Lymphatic Microvessel Density, VEGF-C, and VEGFR-3 Expression in Different Molecular Types of Breast Cancer

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Abstract. *Background: Breast cancer is a heterogeneous disease and five major distinct molecular types have been characterized by gene analysis and immunohistochemistry. The molecular types of breast cancer have different behavior, a particular profile of response to therapy, reflected in the differential survival of patients. Previous findings showed a particular preference for lymph node and distant metastases of different molecular types, but the specific lymphangiogenic profile of these types is lacking. Patients and Methods: We investigated the differential expression vascular endothelial growth factor-C (VEGF-C), vascular endothelial growth factor receptor-3 (VEGFR-3) and D2-40 by immunohistochemistry, to evaluate lymphangiogenesis and the lymphatic microvessel density (LMVD), in patients with breast cancer, stratified according to the molecular classification. Results: There was a differential expression of VEGF-C/VEGFR-3 and D2-40 in different molecular types of breast cancer, with highest level of expression for these markers being found in HER2 and luminal B types and the lowest in basal-like type. The lowest value of both intratumoral and peritumoral LMVD were found in normal-like type breast cancer. VEGF-C expression did not correlate with the grade of the tumor, but a significant correlation was found with lymph node metastasis. VEGFR-3 expression was found in 66.66% of the cases and correlated with the expression of VEGF-C in tumor cells. There was a positive correlation between VEGF-C, VEGFR-3 and LMVD only in the HER2 type, and a positive correlation in HER2 and normal-like types with VEGFR-3 expression in tumor cells. In addition, there was a correlation between HER2 type, VEGF-C and VEGFR-3 expression in tumor cells and lymphatic endothelium, respectively, and LMVD. Conclusion: Our results support a differential signature of lymphangiogenesis in different molecular types of breast cancer and these findings may have a direct impact on prognosis and therapeutic strategy of this disease.*

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Breast cancer is the most frequent malignant tumor in females and its morbidity and mortality continue to increase, despite remarkable progresses in the field of early diagnosis and adjuvant therapy (1). Long-term follow-up studies of patients with breast cancer have shown that a particular histologic subtype of carcinoma or a specific grade have only minimal impact on prognosis and provides little information for the individual therapeutic strategy (2, 3). Moreover, it is very well known that patients with the same pathologic subtype and grade frequently have a different outcome.

Breast cancer is a heterogeneous disease including many morphological and molecular entities. A significant improvement was achieved in the last decade by establishing some pharmacodiagnostically useful markers, such as estrogen receptors (ER), progesterone receptors (PR), Ki67, and HER2 that allow the patients to be stratified on the basis of the expression of these markers, as demonstrated by immunohistochemistry and/or gene expression analysis (4). Much evidence has been accumulated that hormone receptors and HER2 expression have a direct impact on therapy, while no significant correlation was found with conventional subtypes of breast carcinoma. It is very likely that this difference is probably due to the fact that molecularly distinct diseases are grouped into clinical subtypes, based on their morphological aspects (5).

In an attempt to address this question, breast tumors have been classified based on their gene expression profile and immunohistochemical expression of hormone receptors, HER2, cytokeratin 5/6, epidermal growth factor receptor (EGFR), p53 and BCL-2 (6, 7). This classification had an immediate impact on prognosis and therapeutic strategies and it was shown that molecular subtypes showed a

Table I. *Reagent characteristics and technical details for immunohistochemistry.*

