CLINICAL SCIENCE

Expression of Hypoxia-inducible factor 1-α and Vascular endothelial growth factor–C in locally advanced breast cancer patients

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BACKGROUND: Locally advanced breast cancers are more prevalent in underdeveloped countries. Targeted therapy has been improved to identify hallmarks that are specific to these subtypes of tumors.

OBJECTIVES: We aimed to prospectively assess the expression of Hypoxia inducible factor-1 α and vascular endothelial growth factor-C in locally advanced breast cancer patients.

METHODS: Thirty women underwent incisional biopsies for the histopathological diagnosis of breast carcinoma and participated in neoadjuvant chemotherapy. The association of Hypoxia inducible factor-1 α and vascular endothelial growth factor-C with age, tumor size, histological grade, clinical staging, hormonal and axillary status, clinical and pathological response after neoadjuvant chemotherapy, expression of estrogen and progesterone receptors, and the presence of c-erbB-2 antigen was studied.

RESULTS: Hypoxia inducible factor-1 α expression and Vascular endothelial growth factor-C expression were observed in 66.7% and 63.3% of all patients, respectively, and were marginally associated with each other ($p = 0.06$). Among the studied variables, only positive axillary status was associated with the presence of HIF-1α ($p = 0.02$). Complete pathological response was significantly associated ($p = 0.04$) with the expression of vascular endothelial growth factor-C prior to neoadjuvant chemotherapy.

CONCLUSION: We concluded that Hypoxia inducible factor-1 α was associated with a poor prognosis and that vascular endothelial growth factor-C could be used as a predictive factor in locally advanced breast cancer patients with complete pathological response after neoadjuvant chemotherapy.

KEYWORDS: Locally advanced breast cancer; HIF-1α; VEGF; Axillary lymph nodes; Immunohistochemistry.

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INTRODUCTION

Breast cancer is one of the main causes of death in occidental women. Statistics have indicated that the frequency of breast cancer has recently increased in developed and developing countries.[1] In the USA, 192,370 women were diagnosed with breast cancer in 2009, and 40,170 deaths occurred.[1]

Randomized trials performed between 1976 and 1990 have shown that early detection through mammographic examination reduced mortality from breast cancer by 25% in women between 50 and 69 years old.[2] Although the government provides incentives for mammography, approximately 10% of breast tumors are diagnosed as locally advanced tumors (LABC), which have a greater risk of metastasis and a reserved prognosis.[3] Unlike the USA and European countries, where the incidence of breast cancer is increasing and mortality is decreasing, the mortality rate of breast cancer in Brazil is high due to the relatively high percentage (50%) of LABC cases.[4]

Surgery is not the primary recommended method of treatment for LABC. Neoadjuvant therapy reduces the tumor's primary volume and transforms inoperable breasts into operable ones, increasing the conservative surgery rate. By identifying patients who present an optimal response to certain treatments, mechanisms that support tumor growth can be discovered, and novel targeted therapies can be developed. All solid tumors require a microenvironment that promotes angiogenesis, which either maintains tumor viability and its growth or contributes to the spread of the

disease.[5] Vascular endothelial growth factors (VEGFs) constitute a family of potent angiogenic peptides that act on the development of hematopoietic stem cells, remodel the extracellular matrix (ECM) and regenerate inflammatory cytokines. The VEGF family is classified into various subtypes (A to D). VEGF-C and -D are produced by tumor-associated macrophages (TAMs), which express VEGFR-3 (the VEGF-3 receptor). Microvascular lymphatic density and the abovementioned hallmarks promote the lymphatic dissemination of breast tumor cells, which is directly related to the axillary status and prognosis of the patient.[6]

Another important mechanism that leads to angiogenesis is tissue hypoxia. Hypoxia is present in many solid tumors and is caused by abnormal neoplastic vascularization and rapid cell production, which results in apoptosis and areas of necrosis.[7] Based on the results of several studies on the clinical applicability of hallmarks as a targeted therapy, biochemical hallmarks produced in hypoxic environments are related to several cancers (especially breast tumors) and have been considered as prognostic factors for highly undifferentiated tumors.[8,9] However, it is not known if hypoxia is the cause or the effect of the formation of aggressive tumors.

