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*Original Article*

# Up-regulation of SPARC is associated with tumor progression and epithelial SPARC expression is correlated with poor survival and MMP-2 expression in patients with breast carcinoma

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**Abstract:** Objective: To investigate the potential involvement of secreted protein acidic and rich in cysteine (SPARC) in the progression of the breast tumor and to determine its association with outcome variables and matrix metalloproteinases (MMPs) expression in patients with breast carcinoma (BC). Methods: SPARC expression was examined in 8 pairs of BC tissues and surrounding normal tissues at mRNA and protein levels by qRT-PCR, RNAscope in situ hybridization (ISH), Western blotting, and immunohistochemistry techniques. Immunohistochemical staining of SPARC was done in 26 normal breasts, 76 ductal carcinoma in situ (DCIS), and 198 BC samples. In addition, immunohistochemical staining was performed for MMP-2 and MMP-9 in BC. Results: SPARC expression at mRNA and protein levels was significantly increased in BC tissues compared to the surrounding normal tissues ($P < 0.05$ and $P < 0.01$, respectively). RNAscope ISH and immunohistochemistry of SPARC confirmed an increase in SPARC expression in BC tissues compared with the normal tissues. Epithelial SPARC expression increased continuously from normal breast through DCIS to BC ($P < 0.001$). In patients with BC, high epithelial SPARC expression was associated with worse disease-free survival and overall survival ($P = 0.002$ and $P = 0.048$, respectively) and independently predicted worse disease-free survival ($P = 0.002$). Epithelial SPARC expression was significantly correlated with MMP-2 expression ($P < 0.05$). Conclusion: Up-regulation of SPARC contributes to breast tumor progression. SPARC expression may be a useful biomarker for the prognostic prediction in patients with BC. SPARC can control extracellular matrix degradation through up-regulation of MMP-2.

**Keywords:** SPARC, breast, carcinogenesis, prognosis, matrix metalloproteinases

## Introduction

In Korean women, breast cancer has become the second most common malignancy [1]. Despite the significant improvement in breast cancer mortality, breast cancer is still one of the leading causes of cancer death in Korean women. Invasion and metastasis, the direct causes of mortality in women with breast cancer, involve complicated multi-step cascades [2]. Thus, studies exploring the precise molecular mechanisms involved in breast cancer invasion and metastasis are needed to identify novel targets for the treatment of breast cancer [3]. The extracellular matrix (ECM) is a collection of secreted extracellular molecules that provide structural and biochemical scaffolding for the cellular constituents [4]. Modulation of the ECM is critical in the progression of malignancy, and for the complex processes involved in cancer invasion and metastasis [5]. Matrix metalloproteinases (MMPs) are proteases that participate in the degradation of all ECM components [6].

Matricellular proteins are ECM components that modify cell-ECM interactions [7]. Secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin or basement-membrane protein 40, is a collagen-binding matricellular protein that mediates interactions between cells and their surrounding ECM [8]. SPARC is expressed in a variety of tissues during embryonic development and produced postnatally at

sites of ECM remodeling in response to inflammation, tissue injury, invasion, and metastasis [9, 10]. SPARC has been shown to regulate the activity of MMPs that degrade the ECM, thereby facilitating the invasion and metastasis [11-13].

Modulation of ECM by SPARC can promote or inhibit neoplastic progression depending on the involved tissue and tumor cell types [9, 10]. Therefore, it is necessary to evaluate the mechanism of ECM remodeling and its regulation by SPARC for the development of new treatment regimens for patients with breast cancer. Although several studies have already been carried out with an aim to explore the potential role of SPARC in breast cancer, the results are not conclusive [14-23].

It is not clear whether the production and secretion of SPARC occur in breast carcinoma (BC) cells or cancer-associated stromal cells or in both the compartments [14, 17-23]. Although ductal carcinoma in situ (DCIS) is considered as a precursor of BC, little investigation has been done on the profile of SPARC expression during breast neoplastic progression, including DCIS [24, 25]. Furthermore, there have been conflicting reports on the prognostic significance of SPARC expression in BC cells and the surrounding stroma, with some studies demonstrating that high SPARC expression is a risk factor for poor prognosis [14, 16, 18, 21, 23], while other studies have revealed contradictory results [17, 19, 20].

In this study, we aimed to evaluate the potential involvement of SPARC in the progression of the breast tumor and to verify its association with outcome variables and MMPs expression in patients with BC. The mRNA and protein levels of SPARC were examined in 8 pairs of BC tissues and their corresponding normal tissues by quantitative real-time PCR (qRT-PCR), RNAscope in situ hybridization (ISH), Western blotting, and immunohistochemistry techniques. Immunohistochemical staining of SPARC on tissue microarray (TMA) was performed in 300 patients, including 26, 76, and 198 patients with normal breast tissue, DCIS, and BC, respectively. In BC, we also performed immunohistochemical staining for MMP-2 and MMP-9, which are known to be involved in breast cancer progression [26].

## Materials and methods

### *SPARC mRNA and protein expression in BC tissues and their corresponding normal breast tissues*

*Collection of samples:* Frozen samples and their corresponding formalin-fixed-paraffin-embedded (FFPE) samples comprising of BC tissues and their matching normal breast tissues were provided by the Biobank of Chonnam National University Hwasun Hospital, a member of the Korea Biobank Network. We included 8 patients in the present study and informed consent was obtained from all these participants. The resected specimen in a mirror-imaged fashion was alternatively submitted for biobanking and for histological assessment to evaluate the overall suitability of frozen banking tissues. Approximately 1.0 × 1.0 × 0.5 cm of each BC and normal tissue were removed from the resected sample. Specimens for biobanking were divided into smaller fragments and stored within 30 min after resection in a -196°C liquid nitrogen freezer. Specimens for histological evaluation were fixed in 10% neutral buffered formalin and further processed into paraffin blocks.

Frozen samples were used for qRT-PCR and Western blotting and FFPE samples were used for RNAscope ISH and immunohistochemistry.

*Quantitative real-time polymerase chain reaction (qRT-PCR):* The expression of SPARC mRNA was analyzed by qRT-PCR, as previously described [27]. Total RNA was extracted from frozen breast tissues using the RNeasy Mini Kit (Qiagen, Valencia, CA) and was then used for the cDNA synthesis employing GoScript™ Reverse Transcription System (Promega, Madison, WA). The real-time PCR reaction was performed with TaqMan® Gene Expression Master Mix (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions under the following cycling conditions: initial denaturation for 30 s at 95°C, followed by 40 cycles of 95°C for 15 s and of 60°C for 60 s, in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The data were analyzed using the 7500 system SDS software program (v2.0.5; Applied Biosystems). The following probes of TaqMan® Gene Expression Assays (Thermo Fisher Scientific) were used: Hs00234160_m1 (SPARC) and Hs02758991_

g1 (glyceraldehyde 3-phosphate dehydrogenase, GAPDH). All experiments were performed in triplicate. For data analysis, the $2^{-\Delta\Delta Ct}$ method was used and the value of $2^{-\Delta\Delta Ct}$ indicated the fold change in gene expression normalized to GAPDH.

