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Collagen content and C-X-C motif chemokine ligand 12 expression in neoplastic breast stroma

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SUMMARY

OBJECTIVE: This study aimed to evaluate the expression of C-X-C motif chemokine ligand 12 and its C-X-C chemokine receptor type 4, and the tumor-stroma ratio using collagen stromal content of breast cancer samples, correlating it with clinicopathological data.

METHODS: Through a retrospective cohort study, samples were obtained from female patients, over 18 years of age, with the disease in stages 1–4, who underwent mastectomy or lumpectomy. The biopsies were provided by the Oncology sector of the Hospital das Clínicas of Universidade Federal de Pernambuco, Recife city, in 2011–2014, including samples of invasive ductal carcinoma, ductal carcinoma in situ, or benign changes (fibroadenoma and hypertrophy), which were analyzed between 2020 and 2022 by immunohistochemistry for the expression of stromal cell characteristics. Collagen content was tested by Gomori staining and digital analysis of images.

RESULTS: Absence of stromal expression of C-X-C motif chemokine ligand 12 was associated with longer disease-free survival (disease-free survival=0.481), and expression of C-X-C chemokine receptor type 4 was associated with lower disease-free survival. An association was observed between clinicopathological variables and stromal expression of chemokines, that is, an association of stromal C-X-C motif chemokine ligand 12 with histological grade, angiolymphatic invasion, and an association between C-X-C chemokine receptor type 4 expression and histological grade. Analyses of digital pixels images of collagen and tumor cells showed a lower percentage of collagen in the invasive ductal carcinoma samples (39%), unlike samples without neoplasms (78%).

CONCLUSION: Low expression of C-X-C motif chemokine ligand 12 may be associated with a worse prognosis for breast cancer. Collagen staining analyzed using digital images represents an opportunity for clinical application and is indicative of the prognosis of the tumor microenvironment in breast carcinoma.

KEYWORDS: Breast carcinoma *in situ*. Collagen type I. Chemokine CXCL12.

INTRODUCTION

Breast cancer is the main malignant neoplasia among women, and there is still a necessity for a better understanding of this disease[1]. In recent years, the tumoral microenvironment has been considered indispensable for elucidating both the cellular transformation process and the cancer cells spread through the human body[2]. Carcinogenesis of breast cancer is assumed as a progression from hyperproliferation, followed by an evolution to ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC), and finally tumoral metastasis[3].

Tumoral microenvironment refers to a set of different cellular and physical-chemical factors including immune cells, blood vessels, and extracellular matrix (ECM) that interfere with many disease aspects and therapeutic results[4,5]. ECM components take part in cancer development, and special attention has been devoted to tumor-stroma ratio (TSR) as a significant prognosis indicator. Because of this, the measuring of TSR has been used in the evaluation of diverse types of malignant neoplasms[6,7]. The first use of the TSR for breast cancer prognosis was described by Kruijf et al.[8]. Since then, other studies pointed out a significant association between an increase in TSR and worse prognoses. The most abundant constituent of EMC is collagen that can be involved in breast cancer progression by supporting the cancer cell migration or influencing cell differentiation and proliferation[9].

Cytokines participate in cell movement control, adhesion, and proliferation during embryogenesis and tissue repairment

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after damage. These peptides and their receptors are highly expressed in tumor cells affecting their proliferation, survival, and invasiveness[10]. An example is the C-X-C motif chemokine ligand 12 (CXCL12)/stromal-derived factor 1 cytokine that functions by C-X-C chemokine receptor type 4 (CXCR4) and C-X-C chemokine receptor type 7 receptors. When overexpressed in cancer, they can promote greater cell multiplication, increase their survival, and contribute to their invasiveness in adjacent tissues[11,12].

Therefore, the objective of this study was to measure TSR by specific collagen staining and to evaluate the expression of CXCL12 and CXCR4 by immunohistochemistry (IHC) in breast cancer biopsies, correlating it with clinicopathological data.

