Molecular expression of vascular endothelial growth factor, prokineticin receptor-1 and other biomarkers in infiltrating canalicular carcinoma of the breast

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Abstract. Vascular endothelial growth factor (VEGF) is important in the growth and metastasis of cancer cells. In 2001, another angiogenic factor, endocrine gland-derived VEGF (EG-VEGF), was characterized and sequenced. EG-VEGF activity appears to be restricted to endothelial cells derived from endocrine glands. At the molecular level, its expression is regulated by hypoxia and steroid hormones. Although VEGF and EG-VEGF are structurally different, they function in a coordinated fashion. Since the majority of mammary tumors are hormone-dependent, it was hypothesized that EG-VEGF would be expressed in these tumors, and therefore, represent a potential target for anti-angiogenic therapy. The aim of the present study was to assess the expression of VEGF, EG-VEGF and its receptor (prokineticin receptor-1), as well as that of breast cancer resistant protein, estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2, in 50 breast samples of infiltrating canalicular carcinoma (ICC) and their correlation with tumor staging. The samples were analyzed using reverse transcription-quantitative polymerase chain reaction and immunohistochemistry. Both angiogenic growth factors were identified in all samples. However, in 90% of the samples, the expression level of VEGF was significantly higher than that of EG-VEGF (P=0.024). There was no association between the expression of VEGF, EG-VEGF or its receptor with tumor stage. In ICC, the predominant angiogenic factor expressed was VEGF. The expression level of either factor was not correlated with the tumor-node-metastasis stage. Although ICC is derived from endothelial cells, EG-VEGF expression was not the predominant angiogenic/growth factor in ICC.

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Introduction

Tumor neovascularization is a complex process that plays a crucial role in the development of several types of cancer. The mechanism of hematogenous metastasis requires newly formed capillaries and overexpression of ≥1 positive regulators of angiogenesis such as vascular endothelial growth factor (VEGF) (1,2). Previous studies reported the existence of another angiogenic mitogen called endocrine gland-derived VEGF (EG-VEGF), which selectively acts on the endothelium of endocrine gland cells (3-5). Both angiogenic proteins have been identified in a variety of tissues, and are overexpressed in various cancers (6-12). Every year, >1,000,000 women are diagnosed with breast cancer, which is the first cause of mortality in females (13). In the USA, the incidence has been estimated at 12.3%, and in Mexico, at 11.34%, according to the National Cancer Institute of Mexico (13,14). Since breast carcinoma may be asymptomatic and clinically undetectable, numerous women are diagnosed with distant metastasis to the liver, lungs, bones and brain, according to the American Cancer Society (http://www.cancer.org), Centers for Disease Control and Prevention (http://www.cdc.gov/cancer/breast/statistics) and CancerResearchUK(http://www.cancerresearchuk.org/cancer-statistics/statistics) (15-17). The expression of estrogen receptors (ERs), progesterone receptors (PRs) and human epidermal growth factor receptor 2 (HER-2/neu) are among the most prominent predictive and prognostic factors in breast cancer. Chemotherapy is the main treatment modality (17); however, resistance to drugs is inherent in certain cases and acquired during treatment in others (18). The resistance of malignant cells is often the result of the overexpression of specific members of the adenosine triphosphate-binding cassette family of transporters, which actively export cytotoxic drugs out of the tumor cell, thus preventing cell death (18). One of the members of this family is breast cancer resistance protein (BCRP) (19,20). The mechanisms of BCRP

regulation involve diverse factors such as hypoxia and steroid hormones (21-23).

The messenger RNA (mRNA) and/or protein expression of BCRP has been detected in numerous types of human cancer, including pancreatic, gastric, renal, hepatocellular, endometrial and colon carcinoma, as well as melanoma and leukemia (24). In addition, its overexpression has been observed in placental choriocarcinoma (BeWo) (25) and human breast cancer (MCF-7) cell lines (26). The aim of the present study was to assess whether the expression of the angiogenic factors VEGF, EG-VEGF and its receptor [prokineticin receptor-1 (PROKR1)] in breast samples of infiltrating canalicular carcinoma (ICC) correlated with tumor staging and could be used as prognosis factors.