*RNAscope in situ hybridization (ISH):* Serial sections (4 μm thickness) of FFPE blocks of BC tissues and their normal breast tissues were used for in situ detection of SPARC mRNA signal. RNAscope ISH was performed using a human SPARC probe (Advanced Cell Diagnostic, Hayward, CA) specific to the SPARC (Accession #NM_003118.3; probe region, 1467-2515 bp). RNAscope® 2.0 BROWN (Advanced Cell Diagnostics) was used for signal amplification and detection as directed by the manufacturer instructions [28, 29]. Briefly, sectioned slides were deparaffinized and dehydrated. Slides were then pretreated with heat and protease digestion and hybridized with Probe-Hs-SPARC. Chromogenic detection using 3,3'-diaminobenzidine (DAB) was done followed by counterstaining with hematoxylin. A bacterial *DapB* probe and housekeeping gene *peptidylprolyl isomerase B* (*PPIB*) probe were used as a negative and positive control, respectively.

SPARC mRNA amplification was graded in a semi-quantitative manner following scoring guidelines provided by the manufacturer [28, 29]. The SPARC mRNA amplification was graded as score 0 (no staining or less than 1 dot to every 10 cells, 40 × magnification), score 1 (1-3 dots/cell visible at 20-40 × magnification), score 2 (4-10 dots/cell, very few dot clusters visible at 20-40 × magnification), score 3 (> 10 dots/cell, less than 10% positive cells have dot clusters visible at 20 × magnification) or score 4 (> 10 dots/cell, more than 10% positive cells have dot clusters visible at 20 × magnification). We assessed SPARC mRNA amplification in both epithelial (inner luminal cells of normal breast tissue or BC cells) and surrounding stromal compartments.

*Western blot analysis:* The expression level of SPARC protein was determined by Western blot analysis, as described previously [30]. The mouse polyclonal antibodies to SPARC (1:1,000 dilution, US Biological Life Sciences, Salem, CA) and β-actin antibody (1:1,000 dilution; Cell Signaling Technology, Danvers, MA) as an internal loading control were used for immunolabeling. The bands for SPARC and β-actin protein were measured with Multi-gauge V3.0 analysis software (Fujifilm Life-science, Tokyo, Japan) and the SPARC protein levels were normalized to the corresponding β-actin signal.

*Immunohistochemistry:* Serially sectioned slides of FFPE blocks were used for SPARC immunohistochemical staining. Staining was done using a Bond-max automatic device (Leica Microsystems, Bannockburn, IL) as previously described [31]. Mouse polyclonal antibodies to SPARC (1:200 dilution, US Biological Life Sciences) were used. According to the previous report [18], SPARC immunoreactivity in the epithelial compartment and in the surrounding stromal tissue was semi-quantitatively evaluated based on the intensity of staining and the percentage of stained cells. The final combined staining score was the sum of the staining intensity score (0 for no staining, 1 for light staining, 2 for moderate staining, and 3 for strong staining) and the percentage score of immunostained cells (0, none; 1, 1-10%; 2, 11-30%; 3, 31-50%; and 4, ≥ 51%).

*SPARC expression in normal, DCIS, and BC tissues and MMP-2 and MMP-9 expression in BC tissue*

*Patients and tissues:* The FFPE samples of the normal breast with no pathological lesions (n = 26), DCIS (n = 76), and of BC (n = 198) were selected from patients within the Chonnam National University Hospital and Chonnam National University Hwasun Hospital, Korea.

Patients diagnosed with BC during the period from January 1997 to December 2002 were included in the present study and had a minimum of 10 years follow-up. Medical records were reviewed to obtain treatment protocol and follow-up information. Tumor characteristics were obtained from histopathology reports. Estrogen receptor-α (ER-α), progesterone receptor (PR), and HER2/neu expression were assessed by American Society of Clinical Oncology/College of American Pathologists guideline [32, 33].

*Tissue microarray construction:* One representative FFPE block of each case was used to construct TMA blocks. For each BC, three cores of 1-mm diameter were punched from the donor block. Two 2-mm diameter cores were

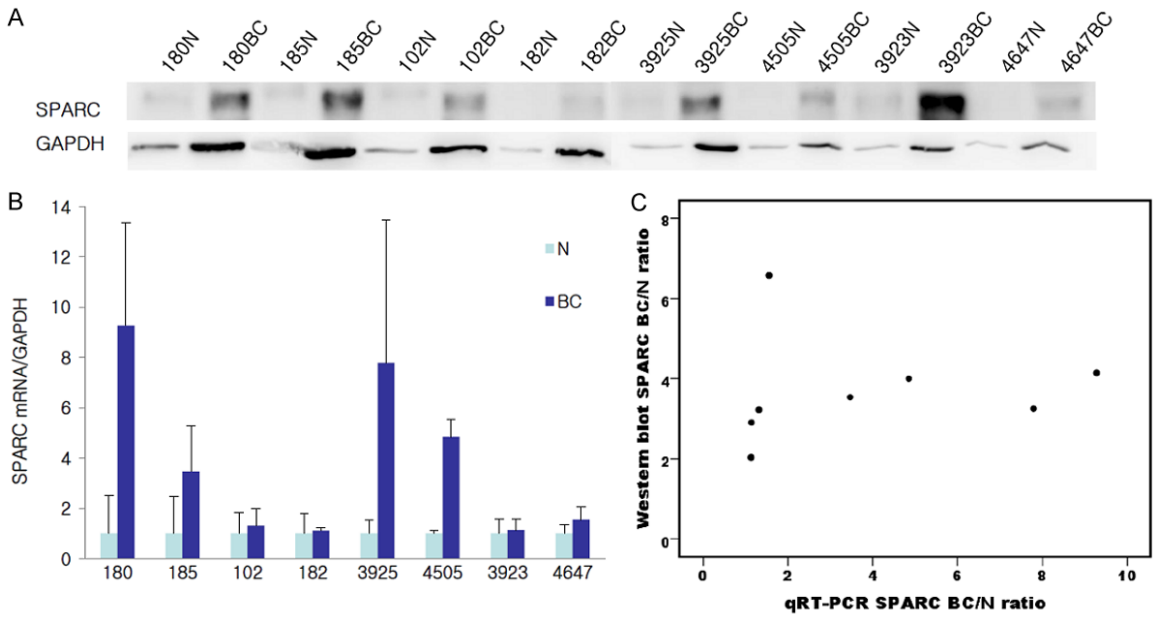


**Figure 1.** Increased SPARC expression in breast carcinoma. Western blot and quantitative real-time PCR analyses of SPARC were performed in eight paired breast carcinoma and adjacent normal specimens. A: SPARC protein was increased in breast carcinoma (BC) tissues compared to that in the corresponding adjacent normal (N) breast tissues. B: qRT-PCR also demonstrated up-regulated SPARC mRNA expression in the BC tissues compared to normal breast tissues. C: A positive correlation was observed between SPARC mRNA and protein expression.