METHODS

Sample's obtainment

The study was approved by the Ethics Committee (protocol number 2.701.211, on June 8, 2018) following Resolution CNS 466/12 which regulates research involving human beings in Brazil. All participants who agreed to participate in the research signed a free and informed commitment term. The sample size was determined according to the demand of the hospital sector, including only samples of women, aged 18 years or older, with a confirmed diagnosis of cancer in stages 1–4, and surgery performed by lumpectomy or mastectomy was included in the study. Patients with unavailable slides and biopsy blocks that did not show a representative tumor in the residual sample were excluded from the study.

Biopsies from 43 diagnosed with breast cancer or with benign alterations (hypertrophy and fibroadenoma) were obtained from 2011 to 2014 in the Oncology Sector, Hospital das Clínicas – Universidade Federal de Pernambuco, Recife City, Brazil. The samples diagnoses were confirmed by an anatomopathologist under optical microscopy (100× magnification) as ICD, DCIS, fibroadenoma, and hypertrophy.

After selection, the samples were analyzed by IHC to determine the expression of estrogen and progesterone receptors, cytokine CXCL12 content, and its respective receptor CXCR4, and the content of collagen was studied by specific stain followed by digital analysis.

Immunohistochemistry

The IHC analysis with anti-ER, anti-RP, anti-CXCL12, and anti-CXCR4 antibodies (Boster Biological Technology) followed the protocol described by Santos et al.[13], with revelation by the streptavidin-biotin-peroxidase complex technique (Kit LSAB+peroxidase, K0690, DAKO; kit Liquid DAB+Substrate, K3468, DAKO). Positive controls were used as indicated by the respective antibody manufacturers, and the antibody was replaced by PBS for negative controls.

Collagen staining and tumor-stroma ratio measuring

Slices with ICD, CIDS, fibroadenoma, and hypertrophy were stained with Gomori's Trichrome according to the manufacturing kit (Leica Biosystems). To quantify collagen and stroma percentual calculation, five interest regions (regions that must contain neoplastic cells) were manually selected per slide, with a field of 0.145 mm^2. A micrograph at 100× magnification was acquired for each IR using a Panthera Series L optical photomicroscope (MOTIC, San Antonio, United States). For micrograph analysis, the standard color thresholding method was used in the ImageJ software (ImageJ 1.53c, National Institutes of Health, United States) with the following parameters: Tone 145–195, Saturation 20–255, and Brightness 0–200 for collagen quantification, while, for measuring the area of tumor cells or normal glands red and pink tones by excluding the Tone ranging between 15 and 220, the parameters for Saturation and Brightness were 20–255 and 0–200, respectively. A total of 40 micrographs were analyzed by two independent observers, and a correlation coefficient was calculated using the SPSS software (Version 20.0. Armonk, New York, USA). The percentage of the area marked in blue or pink/red corresponding to each slide was obtained, and the ratio between the percentage of the pink/red tone area and the area occupied by the blue tone was used to represent the TSR in cases of ICD and stroma gland in the groups without malignancy.

Variables and statistical analysis

Data were collected from the medical records of patients for age, tumor size, lymph node involvement, histological subtype, and ER and PR expressions and analyzed for the CXCL12 and CXCR4 expressions using a logistic regression model and probability analysis, with the following logit model: $A(x'\beta)-\varepsilon x'\beta(1+\varepsilon x'\beta)$, which allows evaluating the multiplicative effect of a single variable on the others, through the Stata statistical package, version 13. Disease-free survival (DFS) was analyzed using the Kaplan-Meier method, and the curves were compared using log rank between CXCL12 positive and negative and CXCR4 positive and negative patients. Statistical significance was considered when $p<0.05$ for two-tailed tests.

