$, Normal vs Lum A: $p < 0.0001$, Normal vs Lum B: $p < 0.0001$). Also, a significant downregulation of

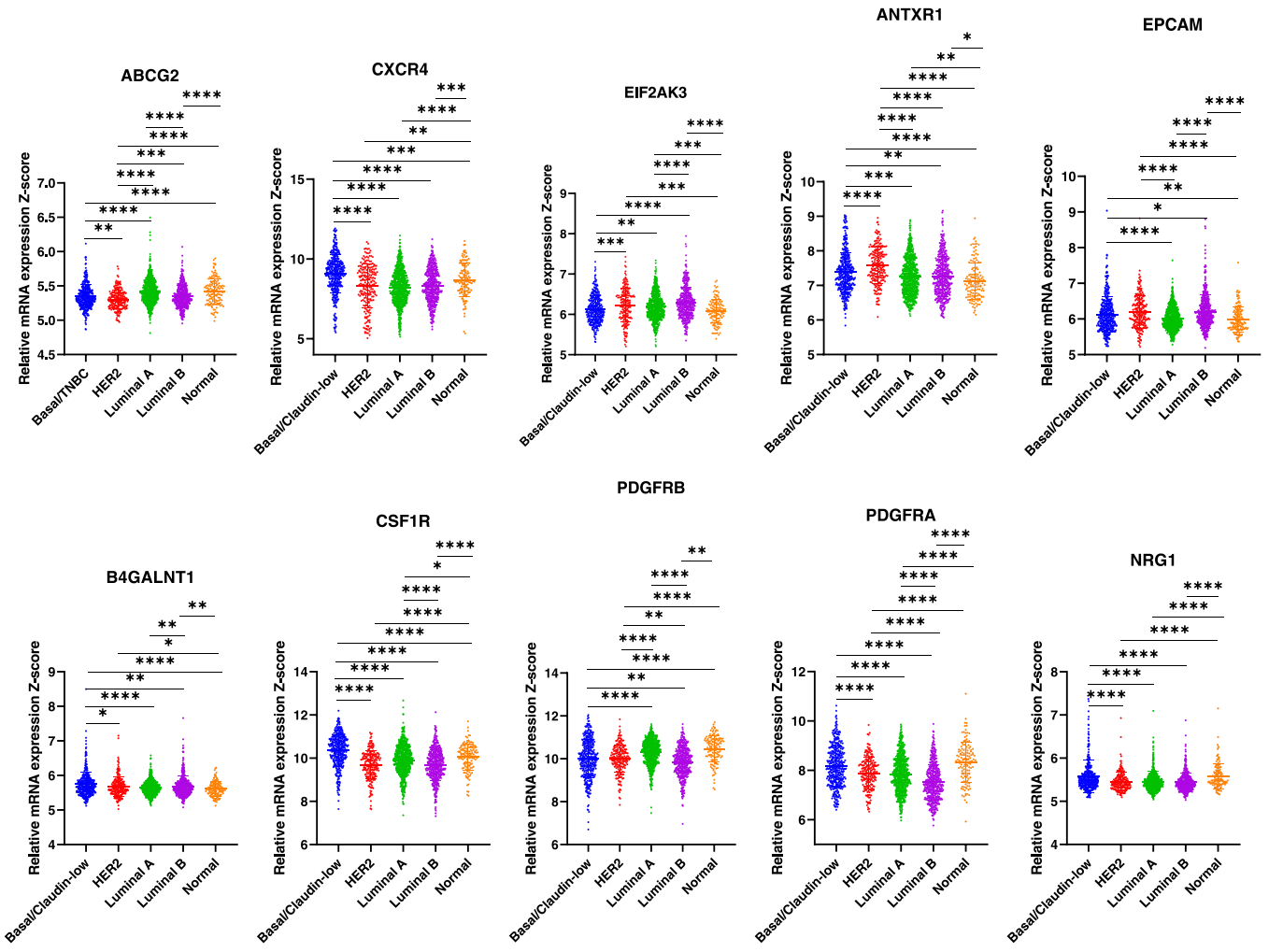

Fig. 3. Differential gene expression pattern of stem-cell related drug resistance factors across different breast cancer intrinsic subtypes. RNA-Seq data for genes involved in breast cancer drug resistance from METABRIC by Pam50 gene expression subtype classification. Scatterplots show that there is a significant association between breast cancer subtypes and the level of gene expression in breast cancer patients. Data were analyzed by one-way ANOVA followed by t-test. Statistically significant values of *$p < 0.05$, * *$p < 0.01$, * **$p < 0.001$ and * ** *$p < 0.0001$ were determined.

PDGFRA in the Lum B subtype vs. HER2 and vs. Lum A subtypes ($p < 0.0001$ for both) was seen.

The results also indicated that the highest expression of the NRG1 gene was observed among the Normal subtype, and the lowest expression was detected among the Lum B subtype. The expression of the NRG1 gene was significantly higher in Basal/Claudin-low compared with HER2 ($p < 0.0001$), Lum A ($p < 0.0001$), and Lum B ($p < 0.0001$) among BC subtypes. In addition, the increased expression level of the Normal subtype was observed compared to HER2 ($p < 0.0001$), Lum A ($p < 0.0001$), and Lum B ($p < 0.0001$), which were statistically significant.

On the other hand, the ABCC1 gene was highly expressed in the Basal/Claudin-low subtype, while it showed the lowest expression in the Lum A subtype. The expression of the ABCC1 gene was significantly decreased in Lum A subtype compared to all other subtypes except the Normal subtype (Basal/Claudin-low vs. Lum A: $p < 0.0001$, HER2 vs. Lum A: $p < 0.0001$, Lum B vs. Lum A: $p = 0.230$, and Normal vs. Lum A: $p = 0.8317$). Additionally, a significant increase in the expression of the ABCC1 gene was detected among the Basal/Claudin-low subtype compared to Lum A ($p < 0.0001$) and Normal ($p = 0.0001$) Subtype of BC. The results also demonstrated an increased expression of the ABCC1 gene in the HER2 subtype compared to Lum B ($p = 0.0025$) and Normal ($p = 0.0021$) subtype.

Our data revealed that the EGFR gene had the highest expression in Basal/Claudin-low and the lowest expression in Lum B. The up-regulation of the EGFR gene in the Basal/Claudin-low subtype was statistically significant compared to all other subtypes of BC (Basal/Claudin-low vs. HER2, Basal/Claudin-low vs. Lum A, Basal/Claudin-low vs. Lum B, and Basal/Claudin-low vs. Normal: $p < 0.0001$). In addition, the expression of Lum B was significantly decreased compared to all other subtypes (Lum B vs. HER2, Lum B vs. Lum A, and Lum B vs. Normal: $p < 0.0001$). Besides, the expression of the EGFR gene was statistically increased in the HER2 subtype compared to Lum A ($p < 0.0001$) and decreased in Lum A compared to Normal ($p < 0.0001$).

Furthermore, the analyses indicated that the PGP gene was highly expressed in the HER2 subtype compared to other subtypes. In contrast, the Basal/Claudin-low subtype had the lowest expression compared to all other subtypes of BC (Basal/Claudin-low vs. HER2: $p < 0.0001$, Basal/Claudin-low vs. Lum A: $p < 0.0001$, Basal/Claudin-low vs. Lum B: $p < 0.0001$, and Basal/Claudin-low vs. Normal: $p = 0.0166$). The up-regulation of the PGP gene in the HER2 subtype was statistically significant when compared to Basal/Claudin-low ($p < 0.0001$), Lum A ($p < 0.0001$), and Normal ($p < 0.0001$) subtypes. Besides, the higher expression of the PGP gene was reported in Lum B compared to Lum A and Normal ($p < 0.0001$) and Lum A compared to Normal ($p = 0.0020$).