punched from the representative regions of each normal breast and DCIS donor block.

*Immunohistochemistry and evaluation of immunohistochemical staining:* Serial sections (4 μm thickness) of TMA blocks were used for immunohistochemistry using a Bond-max automatic device (Leica Microsystems). Mouse polyclonal antibodies to SPARC (1:200 dilution, US Biological Life Sciences, Salem, CA) and rabbit polyclonal antibodies to MMP-2 (1:25 dilution, Thermo Scientific, Fremont, CA) and MMP-9 (1:50 dilution, Thermo Scientific) were used.

The immunostained slides were digitized with a whole slide scanner (Aperio Technologies, Vista, CA), and SPARC immunoreactivity was assessed as described above. For statistical analysis, specimens with final combined staining scores between 0 and 3 were considered as SPARC low expression and scores between 4 and 7 were defined as SPARC-high expression. Immunohistochemical analysis of MMP-2 and MMP-9 expression was done in the BC cells using a semi-quantitative scoring method, as described previously [31]. In brief, the percentage score of immunostained cells (0, none; 1, 1% to 25%; 2, 26% to 50%; 3, 51% to 76%; and 4, 76% to 100%) was added to the staining intensity score (0, no staining; 1, light; 2, moderate; and 3, strong). The sum of the percentage score and intensity score was used as the final staining score. A final combined staining score of > 2 was considered positive for MMP-2 and MMP-9 expression.

*Statistical analysis*

SPARC mRNA expression according to RNAscope ISH and protein expression measured by Western blotting in the BC tissues and the surrounding normal tissues were compared using the *t*-test (two-sided). The correlation between the SPARC mRNA expression and the protein expression was analyzed by the Spearman correlation test. The association between SPARC expression and breast neoplastic progression was analyzed by the $x^2$ test. The clinicopathologic features, and MMP-2 and MMP-9 expression in patients with BC were correlated with the expression of SPARC and checked by $x^2$ test. Disease-free and overall survival curves for each study group were illustrated by Kaplan-Meier method and the differences between the survival curves were compared using the log-

**Table 1.** SPARC mRNA and protein expression in normal and breast carcinoma tissues

| Case No | RNAscope ISH amplification scores | | | | | | Immunohistochemistry staining scores | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Normal | | | Breast carcinoma | | | Normal | | | Breast carcinoma | | |
| | Epi | Stromal | Sum | Epi | Stromal | Sum | Epi | Stromal | Sum | Epi | Stromal | Sum |
| 180 | 1 | 1 | 2 | 3 | 4 | 7 | 1 | 3 | 4 | 7 | 7 | 14 |
| 185 | 1 | 1 | 2 | 2 | 4 | 6 | 0 | 0 | 0 | 3 | 7 | 10 |
| 102 | 2 | 3 | 5 | 1 | 3 | 4 | 1 | 5 | 6 | 0 | 5 | 5 |
| 182 | 1 | 1 | 2 | 1 | 4 | 5 | 0 | 2 | 2 | 0 | 5 | 5 |
| 3925 | 1 | 1 | 2 | 2 | 4 | 6 | 0 | 3 | 3 | 4 | 7 | 11 |
| 4305 | 1 | 2 | 3 | 2 | 4 | 6 | 0 | 3 | 3 | 3 | 7 | 10 |
| 3923 | 1 | 1 | 2 | 1 | 4 | 5 | 1 | 4 | 5 | 2 | 5 | 7 |
| 4647 | 2 | 2 | 4 | 2 | 3 | 5 | 1 | 5 | 6 | 3 | 4 | 7 |

ISH, in situ hybridization; Epi, epithelial.

rank test. For the multivariate analysis, the Cox's proportional hazard model was used. The SPSS system (version 13.5 for windows; SPSS INC., Chicago, IL) was used for all statistical analyses. A *P*-value < 0.05 was considered statistically significant.

*Compliance with ethical standards*

The Institutional Review Board at the Chonnam National University Hwasun Hospital (Jeollanam-do, Korea) approved this study (CNUHH-2014-156).

## Results

*SPARC mRNA and protein expression in BC tissues and their corresponding normal breast tissues*

We examined SPARC mRNA expression in eight frozen BC tissues and their surrounding normal breast tissues by qRT-PCR analysis. SPARC mRNA was detected in all the studied tissues samples. The expression level of SPARC mRNA in BC tissues was elevated compared to the corresponding normal tissues and the difference was significant (3.81 ± 3.22 vs. 1.00 ± 0.01, P < 0.05) (**Figure 1**). To analyze the correlation of SPARC mRNA expression with protein levels, SPARC protein expression was measured by Western blotting in the same samples used for qRT-PCR analysis. The density of SPARC expression measured by quantitative analysis was significantly increased in BC tissues compared with the corresponding normal tissues (0.71 ± 0.57 vs. 0.21 ± 0.20, P < 0.01). SPARC mRNA and protein ratio between BC tissues and corresponding normal tissues in all eight patients was higher than 1. There was a positive correlation between the SPARC mRNA BC/normal ratio and SPARC protein BC/normal ratio (r = 0.690, P < 0.05).

To investigate the expression and location of SPARC mRNA and protein in tissues, ISH and immunohistochemistry analyses were performed (**Table 1** and **Figure 2**). SPARC mRNA ISH signals were detected in the epithelium and stroma of normal and BC tissues. As expected, serial section samples hybridized with PPIB and DapB mRNA probes showed positive and no signal, respectively. Comparative analysis of the sum of epithelial and stromal SPARC mRNA amplification scores between BC tissues and normal breast tissues revealed increased SPARC mRNA expression in BC tissues compared with their corresponding normal tissues (5.5 ± 0.3 vs. 2.8 ± 1.2, P < 0.01). SPARC mRNA expressions were higher in the stromal component than in the epithelial component of normal breast and BC tissues, and the difference was only significant in BC tissues (P < 0.001).

