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ORIGINAL ARTICLE

VEGFR-3 expression in breast cancer tissue is not restricted to lymphatic vessels

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Abstract

We examined the immunohistochemical reactivity for vascular endothelial growth factor receptor 3 (VEGFR-3), a protein playing an important role in lymphangiogenesis, in breast cancer. A retrospective series of 77 invasive ductal breast carcinomas was investigated. The relationship between VEGFR-3 expression and clinicopathologic parameters was examined for statistical significance using Pearson's chi-square (χ^2) test and Fisher's exact test (when $n<5$). Threshold for significance was $p<0.05$. Patient age ranged from 31 to 77 years (mean: 55 years). The VEGFR-3 immunoreactivity was as follows: 5 cases were negative (6.5%), 35 + (45.4%), 27 + + (35.1%), and 10 + + + (13.0%). Reactions were positive for both lymphatic and blood vessels in several cases. VEGFR-3-positive reactions were more frequent in the tumor periphery than within the tumor. Immunoreactivity was also observed in myoepithelial cells surrounding both normal ducts and ducts with ductal carcinoma in situ. Statistical analysis of VEGFR-3 reactions was not significantly related to node status, microvessel density, and tumor grade. Ploidy showed a tendency towards significance ($p = 0.063$); however, owing to the limited number of cases, statistical significance was not reached. VEGFR-3 lacks lymphatic vessel specificity and is also expressed in blood vessels, myoepithelial cells, and neoplastic cells.

Keywords: Lymphangiogenesis; Angiogenesis; Vascular endothelial growth factor receptor 3; Breast cancer

Introduction

The vascular growth factor family (VEGF-A, -B, -C, -D, and placenta growth factor — PlGF) is involved in angiogenesis and lymphangiogesis [30]. This has led to the investigation of metastasis mechanisms with the purpose to block the growth of new blood vessels (BVs) and lymphatic vessels (LVs) in order to inhibit neoplastic dissemination [9]. However, it has been shown that VEGF-D may have different biologic functions in mouse and man [2]. VEGF-D is recognized as an angiogenic promoter, mitogenic for endothelial cells in vitro. It is expressed at different embryonic sites and in human tumors, and also induces lymphangiogenesis and metastatic spread via LVs in mouse tumor models [2].

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Vascular endothelial growth factor receptor 3 (VEGFR-3, Flt-4) is a tyrosine kinase receptor for VEGF-C and VEGF-D expressed in LVs [22]. The interaction between VEGF-C and VEGFR-3 plays an important role in the preservation of LVs and lymphangiogenesis [22,33]. VEGFR-3 activity is supposed to induce lymphangiogenesis and LV hyperplasia, but it is also upregulated in tumor blood vessels and is involved in neoplastic angiogenesis [11,18]. VEGFR-3 is expressed preferentially in LVs, although Patanen et al. [21] have described its consistent expression in other endothelial cells, such as the lining of fenestrated capillaries in bone marrow, splenic and hepatic sinusoids, kidney glomeruli, adenohypophysis, thyroid gland, parathyroid gland, adrenals, and choroids plexus. Moreover, Valtola et al. [29] have demonstrated VEGFR-3 expression in blood capillaries of normal and neoplastic breast tissue.

Identification of intra-tumoral LVs can be very difficult, even impossible with the use of the available means. This is partly due to the mechanical stress caused by neoplastic expansion in a confined area, compressing the newly formed channels [1].

Increased lymphatic vasculature was observed in in situ and in invasive ductal breast carcinoma [26]. Patients with the highest relative VEGFR-3-positive immunostaining most likely have lymph node metastasis [17]. Intratumoral lymphangiogenesis with significantly enhanced lymph node and lung metastases was observed in human breast cancer transplanted onto nude mice [27]. However, an experimental tumor model has demonstrated that lymphatic metastasis occurs in the absence of functional intratumoral lymphatics; the functional LVs surrounding the tumor are sufficient to allow for metastatic dissemination of malignant cells [19].

Thus, we investigated VEGFR-3 immunoreactivity in invasive ductal breast carcinoma to evaluate this lymphangiogenic marker for its usefulness under routine conditions using a commercially available antibody, and to analyze its importance for breast cancer diagnosis and prognostic evaluation.

