Cytokine 55 (2011) 260–265

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/issn/10434666

CXCL12 rs1801157 polymorphism and expression in peripheral blood from breast cancer patients

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ARTICLE INFO

Article history:
Received 5 January 2011
Received in revised form 13 April 2011
Accepted 19 April 2011
Available online 17 May 2011

Keywords:
CXCL12 rs1801157 polymorphism
Estrogen receptor
Breast cancer

ABSTRACT

The role of chemokines has been extensively analyzed both in cancer risk and tumor progression. Among different cytokines, CXCR4 and its ligand CXCL12 have been recently subjected to a closer examination. The single-nucleotide polymorphism (SNP) rs1801157 (previously known as CXCL12-A/SDF1-3′A) in the CXCL12 gene and the relative expression of mRNA CXCL12 in peripheral blood were assessed in breast cancer patients, since the chemokine CXCL12 and its receptor CXCR4 regulate leukocyte trafficking and many essential biological processes, including tumor growth, angiogenesis and metastasis of different types of tumors. Genotyping was performed by PCR-RFLP (polymerase chain reaction followed by restriction fragment length polymorphism) using *MspI* restriction enzyme and the expression analyses by quantitative RT-PCR. No difference in GG genotype and allele A carrier frequencies were observed between breast cancer patients and healthy blood donors and nor when CXCL12 mRNA expression was assessed among patients with different tumor stages. However a significant difference was observed when CXCL12 mRNA relative expression was analyzed in breast cancer patients in accordance to the presence or absence of the CXCL12 rs1801157 allele A. Allele A breast cancer patients presented a mRNA CXCL12 expression about 2.1-fold smaller than GG breast cancer patients. Estrogen positive patients presenting CXCL12 allele A presented a significantly lower expression of CXCL12 in peripheral blood ($p = 0.039$) than GG hormone positive patients. Our findings demonstrated that allele A is associated with low expression of CXCL12 in the peripheral blood from ER-positive breast cancer patients, which suggests implications on breast cancer clinical outcome.

1. Introduction

Breast cancer is one of the most serious and prevalent diseases affecting women worldwide [1]. In Brazil, estimates for the year 2010 indicate that 49.240 new cases of breast cancer will occur, with an estimated risk of 49 cases to 100 thousand women [2].

CXC chemokine ligand 12 (CXCL12), previously designated stromal cell-derived factor 1 (SDF-1) is a small chemotactic cytokine belonging to the CXC chemokine family and it is constitutively expressed in various organs [3]. It was first cloned from a bone marrow-derived stromal cell line [4] and was later identified as a pre-B-cell growth stimulating factor [5]. CXCL12 is secreted by marrow stromal and endothelial cells [5], heart [6] and skeletal muscle [7], liver [8], brain [9], kidney parenchymal [10], and osteoblasts [11] but it is mainly produced by osteoblasts, fibroblasts and endothelial cells in the bone marrow [12]. It regulates leukocyte trafficking and many essential biological processes, including cardiac and neuronal development, stem cell motility, neovascularization and tumorigenesis [13–17].

In cooperation with its cognate receptor, CXCR4 [chemokine (C–X–C motif) receptor 4], the CXCR4/CXCL12 axis plays various roles in many normal and pathological processes including embryogenesis, hematopoiesis, immunological homeostasis, human immunodeficiency virus infection, and the progression of rheumatoid arthritis. Furthermore, in various types of cancer, including breast cancer, CXCR4 on tumor cells has been shown to

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doi:10.1016/j.cyto.2011.04.017

be critically involved in tumor progression [18]. CXCR4/CXCL12 interaction produces pleiotropic effects in stem cells and plays a pivotal role in several processes related to the development, tissue regeneration and CXCR4/CXCL12 axis is involved in the progression of malignancies since they may contribute to increase the metastatic properties, growth, and/or survival of cancer cells [19]. In contrast, only a limited number of studies have investigated tumor-derived CXCL12, and the significance of this molecule in tumor biology is not fully understood [20].