RESULTS

Anatomopathological analysis showed the following diagnosis: 30 (69%) patients with IDC, 3 (7%) with CDIS, 6 (13%) with hypertrophy, and 4 (9%) cases of fibroadenoma. Of note, 21 (48%) patients were >50 years old, 20 (46%) patients were <50 years old, and 2 patients without age information. Most IDC (60.6%) presented tumor size ≥2 cm. The same percentage showed axillary lymph node involvement. The most prevalent histological grades were II and III (42% for both). There is no information about tumoral size, lymph node commitment, and histological grades for benign alterations of fibroadenoma and hypertrophy samples (n=10). In relation to the IHC results, 44% of patients showed positivity for ER and 33% for PR, while, concerning the cytokine evaluation, 29% of patients were positive for CXCL12 expression and 25% of patients were positive for CXCR4 (Figure 1). According to the logistic analysis, the absence of stromal CXCL12 expression was associated with a higher DFS 0.481 (95%CI 0.08–2.72), without statistical significance (p=0.408). CXCR4 expression was related to a higher DFS with OR=1.7 (95%CI 0.18–16.42), also without statistical significance (p=0.643). These results were limited by the small sample amount. There was an association between clinicopathological variables, histological grade, angiolymphatic invasion, and chemotherapy with stromal expression of CXCL12 and between CXCR4 and histological grade (Table 1).

Cellular CXCL12 expression did not show significant association with any variable. No expression of stromal CXCL12 was associated with high histological grades. After robustness test, there was confirmation of the result for grades 1–3. For CXCR4, no expression was influenced by histological grade 1 only. However, the stromal CXCL12 positivity was correlated with angiolymphatic invasion and influenced by chemotherapy, according to Table 1.

The collagen content and the ratio average were calculated for each group of patients (Table 2). The TSR results indicate a consistent relationship for the percentage of collagen area, especially in the groups with and without malignancy, being remarkably reduced in patients with IDC (39%) considering the markedly high value (78%) in the benign cases. TSR showed no significant association with prognosis, adjuvant chemotherapy, or TNM staging.

DISCUSSION

According to our results, the sample mainly consisted of patients with ICD, as typically found in the literature[14], most of whom were diagnosed after menopause[15]. Furthermore, tumor size corresponds to the diameter of the primary tumor and is an important negative prognostic factor. In our study, most patients had a size greater than 2 cm, which reveals late diagnosis, increasing the chance of metastasis, treatment ineffectiveness, and probability of recurrence[16]. In view of this, as tumor size is fully correlated with lymph node involvement, most patients presented metastasis.

The release of growth factors that function as chemoattractant occurs in tumoral microenvironment which modulates the cell behavior through positive feedback, including the cytokine production[17]. In our study, the lowest expression of the chemokines CXCL12 and its receptor CXCR4 was observed in larger tumors and with lymph node metastases. In addition, high histological grades significantly correlated with lower expression of CXCL12. According to this result, we realized that, for breast cancer, the lower expression of these chemokines can worsen the prognosis for patients, as it increases the malignancy of the tumor[18].

Collagen is the main component of the cellular matrix, and its increased deposition favors greater tumor aggressiveness and the occurrence of metastasis[19,20]. Due to its importance in pathological processes, several methods can be used for its evaluation[21,22]. Here, we used Trichrome Gomori staining and the collagen content served to calculate the TSR. Through the TSR analysis, it is possible to perform a risk stratification and predict the prognosis and the highest value in the quantity parameters[23]. In addition, the uniformity of collagen fibers is associated with the worst prognosis in IDCs. Our data revealed that collagen deposition in tumor lesions occurs because tumors with a high incidence of metastases may have less adhesion between cells. This result infers that tumor behavior depends not only on genetic characteristics but also on the ECM and collagen framework[24]. Lower collagen deposition in the ECM and the increase in TSR in the IDC can favor a more aggressive behavior of neoplastic cells, with an increase in the chances of metastases and higher histological grade. The TSR assessment is a simple, reproducible, and predictor variable for the diagnosis of malignancy and can be incorporated into the pathological assessment given the reduced cost[25].