Marker	Clone	Source	Dilution	HIER	WS
ER	1D5	Dako Cytomation, CA, USA	RTU	MW, 30', pH 6	LSAB-HRP
PR	PgR636	Dako Cytomation, CA, USA	RTU	MW, 30', pH 6	LSAB-HRP
HER2	Polyclonal	Dako Cytomation, CA, USA	RTU	MW, 30', pH 6	EnVision-HER
CK5/6	D5/16B4	Dako Cytomation, CA, USA	1:75	MW, 30', pH 6	LSAB-HRP
EGFR	Polyclonal	Dako Cytomation, CA, USA	RTU	MW, 30', pH 6	EGFR kit
p53	DO7	Dako Cytomation, CA, USA	RTU	MW, 30', pH 6	LSAB-HRP
BCL-2	124	Dako Cytomation, CA, USA	RTU	MW, 30', pH 6	LSAB-HRP
VEGF-C	Polyclonal	Santa Cruz Biotechnology, CA, USA	1:200	MW, 15', pH 9	LSAB-HRP
VEGFR-3	Polyclonal	Neomarkers, Fremont, CA, USA	1:200	MW, 15', pH 9	LSAB-HRP
D2-40	D2-40	Dako Cytomation, CA, USA	RTU	MW, 30', pH 6	LSAB-HRP

HIER, Heat-induced epitope retrieval; RTU, ready-to-use; WS, working system; MW, microwave; ER, estrogen receptor; PR, progesterone receptor; CK, cytokeratin; EGFR, epidermal growth factor receptor; LSAB-HRP, labeled streptavidin biotin-horseradish peroxidase.

different response to preoperative chemotherapy and to adjuvant postoperative therapy (8-10). Five distinct molecular subclasses of breast cancer have been identified by genomic analysis: ER-positive, which include luminal A and B tumors; and ER-negative that include HER2 type, basal-like tumors and normal-like breast tumors (11-13). Results obtained with gene analysis overlap with immunohistochemical findings, based on the expression of hormone receptors (ER and PR), HER2, EGFR, monoclonal cytokeratin, and BCL-2. Although less sensitive, immunohistochemistry can be a useful surrogate for gene expression analysis.

Extensive evaluation of the molecular types of breast cancer has shown significant differences not only in their molecular profile, but also in their metastasis susceptibility (14). Lymphangiogenesis in breast cancer was extensively investigated in recent years, and lymphatic microvascular density (LMVD) has been shown to be a good predictor of lymph node metastasis (15, 16). Although the role of vascular endothelial growth factor-C and D (VEGF-C and VEGF-D), and their binding to the cognate receptor VEGFR-3 in the induction of lymphangiogenesis is largely accepted, their predictive value for lymph node metastasis in breast cancer is still controversial (17-19). Recently, it has been shown that lymphangiogenesis assessed by LMVD predicts lymph node metastasis in ductal carcinoma *in situ* with microinvasion (20).

Few data are available concerning the specific profile of lymphangiogenesis in different molecular subtypes of breast cancer, despite it has been shown that their metastatic behaviour is different (21). More recently, it has been shown that triple-negative breast cancer correlates with higher LMVD and expression of VEGF-C and D (22).

In the present study, we have investigated the relationships between VEGF-C/VEGFR-3 expression, LMVD, and different molecular types of breast cancer by immunohistochemistry.

Patients and Methods

Patients and samples. Ninety-six patients affected by breast cancer, aged between 26 and 81 years, were examined. Specimens from the primary tumor and axillary lymph nodes were available in all cases and lymph node metastases were found in 47 (49.47%) cases by routine examination. Based on the conventional pathological report, 92 cases were diagnosed as ductal invasive carcinoma and 4 as lobular invasive carcinoma. The cases with ductal invasive carcinoma presented associated lesions, as follows: ductal *in situ* carcinoma (n=23), atypical ductal hyperplasia (n=4), apocrine metaplasia (n=11), and intraductal papilloma (n=2). Apparently normal mammary tissue adjacent to the tumor was available in 68 (70.83%) cases.

Specimens were fixed in buffered formalin, pH 7.6, for 48 hours and embedded in paraffin using an automated system (Thermo-Shandon, UK). Five-μm-thick sections were stained with hematoxylin-eosin method and slides were reviewed for the pathologic diagnosis and tumors were graded according to the Nottingham modification of the Scarf-Bloom-Richardson method (23). According to this system, 19 cases were classified as G1, 53 as G2, and 24 as G3. A representative sample containing the invasive component was selected for the immunohistochemical evaluation. There was no attempt to select areas characterized by any particular growth pattern.