Chemokine receptors have been implicated in the pathogenesis of many diseases. Kobayashi et al. [21] examined the expression of SDF-1 protein using immunohistochemistry and of SDF-1 mRNA by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) in surgically resected specimens of invasive breast cancer. They showed that high SDF-1 expression was significantly correlated with nuclear expression of CXCR4 in all breast cancers assessed. This finding was concordant with previously reported results indicating that SDF-1 stimulation induced rapid nuclear internalization of CXCR4 [22,23], and confirmed that the CXCR4/SDF-1 axis plays an important role in progression of breast cancer.

Peripheral blood is historically one of the most important diagnostic specimens. For instance, circulating tumor markers have been monitored in serum for years to provide indicative values about metastatic or emerging primary breast cancer. It has been reported that a low plasma CXCL12 level in breast cancer is predictive of distant metastasis and poorer survival [24]. Many authors have investigated genetic polymorphisms of CXCL12 involving variables factors in the diseases pathogenesis [25–27]. Therefore, the aim of this study was to analyze the CXCL12 genotype and its expression in the peripheral blood cells of breast cancer patients according to clinic-pathological data and in healthy blood donors, and to examine the relationship between these parameters.

2. Material and methods

Following approval from the Human Ethics Committee of the State University of Londrina, peripheral blood was collected from breast cancer patients and normal healthy blood donors. A Term of Free Informed Consent was signed by all sample donors and doctors involved prior to blood collection. Clinical staging was determined according to the Union of International Control of Cancer (UICC, 2002) classification criteria.

Peripheral blood was drawn in sterile syringes containing heparin, as anticoagulant, from 55 untreated breast cancer patients with a histopathological diagnosis of ductal and lobular carcinoma, according to World Health Organization (1993) classification system. Blood samples from breast cancer patients were provided by the Londrina Cancer Hospital, Parana State, Brazil. Samples from 54 healthy blood donors were obtained from the Blood Center of the University Hospital of Londrina, Parana State, Brazil.

2.1. DNA extraction

Genomic DNA was isolated from peripheral blood cells using the technique described by Kirby [28]. Briefly, DNA was extracted from whole blood in the presence of 0.2 M NaCl and 0.25% SDS, for 4 h at 37 °C. After precipitation with ethanol, the pellet was dried and resuspended in 50 μL milli Q water.

2.2. Polymerase chain reaction (PCR) – CXCL12

DNA (100 ng) was analyzed using PCR with specific primers for CXCL12 3′UTR-F1 (forward 5′-CAGTCAACCTGGGCAAAGCC-3′) and CXCL12 3′UTR-R2 (reverse 5′-CCTGAGAGTCCTTTTGCGGG-3′) (GenBank accession number L36033). Samples were amplified using the kit buffer plus 1.25 units of Taq polymerase (Invitrogen™, Carlsbad, California, USA). PCR conditions were: 5 min denaturation at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C, and 10 min elongation at 72 °C in a thermocycler (PCR-Sprint Hybaid – Guelph, Ontario, Canada). Amplicons of 293 base pairs were analyzed by electrophoresis in 2% agarose gel and visualized using UV fluorescence after staining with ethidium bromide.

2.3. CXCL12 rs1801157 genotyping

PCR products were subjected to restriction digestion by incubating with *Msp*I (Promega, Madison, WI, USA) for 3 h at 37 °C. The restriction digestion products were analyzed by electrophoresis on 10% acrylamide gel and detected by a nonradioisotopic technique using a commercially available silver staining method. The CXCL12 GG genotype yielded 100 and 193 bp products, while the AA genotype yielded a 293 bp product.