Materials and methods

Reagents. TRIzol reagent was acquired from Ambion (Thermo Fisher Scientific, Inc., Waltham, MA, USA), while Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) culture medium, fetal bovine serum (FBS), oligonucleotides, molecular probes and secondary antibodies were obtained from Invitrogen (Thermo Fisher Scientific, Inc.). TaqMan® Reverse Transcription kit, TaqMan® Universal PCR Master Mix and TaqMan® probes were purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.). Capillaries were obtained from Roche Applied Science (Pleasanton, CA, USA) and primary antibodies from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and Sigma Aldrich (St. Louis, MO, USA). Paraformaldehyde, 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI), ProLong® Gold antifade reagent and phosphate-buffered saline (PBS) were acquired from Sigma-Aldrich.

Patients and tissue collection. Breast carcinomas from 50 patients treated at the Department of Mammary Tumors, National Cancer Institute (Mexico City, Mexico) were analyzed. Ethical approval for the present study protocol was obtained from the Human Research Ethics Committee of the National Cancer Institute. Written informed consent was obtained from all subjects prior to tissue collection, and the study was conducted in accordance with the guidelines stipulated in the Declaration of Helsinki. Breast carcinoma (n=50) full-thickness biopsies were obtained during diagnostic procedures between October 2008 and October 2010. A portion of each harvested tissue sample was immediately frozen to -75˚C in liquid N_2 to await RNA extraction. The remaining tissue was either fixed in 4% paraformaldehyde and analyzed by immunohistochemistry (IHC), or placed in DMEM-HG supplemented with 10% FBS, 250 mg/l penicillin, 50 mg/l streptomycin and 10^{-9} M estradiol (E_2) (Steraloids, Inc., Wilton, NH, USA), and transported to the Department of Reproductive Biology, National Institute of Medical Sciences and Nutrition Salvador Zubirán (Mexico City, Mexcio) for *in vitro* culture. Patient information was obtained from clinical charts, including age, size of tumors, clinical presentation, family history and reproductive factors.

Histology. For all patients, paraffin slides were used for typing and grading the tumor. Histological grading was performed according to the Scarff-Bloom-Richardson (SBR) histologic grading system, which recommends the sum of individual scores for three variables: i) Percentage of tubule differentiation; ii) degree of nuclear pleomorphism; and iii) mitotic count within a defined field area (27). From the total of samples, 28 were classified as SBR grade 8, 9 as SBR grade 6 and 22 as SBR grade 7.

Primer design. Specific oligonucleotide primers for human VEGF, EG-VEGF, PROKR1 and BCRP were designed based on published sequences (Table I). To avoid false positives due to the amplification of contaminating genomic DNA in the complementary DNA (cDNA) preparation, all primers were designed to anneal to exons separated by an intron. Hence, the primers generated short amplicons (65-100 bp) that crossed an intron/exon boundary within the PCR fragment. The VEGF, EG-VEGF, PROKR1 and BCRP amplicons spanned the boundary of exons 7, 1, 2 and 16, respectively. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as control, amplified a region between exons 2 and 3.

Total RNA isolation and qPCR analysis. Total RNA was isolated from breast tissue using TRIzol reagent according to the manufacturer's protocol. The quantity and quality of RNA was determined by measuring the optical density (OD) at 260 nm. The OD260/OD280 ratio of all RNA samples was determined to be between 1.7 and 2.0, indicating that the samples were exceptionally pure. RNA integrity was examined using 1.5% agarose gel electrophoresis with ethidium bromide staining (data not shown). Single-strand cDNA was synthesized from 3.0 μg purified total RNA using TaqMan® Reverse Transcription kit in a reaction volume of 22 μl. RT-qPCR was performed using TaqMan® Universal PCR Master Mix and in a LightCycler® 2.0 instrument (Roche Diagnostics GmbH, Mannheim, Germany). Each reaction mixture included 10 μl 2X TaqMan® Universal PCR Master Mix, 5.2 μl sterile EMD Millipore water (Billerica, MA, USA), 0.1 μl forward primer (20 nM), 0.1 μl reverse primer (20 nM), 0.1 μl TaqMan® probe (10 nM) and 2.5 μl RT products. The PCR cycling conditions included denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 10 sec. The sizes of the resulting amplicons were 84, 88, 62, 94 and 66 bp, and the probes utilized were numbered 63, 22, 45, 12 and 60, respectively, in the Universal ProbeLibrary (Roche Diagnostics GmbH). All studies were performed at least in duplicate. Quantification of relative mRNA levels was conducted by determining the threshold cycle (Cq), which is defined as the cycle at which the fluorescence emission intensity of the 6-carboxyfluorescein reporter exceeds the standard deviation of the mean baseline emission intensity for cycles 3 to 10 by a factor of 10 (25). Normalization of the cDNA load was performed against the housekeeping gene GAPDH according to the following formula: Cq (VEGF, EG-VEGF, PROKR1 or BCRP) - Cq (GAPDH) = ΔCq.