Immunohistochemistry. To classify the specimens according to the molecular profile, immunohistochemical staining was performed to detect hormone receptors (ER and PR), HER2 protein, cytokeratin 5/6, EGFR, p53, and BCL-2 expression. Details concerning the clone, source, dilution, antigen retrieval, and working system have been summarized in Table I. 3,3'Diaminobenzidine tetrahydrochloride was used as chromogen and nuclei were stained with Lillie's modified hematoxylin. Staining for ER and PR were scored accordingly to Allred *et al*. (24), for HER2 accordingly to the criteria used for HercepTest from 0 to +3, while cytokeratin 5/6, p53, EGFR and BCL-2 were considered positive if more than 10% of the tumor cells were stained with a cytoplasmic (for cytokeratin 5/6), membranous (for EGFR and BCL-2) and nuclear (for p53) patterns.

VEGF-C and VEGFR-3 scoring. VEGF-C positivity was scored based on the percentage of positive cells and intensity of the final

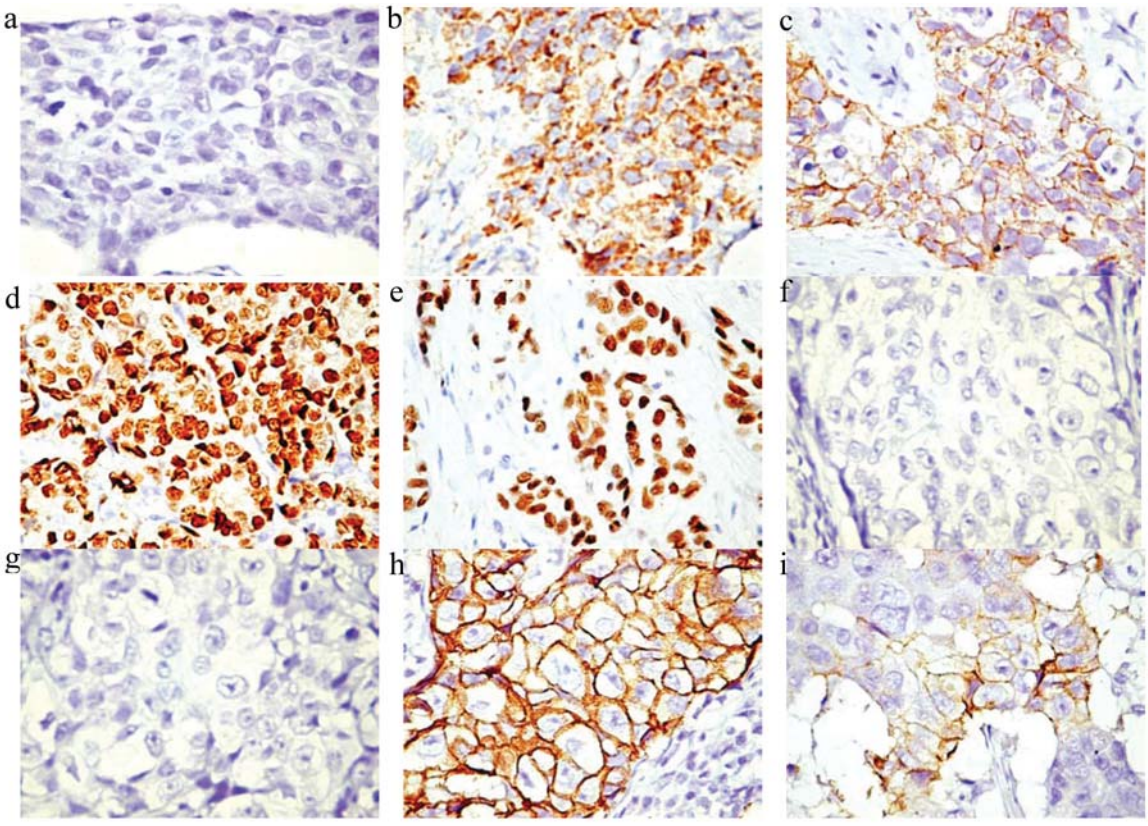

Figure 1. *Basal-like breast carcinoma: ER-negative (a), cytokeratin 5/6-positive (b), EGFR-positive (c). Luminal A type breast cancer: ER-positive (d), PR-positive (e), HER2-negative (f). HER2 type breast cancer: ER-negative (g), HER2-positive +3 (h), focal expression of EGFR (i). Original magnification: ×400.*

