Original Study

Association Between Ligands and Receptors Related to the Progression of Early Breast Cancer in Tumor Epithelial and Stromal Cells

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Abstract

We have demonstrated the association between RANKL, IL-6, SDF-1, and CCL-2 in tumor epithelial cells and their receptors in spindle-shaped stromal cells, as well as between TRAIL, RANKL, and CCL-2 in spindle-shaped stromal cells and their receptors in tumor epithelial cells from 63 patients with breast cancer (I/II stage). These molecules may be implicated in breast cancer progression during the early stage of the disease.

Background: Despite advances in the study of breast cancer (BC), it remains the second leading cause of mortality among women. BC is a heterogeneous system, mainly composed of tumor epithelial cells (TEpCs) and stromal cells (SCs); the interaction through the ligands and their receptors (Rs) plays a major role in BC progression. The aim of the present study was to evaluate the association between ligands, such as osteoprotegerin, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), receptor activator of nuclear factor kappa B ligand (RANKL), stromal cell–derived factor (SDF)-1, interleukin (IL)-6, macrophage colony stimulating factor, chemokine (C-C motif) ligand-2 (CCL-2), and their Rs in TEpC and spindle-shaped SCs not closely associated with the vasculature. **Patients and Methods:** We studied the expression of all those factors in 63 primary tumors of untreated patients with BC with infiltrative ductal carcinoma (I/II stage) and 10 non-neoplastic tissues. The percentage of positive cells and the staining intensity were analyzed by immunohistochemistry. Mann–Whitney test and Spearman's rank correlation coefficient were used ($P \leq .05$). **Results:** We found a significant association between the expression of RANKL, IL-6, SDF-1, and CCL-2 in TEpC and the receptor activator of nuclear factor kappa B (RANK), IL-6R, C-X-C chemokine R type 4, and chemokine (C-C motif) R-2 (CCR-2) in spindle-shaped SC. The expression of TRAIL, RANKL, and CCL-2 in spindle-shaped SC also was associated with the expression of TRAIL-receptor 1, TRAIL-receptor 4, RANK, and CCR-2 in TEpC. **Conclusions:** Because the described ligands and Rs are implicated in BC progression, our results suggest that these factors could be involved in the crosstalk between TEpC and SC in the early stages of BC.

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Introduction

Statistics indicate that the breast cancer (BC) mortality rate in 2000 was 26.7 in Argentina and 24.2 in the United States (Bulletin N° 96 of malignant tumor mortality: National Programme of Health Statistics, Department of Statistics and Health Information, Ministry of Health, Argentina, 2002). In both cases, the specific

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rates are expressed as the number of deaths per year and per 100,000 inhabitants. Moreover, a study conducted in Argentina between 2008 and 2010 indicated that the highest cancer mortality in women corresponded to BC, with an annual average of cases of 5367 per 25,618 (21%) (Bureau of Health Information Statistics and Nation, Department of Statistics and Health Information, Ministry of Health, Argentina, 2013). Although different treatment options for early BC exist, they are not totally effective.

BC tissue is a heterogeneous system, mainly composed of tumor epithelial cells (TEpCs) and stromal cells (SCs). The interaction between malignant and nonmalignant cells takes place in the breast tumor microenvironment at the primary site and plays a major role in the various steps of BC progression.[1-5] Therefore, the bidirectional crosstalk between the BC cells and the microenvironment components (immune cells, mesenchymal stem cells [MSCs], tumor-associated fibroblasts [TAFs], fibroblasts, tumor vasculature, and extracellular matrix) cannot be overlooked, because cellular interactions in vivo have a strong influence on the biological behavior of cancer cells. This interaction, mainly driven by soluble secreted factors, allows tumor cells to modify the stroma via tissue remodeling and gene expression and vice versa.[4] It is known that the network orchestrated by BC cells, SC, and other components of the tumor microenvironment contributes to tumorigenesis, progression, local relapse, and metastases.[6] Therefore, defining the nature of the signals exchanged between the tumor microenvironment and the tumor cells should provide insights into how BC develops and progresses, and may help to reveal therapeutic modalities based on intercepting the tumor-stroma crosstalk.[7]

Emulating wounds, BC cells emit systemic signals that attract other cells, including bone marrow mesenchymal SCs.[7,8] The recruitment of bone marrow mesenchymal SCs to tumors is enhanced by the binding of TEpC's interleukin (IL)-6, stromal cell–derived factor (SDF)-1, tumor necrosis factor (TNF)-α, TNF-related apoptosis-inducing ligand (TRAIL), chemokine (C-C motif) ligand-2 (CCL-2), transforming growth factor (TGF)-β, and platelet-derived growth factor AB (PDGF-AB) to the corresponding receptor (R) on the mesenchymal SCs (IL-6R, C-X-C chemokine R type 4 [CXCR-4], tumor necrosis factor-α receptor [TNF-αR], TRAIL-R2, TRAIL-R4, chemokine [C-C motif] R-2 [CCR-2], TGF-βR, and platelet-derived growth factor R type alpha or beta [PDGF-αβR]).[9,10] Moreover, the ability of mesenchymal SCs to increase tumor development is mediated by a number of different factors, that is, cytokines and chemokines, such as TRAIL, osteoprotegerin (OPG), chemokine (C-C motif) ligand-5 (CCL-5), CCL-2, SDF-1, IL-17B, IL-6, IL-8, macrophage colony stimulating factor (M-CSF), and receptor activator of nuclear factor kappa B ligand (RANKL),[6,7] that regulate BC cell proliferation, survival, invasion, migration and intravasation processes, and tumor angiogenesis (Figure 1).

BC cells and mesenchymal SCs may act as a stimulus to induce epithelial-mesenchymal transition (EMT) by TNF-α, IL-1β, IL-6, SDF-1, M-CSF, RANKL, macrophage migration inhibitory factor, and CCL-2, and to increase BC metastatic potential,[14] promoting tumor hormone independence and endocrine therapy resistance (Figure 1).[4]

In addition, MSC could differentiate into TAF under the induction of tumor microenvironment factors, such as TGF-β, PDGF-AB, vascular endothelial growth factor, and IL-6.[11,12]

Given all these observations, the aim of the present study is to evaluate the possible association of ligands and R' expression (OPG, TRAIL, TRAIL-R [R1, R2, R3, and R4], RANKL, RANK [RANKL-R], SDF-1, CXCR-4 [SDF-1-R], IL-6, IL-6R (IL-6-R), M-CSF, CD115 [M-CSF-R], CCL-2, and CCR-2 [CCL-2-R]) between TEpC and spindle-shaped SC in primary tumors of untreated patients with early BC with infiltrative ductal carcinoma.