2.4. RNA isolation and reverse transcriptase reaction

Total cellular RNA was extracted from peripheral white blood cells with TRIzol LS reagent (Invitrogen™, Carlsbad, California, USA) according to the manufacturer's instructions. Purified total RNA was measured and assessed for purity by determining absorbance at 260 and 280 nm and then was stored at −80 °C until testing.

Reverse transcriptase reaction was performed using 500 ng of RNA, 20 units of cloned Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Invitrogen™), 4 units of Recombinant Ribonuclease Inhibitor (RNaseOUT™; Invitrogen™) under the following conditions: 2.5 μM oligo dT, 50 mM Tris–HCl pH 8.3, 75 mM KCl, 1.5 mM $MgCl_2$, 1.25 mM of dNTP, at 42 °C for 60 min in a Hybaid PCR Sprint Thermal Cycler (Biosystems, Guelph, Ontario, Canada).

2.5. Molecular analysis of beta-actin mRNA

PCR for beta-actin cDNA was determined as described by Amarante et al. [29]. Briefly, cDNA synthesis was carried as previously described, and PCR conditions were: 94 °C for 1 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and finally, 72 °C for 10 min in a Hybaid PCR Sprint Thermal Cycler (Biosystems, Guelph, Ontario, Canada).

2.6. Quantitative real-time PCR for CXCL12 mRNA

Real-time PCR using SYBR green fluorescence was performed with 20 ng of cDNA in a total volume of 20 μL. Quantitative real-time PCR reaction was carried out using Platinum®SYBR Green qPCR SuperMix UDG (Invitrogen™) using 0.25 nm of each sense and antisense primer. The amount of CXCL12 cDNA was estimated by quantitative polymerase chain reaction (qPCR) amplified using the sense primer 5′-TTA CCC GCA AAA GAC AAG T-3′ and the antisense primer 5′-AGG CAA TCA CAA AAC CCA GT-3′, human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified using the sense primer 5′-GAAGGTGAAGGTCGGA-3′ and the antisense primer 5′-GGGTCATTGATGGCAAC-3′. The PCR reaction was performed for 40 cycles as follows: 95 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s in a Chromo4™ Real Time PCR Detection (Bio-Rad, Hercules, USA).

In quantitative RT-PCR analysis the expression level of SDF-1 mRNA was calculated according to the Pfaffl method [30], in which Ct values for CXCL12 were the mean fold change + SEM for three independent determinations corrected by GAPDH C_t values from control samples, considering efficiency values.

2.7. Statistical analysis

RS1801157 allele frequency was calculated as: $[1 \times (h + 2H)]/2N$, where h represents the heterozygous genotype, H the homozygous genotype and N the sample size for each population. SNP allele frequencies were tested against the Hardy–Weinberg equilibrium by comparing observed with expected genotype frequencies using a χ^2 test. Genotype data were analyzed by the chi-square (χ^2) test with the level of significance set at $p < 0.05$. Statistical analysis was realized using the SPSS Statistics 17.0 software (SPSS Inc., Chicago, Illinois, USA). A p value $\leqslant 0.05$ was considered statistically significant.

3. Results

In the present study, the frequency of CXCL12 rs1801157 genotypes and CXCL12 mRNA peripheral expression were assessed in 55 women patients presenting breast cancer, 18–76 years old, the median age of the patients was 52 years, diagnosed at the Londrina Cancer Hospital, Parana, Brazil, and 54 healthy women, blood donors with negative serology for HIV, HBV and HCV from the University Hospital of the State University of Londrina, PR Brazil, aged 25–56 years old median age 36 years.

The majority of the patients (87.27%; 48/55) were diagnosed with ductal carcinoma, according to the clinical criteria determined by the Union of International Control of Cancer (UICC, 2002). A larger number of patients included in this work presented stages II and III (81.8%; 45/55), while the number of patients who presented stages I and IV was relatively small (18.2%; 10/55).

