Osteopontin Regulates VEGFA and ICAM-1 mRNA Expression in Breast Carcinoma

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Key Words: Bcl2; Breast carcinoma; ICAM-1; mRNA expression; Osteopontin; VEGFA

ABSTRACT

Objectives: To analyze the regulatory role of osteopontin on biomarkers associated with cell survival, invasiveness, and angiogenesis mechanisms in a clinical series and breast cancer cell lines.

Methods: We analyzed by quantitative real-time polymerase chain reaction the messenger RNA (mRNA) expression of osteopontin, Bcl2, intercellular adhesion molecule 1 (ICAM-1), and vascular endothelial growth factor A (VEGFA) in several breast cancer cell lines and in 148 breast carcinomas classified into intrinsic subtypes.

Results: We found coexpression of osteopontin, Bcl2, ICAM-1, and VEGFA in triple-negative MDA-MB-468 and MDA-MB-231 cell lines. Furthermore, osteopontin silencing by small interfering RNA inhibited ICAM-1 and VEGFA expression and cell proliferation in MDA-MB-468 cells. In breast cancer specimens, we found a positive correlation between osteopontin, ICAM-1, and VEGFA mRNA expression, especially in triple-negative/basal-like tumors. Among patients with osteopontin-overexpressing tumors, VEGFA remained an independent prognostic indicator for recurrence (hazard ratio, 2.95; 95% confidence interval [CI], 1.48-5.87; P = .002) and death (hazard ratio, 3.25; 95% CI, 1.48-7.11; P = .003) (multivariate analysis, Cox regression).

Conclusions: Our results support that osteopontin regulates ICAM-1 and VEGFA expression mainly in triple-negative/basal-like breast carcinomas, suggesting a relevant role in the pathogenesis and tumor progression of this molecular subtype. Moreover, VEGFA mRNA levels showed an independent prognostic value in patients with breast cancer.

Breast cancer is the most commonly diagnosed neoplasm among women and the second major cause of cancer death worldwide. Classical clinicopathologic factors and, more recently, intrinsic subtypes have shown correlation with prognosis. It is well known that patients’ outcome depends primarily on the development of distant metastasis. This is the mechanism by which tumor cells acquire capabilities to survive in stressed conditions and invade new tissues.

SPPI (secreted phosphoprotein 1) is a metastasis-associated gene located on chromosome 4 that encodes osteopontin, a glycoprophosphoprotein highly expressed in cancer cells. Secreted osteopontin interacts with cell receptors such as integrins and CD44, thus regulating cell signaling pathways and gene expression. Thereby, osteopontin orchestrates several cellular processes, inducing an increased metastatic potential. Experimental studies with breast cancer cells have shown that high levels of osteopontin promote cell survival, activating the PI3K/Akt and JAK2/STAT3 pathways.
signaling pathways and suppressing apoptosis by increasing the expression of Bcl2.\textsuperscript{8,9}  
Cancer cells establish cell-cell and cell-extracellular matrix unions in a tumor microenvironment through a variety of cell surface proteins, such as integrins, cadherins, and immunoglobulins. Breast cancer cells express the intercellular adhesion molecule 1 (ICAM-1) that belongs to the immunoglobulin superfamily, which induces cell motility and invasion.\textsuperscript{10} Ahmed and Kundu\textsuperscript{11} reported in breast cancer cells that osteopontin binding to \( \alpha \beta \) integrin through nuclear factor (NF)-\( \kappa \)B activates AP-1 transcriptional activity, which in turn increases ICAM-1 expression. In human breast carcinomas, elevated ICAM-1 levels have been correlated with high-grade lymph node metastasis and increased relapse rates.\textsuperscript{12}  
Vascular endothelial growth factor (VEGF) is one of the main regulators of neoangiogenesis, which is essential for tumor growth. Its regulation depends on oxygen levels, growth factors, and oncogenes.\textsuperscript{13,14} Chakraborty et al\textsuperscript{15} showed in breast cancer cells that osteopontin stimulates VEGF expression at both messenger RNA (mRNA) and protein levels. Furthermore, osteopontin-induced VEGF enhanced aggressive tumor features such as poor differentiation, vessel formation, and cell infiltration in mice models. However, these results were confirmed in only a small series of 14 human breast carcinomas.