Hypoxia inducible factor-1 (HIF-1) is a heterodimeric nuclear transcription factor that is divided into two subunits (alpha [α] and beta [β]). Subtype 1α is overexpressed in breast tumors [10] and is of functional importance. Under normoxic conditions, HIF-1α is recognized by von Hippel-Lindau (pVHL) proteins, hydroxylated by proline hydroxylases (PHDs) and factor-inhibiting HIF (FIH), and is ubiquitinated by the proteasome.[10] However, the PHD and FIH levels is low during hypoxia. Thus, HIF-1 migrates from the cytoplasm to the nucleus and binds to hypoxia response elements (HREs), leading to the expression of targeted genes.[10] Specifically, increases in the activation of genes that control glucose transportation, glycolysis, growth factor production, high-energy phosphate metabolism, erythropoiesis, heme metabolism, iron transport and nitric oxide synthesis are observed.

The role of HIF-1 in breast carcinogenesis is related to the induction of VEGF transcription, which leads to a greater rate of tumor angiogenesis.[11,12] In preclinical studies with metastatic patients, monoclonal antibodies against VEGF, such as bevacizumab, were used to control tumors.[13] HIF-1α can also be overactivated by vascular growth factors, such as PDGF (platelet-derived growth factor), EGF (epidermal growth factor), FGF-2 (fibroblast growth factor type 2), TGF-1β (transforming growth factor type 1 beta), IGF (insulin growth factor) and cytokines, such as TNF-α (tumor necrosis factor alpha) and interleukin (IL) 1β. Overactivation occurs through two important signaling pathways, the RAS/MEK/MAPK and PI3K/AKt/mTOR cascades.[10]

Several investigations on the prognostic value of HIF-1α in breast cancer have been conducted. Schindl et al.[14] showed that HIF-1α overexpression was associated with poor overall survival rates in LABC patients. However, Bos et al.[5] identified an association between HIF-1α expression and survival in negative lymph node patients. In our previous study, significant differences between the serum levels of HIF-1α and VEGF were not observed before or after neoadjuvant chemotherapy (NACT).[16] Studies on the modifications of the immunohistochemical and serum expression of HIF-1α and VEGF after NACT are scarce. Although VEGF is a classical prognostic and predictive factor for breast cancer, the relationship between VEGF and HIF-1α has not yet been established. Therefore, we analyzed the expression of HIF-1α and VEGF in LABC patients with the goal of identifying associations between the expression of these two genes and other prognostic factors.

METHODS AND MATERIALS

Patients

The present investigation was a prospective study. Consecutive sampling was conducted in the outpatient clinic of the Breast Division of the Hospital das Clínicas, Ribeirão Preto School of Medicine – University of São Paulo (HC-FMRP-USP) from March to November of 2009, and 30 locally advanced breast cancer patients were evaluated. All the patients signed informed consent forms before participating in the study. The Ethics Research Committee of HC-FMRP-USP approved this research.

The following inclusion criteria were applied: no previous treatment for breast cancer, between 25 and 80 years of age, and with an indication for NACT (the presence of stage II and III tumors, where the tumor volume/breast volume ratio did not allow for conservative surgery), as assessed by our clinic. The exclusion criteria were as follows: patients with a reduced Karnofsky performance status scale, previous malignant neoplasm, pregnancy, and confirmed metastatic disease.

Prior to chemotherapy, an incisional biopsy was performed for histopathological diagnosis. During the procedure, a 1-cm^3 tissue block was removed and stored in Tissue-Tek O.C.T. (Qiagen, USA). Except for one patient, who was diagnosed with lobular carcinoma, all of the patients were diagnosed with invasive ductal carcinoma (WHO criteria).

Baseline characteristics

The following clinical variables were studied: age (years), tumor size (in centimeters), histological grade,[17] clinical staging,[18] and menopausal status (pre- and post-menopause). Clinical and pathologic responses were evaluated in all of the patients. The clinical assessment of tumor features was based on RECIST criteria;[19] however, the following modified definitions were employed: complete response: no existence of clinical disease; partial response: less than a 50% reduction in lesion measurements; minor response/progression: less than 25% reduction or an increase in tumor volume. Pathologic response was determined according to an identical rubric.