The analysis of the CXCL12 rs1801157 genotype frequencies in breast cancer patients and the controls did not show a statistically significant difference. As a result, 32 (58.18%) patients and 37 (68.52%) controls were detected with the GG genotype, 21 (38.18%) patients and 15 (27.78%) controls had the GA genotype whereas 2 (3.64%) patients and 2 (3.7%) controls had the AA genotype. Consequently, there was no difference in AA and GA genotype frequencies between patients and healthy subjects ($p > 0.05$) (Table 1; Fig. 1). Genotype frequency distributions were in Hardy–Weinberg equilibrium in both groups.

Before the CXCL12 mRNA assays, the viability of the RNA samples and cDNA quality were analyzed by conventional PCR for beta-actin, performed with specific primers. All the RNA samples presented detectable quantities of beta-actin mRNA and acceptable integrity during amplification. No contamination with genomic DNA was verified, since all the amplified products presented a fragment correspondent to 353 bp.

The expression of CXCL12 mRNA was investigated by quantitative real time PCR in human peripheral blood cells, and correlated to clinicopathological features of the patients. No statistically significant difference was observed when age range ($p = 0.886$), estrogen receptor status ($p = 0.782$), progesterone receptor status ($p = 0.782$), tumor histology ($p = 0.605$) and tumor state ($p = 0.596$) were compared to CXCL12 mRNA expression independently of the CXCL12 genotype in an univariate analysis, nor in a multivariate analysis comparing these features in accordance to CXCL12 rs1801157 allele A absence or presence (Table 2).

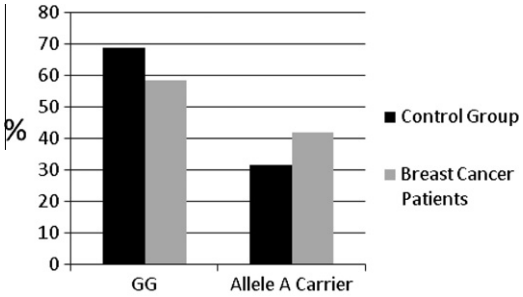

Fig. 1. CXCL12 rs1801157 genotypic distribution among the control group and breast cancer patients. No difference genotypes frequencies between patients and healthy subjects were observed. $\chi^2 = 1.2533$(one degree of freedom; $p > 0.05$).

Although the Kruskal–Wallis Test showed no difference among CXCL12 mRNA expression in the patients with different tumor stages ($p = 0.596$), a significant difference was observed when CXCL12 mRNA relative expression was assessed in breast cancer patients in accordance to the presence or absence of CXCL12 rs1801157 allele A ($p = 0.017$). Allele A breast cancer patients presented a mRNA CXCL12 expression about 2.1-fold smaller than GG breast cancer patients (Fig. 2).

No difference in CXCL12 expression was observed between positive and negative estrogen receptor patients ($p = 0.782$) nor when CXCL12 expression was assessed in accordance to the presence or absence of CXCL12 allele A. However, considering only estrogen positive patients, a significantly lower expression of CXCL12 in peripheral blood from CXCL12 allele A carriers was observed when compared to GG patients ($p = 0.039$) (Fig. 3). But no significance was observed among estrogen negative breast cancer patients ($p = 0.617$).

Positive progesterone receptor (PR) breast cancer patients showed the same statistically significant CXCL12 mRNA relative expression pattern in accordance to CXCL12 genotype than positive estrogen receptor patients ($p = 0.039$), since all the positive estrogen receptor patients in the present study also presented progesterone receptors in their breast cancer cells.

4. Discussion

The roles of the genetic background and environmental factors have been firmly established as causes of breast cancer, which is a genetically heterogeneous malignancy [31–33].

It has been reported that immune system could be altered with aging, contributing to tumorigenesis [34]. The association between cancer and age can be explained by a more prolonged exposure to carcinogens in older individuals, what would lead to age-associated tissue dysfunction caused by the accumulation of molecular and cellular damage [35,36] since aging is associated with the inability to maintain and repair somatic cells [37].