Similar results were observed when the SPARC immunohistochemically stained slides were scored. The immunolocalization of SPARC showed cytoplasmic staining in the epithelium and stroma of normal and BC tissues. The sum of epithelial and stromal SPARC staining scores was significantly higher in BC tissues compared with adjacent normal tissues (8.6 ± 3.2 vs. 3.6 ± 2.1, P < 0.05). Among normal or BC tissues, the staining scores of SPARC expression were significantly higher in the stromal compartment as compared to the scores in epithelial com-

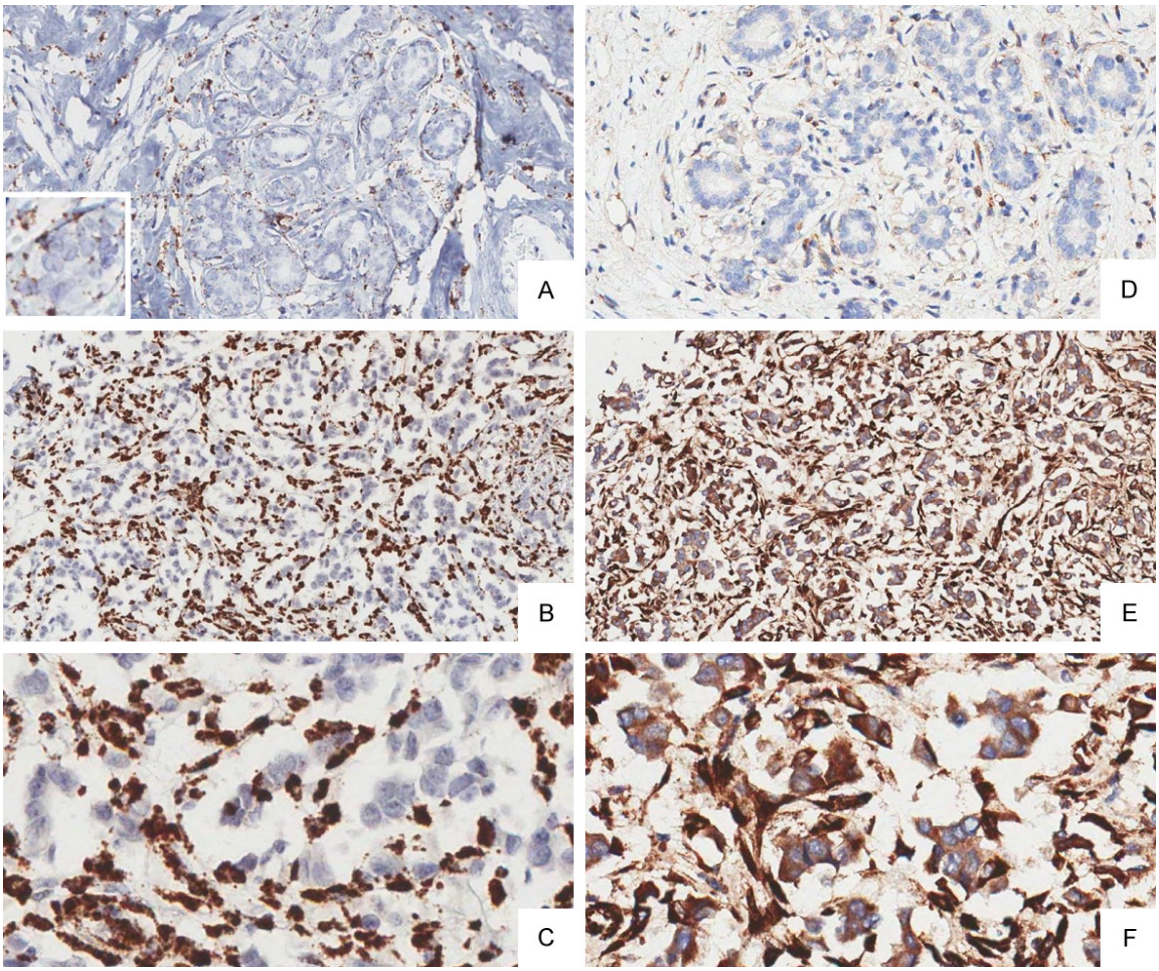


**Figure 2.** Expression and localization of SPARC mRNA and protein in the breast carcinoma tissues and the corresponding normal breast tissues of serial sectioned samples. (A: inlet) Normal breast (RNAscope in situ hybridization), (B, C) breast carcinoma (RNAscope in situ hybridization; B: low magnification; C: high magnification); (D) Normal breast (immunohistochemistry), and (E, F) breast carcinoma (immunohistochemistry; E: low magnification; F: high magnification). The expression pattern of SPARC mRNA and protein was similar. The expression of SPARC mRNA and protein was higher in breast carcinoma tissues compared with corresponding normal tissues and was higher in stromal area than in epithelia area of breast carcinoma tissues.

partment ($P < 0.05$ and $P < 0.01$, respectively). There was a significant positive correlation between the mRNA RNAscope ISH SPARC expression and the immunohistochemistry SPARC expression in BC tissues ($r = 0.943$, $P < 0.001$).

*SPARC expression in the epithelial and stromal compartments of normal, DCIS, and BC tissues*

SPARC immunostaining data were available for all 26 (100%) normal breast tissues, 75 of 76 (98.7%) DCIS cases, and 189 of 198 (95.5%) BC cases, after omission of the cases with non-informative cores. Although some SPARC immunoreactivity was detected in luminal epithelial cells of normal breast tissues, predominant expression was observed in myoepithelial cells and in some stromal cells (**Figure 3A**, **3B**). The endothelial cells and inflammatory cells (macrophages and lymphocytes) also showed SPARC expression. In DCIS and BC tissues, the epithelial and stromal compartments displayed variable SPARC expression (**Figure 3C-F**). The immunoreactivity of SPARC was heightened not only in the stroma but also in the cytoplasm of the carcinoma cells (**Figure 3C**, **3E**). In the DCIS lesions, the SPARC-positive stroma formed a well-defined narrow band around the DCIS