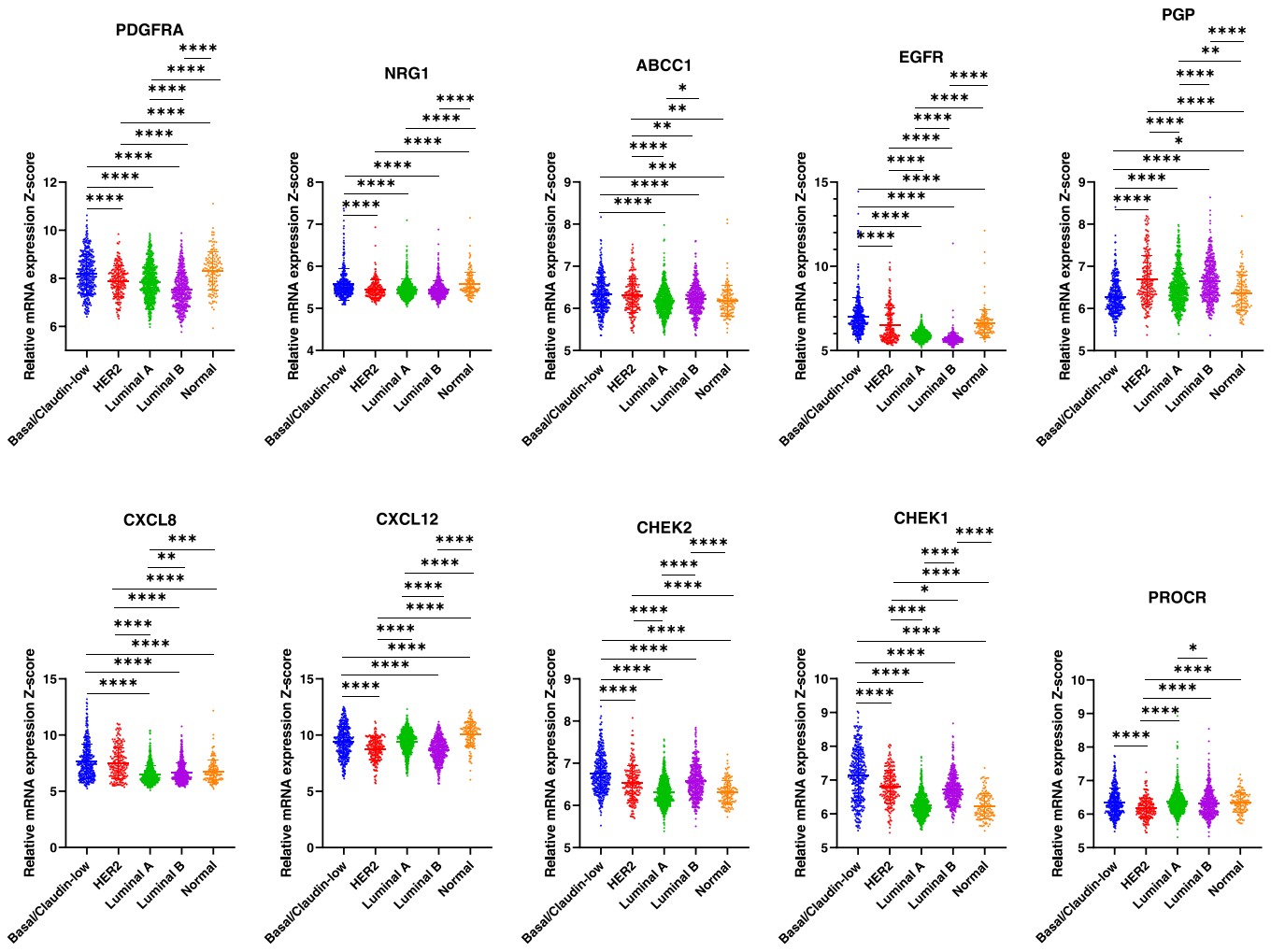

Fig. 3. (*continued*).

The expression of the CXCL8 was the highest in Basal/Claudin-low and the lowest in Lum A subtype of BC. The higher expression of the CXCL8 was reported as statistically significant in Basal/Claudin-low compared to Lum A, Lum B, and Normal subtypes ($p < 0.0001$). However, the lower expression of CXCL8 was detected in Lum A compared to all other subtypes (Lum A vs. HER2: $p < 0.0001$, Lum A vs. Lum B: $p = 0.0046$, and Lum A vs. Normal: $p = 0.0007$). In addition, the expression of CXCL8 was significantly increased in the HER2 subtype compared to Lum B and Normal subtypes ($p < 0.0001$).

The Normal and the Lum B subtypes presented the highest and lowest expression of the CXCL12 gene among BC subtypes, respectively. The expression of CXCL12 was significantly higher in the Normal subtype compared to all other subtypes of BC ($p < 0.0001$). The CXCL12 gene was significantly downregulated in the Lum B subtype compared to all other subtypes except HER2 ($p < 0.0001$). Additionally, the expression of CXCL12 was significantly lower in HER2 compared to Basal/Claudin-low and Lum A subtypes ($p < 0.0001$).

The data showed that the expression of CHEK1 was significantly elevated in Basal/Claudin-low compared to all other subtypes of BC ($p < 0.0001$). In addition, the intensified expression of CHEK1 could be observed in Lum B compared to Lum A and Normal ($p < 0.0001$). Likewise, the expression of CHEK1 was increased in HER2 compared to Lum A ($p < 0.0001$), Lum B ($p = 0.0121$), and Normal ($p < 0.0001$) subtypes.

The expression of CHEK2 had a similar pattern as CHEK1 and was significantly up-regulated in Basal/Claudin-low compared to all other subtypes of BC ($p < 0.0001$). On the other hand, the increased amount of CHEK2 in HER2 and Lum B subtypes was detected compared to Lum A and Normal Subtypes (HER2 vs. Lum A: $p < 0.0001$, HER2 vs. Normal: $p < 0.0001$, Lum B vs. Lum A: $p < 0.0001$, and Lum B vs. Normal: $p < 0.0001$).

The PROCR gene was statistically decreased in the HER2 subtype compared to all other BC subtypes ($p < 0.0001$). Besides, the PROCR gene was significantly downregulated in Lum B compared to Lum A ($p = 0.0182$).

3.4. Deregulation of drug resistance genes in various stages of breast cancer

We compared the expression levels of all drug resistance genes in several stages of BC (stage I vs. II, stage I vs. III, stage I vs. IV, stage II vs. III, stage II vs. IV, stage III vs. IV) (Fig. 4). mRNA level of EIF2AK3 was significantly lower in stage II than in stage I and III BC samples ($p = 0.0389$, $p = 0.0052$, respectively). There was a significant decrease in the transcript level of CXCR4 in stage I compared to stage III and IV of BC ($p = 0.0015$, $p = 0.0042$, respectively), and the same trend was observed in stage II BC in comparison with stage III and IV ($p = 0.0103$, and 0.0105). EPCAM had higher mRNA expression in stage III breast tumors than in stage I and II samples ($p = 0.0269$, $p = 0.0107$, respectively). Moreover, a significant increase in B4GALNT1 and CHEK2 levels was observed in stage III of BC vs. stage I ($p = 0.0067$, $p = 0.443$). PDGFRB and PDGFRA mRNA expressions in stage I were substantially higher in comparison with stage II ($p = 0.0005$, $p < 0.0001$, respectively), while stage III BC tissues expressed a higher level of PDGFRA