IHC. Serial sections (5-μm thick) were prepared from paraffin-embedded tissues, and the sections were deparaffinized in xylene and then rehydrated through decreasing concentrations of ethanol. Antigen retrieval was performed by treating the sections for 10 min in a 0.01 M citrate buffer, and endogenous peroxidase activity was quenched with 10%

Table I. Primers used in the present study.

Gene	Sequence
VEGF (GenBank accession No. NM_001025366)	Forward: 5'-GCAGCTTGAGTTAAACGAACG-3' Reverse: 5'-GGTTCCCGAAACCCTGAG-3'
EG-VEGF (GenBank accession No. NM_32414)	Forward: 5'-CCACGCGAGTCTCAATCA-3' Reverse: 5'-ACTGGACATCCCGCTCAC-3'
PROKR1 (GenBank accession No. NM_138964)	Forward: 5'-ACCTGCGCACTGTCTCTCTC-3' Reverse: 5'-CTCAGCGGATGGACAATAGC-3'
BCRP (GenBank accession No. NM_004827)	Forward: 5'-ATTTGGTAAAGCAGGGCATCC-3' Reverse: 5'-CAAGGCCACGTGATTCTT-3'
GAPDH (GenBank accession No. NM_002046)	Forward: 5'-AGCCACATCGCTGAGACAC-3' Reverse: 5'-GCCCAATACGACCAAATCC-3'

VEGF, vascular endothelial growth factor; EG-VEGF, endocrine gland-derived vascular endothelial growth factor; PROKR1, prokineticin receptor-1; BCRP, breast cancer resistant protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table II. Antibodies and spectral characteristics of rhodamine, Alexa Fluor® 532, Alexa Fluor® 647, FITC and DAPI dyes.

Primary antibody/dye	Secondary dye	Color	Abs[a]	Em[a]	Extinction coefficient[b]
Anti-EG-VEGF	Rhodamine	Red	550	600	91,000
Anti-PROKR1	Alexa Fluor® 532	Yellow	532	553[c]	81,000
Anti-HER-2/neu	Alexa Fluor® 647	Pink	650	665[c]	239,000
Anti-cytokeratin-7	FITC	Green	494	518	>70,000
DAPI	Nucleus counterstaining	Blue	350	461	-

[a]Maximum absorbance and fluorescence emission wavelengths in nm. [b]Extinction coefficient at λmax in $cm^{-1}M^{-1}$. [c]Human vision is insensitive to light beyond ~650 nm (www.probes.com); therefore, it is not possible to visualize far red fluorescent dyes by looking through the eyepiece of a conventional fluorescence microscope (28). FITC, fluorescein isothiocyanate; DAPI, 4'-6-diamidino-2-phenylindole dihydrochloride; Abs, absorbance; Em, emission; EG-VEGF, endocrine gland-derived vascular endothelial growth factor; PROKR1, prokineticin receptor-1; HER-2/neu, human epidermal growth factor receptor 2.

(vol/vol) H_2O_2/methanol at room temperature. To prevent the nonspecific binding of antibodies, the sections were preincubated with protein blocking buffer diluted in PBS/bovine serum albumin (BSA; 1%; Sigma-Aldrich) for 1 h prior to overnight incubation at 4˚C with the corresponding primary antibody. Anti-EG-VEGF (A-12; sc-30343; dilution, 1:300), anti-PROKR1 (HPA029396; dilution, 1:300), anti-HER-2/neu (C-18; sc-284; dilution, 1:100) and anti-cytokeratin-7 (5F282; sc-70936; dilution, 1:100) were used. The slides were washed three times for 5 min in PBS. Antibody binding was visualized with anti-goat-immunoglobulin G (IgG)-rhodamine (sc-3945), anti-rabbit-IgG-Alexa Fluor® 532 (A11009), anti-rabbit-IgG-Alexa Fluor® 647 (A31573) and anti-mouse-fluorescein isothiocyanate (sc-2010) secondary antibodies at 1:200 dilution at 4˚C for 2 h. Subsequently, the sections were washed and mounted with ProLong® Gold antifade reagent prior to be visualized and photographed using a confocal laser scanning microscope (TCS SP5; Leica Microsystems, Inc., Buffalo Grove, IL, USA). In each case, negative controls without the primary antibody were included (data not shown).