As complex biological phenomena, susceptibility to cancer and its age-dependent increase is thought to include mixed genetic and environmental components [38]. In the present, the age range in breast cancer patients and healthy women was 18–76 years. Women aged over 40 years formed the greater part of the breast cancer population (Table 2).

Table 1
Genotypic and allelic frequencies for CXCL12 rs1801157 allele A and G in breast cancer patients.

Study subjects	Number of samples	Genotype[a]			Allelic frequency[b]	
		GG	AG	AA	G	A
Control group	54*	37 (68.52%)	15 (27.78%)	2 (3.7%)	0.82	0.18
Breast cancer patients	55**	32 (58.18%)	21 (38.18%)	2 (3.64%)	0.77	0.23

* χ^2 in HWE = 0.095, one degree of freedom; $p > 0.05$.
** χ^2 in HWE = 0.416, one degree of freedom; $p > 0.05$.
[a] Breast cancer patients × control group 1; $\chi^2 = 1.3533$ (two degrees of freedom; $p > 0.05$).
[b] Breast cancer patients × control group 1; $\chi^2 = 0.8918$ (one degree of freedom; $p > 0.05$).

Table 2
Clinicopathological features of breast cancer patients according to the CXCL12 rs1801157 genotype and CXCL12 mRNA expression ($n = 55$).

		Total *N* (%) ($n = 55$)	Univariate analysis	CXCL12 genotype		Multivariate analysis
			p value	GG *N* (%)	Allele A carrier *N* (%)	*p* value
Age (years)	18–40	9 (16.36)		4 (7.27)	5 (9.09)	
	41–50	16 (29.09)		11 (20.0)	5 (9.09)	
	51–60	17 (30.90)	0.886	9 (16.36)	8 (14.54)	0.797
	>60	13 (23.63)		8 (14.54)	5 (9.09)	
Estrogen receptor (ER) status	Positive	35 (63.63)		24 (43.63)	11 (20.0)	
	Negative	10 (18.18)	0.782	4 (7.27)	6 (10.9)	0.175
	Unknown	10 (18.18)		4 (7.27)	6 (19.9)	
Progesterone receptor (PR) status	Positive	35 (63.63)		24 (43.63)	11 (20.0)	
	Negative	10 (18.18)	0.782	4 (7.27)	6 (10.9)	0.175
	Unknown	10 (18.18)		4 (7.27)	6 (19.9)	
Tumor histology[a]	IDC	48 (87.27)		27 (49.09)	21 (38.18)	
	ILC	4 (7.27)	0.605	3 (5.45)	1 (1.82)	0.754
	Special	3 (5.45)		2 (3.63)	1 (1.82)	
Tumor stage	I	5 (9.09)		2 (3.63)	3 (5.45)	
	II	26 (47.26)	0.596	17 (30.90)	9 (16.36)	0.919
	III	19 (34.54)		9 (16.36)	10 (18.18)	
	IV	5 (9.09)		4 (7.27)	1 (1.82)	

[a] IDC – invasive ductal carcinoma; ILC – invasive lobular carcinoma; special – adenocarcinoma.

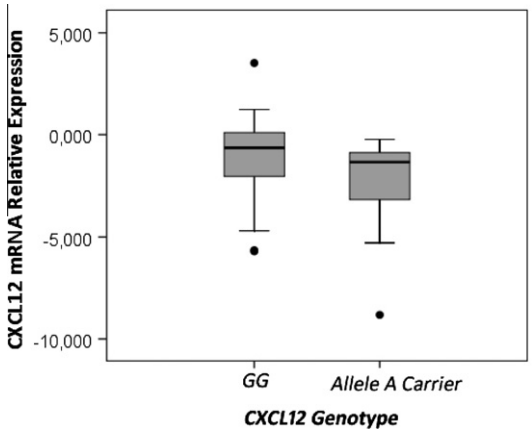

Fig. 2. CXCL12 relative mRNA expression in accordance to CXCL12 genotype. The Mann–Whitney Test demonstrated that CXCL12 mRNA levels differed significantly between GG patients (−1.28 ± 0.49; mean ± SE) and allele A carriers (−2.75 ± 0.65; mean ± SE) ($p = 0.017$). *Boxes and whiskers*, 25th–75th and 10th–90th percentiles, respectively; the median is the central line in each box, circles, outliers.