Materials and methods

Patients and tumor samples

Formalin-fixed, paraffin-embedded samples obtained from 77 invasive ductal carcinomas were retrieved from the pathology files of the IPATIMUP, Porto, Portugal. One of the authors reviewed all the cases classified previously on the basis of the 1981 WHO recommendations [31] and now revised according to the most recent WHO classification [28]; histologic grading was performed using the modified criteria of Bloom and Richardson in the light of the advances achieved in the classification of breast cancer, described by Elston and Ellis [5].

VEGFR-3 Immunohistochemical procedure

Immunohistochemistry was carried out with the streptavidin-biotin-peroxidase complex technique using a primary antibody raised against VEGFR-3 (Zymed Laboratories Inc., CA, USA) diluted 1:200. Briefly, deparaffinized and rehydrated sections were immersed in 0.01 M citrate buffer (pH 6.0) and microwaved at 700w for 15 min. The slides were then incubated with 3% hydrogen peroxide in methanol for 10 min and in Ultravision Block Solution (Neomarkers, Freemont, CA, USA) for 10 min at room temperature before 30 min-incubation with the primary antibody. Sections were sequentially washed in PBS 1X with 0.02% Tween 20 and incubated with biotinylated goat anti-polyvalent antibody for 10 min, streptavidin peroxidase for 10 min, and developed with 3,3′-diamino-benzidine for 10 min. The slides were counterstained with Mayer's hematoxylin (Merck, Dermstadt, Germany). Negative controls were carried out by omitting primary antibody; regarding the positive controls, we used invasive ductal breast carcinoma tissue as indicated by the manufacturer, leading to satisfactory immunostaining in the LVs.

Assessment of positive reactions

For immunohistochemistry, the expression of VEGFR-3 was evaluated considering cytoplasmic staining and, eventually, nuclear staining. The slides were analyzed by two pathologists using a double blind method.

We semiquantitatively assessed the distribution of the marker in endothelial and epithelial cells independently. For this, the following grading system was used: negative (−), absence of expression; slightly positive staining (+), expression in up to 10% of cells; moderately positive (++), expression in over 10% up to 50% of cells; strongly positive (+++), expression in over 50%. The positive reactions were assessed in hot spot areas where proliferating vascular structures and epithelial malignant cells were present and stained. The positive reactions were discriminated according to tissue localization: peri- and intratumoral for LVs and BVs and neoplastic cells. To clear up disagreements, the cases were analyzed by the two observers using a double-head microscope, and a final consensus was reached.

Clinical and pathologic parameters

The prognostic factors herein studied were reported by our group previously [14]. The VEGFR-3 results were correlated with some clinical and pathologic parameters as follows:

- Histologic grade: grade I, II, or III [5]: tubule and gland formation $>75\%$, 10–75%, or $<10\%$, nuclear pleomorphism (small-regular uniform cells, moderate increase in size and variability or marked variation), and mitotic counts.
- Axillary lymph node status: negative or positive for metastasis.
- Proliferative index assessed by MIB-1 immunohistochemical reaction: the score was determined by two pathologists counting at least 1000 neoplastic cells in 10–20 fields in a double blind method; cases with $\leqslant 15\%$ of positive tumor cells were considered to have a low proliferative index, cases with $>15\%$ of positive cells were considered to have a high proliferative index, as described previously [25].
- Microvessel density: microvessel density was determined by counting vessel structures positive for factor VIII-related antigen. If single cells or clusters of cells clearly separated from adjacent clusters and background, with or without lumen, were positive, we considered these an individual vessel, as recommended in previous studies [14]. Areas of fibrosis, necrosis and inflammation, as well as vessels with a muscle wall, were excluded from counting. Two observers simultaneously counted the microvessels in the three most vascularized areas in a X200 field ($0.74\,mm^2$). As there was no statistical difference when considering the highest count of each case or its average count, the results were analyzed for the average vessel count of an individual case.
- Ploidy: Ploidy was assessed in material obtained from paraffin sections, stained by the Feulgen method, and measured using an image analysis system (Ahrens System, Bargteheide/Hamburg, Germany).

Statistical analysis

For statistical analysis, some parameters were grouped as follows:

1. VEGFR-3
   - VEGFR-3 expression: 0 and 1+ were evaluated as negative;
   - 2+ and 3+ were positive.
2. Histologic grade I and histologic grade II /III.
3. Microvessel density (MVD) was subdivided into two categories: $\leqslant 30$ and >30 (mean microvessels in three fields).
4. MIB immunoreactions were subdivided into two groups of positive cells: $\leqslant 15\%$ and $>15\%$.