product of reaction. The percentage of positive cells was evaluated as 0 in negative cases, as 1 when <10%, as 2 when 10-50%, and as 3 when >50% of tumor cells were positively stained. In terms of intensity of reaction, a scale from 0 to 3 for negative, weak, moderate, and strong reaction, respectively, was used. The final score was 0-6, and cases scored as 0-2 were considered negative (0, +1), while cases scored as 3-6 were considered positive (+2, +3). VEGFR-3 expression was evaluated in the endothelium and scored as 0 (negative) or +1 (positive), and in tumor cells, using the same system used to score VEGF-C.

Lymphatic vessel count. Lymphatic vessels (LVs) were identified on slides stained with anti-D2-40 antibody, which recognizes the formalin-insensitive epitope of podoplanin. D2-40 is a highly specific marker of the lymphatic endothelium and it is not expressed by the blood vessels' endothelium. Lymphatic vessels were counted in the tumoral and peritumoral areas at ×200 magnification (covering an area of 0.74 mm^2) by two independent observers (MR and AMC), using an Eclipse E600 bright-field microscope and the hot-spot method. In each case, for both intra- and peritumoral areas, three fields with maximum density of LVs were chosen. Fields containing clusters of myoepithelial and/or myofibroblasts were excluded in order to avoid an overestimation of the LVMD. Results were compared with the molecular type of breast cancer, VEGF-C and VEGFR-3 expression.

Statistical analysis. Statistical analysis was performed by means of the commercially available software SPSS version 17.0. Pearson, Spearman, and Student's *t*-tests were performed, and $p<0.05$ was considered as significant.

Results

According to the molecular classification and on the basis of the immunohistochemical expression of the different markers, 11 cases (11.45%) of basal-like carcinoma, 50 cases (52.08%) of luminal A type, 8 cases (8.33%) of luminal B type, 20 cases (20.83%) of HER2 type, and 7 cases (7.29%) of normal-like tumors were identified. The immunohistochemical profile of an example of basal-like, luminal A and HER2 types is shown in Figure 1. Lymph node metastasis (n=47) was distributed as follows: 22/50 cases with luminal A type; 18/20 cases with HER2 type; 5/8 with luminal B type; 1/11 cases with basal-like carcinoma, and 1/7 of normal-like tumors.

VEGF-C expression. The final product of reaction for VEGF-C had a granular cytoplasmic pattern. In the normal mammary tissue adjacent to the tumor, a weak expression of VEGF-C was recognizable in epithelial cells (Figure 2a). In