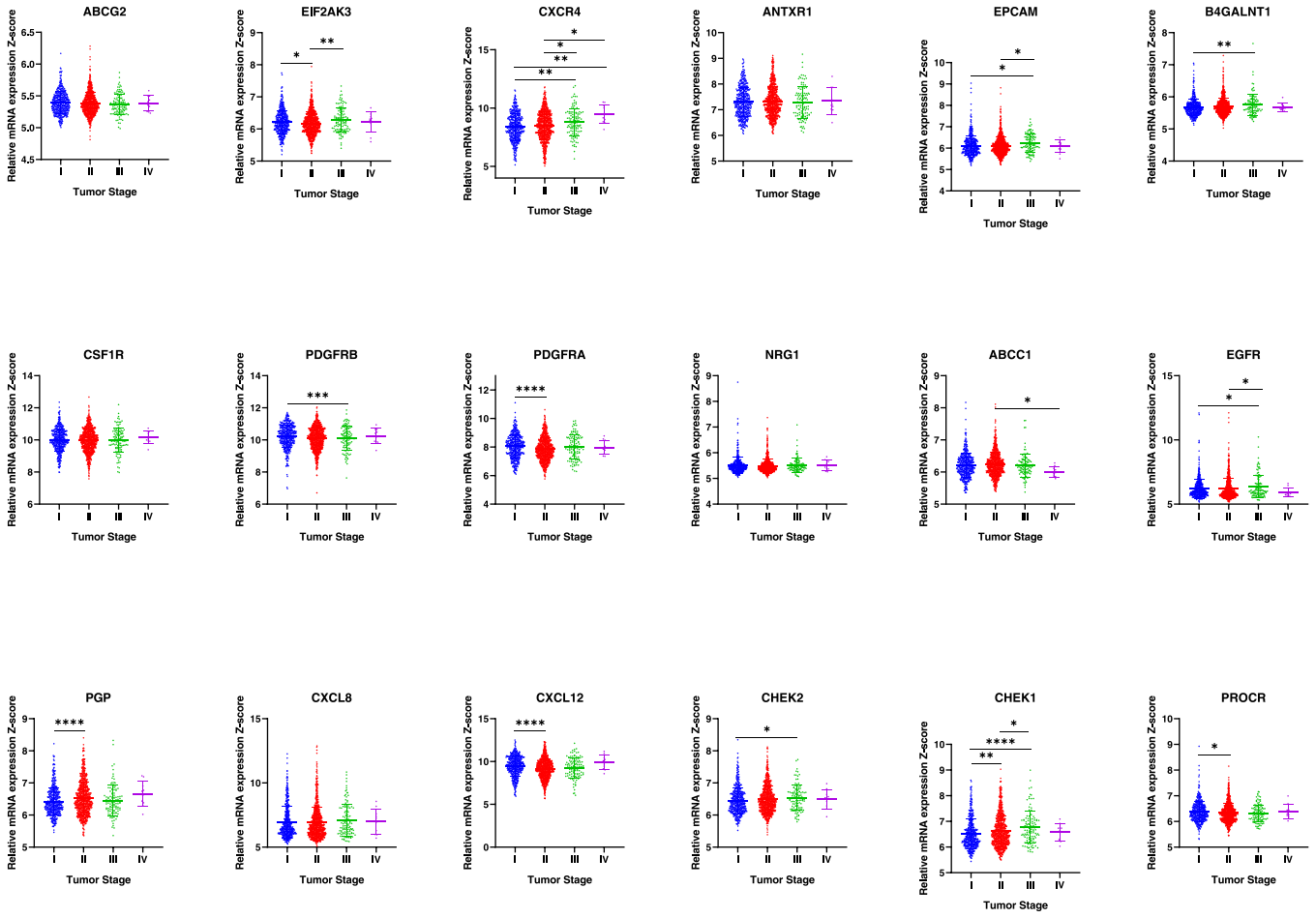

Fig. 4. Relative expression of stem-cell related drug resistance factors across different breast cancer tumor stages. Data were extracted from the METABRIC dataset and analyzed by one-way ANOVA followed by t-test. Statistically significant values of (p-value*: p-value < 0.05, **: p-value < 0.001, ***: $0.0001 <$ p-value $= 0.0001$, ****: p-value < 0.0001) were determined.

compared to stage II of BC ($p = 0.0226$). mRNA expression of EGFR increased significantly according to the stage progression of BC, with EGFR showing a higher level of expression in stage III compared to stages I and II ($p = 0.0286$, and $p = 0.0260$). Similarly, CHEK1 exhibited higher mRNA expression in stage III than in stages I and II ($p < 0.0001$, and $p = 0.0128$) and also in stage II vs. stage I ($p = 0.0016$). Stage IV breast tissues expressed a lower level of ABCC1 compared to stage II of the patients ($p = 0.0381$), and the PGP transcript level was lower in stage I vs. stage II ($p = 0.0002$). There was a significant decrease in CXCL12 and PROCR expressions in stage II BC patients when compared to stage I cases ($p < 0.0001$, and $p = 0.0312$). However, no significant differences in ABCG2, ANTXR1, NRG1, CSF1R, and CXCL8 expressions were found among various stages of BC.

3.5. *Drug resistance genes methylation status in breast cancer and its correlation with gene expression*

We further examined the correlation between mRNA levels of the genes causing drug resistance and their methylation levels among BC samples. The results indicated that there was a significant negative correlation between mRNA expression levels of CXCR4 (r:−0.4046), EPCAM (r:−0.3051), B4GALNT1 (r:−0.1318), CSF1R (r:−0.4754), PDGFRA (r:−0.1961), and PDGFRB (r:−0.6197) and their methylation status ($p < 0.0001$ for all of them except for B4GALNT1 with $p = 0.0002$). Moreover, the available data demonstrated that there was a significant negative correlation between the methylation status of ABCC1 (r:−0.2575), EGFR (r:−0.1523), PGP (r:−0.1423), CXCL12 (r:−0.3266), CHEK1 (r:−0.3668), CHEK2 (r:−0.2081), and PROCR (r:−0.2390) and their mRNA expression levels ($p < 0.0001$ for all of them) (Fig. 5). The data suggested that the altered methylation of the genes involved in drug resistance of patients with BC or the aberrant response to that methylation could result in a deregulated expression among the components causing drug resistance in tumor tissues compared to normal tissues.

3.6. *The drug resistance is correlated to many tumor evasion, metastasis, angiogenesis biomarkers in breast cancer*

Immune evasion is a key event in tumor progression [24] BC cells evade immune surveillance through changes in the tumor immune microenvironment and other mechanisms such as downregulating their antigen presentation [25,26]. Aberrant angiogenesis is critical in BC metastasis [27,28]. BC metastasis is still attributable to a considerable mortality rate [29]. Using the clinical data from the METABRIC study, we showed that the drug resistance promoting genes have a significant correlation with many metastases, angiogenesis, and tumor evasion markers in BC (Figs. 6 and 7).