In vitro experiments using cell lines\textsuperscript{6,7,9,11} and in vivo studies with mouse models\textsuperscript{15,16} support the role of osteopontin in breast cancer progression. Nevertheless, there are no published data about the relationship between osteopontin and the above related biomarkers in clinical series of breast carcinoma. Therefore, we conducted a retrospective analysis of \( SPP1 \), \( BCL2 \), \( ICAM1 \), and \( VEGFA \) mRNA transcript levels in breast cancer cells and in a clinical series of intrinsic subtypes of breast carcinoma in relation to clinicopathologic features and outcome. On the basis of prior experimental evidence, we hypothesized that osteopontin mRNA expression levels correlate with the expression of \( BCL2 \), \( ICAM1 \), and \( VEGFA \) and more aggressive phenotypes. Our results showed a positive association between \( SPP1 \), \( ICAM1 \), and \( VEGFA \) mRNA expression levels in breast tumors, especially in the triple-negative/basal-like subtype. Moreover, among osteopontin-overexpressing tumors, vascular endothelial growth factor A (VEGFA) showed an independent poor prognostic value.

### Materials and Methods

#### Cell Lines

Human breast cell lines were obtained from American Type Culture Collection (Manassas, VA): MCF-7 (luminal A), BT-474 (luminal B), SK-BR-3 (human epidermal growth factor receptor 2 [HER2] positive), MDA-MB-468 (triple-negative/basal-like), and MDA-MB-231 (triple-negative/claudin-low) cancer cells\textsuperscript{17} and the non–tumor-derived epithelial breast cell line 184A1. Cells were maintained in Dulbecco modified Eagle medium/Ham F12 (1:1) with L-glutamine and HEPES media supplemented with 10% vol/vol fetal bovine serum (PAA, Pasching, Austria), 50 U/mL penicillin, and 50 mg/mL streptomycin and incubated in a humidified 5% CO\textsubscript{2} air atmosphere at 37°C.

#### Tumor Sample Collection

This retrospective study included a nonconsecutive series of 177 patients with stage I to III primary breast carcinoma diagnosed and treated at the University General Hospital of Alicante, Spain, between January 1994 and December 2007. Clinical data were available in all cases included. Inclusion criteria were as follows: patients with infiltrating breast carcinoma, complete pathologic data, presence of the expression of the endogenous control gene in tumor tissue, and follow-up information. Exclusion criteria were the following: preoperative chemo- or radiation therapy, patients in stage IV, a quantity of RNA lower than 1,000 ng, and an RNA ratio of 260/280 nm or 260/230 nm lower than 1.70. Clinicopathologic and molecular features recorded were age, tumor size, histologic grade according to the Elston and Ellis method,\textsuperscript{18} nodal status, breast cancer intrinsic phenotype, and the mRNA expression fold change of \( SPP1 \), \( BCL2 \), \( ICAM1 \), and \( VEGFA \) genes. A subset of patients in the current study has been included in previous studies, and part of the results have been published.\textsuperscript{19,20}

Patients underwent breast-conserving surgery (\( n = 90 \) [51%]) or mastectomy (\( n = 87 \) [49%]). Among patients who received breast-conserving surgery, those with hormonal receptor (HR)–positive status tumors received tamoxifen for 2 to 5 years, and postlumpectomy radiotherapy was given at a dose of 50 Gy, including a boost (median, 10 Gy). Patients with high-risk factors (young age, high histologic grade, positive lymph nodes, or HR-negative tumors) were treated