Patients and Methods

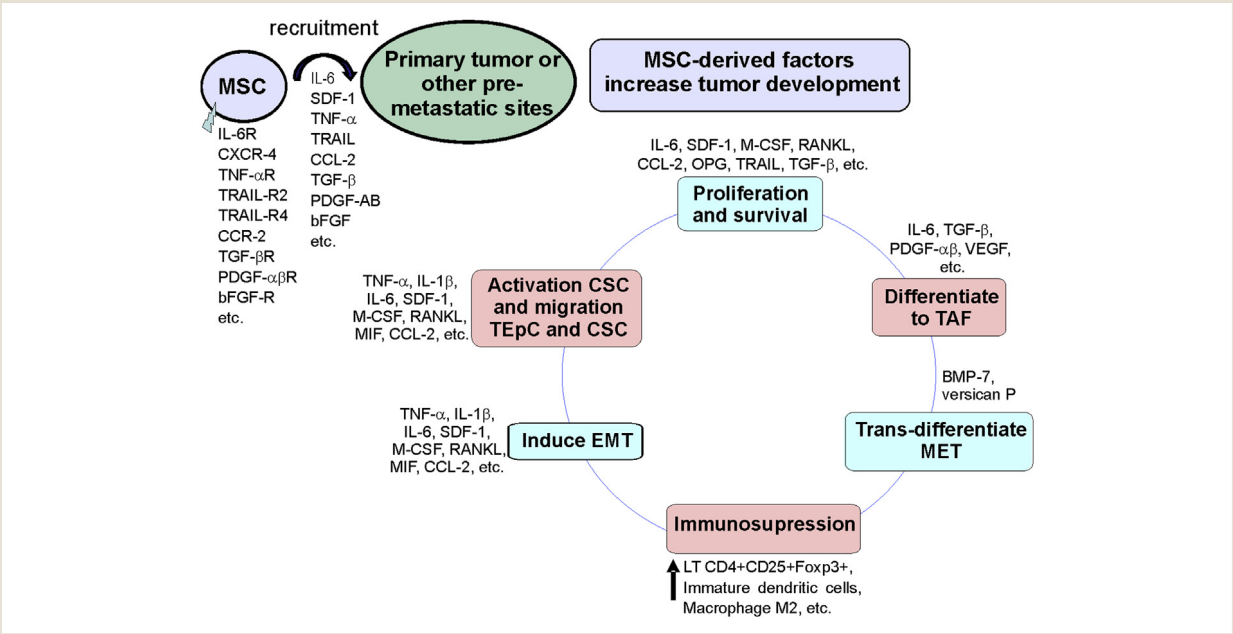
Study Design

This study enrolled surgical biopsy samples from unselected consecutive patients in whom BC diagnosis had been confirmed histologically. A total of 63 biopsies from women who had undergone surgery in the Italian Hospital were included in the study. These patients were diagnosed with infiltrative ductal BC, in early clinical-pathologic stages I and II, and free of neoadjuvant therapy. Samples of non-neoplastic breast tissues from 10 women who presented negative BC results were used as controls. The International Union Against Cancer TNM classification system was used. The study was approved by the Experimental Biology and Medicine Institute and the Italian Hospital Ethical Committees and was performed in accordance with the principles of the Declaration of Helsinki. Informed consent statement was obtained, and the information of each patients and controls was made anonymous and coded. Exclusion criterion in both groups was insufficient tissue availability for immunohistochemistry (IHC).

Evaluation of Ligands and Receptors by Immunohistochemical Assay

Biopsies from BC and non-neoplastic tissues were used to evaluate by IHC the expression of OPG, TRAIL, TRAIL-R (R1, R2, R3, and R4), RANKL, receptor activator of nuclear factor kappa B (RANK), SDF-1, CXCR-4, IL-6, IL-6-R, M-CSF, CD115, CCL-2, and CCR-2 in TEpC, epithelial cells (EpCs), and spindle-shaped SC, not closely associated to vasculature. Tissues were fixed in 10% neutral buffer formalin, embedded in paraffin, and sectioned at 4-μm thickness. After de-paraffination, rehydration, and antigen recovery, the endogenous peroxidase activity was blocked with 3% H_2O_2, and nonspecific sites were blocked with phosphate-buffered saline/bovine serum albumin 1%. Samples were incubated overnight at 4°C with primary human antibodies (Abs) against: OPG (goat immunoglobulin [Ig]G, AF805, R&D Systems Inc, Minneapolis, MN), TRAIL (mouse IgG_1, MAB687, R&D Systems Inc), TRAIL-R1 (goat IgG, AF347, R&D Systems Inc), TRAIL-R2 (mouse IgG_{2B}, MAB6311, R&D Systems Inc), TRAIL-R3 (mouse IgG_1, MAB6301, R&D Systems Inc), TRAIL-R4 (goat IgG, AF633, R&D Systems Inc), RANKL (mouse IgG_{2B}, AB1862, Chemicon, Billerica, MA), RANK (mouse IgG_1, MAB683, R&D Systems Inc), SDF-1 (mouse IgG_1, MAB350, R&D Systems Inc), CXCR-4 (mouse IgG_{2B}, MAB172, R&D Systems Inc), IL-6 (mouse IgG_{2B}, MAB, R&D Systems Inc), IL-6R (mouse IgG_1, AHR, 0961), M-CSF (rabbit IgG, ab9693, Abcam, Cambridge, England), CD115 (rat IgG_1, MAB350, R&D Systems Inc), CCL-2 (mouse IgG_{2B}, MAB2791, R&D Systems Inc), CCR-2 (goat IgG, ab1668, Abcam), and anti-cytokeratin monoclonal Abs (AE1AE3) as a positive control (EpC) (mouse IgG_1, N1590, Dako, Carpinteria, CA) overnight at 4°C in a humidity environment. According