EG-VEGF, HER-2/neu and cytokeratin-7 in cell culture. A total of 10 random samples of breast cancer of various degrees were obtained in order to study cultured cells on glass chamber slides (Nalge Nunc International; Thermo Fisher Scientific, Inc.). The cells were maintained in monolayer culture in DMEM-HG supplemented with 10% FBS, 250 mg/l penicillin, 50 mg/l streptomycin and 10^{-9} M E_2. The cultures were incubated at 37˚C in a 5% CO_2 humidified incubator, and fresh medium was provided every other day. The cells were washed twice with PBS and fixed in 4% paraformaldehyde in PBS for 10 min, followed by a PBS wash and subsequent treatment with 50 mM ammonium chloride in PBS for 10 min

to reduce the auto-fluorescence of aldehyde groups during immunofluorescence microscopy. The cells were then washed again with PBS and incubated in permeabilization buffer (0.2% Triton X-100 in PBS) for 5 min. To prevent the nonspecific binding of antibodies, the cells were incubated with blocking buffer containing 10% BSA diluted in PBS for 30 min at room temperature. Next, the slides were incubated overnight at 4˚C with antibodies against EG-VEGF (gland-derived endothelial cell marker), HER-2/neu (oncoprotein) and cytokeratin-7 (epithelial tumor marker) at the aforementioned dilutions.

Following three washes for 5 min each, the slides were incubated with the corresponding secondary antibody at 1:200 dilution at 25˚C for 2 h. The sections were then washed, the nuclei were counterstained with DAPI, and coverslips were attached. Digitized images of the same microscopic field were captured using four specific band-pass filters. The wavelength of the emitted light are shown in Table II. Images were obtained using a TCS-SP5 confocal laser scanning microscope with 20X and 40X objectives, 1.4 oil immersion lens, and identical exposure times. Simultaneous evaluation of the negative control (without primary antibody) confirmed the absence of nonspecific immunofluorescent staining, cross-immunostaining or fluorescence bleed-through. Representative photomicrographs were processed using Adobe Photoshop (version CS6; Adobe Systems, Inc., San Jose, CA, USA) without any further adjustment to maintain the veracity of the findings.

Results

Although breast cancer is a heterogeneous disease, the present study was conducted exclusively with the ICC histologic phenotype (17). In accordance with SBR scoring, there were 22 samples (44%) of grade 6/7 and 28 (56%) of grade 8/9. The patients had a mean age of 53.4 years (ranging from 26 to 86 years), and no significant differences were noticed when comparing this parameter with tumor size. A family history of breast cancer was recorded for 4 patients, and a history of relatives with cancer at other sites was recorded for 10 patients. Clinical staging criteria were based on the tumor-node-metastasis (TNM) system, which considers the size of the tumor (T), lymph nodes (N) and metastases (M) (17). The size of the majority of tumors (32 samples, 64%) was classified as T2/T3, being >20 and ≤50 mm, while in 16 samples (32%), it was graded as T4. There were 34 patients (68%) presenting infiltrated ipsilateral lymph nodes.

Regarding metastasis, 17 patients (34%) were positive and 33 (66%) negative, according to the computed tomography scan. Additional data were obtained, including the status of the receptors for steroid hormones and two oncogenic markers (Table III). Molecular studies conducted on the mRNA expression of EG-VEGF and PROKR1 exhibited variable results. Whereas low levels of EG-VEGF were identified in 28 samples, this protein was undetectable in 22 samples. Although PROKR1 is required for EG-VEGF to exert its function, the mRNA of this receptor was only detectable in 17 samples, 14 of which were positive for EG-VEGF. To confirm the expression of both proteins, IHC studies were conducted in tumoral tissue and cell culture. EG-VEGF and PROKR1 were localized in the cytoplasm (Fig. 1A and C, respectively). Additionally, the expression of cytokeratin-7 (Fig. 1B) and HER-2/neu (Fig. 1D) was analyzed, since cytokeratin-7 expression is considered particularly useful for the diagnosis of poorly differentiated tumors (29). In cultures of several randomly selected samples that were grown to 80% confluence, positive immunostaining of EG-VEGF, HER-2/neu and cytokeratin-7 was co-localized (Fig. 2).

