Epigenetic Changes of CXCR4 and Its Ligand CXCL12 as Prognostic Factors for Sporadic Breast Cancer

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Abstract

Chemokines and their receptors are involved in the development and cancer progression. The chemokine CXCL12 interacts with its receptor, CXCR4, to promote cellular adhesion, survival, proliferation and migration. The CXCR4 gene is upregulated in several types of cancers, including skin, lung, pancreas, brain and breast tumors. In pancreatic cancer and melanoma, CXCR4 expression is regulated by DNA methylation within its promoter region. In this study we examined the role of cytosine methylation in the regulation of CXCR4 expression in breast cancer cell lines and also correlated the methylation pattern with the clinicopathological aspects of sixty-nine primary breast tumors from a cohort of Brazilian women. RT-PCR showed that the PMG-42, MCF7 and MDA-MB-435 breast tumor cell lines expressed high levels of CXCR4. Conversely, the MDA-MB-435 cell line only expressed CXCR4 after treatment with 5-Aza-CdR, which suggests that CXCR4 expression is regulated by DNA methylation. To confirm this hypothesis, a 184 bp fragment of the CXCR4 gene promoter region was cloned after sodium bisulfite DNA treatment. Sequencing data showed that cell lines that expressed CXCR4 had only 15% of methylated CpG dinucleotides, while the cell line that not have CXCR4 expression, had a high density of methylation (91%). Loss of DNA methylation in the CXCR4 promoter was detected in 67% of the breast cancer analyzed. The absence of CXCR4 methylation was associated with the tumor stage, size, histological grade, lymph node status, ESR1 methylation and CXCL12 methylation, metastasis and patient death. Kaplan-Meier curves demonstrated that patients with an unmethylated CXCR4 promoter had a poorer overall survival and disease-free survival. Furthermore, patients with both CXCL12 methylation and unmethylated CXCR4 had a shorter overall survival and disease-free survival. These findings suggest that the DNA methylation status of both CXCR4 and CXCL12 genes could be used as a biomarker for prognosis in breast cancer.

Introduction

Breast cancer is a major public health issue worldwide. In 2004, the most recent year available for global data, there were 1.15 million new breast cancer cases and over 500,000 deaths reported worldwide [1]. Although advances have been made in reducing the mortality rates and improving survival, cancer is still the leading cause of death among men and women under 85 years of age in the United States [2]. In Brazil, 49,420 new cases of breast cancer have been estimated to occur between 2010 and 2011 [3]. Data from the Unique System of Heath (SUS) demonstrated that the mortality rates for breast cancer are 12.6 out of every 100,000 cases in Brazilian women [http://mortalidade.inca.gov.br]. Metastases cause 90% of human cancer deaths [4]. For breast cancer, due to the inability to accurately predict the risk of metastasis, more than 80% of patients receive adjuvant chemotherapy. However, approximately 40% of these patients still relapse and die of metastatic breast cancer within five years [4]. Generally, cancer is described as a disease driven by progressive genetic abnormalities involving mutations in oncogenes and tumor suppressor genes as well as other chromosomal aberrations [3]. Breast cancer, similar to other types of cancer, is driven by epigenetic alterations, which do not affect the primary DNA sequence [6,7]. These alterations lead to aberrant transcriptional regulation, which results in changes in the expression pattern of genes implicated in many cellular functions. These epigenetic alterations include changes in DNA methylation and histone modifications [7]. DNA hypermethylation is frequently associated with gene repression and genomic instability through silencing of the DNA repair genes, and several genes have been shown to be silenced in different steps of breast cancer [9,10]. Although the list of hypermethylated genes involved in the tumorigenesis of breast cancer has increased, much of the focus has remained on the estrogen receptor alpha (ESR1) and progesterone receptor (PGR) as these proteins have been implicated in breast cancer development and progression [7].

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These genes are viable prognostic markers, and approximately 70% of patients are suitable candidates for endocrine therapy [11]. The HER2 protein, which is present in approximately 30% of patients, serves as another important molecular prognosis marker for breast cancer and makes tumors suitable for herceptin antibody treatment [12]. However, despite the existence of well-documented molecular markers, breast cancer deaths remain a major public health issue.