4. Discussion

Women are at risk from BC, which has a high variability in response to treatment [30]. While advances have been made in the treatment of BC and the prognosis of the disease, multidrug resistance and subsequent relapses remain the most major obstacles to successfully

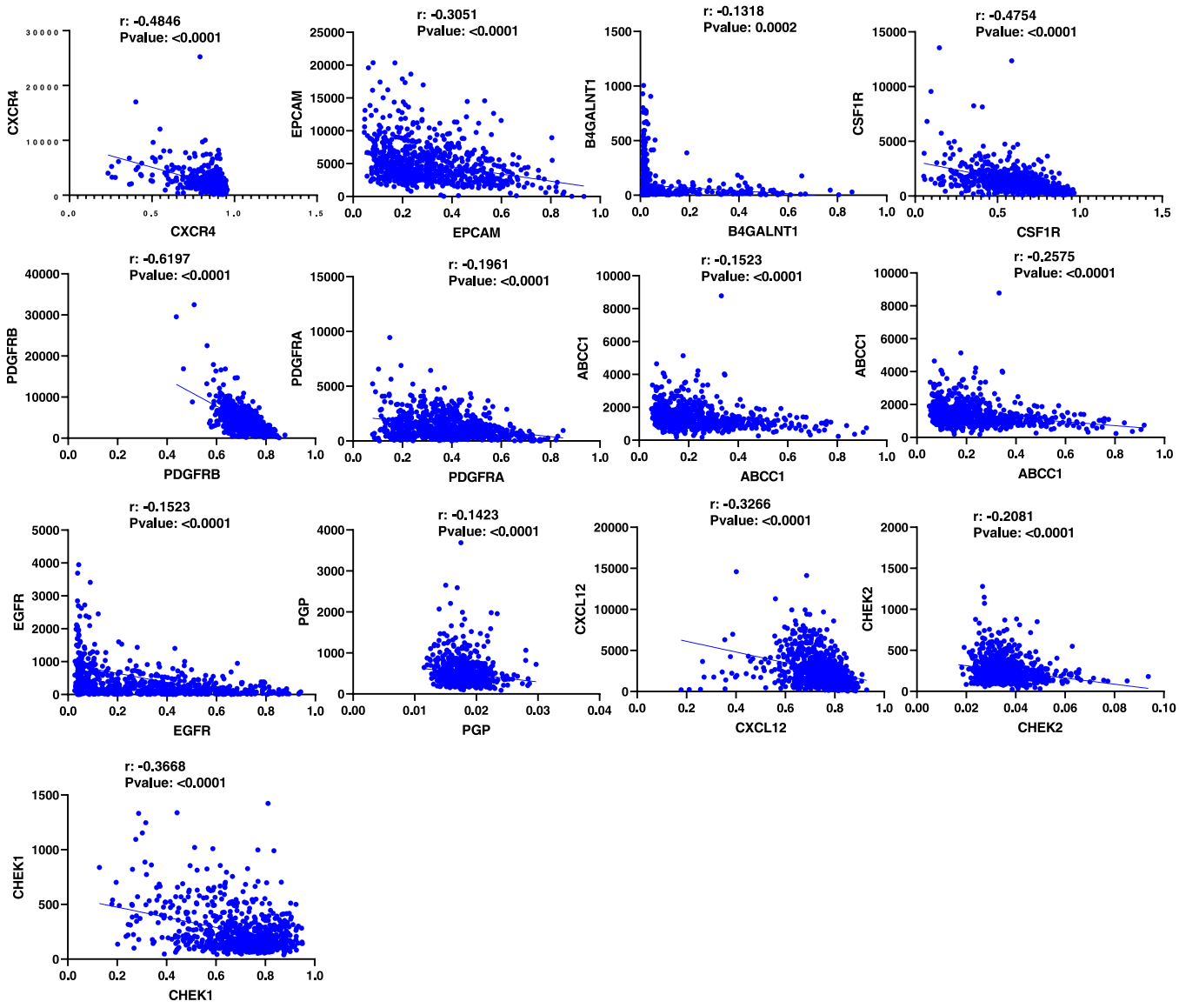

Fig. 5. Correlation between drug resistance factors and DNA methylation status in breast cancer. The Pearson correlation coefficients (r) and the relative p-values are shown. The association between genes was measured using the Pearson correlation coefficient (r) and respective computed p-value.

managing BC [10]. In spite of the fact that the molecular basis of multidrug resistance (MDR) is unclear, an accumulating body of evidence links genetic and epigenetic changes, including the overexpression of certain drug resistance genes, with the response to therapy [31]. The purpose of the present study was to examine the association between the expression levels of drug resistance genes and different subtypes of BC, stages, grades, and methylation genes. As a comprehensive summary of the potential inhibitors and/or pharmaceutical agents for the genes examined in this study, please refer to Table 1, which provides a succinct overview of the function of each gene, as well as information on the status of approved and investigational medications.

Based on their genome sequence and transmembrane domain (TMD) structure, the ATP-binding cassette (ABC) transporter family is composed of 49 energy-dependent membrane proteins classified into seven subfamilies (A-G). [32]. The ABC transporters are responsible for pumping out various drugs and metabolites across the cellular membranes and play a crucial role in monitoring the levels of endogenous compounds and protecting cells from xenobiotics [33]. Overexpression of specific ABC transporters has been observed in cancer cell lines, resulting in resistance to therapeutic agents that are the backbone of cancer treatments. ABCB1, ABCC1, and ABCG2 are the three most important ABC transporters, and their implication in the development of MDR has been extensively studied [34]. P-gp, or ABCB1, was the first ABC transporter discovered, and it is responsible for transporting neutral and positively charged molecules. A group of closely related genes encode the different isoforms of this protein (MDR1, MDR2/3). Drug resistance is solely a function of MDR1 [35]. Tumors, for example, can modulate the promoter region of the MDR1 gene and result in upregulation of P-gp, which can predict relapse, decreased survival rates, and resistance to chemotherapy [36,37]. Different malignancies with poor prognoses have been reported to overexpress P-gp, such as neuroblastoma, soft tissue sarcoma, and acute myeloid leukemia (AML) [38]. Additionally, several lines of evidence have demonstrated that the expression level of P-gp increases after neoadjuvant therapy or preoperative chemotherapy in patients with BC [37]. There was a 2.7-fold increase in the expression of P-gp in MDR BC cell lines compared to sensitive cell lines [36]. According to a meta-analysis, 41% of BC cases expressed P-gp and were three times more likely to be resistant to chemotherapy [38]. Compared with normal tissues, BC tissues expressed significantly higher levels of P-gp. Subtypes with HER2 positivity expressed the protein the most, while those with TNBC expressed the