<table>
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<td>ABR</td>
<td>Polyclonal</td>
<td>Prediluted</td>
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\textsuperscript{a} Dako (Glostrup, Denmark); Neomarkers (Fremont, CA); Pierce Biotechnology (Rockford, IL); ABR: Affinity BioReagents (Golden, CO).
with systemic chemotherapy with six cycles of cyclophosphamide, methotrexate, and 5-fluorouracil or four cycles of doxorubicin plus cyclophosphamide after surgery. Starting in 2005, trastuzumab (Herceptin) was added to chemotherapy protocols to treat patients with HER2-positive metastatic breast carcinoma (first-line treatment). Therefore, none of the patients included in this study received trastuzumab in the adjuvant setting. Disease-free survival (DFS) was defined as the time from surgery to local and/or distant recurrence and overall survival (OS) as the time from surgery to patient death from breast cancer. Follow-up information was obtained from clinical records, ranging from 1 to 259 months (median, 86 months). Forty-four patients developed local recurrence and/or distant metastasis, and 32 patients died of the disease. This project was approved by the ethics committee of the University General Hospital of Alicante, and patients’ data were anonymized. We followed the Reporting Recommendations for Tumor Marker Prognostic Studies criteria to perform this study.

Immunohistochemistry and Fluorescence In Situ Hybridization

Immunohistochemistry was performed on paraffin-embedded tissue from tissue microarrays using standard techniques, with antibodies and conditions detailed in Table 1. The staining was scored as positive as follows: estrogen receptor (ER)/progesterone receptor (PR), 1% or more (nuclei); Bcl2, 50% or more (cytoplasm); p53, 20% or more (nuclei); Ki-67, 14% or more (nuclei); any degree for CK5/6 (cytoplasmic) or epidermal growth factor receptor (EGFR) (membranous in >10% of cells 3+22; and HER2 according to the scoring guidelines (>10% 3+).23 Image 1A. ERBB2 gene status was confirmed by fluorescence in situ hybridization (FISH) (pharmDx; Dako, Glostrup, Denmark) or chromogenic in situ hybridization (CISH) (SPOT-Light; Zymed, South San Francisco, CA) in nondefinite cases (2+ and <10% 3+ cells).19,23 Classification of intrinsic subtypes was based on the immunohistochemical results according to the St Gallen recommendation.24 Of note, based on our results of immunohistochemistry, the different categories were defined as follows: luminal A (ER/PR, >20%; Bcl2, >50%; p53, <20%; or Ki-67, <14%) and luminal B (ER and/or PR, <20%; Bcl2, <50%; p53, >20%; Ki-67, >14%; and HER2-negative), HER2-positive (3+ in >10% cells by immunohistochemistry or amplification by FISH/CISH), and triple-negative/basal-like (ER/PR/HER2-negative, CK5/6 and/or EGFR-positive). For the purpose of the study, tumors were stratified into three groups: luminal A and B/HER2-negative, HER2-positive (independent of the HR status), and triple-negative/basal-like.

RNA Extraction and Complementary DNA Synthesis

RNA was extracted from three 1-mm-thick/4-mm-deep cores of formalin-fixed, paraffin-embedded tissue from preselected tumor areas with at least 30% tumor cell content. Normal breast tissue RNA from reduction mammoplasties was used as a calibrator sample. TissueLyser (Qiagen, Hilden, Germany) was used to disrupt samples. RNA was isolated using the RNeasy Mini kit (Qiagen) for cells and the RNeasy FFPE Kit (Qiagen) for tissue and was stored at –80°C. RNA concentration and ratios regarding proteins (260/280) and salts (260/230) were measured by a NanoDrop spectrophotometer (Thermo Scientific, Waltham,
MA). Then, 2 μg RNA was used in each reverse-transcription reaction according to the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA).