Understanding the molecular mechanisms involved in breast cancer initiation and progression could provide strategies to identify new diagnostic and prognostic markers as well as better treatment for the disease. Thus, we evaluated the expression pattern and methylation status of the CXCR4 gene, which encodes a well-known protein involved in breast cancer. The CXCR4 chemokine together with its ligand, CXCL12, are involved in the mechanism of breast cancer metastasis. Breast cancer cells from primary tumors over-expressing CXCR4 are attracted to CXCL12 expressing cells in the lung, lymph nodes, liver or bones, which leads to the metastasis of detached tumor cells [13]. Immunohistochemical analyses have shown that specific patterns of CXCR4 expression (i.e., in the nucleus or cytoplasm) are correlated with a high nuclear grade [14] or lymph node metastasis [15,16]. Recent studies have indicated that the epigenetic mechanisms that negatively regulate the expression of CXCR4 and ESR1 are involved in breast cancer metastasis and correlate with poor survival of patients [17]. Additionally, in melanoma and pancreatic cancer, the CXCR4 promoter is regulated by increased DNA methylation, which results in lower CXCR4 mRNA expression [18,19].

In this study, we evaluated the methylation pattern of the CXCR4 gene promoter in breast tumor cell lines and primary tumor samples and correlated this pattern with clinicopathological data. We also compared the results from the CXCR4 DNA methylation study with the results from our previous CXCL12 study [17]. Together, these results suggest that the epigenetic regulation by DNA methylation of both the CXCR4 and CXCL12 genes in breast cancer could serve as a potential biomarker to indicate patient prognosis.

Results

CXCR4 expression in breast tumor cell lines

The expression pattern of CXCR4 in four breast tumor cell lines was evaluated using RT-PCR. A 389 bp transcript corresponding to the CXCR4 gene was detected in the PMC-42, MCF-7 and MDA-MB-436 cell lines (Fig. 1A). In contrast, CXCR4 expression was not detected in the MDA-MB-435 cell line. To determine if CXCR4 expression was lost, all analyses were repeated at least twice. GAPDH expression was detected in all samples tested (Fig. 1A).

To confirm the epigenetic transcriptional silencing of CXCR4 in breast cancer, we treated the MDA-MB-435 cell line with the demethylating agent 5-aza-2′-deoxycytidine (5-aza-CdR). As previously demonstrated in pancreatic and melanoma cell lines, the expression of CXCR4 was restored in the MDA-MB-435 cells upon 5-aza-CdR treatment (Fig. 1B) [18,19].

CXCR4 silencing by DNA methylation

Sato et al. (2005) [18] analyzed four DNA areas within the 5′ upstream region of the CXCR4 gene using a combined bisulfite restriction analysis (COBRA) method. Their work demonstrated that CXCR4 is regulated by DNA methylation in human pancreatic cancer cell lines within the TSS region, which contains the majority of the methylated CpG dinucleotides. In our work, we therefore selected the TSS region, comprised of nucleotides from the positions −173 to +11 in the CXCR4 promoter, to analyze in our breast tumor cell lines and tumor samples.

Sodium bisulfite sequencing was performed on 184 bp DNA fragment containing 19 CpG dinucleotides. The methylation patterns of eight independent CXCR4 alleles in the PMC-42,

Figure 1. CXCR4 expression analysis using semi-quantitative RT-PCR in breast cancer tumor cell lines and CXCR4 expression after 5-aza-2′-deoxycytidine (D-Aza) treatment. (A) The bands represent CXCR4 expression in the PMC-42, MCF7, MDA-MB-436 cell lines and (B) MDA-MB-435 mock or MDA-MB-435 D-Aza represent the MDA-MB-435 cell line before and after treatment with 5-aza-2′-deoxycytidine, respectively. The GAPDH gene was used as a positive control in both experiments. MW, Molecular Weight, NC represents the PCR reaction without DNA (negative control).

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MCF7, MDA-MB-436 and MDA-MB-135 cell lines were analyzed. The CXCR4-negative cell line, MDA-MB-435, demonstrated a hypermethylation of 91% of the CpG dinucleotides (Fig. 2). The high density of cytosine methylation explains the inactivation of CXCR4 in the MDA-MB-435 cell line, which was demonstrated by RT-PCR (Fig. 1A). This inactivation due to hypermethylation was confirmed by treatment with 5-aza-2'-deoxycytidine, a demethylating agent, which resulted in the subsequent expression of CXCR4 (Fig. 1B). In contrast, the cell lines that expressed CXCR4, which were PMC-42, MCF-7 and MDA-MB-436, had lower levels of CpG dinucleotide methylation (i.e., 17%, 20% and 9%, respectively) (Fig. 2). The presence of a greater number of unmethylated CpG dinucleotides may explain the expression of the CXCR4 gene in these cell lines, which was verified by RT-PCR (Fig. 1A).