Table III. Clinical and histological data.

Characteristics	No. of patients (%)
Total no. of patients	50 (100)
Mean age, years (range)	53.4 (26-86)
Mean tumor size, mm (range)	20 (2-12)
Menopausal status	
Pre	7 (14)
Post	43 (86)
Histology, ICC	50 (100)
TNM stage	
Tumor size	
pT1	2
pT2	20
pT3	12
pT4	16
Nodal status	
pN0	8
pN1	16
pN2	18
pN3	8
Metastatic status	
pM0	33
pM1	17
Hormone receptor status	
Positive ER	6
Positive PR	1
Positive ER and PR	18
Positive HER-2/neu	5
Negative ER, PR and HER-2/neu	11
Positive ER and HER-2/neu	3
Positive PR and HER-2/neu	2
Positive ER, PR and HER-2/neu	4

ICC, infiltrating canalicular carcinoma; TNM, tumor-node-metastasis; ER estrogen receptor; PR, progesterone receptor; HER-2/neu, human epidermal growth factor receptor 2.

Discussion

In the normal human breast, two cell types have been morphologically described, inner luminal cells (parenchyma) and outer myoepithelial cells (stroma) (30). This anatomical distinction is important for understanding the interactions between both cell types during breast tumorigenesis. The majority of breast malignancies (>95%) are derived from an epithelial lineage (31). The epithelial-mesenchymal interactions and the tissue-specific microenvironment modulate the growth, progression and

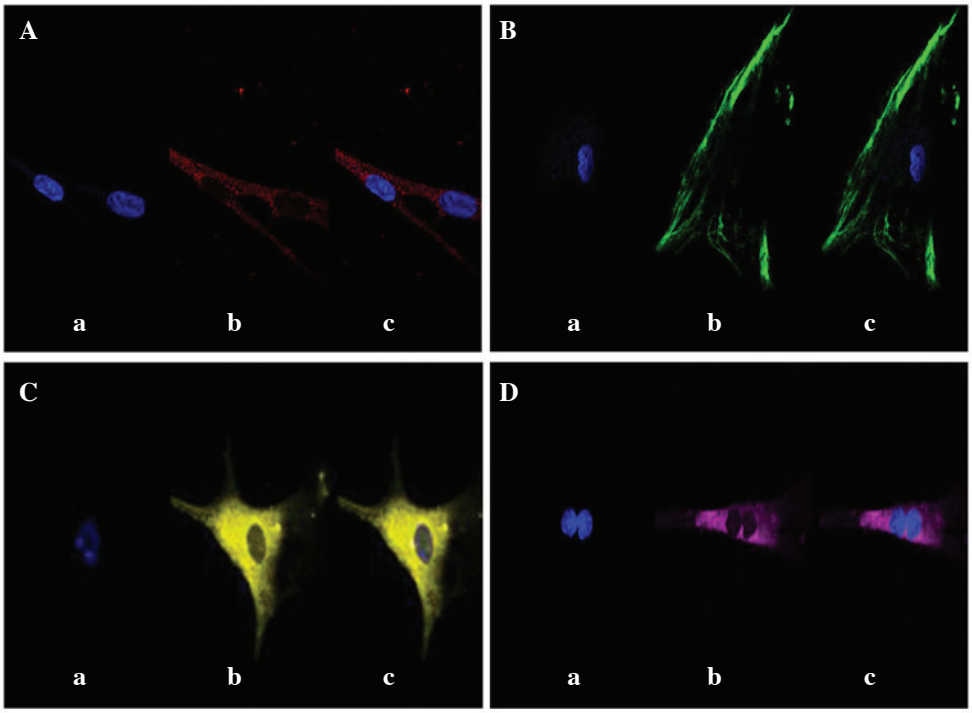

Figure 1. Representative photomicrographs of monolayer-cultured cells. Images were obtained using band-pass filters specific for a) 4'-6-diamidino-2-phenylindole dihydrochloride (blue labeling), b) specific fluorophore and c) co-localization of both fluorophores. Positive staining for (A) endocrine gland-derived vascular endothelial growth factor (red), (B) cytokeratin-7 (green), (C) prokineticin receptor-1 (yellow) and (D) human epidermal growth factor receptor 2 (pink). Magnification, x100.

