Evaluation of Prognostic Value of VEGF-C and VEGF-D in Breast Cancer – 10 Years Follow-up Analysis

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Abstract. *The aim of the study was to evaluate the prognostic value of vascular endothelial growth factor C (VEGF-C) and VEGF-D expression in stage II, grade 2 and 3, ductal breast cancer patients. Patients and Methods: The immunohistochemical staining of 98 tumor samples and 5- and 10-year overall (OS) and disease-free survival (DFS) were analyzed. Results: A significant relationship between VEGF-C and VEGF-D expression (p=0.000002) was noted. No correlations between protein expression and clinical parameters (tumor size, grade, estrogen receptor status, axillary lymph node metastases and age) or 5- and 10-year DFS or OS were demonstrated. A close to significant correlation (p=0.084) was observed between high expression of VEGF-C and 5-year OS. Conclusion: Our study did not reveal any prognostic value of VEGF-C or VEGF-D. Therefore they are not useful as markers for patients with poor prognosis. Unlike in other studies, our patient group was homogenous which might have contributed to the results obtained.*

While patient qualification for further treatment is obvious at the extreme stages of breast cancer (I, III and IV), the position in stage II cases is still undefined (1). It has been estimated that only a low percentage of stage II patients benefits from aggressive chemotherapy (2). Consequently, it is of major importance to define the immunohistochemical features of this group which could make patient stratification and individualization of treatment possible. Vascular endothelial growth factor, which is also referred to as VEGF-A, belongs to the VEGF-platelet-derived growth factor (PDGF) supergene family; other family members are VEGF-B, VEGF-C, VEGF-D and VEGF-E. All of them show varying degrees of homology with VEGF-A (3). VEGF-C and VEGF-D are peptide growth factors capable of inducing growth of new lymphatic vessels *in vivo* in a process called lymphangiogenesis. They are ligands for the endothelial cell specific tyrosine kinase receptors VEGFR-2 and VEGFR-3. While VEGFR-2 is thought to be the major regulator of angiogenesis and is expressed by both blood vascular and lymphatic endothelium (4), VEGFR-3 signaling is crucial for the development of the lymphatic vessels (5, 6). In normal adults, VEGF-C appears to be a lymphangiogenic factor. VEGF-C also induces the formation of new blood vessels, but it appears to be restricted to early development and certain pathological settings such as tumorigenesis (7). Functionally, VEGF-D stimulates endothelial cell migration and proliferation *in vitro*. In animal tumor models VEGF-D induces both tumor angiogenesis and lymphangiogenesis, promoting lymphatic spread (8). Furthermore, neutralizing antibodies against VEGF-D reduce the number of lymphatic metastases, and soluble VEGFR-3 inhibits tumor- associated lymphangiogenesis (9).

In this study VEGF-C and VEGF-D expression, 5- and 10-year overall and disease-free survival (OS, DFS) and clinico-pathological factors were analyzed in highly homogenous group of 98 patients with TNM stage II, histological grade 2 and 3 ductal breast carcinomas. The aim was to determine whether the expressions of VEGF-C and/or VEGF-D in primary tumors were correlated with lymph node metastasis and patient prognosis and could be helpful in defining a subgroup of patients for more or less aggressive treatment.

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Patients and Methods

Patients and tumor samples. The present study included archival tumor samples from 98 patients of the Lower Silesian Oncology Center (Wroclaw, Poland, radically treated for stage II ductal breast cancer in 1993-1994. The study was approved by a regional Institutional Review Board. The median age of the patients was 56, range from 29 to 86 years. All patients underwent surgery (radical modified Patey-Madden mastectomy) with or without adjuvant treatment. The patients' clinical history and clinical and pathological variables were obtained from the medical records and during follow-up visits. The size of the primary tumor was evaluated from the surgical specimen. Lymph node status was determined by lympha-denectomy of the axillary lymph nodes and by histological evidence of metastatic breast carcinoma. OS and DFS (in weeks) were established for all the patients with follow-up periods of 5 years (261 weeks) and 10 years (522 weeks). Microscopic studies were performed on formalin-fixed, paraffin-embedded cancer tissues, obtained during surgery and stained routinely with haematoxylin and eosin. Histopathological type according to the World Health Organization (10) (ductal breast cancer in all the cases), grade (only G2 and G3 were included in this study) and stage according to the TNM classification were determined during microscopic examination. The tumour grade was estimated according to Bloom-Richardson in the Elston and Ellis modification (11). The detailed characteristics of the patients are given in Table I and II.