The methylation profile comparing the CXCR4-expressing cell lines (MCF7, PMC-42 and MDA-MB-436) to the non-expressing cell line (MDA-MB-435) demonstrated that the differentially methylated dinucleotides were CpGs 1–4 and 9–16 (Fig. 2). Since these differentially methylated CpGs may regulate the silencing of the CXCR4 gene, these regions were subsequently analyzed in primary breast tumor samples using Methylation-Specific PCR (MSP).

MSP analysis in breast tumor cell lines

CpG dinucleotides 1 to 4 and 9 to 16, which lie within a region that is differentially methylated, were chosen for MSP analysis (as described in material and methods) (Fig. 2). The MSP technique was tested with DNA from the tumor cell lines to confirm if this DNA region could be used to analyze the CXCR4 methylation pattern in primary tumors (Fig. 3A). RT-PCR results from the cell lines were then used to compare the pattern of gene expression to the presence or absence of DNA methylation detected by the MSP technique. The MDA-MB-435 breast tumor cell line showed a methylated fragment in the CXCR4 CpG island, which correlated with the lack of CXCR4 expression in this cell line. In contrast, the PMC-42, MCF7 and MDA-MB-436 cell lines, which express CXCR4, demonstrated only unmethylated fragments (Fig. 3A). Therefore, the MSP results from the breast tumor cell lines corroborated with both the RT-PCR and sequencing data.

MSP analysis in primary breast tumors

The MSP assay was subsequently used to analyze the methylation of the CXCR4 gene in primary breast tumor samples. For the methylated and unmethylated conditions, thirteen representative tumor samples are shown (Fig. 3B). From all the samples tested (69), only three contained both methylated and unmethylated CpG dinucleotides. Based on this result, we concluded that CXCR4 could be partially silenced, or the mechanism of silencing could progress during the tumorigenesis process (Fig. 3B). However, a lack of methylation of the CXCR4 gene was found in the majority of the samples with 46 out of the 69 samples (67%) not showing CpG methylation in the region evaluated by MSP.

Correlations between the CXCR4 promoter methylation status and clinicopathological data

Sato et al. (2003) [18] analyzed a DNA region comprised of nucleotides from the −173 to +11 positions in the CXCR4 promoter. This region was shown to contain 19 CpGs, which were used to determine the methylation pattern of the CXCR4 gene and correlates this pattern with CXCR4 gene silencing in human pancreatic cancer cell lines. In this study, we used this region to correlate the CpG island methylation pattern of the CXCR4 gene with the clinical and pathological parameters shown in Table 1. Unmethylated CXCR4 was not significantly associated with the age of disease onset (p = 0.466), estrogen receptor status (p = 0.310), HER2 expression (p = 0.276), progesterone receptor status (p = 0.276), tumor recurrence (p = 0.276) and tumor stage (p = 0.276).

Figure 2. Bisulfite sequencing of the CXCR4 gene promoter in the breast cancer cell lines. The cell lines used are shown. The nineteen dinucleotides are numbered in agreement with the sequence. The open circles represent the unmethylated dinucleotides while the gray to black portion represents the percentage of methylation. On the right side methylation pattern are represented according to data of RT-PCR and the absolute percentage value. The arrows below the CpG dinucleotides represent the MSP primers that were used.

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Table 1. Presence of unmethylated CpG dinucleotides in the CXCR4 gene promoter of primary breast tumor samples

| Parameter                  | MDA-MB-435 (%) | MDA-MB-135 (%) | PMC-42 (%) |
|----------------------------|----------------|----------------|------------|
| Age of disease onset       | 17             | 20             | 9          |
| Estrogen receptor status   | 95             | 95             | 95         |
| HER2 expression            | 95             | 95             | 95         |
| Progesterone receptor status | 95          | 95             | 95         |
| Tumor recurrence           | 95             | 95             | 95         |
| Tumor stage                | 95             | 95             | 95         |
| Lymph node status          | 95             | 95             | 95         |