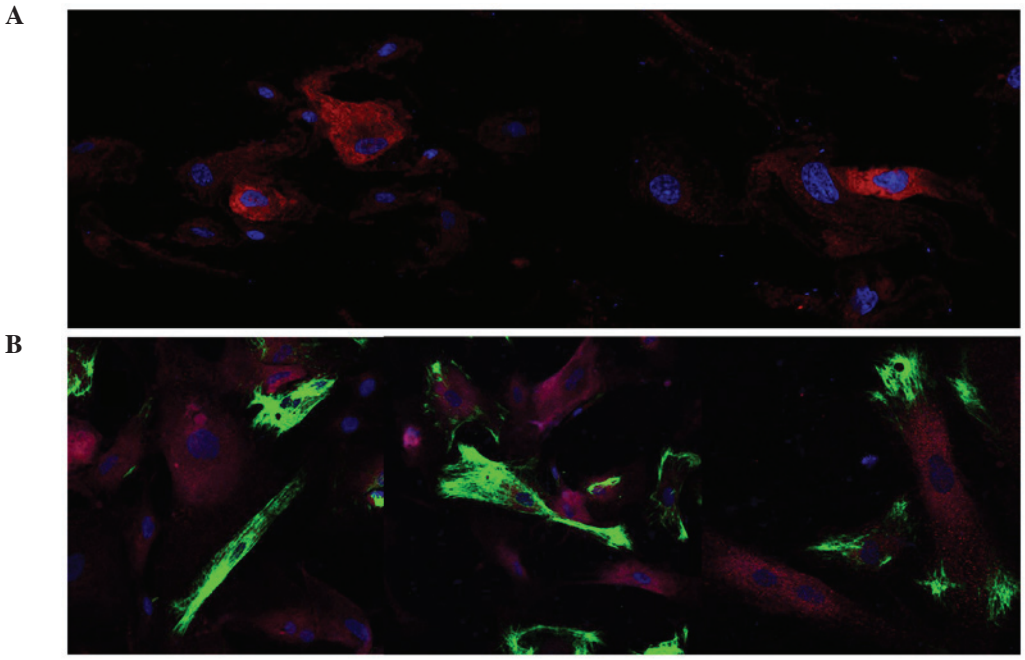

Figure 2. (A) Representative photomicrographs of breast carcinoma cells exhibiting immunofluorescence staining for EG-VEGF (red). (B) Co-localization of EG-VEGF (red staining), human epidermal growth factor receptor 2 (pink staining) and cytokeratin-7 (green staining). The images were acquired using band-pass filters for rhodamine, Alexa Fluor® 647 and fluorescein isothiocyanate. The nuclei were stained with blue-fluorescent 4'-6-diamidino-2-phenylindole dihydrochloride. EG-VEGF, endocrine gland-derived vascular endothelial growth factor.

metastatic behavior of cancer cells (31). The expression levels of several biomarkers, including ERs, PRs and HER-2/neu, play a critical role in the therapy and prognosis of breast tumors (17).

Previous reports on human breast cancer cell lines, patient tumor samples and clinical studies have all indicated that progesterone is a risk factor for breast cancer, and that changes in progesterone signaling pathways contribute to the early stage of tumor progression (22,25). PR signaling stimulates epithelial cell proliferation via an unknown mechanism in pre-neoplastic lesions and mammary tumors (32). However,

primary tumors with negative PR expression have been associated with a less differentiated, more invasive phenotype and a worse prognosis than those expressing PR (32). The data from the current study differs from the aforementioned previous evidence in the aggressiveness of PR-negative tumors. Of 25 such tumors analyzed in the present study, only 10 were poorly differentiated and 7 had an invasive phenotype. In addition, of the 31 ER-positive tumors identified in the present study, only 18 were moderately differentiated.