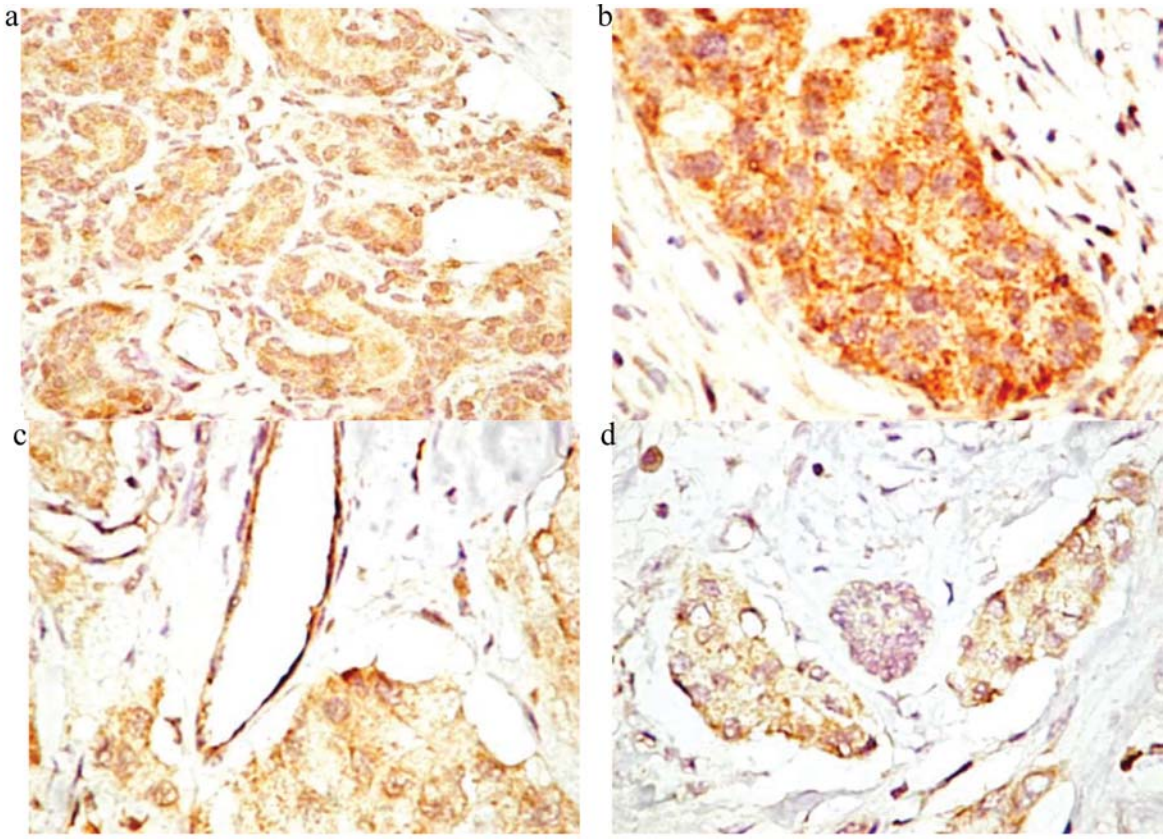

Figure 2. *Immunohistochemical expression of VEGF-C in normal mammary tissue (a), invasive carcinoma (b), tumor cells and endothelium (c), and tumor cells and stromal cells (d). Original magnification: ×400.*

both normal and tumor samples, no positive reaction was detectable in endothelial cells and the perivascular coverage of blood vessels. Positive reaction of tumor cells to VEGF-C was detectable in 62 cases [64.58%; 18 cases (9 HER2 type) scored as +3; 44 as +2; 19 as +1; 15 as 0], with a homogenous distribution within the tumor mass (Figure 2b). LVs were also positive for VEGF-C (Figure 2c). In ductal carcinoma *in situ*, atypical hyperplasia, apocrine metaplasia and intraductal papilloma associated with the carcinoma, the intensity of the reaction was similar to that found in neoplastic cells of the overt invasive tumor. The highest average score for VEGF-C expression by tumor cells was found in the HER2 type and the lowest in basal/like carcinoma (Table II).

In basal-like carcinoma, VEGF-C expression in tumor cells correlated with VEGF-C expression in stromal cells ($p<0.007$). In HER2 type breast cancer, VEGF-C expression in tumor cells correlated with VEGFR-3 expression in tumor cells ($p=0.033$) and with intratumoral LMVD ($p=0.042$). In luminal A type breast cancer, VEGF-C expression in tumor cells correlated with VEGFR-3 expression in tumor cells ($p=0.001$) and with both peritumoral and intratumoral LMVD ($p=0.034$, and $p=0.006$, respectively). In luminal B type breast cancer, VEGF-C expression in tumor cells correlated with VEGFR-3 expression in tumor cells ($p=0.02$), and in normal-like tumors, with peritumoral LMVD ($p=0.012$).

In 25 cases, VEGF-C-positive reaction was noticed in the tumor stroma, associated (n=17) or not (n=8) with positive reaction in tumor cells (Figure 2d). Positive stromal cells were polygonal or spindle-shaped. VEGF-C expression in stromal cells correlated only with VEGFR-3 expression in endothelial cells ($p=0.025$) in luminal A type.