The histopathological results of lymph node dissection after NACT were considered when the axilla was clinically negative. Alternatively, if the physical examination indicated that the cancer had metastasized to the lymph nodes, NACT was immediately conducted. NACT consisted of docetaxel (75 mg/m^2) and epirubicin (60 mg/m^2) on the first day, preceded by hydration with isotonic fluid, dexamethasone and antiemetic (ondansentron). The procedure was repeated every three weeks.

The mean/standard deviation and median age of all 30 patients were 51.96 ± 12.65 and 52 years, respectively. Thus, most (66.7%) of the patients were more than 50 years old. The mean/standard deviation and median size of breast lumps were 5.85 ± 4.19 (range 2.7 to 25) and 5 cm, respectively. Table 1 shows the baseline features of the

Table 1 - Baseline characteristics of the studied patients (Ribeirão Preto, Brazil, 2010).

Variables	n	%
Age (years)		
<50	10	33.3
50+	20	66.7
Tumor size (cm)		
≤5	15	50
5.1-10	13	43.3
>10	2	6.7
Menopausal status		
Pre-menopause	14	46.7
Post-menopause	16	53.3
Histological subtype		
Ductal	28	93.3
Lobular	2	6.7
Histological grade		
I	3	10
II	16	53.3
III	11	36.7
Clinical staging (TNM)		
IIA	4	13.3
IIB	4	13.3
IIIA	10	33.4
IIIB	7	23.3
IIIC	5	16.7
Estrogen receptor (ER)		
Positive	19	63.3
Negative	11	36.7
Progesterone receptor (PR)		
Positive	14	46.7
Negative	16	53.3
c-erbB-2		
Positive	10	33.3
Negative	20	66.7
HIF-1α		
Positive	20	66.7
Negative	10	33.3
VEGF-C		
Positive	19	63.3
Negative	11	36.7
Total	30	100.0

patients. Most of the women were postmenopausal and possessed aggressive histological tumors in advanced clinical stages (IIIA: 33%). In total, 54.2% displayed complete clinical response, and 28.6% displayed complete pathological response. Half of the patients possessed a positive axillary status.

Immunohistochemistry and quantification of ER, PR, C-erbB-2, VEGF-C and HIF-1-α

Tissue sections with a diameter of 4 micrometers were obtained with a cryostat, and immunohistochemistry was performed according to the avidin-biotin-peroxidase complex (ABC) method. The tissue sections were fixed in 100% acetone for 10 minutes at 20℃. Endogenous peroxidase activity was blocked by incubating the slides in a solution of hydrogen peroxide (0.3%) and PBS for 30 minutes. After antigen retrieval, the sections were cooled for 20 minutes and then incubated with the primary antibody for two hours at 37℃. The specimens were washed and incubated for 45 minutes with biotinylated secondary antibodies. When the incubation was complete, the signal was amplified by the formation of an avidin-biotin complex and was developed with diaminobenzidine and Mayer's hematoxylin counterstain (Zymed Laboratories Inc., CA, USA).

HercepTest (Dako System) was employed to immunohistochemically quantify the HER-2 antigen. A score of +3 (10% of the tumor cells showed strong and complete nuclear membrane staining) was considered positive for HER-2. The test results were considered negative when membrane staining did not occur or was absent in less than 10% of the tumor cells.[20] When intermediate tumors were identified (2+), CISH (chromogenic in situ hybridization) was conducted. Estrogen and progesterone receptor (ER and PR) status was determined when nuclear staining was visualized and was considered positive when more than 10% of the cells were stained.[21] Mouse MAb was applied to analyze HIF-1α (Abcam Company), and quantification of darkly stained epithelial nuclei was performed.[22] Cytoplasmic stainings were ignored, and the scoring rubric applied to ER/PR was employed. An anti-VEGF-C antibody was used (BD Biosciences) to analyze VEGF-C. To score VEGF-C stains, the percentage of strongly stained tumor cells was assessed.[12] Staining was scored by three observers (L.G.O.B, V.F.S, and H.R.C.M), who were blinded to the clinical outcomes.