Immunohistochemistry. Formalin-fixed paraffin-embedded freshly-cut 4-μm tissue sections were mounted on Superfrost slides (Menzel Glaeser, Germany), dewaxed with xylene, and gradually rehydrated. The sections were incubated with citrate buffer at 98˚C to unmask the epitopes and treated with 1% hydrogen peroxide (H_2O_2) for 10 min to block endogenous peroxidase. The sections were then incubated with monoclonal antibodies against VEGF-C and VEGF-D (goat anti-human VEGF-C and goat anti-human VEGF-D, from R&D Systems, UK). The sections were further incubated with biotin-labeled secondary antibody and streptavidin-biotin-peroxidase, for 20 min each. The tissue was stained for 5 min with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB), counterstained with haematoxylin, dehydrated and mounted. The intensity of the immunocytochemical reactions was estimated independently by two pathologists. In cases of controversy, a re-evaluation was performed with the use of a double-headed microscope. In order to evaluate VEGF-C and -D expression a semiquantitative scale was applied. Finally results were divided into four groups according to the intensity and extent of staining as follows: no reaction =0; weak/very limited moderate staining =1; moderate widespread/strong localized staining =2 and strong widespread =3.

Statistics. The association between VEGF-C and VEGF-D expression and between their expression and clinicopathological parameters was tested using the Spearman correlation coefficient. The Cox proportional hazard regression model was used for multivariate analysis of survival. The following parameters were considered: age and hormonal status of the patients; axillary lymph node metastasis; histological grade (G2 *vs.* G3); estrogen receptor (ER) alpha status; 5-year and 10-year overall survival (5-OS, 10-OS); 5-year and 10-year disease-free survival (5-DFS, 10-DFS) and VEGF-C and -D expressions. The Cox-Mantel and Fisher test was used for the statistical analysis and statistical significance was defined as $p<0.05$.

Results

VEGF-C expression in breast cancer tissue. In the breast cancer cells, expression of VEGF-C protein was observed in the cytoplasm (Figure 1). VEGF-C protein positive tumor tissue was found in 83.7% (82/98) of the samples. The level 1 expression was noted in 29.6% (29 patients), level 2 in 38.8% (38 patients) and level 3 in 15.3% (15 patients).

VEGF-D expression in breast cancer tissue. In the breast cancer cells, expression of VEGF-D was observed in the cytoplasm (Figure 2). VEGF-D protein positive tissue was found in approximately 50% (49/98) of the tumor tissue samples. In 40.8% (40/98) of the samples the expression was level 1 and in 9.2% (9/98) it was level 2.

Association between VEGF-C and VEGF-D expression and clinicopathological factors. The correlations between VEGF-C, VEGF-D expression and clinicopathological data are shown in Table I. No associations were observed between VEGF-C and VEGF-D expression and patient age, tumor size, tumor grade, ER status or axillary lymph node metastases.

Correlation between VEGF-C expression and VEGF-D expression. There was a statistically significant relationship between VEGF-C and VEGF-D expression ($p<0.001$, Spearman correlation coefficient =0.455).

Prognostic value of VEGF-C and VEGF-D in breast carcinoma patients. Five- and 10-year OS and DFS were compared between groups with different VEGF-C and VEGF-D expression levels. No significant difference was found in OS or DFS. The details are included in Table II and Table III. The only correlation approaching significance ($p=0.084$) was the association between high expression of VEGF C and very good 5-year OS (100% of patients with level 3 expression of VEGF C survived 5 years compared to 81.25% with level 0, 82.76% with level 1 and 81.58 with level 2).

Discussion

In this series of breast carcinomas, 83.7% tumors expressed the VEGF-C protein, which is in agreement with data reported by Nakamura *et al.* (12). Other authors have found that VEGF-C was expressed in 39.8%-90.8% of studied breast carcinomas (13-15). Fewer patients with VEGF-D expression (50%), were found in this study compared to the study by Nakamura *et al.* (16, 17) who observed VEGF-D positive

Table I. *Distribution of breast ductal cancer patients according to VEGF-C and VEGF-D protein expression in tumor tissue.*