For the methylated and unmethylated conditions, thirteen CpG dinucleotides were chosen for MSP analysis (as described in material and methods) (Fig. 2). The MSP technique was subsequently used to analyze the methylation of the CXCR4 gene promoter in the breast cancer cell lines. In this study, we used this region to correlate the CpG island methylation pattern of the CXCR4 gene with the clinical and pathological parameters shown in Table 1. Unmethylated CXCR4 was not significantly associated with the age of disease onset (p = 0.466), estrogen receptor status (p = 0.310), HER2 expression (p = 0.276), progesterone receptor status (p = 0.276), tumor recurrence (p = 0.276) and tumor stage (p = 0.276).
Discussion

Breast cancer is the most common malignant tumor affecting women worldwide. Metastasis is an important feature of malignant tumors and has a major impact on the prognosis and therapeutic decisions for patients. The metastatic process is multi-factorial, non-random and exhibits organ selectivity. Lymph node metastases are the most frequently occurring type of metastatic lesion [20]. Chemokine receptors are defined by their ability to induce the directional migration of cells toward a chemotactic cytokine gradient, and the CXCR4 receptor is essential for development, hematopoiesis, organogenesis and vascularization [21]. Muller et al. demonstrated that CXCR4 is undetectable in normal mammary gland tissue but is consistently expressed in human breast cancer cells and metastases. The ligand of CXCR4, CXCL12, is preferentially expressed in organs targeted by metastases, such as the lungs, liver, bone marrow and lymph nodes [13]. Additional reports have demonstrated that a high CXCR4 expression pattern correlates with lymph node metastases from invasive ductal breast cancer [15,16].

Recent emphasis has been placed on the critical role of epigenetic changes, especially DNA methylation and histone modifications, in human carcinogenesis. Epigenetic changes differ from genetic changes as they occur at a higher frequency, are reversible upon treatment with pharmacological agents and occur at defined regions within genes [7]. The CXCR4 gene has been shown to be epigenetically regulated in endometrial carcinoma [22], melanoma [19], colonic carcinoma [23] and pancreatic cancer [18]. In this study, we investigated the methylation status of the 5' TSS region of the CXCR4 promoter in primary breast tumor samples.

First, we evaluated the regulation of the CXCR4 gene by DNA methylation in breast cancer cell lines. MDA-MB-435 demonstrated a repression of CXCR4 gene expression, which was restored after 5-aza-CdR treatment (Fig. 1A and 1B). This result agrees with the data from pancreatic cancer cell lines [18]. Then, a 184 bp fragment of the CXCR4 promoter, which harbored the TSS motif that contains 19 CpGs, was sequenced in breast cancer cell lines. This region was differentially methylated according to the CXCR4 expression levels (Fig. 2). The MSP technique was then used to demonstrate that 46 of the 69 samples analyzed were not methylated (67%). These data are novel for breast cancer since only pancreatic primary carcinomas have previously been studied for CXCR4 DNA methylation. In pancreatic cancer, CXCR4 methylation occurred in 46% of the tumors but did not display any significant associations with common clinicopathological factors, such as age, gender, stage or lymph node metastasis [18].

The importance of oncogene methylation in cancer is still poorly understood. The inactivation of oncogenes confers a selective disadvantage to tumor cells by threatening the survival of the cell and negatively affecting carcinogenesis [24]. Muller et al. [13] showed that CXCR4 gene expression is absent or down regulated in normal breast cells, and this result was also confirmed in other tumor cell types. Singh et al. (2004) observed that the CXCR4 mRNA and protein levels were significantly higher in prostate cancer cell lines (PC3 and LNCaP) compared to normal prostate epithelial cells (PrEC) [25]. A similar finding was reported by Meier et al. (2007) in neuroblastoma cell lines where invasive cells lines (IGR-N91, SH-SY5Y) had high expression levels of CXCR4, whereas a non-invasive neuroblastoma cell line (IGR NB8) expressed low levels of the CXCR4 gene [26]. These data suggest that mechanisms, likely including DNA methylation, exist in normal cells to reduce the expression of CXCR4. Thus, cancer progression could lead to the demethylation of the CXCR4 promoter to selectively favor tumor growth and cell migration.