The relationship between VEGFR-3 expression and clinic-pathologic parameters was examined for statistical significance using Pearson's chi-square (χ^2) test and Fisher's exact test (when $n<5$), the threshold for significance being $p<0.05$.

Results

VEGFR-3 immunostaining was observed both in LVs and BVs in several cases. The overall VEGFR-3 reactions were as follows: five cases were negative (6.5%), 35+(45.4%), 27++ (35.1%) and 10+++ (13.0%). VEGFR-3-positive reaction was more frequent in the tumor periphery than within the tumor. Intratumoral-positive reactions were recognized in filamentous deformed structures compressed by neoplastic masses resembling lymphatics and by small vessels similar to BVs.

There were different combinations of VEGFR-3-positive reactions in LVs and BVs in the periphery of the tumor and within. In most cases, VEGFR-3 was concomitantly expressed by LVs and BVs in the periphery of the tumor. There were a few cases in which only LVs were positive for VEGFR3, and the BVs were negative. We also observed positivity for VEGFR-3 in myoepithelial cells surrounding normal ducts and ducts with ductal carcinoma in situ (Figs. 1 and 2). In some cases, VEGFR-3 was expressed in the cytoplasm of carcinoma cells, there being no relationship with the positivity in the respective LVs and BVs.

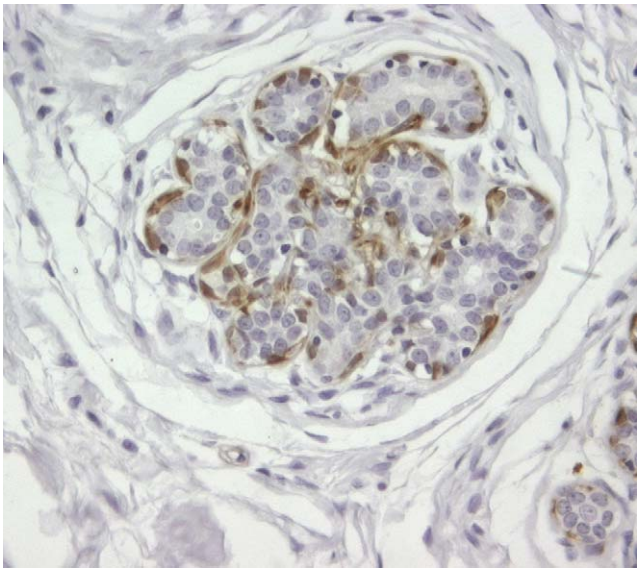

Fig. 1. VEGFR-3 expression in myoepithelial cells of normal lobules.

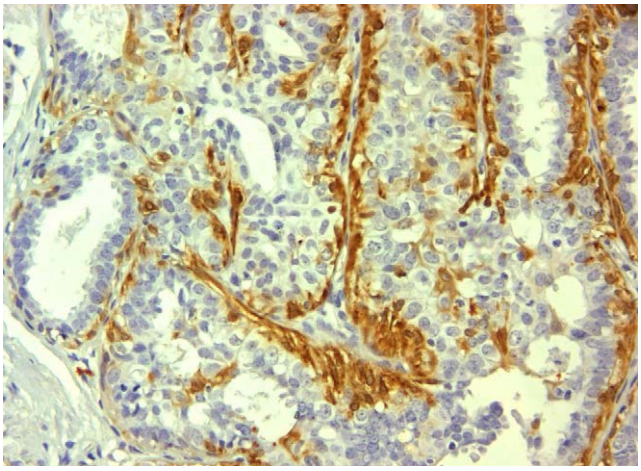

Fig. 2. Positive VEGFR-3 reaction in myoepithelial cells surrounding ducts with ductal carcinoma in situ.

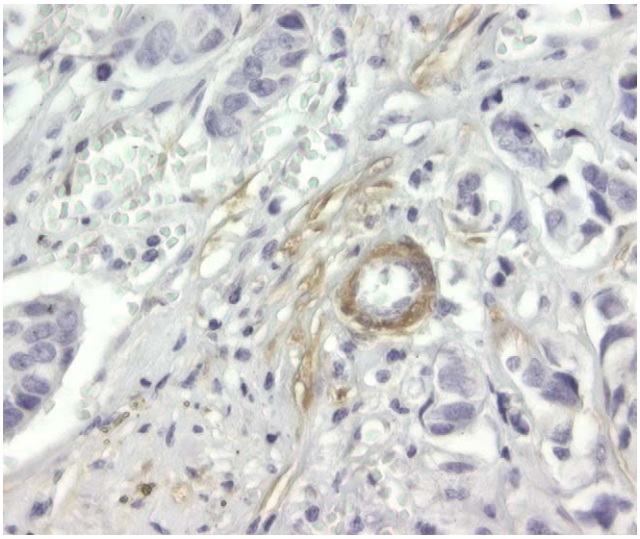

Fig. 3. Intratumoral lymphatics marked for VEGFR-3 compressed within neoplastic cells. Note the small BVs also positive for VEGFR-3.