Patient characteristics	n (%)	VEGF-C		*p*-value	VEGF-D		*p*-value
		negative	positive		negative	positive	
Total		98 (100%)	16 (16.33%)	82 (83.67%)	49 (50%)	49 (50%)	
Age							
≤50	31 (31.63%)	3 (9.68%)	28 (90.32%)		15 (48.39%)	16 (51.61%)	
>50	67 (68.37%)	13 (19.40%)	54 (80.60%)		34 (50.75%)	33 (49.25%)	
Nodal status							
Negative	58 (59.18%)	12 (20.69%)	46 (79.31%)	0.60	28 (48.28%)	30 (51.72%)	0.54
Positive	40 (40.82%)	4 (10%)	36 (90%)		21 (52.50%)	19 (47.50%)	
Tumor size							
≤20 mm	12 (12.24%)	1 (8.33%)	11 (91.67%)	0.83	7 (58.33%)	5 (41.67%)	0.22
>20 mm	86 (87.76%)	15 (17.44%)	71 (82.56%)		42 (48.84%)	44 (51.16%)	
Grade							
II	67 (68.37%)	10 (14.93%)	57 (85.07%)	0.62	32 (47.76%)	35 (52.24%)	0.50
III	31 (31.63%)	6 (19.35%)	25 (80.65%)		17 (54.84%)	14 (45.16%)	
Estrogen receptor							
Negative	20 (20.41%)	4 (20%)	16 (80%)	0.85	12 (60%)	8 (40%)	0.20
Positive	78 (79.59%)	12 (15.38%)	66 (84.62%)		37 (47.44%)	41 (52.56%)	
Menopausal status							
Postmenopausal	68 (69.39%)	12 (17.65%)	56 (82.35%)		34 (50 %)	34 (50 %)	
Premenopausal	30 (30.61%)	4 (13.33%)	26 (86.67%)		15 (50%)	15 (50%)	

staining in 81.9% and 81% cases. Although tumor cell immunoreactivity was generally homogenous throughout most samples, some of them did display increased staining at the invasive edge. A study of colorectal carcinoma has shown that VEGF-C expression at the deepest edge of the tumor was associated with lymphatic and vascular invasion, whilst no such correlations existed in the central part of the tumor (18).

Recently, Li *et al.* (19) examined the expression of VEGF-C and lymphatic vessel density (LVD) in 40 cases of invasive micropapillary carcinoma (IMPC), a rare tumor of the human breast, with high potential to metastatise to the lymph nodes. Involvement of the axillary lymph nodes is considered to be the most important prognostic factor in breast carcinoma. Cytoplasmic expression of VEGF-C was significantly associated with higher peritumoral LVD, lymphatic invasion and number of lymph node metastases in IMPC. These findings indicated, that VEGF-C indeed promotes the proliferation of peritumoral lymphatic vessels.

Some clinical analyses have revealed that expression of VEGF-C or VEGF-D in breast, ovarian and colorectal cancer may be an independent prognostic indicator of survival and that the expression of lymphangiogenic growth factors promoted metastatic spread of tumor cells *via* the lymphatics (20). A number of studies have demonstrated an association between VEGF-C and VEGF-D expression and regional nodal metastases in colorectal, gastric and cervical carcinomas (21-23). Conflicting evidence of VEGF-C and VEGF-D expression in relation to lymph node metastasis has been reported in the literature. While Nakamura *et al.* (12, 17) and Hu *et al.* (15) reported an association Kinoshita *et al.* (14), Gunningham *et al.* (24) and Yang *et al.* (25) did not find any significant relationship. One possible explanation, as suggested by Kinoshita *et al.* (14), is that VEGF-C might be responsible for early invasive events, but might not be sufficient to produce lymph node metastasis. Another reason for the discrepancy might be due to the use of different antibodies.

It is very important as a practical medical goal to determinate whether VEGF-C and VEGF-D expression prove to be prognostic indicators in breast cancer. Nakamura *et al.* (12) have shown in their results, that VEGF-C expression was associated with both DFS and OS. However, they could not identify VEGF-C expression as an independent prognostic factor for DFS and OS. The median follow-up of their patients was 109 months (range 5.5 to 230 months). Similar data reported by Kinoshita *et al.* (14) indicated that the VEGF-C positive group had a significantly poorer DFS rate, but there was no significant correlation between the expression of VEGF-C protein and survival rates. Nakamura *et al.* (16) have also shown that the expression of VEGF-D was associated with shorter DFS and OS. Furthermore, multivariate analysis demonstrated that the VEGF-D expression was still related to poor DFS, after consideration of other prognostic factors. Yang *et al.* (25) indicated a trend for worse survival for patients with a high VEGF-C or VEGF-D expression level, but the difference was not significant. The lack of correlation between VEGF-C expression and DFS or OS in 91 patients with breast cancer using 10 year

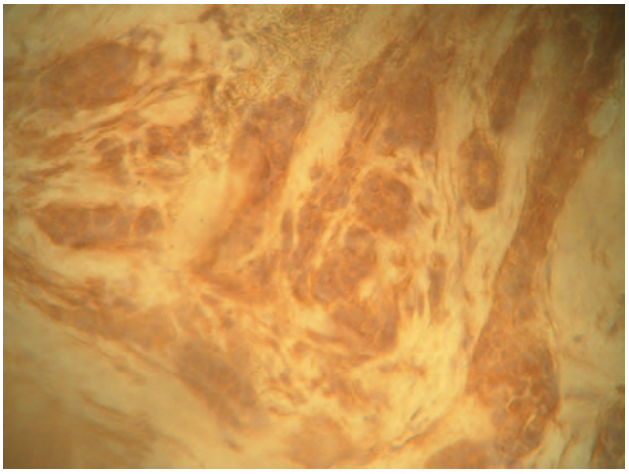

Figure 1. *Immunohistochemical staining of primary tumor tissue section with the monoclonal antibody. Expression of vascular endothelial growth factor-C (VEGF-C) in ductal carcinoma of the breast. VEGF-C is strongly expressed in the cytoplasm of most of the tumor cells.*