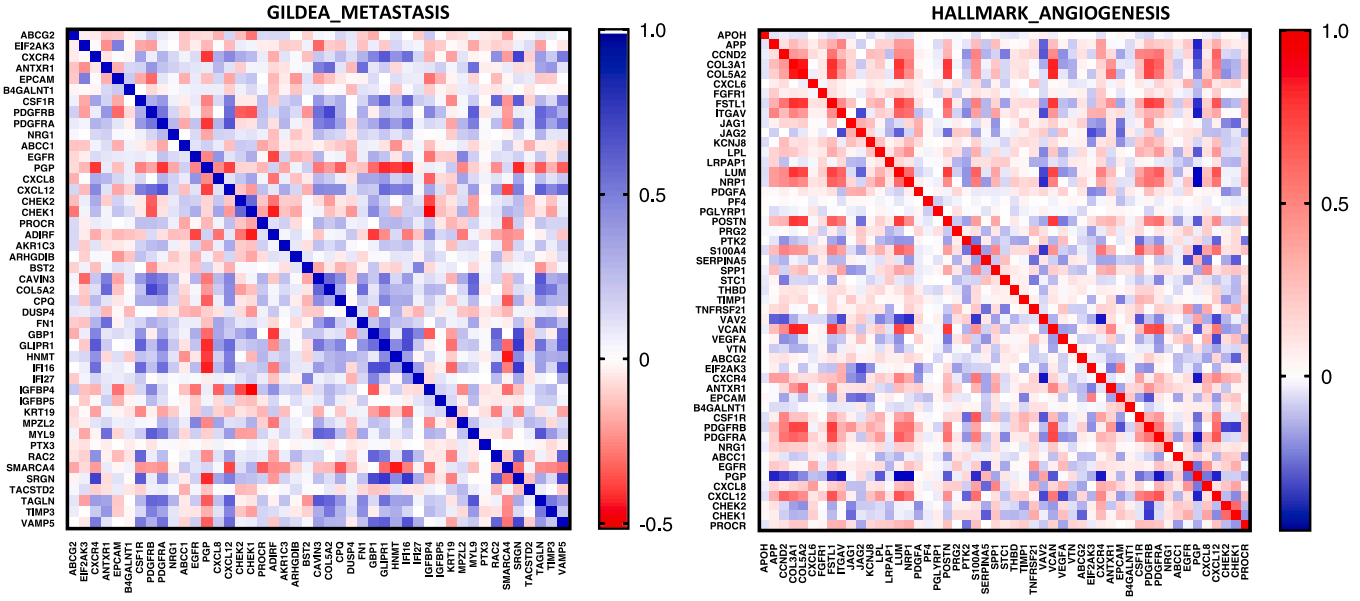

Fig. 6. Co-expression correlation analysis between genes involved in drug resistance and the (A) Metastasis (GILDEA_METASTASIS), and (B) Angiogenesis (HALLMARK_ANGIOGENESIS), and (C) immune evasion (LIN_TUMOR_ESCAPE_FROM_IMMUNE_ATTACK) gene sets (https://www.gsea-msigdb.org/gsea/msigdb). Correlation heatmap (Pearson r) of the transcriptomes from METABRIC breast cancer project samples (n = 1985). Red color refers to negative correlation, and the blue color indicates positive correlation.

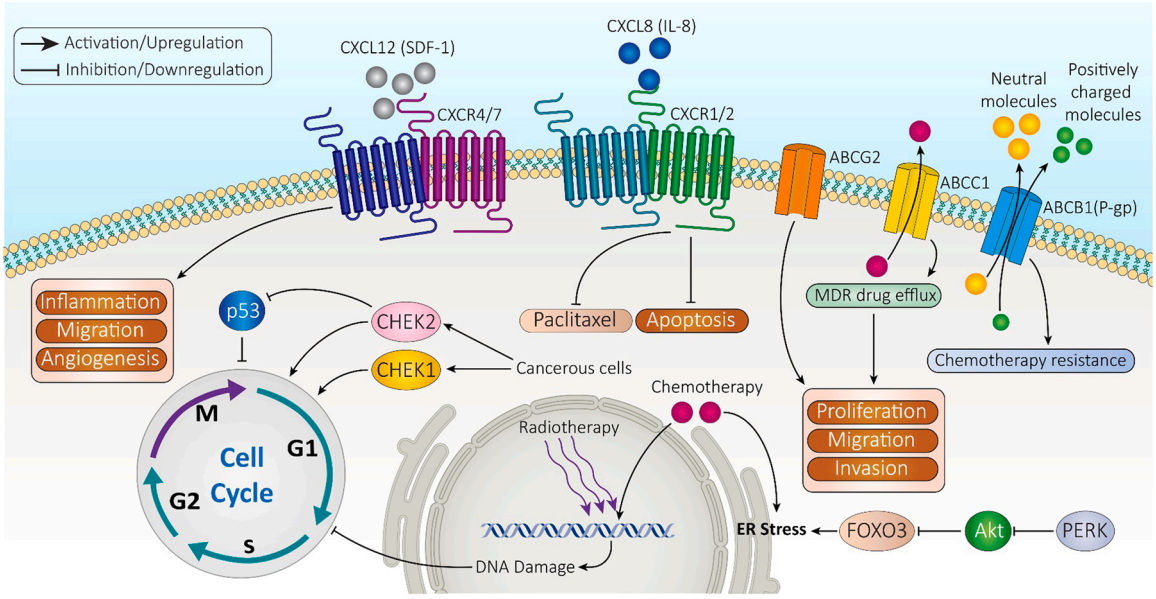

Fig. 7. An overview of the function of cancer stem cells in regulating drug resistance.

protein the least. As opposed to a previous study that found that P-gp was more prevalent in poorly differentiated and aggressive BC types, such as TNBC, when compared to other types [39]. Moreover, we found that P-gp expression was lower in stage I and that it was inversely correlated with the level of gene methylation.

MRP1, also known as ABCC1, is the second ABC efflux transporter associated with MDR in cancerous cells. The substrates of P-gp and ABCC1 for chemotherapy are identical with the exception of taxanes, which are poor substrates of ABCC1 [35]. Even though ABCC1 plays a significant role in MDR drug efflux, it plays a physiological role in cancer development, affecting cell proliferation, migration, and invasion. This protein has been demonstrated to play an important role in the proliferation of BC cells in previous studies. ABCC1 expression is increased in neuroblastoma and AML as well as P-gp, and its overexpression is related to the most aggressive subtypes of BC [38,40]. Findings in several studies emphasize the association between ABCC1 overexpression and metastatic breast cancer and poor outcome [34]. Based on our analysis, ABCC1 had a high expression level in the TNBC subtype, which confirms previous reports, and the lowest expression level in luminal A compared to all other BC subtypes. In comparison with healthy individuals, ABCC1 showed a slight up-regulation in breast tumors, although this was not statistically significant. The upregulation of ABCC1 correlates with grades II and III of BC, whereas the expression of ABCC1 is lower in stage IV compared to stage II. ABCC1 expression also correlates negatively with methylation level.

ABCG2, or BC resistance protein (BCRP), represents the last

Table 1
Genes and related drugs*.

Gene	Possible drug/inhibitor	Gene description
ABCG2	Tariquidar	encoding gene facilitates intra- and extra-cellular molecular transport.
CXCR4	Balixafortide	encodes SDF-1-specific protein with 7 transmembrane regions, facilitates HIV entry via CD4 interaction, and highly expressed in breast cancer cells.
EIF2AK3	3,5-dibromosalicylaldehyde	encodes protein that phosphorylates EIF2α, inhibiting translation initiation, repressing global protein synthesis, and potentially modulating mitochondrial function.
ANTXR1	6-thioguanosine	encodes tumor-specific endothelial marker linked to colorectal cancer, serves as receptor for Bacillus anthracis toxin causing anthrax.
B4GALNT1	TL-ADCs	sialic acid-containing glycosphingolipids, synthesized by GalNAc-T enzyme via β-1,4-linked GalNAc transfer to GM3/GD3, producing GM2/GD2.
CSF1R	Anti-CSF-1R monoclonal	encodes protein for colony stimulating factor 1, regulating macrophage production, differentiation, and function, mediating cytokine's biological effects.
PDGFRA	Avapritinib	produces cell surface receptor for platelet-derived growth factors, mitogenic for mesenchymal cells, forms homodimers or heterodimers with PDGFRA and PDGFRB.
PDGFRB	Imatinib	generates cell surface receptor for platelet-derived growth factors, mitogenic for mesenchymal cells, crucial for cardiovascular development, and involved in actin cytoskeleton rearrangement.
EPCAM	Adecatumumab	member of type I membrane protein family, expressed on normal epithelial cells and gastrointestinal carcinomas, enables homotypic calcium-independent cell adhesion, and targeted in immunotherapy for carcinomas.
NRG1	Duligotuzumab	mediates cell-cell signaling, crucial for multi-organ growth and development, generates diverse isoforms via alternative promoter usage and splicing.
ABCC1	Doxorubicin	encodes protein for ATP-binding cassette superfamily, transports molecules across intra- and extra-cellular membranes, associated with multi-drug resistance.
EGFR	Cetuximab	generates transmembrane glycoprotein in protein kinase superfamily, receptor for epidermal growth factors, promotes dimerization and tyrosine autophosphorylation, leading to cell proliferation.
PGP	Perospirone	involved in glycerol biosynthesis, glycerophospholipid metabolism, and negative regulation of gluconeogenesis
CXCL8	Recombinant Tumor Necrosis Factor-Alpha	encodes inflammatory response mediator, functions as chemotactic factor guiding neutrophils to infection site.
CXCL12	Motixafortide	produces intercrine family member, ligand for CXCR4 receptor, involved in embryogenesis, immune surveillance, inflammation, tissue homeostasis, and tumor growth/ metastasis.
CHEK2	Olaparib	generates putative tumor suppressor protein with forkhead-associated domain, activated in response to DNA damage and replication blocks, rapidly phosphorylated.
CHEK1	Rabusertib	involved in checkpoint-mediated cell cycle arrest, integrates signals from ATM/ATR in DNA damage response, associates with chromatin during meiotic prophase I.
PROCR	Thrombin	produces N-glycosylated type I membrane protein, involved in blood coagulation pathway, enhances activation of protein C.