Statistical analysis

The intercooled Stata statistical package version 8.0 (Stata Corporation, Texas, USA) was used to interpret the data. A Fisher's test was performed to assess the link between ER, PR, c-erbB-2 and the presence or absence of HIF-1α and VEGF-C. The mean, median and standard deviation of the continuous variables were also calculated. A significance level of 5% was used in two-tailed tests.

RESULTS

Table 2 displays the positivity/negativity ratio of the immunohistochemical variables. Approximately 30% of all patients were positive for c-erbB-2 antigen. HIF-1α expression was observed in 66.7% of the patients; however, the number of patients with HIF-1α expression was reduced to 25.9% when only strong and diffuse staining was considered (Figure 1). VEGF-C was expressed in two-thirds of all cases, primarily in the cytoplasm of tumor cells (Figure 2).

A trend between HIF-1α expression and age less than 50 years ($p = 0.08$) was observed, as was a statistically significant association with positive axillary status ($p = 0.02$) (Table 2). A marginally significant trend between elevated HIF-1α levels and the following variables was also observed: pre-menopausal women ($p = 0.10$), reduced pathologic response ($p = 0.12$) and higher clinical response ($p = 0.09$). A statistically significant association between HIF-1α expression and immunohistochemical markers (ER, PR and c-erbB-2) was not observed (Table 2).

When VEGF-C expression was correlated with other variables, only the complete pathological response was associated with it ($p = 0.04$). Histological grading, axillary status, tumor size, age, menopausal status, ER, PR and c-erbB-2 were not associated with VEGF-C. HIF-1α expression was marginally associated with VEGF staining ($p = 0.06$).

DISCUSSION

The objectives of the present study were to assess the expression of HIF-1α and VEGF-C in LABC patients,

Table 2 - The association between HIF-1α/VEGF-C expression and clinical variables (Ribeirão Preto, Brazil, 2010).

Variables	HIF-1α expression (%)		*p*	VEGF-C expression (%)		*p*
	Negative	Positive		Negative	Positive	
Age (years)			0.06			0.79
<50	6 (60)	4 (40)		4 (40)	6 (60)	
50+	4 (20)	16 (80)		7 (35)	13 (65)	
Tumor size (cm)			1.0			0.76
<5	4 (33.3)	8 (66.7)		4 (33.3)	8 (66.7)	
5+	6 (33.3)	12 (66.7)		7 (38.8)	11 (61.2)	
Clinical staging (TNM)			0.56			0.74
II	2 (25)	6 (75)		4 (50)	4 (50)	
III	8 (36.4)	14 (63.6)		7 (31.8)	15 (68.2)	
Histological grade*			0.18			0.68
I	0 (0)	3 (100)		1 (33.3)	2 (66.7)	
II	8 (50)	8 (50)		7 (43.8)	9 (56.2)	
III	2 (18.2)	9 (81.8)		3 (27.3)	8 (72.7)	
Menopausal status			0.07			0.91
Menopause	3 (18.7)	13 (81.3)		6 (37.5)	10 (62.5)	
Pre-menopause	7 (50)	7 (50)		5 (35.7)	9 (64.3)	
Clinical response			0.10			0.46
Complete	2 (15.4)	11 (84.6)		4 (30.8)	9 (69.2)	
Incomplete/no response	5 (45.5)	6 (54.5)		5 (45.5)	6 (54.5)	
Pathological response			0.10			0.04
Complete	0 (0)	6 (100)		0 (0)	6 (100)	
Incomplete/no response	5 (33.3)	10 (66.7)		7 (46.6)	8 (53.4)	
Axillary status			0.02			0.70
Positive	8 (53.3)	7 (46.7)		5 (33.3)	10 (66.7)	
Negative	2 (13.3)	13 (86.7)		6 (40)	9 (60)	
ER			0.59			0.98
Positive	7 (36.8)	12 (63.2)		7 (36.8)	12 (63.2)	
Negative	3 (27.3)	8 (72.7)		4 (36.4)	7 (63.6)	
PR			0.30			0.92
Positive	6 (42.9)	8 (57.1)		5 (35.7)	9 (64.3)	
Negative	4 (25)	12 (75)		6 (37.5)	10 (62.5)	
c-erbB-2			0.78			0.79
Positive	3 (30)	7 (70)		7 (35)	13 (65)	
Negative	7 (35)	13 (65)		4 (40)	6 (60)	
Total	10 (33.3)	20 (66.7)		11 (36.3)	19 (63.3)	

*The second and third histological grades were clustered into one category for the Fisher's test.

determine possible prognostic and/or predictive functions, and identify associations with clinical and immunohistochemical markers.