Quantitative Real-Time Polymerase Chain Reaction

We used TaqMan Gene Expression Assays (Life Technologies) for specific complementary DNA retrotranscribed from mRNA since these assays do not detect genomic DNA. Selected assays were ACTB (Hs99999903_m1), BCL2 (Hs00608023_m1), ICAM1 (Hs00164932_m1), PUM1 (Hs00472881_m1), SPP1 (Hs00959010_m1), and VEGFA (Hs00900055_m1) for carcinoma specimens and both ACTB and PUM1 for cancer cells were selected as reference genes for gene expression normalization. As calibrators (reference samples), we used a pool of RNA from 10 normal mammary tissues for breast carcinomas and 184A1 cells for cancer cell lines. The maximum cycling threshold (Ct) value accepted was 40 or less. Relative gene expression was calculated by the 2^-ΔΔCt method, and data were analyzed using the 7500 Software v2.0.6 (Life Technologies). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in a 7500 Fast Real-Time PCR System (Life Technologies). Normal breast RNA (Agilent Technologies, Santa Clara, CA) was used as a positive control and biology grade water as a negative control during reverse transcription and qRT-PCR.

Treatment With Small Interfering RNA and Recombinant Osteopontin

To downregulate osteopontin mRNA expression, 5 × 10^4 to 1 × 10^5 cells per well were harvested in six-well plates. Cells were treated with Hs_SPP1.6 FlexiTube siRNA GeneSolution (GeneGlobe; Qiagen), a gene-specific package of four small interfering RNA (siRNA) oligonucleotides that target the three splice variants of osteopontin (see supplement at www.ascp.org/docs/default-source/pdf/press/oritz-martinezjun15.pdf), at a concentration of 5 nmol/L for 48 hours. Cells were also transfected with AllStars Negative Control siRNA (negative control) and AllStars Hs Cell Death Control siRNA (positive control) (GeneGlobe, Qiagen) at the same conditions (sequences not provided). Transfection was performed using HiPereFect Transfection Reagent (Qiagen). Recombinant osteopontin (Prospec, Rehovot, Israel) was added 8 hours before RNA extraction at 250 ng/mL, a concentration that has a prognostic value for poor survival in plasma of patients with breast cancer. Dimethyl sulfoxide (DMSO) was used as a negative control for recombinant osteopontin treatment.

Proliferation Assay

Cells (2 × 10^4 to 3 × 10^5) were harvested in 96-well plates, using at least four wells per treatment. Cells were treated with siRNA and recombinant osteopontin for 72 to 96 hours. AllStars Negative Control siRNA and AllStars Hs Cell Death Control siRNA were used as negative and positive controls, respectively, while DMSO was used as a negative control for recombinant osteopontin treatment. Afterward, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) reagent was added at 0.25 mg/mL, and cells were incubated for 3 hours. Then, cell media were replaced by 200 μL DMSO to dissolve the formazan product. After 30 minutes of shaking, the absorbance was measured in a microplate reader (Tecan, Männedorf, Switzerland) at 570 nm.

Statistical Analysis

Statistics were performed using SPSS version 19.0 (SPSS, Chicago, IL). Mann-Whitney or Kruskal-Wallis tests were used to compare the median and t test to compare the mean. Correlation between quantitative variables was tested by the Spearman rank coefficient. Kaplan-Meier survival plots were compared using the log-rank test. A Cox proportional hazards model was used for univariate and multivariate analyses. For the purpose of the study, we defined high vs low mRNA levels based on the median fold change for each gene. We considered statistical significance when P < .05.

Results

ICAM-1 and VEGFA mRNA Levels Depend on Osteopontin in MDA-MB-468 Cells

First, we analyzed the mRNA levels of the selected genes in a panel of cell lines representing the different breast cancer subtypes and in a normal breast epithelial cell line. Compared with 184A1 cells, osteopontin mRNA expression was similar in SK-BR-3 (fold change = 0.88) and MDA-MB-468 (fold change = 0.67), low in MDA-MB-231 (fold change = 0.10) and MCF-7 (fold change = 0.07), and absent in BT-474. Bcl2, ICAM-1, and VEGFA mRNA levels were higher in MDA-MB-468 and MDA-MB-231 cells

Figure 1A.