Overall, VEGF-C expression did not correlate with the grade of the tumor, but a significant correlation was found with lymph node metastasis ($p<0.001$).

VEGFR-3 expression. VEGFR-3 expression was assessed in the tumor cells (Figure 3a) and lymphatic endothelium (Figure 3b). VEGFR-3 expression in tumor cells was found in 64 cases (66.66%; 3 basal-like, 19 HER2, 35 luminal A, 5 luminal B, and 2 normal-like). The highest average score was found in the HER2 type and the lowest in the basal-like carcinoma (Table II). VEGFR-3 expression in tumor cells

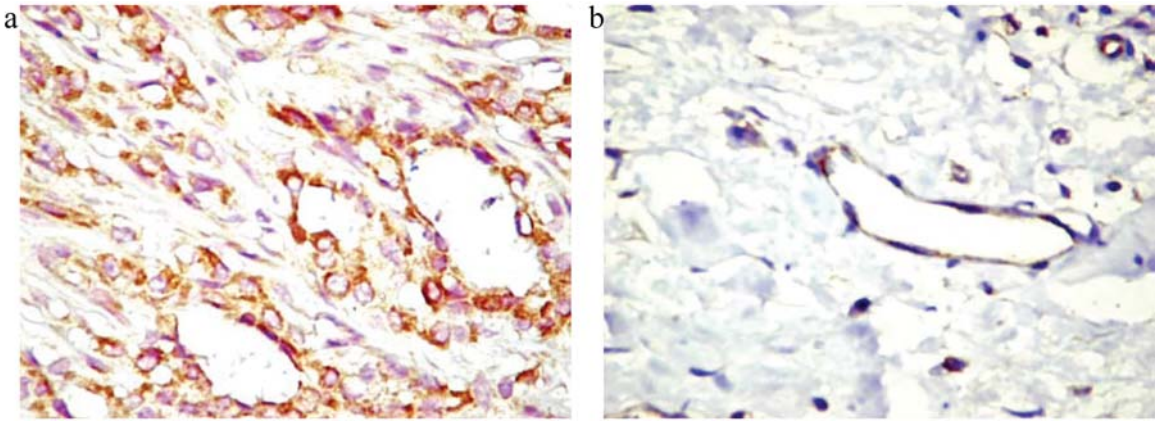

Figure 3. *VEGFR3 expression in tumor cells (a) and in lymphatic endothelial cells (b). Original magnification: a, ×200: b, ×400.*

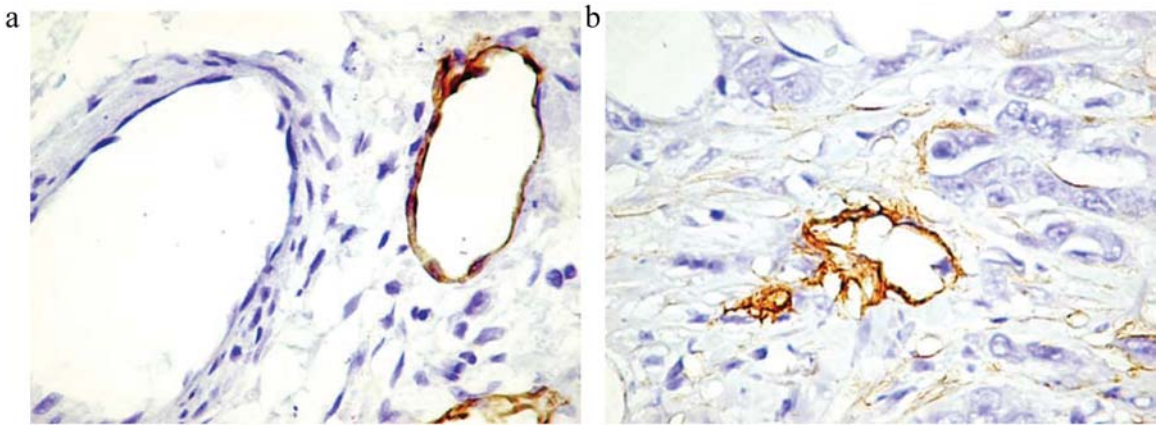

Figure 4. *D2-40-positive peritumoral (a) and intratumoral (b) lymphatic vessels. The endothelium of the blood vessel is negative (a). Original magnification: ×400.*