The expression level of HIF-1α (66.7%) was similar to those observed in other studies. For instance, Gruber[23] and Bos[12] observed an expression rate of 56%, and Kronblad[24] obtained an expression level of 67%. However, some deviations were observed due to the diversity of cut-off points used in the aforementioned studies.[10] Subjectively, a higher concentration of HIF-1α was detected in perinecrotic areas, which likely corresponds to activation by hypoxia. However, Vleugel et al.[25] showed that 44% of their samples

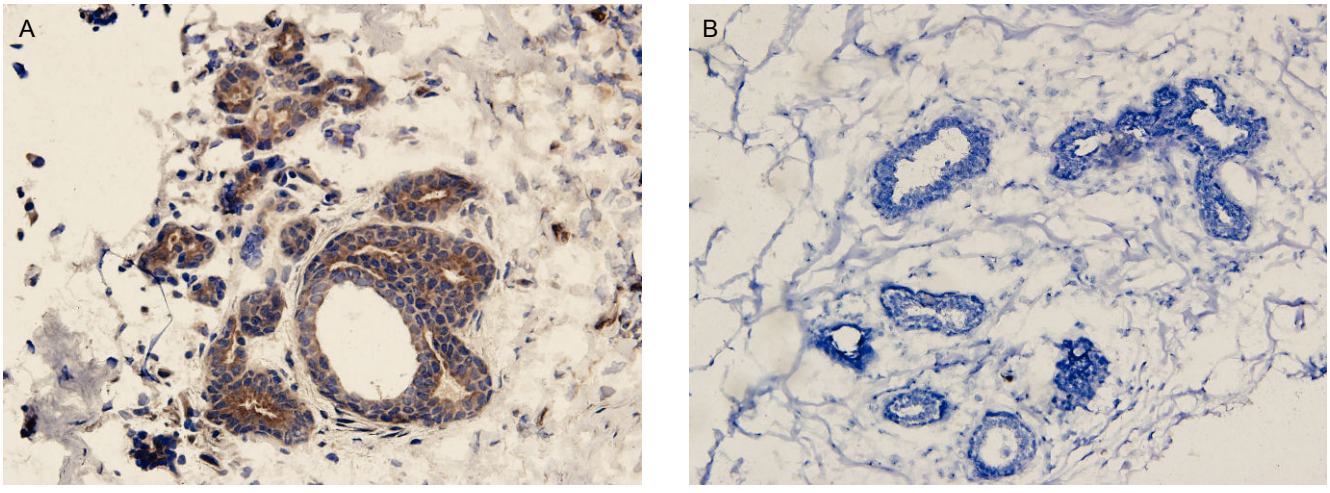

Figure 1 - The presence (a) and absence (b) of HIF-1α expression in breast cancer cells (DAB antibody, 200x).