To determine whether Bcl2, ICAM-1, and VEGFA mRNA expression depend on osteopontin, we knocked down osteopontin mRNA expression using siRNA. Due to the absent mRNA levels of osteopontin in BT-474 and the low mRNA levels of the selected genes in MCF-7, we did not perform experimental treatment in these cells. Osteopontin-specific siRNA silenced osteopontin mRNA expression in SK-BR-3, MDA-MB-468, and MDA-MB-231 cells (all P < .05). We observed that osteopontin silencing decreased ICAM-1 (fold change = 0.69, P = .013) and VEGFA (fold change = 0.57, P = .004) expression in MDA-MB-468 cells, whereas there was a trend in ICAM-1 (fold change = 0.86, P
Osteopontin Silencing Reduces Proliferation in Triple-Negative Breast Cancer Cells

To assess whether osteopontin affects breast cancer cells’ viability and proliferation, we knocked down its expression for 72 to 96 hours. We could confirm in MDA-MB-468 cells that osteopontin silencing (72.61% ± 7.58%, \( P = .033 \)) and siRNA positive control (40.11% ± 10.45%, \( P = .004 \)) significantly inhibited cell growth compared with the siRNA negative control (94.95% ± 4.07%). Similarly, in MDA-MB-231 cells, osteopontin silencing (29.33% ± 7.02%, \( P = .043 \)) and siRNA positive control (23.45% ± 9.12%, \( P = .040 \)) showed significant differences compared with siRNA negative control (73.66% ± 13.72%). Although osteopontin silencing decreased cell growth (66.17% ± 9.28%, \( P = .226 \)) in SK-BR-3, it lacked statistical significance compared with the siRNA negative control (73.66% ± 13.72%). Although osteopontin silencing decreased cell growth (66.17% ± 9.28%, \( P = .226 \)) in SK-BR-3, it lacked statistical significance compared with the siRNA negative control (80.03% ± 5.40%). \( \text{Figure 1C} \). Furthermore, recombinant osteopontin did not influence cell growth in any cell line (data not shown).

Patients and Tumor Characteristics

We included 148 patients who fulfilled the established criteria. Patients’ age ranged from 32 to 89 years (mean, 57 years). The median time from surgery to local recurrence or distant metastasis was 77 months (range, 1-259 months), whereas the median time from surgery to patient death was 86 months (range, 1-259 months). Tumors were predominantly larger than 20 mm (75/148 [51%]), were grade 3 (84/148 [57%]), and had a negative nodal status (95/142 [67%]). The molecular subtype classification according to a profile-validated immunohistochemical panel\(^2\) showed that 32% (47/148) were luminal (A and B), 30% (45/148) HER2-positive, and 38% (56/148) triple-negative/basal-like.

Clinicopathologic and Molecular Correlations in Breast Carcinomas

To obtain translational information regarding whether osteopontin regulates the studied genes, we analyzed their expression in a series of breast carcinomas. Compared with normal breast tissue, the median (P\(_{25}\)-P\(_{75}\)) values of the mRNA fold change for the analyzed genes in breast carcinomas were as follows: osteopontin, 14.32 (3.96-36.35); Bcl2, 0.18 (0.04-0.54); ICAM-1, 1.67 (0.54-3.30); and VEGFA, 3.16 (1.62-5.31). The cutoff fold change to consider high osteopontin mRNA expression was set at the osteopontin median value: more than 14.32 for all analyzed tumors, being specifically more than 5.23 in luminal tumors, more than 33.87 in HER2-positive cases, and more than 17.32 in triple-negative/basal-like tumors. The protein expression levels of osteopontin in the current series of breast carcinoma were analyzed by immunohistochemistry \( \text{Image 2} \) and reported in a previous study by our group.\(^2\) The median of osteopontin mRNA fold change was higher among HER2-positive and triple-negative/basal-like phenotypes and in tumors with positive lymph node status. The Bcl2 median mRNA fold change was higher among tumors with low (1+2) histologic grade and luminal phenotype. In contrast, the median of ICAM-1
and VEGFA mRNA fold change was higher among the triple-negative/basal-like phenotype and tumors with high expression of osteopontin. Moreover, higher VEGFA mRNA levels were found among tumors larger than 20 mm (all \( P < .05 \)).