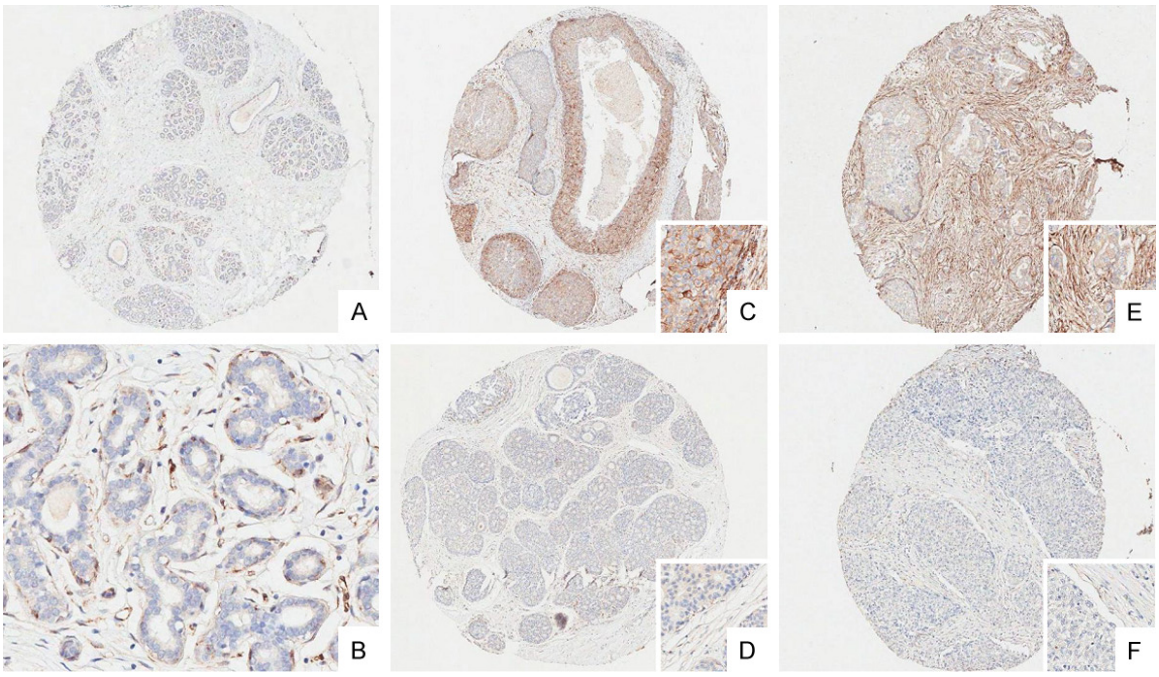


**Figure 3.** SPARC expression in normal breast (A, B), ductal carcinoma in situ (DCIS) (C, D), and breast carcinoma (BC) (E, F). In normal breast tissue, positive staining was mainly restricted to the stromal connective tissue, whereas the epithelial component was devoid of SPARC (A: low magnification; B: high magnification). In DCIS (C: inlet) and IBC (E: inlet), immunoreactivity of SPARC was heightened not only in stroma but also in the cytoplasm of cancer cells. DCIS (D: inlet) and IDC (F: inlet) show low SPARC expression.

**Table 2.** SPARC expression in the epithelial and stromal compartment in normal breast, ductal carcinoma in situ, and breast carcinomas

| Histologic stage | High epithelial SPARC expression N/total N (%) | *P* value | High stromal SPARC expression N/total N (%) | *P* value |
|---|---|---|---|---|
| Normal | 0/26 (0) | < .001* | 10/26 (38.5) | .055 |
| Ductal carcinoma in situ | 8/75 (10.7) | | 49/75 (65.3) | |
| Breast carcinoma | 49/189 (25.9) | | 107/189 (56.6) | |

N, numbers, *Linear-by-linear association.

malignant epithelium (**Figure 3C**). In the BC, stromal SPARC staining was more diffuse and intense, and not just juxtaposed with the malignant epithelium as observed in the DCIS lesions (**Figure 3E**).

SPARC expression in the epithelial and stromal compartments of normal breast, DCIS, and BC tissues is summarized in **Table 2**. Differential expression of SPARC was noted in the normal, DCIS and BC groups. High epithelial and stromal SPARC expression was detected in 0 (0%) and 10 (38.5%) of normal breast tissues, 8 (10.7%) and 49 (65.3%) of 75 DCIS cases, and 49 (25.9%) and 107 (56.6%) of 189 BC cases, respectively. The expression of SPARC in the stromal compartment was significantly higher than the corresponding expression in the epithelial compartment of each group (normal breast tissue, $P < 0.01$, DCIS tissue, $P < 0.001$; and BC tissue, $P < 0.001$, respectively). Epithelial SPARC expression increased progressively from normal breast through DCIS to BC ($r = 0.216$, $P < 0.05$) and epithelial SPARC expression in BC was significantly higher than in DCIS ($P < 0.01$). Stromal expression of SPARC in DCIS and BC was significantly higher as compared to the normal breast ($P < 0.01$, for both). No significant difference in stromal SPARC expression between DCIS and BC was noted.

*Relationship between SPARC expression and clinicopathologic features of BC*

Associations between SPARC expression and the clinicopathologic features in patients with