Figure 1 Contribution of MSCs to Breast Cancer Progression. The Recruitment of MSCs to the BC Site Is Shown. Once in the Tumor Microenvironment, MSCs are Able to Increase Proliferation and Survival of TEpC, Differentiate Into SCs (eg, TAF, MET), Suppress Immune Responses, Promote Tumor Metastasis by EMT Induction, and Increase Migration of Cancer Stem Cells and TEpC



Abbreviations: BC= breast cancer; bFGF-R = basic fibroblast growth factor receptor; BMP = bone morphogenetic protein; CCL-2 = chemokine (C-C motif) ligand 2; CCR-2 = chemokine (C-C motif) receptor 2; CSC = cancer stem cell; CXCR-4 = chemokine receptor type 4; EMT = epithelial-mesenchymal transition; IL = interleukin; IL-6R = interleukin-6 receptor; LT = T lymphocyte; M-CSF = macrophage colony-stimulating factor; MET = mesenchymal-epithelial transition; MIF = migration inhibitory factor; MSC = mesenchymal stem cell; OPG = osteoprotegerin; PDGF-AB = platelet-derived growth factor AB; R = receptor; RANKL = receptor activator of nuclear factor kappa-B ligand; SDF = stromal cell—derived factor; TAF = tumor-associated fibroblast; TEpC = tumor epithelial cell; TGF = transforming growth factor; TNF = tumor necrosis factor; TNF-αR = tumor necrosis factor-α receptor; TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; VEGF = vascular endothelial growth factor.

to the manufacturer's recommendations, a peroxidase-based IHC staining method (K0690, Dako) was used for primary Ab detection, and a 3,3′-diaminobenzidine tetrahydrochloride substrate system (K3468, Dako) was used as the chromogen. Hematoxylin (Biopur Company, Rosario, Santa Fé, Argentina) was used for counterstaining, followed by mounting with Canada Balsam (Canadax; Biopur). Negative controls without primary Abs or with an irrelevant Ab as a negative isotype control (mouse IgG_1 isotype [X0931, Dako], normal mouse Igs [08-6599, Zymed, South San Francisco, CA], normal goat IgG [AB-108-C, R&D Systems Inc], normal rabbit Igs [X0936, Dako], or normal rat IgG_1 [MAB005, R&D Systems Inc]) were used to assess nonspecific staining. Appropriate positive and negative controls were included in each Ab run according to the manufacturer's recommendations. Each sample was assayed in duplicate.

The IHC reactions were estimated independently by 2 pathologists. In doubtful cases, a reevaluation was performed using a double-head microscope, and staining was discussed until a consensus was achieved.

Each slide was initially examined at 10 × magnification for an overall view. In each slide, 5 different fields along a projected Z-line at 400 × magnification composed of both TEpC and SC were systematically evaluated. The expression of the OPG, TRAIL, TRAIL-R (R1, R2, R3, and R4), RANKL, RANK, SDF-1, CXCR-4, IL-6, IL-6R, M-CSF, CD115, CCL-2, and CCR-2 was evaluated in TEpC, EpC, and spindle-shaped SC, on the basis of the Allred score compared with cytokeratin AE1AE3 expression.[13] Briefly, the percent of positive cells was defined as 0 (< 10%), 1 (10%-30%), 2 (30%-60%), 3 (60%-90%), and 4 (> 90%). The staining intensity was scored as 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong). Thus, we obtained the final score as the sum of the quantitative score and intensity score, which ranged from 0 to 7.

Spindle-shaped SC, not closely associated with the vasculature included in this study, were positive for alpha smooth muscle actin, fibroblast surface protein, CD105, and CD146, and negative for CD34.[14]

Statistical Analysis

The 2-tailed nonparametric Mann—Whitney test for unpaired or paired samples was used to determine differences (ligands and R) between BC and non-neoplastic tissues, and between TEpC and spindle-shaped SC. Spearman's rank correlation coefficient was used to analyze the association between ligands and their R in TEpC and spindle-shaped SC. All statistical analysis was performed by an expert statistician using SSPS software version 18.00 (SPSS Inc, Chicago, IL). P values $\leq .05$ are considered statistically significant.

Results

Expression of Ligands and Receptors in Breast Cancer and Non-neoplastic Tissues

The expression (as final score) of OPG, TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, RANKL, RANK, SDF-1, CXCR-4, IL-6, IL-6R, CCL-2, and CCR-2 was significantly higher in TEpC of patients with BC than in EpC of non-neoplastic breast tissues ($P < .05$ by Mann–Whitney test) (Table 1). Expression of OPG, TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, RANKL, RANK, CXCR-4, IL-6, IL-6R, and CCL-2 was significantly higher in spindle-shaped SC from patients with BC than in non-neoplastic tissue SC ($P < .05$ by Mann–Whitney test) (Table 2). Figure 2A shows representative IHC staining for ligands and R in BC and nonmalignant tissues. Negative or low staining was observed in non-neoplastic tissues for all the ligands and R shown (Figure 2A). In the samples in which the primary Ab was omitted or isotype controls were used, negligible staining was observed (Figure 2B).

Percentage of Tumor Epithelial and Spindle-Shaped SCs That Express Ligands and Percentage of BC Samples With Different Staining Intensity of Each Ligand

Figure 3 illustrates the percent of positive cells and the intensity pattern of OPG, TRAIL, RANKL, SDF-1, IL-6, M-CSF, and CCL-2 in TEpC and spindle-shaped SC. The percentage of cells that express OPG, TRAIL, SDF-1, IL-6, M-CSF, and CCL-2 was highest in TEpC compared with spindle-shaped SC ($P < .0001$) (Figure 3).

The percent of BC samples that presented moderate and strong ligand intensity in TEpC and spindle-shaped SC, respectively, was as follows: OPG: 76% (46/61) versus 31% (19/61); TRAIL: 88% (53/60) versus 68% (41/60); RANKL: 69% (43/62) versus 62% (37/62); SDF-1: 86% (53/62) versus 33% (19/62); IL-6: 42% (25/60) versus 22% (12/60); M-CSF: 7% (5/60) versus 9% (4/59); and CCL-2: 48% (28/58) versus 21% (12/59).