Tumors with a poor prognosis in our study, such as stage III (94%), tumor size T3/T4 (82%) or SBR III (95%), had unmethylated CXCR4 (p<0.001). The demethylation of an oncogene, such as CXCR4, could be involved in processes such as cell migration and metastasis. Thus, the regulation of this gene deserves attention for its involvement in disease progression.
Recent work by Hiller et al. showed an association between CXCR4 over-expression and patient outcome [27]. This group analyzed the association of locally advanced breast cancer (stages IIb or III of the TNM staging system) and CXCR4 expression after neoadjuvant therapy. The survival was poor for patients whose CXCR4 expression levels remained high following neoadjuvant therapy [27].

Our study showed a statistical correlation between a positive lymph node status and unmethylated CXCR4 (p = 0.002). A similar correlation was observed between unmethylated CXCR4 and the presence of metastases (86% of the samples were unmethylated) (p = 0.026) and non-survival (82% of the samples were unmethylated) (p = 0.039). These results showed a correlation between a poor prognosis and an unmethylated CXCR4 promoter. Data discussing the expression of CXCR4 and the association of the CXCR4 with metastasis in the literature are somewhat controversial. Andre et al. (2006) found a correlation between CXCR4 expression and liver metastases but no correlation was found between the expression of the CXCR4 protein with various clinicopathological variables, such as age, tumor grade, estrogen receptor status or HER2 expression [28]. Kang et al. (2005) reported an association between high CXCR4 protein levels and lymph node metastasis but not with distant metastases [29]. Kato et al. (2003) examined the CXCR4 staining patterns in focal and diffuse-type tumors and found no significant differences in the pathological types, histological grades or estrogen receptor statuses of the tumor types [14]. However, a significant correlation was observed between the CXCR4 protein level and the degree of lymphatic spread but not hematogenous metastases [15]. Holm et al. (2007), opposed to Andre et al. (2006) [28], found a significant correlation between a high CXCR4 protein expression level and a HER2-negative status [30]. Conversely, Woo et al. (2008) found a significant association between a high nuclear expression of CXCR4 with the occurrence of metastasis in the lymph nodes. According to this study, tumors that were CXCR4+/lymph node+ were associated with a negative ER and PR status [31]. Kang et al. (2005) found no correlation between CXCR4 expression and overall survival or disease-free survival of patients but found statistically significant higher levels of CXCR4 protein in node-positive tumors [29]. The expression of CXCR4 was also higher among patients with distant metastases, but no significant correlation between these factors was found [29].

The lack of correlation between CXCR4 protein expression and a positive lymph node status or distant metastases was discussed by Shim et al. (2006) [32]. They observed a high expression of CXCR4 in primary tumors, whereas cytoplasmic expression of this receptor was undetected in most secondary lymph nodes tumors. The reduced expression of CXCR4 on the cell surface can be justified by the high expression of the CXCL12 protein in the lymph nodes [32] as CXCL12 stimulates the internalization and subsequent lysosomal degradation of CXCR4.

Furthermore, we evaluated the correlation between unmethylated CXCR4 and the hypermethylation of other genes strongly associated with breast cancer. Our previous results with the same

**Table 1. Clinicopathological features of 69 patients with primary breast carcinomas and methylation status of CXCR4 gene.**