References
https://www.coremine.com
https://www.genecards.org

discovered ABC transporter involved in MDR. BCRP overexpression has been observed in several drug-resistant cell lines and all types of tumors such as non-small cell lung cancer (NSCLC) [41], ALL [42], endothelial cells of the adenocarcinomas of the digestive tract, lung, and endometrium [43]. Using cancer cell lines, Chen et al. concluded that silencing or inhibiting BCRP suppressed cellular proliferation [44]. Another study found a correlation between ABCG2 expression and the grade, N-stage, and TNM stage of invasive BCs [45,46]. The ABCG2 transporter is enriched in TNBC, as well as other subtypes of BC [47]. Overall, ABCB1, ABCC1, and ABCG2 overexpression levels differ by subtype and are highly correlated with TNBCs. According to Liaghati et al., ABCG2 expression levels were compared between BC tissues and adjacent non-cancerous tissues. Neither tumoral tissues nor ANCTs were significantly different in terms of ABCG2 expression [48]. Compared to grades II and III, grade I exhibited higher mRNA expression of ABCG2. It should be noted, however, that ABCG2 expression levels in BC tumors were significantly reduced when compared with control tissues from healthy individuals.

During chemotherapy and radiotherapy, DNA is damaged, cancer cells are inhibited from proliferating and cell cycle arrest is induced [49]. On the other hand, cancerous cells utilize DNA repair mechanisms, such as checkpoint activation, to escape these therapies and become resistant to chemotherapy. CHEK1 is a conserved protein kinase that is required for the speed limit in phase G2 of the cell cycle as well as mitosis during the cell cycle. [50]. As a result of overexpression of CHEK1, many human malignancies can develop, including lung, stomach, colon, bladder, ovarian, and cervical cancers [51]. CHECK1 has been reported to play either an oncogenic or an anti-oncogenic role depending on the type of cancer since reduced levels of CHEK1 expression have been observed in brain and central nervous system tumours [50]. CHEK1 overexpression correlates with tumor grade and disease recurrence [52,53] and reduced survival rates are more possible in patients with high expression of CHEK1 in bladder, brain, lung, ovary, and BCs compared with those with low expression [50]. In BC tissues, there is a high expression of CHEK1 mRNA levels, and it is more strongly expressed in triple-negative cancers than other subtypes [54]. Compared to normal tissues, mRNA levels of CHEK1 were significantly higher in BC tissues, and higher CHEK1 levels were related to the TNBC subtype, in keeping with the previous report. A higher level of CHEK1 expression was negatively associated with methylation status as well as grade II, III and stage III of BC.

CHEK2 is a tumor suppressor gene that functions as a transducer of DNA damage response to maintain genome integrity. [55]. In the cell cycle phase G2/mitosis, CHEK2 phosphorylates the tumor suppressor gene *p53* and improves its stability during DNA double-strand breaks

[56]. Despite the similarity in the name of CHEK1 and CHEK2, they are different in their kinase pocket structure [54]. According to the results of a study in 2021, the CHEK2 expression level is significantly elevated in both early-onset and conventional subtypes of gastric cancer. [57]. As opposed to this, previous studies have demonstrated a low expression level of the CHEK2 protein in breast tumors, with a special emphasis on ER-positive tumors [58]. As a whole, BCs associated with CHEK2 are considered to be more likely to be ER positive, PR positive, and grade II [59]. CHEK2 is not a good prognostic marker for predicting metastasis in BC [60]. A significant increase in CHEK2 expression was observed in BC tissues as compared with normal tissues in the current study, and, in contrast with the previous studies, there was a higher expression of CHEK2 in the TNBC subtype. Grade II and III showed higher levels of mRNA for the gene, as well as stage III, and methylation status was negatively correlated with gene expression.

Chemokines and their cognate receptors have always been the focus of attention not only for their role in immunological processes but also because of their involvement in responses to chemotherapy and radiation, cancer progression and metastasis by promoting cell growth, survival, and angiogenesis [61]. CXCL8, also known as Interleukin-8 (IL-8) is a pro-inflammatory chemokine that plays its biological function as a leukocyte chemoattractant by binding to its receptors, CXCR1 and CXCR2 [62,63]. The CXCL8-CXCR1/2 axis attracts neutrophils to the site of infection to eliminate inflammatory stimulus by means of neutrophil oxidative burst [64]. Abnormal regulation of CXCL8 can lead to many inflammatory diseases such as cystic fibrosis, asthma, psoriasis and rheumatoid arthritis and multiple human cancers such as prostate, ovarian, breast, lung, colon, and skin cancer [64,65]. Overexpression and high levels of CXCL8 secretion have been reported in BC both in vivo and in vitro [66,67]. CXCL8 level is significantly upregulated in patients with bone metastasis and correlates with bone resorption and disease stage [68,69]. Overexpression of CXCL8 in TNBC tissues and cells was demonstrated which was associated with a poor prognosis. It also results in a decrease in cell apoptosis and resistance to paclitaxel [70]. CXCL8 also is able to elevate the aggressiveness of ER+ BC cells and empower the activity of BC stem-like cells (CSCs) by transactivating HER2 [71]. Database analysis of the current study showed that the expression level of CXCL8 is the highest in TNBC and the lowest in luminal A subtype and higher expression levels were related to grade III. It also had a slight up-regulation in breast tumors compared to healthy individuals, although it was not statistically significant.

CXCL12, also known as stromal cell-derived factor 1 (SDF-1), is another member of the chemokine family which binds to CXCR4 and CXCR7 [72]. The major function of CXCL12 is to maintain tissue hemostasis and regulate cell migration as an attractant and thus can be effective in the development and metastatic progression of cancers [73]. CXCL12 is highly expressed in bone marrow, liver and lung and attracts tumor cells supporting the hypothesis that these organs are the common targets for many tumors to metastasize. Increased expression of the CXCL12-CXCR4 axis and its role in metastasis has been improved in colorectal carcinoma, hepatocellular carcinoma, pancreatic cancer, melanoma and BC [74]. Especially in BC, CXCL12 is an extensively studied chemokine because high expression of that is reported in patients with lymph node and brain metastasis and a low overall survival rate. Also, the CXCL12-CXCR4 axis is a detrimental stimulus in transforming fibroblasts into cancer-associated fibroblasts (CAFs) that can trigger inflammation, angiogenesis, metastasis and chemoresistance and enhances BC invasiveness specifically in TNBC [75]. Although, some studies have reported opposite results which indicate that CXCL12 is a good prognostic factor in patients with BC and its overexpression correlates with small tumor size, positive ER status, and negative HER-2 status [76]. In the present study, the expression level of the CXCL12 gene was significantly decreased in BC tissues compared to normal tissues and normal subtypes had the highest expression level compared with other subtypes. Luminal B had the lowest expression level among the other BC subtypes. High levels of mRNA were associated with grade I and a significant decrease in expression level were observed in stage II. There was a negative correlation between CXCL12 methylation level and corresponding mRNA expression.