Analysis of Association Between Expression of Ligands and Receptors in Tumor Epithelial Cells From Patients With Breast Cancer

Spearman rank correlation analysis showed a significant positive association among the expression of RANKL/RANK (Spearman K = 0.40, $P = .0012$), TRAIL/TRAIL-R1 (Spearman K = 0.34, $P = .0093$), TRAIL/TRAIL-R2 (Spearman K = 0.31, $P = .0195$), TRAIL/TRAIL-R4 (Spearman K = 0.43, $P = .0006$), IL-6/IL-6R (Spearman K = 0.36, $P = .0075$), SDF-1/CXCR-4 (Spearman K = 0.30, $P = .0193$), M-CSF/CD115 (Spearman K = 0.49, $P = .0001$), and CCL-2/CCR-2 (Spearman K = 0.38, $P = .0035$) in TEpC (Figure 4).

Table 1 Expression (as Score Final) of OPG, TRAIL, TRAIL-R (R1, R2, R3, and R4), RANKL, RANK, SDF-1, CXCR-4, IL-6, IL-6R, M-CSF, CD115, CCL-2, and CCR in TEpCs of Breast Cancer Tissues and in EpCs of Non-Neoplastic Tissues

Ligands and Receptors	BC-TEpC	Non-Neoplastic Tissue-EpC	*P*
OPG	6 (4-6); n = 61	3 (0-4); n = 10	**.0004**
TRAIL	6 (5-6); n = 59	4.5 (0-5); n = 10	**.0233**
TRAIL-R1	4 (0-5); n = 60	0 (0-0); n = 10	**.0036**
TRAIL-R2	5 (3-6); n = 61	0 (0-0); n = 10	**.0001**
TRAIL-R3	5 (3-6); n = 63	2.5 (0-5); n = 10	**.0194**
TRAIL-R4	6 (5-7); n = 63	2.5 (0-5); n = 10	**.0060**
RANKL	5 (3-5); n = 62	2 (0-3); n = 10	**.0032**
RANK	7 (6-7); n = 62	5 (3-5); n = 10	**.0004**
SDF-1	6 (5-7); n = 62	3.5 (0-5); n = 10	**.0031**
CXCR-4	5 (4-6); n = 62	0 (0-3); n = 10	**<.0001**
IL-6	4 (2-5); n = 60	0 (0-3); n = 10	**.0075**
IL-6R	6 (5-7); n = 56	3 (2-6); n = 9	**.0036**
M-CSF	0 (0-1); n = 59	0 (0-0); n = 10	.0650
CD115	0 (0-0); n = 59	0 (0-0); n = 10	.1212
CCL-2	4 (3-6); n = 58	0 (0-1); n = 8	**.0003**
CCR-2	5 (4-6); n = 59	2 (0-3); n = 8	**.0007**

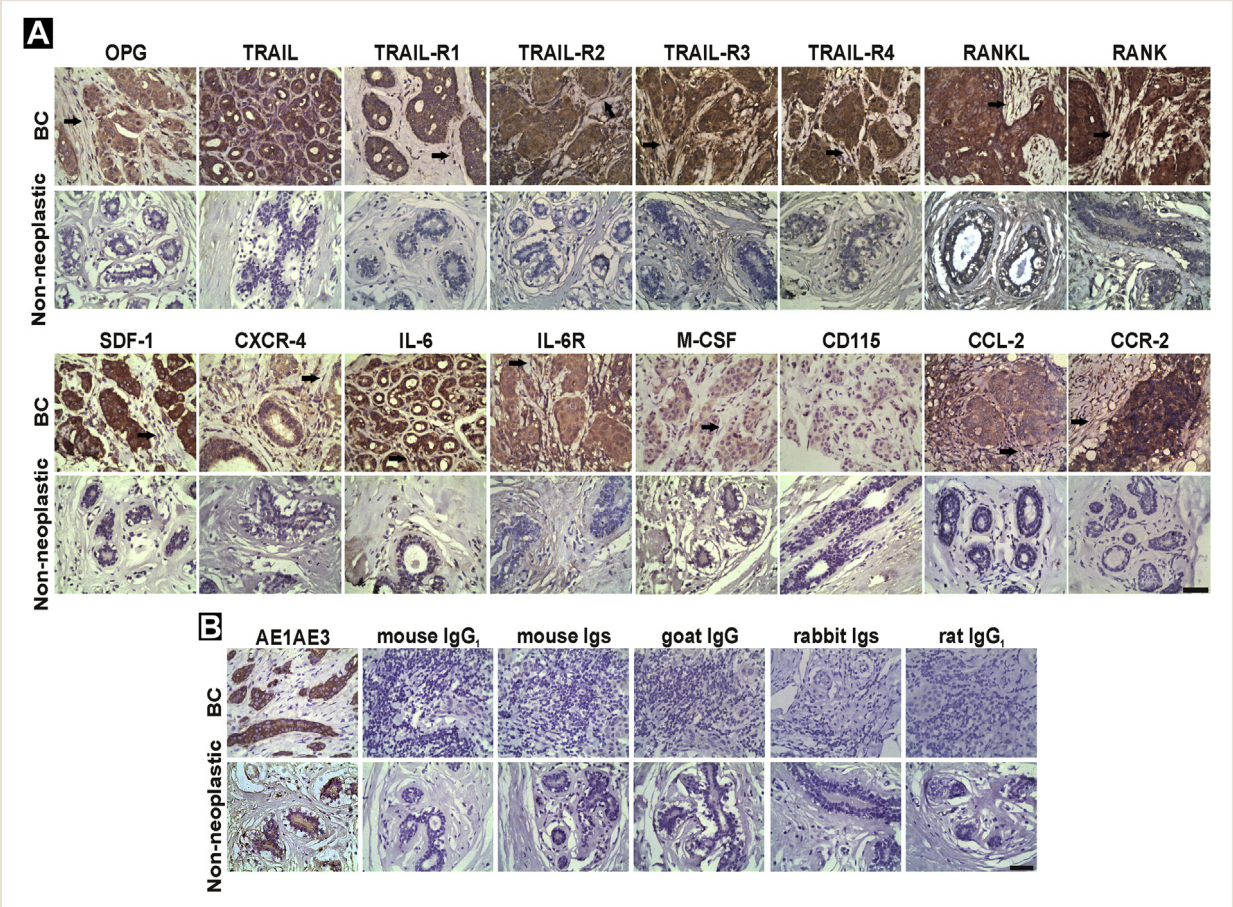
Data are expressed as median (interquartile range). Statistical analysis was performed using the Mann–Whitney test, and the level of statistical significance was set at $P \leq .05$.
Abbreviations: BC-TEpC = breast cancer tumor epithelial cell; CCL-2 = chemokine (C-C motif) ligand 2; CCR-2 = chemokine (C-C motif) receptor 2; CXCR-4 = chemokine receptor type 4; IL = interleukin; M-CSF = macrophage colony-stimulating factor; OPG = osteoprotegerin; SDF = stromal cell–derived factor; TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; TRAIL-R1 = tumor necrosis factor-related apoptosis-inducing ligand receptor 1; TRAIL-R2 = tumor necrosis factor-related apoptosis-inducing ligand receptor 2; TRAIL-R3 = tumor necrosis factor-related apoptosis-inducing ligand receptor 3; TRAIL-R4 = tumor necrosis factor-related apoptosis-inducing ligand receptor 4.