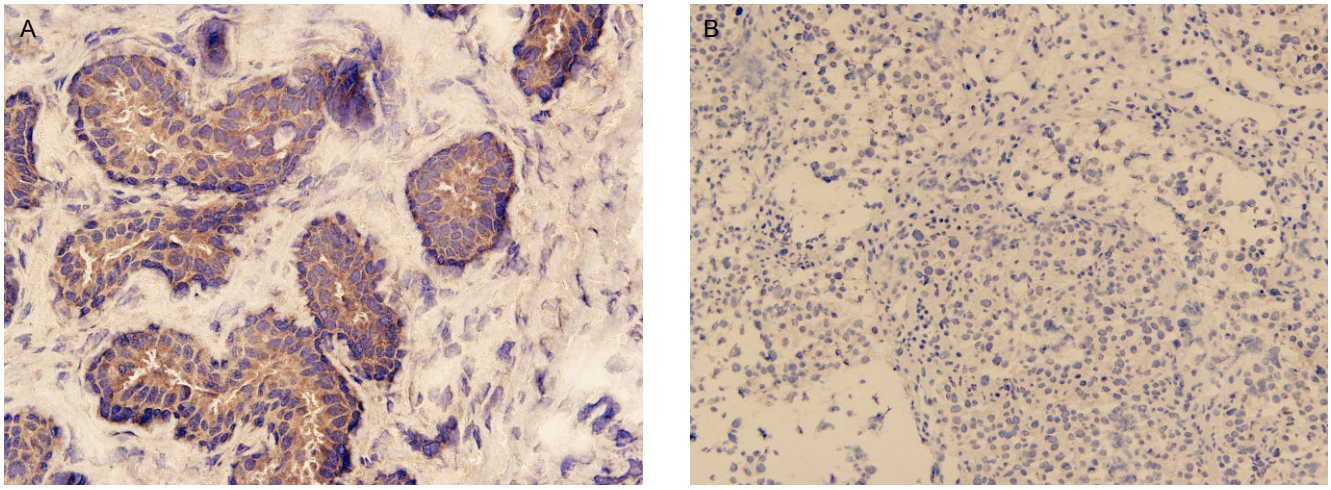

Figure 2 - The presence (a) and absence (b) of VEGF-C expression in breast cancer cells (Anti-VEGF-C antibody mouse isotype IgG2β,κ, 200x).

were positive for HIF-1α, and 13.5% of HIF-1α was distributed perinecrotically. By contrast, 30.5% of HIF-1α had a more diffuse expression, which was indicative of alternative activation (of p53 mutation or HER-2 over-expression, for example). The effect of HER-2 on HIF-1α activation has been determined by identifying significant associations (with the expression of the c-erbB-2 antigen) and has been demonstrated using plasma analyses on locally advanced tumors conducted by our institution.[16] Moreover, the effect of HER-2 on HIF-1α activation has been extensively described.[10, 15, 22, 24] However, in the present study, an association between HER-2 and HIF-1α activation was not observed due to limited sampling.

The expression level of VEGF-C (63.3%) in the present investigation was similar to the results obtained in previous studies[15, 26-28] on breast ductal carcinomas. In our study, staining was localized to the cytoplasm, which is in accordance with the results of previous studies.[29] Although VEGF-C expression could not be correlated with clinical parameters (except for complete pathological response), angiogenesis is extremely important for tumor growth. The level of VEGF-C expression was expected to be reduced, leading to rapid cell apoptosis. Singh et al.[27] observed reduced levels of VEGF expression in the tissues of patients who displayed excellent clinical responses after NACT.

A significant association between positive axillary status and strong/diffuse HIF-1α staining was observed in locally advanced tumors. This finding is in accordance with the results of a previous study conducted by Gruber et al., who evaluated 77 patients.[23] In the abovementioned investigation, HIF-1α was expressed in the majority of patients with positive axilla and served as a poor prognostic factor, especially for T1/T2 tumors. In particular, upon association with HIF-1α, no variables had significant association in the multivariate analysis. Schindl and colleagues[14] obtained similar results in patients with positive axilla or T1/T2 tumors (192/206 patients) and demonstrated that HIF-1α expression was highly significant. Moreover, Schoppmann et al.[30] found a significant association between HIF-1α expression and the amount of peritumoral lymphangiogenesis in breast cancer patients.

Similar to previous reports,[15,23] a significant association between HIF-1α and large tumors and more advanced clinical staging was not observed in the present study. Conversely, Kronblad et al.[24] observed a significant positive correlation, especially in tumors with diameters greater than 5 centimeters. However, this association did not remain after multivariate analysis. Logistic regression could not be performed in our study due to limited sampling. Nevertheless, we concur with Gruber's hypothesis,[23] which states that the impact of HIF-1α is minimized in more advanced tumors due to repression by adaptive mechanisms.