Table II. *Average scores for VEGF-C, VEGFR-3, and LMVD in different molecular types of breast cancer.*

Type	VEGFC		VEGFR-3		LMVD	
	Tumor cells	Stromal cells	Tumor cells	Stromal cells	Intratumoral	Peritumoral
Basal-like (n=11)	0.72	0.54	0.45	0.09	1.60	4.84
HER2 (n=20)	2.2	1.05	2	0.85	2.78	6.69
Luminal A (n=50)	1.62	0.74	1.34	0.48	1.83	7.64
Luminal B (n=8)	2.12	0.87	1.25	0.37	2.12	6.28
Unclassified (n=7)	1.28	0.42	0.57	0.28	0.57	3.71

correlated with VEGF-C expression by tumor cells (p=0.033), VEGFR-3 expression in lymphatic vessels in HER2 type (p=0.038), VEGF-C expression in tumor cells (p=0.001) and peritumoral LMVD in luminal A type breast cancer (p=0.001).

VEGFR-3 expression in the lymphatic endothelium was found in 30 cases (31.25%). The highest average score was noticed in the HER2 type and the lowest in the basal-like carcinoma (Table II). VEGFR-3 expression in endothelial cells correlated with VEGF-C expression by stromal cells

(p=0.025) in luminal A carcinoma and with intratumoral LMVD (p=0.002), while in luminal B carcinoma, VEGFR-3 correlated only with intratumoral LMVD (p=0.015). No correlation between VEGFR-3 expression and the other markers was found in basal-like and normal-like carcinomas.

Lymphatic microvascular density. LVs were identified in the peritumoral tissue of 95 cases (Figure 4a). No LVs were found in a single case of basal-like carcinoma. Peritumoral LVs were characterized by relatively large lumen, regular borders and lymphovascular invasion in 13 cases (13.54%). Peritumoral LMVD ranged between 0 and 15.33, with the highest average score in luminal A carcinoma, HER2 type and luminal B carcinoma, as compared to basal-like and normal-like carcinomas (Table II). Peritumoral LMVD correlated with VEGF-C and VEGFR-3 expression in tumor cells in luminal A type (p=0.006, and p=0.001, respectively), and with VEGF-C expression in stromal cells in the normal-like type breast cancer (p=0.012). No significant correlation was found between peritumoral LMVD and the other molecular types of breast cancer.

LVs were found in the intratumoral tissue of 68 cases (70.83%), and were significantly smaller than peritumoral LVs, with irregular, narrow lumen (Figure 4b); tumor cells within the lumen were found in 9 cases (9.37%). Intratumoral LMVD ranged between 0 and 6.33, with the highest average score in the HER2 type, and the lowest in the normal-like type (Table II). Intratumoral LMVD correlated with VEGF-C expressed by tumor cells in HER2 and luminal A types (p=0.042, and p=0.034, respectively), and with VEGFR-3 expression in the lymphatic endothelium in the luminal B type (p=0.015). No significant correlation was found between both peritumoral and intratumoral LMVD and basal-like carcinoma.

Discussion

In this study, we have shown a differential expression of VEGF-C, VEGFR-3 and D2-40 in molecular types of breast cancer and found the highest level of expression for these markers in the HER2 and luminal B subtypes, and the lowest in the basal-like subtype. The lowest values for both intratumoral and peritumoral LMVD were found in the normal-like subtype.

Breast cancer is the most frequent malignancy in female and lymph node metastasis occurs in more than one third of the cases. It has been well established that in breast cancer, increased LMVD correlates with lymph node metastasis (25, 26) and VEGF-C is expressed in tumor in more than half of the patients with invasive breast cancer, and correlates with lymph node metastasis, disease-free survival and overall survival (27). In the present study, we found VEGF-C expression in 64.58% of the cases and a strong correlation with lymph node metastasis, confirming both clinical observations and experimental studies (28).