Table 2 Expression (as Score Final) of OPG, TRAIL, TRAIL-R (R1, R2, R3 and R4), RANKL, RANK, SDF-1, CXCR-4, IL-6, IL-6R, M-CSF, CD115, CCL-2, and CCR-2 in Spindle-Shaped SCs of Breast Cancer Tissue and Non-Neoplastic Tissue

Ligands and Receptors	BC-SC	Non-Neoplastic Tissue-SC	*P* Value
OPG	2 (1-4); n = 61	0 (0-0); n = 10	**.0010**
TRAIL	4 (2-5); n = 60	0 (0-2); n = 10	**.0002**
TRAIL-R1	1 (0-3); n = 60	0 (0-0); n = 10	**.0368**
TRAIL-R2	4 (3-5); n = 61	0 (0-1); n = 10	**.0002**
TRAIL-R3	5 (3-6); n = 63	1 (0-4); n = 10	**.0041**
TRAIL-R4	5 (4-6); n = 63	1 (0-2); n = 10	**.0002**
RANKL	4 (2-5); n = 62	2 (0-4); n = 10	**.0343**
RANK	6 (5-7); n = 62	3.5 (0-5); n = 10	**.0002**
SDF-1	3 (2-4); n = 62	1 (0-4); n = 10	.1996
CXCR-4	5 (3-6); n = 62	2 (0-2); n = 10	**<.0001**
IL-6	2 (0-4); n = 60	0 (0-0); n = 10	**.0115**
IL-6R	5 (4-6); n = 56	2 (2-3); n = 10	**.0023**
M-CSF	0 (0-0); n = 59	0 (0-0); n = 10	.3431
CD115	0 (0-0); n = 59	0 (0-0); n = 10	.6831
CCL-2	2 (0-3); n = 58	0 (0-0); n = 8	**.0501**
CCR-2	2 (2-5); n = 59	2 (1-2); n = 8	.9120

Data are expressed as median (interquartile range). Statistical analysis was performed using the Mann–Whitney test, and the level of statistical significance was set at $P \leq .05$.
Abbreviations: BC-SC = breast cancer stromal cell; CCL-2 = chemokine (C-C motif) ligand 2; CCR-2 = chemokine (C-C motif) receptor 2; CXCR-4 = chemokine receptor type 4; IL = interleukin; M-CSF = macrophage colony-stimulating factor; OPG = osteoprotegerin; RANK = receptor activator of nuclear factor kappa B; RANKL = receptor activator of nuclear factor kappa-B ligand; SDF = stromal cell–derived factor; TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; TRAIL-R1 = tumor necrosis factor-related apoptosis-inducing ligand receptor 1; TRAIL-R2 = tumor necrosis factor-related apoptosis-inducing ligand receptor 2; TRAIL-R3 = tumor necrosis factor-related apoptosis-inducing ligand receptor 3; TRAIL-R4 = tumor necrosis factor-related apoptosis-inducing ligand receptor 4.

Figure 2 Expression of Ligands and Receptors in Breast Cancer and Non-Neoplastic Tissues. A, Representative IHC Staining for OPG, TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, RANKL, RANK, IL-6, IL-6R, SDF-1, CXCR-4, M-CSF, CD115, CCL-2, and CCR-2 in TEpC and EpC of BC and Non-Neoplastic Tissues, Respectively, and in Spindle-Shaped SC From Both Tissues. B, No Staining was Observed in Both Types of Tissues When Incubated With an Irrelevant IgG_1 (for TRAIL, TRAIL-R3, RANK, SDF-1, IL-6R, CD115, AE1AE3), Mouse Igs (for TRAIL-R2, RANKL, CXCR-4, IL-6, CCL-2), Goat IgG (for OPG, TRAIL-R1, TRAIL-R4, CCR-2), Rabbit Igs (for M-CSF), and Rat IgG_1 (for CD115) as a Negative Isotype Control. Nuclei were Counterstained With Hematoxylin (*purple*). AE1AE3 was Run as a Positive Control of Breast EpC. *Arrows* Indicate Positively Stained Spindle-Shaped SC. Negative or Low Staining was Observed in Non-Neoplastic Samples (Magnification × 400, the Scale bar Represents 50 μm)



Abbreviations: BC = breast cancer; CCL-2 = chemokine (C-C motif) ligand 2; CCR-2 = chemokine (C-C motif) receptor 2; CXCR-4 = chemokine receptor type 4; Ig = immunoglobulin; IL = interleukin; M-CSF = macrophage colony-stimulating factor; OPG = osteoprotegerin; RANK = receptor activator of nuclear factor kappa B; RANKL = receptor activator of nuclear factor kappa-B ligand; SDF = stromal cell–derived factor; TRAIL = tumor necrosis factor-related apoptosis-inducing ligand.