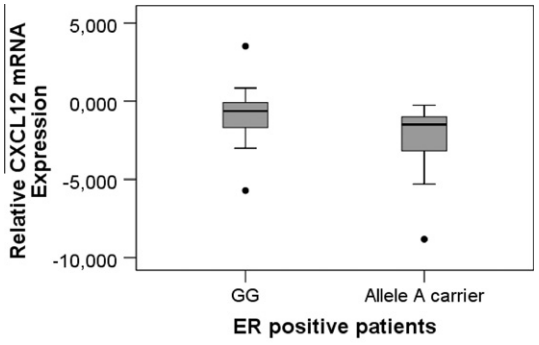

Fig. 3. CXCL12 mRNA relative expression in accordance to the presence of allele A in ER-positive patients. The Mann–Whitney Test demonstrated that CXCL12 mRNA levels differed significantly between GG patients (−1.24 ± 0.598; mean ± SE) and allele A carriers (−2.60 ± 0.766; mean ± SE) ($p = 0.039$) among ER-positive patients. *Boxes and whiskers*, 25th–75th and 10th–90th percentiles, respectively; the median is the central line in each box, circles, outliers.

There is an extensive interplay between tumor cells and signaling molecules such as chemokines [39]. This study investigated the CXCL12 gene in a Brazilian breast cancer population and compared the findings with healthy control subjects. The examination of 55 breast cancer patients for the CXCL12 genotype showed no difference in GG, AA and GA genotype frequencies between patients and healthy subjects ($p > 0.05$).

During the last years, the number of single markers that have been evaluated for disseminated tumor cell detection, mainly by nucleic acid-based techniques, has considerably increased [40]. Many studies have investigated the association of CXCL12 rs1801157 polymorphism in breast cancer and no association has been described either in the distribution of the allele A or G in genotype distribution among patients and healthy individuals [41,25].

Zafiropoulos et al. [42] investigated the potential involvement of CCR5, CCR2, and CXCL12 gene polymorphisms as markers for genetic events contributing to the appearance of breast, bladder, and non-melanoma skin cancer. They observed a significant association for CXCL12 and CCR2 polymorphisms exclusively in breast cancer. Razmkhah et al. [43] also reported that CXCL12 rs1801157 polymorphism was associated with an increased susceptibility to breast cancer development.

Many others studies have also not recognized the CXCL12 rs1801157 polymorphism as a risk factor in the incidence of breast cancer, multiple myeloma, colorectal or cervical and bladder carcinomas [25,44–47]. In agreement with these authors, the present study did not observe any difference in the CXCL12 genotype when healthy blood donors (controls) were compared to breast cancer group (Table 1).

Considerable knowledge of the structures, activities, receptor selectivity and expression of human chemokines exists, but information concerning their role in human pathology is largely limited to studies of their occurrence and distribution in specimens of diseased tissues and their concentrations in plasma, exudates and other body fluids [48].

The role of the CXCL12 rs1801157 polymorphism in the production of CXCL12 protein is controversial. Many studies suggest that the rs1801157 polymorphism in the CXCL12 gene may influence the pathogenesis of the disease [49–53].