**Table 3.** Relationship between SPARC expression and clinicopathologic parameters in breast carcinomas

| Characteristics | High epithelial SPARC expression N/total N (%) | *P* value* | High stromal SPARC expression N/total N (%) | *P* value* |
|---|---|---|---|---|
| Age (years) | | 0.133 | | 0.374 |
| ≤ 46 | 23/107 (21.5) | | 64/107 (59.8) | |
| > 46 | 26/82 (31.7) | | 43/82 (52.4) | |
| Histopathologic type | | 0.677 | | 0.631 |
| Invasive ductal carcinoma, NOS | 43/162 (26.5) | | 94/162 (58.0) | |
| Invasive lobular carcinoma | 6/25 (24.0) | | 12/25 (48.0) | |
| Mucinous carcinoma | 0/2 (0) | | 1/2 (50.0) | |
| Tumor size (cm) | | 0.070 | | 0.104 |
| ≤ 2 | 13/32 (40.6) | | 25/32 (78.1) | |
| 2-5 | 30/128 (23.4) | | 65/128 (50.8) | |
| > 5 | 6/29 (20.7) | | 17/29 (58.6) | |
| Number of nodal metastasis | | 0.590 | | 0.393 |
| 0 | 26/99 (26.3) | | 57/99 (57.6) | |
| 1-3 | 10/50 (20.0) | | 31/50 (62.0) | |
| 4-9 | 8/24 (33.3) | | 11/24 (45.8) | |
| ≥ 10 | 5/16 (31.3) | | 8/16 (50.0) | |
| Histologic grade | | 0.397 | | 0.219 |
| 1 | 4/23 (17.4) | | 17/23 (73.9) | |
| 2 | 26/98 (26.5) | | 53/98 (54.1) | |
| 3 | 19/68 (27.9) | | 37/68 (54.4) | |
| Stage | | | | |
| I | 13/32 (40.6) | 0.539 | 25/32 (78.1) | 0.052 |
| II | 21/108 (19.4) | | 56/108 (51.9) | |
| III | 15/49 (30.6) | | 26/49 (53.1) | |
| Estrogen receptor-α | | 0.096 | | 0.555 |
| Negative | 24/84 (28.6) | | 50/84 (59.5) | |
| Positive | 28/105 (26.7) | | 57/105 (54.3) | |
| Progesterone receptor | | 0.318 | | 0.464 |
| Negative | 25/84 (29.8) | | 45/84 (53.6) | |
| Positive | 24/105 (22.9) | | 62/105 (59.0) | |
| HER-2 | | 0.675 | | 0.854 |
| Negative | 41/153 (26.8) | | 86/153 (56.2) | |
| Positive | 8/36 (22.2) | | 21/36 (58.3) | |
| Molecular subtypes | | 0.152 | | 0.723 |
| Luminal | 28/129 (21.7) | | 72/129 (55.8) | |
| HER2 | 7/20 (35.0) | | 13/20 (65.0) | |
| Triple negative | 14/40 (35.0) | | 22/40 (55.0) | |
| Distant metastatic relapse | | 0.015 | | 0.325 |
| No | 29/138 (21.0) | | 75/138 (54.3) | |
| Yes | 20/51 (39.2) | | 32/51 (62.7) | |

N, number; NOS, Not otherwise specified; ns, not significant. *Analyzed by $\chi^2$ test.

BC are summarized in **Table 3**. High epithelial SPARC expression was significantly associated with distant metastatic relapse (present, 39.2% vs. absent, 21.0%, $P < 0.05$). Other clinicopathologic features, including age, histologic type, tumor size, nodal metastasis, grade, stage,

**Table 4.** Univariate analysis of prognostic factors in patients with breast carcinoma

| | Survival | |
|---|---|---|
| | Disease-free | Overall |
| Age | 0.921 | 0.490 |
| Histologic type | 0.084 | 0.439 |
| Tumor size | 0.001 | < 0.001 |
| Lymph node status | < 0.001 | < 0.001 |
| Histologic grade | 0.533 | 0.110 |
| Stage | < 0.001 | < 0.001 |
| Hormonal therapy | 0.386 | 0.310 |
| Chemotherapy/radiotherapy | 0.027 | 0.017 |
| Estrogen receptor-α status | 0.446 | 0.411 |
| Progesterone receptor status | 0.565 | 0.658 |
| HER-2 status | 0.911 | 0.667 |
| Epithelial SPARC expression | 0.002 | 0.048 |
| Stromal SPARC expression | 0.107 | 0.438 |

ER-α, PR, HER2/neu, and molecular subtypes were not found to be associated with SPARC expression.

*Summary of survival analysis*

In univariate analysis, tumor size, lymph node status, stage, and chemotherapy/radiotherapy status were observed as statistically significant risk factors affecting the disease-free and overall survival of patients with BC (**Table 4**). Patients with high epithelial SPARC expression showed a worse prognosis for disease-free and overall survival compared to those with low expression (P = 0.002 and P = 0.048, respectively) (**Figure 4**). However, stromal SPARC expression was not associated with survival. We categorized SPARC expression as high or low for both the epithelial and stromal compartments of BC: epithelial low/stromal low, epithelial high/stromal low, epithelial low/stromal high, and epithelial high/stromal high. However, this categorization did not enhance the statistical performance in terms of its ability to estimate survival (data not shown). A multivariate survival analysis demonstrated tumor size, stage, and epithelial SPARC expression as independent prognostic factors for disease-free survival (**Table 5**). Lymph node status was the only independent poor prognostic factor for overall survival.

*Correlation between SPARC expression, and MMP-2 and MMP-9 expression in BC*

Immunostaining MMP-2 and MMP-9 data for all 189 BC cases were available. Since carcinoma cells showed greater expression of MMP-2 and MMP-9 than stromal cells, MMP-2 and MMP-9 immunoreactivity was only evaluated in the BC cells. MMP-2 and MMP-9 positivity was present in 37.6% (71/189) and 30.2% (57/189) of BC cases, respectively. Correlation analysis of SPARC expression and MMP-2 and MMP-9 expression showed a strong positive correlation between epithelial SPARC expression and MMP-2 expression (P < 0.05, **Table 6**; **Figure 5**).

## Discussion

SPARC expression in tumor cells and/or in the surrounding stromal cells is correlated with tumor progression, clinical outcome, and MMPs expression in various types of malignancies. In this study, we demonstrated up-regulation of SPARC mRNA and protein expression in BC tissues as compared with their corresponding normal breast tissues. Epithelial SPARC expression was associated with breast tumor progression and was correlated with poor survival as well as MMP-2 expression in BC.

ECM is an extracellular protein scaffold that provides structural and biochemical support for the connective tissues. Modulation of the ECM is an important step in tumor development and progression [5]. ECM deposition and remodeling is regulated by a family of extracellular proteins known as matricellular proteins [7]. SPARC, a small ECM-associated matricellular glycoprotein, mediates interactions between cells and their microenvironment, including the ECM [8]. During extensive matrix remodeling in tumor progression, SPARC is differentially expressed in tumor-associated stroma or in tumor cells, and SPARC can promote or inhibit tumor progression depending on the tissue and cell type [9, 10].