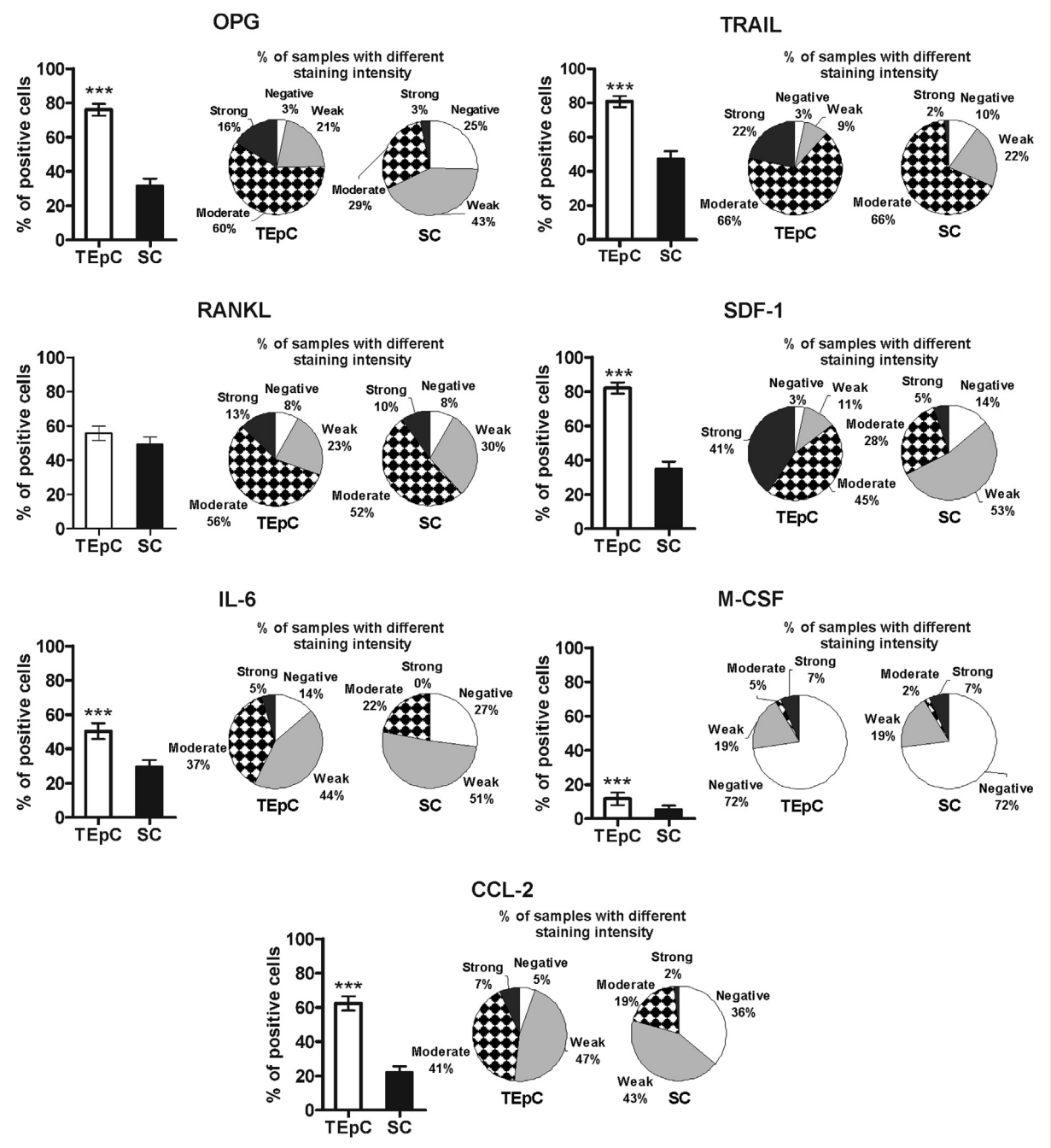
Analysis of Association Between Expression of Ligands and Receptors in Spindle-Shaped SCs From Patients With Breast Cancer

Spearman rank correlation analysis showed a significant positive association between the expression of RANKL/OPG (Spearman K = 0.39, P = .0021), RANKL/RANK (Spearman K = 0.49, P = .0001), TRAIL/TRAIL-R1 (Spearman K = 0.46 P = .0003), TRAIL/TRAIL-R2 (Spearman K = 0.27, P = .0398), IL-6/IL-6R (Spearman K = 0.33, P = .0127), SDF-1/CXCR-4 (Spearman K = 0.43, P = .0004), and CCL-2/CCR-2 (Spearman K = 0.49, P = .0001) in spindle-shaped SC from patients with BC (Figure 5).

Analysis of Association Between Expression of Ligands and Receptors in Tumor Epithelial and Spindle-Shaped SCs From Patients With Breast Cancer

Spearman rank correlation analysis showed a significant positive association between RANKL, IL-6, SDF-1, and CCL-2 expression in TEpC and RANK, IL-6R, CXCR-4, and CCR-2 in spindle-shaped SC from patients with BC (Spearman K = 0.34, P = .0076; K = 0.29, P = .0321; K = 0.38, P = .0022; K = 0.33, P = .0117, respectively) (Figure 6). In addition, Spearman rank correlation analysis showed a significant positive association between TRAIL, RANKL, and CCL-2 in spindle-shaped SC from patients with BC and TRAIL-R1, TRAIL-R4, RANK, and CCR-2

Figure 3 Percentage of Tumor Epithelial Cells and SCs That Express OPG, TRAIL, RANKL, SDF-1, IL-6, M-CSF, and CCL-2, and Percentage of BC Samples With Different Staining Intensity of Each Ligand. Data are Expressed as Mean % ± Standard Error. Statistical Analysis was Performed Using the Mann–Whitney Test. The Level of Statistical Significance was Set at $P \leq .05$ (***$P < .0001$). The Circle Graph Illustrates the Percentage of BC Samples With Different Staining Intensity of Each Ligand



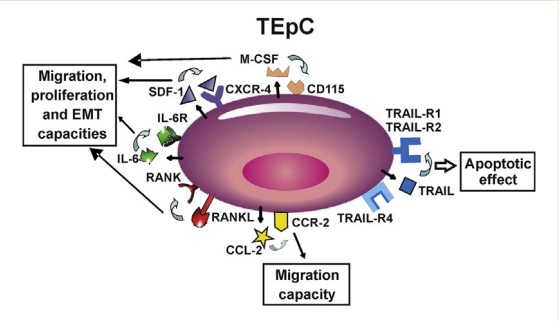
Abbreviations: CCL-2 = chemokine (C-C motif) ligand 2; IL = interleukin; M-CSF = macrophage colony-stimulating factor; RANKL = receptor activator of nuclear factor kappa-B ligand; SC = stromal cell; SDF = stromal cell–derived factor; TEpC = tumor epithelial cell.

expression in TEpC (Spearman K = 0.47, $P = .0002$; K = 0.37, $P = .0040$; K = 0.33, $P = .0098$; K = 0.33, $P = .0114$, respectively) (Figure 6).