Regarding molecular subtypes, VEGFA mRNA expression was higher among HER2-positive tumors with high expression of osteopontin (\( P = .006 \)). Similarly, ICAM-1 (\( P = .101 \)) and VEGFA (\( P = .080 \)) increased expression showed a trend in triple-negative/basal-like tumors with high osteopontin levels (Mann-Whitney \( U \) and Kruskal-Wallis tests) [Table 2].

Using Spearman tests, we observed a positive correlation of osteopontin with both ICAM-1 (\( r = 0.225, P = .006 \)) and VEGFA (\( r = 0.283, P < .001 \)) mRNA levels in all tumors but not with Bcl2. As expected, ICAM-1 and VEGFA also correlated positively (\( r = 0.364, P < .001 \)). Further analysis stratified according to tumor phenotypes showed that in the
HER2-positive subtype, osteopontin correlated positively only with VEGFA mRNA levels \((r = 0.303, P = .045)\). However, in triple-negative/basal-like tumors, osteopontin correlated with both ICAM-1 \((r = 0.409, P = .002)\) and VEGFA mRNA levels, the latter only as a trend \((r = 0.202, P = .138)\) (Spearman tests) \[Table 3\].

**Univariate Analysis for DFS and OS**

All clinicopathologic features, except age, achieved prognostic significance for DFS and OS of patients (all \(P < .05\), Cox regression) \[Table 4\]. To evaluate the impact of osteopontin, Bcl2, ICAM-1, and VEGFA gene expression on DFS and OS, we defined the cutoffs by their median fold change in tumors with high expression of osteopontin. Therefore, they were set as follows: osteopontin fold change, 36.28 or more; Bcl2 fold change, 0.15 or less; ICAM-1 fold change, 2.08 or more; and VEGFA fold change, 4.12 or more (Table 2). We observed that osteopontin, Bcl2, and ICAM-1 mRNA levels did not predict DFS or OS (all \(P = NS\)). However, high VEGFA mRNA levels had a significant impact on DFS (hazard ratio, 2.45; 95% confidence interval [CI], 1.34-4.49; \(P = .004\)) and OS (hazard ratio, 3.15; 95% CI, 1.55-6.38; \(P = .001\)) (Table 4). Relapses occurred in 38% (20/53) of patients with VEGFA-overexpressing tumors compared with 26% (24/93) in those with low expression \((P = .004)\) \[Figure 2A\]. Regarding patients’ OS, 31% (16/52) of deaths occurred in patients with VEGFA-overexpressing tumors compared with 17% (16/93) in those with low expression \((P = .001)\) (Kaplan-Meier, log-rank test) \[Figure 2B\].

**Multivariate Analysis for DFS and OS**

The hazard of relapse was significantly higher for patients with positive nodes (hazard ratio, 2.24; 95% CI, 1.08-4.62; \(P = .030\)) and tumors with high VEGFA mRNA levels (hazard ratio, 2.95; 95% CI, 1.48-5.87; \(P = .002\)). Regarding OS, high VEGFA mRNA levels (hazard ratio, 3.25; 95% CI, 1.48-7.11; \(P = .003\)) remained a significant independent factor to predict patients’ death, whereas lymph node status showed only a trend toward shorter survival (Table 4).