Published data reveal the oncogenic properties of SPARC in BC [14-23]. SPARC is nearly absent or undetectable in normal human breast tissues while its expression is increased in BC [18, 22]. However, published data on the compartmentalization of SPARC expression in BC are inconsistent and often contradictory. Several studies have shown that SPARC overexpression appears to occur mainly in cancer-associated stroma [19, 22]. This finding is in contrast to the findings stated by Nagai et al. [17] and Hsiao et al. [18], which revealed cancer cells as a unique source of SPARC expression. On the contrary, Zhu et al. [14] and

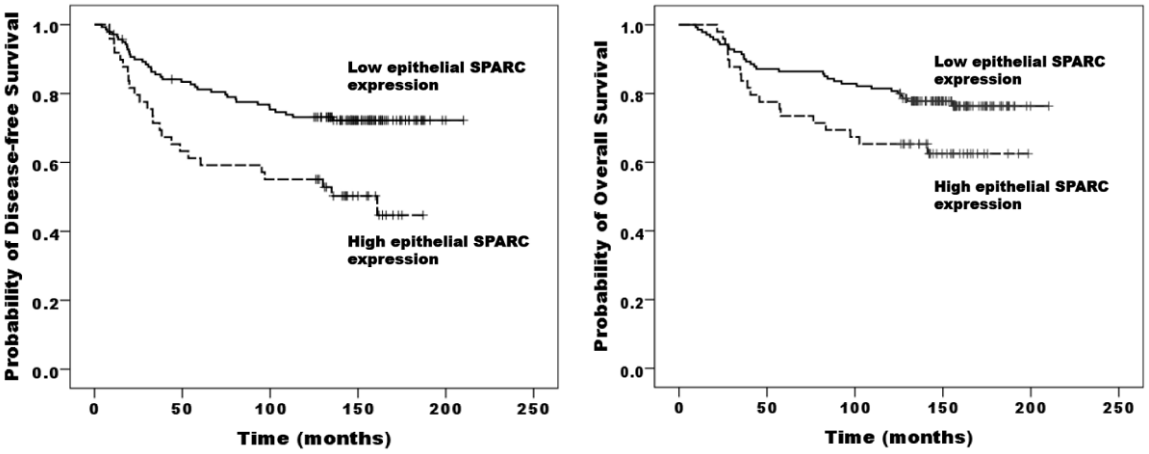


**Figure 4.** Survival of patients grouped according to SPARC expression. High epithelial SPARC expression predicts significantly poor disease-free and overall survival ($P = 0.002$ and $P = 0.048$, respectively).

**Table 5.** Multivariate analysis with Cox's proportional hazard model for prognostic factors in patients with breast carcinoma

| | Disease-free survival | | | Overall survival | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *P* value | HR | 95% CI | *P* value |
| Age (≤ 46 v > 46 years) | 0.111 | 0.550-1.528 | 0.739 | 0.001 | 0.551-1.783 | 0.992 |
| Tumor size (≤ 5 v > 5 cm) | 5.236 | 0.227-0.892 | 0.022 | 2.815 | 0.260-1.110 | 0.093 |
| Lymph node status (negative v positive) | 8.621 | 0.184-0.714 | 0.003 | 6.203 | 0.152-0.799 | 0.013 |
| Stage (I/II v III) | 0.125 | 0.432-1.791 | 0.724 | 1.442 | 0.279-1.359 | 0.230 |
| Chemotherapy/radotherapy (No v Yes) | 0.889 | 0.212-1.723 | 0.346 | 1.294 | 0.100-1.841 | 0.255 |
| Epithelial SPARC expression (low v high) | 9.792 | 0.259-0.733 | 0.002 | 3.351 | 0.318-1.040 | 0.067 |

**Table 6.** Relationship between SPARC expression and MMPs expression in breast carcinomas

| Characteristics | High epithelial SPARC expression N/total N (%) | Low epithelial SPARC expression N/total N (%) | *P* value* | High stromal SPARC expression N/total N (%) | Low stromal SPARC expression N/total N (%) | *P* value* |
|---|---|---|---|---|---|---|
| MMP-2 positive | 25/49 (51.0) | 46/140 (32.9) | 0.027 | 46/107 (43.0) | 25/82 (30.5) | 0.096 |
| MMP-9 positive | 16/49 (32.7) | 41/140 (29.3) | 0.718 | 32/107 (29.9) | 25/82 (30.5) | 1.000 |

*Analyzed by $\chi^2$ test.

Watkins et al. [23] detected increased SPARC expression in both cancer and cancer-associated stromal cells when compared to normal tissue. Although the reason for these discrepancies is unclear, it could be explained by differences in methodology, scoring systems, and protein expression cutoff levels.

For higher accuracy, we first assessed mRNA and protein levels of SPARC in frozen BC tissues and their corresponding normal breast tissues using qRT-PCR and Western blotting. Our data showed that SPARC mRNA and protein expression was significantly up-regulated in BC tissues compared with the surrounding normal breast tissues. To investigate the expression and location of SPARC mRNA in BC tissues, we used RNAscope ISH, which is a novel non-radioisotopic RNA ISH technology, in FFPE samples. This technique can detect mRNA as a single gene copy and can be applied to routine clinical samples for biomarker analysis [28, 29]. There exists no previous data about the use of this method for the detection SPARC mRNA in BC tissues. RNAscope ISH of SPARC confirmed that SPARC mRNA expression was increased in BC tissues compared with their normal tissues counterpart. In addition, SPARC mRNA was

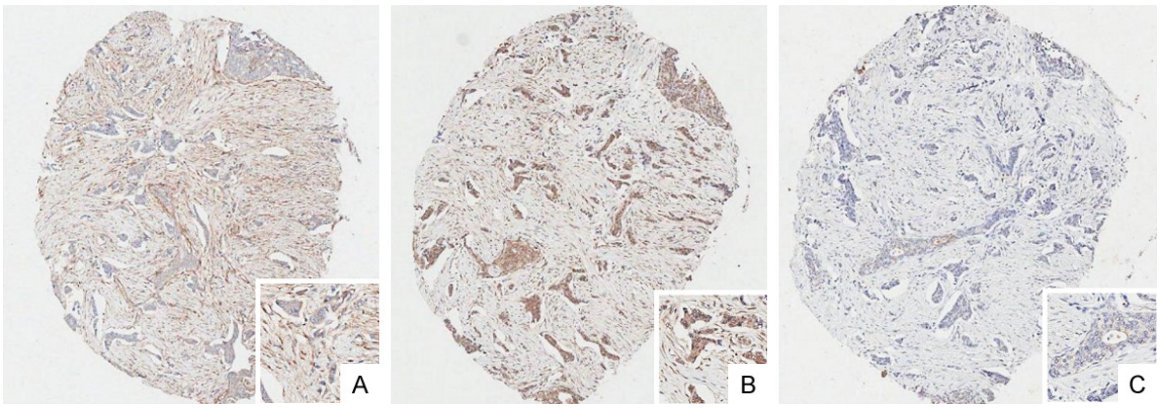


**Figure 5.** MMP-2 (B: inlet) and MMP-9 (C: inlet) expression based on high SPARC (A: inlet) expression in invasive breast carcinoma. Immunostaining for MMP-2 and MMP-9 protein was not only localized in neoplastic cells but also in stromal cells around the tumor. Co-expression of SPARC and MMP-2 proteins is noted in invasive breast carcinoma.

more pronounced in the stromal compartment than in the epithelial compartment in both normal and BC tissues. These SPARC RNAscope ISH results were similar to those of immunohistochemical staining of SPARC protein. SPARC mRNA levels correlated with SPARC protein levels regardless of the employed method of quantification. Although the underlying mechanism of SPARC up-regulation in BC cells remains speculative, our data confirmed that up-regulation of SPARC mRNA and protein expression occurs in both cancer cells and cancer-associated stromal cells of BC tissues. Furthermore, it is proposed that SPARC protein expression in BC tissues is regulated mainly at the transcriptional level.