| Variables               | Samples (%) | CXCR4 Methylation | p value |
|-------------------------|-------------|-------------------|---------|
|                         |             | Yes (%) | No (%) |         |
| **Age**                 |             |         |        |         |
| <45                     | 9 (13)      | 2 (22)  | 7 (78) | 0.466   |
| ≥45                     | 60 (87)     | 24 (40) | 36 (60)|         |
| **Stage**               |             |         |        |         |
| I                       | 19 (27)     | 14 (74) | 5 (26) | <0.001  |
| II                      | 33 (48)     | 6 (18)  | 27 (82)|         |
| III/IV                  | 17 (25)     | 1 (6)   | 16 (94)|         |
| **Tumour size**         |             |         |        |         |
| pT1                     | 17 (25)     | 12 (71) | 5 (29) | <0.001  |
| pT2                     | 35 (50)     | 7 (20)  | 28 (80)|         |
| pT3/pT4                 | 17 (25)     | 3 (18)  | 14 (82)|         |
| **SBR**                 |             |         |        |         |
| I                       | 19 (28)     | 14 (74) | 5 (26) | <0.001  |
| II                      | 32 (46)     | 7 (22)  | 25 (78)|         |
| III                     | 18 (26)     | 1 (5)   | 17 (95)|         |
| **Lymph node status**   |             |         |        |         |
| Positive                | 33 (48)     | 6 (18)  | 27 (82)| 0.002   |
| Negative                | 35 (52)     | 19 (42) | 16 (58)|         |
| **Estrogen receptor (RE)** |         |        |        |         |
| Positive                | 57 (84)     | 21 (37) | 36 (63)| 0.310   |
| Negative                | 11 (16)     | 2 (18)  | 9 (82) |         |
| **HER-2**               |             |         |        |         |
| Positive                | 19 (31)     | 4 (21)  | 15 (79)| 0.276   |
| Negative                | 43 (69)     | 15 (35) | 28 (65)|         |
| **Progesterone receptor (PR)** |     |        |        |         |
| Positive                | 46 (74)     | 16 (35) | 30 (65)| 0.117   |
| Negative                | 16 (26)     | 2 (12)  | 14 (88)|         |
| **ESR1 Methylation**    |             |         |        |         |
| M                       | 28 (41)     | 6 (21)  | 22 (79)| 0.006   |
| U                       | 40 (59)     | 22 (55) | 18 (45)|         |
| **CXCL12 Methylation**  |             |         |        |         |
| M                       | 37 (54)     | 12 (32) | 25 (73)| 0.001   |
| U                       | 32 (46)     | 21 (67) | 11 (33)|         |
| **Metastasis**          |             |         |        |         |
| Positive                | 21 (30)     | 3 (14)  | 18 (86)| 0.026   |
| Negative                | 48 (70)     | 20 (42) | 28 (58)|         |
| **Death**               |             |         |        |         |
| Positive                | 17 (25)     | 3 (18)  | 14 (82)| 0.038   |
| Negative                | 50 (75)     | 23 (46) | 27 (54)|         |
| **Recurrence**          |             |         |        |         |
| Positive                | 10 (14)     | 3 (30)  | 7 (70) | 1.000   |
| Negative                | 59 (86)     | 20 (34) | 39 (66)|         |
| **Histological type**   |             |         |        |         |
| Ductal Carcinoma Invasive | 50 (72)    | 17 (34) | 33 (66)| 0.849   |
| Lobular Carcinoma Invasive | 19 (28)    | 6 (32)  | 13 (68)|         |

**Table 1. Cont.**

**Abbreviations:** p, value from statistical analysis of test and Fisher’s exact test; M, methylated; U unmethylated; significant data are in bold. *CXCL12 and ESR1 methylation data were used from a previous study published by our group [17].
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patients showed that the methylation of ESR1 and CXCL12 occurred at higher frequencies in patients with metastases and death [17]. In this study, we evaluated whether unmethylated CXCR4 with concurrent CXCL12 hypermethylation produced a more aggressive disease phenotype. Tumors from patients with an unmethylated CXCR4 gene had a significantly poorer OS and DFS compared to patients with tumors containing methylated CXCR4 (p = 0.038 and p = 0.009, respectively) (Fig. 4A and B). However, Kaplan-Meier analysis demonstrated that patients with unmethylated CXCR4 and methylated CXCL12 had shorter overall and disease free survivals (p = 0.045 and p = 0.016, respectively) (Fig. 4C and D). The molecular mechanisms facilitated by CXCL12 and its partner, CXCR4, which result in the poor prognosis for these patients remain obscure.

Previously, with the same patient cohort, we demonstrated that ESR1 was inactivated by DNA hypermethylation, which resulted in the loss of the receptor for the estrogen protein (ER) [17]. We hypothesized that the decrease of estrogen would lead to all ER target genes becoming susceptible to epigenetic silencing [33], including CXCL12 [17] and the unmethylated CXCR4 promoter, which would thus lead to a more aggressive disease.

The main limitation of our study was the small sample size. However, even with the limited number of samples, we were able to observe strong correlations between epigenetic changes in both CXCR4 and CXCL12 and a poor prognosis. We believe that the statistical differences found here underline the importance of changes in the DNA methylation of chemokines and their receptors in the process of tumor progression. These new discoveries may provide a molecular prognostic factor for breast cancer and may help to develop therapies that are more effective for this type of cancer. Our results could also open new avenues for a more efficient management of metastatic disease in breast cancers.