**Discussion**

It is known that osteopontin promotes tumor progression through binding cell receptors activating signaling pathways and angiogenesis. However, the available data are based mainly on experimental research, and the results have not been validated in clinical studies. Of note, cell lines lack the molecular and cellular complexity of solid tumors, and xenograft models show some shortages such as deficient immune system, species stroma differences, and lack of translational information. Therefore, we used breast cancer samples with clinical information of the patients, and we complemented our study with breast cancer cell lines. Our results suggest that osteopontin regulates ICAM-1 and VEGFA mRNA expression in breast carcinomas, especially in the triple-negative/basal-like subtype.

Osteopontin mRNA was expressed in all breast cell lines except in BT-474. Interestingly, Bcl2, ICAM-1, and VEGFA mRNA levels were increased only in the triple-negative cell lines, suggesting that osteopontin upregulates these genes in this subset of tumors. These results were supported by further experiments in which silencing of osteopontin downregulated ICAM-1 and VEGFA expression. The mRNA reduction of ICAM-1 and VEGFA was small (30%-40%) but consistent with the role of osteopontin in metastasis. We did not expect a high reduction since osteopontin is a secondary inductor of these genes rather than the main regulator. Although recombinant osteopontin showed biological effects in previous studies, we were not able to demonstrate any change in the mRNA expression of the analyzed genes. This may be due to the lack of posttranslational modifications in the recombinant protein that are present in the native human osteopontin.

The cell-proliferative effect of osteopontin in breast cancer cells as well as in xenografted mice is also supported by our data. In the current study, cell growth decreased only in triple-negative cells after treatment with siRNA against osteopontin. SK-BR-3 cells, representative of HER2-positive tumors, showed a high expression of osteopontin, but their proliferation was not affected when it was knocked down. This result suggests that osteopontin does not play a pivotal role in the proliferation control of...
This tumor phenotype. On the contrary, MDA-MB-231, representative of triple-negative/claudin-low tumors, showed a low expression of osteopontin, but their proliferation was affected when it was knocked down. This result points out that osteopontin plays a key role in the cell proliferation of this tumor phenotype. Of note, the siRNA negative control in MDA-MB-231 showed a decrease in cell proliferation, which means certain toxicity of the transfection reagent in these cells. The intercellular differences in the response to osteopontin could be due to its cell receptor status\(^a\) as well as the expression of the osteopontin splicing variants. Indeed, we have recently reported that HER2-positive and triple-negative/basal-like cells express high levels of the isoform osteopontin-c,\(^{20}\) which is more soluble and, therefore, more available for receptor ligation.\(^{35}\)

Bcl2 is an antiapoptotic protein usually associated with the expression of hormone receptors in breast neoplasms.\(^{36}\)

In our clinical series, Bcl2 mRNA levels were lower in grade 3 tumors and HR-negative immunophenotypes, with a trend to positive lymph node status, in line with a recent
We did not find correlations between osteopontin silencing and Bcl2. However, prior investigators reported that activation of the JAK2/STAT3 pathway by osteopontin induced Bcl2 protein expression. Two possible reasons that may explain these contradictory results are as follows: (1) osteopontin-induced Bcl2 expression was regulated at the protein level, or (2) Behera et al\(^9\) induced Bcl2 expression by exposing cells to 0.5 μmol/L osteopontin, which, through receptor binding, activated the JAK/STAT3 pathway and eventually Bcl2 expression. In contrast to the latter data, we found that silencing osteopontin did not modify the basal mRNA Bcl2 expression, suggesting the existence of alternative mechanisms in Bcl2 regulation.