CXCR4 as a receptor of CXCL12 is the most common chemokine receptor expressed in various types of cancers [77]. CXCR4 is overexpressed in more than 23 different types of human cancers including kidney, lung, brain, prostate, breast, pancreas, ovarian, and melanomas and contributes to the tumor growth, angiogenesis, metastasis, and therapeutic resistance as same as the CXCL12 [78]. BC cells use CXCR4 to proliferate and metastasize to other organs. As a result, overexpression of CXCR4 is associated with a poor prognosis in patients with BC [75,79]. High expression of CXCR4 has been reported in 75% of patients with TNBC [75]. Kang et al. evaluated the levels of CXCR4 transcript in human BC tissues and corresponding normal tissues. The results showed that BCBC tissues highly expressed CXCR4 compared with corresponding normal tissues [80]. The results of the present study showed that CXCR4 was significantly downregulated in BC tumors compared to control tissues from normal individuals. Transcript levels of CXCR4 were significantly higher in grade III vs. grade I. there was a significant negative correlation between mRNA expression levels of CXCR4 and methylation status. Like previous studies, CXCR4 may be a useful prognostic indicator.

Protein folding is an important function of the eukaryotic endoplasmic reticulum (ER); interference with the protein homeostasis (proteostasis) or normal functioning of the ER under stress conditions leads to the accumulation of misfolded or unfolded proteins in the ER lumen, which will cause ‘ER stress’. This response triggers the unfolded protein response (UPR), a tightly protected signaling pathway [81]; Three ER-resident transmembrane proteins in ER are ER stress sensors. One of these proteins is PERK (EIF2AK3 or eukaryotic translation initiation factor 2-alpha kinase 3). Activation of these sensors increases protein folding and decreases protein load, but long-term leads to cell death [82,83]. The key role of the ER stress signaling pathway in the spread of cancer was first proposed in 2004 and its activation represents a hallmark of various human cancers that enable cancer cells to survive in adverse environmental conditions and have the ability to cope with stress. This leads to resistance to chemotherapy drugs [84,85]. Recently, it was shown that PERK also promotes resistance to ER stress and cytotoxic drugs through the repression of FOXO3 by promoting AKT activation in BC cells [83]. In this study, database analyses showed that BC samples express significantly higher levels of EIF2AK3 than normal breast tissue ($p < 0.0001$). However, no significant differences were observed in mRNA expression of EIF2AK3 among various BC grades.

Anthrax toxin receptor 1 (ANTXR1 or TEM8) is a protein encoded in humans by the highly conserved tumor endothelial marker 8 gene. TEM8 is an integrin-like cell surface protein and has been shown to play a role in endothelial cell migration and invasion. Blocking and knocking out TEM8 resulted in a decline in tumor growth in several preclinical cancer models [86]. According to studies, antibodies against the extracellular domain of the ANTXR1 gene reduced tumor-induced angiogenesis and increased the susceptibility of tumor types to anticancer agents; reports indicated that ANTXR1 is expressed on cancer cells of different tumors, including breast, neuroblastoma, and melanoma ANTXR1 expression in BC cells correlating with shorter survival outcome [87,88]. In the current study, BC samples showed a significant rise in mRNA level ANTXR1 gene in BC tissues than in normal samples ($p < 0.0001$). Transcript levels of ANTXR1 were significantly higher in grade III vs. grade I ($p = 0.0011$).

EpCAM (epithelial cell adhesion molecule) is a cell surface molecule encoded by the GA733–2 gene on the long arm of chromosome4. EpCAM plays an essential role in forming adhesive structures and is involved in cell-to-cell adhesion. The first described tumor antigen is much more highly expressed in epithelial cancers than in normal epithelial, and the tumor tissues usually lose organized adhesive structures. EpCAM is frequently overexpressed in human invasive BC. Also, this gene is associated with enhanced proliferation and malignant potential [89,90].

We found a significant high mRNA levels of the EpCAM gene in BC tissues than in normal samples ($p = 0.0007$). Transcript levels of the EpCAM were significantly higher in grade III vs. grade I ($p = 0.0003$). A significant negative correlation was shown between EpCAM gene methylation and mRNA expression level.

There are four β-1.4-glycosyltransferases in the B4GALNT family identified in human tissues; β-1,4-N-Acetyl-Galactosaminyltransferase1 (B4GALNT1) encodes the key enzyme B4GALNT1 for the biosynthesis of complex gangliosides, also known as GM2/GD2 synthase [91]. B4GALNT1 gene is considered to be key tumor-associated antigen and is highly expressed in the progression of various cancers. There is few evidence investigating the silencing of B4GALNT1 that can impact cell cycle. Some results revealed that the knockdown of B4GALNT1 resulted in cell cycle arrest at the G1 phase [92], and B4GALNT1 overexpressed in BC stem cells [93]. In this result, transcript levels of B4GALNT1 were reported to be significantly higher in BC tissues than in normal mammary tissue ($p = 0.0017$), and transcript levels of B4GALNT1 were significantly higher in grade III vs. grade I ($p = 0.0063$). There was a significant negative correlation between mRNA expression levels of B4GALNT1 methylation status.

CSF1R (colony-stimulating factor receptor) belongs to the type III protein tyrosine kinase receptor family; CSF1R-mediated signaling is critical for the differentiation and survival of the mononuclear phagocyte system. CSF1R and its ligand (CSF1) regulate proliferation and differentiation of the monocytes-macrophage lineage, which are abnormally expressed in many cancer types, including breast, prostate, ovarian, and endometrial cancer and CSF-1 and CSF-1R have an important role for tumor invasion and metastasis, also evidence suggests that the CSF-1/CSF-1R autocrine loop contributes to tumor invasion and metastasis to breast, so CSF1R inhibitors represent a class of immunemodulatory drugs [94–97]. This study shows that the expression levels of CSF1R were significantly downregulated in BC tumors compared to control tissues from healthy individuals ($p = 0.0352$). No significant differences were observed in CSF1R mRNA expression among various BC grades. Cancer is a systemic disease and tumor induces changes in the immune system to facilitate cancer progression and metastasis, especially in peripheral blood and distant lymphoid organs and subsequently dysregulated cytokine signaling.

Platelet-derived growth factor (PDGF) ligands and their receptors (PDGFRs) have been shown to be key regulators of cell growth and division [98]. PDGFs are members of the mitogen family; PDGFRs are receptors with intrinsic tyrosine kinase activity that regulate several functions in normal cells such as PDGFR signaling which has an important role during embryogenesis and is widely expressed in a variety of malignancies and its overexpression is associated with unfavorable outcome in several cancers [99]. Signaling through PDGFR-β is essential for maturation of blood vessels, white adipocytes, and kidneys, Several studies have shown that PDGFs/PDGFRs are often expressed in diverse tumors, and their expression levels correlate with tumor growth, drug resistance, and poor clinical outcomes [100,101]. PDGFRB mRNA significantly increased in the stroma of invasive BC versus normal breast stroma, while PDGFRA expression did not change dramatically [102]. BCPDGFRA was upregulated in paclitaxel-resistant BC cells. Hence, it was concluded that PDGFRA is a critical mediator of chemoresistance associated with EMT in BC [103]. However, our study demonstrated that the expression levels of PDGFRB ($p < 0.0001$) and PDGFRA ($p < 0.0001$) were significantly downregulated in BC tumors compared to healthy tissues. Also, we detected mRNA expression of PDGFRB and PDGFRA in various grades of BC. There was a significant rise in mRNA expression of PDGFRB in grade I compared to grade III ($p < 0.0001$), and mRNA level of PDGFRA was significantly higher in the first grade of BC in comparison with second-and third-grade tumors ($p = 0.0040$). They also identified that the expression of these genes negatively correlates with their methylation level.