The HER-2/neu oncogene is amplified/overexpressed in 15-30% of breast cancers (17). This overexpression/amplification of the HER-2/neu protein appears to be of importance for the therapeutic benefit of anthracycline-based treatments, and it is the target for trastuzumab (Herceptin®), a humanized monoclonal antibody designed as a therapy for metastatic breast cancer (33). The absence of ER, PR and HER-2/neu is defined as triple negative breast cancer (TNBC), which is regarded as an aggressive disease that affects young patients, and is characterized by early relapse, particular visceral metastasis and poor prognosis (34). In the current study, 11 patients (22%) were TNBC, of which, 3 were young women (<40 years of age), and 4 developed metastatic tumors. This percentage is similar to that reported in previous studies (35). However, other studies have suggested that Hispanic women are more likely to present TNBC than Caucasian women (36). In 2009, Linderholm *et al* reported higher levels of VEGF in TNBC than non-TNBC patients (37). The present study obtained similar results, finding that all TNBC patients exhibited slightly greater VEGF expression than non-TNBC patients. Regarding the two angiogenic proteins assessed in the current study, VEGF was expressed at a significantly higher level than EG-VEGF. To the best of our knowledge, the present study is the first to report the expression of EG-VEGF in mammary gland tumors. A previous study demonstrated the expression of this protein in a wide variety of human tissues, but did not include the mammary gland (38).

Breast cancer is a heterogeneous disease that presents different biological patterns and histologically diverse subtypes (17). The development of resistance to multiple chemotherapeutic drugs suggests the involvement of BCRP during the treatment of numerous breast carcinomas (18). Unexpectedly, the expression of BCRP was detected in all tumors, independently of TNM and the expression of steroid receptors. In 2011, Moitra *et al* explained that the BCRP phenotype can be produced by an extensive population of tumor cells, cancer stem cells, cells with acquired resistance in chemotherapy and cells with induced genetic changes (39). Large progress has been made in recent years in countering BCRP-induced drug resistance, and ~20 molecules and 6 steroids have been identified that can inhibit BCRP activity (18,40-42). The level of expression of BCRP and the treatment outcome in the present series deserves further analysis.

The differential expression of the angiogenic factors evaluated in the present study could be attributed to the cancer molecular subtype, which is based on gene expression profiles. Recent research has indicated that human breast cells can exhibit extensive lineage plasticity (43), which may explain why marker profiles have been difficult to associate with distinct tumors subtypes. In 2014, Santagata *et al* analyzed normal breast cells and identified 11 cell subtypes in the luminal layer; in the case of breast tumors, none of them exhibited a purely basal-like phenotype (30).

The present IHC data from human breast cancer biopsies indicate that only certain cells were positively stained for EG-VEGF and PROKR1, while others exhibited abundant staining for cytokeratin-7 and HER-2/neu.

Several antineoplastic therapies are aiming to block the function of VEGF (44). However, in the majority of cases, tumors produce a large number of other angiogenic factors, indicating that angiogenesis is a complex process involving multiple signaling pathways (45). Over the last two decades, researchers around the world have developed new techniques involving drugs that target VEGF, including aflibercept and metronomic chemotherapy (46). Aflibercept binds to and inhibits all isoforms of VEGF, and also binds to placental growth factor. Metronomic chemotherapy blocks proliferating tumor cells (47) and is important in breast cancer metastasis (48). Bergers and Hanahan (46) and Dempke and Heinemann (44) reported the results of preclinical studies indicating that certain mechanisms of tumor adaptation and resistance are based on increased tolerance to hypoxia, which leads to a decreased dependence on neovascularization. The role of hormones in the regulation of VEGF is controversial. Numerous studies on estrogen and progesterone have demonstrated that both are able to increase VEGF mRNA and/or protein expression (49,50). In contrast, other studies do not support these findings (51-53).

The regulation of VEGF expression and function by steroid hormones may act through distinct mechanisms in the various cells types involved in breast cancer. Further studies are required to elucidate the mechanism through which EG-VEGF expression is reduced or absent relative to the expression of VEGF in steroid hormone-dependent tumors. These factors could conceivably be used as alternative targets to modulate angiogenesis. The development of novel therapeutic drugs, anti-angiogenic molecules, hormonal agents and biomarkers is important for a better understanding of the molecular mechanisms involved in breast cancer. Successful treatment of patients may depend on addressing the combination of individual genotypes and alternative targets to modulate angiogenesis and reduce drug resistance to chemotherapy.

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