Hassan et al. [24] verified that low plasma CXCL12 is an independent host-derived predictive marker of distant metastasis in breast cancer. The prognostic value of the combination of a low plasma CXCL12 level and the polymorphism identifies a cohort of patients with an intrinsic susceptibility for poorer survival. It has been hypothesized that high plasma CXCL12 levels in the blood would serve to retain tumor cells within the circulation and out of the metastatic organ site, and thus, low plasma CXCL12 levels would serve as a predictive marker for distant metastasis. Moreover, CXCL12 mRNA may be regulated by a common polymorphism of CXCL12 rs1801157 [54].

In the present study, although there was no difference in the CXCL12 genotype when healthy blood donors (controls) were compared to the breast cancer group, analysis of the results showed a significantly lower CXCL12 mRNA expression in the peripheral blood samples of CXCL12 rs1801157 allele A carriers than in GG breast cancer patients. In this context, it has been reported that studies of anti-CXCR4 therapy in breast cancer should consider selecting not only patients whose breast tumors overexpress the CXCR4 receptor, but those patients carrying the SDF-1-3′A (CXCL12 rs1801157 allele A) polymorphism and whose plasma SDF-1 levels are low. The predictive value of plasma SDF-1 offers a direct view of the physiology of metastatic disease in the blood of cancer patients [24]. Moreover, the contribution of tumor-derived CXCL12 to plasma levels is apparently negligible [55].

Understanding the interplay between molecular endocrinology and tumor biology has provided experimental therapeutic insights [56]. Approximately 70% of breast cancers are known to express estrogen receptor (ER) alpha and are considered to be hormone-dependent [57]. In accordance with this study, in our cohort 63.63% of the breast cancer patients expressed estrogen receptor.

CXCL12 has been identified as an estrogen-regulated gene in ER alpha-positive ovarian and breast cancer cells, suggesting a direct pathway by which estrogen may induce CXCL12 production through ER alpha [58].

Kobayashi et al. [21] verified that 82% of ER-positive breast cancer showed a high CXCL12 expression, and demonstrated the importance of tumor-derived CXCL12 in ER-positive breast cancers as an indicator of better prognosis, and no significance was observed among ER-negative cases. Habauzit et al. [59] demonstrated that CXCL12 secretion is regulated by estrogenic compounds in a dose-dependent way in ER-positive breast cancer cell lines (MCF-7 and T47D).

Both estrogen receptors (ERs) and the CXCR4/CXCL12 axis play pivotal roles in breast cancer. Sauvé et al. [60] showed that not only do ERs activate the CXCR4/CXCL12 pathway but, conversely, CXCR4 signaling also promotes ER transcriptional activation, thereby establishing a complete autocrine loop for breast cancer cell growth.

In the present study CXCL12 was assessed in peripheral blood of breast cancer patients, and it was observed that estrogen positive patients presenting CXCL12 allele A showed a significantly lower expression of CXCL12 in peripheral blood although no significance was observed among estrogen negative breast cancer patients. The same expression pattern was observed when positive progesterone receptor patients were analyzed, that is due to the fact that our positive estrogen receptor patients were also positive progesterone receptor patients, and estrogens regulate the expression of various genes such as progesterone receptor, transforming growth factor (TGF)-alpha, cyckin D1, bcl-2 [58].

This is the first study to demonstrate the association of the CXCL12 genotype and peripheral blood CXCL12 mRNA expression in ER-positive breast cancers. Our findings demonstrated that allele A is associated with low expression of CXCL12 in the peripheral blood from ER-positive breast cancer patients, which suggests implications on breast cancer clinical outcome.

Conflict of interest

The authors declare no conflicts of interests.

Acknowledgements

The authors would like to acknowledge the volunteers who made this study possible and Cancer Institute of Londrina, PR, Brazil for their collaboration. This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação Araucária, Programa Pesquisa para o SUS: gestão compartilhada em saúde (PPSUS) and the Londrina State University Coordination for Postgraduation (PROPPG-UEL). The authors would like to express their gratitude to GENOPAR for supplying laboratory equipment. The entire article was revised by Adrienne Toledo, a British-born scientific editor.

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