VEGFR-3 and LVs

Lymphatic vessels were stained mainly in the periphery of the tumor, including adipose tissue. Intratumoral lymphatics predominantly presented as compressed structures faintly marked for VEGFR-3 and frequently associated with small BVs also positive for VEGFR-3 (Fig. 3). In 55 cases (71.4%) LVs were positive; from these cases, 22 (28.6%) were also positive for LVs within the tumor. Immunoreactive intra-tumoral LVs were observed in 25 cases (32.4%).

VEGFR-3 and BVs

BVs were positive in the periphery of the tumor in 52 cases (67.5%) and within the tumor in 22 (28.6%). Concomitant BV-positive reactions within and in the periphery of the tumor were observed in 20 cases (25.9%). Immunoreactive BVs within the tumor frequently presented as small structures with one or two nuclei. BVs in the periphery were frequently larger, and VEGFR-3 staining was also observed in an elastic portion of these vessels (Fig. 4).

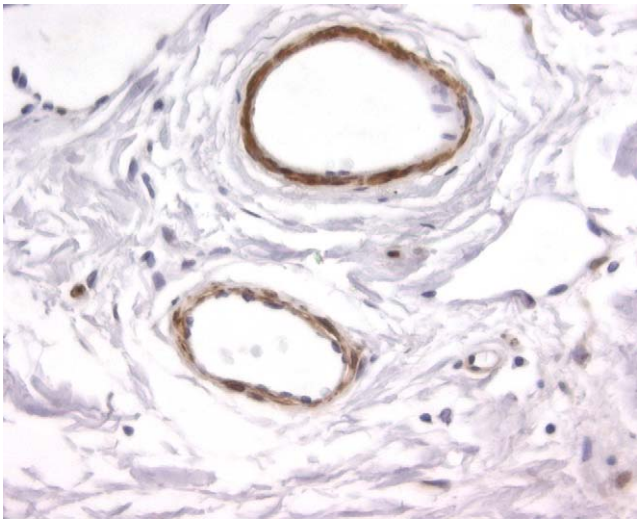

Fig. 4. In the periphery of neoplastic tumor, BVs were frequently large, and positive reactions frequently occurred in an elastic portion of the vessels.

VEGFR-3 general reactions correlated with clinical parameters

Dichotomized negative and positive VEGFR-3 immunoreactions were considered for the evaluation of VEGFR-3 expression.

Patient age ranged from 31 to 77 years (mean: 55 years). Table 1 shows the association between prognostic parameters and VEGFR-3 immunoreactivity. The prognostic parameters were not complete for all the 77 cases studied. Of these 77 cases, clinical information about nodal status and MIB-1 was available for 69 cases; ploidy for 61; MVD for 74; and grading for 68. Statistical analyses of the VEGFR-3 reactions were not significant for nodal status ($p = 0.256$), microvessel density (MVD) ($p = 0.632$), and tumor grade ($p = 0.493$). Ploidy showed a tendency towards significance ($p = 0.063$); however, as the diploid samples comprised a very small series, Fisher's exact test revealed no significant correlation ($p = 0.115$), nor did MIB-1 immunoreactivity ($p = 0.264$).

Table 1. The frequency (%) of the prognostic parameters correlated with the general VEGFR-3-positive and -negative immunoreactions

VEGFR-3	Nodal status		*p* value	Ploidy		*p* value	MIB-1		*p* value	MVD		*p* value	Grade		*p* value
	Negative	Positive		Negative	Positive		Negative	Positive		⩽30	>30		Grade I	Grade II/III	
Negative	$11^{40.7\%}$	$23^{54.8\%}$	0.256	$3^{23.1\%}$	$25^{52.1\%}$	0.063*	$22^{55.0\%}$	$12^{41.4\%}$	0.264	$15^{53.6\%}$	$22^{47.8\%}$	0.632	$6^{60.0\%}$	$28^{48.3\%}$	0493
Positive	$16^{59.3\%}$	$19^{45.2\%}$		$10^{76.9\%}$	$23^{47.9\%}$		$18^{45.0\%}$	$17^{58.6\%}$		$13^{46.4\%}$	$24^{52.2\%}$		$4^{40.0\%}$	$30^{51.7\%}$	

*Fisher's exact test: *p* value = 0.115..