EGFR is a member of the transmembrane tyrosine kinase receptors family and binds to epidermal growth factor (EGF), which is motogenic and mitogenic in various cell types [104]. EGF-EGFR binding results in cell survival and proliferation and functions as a promotor in tumorigenesis [105]. This receptor can be activated through different situations such as ligand-dependent or ligand-independent mechanisms and receptor overexpression which frequently occurs in cancer. Overexpression of EGFR has been considered a consequence of gene amplification in many cancer types including breast, lung, ovarian, cervical, bladder, esophageal, brain and head and neck cancers. A high level of EGFR in these cancers is associated with higher aggressiveness and poor prognosis [106]. Zhang et al. found that EGFR is overexpressed during the occurrence and development of esophageal carcinoma and is associated with cancer progression and unfavorable prognosis [104]. EGFR expression level is also elevated in 15–30% of breast carcinoma and correlates with large tumor size and poor clinical outcome [107]. In a cohort study of 47 cases of breast carcinoma, EGFR overexpression following the EGFR gene amplification was reported in 23% of the cases [108]. In another study, EGFR expression was 1.2 times greater in BC than in precancerous tissues and was closely related to clinical staging, tumor differentiation, and lymphatic metastasis of patients [109]. Unlike previous studies, the results showed that EGFR expression was significantly downregulated in BC tumors compared to healthy tissues. It has the highest expression in the TNBC subtype and the lowest expression level in the luminal B subtype. An affirmative study had previously reported that EGFR is overexpressed in 50–75% of cases with the TNBC subtype and is associated with poor prognosis [110]. EGFR expression was upregulated in grade III and was increased according to the stage progression with the highest expression level in stage III. The expression level of EGFR was also negatively correlated with gene methylation status.

Protein C receptor (PROCR) also known as epithelial protein C receptor (EPCR) is a membrane protein that provides an important balance in the coagulation process by binding to coagulation proteases such as protein C [111]. This receptor is expressed on the cell surface of stem cells in different tissues, including the mammary gland, hematopoietic system, and vascular endothelial cells. There are contradictory reports about PROCR suggesting that its expression can promote tumor growth and prevent tumor progression [112]. Overexpression of PROCR in vascular epithelial cells can presumably reduce metastasis by decreasing thrombin generation, which is vital for the survival of metastatic tumor cells [113]. In contrast, PROCR expression in lung cancer prevents apoptosis and stimulates cell migration in BC, leading to enhanced metastasis and tumor progression in both cancers [114]. In a cohort study of 207 cases with nasopharyngeal carcinoma (NPC), PROCR overexpression was associated with tumor metastasis and recurrence and also resulted in clinically poor prognosis and maintenance of stemness potential in NPC cells [115]. Elevated expression of PROCR results in poor prognosis in patients with ovarian cancer [116]. In BC, PROCR is a potential biomarker of cancer stem cells which is used to isolate subpopulations affecting recurrence and tumor growth [114]. PROCR is also a promising cell surface marker in highly aggressive TNBC subtypes and has a key role in tumorigenesis [117]. Our current findings revealed that PROCR was statistically decreased in the HER2 subtype compared to all other BC subtypes and despite having statistically no significance, the mRNA level of PROCR was slightly decreased in BC samples as compared to healthy normal tissues. PROCR expression level was higher in stage I and reversely had a significant decrease in stage II of BC. PROCR gene methylation and mRNA expression level showed a significant negative correlation.

The neuregulin 1 (NRG1) is a family member of epidermal growth factor (EGF) ligands which release the EGF-like domain in extracellular space under proteolytic activities [118]. NRG1 is the major activating ligand for HER3 receptors, which cannot activate other tyrosine kinases due to the lack of certain amino acid residues [18]. However, when HER3 receptors are activated by NRG1, they can form heterodimers with other HER family members, including HER2, and impact downstream oncogenic signaling pathways resulting in tumor progression and

therapy resistance [119,120]. Several studies suggest that NRG1 has an important role in the development and progression of various tumor types and its overexpression is associated with poor prognosis in pancreatic cancer, head and neck squamous cell carcinoma and BC [119]. NRG1 secreted from cancer-associated fibroblasts (CAFs) in the tumor microenvironment promotes anti androgen resistance in prostate cancer through the activation of HER3 [121]. NRFG1 can also induce cell proliferation in the colon and ovarian cancers through autocrine and paracrine modes [122,123]. In the case of BC, NRG1 upregulates the expression of proteins like matrix metalloproteinases that promote invasion and metastasis in HER2-overexpressing BC subtypes and results in tyrosine kinase inhibitor-resistant growth [18,120]. HER2/HER3 heterodimer is a powerful carcinogenic factor in promoting the proliferation of HER2-overexpressing BC cells [124]. However, a study demonstrated that the expression level of NRG1 in BC cells is usually low because the gene is frequently silenced by DNA methylation [125]. The results of the present study implied that NRG1 expression level was significantly lower in BC tissues related to healthy tissues and the normal subtypes had the highest expression level. The luminal B subtype had the lowest expression; among the BC subtypes, the TNBC subtype had a higher expression level.

According to studies, new therapies targeting BCSCs are critical because recent analyses of BCSCs in breast tumors have found a link between the ratio of BCSCs and poor prognosis [126]. Notch, Hedgehog, and Wnt pathways have a role in an increased number of BCSCs during and post-treatment. Hedgehog activates Gli1- and Ptch1-positive modulators, leading to BCSC proliferation. Transcription factors such as cyclinD1, c-myc, CDKN1A, and HES-related repressor protein target in the Notch pathway, and this pathway has been reported to act in BCSCs. Notch pathway targets genes that result in high proliferation and apoptosis prevention [127]. We investigated whether these studied genes induce CSCs. NRG1 treatment was reported to induce CSC characteristics in BC cell lines, expression levels of CSC markers were observed after NRG1 treatment [120]. It seems that ANTXR1 controls signaling in stem/progenitor cells of both normal and cancerous breast and is identified as a functional biomarker of normal stem cells and BC stem-like cells. EpCAM is widely expressed on CSCs, and also presents in bulk cancer cells. EpCAM is also a marker for CSCs in cancers of the prostate, colon, pancreas, breast, ovary, lung, and stomach/intestine [128,129]. Therefore, due to the characteristics of BC stem cells, and their role in drug and chemotherapy resistance for more effective therapies, BC disease may need to target therapy in this cell population.

CRediT authorship contribution statement

Pedram Torabian and Hassan Yousefi: The authors were responsible for conceiving and designing the experiments, as well as conducting the data analysis. **Aysan Fallah, Zahra Moradi, Tohid Naderi, & Mahsa Rostamian Delavar:** They were involved in writing the manuscript. **Yavuz Nuri Ertas & Ali Zarrabi:** They were involved in creating the graphical abstract and illustrating the signaling pathways. **Amir Reza Aref:** Supervised the project, provided critical feedback, and revised the manuscript.

Declaration of Competing Interest

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