The main limitations of the study are related to data from retrospective studies and the limited sample size, which made it impossible to perform some more robust statistical analyses. In addition, the reduced sample size may have caused the non-observance of statistically significant results in relation to collagen content and TSR with clinicopathological variables, which is one of the main limitations of the study. However, it was possible to demonstrate how to calculate TSR by specific collagen staining using the color threshold method, which is an important prognostic factor.

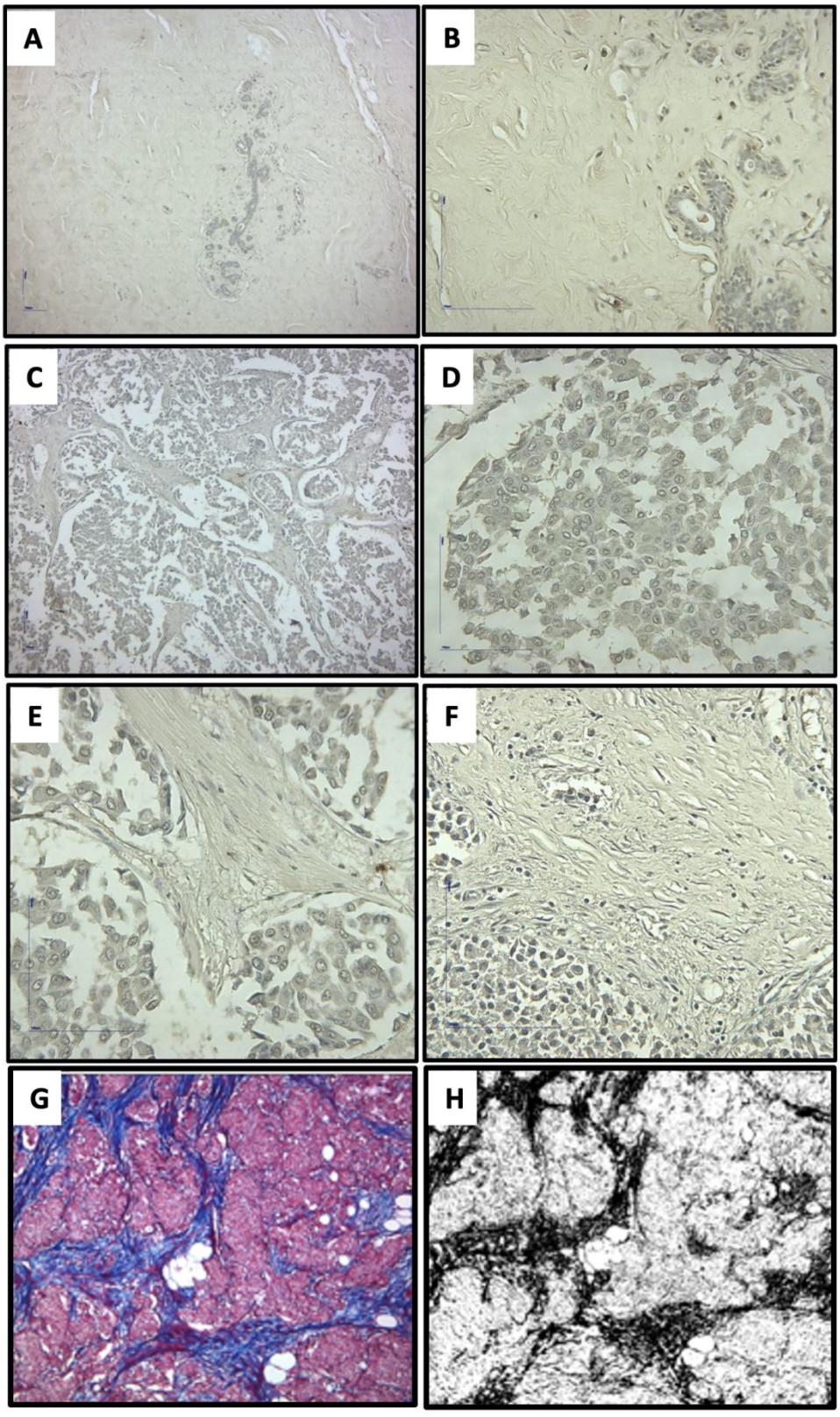

Figure 1. Immunohistochemistry staining pattern of breast cancer for C-X-C motif chemokine ligand 12 and collagen stain. (A,B) Normal breast tissue (100× and 400×, respectively) showing stroma and cellular positivity. (C–F) Invasive ductal carcinoma staining (100×) showing the cytoplasmatic and stromal positivity (400×). (G) Micrograph of the region of interest from tissues stained by Gomori, 100× magnification. (H) Image obtained for stroma quantification by collagen labeling.

Table 1. Correlation between cellular and stromal expression of C-X-C motif chemokine ligand 12 and C-X-C chemokine receptor type 4 with clinicopathological variables by the logit method, demonstrating an association between stromal C-X-C motif chemokine ligand 12 expression and histological grade, amphiolymphatic invasion, and chemotherapy, and an association between C-X-C chemokine receptor type 4 expression and grade histological.

Variables	Cellular CXCL12		Stromal CXCL12		p	CXCR 4
	p	(95%CI)	p	(95%CI)		(95%CI)
Tumoral size	0.45	(-6.01 to 2.69)	0.75	(-3.38 to 4.64)	0.14	(-3.97 to 2.78)
Lymph node commitiment	0.22	(-7.65 to 1.76)	0.25	(-4.38 to 16.44)		