Bos et al.[15] conducted a study on 81 negative axilla and 69 positive axilla patients and concluded that high HIF-1α levels had a profound impact on the overall survival and disease-free survival of patients with negative axilla. Alternatively, high HIF-1α levels did not have an effect on positive axillary patients. In the abovementioned study, patients did not receive NACT because all locally advanced tumors were excluded; however, NACT would likely affect patients in more advanced stages.

Tumor volume has been associated with a determined factor. For example, larger breast tumors are positively related to VEGF levels.[22] Furstenberger et al.[31] analyzed patients in diverse clinical stages and found that the VEGF values of subjects in the control group and patients with tumors were 92 pg/ml and 132 pg/ml, respectively. However, Marana et al.[16] did not observe any significant relationship between tumor volume and plasmatic VEGF level ($p = 0.736$). This result may be attributed to the occurrence of locally advanced breast cancers, not to the presence of tumors with different sizes. Similar results were observed in the present study.

Histological grade was not associated with HIF-1α, even when this variable was divided into low and high grades. This result may be attributed to the sample used in the current investigation, which was different from that of other studies.[15, 24] Another possibility is that the patients in our sample had a high percentage of positive lymph nodes (50%). This result could also explain the results obtained from previous studies conducted on patients with positive axillary status.[14,23] However, Kronblad et al.[24] evaluated 564

patients and observed a positive correlation between higher histological grade and HIF-1α expression ($p = 0.003$).

In the present study, age less than 50 years and premenopausal status were slightly associated with increased HIF-1α expression; however, the crude odds ratio did not corroborate this association, which is similar to the results of previous studies.[15, 23] A similar correlation with VEGF was not observed.

HIF-1α expression did not have an effect on the clinical and pathological responses of patients who underwent NACT; thus, HIF-1α is not a predictive factor. Few studies aiming to establish a predictive value of HIF-1α in neoadjuvant treatments have been conducted; however, further investigations would stimulate research on targeted therapies.

Neither a positive nor a negative association between HIF-1α expression and the estrogen receptor concentration was observed in the present study. The expression of estrogen receptors in mammary cancer cells is reduced under hypoxic conditions (MCF-7 and CAMA-1). The observed reduction in protein production in these cells and the oxygen level are directly related.[10] Compared to normoxic conditions, tamoxifen response in breast cancer cells of premenopausal patients is reduced under hypoxic conditions.[32] Thus, proteosomic degradation occurs at these receptors, reducing the concentration of activated forms. Interestingly, Generali et al.[33] found that HIF-1-α was an independent predictor of poor response, especially in ER-positive patients.

Similar to the results obtained from other authors, VEGF expression was not correlated with ER and PR status in the present study;[27] however, VEGF production in breast cancer cells was stimulated by estrogen and progestin. Foekens et al.[29] studied breast cancer cell lines and suggested that this discrepancy might be due to the constitutive expression of high levels of VEGF by ER-negative breast cancer cells. Alternatively, the expression of VEGF is well-controlled in better-differentiated ER-positive breast cancer cells.

The expression of c-erbB-2 antigen in patients with HIF-1α expression has been well documented. Giatromanolaki et al.[34] showed that patients with high HIF-1α expression and HER-2 overexpression had a lower overall survival rate. In our study, no correlation with HIF-1α or VEGF-C expression was observed. Assuming that HER2/neu protein expression could affect cell migration and proliferation, as well as the spread of lymphangiogenic tumors via VEGF-C upregulation, Schoppmann et al.[35] demonstrated that HER2/neu expression was related to significantly stronger VEGF-C expression and lymphangiogenesis in lymph node-positive breast cancer patients.

The results of the present study emphasized the role of HIF-1α as a poor prognostic factor and demonstrated that increased levels of this protein are associated with positive axillary lymph nodes. A connection between the expression of hallmarks related to hypoxia and poor outcomes was also observed, which reinforces the need for studies concerning the role of HIF-1α as a predictive factor, the creation of new HIF-1α inhibitors and their genic products, the identification of a subpopulation of patients who will benefit from this therapy (especially previously treated patients), and the attainment of knowledge about a hallmark that may be used in targeted therapies. The VEGF-C expression results obtained in the present investigation were in accordance with those of previous studies. In particular, VEGF-C expression was higher in patients with complete pathological response, which confirms its ability to act as a predictive factor.

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