Discussion

Dynamic and reciprocal interactions between BC cells and spindle-shaped SC are able to orchestrate critical events regarding

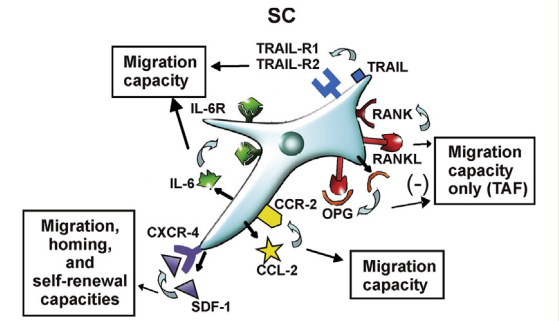
Figure 4 Analysis of Association Between Expression of Ligands and Receptors in Tumor Epithelial Cells From Patients With Breast Cancer The Correlation Between the Ligands and the Rs in TEpC Is Shown. The Ligands (TRAIL, RANKL, IL-6, SDF-1, M-CSF, and CCL-2) Correlate With Their Rs (TRAIL-R1, TRAIL-R2, TRAIL-R4, RANK, IL-6R, CXCR-4, CD115, and CCR-2, Respectively) in TEpC. Correlation Analysis was Performed Using Spearman Rank Correlation. The Level of Statistical Significance was Set at $P \leq .05$



Abbreviations: CCL-2 = chemokine (C-C motif) ligand 2; CCR-2 = chemokine (C-C motif) receptor 2; CXCR-4 = chemokine receptor type 4; EMT = epithelial-mesenchymal transition; IL = interleukin; M-CSF = macrophage colony-stimulating factor; SDF = stromal cell–derived factor; RANK = receptor activator of nuclear factor kappa B; RANKL = receptor activator of nuclear factor kappa-B ligand; TEpC = tumor epithelial cell.

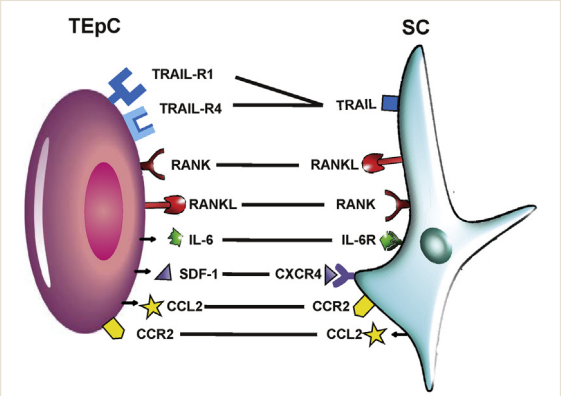
tumor evolution. During the last few years, SC and molecular elements of the microenvironment have been considered as attractive targets for therapeutic strategies.[15,16] However, the association

Figure 5 Analysis of Association Between Expression of Ligands and Receptors in Spindle-Shaped SCs From Patients With Breast Cancer. The Correlation Between the Ligands and the Rs in Spindle-Shaped SC Is Shown. The Ligands (TRAIL, RANKL, IL-6, SDF-1, and CCL-2) Correlate With Their R (TRAIL-R1 and R2, RANK, IL-6R, CXCR-4, and CCR-2, Respectively) in SC (SC: MSC or TAF). Correlation Analysis was Performed Using Spearman Rank Correlation. The Level of Statistical Significance was Set at $P \leq .05$



Abbreviations: CCL-2 = chemokine (C-C motif) ligand 2; CCR-2 = chemokine (C-C motif) receptor 2; CXCR-4 = chemokine receptor type 4; IL = interleukin; OPG = osteoprotegerin; RANK = receptor activator of nuclear factor kappa B; RANKL = receptor activator of nuclear factor kappa-B ligand; SC = stromal cell; SDF = stromal cell–derived factor; TAF = tumor-associated fibroblast; TRAIL = tumor necrosis factor-related apoptosis-inducing ligand.

Figure 6 Analysis of Association Between Expression of Ligands and Receptors in Tumor Epithelial and Spindle-Shaped SCs From Patients With Breast Cancer. The Correlation of the Ligands RANKL, IL-6, SDF-1, and CCL-2 in TEpC With Their Receptors (Rs) RANK, IL-6R, CXCR-4, and CCR-2, Respectively, in Spindle-Shaped SCs Is Shown. The Ligands TRAIL, RANKL, and CCL-2 in Spindle-Shaped SC Correlate With Their Rs TRAIL-R1 and R4, RANK, and CCR-2 in TEpC, Respectively. Correlation Analysis was Performed Using Spearman Rank Correlation. The Level of Statistical Significance was Set at $P \leq .05$



Abbreviations: CCL2 = chemokine (C-C motif) ligand 2; CCR2 = chemokine (C-C motif) receptor 2; CXCR4 = chemokine receptor type 4; IL = interleukin; RANK = receptor activator of nuclear factor kappa B; RANKL = receptor activator of nuclear factor kappa-B ligand; SDF = stromal cell–derived factor; TRAIL = tumor necrosis factor-related apoptosis-inducing ligand.

between the expression of some ligands and the Rs that are involved in BC proliferation, survival, invasion, migration and intravasation in TEpC and spindle-shaped SC in the early stage of BC is not well known. First, in agreement with other authors, we found that the expression of OPG, TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, RANKL, RANK, SDF-1, CXCR-4, IL-6, IL-6R, CCL-2, and CCR-2 was significantly higher in TEpC from patients with BC compared with EpC in non-neoplastic tissues.[17-23]

Furthermore, Spearman rank correlation analysis showed a significant positive association in the expression of RANKL/RANK, TRAIL/TRAIL-R1, TRAIL/TRAIL-R2, TRAIL/TRAIL-R4, IL-6/IL-6R, SDF-1/CXCR-4, M-CSF/CD115, and CCL-2/CCR-2 in TEpC from patients with BC.