ICAM-1 induces cell invasion and promotes metastasis in breast cancer cells,\(^38\) whereas previous clinical studies have associated its overexpression with poor prognosis in patients with breast cancer.\(^12\) Interestingly, we demonstrated that ICAM-1 mRNA expression depended on osteopontin mRNA in MDA-MB-468 cells and in triple-negative/basal-like tumors (Spearman test), a subtype that has a high metastatic potential. In line, a previous study showed that osteopontin induces ICAM-1 expression through NF-κB

**Table 4**

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<td>VEGFA FC, ≥4.12 vs &lt;4.12</td>
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CI, confidence interval; FC, fold change; HR, hazard ratio; ICAM-1, intercellular adhesion molecule 1; OPN, osteopontin; VEGFA, vascular endothelial growth factor A.

\(^a\) Cox model.
activation. Therefore, our results support that osteopontin-induced ICAM-1 might be one of the effectors whereby osteopontin participates in the mechanisms of metastasis.

Chakraborty et al. showed that exogenous osteopontin induced VEGF protein expression in MDA-MB-231 cells. In line, osteopontin silencing reduced VEGFA mRNA levels in our MDA-MB-468 cells. This fact was further confirmed in our series showing increased VEGFA mRNA levels among tumors larger than 2 cm, HER2-positive tumors, triple-negative/basal-like subtype as a trend, and with osteopontin overexpression. Similar results have been reported in previous translational studies. Strikingly, we observed a strong association between osteopontin and VEGFA expression in HER2-positive tumors but not in SK-BR-3 cells. This could be an example of the discordance between tumors and cell lines, since tumors show higher heterogeneity and complexity compared with cell lines.

According to our results, triple-negative/basal-like and HER2-positive tumors express more osteopontin that functions as a critical proangiogenic factor by induction of VEGFA, imparting a more aggressive clinical behavior and poorer survival. Moreover, we found that mRNA levels of VEGFA showed an independent prognostic value for DFS and OS for patients with breast cancer, in line with previous studies that determined VEGF at the protein level. Although we observed an association between VEGFA and osteopontin mRNA levels, the latter did not show prognostic value, probably because osteopontin-c has more prognostic relevance than all isoforms.

Of note, therapies against ICAM-1 and VEGFA have shown promising results in prior experimental and clinical studies. In fact, recent data suggest that treatment with bevacizumab, an antibody anti-VEGFA, had better rates of pathologic complete response among patients with triple-negative tumors. Our results show that VEGFA expression appears to depend on osteopontin; therefore, osteopontin blockade might increase the antiangiogenic effect of VEGFA inhibitors. Interestingly, osteopontin mRNA inhibition has been shown to increase tumor cell chemosensitivity and cell radiosensitivity, as well as decrease tumor progression.

The analysis regarding the regulatory role of osteopontin on ICAM-1 and VEGFA protein expression was beyond the scope of the current study. Nevertheless, published data in some in vitro studies have shown in MDA-MB-468 that treatment with a higher concentration of osteopontin resulted in higher ICAM-1 protein levels, whereas a weaker response was observed in MCF-7 cells. Similarly, VEGF protein levels showed a dependence when MDA-MB-231 cells were treated with osteopontin. However, further studies including larger clinical cohorts are needed to confirm our results in the different breast cancer subtypes.

In conclusion, our study provides further insight into the mechanism of action of osteopontin in breast carcinoma through the regulation of invasion (ICAM-1) and angiogenesis (VEGFA) pathways, leading to a more aggressive tumor phenotype. In addition, we identified the VEGFA mRNA levels as an independent poor prognostic factor for patients with breast cancer. Importantly, osteopontin might be considered a new biomarker that could improve the clinical management with specific therapies, as well as the outcome in those patients with high-risk breast carcinomas.

References

10. Gritsenko PG, Ilina O, Friedl P. Interstitial guidance of invasion (ICAM-1) and angiogenesis (VEGFA) pathways, leading to a more aggressive tumor phenotype. In addition, we identified the VEGFA mRNA levels as an independent poor prognostic factor for patients with breast cancer. Importantly, osteopontin might be considered a new biomarker that could improve the clinical management with specific therapies, as well as the outcome in those patients with high-risk breast carcinomas.

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