According to the new molecular classification of breast cancer, five major classes have been identified by gene analysis, namely luminal A and B, basal-like, HER2 and normal-like (29). The advantages of this classification are closely related to the prognosis and to the potential response to adjuvant chemotherapy (30). Clinical and pathologic observations have shown a particular pattern of lymph node and distant metastasis in molecular types of breast cancer.

Few data are available concerning VEGF-C and VEGFR-3 expression in breast cancer according to this classification. In this study, we have demonstrated the highest VEGF-C expression in the HER2 subtype and the lowest in the basal-like subtype. Recently, Liu *et al.* (22) showed that triple-negative breast cancer, including basal-like, HER2 and normal-like subtypes, correlates with both intratumoral and peritumoral LMVD, and with the expression of VEGF-C and VEGF-D. In contrast, we found a positive correlation between VEGF-C, VEGFR-3 and LMVD only in the HER2 subtype, and a positive correlation in HER2 and normal-like types for VEGFR-3 expression in tumor cells.

The HER2 subtype is one of the most aggressive molecular variants of breast cancer, frequently associated with lymph node metastasis and poor prognosis. The aggressive behaviour of these tumors may be explained in part by VEGF-C expression in tumor cells (31). More recently, Schoppmann *et al.* (32) have shown that HER2 overexpression is associated with high VEGF-C expression and LMVD. These data support the clinical relevance of the association between HER2 and VEGF-C expression, and blocking HER2 may reduce not only tumor progression, but also lymphangiogenic metastasis. In this study, we have demonstrated a correlation between HER2 subtype and VEGF-C and VEGFR-3 expression in tumor cells and lymphatic endothelium, respectively, and LMVD.

Expression of VEGF-C and VEGFR-3 by tumor cells is not a characteristic of invasive tumors, as they were also detected in ductal carcinoma *in situ* of the breast (33). This suggests that lymphangiogenesis could be an early event during breast carcinogenesis; but on the other hand, precursor lesions for some molecular types have not yet been characterized. Moreover, VEGF-C and its specific receptors promote not only lymphangiogenesis, but also migration of human breast cancer cells (34). The novel role of VEGF-C indicates that breast cancer metastasis can be orchestrated by lymphangiogenesis and enhanced migratory activity of tumor cells.

Based on these data, lymphangiogenic factors, and particularly VEGF-C and VEGFR-3, together with lymphatic vessels could be attractive targets for anti-lymphangiogenic therapy in breast cancer. In an experimental model of breast cancer, it has been shown that inhibition of VEGFR-3 activation suppresses regional and distant metastasis, and the

combination treatment with anti-VEGFR-3 and anti-VEGFR-2 antibodies more potently reduced lymph node and lung metastasis than each antibody alone (35). These data are supported by the complete and specific inhibition of VEGF-C-enhanced lymphangiogenesis in adult mice using a VEGFR-3 neutralizing antibody (36). The problem of the basic mechanisms involved and the strategy for an anti-lymphangiogenic therapy is even more complicated, as it was shown that the neutralizing anti-VEGF-A antibody suppresses tumor lymphangiogenesis and reduces VEGFR-3 expression in an orthotopic breast tumor model (37). Although a lot of evidences has been accumulated in recent years to support the contribution of the VEGF-C/VEGFR-3 axis to lymphangiogenesis in breast cancer, the clinical significance remains controversial and the results of experimental studies have not yet been translated into practice.

In summary, we have shown a differential expression of VEGF-C, VEGFR-3 and D2-40 in molecular types of breast cancer. We found the highest level of expression for these markers in HER2 and luminal B types and the lowest in basal-like carcinoma. The lowest value of both intratumoral and peritumoral LMVD were found in normal-like tumors. These data provide evidence not only for organ-specific lymphangiogenesis, but also for tumor type-specific lymphangiogenesis. Further studies are necessary to investigate the contribution of VEGF-C and VEGFR-3 in the promotion and maintenance of lymphangiogenesis in breast cancer, in order to define specific targets for therapy.

Conflict of Interest

None to declare.

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