In summary our data demonstrate for the first time that CXCR4 gene expression in primary breast tumors is regulated by DNA methylation, and CXCR4 methylation associates with several clinicopathological parameters. Loss of DNA methylation in the promoter region of CXCR4 correlated with a more aggressive disease in terms of tumor stage, tumor size, SBR grade, and demonstrated that concurrent epigenetic changes of CXCR4 and its ligand, CXCL12, correlated with shorter disease-free and overall survivals. We believe that our findings will be important for

Figure 4. Kaplan Meier curves for overall survival and disease-free survival according to the methylation status of CXCR4 and CXCL12. CXCR4 methylation status and the correlation with (A) overall survival (OS) and (B) disease-free survival (DFS) are shown. CXCL12 methylation status and association to CXCR4 methylation for (C) OS and (D) DFS are shown. doi:10.1371/journal.pone.0029461.g004
a better understanding of metastatic disease; however, more research is needed to unveil additional molecular mechanisms associated with the metastatic process.

**Materials and Methods**

**Cell lines**

Breast tumor cell lines were obtained from the Ludwig Institute for Cancer Research (São Paulo, Brazil). The following cell lines were used: MDA-MB-436, MDA-MB-435, MCF7 and PMC-42. The cell lines were cultured at 37°C in a humidified incubator with 5% CO₂ in RPMI 1640 medium containing 10% fetal bovine serum supplemented with 0.2 mM glutamine and 40 µg/mL gentamicin.

**Patient samples**

For the methylation analysis, frozen samples of breast tumors (n = 69) were obtained from breast cancer patients treated by primary surgery at the Nossa Senhora das Graças Hospital, Curitiba, PR, Brazil, with institutional approval. The study included female patients with invasive breast tumors. All patients gave informed consent for their tissue to be retained and analyzed for research purposes. The ages of the patients ranged from 27 to 84 years (mean 57.8 ± 14.7). The histological types of the tumors were either infiltrative ductal carcinoma (IDC) (n = 51, 74%) or infiltrative lobular carcinoma (ILC) (n = 18, 26%). The lymph node statuses of the patients were determined and included 51% positive (n = 35) and 49% negative (n = 33) samples. The histological grades of the tumors were determined according to the modified Bloom-Richardson criteria. Of the patients analyzed, 28% were Grade I, 48% were Grade II and 24% were Grade III. TNM staging was determined according to the World Health Organization (WHO) classification [34]. The tumor samples were the same samples used by Ramos et al. (2010) [17]. The patients’ clinicopathological data are shown in Table 1.

**Ethics Statement.** All patients gave their informed written consent for their tissues to be retained and analyzed for research purposes. All signed consent forms are in the custody of the corresponding author. This study was approved by the Ethics Committee of Human Beings Research from the Federal University of Paraíba (UFPR) with the register number 7220-251/2003 (20/02/2003).

**Immunohistochemistry**

Immunohistochemical (IHC) staining of the tumor samples was evaluated and scored by two pathologists who were also responsible for generating the clinicopathological data. The estrogen receptor (ER) and progesterone receptor (PR) were detected using the specific monoclonal antibodies 1D5 and PgR 636 (DAKO), respectively. The cut-off values for the ER and PR statuses were 10% positively stained cells. The HER2 analysis was performed using the HercepTest™ (DAKO CYTOMATION code K5204). When a result of +2 positive was obtained, an in situ fluorescent hybridization (FISH) assay was performed to confirm the result. Other clinicopathological data (e.g., tumor size, local recurrence, metastasis and death) are summarized in Table 1.

**RNA extraction and reverse transcription**

Total RNA was isolated using the TRIzol® Reagent (Invitrogen) according to the manufacturer’s protocol. Reverse transcription reactions were performed using 500 ng of DNA-free RNA, an oligo (dT)12-18 primer and SuperScript II Reverse Transcriptase (Gibco, BRL). PCR was performed using CXCR4-specific primers and GAPDH-specific primers as a positive control (Table 2). The PCR was performed in a 20 µl volume containing 1× PCR buffer (Invitrogen), 1.5 mM of MgCl2 (Invitrogen), 200 µM dNTPs, 0.3 µM of each primer and 1 U of Premix Taq (Takara). The PCR conditions were as follows: 95°C for 10 min, 94°C for 45 s, the appropriate annealing temperature for 45 s, 72°C for 1 min and a final extension of 72°C for 5 min. PCR products were resolved on 1% agarose gels and stained with ethidium bromide.