Discussion

Our results, obtained using a commercially available VEGFR-3 antibody under routine conditions in paraffin-embedded samples of invasive breast ductal carcinoma, have revealed interesting points that might be considered a critical approach regarding the regulation of lymphangiogenesis in malignant tumors.

VEGFR-3 is expressed in vascular endothelium early in development and in angiogenic endothelium, but is almost restricted to LVs in adults. Conversely, it lacks specificity in tumors and is upregulated in angiogenic endothelium [4]. Furthermore, VEGFR-3 was recently demonstrated in lymphatic and vascular precursor cells [24].

VEGFR-3 upregulation in BVs has previously been documented in breast carcinomas, and the consistency of this finding is well established [29]. VEGFR-3 expression in tumor blood and LVs is believed to play an important role in mediating lymphangiogenic factor-induced neovascularization, and is significantly correlated with VEGF-D expression, nodal status, and prognosis [16]. In addition, VEGFR-3 expression was also detected in cancer cells of colon-rectal adenocarcinomas [34], as well as in normal-hyperplastic and malignant prostatic cells [13], myeloid leukemia cells [6], and pancreatic endocrine malignant cells [7]. These studies certainly corroborate in part our observations.

Surprisingly, we came upon an unexpected finding related to the strong expression of VEGFR-3 in myoepithelial cells. As shown in Fig. 1, the myoepithelial cells were uniformly stained with anti-VEGFR-3, outlining a subtle drawing of the cytoplasmic limits of the cells. VEGFR-1 and -2 expressions have previously been demonstrated in smooth muscle cells of vessel walls in the porcine placenta and non-pregnant uterus [32], which leads to the speculation that VEGFR-3 might exert a similar regulation. VEGFR-3 ligands, VEGF-C, and -D were found to be expressed in vascular smooth muscle vessels of BVs [21]. Most recently, Rutanen et al. [23] have corroborated the finding of VEGF-D expression in smooth muscle cells mainly in large arteries of atherosclerotic lesions, and they have also reported that VEGFR-2, but not VEGFR-3, is expressed in lamina intima and lamina media of larger arteries. In addition, VEGFR-3 was found in endothelial cells of adventitial LVs. Curiously, cells migrating from the lateral somatic edge into the limb buds can differentiate into three cell populations: myocytes, blood and lymphatic endothelial cells [8]. In vivo experiments with non-neoplastic conditions have demonstrated that smooth muscle cells downregulate VEGFR-3 expression, thus reducing the required signaling capacity of developing LVs [30]. We hypothesize that smooth muscle cells and myoepithelial cells can upregulate VEGFR-3 in breast cancer.

Recently, Padera et al. [20] demonstrated that tumor compression impairs intratumoral lymphatic activity,

probably because of permanent damage of LV structures, but not of the LVs in the tumor periphery, most likely caused by less pressure. This complicated issue must be carefully evaluated for the role of intra- and peritumoral vasculature in terms of therapeutic targets and metastatic conduit for malignant neoplastic cells [12]. The function of lymphangiogenesis in breast cancer must be evaluated carefully. The increasing knowledge encourages research that aims at establishing therapeutic strategies and aid for evaluation of prognosis [3,12]. Quantification of lymphatics seems to be restricted because of the great difficulty in counting intratumoral vessels. Firstly, as reported previously [20], the neoplastic pressure deforms the vessels, making the identification of lymphatics doubtful. Secondly, the lack of VEGFR-3 specificity does not allow for the discrimination between small LVs and BVs, which are presumed to occur in active angiogenic processes as in neoplastic conditions. Finally, the assessment of vessel quantification has a number of different protocols, with significant [15] and non-significant results achieved in breast cancer patients [14]. The use of VEGFR-3 in breast carcinoma has been reported previously, and, in spite of the optimism concerning VEGFR-3 specificity for lymphatics, the microlymphatic count had neither a prognostic value nor did it correlate with the axillary's lymph node status [10].

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