Schramek et al[24] demonstrated that the RANKL-RANK system appears to regulate the proliferation of mammary TEpCs.[25] Our study shows the RANKL-RANK interaction as an important event during the early stage of BC. Nevertheless, we cannot exclude a dynamic system that can be modulated and changed during the progression of the disease because it has been reported, for example, that RANKL expression is sometimes lost during tumor progression correlating with a metastatic phenotype.[24]

On the other hand, we found that TRAIL expression is associated with proapoptotic TRAIL R (TRAIL-R1 and TRAIL-R2) and with the decoy R (TRAIL-R4), which antagonizes the proapoptotic actions of TRAIL. Even if recent studies showed that TRAIL behaves as a metastasis suppressor by activating proapoptotic TRAIL-R1 and

TRAIL-R2,[26] we believe that TRAIL correlation with its R (TRAIL-R1, TRAIL-R2, and TRAIL-R4) supports the idea of an autocrine regulation of the apoptotic process. In regard to IL-6, our results are in accordance with the work of Sansone et al,[27] which demonstrates the autocrine IL-6 loop in BC cells, suggesting that IL-6 triggers self-renewal and invasive capacity. In the case of SDF-1/CXCR-4 axis, our results may be related to tumor progression, because SDF-1 signaling through CXCR-4 present in carcinoma cells surface directly boosts the proliferation and EMT of these cells.[28]

Secretion of M-CSF from BC cells is accompanied by the expression of its own R (CD115, in ~50% of all tumors and 90% metastatic tumors), suggesting that this cytokine plays a role in the invasive process of BC cells.[29]

Our observations related to CCL-2/CCR-2 are in accordance with those made by other authors that showed this chemokine interaction as a promoter of BC cell survival and motility.[30,31]

On the other hand, we found that the expression of OPG, TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, RANKL, RANK, CXCR-4, IL-6, IL-6R, and CCL-2 was significantly higher in spindle-shaped SC from patients with BC compared with the expression in non-neoplastic tissues. These results, together with those obtained over TEpC, suggest that the spindle-shaped SC are involved in the evolution of primary tumors through all the factors described.

With respect to spindle-shaped SC from patients with BC, Spearman rank correlation analysis showed a significant positive association among RANKL/RANK, RANKL/OPG, TRAIL/TRAIL-R1, TRAIL/TRAIL-R2, IL-6/IL-6R, SDF-1/CXCR-4, and CCL-2/CCR-2. These associations among RANKL/RANK, IL-6/IL-6R, SDF-1/CXCR-4, and CCL-2/CCR-2 could be important in the context of SC proliferation and migration.[23,28,32-35] However, future studies need to be undertaken to further describe the exact role and mechanism driven by these associations.

Spearman rank correlation also showed a significant positive association between RANKL, IL-6, SDF-1, and CCL-2 expression in TEpC and RANK, IL-6R, CXCR-4, and CCR-2 in spindle-shaped SC, respectively. These results are interesting because many authors have confirmed that RANKL, IL-6, SDF-1, and CCL-2 released by tumor cells (among others) are spindle-shaped SC-attracting factors (eg, MSC or TAF).[33,34,36-39] Finally, we found a significant positive association between TRAIL, RANKL, and CCL-2 expression in spindle-shaped SC and TRAIL-R1 and R4, RANK, and CCR-2 in TEpC, respectively. These last results, along with the findings of other authors, suggest that the spindle-shaped SC through RANKL and CCL-2 could influence the proliferation, survival, invasion, migration, and intravasation of TEpC at an early stage of the disease.[33,34,40-42] In addition, these factors could induce the EMT of TEpC, a phenotype that induces hormone therapy resistance.[33,40,41]

Our data show that breast tumorigenesis is a multifaceted process involving change in the expression of ligands and R in both TEpC and spindle-shaped SC. Although these alterations have been identified in both stromal and epithelial compartments early in the carcinogenic process, it is still unclear which compartment is affected first, the epithelium or the stroma, or if they are affected simultaneously. A deep understanding of the epithelial-stromal biochemical interactions and molecular reciprocal heterotypic signaling is mandatory for developing preventive strategies and new therapeutic targets that take into consideration the procarcinogenic actions of the microenvironment.

Conclusions

We found that the expression of OPG, TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, RANKL, RANK, SDF-1, CXCR-4, IL-6, IL-6R, CCL-2, and CCR-2 was significantly higher in TEpC from patients with BC than in EpC of non-neoplastic breast tissues. Moreover, the expression of OPG, TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, RANKL, RANK, CXCR-4, IL-6, IL-6R, and CCL-2 was significantly higher in spindle-shaped SC from patients with BC than in non-neoplastic tissue SC.

On the other hand, an association among RANKL/RANK, TRAIL/TRAIL-R1, TRAIL/TRAIL-R2, TRAIL/TRAIL-R4, IL-6/IL-6R, SDF-1/CXCR-4, M-CSF/CD115, and CCL-2/CCR-2 was found in TEpC. In addition, we obtained an association among RANKL/OPG, RANKL/RANK, TRAIL/TRAIL-R1, TRAIL/TRAIL-R2, IL-6/IL-6R, SDF-1/CXCR-4, and CCL-2/CCR-2 in spindle-shaped SC of these patients. Finally, we observed a significant positive association between RANKL, IL-6, SDF-1, and CCL-2 expression in TEpC and RANK, IL-6R, CXCR-4, and CCR-2 in spindle-shaped SC. Furthermore, TRAIL, RANKL, and CCL-2 in spindle-shaped SC are associated with TRAIL-R1, TRAIL-R4, RANK, and CCR-2 in TEpC. Our observations may represent an important tool to address new therapeutic targets for BC progression during the early stage of this disease. Further work detailing the complexities of the bidirectional crosstalk between cancer and SCs, not closely associated with vasculature, is required.

Clinical Practice Points

- The interaction, mainly driven by soluble secreted factors, allows tumor cells to modify the stroma via tissue remodeling and gene expression and vice versa. It is known that the network orchestrated by BC cells, SC, and other components of the tumor microenvironment contributes to tumorigenesis, progression, local relapse, and metastases.
- The main result from our study showed the existence of a significant positive association between RANKL, IL-6, SDF-1, and CCL-2 expression in TEpC and RANK, IL-6R, CXCR-4, and CCR-2 in spindle-shaped SC, respectively. Furthermore, we found a significant positive association between TRAIL, RANKL, and CCL-2 expression in spindle-shaped SC and TRAIL-R1 and R4, RANK, and CCR-2 in TEpC, respectively.
- Such evidence may represent an important tool to study tumor inhibition or regression during the early stages of BC, when the association between ligands and R in the tumor epithelial and SCs could be modified.

Disclosure

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The authors have stated that they have no conflicts of interest.

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