Although BC is believed to develop from histologically identifiable non-invasive DCIS, there have been few studies examining the role of SPARC in DCIS lesions. Witkiewicz et al. immunohistochemically evaluated SPARC expression in 97 cases of DCIS [25]. SPARC expression was observed in both tumor and peritumoral stromal cells in DCIS lesion. Stroma distant to the DCIS lesion showed absent or weak SPARC expression. Szynglarewicz et al. reported positive SPARC expression in DCIS tumor cells, stromal fibroblasts, and myoepithelial cells in 38%, 62%, and 61% of 209 DCIS cases, respectively [24]. Strong SPARC expression in tumor cells and stromal fibroblasts was observed as most significant and independent predictive factor for postoperative invasion.

To evaluate the potential involvement of SPARC in the progression of breast tumor, we included normal breast, DCIS, and BC tissues, and evaluated immunohistochemical SPARC expression in the epithelial compartment and in the surrounding stromal compartment using TMA technology. Stromal SPARC expression in the normal breast, DCIS, and BC groups was significantly higher than epithelial SPARC expression. These findings suggest that SPARC is produced and secreted by both epithelial and stromal cells of normal and cancerous breast tissues and stromal overexpression is far more frequent than epithelial overexpression. Epithelial SPARC expression was found to increase progressively from normal breast tissue through DCIS to BC. Epithelial SPARC expression in BC tissue was significantly higher compared to DCIS tissue. Stromal SPARC expression was higher in DCIS and BC tissues than in normal breast tissue. Although there was no significant difference in stromal SPARC expression between DCIS and BC tissues, stromal SPARC staining in BC tissue was more diffuse and intense, and not just juxtaposed with the malignant epithelium as observed in DCIS lesions. These results suggest that SPARC plays an imperative role in all stages of breast carcinogenesis and is especially activated during the process of DCIS development. SPARC might play a different biological role in breast carcinogenesis, depending on its compartmentalization and redistribution of stromal SPARC expression may be involved in BC invasion.

Up-regulation of SPARC expression in cancer cells or in surrounding stromal cells can promote metastasis and could be associated with the clinical outcomes of cancer patients [9, 10].

However, there have been conflicting reports on the prognostic significance of SPARC expression in BC cells and the surrounding stroma [14-23]. SPARC gene expression analysis showed that high expression in BC was associated with poor prognosis [16, 23]. Conversely, Bergamaschi et al. defined ECM signatures by evaluating the gene expression profiles of 278 ECM-related genes derived from the literature, and found that high SPARC expression was associated with improved outcome in BC [20]. Immunohistochemical data from several studies have demonstrated a significant association of SPARC expression in cancer cells with poor prognosis in patients with BC [18, 21]. Recently Zhu et al. also showed that high SPARC expression in cancer cells was an independent prognostic factor for both disease-free survival and overall survival in triple-negative breast cancer patients [14]. Lindner et al. performed the immunohistochemical evaluation of SPARC expression in the pretherapeutic core biopsies of 667 BC patients treated with neoadjuvant chemotherapy [15]. Although there was no significant correlation between SPARC expression and overall or disease-free survival, high epithelial SPARC expression in BC was found to be associated with an increased pathological complete response after neoadjuvant anthracycline-taxane-based chemotherapy. On the contrary, patients with low-expressing levels of SPARC in cancer cells had worse disease-free and overall survival compared to those with high expressing levels of SPARC [17]. With respect to the prognostic relevance of SPARC in the cancer-associated stroma, patients with strong stromal SPARC positivity showed a trend for increased disease-specific survival in univariate analysis [19]. However, stromal SPARC staining showed no significant association with disease-specific survival in the multivariate analysis.

Furthermore, we observed a significant association between the expression of epithelial SPARC and metastasis. High epithelial SPARC expression in BC tissues was more frequent in the distant metastatic relapse-positive group than in the relapse-negative group. Disease-free and overall survival was significantly poorer in BC patients with high epithelial SPARC expression compared to the patients with low epithelial SPARC expression. Multivariate analyses have confirmed that high epithelial SPARC expression was an independent poor prognostic indicator for disease-free survival. Despite the high predominance of stromal SPARC expression observed in our study, the results were not in correlation with the survival of BC patients. These results support the findings of Zhu et al. [14], Hsiao et al. [18], and Lien et al. [21], and suggest that epithelial SPARC overexpression may be associated with a more aggressive phenotype in BC, and may be used as a poor prognostic marker in patients with BC.

SPARC has been shown to modulate the activity of MMPs, a family of enzymes considered as the primary mediators of ECM degradation and turnover [11-13]. MMP-2 and MMP-9 are efficient in degrading type IV collagen, the major component of the basement membrane, which is invaded during BC progression [26]. Previous research stated that SPARC could induce MMP-2 activation in two breast cancer cell lines (MDA-MB-231 and BT549), thereby increasing invasion and metastasis [11]. Moreover, Zhao et al. evaluated the immunohistochemical expression of SPARC and MMP-2 in 436 gastric cancer specimens, and found a significant correlation between SPARC and MMP-2 expression in cancer cells [34].

In the present study, MMP-2 and MMP-9 expressions in cancer cells were positive in 37.6% and 30.2% of BC samples, respectively. The study also demonstrated that SPARC expression was significantly correlated with MMP-2 expression in BC cells. In accordance with the findings of the aforementioned studies, our results indicate that up-regulation of SPARC in BC cells can induce MMPs (probably chiefly MMP-2), providing a mechanism for SPARC to facilitate the progression of BC and to confer a worse prognosis in patients with BC. For the reason that other MMPs such as MMP-1, MMP-3, and MMP-11 have been demonstrated to correlate with SPARC expression, further studies should be performed to evaluate the role of SPARC in ECM turnover in the BC microenvironment [35, 36].

In conclusion, our results suggest that up-regulation of SPARC plays an important role in breast tumor progression. Epithelial SPARC expression may be a useful biomarker to predict prognosis in patients with BC. In addition,

SPARC can control ECM remodeling through up-regulation of MMP-2.

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## Disclosure of conflict of interest

None.

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