**5-aza-2’-deoxycytidine (5-aza-CdR) treatment**

The MDA-MB-435 cell line were plated (1×10⁵ cells/ml) and treated for 7 days with 1 µM 5-aza-CdR (Sigma Aldrich, Germany) or left untreated for an equivalent time. The media was changed daily, and no significant cell death was observed. After treatment for 7 days, total RNA was isolated. The expression of CXCR4 in breast tumor cells was analyzed using semi-quantitative RT-PCR with GAPDH as an internal control. The PCR products were resolved on a 1% agarose gel and stained with ethidium bromide.

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**Table 2. Sequence of the primers used for RT-PCR, nested-PCR and MSP.**

| Application and specificity | Forward primer (5’-3’) | Reverse primer (5’-3’) | Product size (bp) | Annealing Temperature (°C) |
|---------------------------|------------------------|------------------------|-------------------|---------------------------|
| **RT-PCR**                |                        |                        |                   |                           |
| CXCR4                     | CAGCGAGTGACAAAGTGA     | AGCGTGTGACAAAGAGG     | 389               | 58                        |
| GAPDH nested-PCR          | CTGACACCAAGCACTGCTTA   | CATGACGGGAGGTCAGTC    | 296               | 63                        |
| CXCR4 nested              | AGGAAATGTTGGAGGTTTGG   | TTTGATTGGAATGATGATTAGG | -                 | 50, 52, 54                |
| SATR-1                    | GTATTAATTATTATTATTG    | ACATCTTCCATATATTAT    | 184               | 52, 54, 56                |
| SATR-1 nested             | TATATGTTGGGTTGTTAGTT   | CACCTAACCTATATATTCTTC | 690               | 52, 54, 56                |
| **MSP-PCR**               |                        |                        |                   |                           |
| CXCR4 – M                 | CGGCGATTTTGTGTTTGC    | AATGCGGCGATACCGACG    | 99                | 61                        |
| CXCR4 – U                 | AAGTGTGGTTTTTGGTTTGTG | ACATACACACCAAAACCTCAC | 110               | 50                        |

**Abbreviations:** M, specific for methylated condition; U, specific for unmethylated condition. doi:10.1371/journal.pone.0029461.t002

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DNA isolation and sodium bisulfite treatment
Genomic DNA was isolated from the MDA-MB-436, MDA-MB-435, MCF7 and PMC-42 breast cancer cell lines or frozen tumor samples using a phenol/chloroform extraction [35]. The DNA was then subjected to sodium bisulfite treatment using an Epitect® Bisulfite Kit (Qiagen) according to the manufacturer’s instructions.

CXCR4 CpG island methylation analysis
The DNA region located between positions −173 and +11 from the 5′-flanking region of the CXCR4 gene, which contained a 184 bp fragment with 19 CpG dinucleotides, was examined. The DNA fragment was amplified from bisulfite-treated DNA of breast tumor cell lines and tumor samples using a nested-PCR amplification protocol. We designed primers using the Methprimer program (http://www.urogene.org/Methprimer/index1.8.html). Briefly, two sets of primers were used for the nested PCR reactions at their appropriate annealing temperatures. The primer sequences are shown in Table 2. The amplified products were purified using a QIAquick Gel Extraction Kit (Qiagen) and cloned into the pCR2.1 cloning vector (Invitrogen). Eight clones were sequenced for each cell line using the universal or reverse primers.

Methylation-specific PCR (MSP)
After sequencing a 184 bp fragment from the bisulfite treated DNA, we identified the differentially methylated CpG dinucleotides in samples that expressed and did not express the CXCR4 gene, which contained a methylated (M) gene, and new therapies. Drug Discov Today 16: 626–635.

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Author Contributions
Carried out the experimental data acquisition, performed data analyses and interpretation and drafted the manuscript: EASR. Sequencing data collection: MG. Biostatistician in the study: KB. Processed cell cultures and DNA extractions: GGS. Provided patient material and clinicopathological data and critically revised the manuscript: EMSFR IJC. Critically revised the manuscript and gave partial financial support: FFC. Designed and coordinated the study, supplied administrative support and critically revised the manuscript: GK. Performed the experiments: EASR MSG GGS GK. Analyzed the data: EASR KB FFC GK. Contributed reagents/materials/analysis tools: IJC EMSFR AAC. Wrote the paper: EASR FFC GK. All authors read and approved the final manuscript.

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