Histological grade	0.45	(-5.35 to 2.40)	<0.001	(-28.58 to -10.13)	0.003	(-6.52 to -1.37)
Inflammation	0.95	(-3.59 to 3.40)	0.11	(-1.28 to 11.19)	0.62	(-3.79 to -2.26)
Angiolymphatic invasion	0.14	(-0.97 to 6.53)	<0.001	(28.33 to 52.98)	0.26	(-1.0 to 3.70)
Histological subtype	0.08	(-0.13 to 1.82)	0.67	(-9.47 to 1.46)	0.99	(-1.17 to 1.19)
Chemotherapy	0.33	(-5.87 to 1.99)	<0.001	(-25.56 to -14.34)		

CXCR4: C-X-C chemokine receptor type 4; CXCL12: C-X-C motif chemokine ligand 12.

Table 2. Analysis of collagen and tumor/normal cells areas in the indicated groups.

	Invasive ductal carcinoma	Hypertrophy	Fibroadenoma
	Average±standard deviation		
Percentage of collagen area (%)	39.029±13.442	78.929±20.924	59.378±17.034
Percentage of tumor cells or normal cells (%)	43.463±9.2	10.234±3.394	25.535±17.121
Tumor-stroma ratio	1.263±0.899	7.802±0.542	3.519±2.545

CONCLUSION

In our study, the immunohistochemical evaluation of CXCL12 and its CXCR4 receptor was correlated with clinical pathological data in breast cancer, indicating that the low expression of both chemokines promotes worse tumor biology. However, further studies are needed to better understand the functioning of these chemokines in the breast tumor microenvironment. The collagen content that was used to calculate the TSR through digital image analysis represents an excellent opportunity for clinical application and may be indicative of the prognosis of the tumor microenvironment in breast carcinoma.

AUTHORS' CONTRIBUTIONS

CJSF: Investigation, Formal Analysis, Writing – original draft. **IQSC**: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology. **WJBCN**: Formal Analysis, Methodology, Writing – original draft. **SMVA**: Project administration, Resources, Supervision, Validation, Writing – review & editing.

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