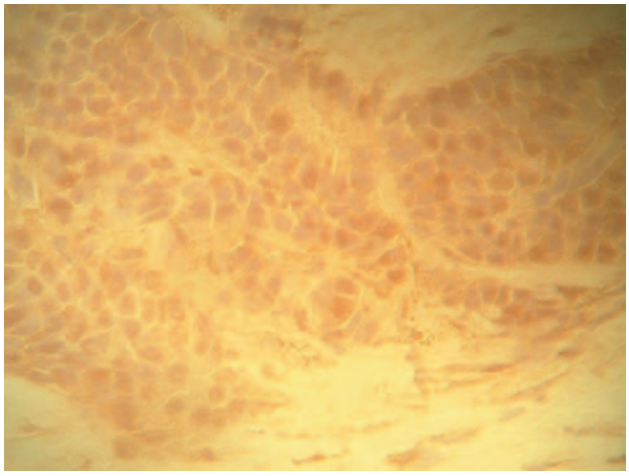

Figure 2. *Immunohistochemical staining of primary tumor tissue section with the monoclonal antibody. Expression of vascular endothelial growth factor-D (VEGF-D) in ductal carcinoma of the breast. VEGF-D is expressed in the cytoplasm of most of the tumor cells.*

Table II. *The 5- and 10-year disease-free (DFS) and overall (OS) survival rates of 98 breast ductal carcinoma cancer patients.*

Patient characteristics	Total	5-year DFS		10-year DFS		5-year OS		10-year OS	
	n	n	%	n	%	n	%	n	%
Age	98	77	78.57	62	63.27	83	84.69	70	71.43
≤50	31	27	87.10	23	74.19	28	90.32	25	80.65
>50	67	50	74.63	39	58.21	55	82.10	45	67.16
Nodal status	98	77	78.57	62	63.27	83	84.69	70	71.43
Negative	58	51	87.93	42	72.41	55	94.83	48	82.76
Positive	40	26	65.00	20	50.00	28	70.00	22	55.00
Tumor size	98	77	78.57	62	63.27	83	84.69	70	71.43
≤20 mm	12	10	83.33	9	75.00	10	83.33	10	83.33
>20 mm	86	67	77.91	53	61.63	73	84.88	60	69.77
Grade	98	77	78.57	62	63.27	83	84.69	70	71.43
II	67	54	80.60	43	64.18	58	86.57	49	73.13
III	31	23	74.19	19	61.29	25	80.65	21	67.74
Estrogen receptor	98	77	78.57	62	63.27	83	84.69	70	71.43
Negative	20	15	75.00	12	60.00	16	80.00	13	65.00
Positive	78	62	79.49	50	64.10	67	85.90	57	73.07
VEGF-C	98	77	78.57	62	63.27	83	84.69	70	71.43
0	16	12	75.00	10	62.50	13	81.25	11	68.75
1	29	22	75.86	16	55.17	24	82.76	20	68.97
2	38	29	76.32	26	68.42	31	81.58	29	76.32
3	15	14	93.33	10	66.67	15	100.00	10	66.67
VEGF-D	98	77	78.57	62	63.27	83	84.69	70	71.43
0	49	38	77.55	31	63.27	41	83.67	35	71.43
1	40	31	77.50	25	62.5	34	85.00	28	70.00
2	9	8	88.89	6	75.00	8	88.89	7	77.78
3	0	0	–	0	–	0	–	0	–

follow-up was reported by Watanabe *et al.* (26). Their findings are very similar to our data, since we did not find any statistically significant association between VEGF-C nor VEGF-D expression and DFS or OS, after 5- and 10-years follow-up. However, our group of patients was very homogenous (similar treatment, grade and type stage II carcinomas) while other authors investigated more heterogeneous groups of patients. Our study did not

Table III. *Multivariate regression analysis of survival according to VEGF-C and VEGF-D protein expression level in breast tumor tissue (p-value is shown).*

Type of marker	Expression level	5-year DFS	10-year DFS	5-year OS	10-year OS
VEGF-C	0 *vs.* 1-3	0.76	0.92	0.53	0.71
	0-1 *vs.* 2-3	0.60	0.70	0.65	0.66
	0-2 *vs.* 3	0.12	0.85	0.084	0.84
VEGF-D	0 *vs.* 1-2	0.93	0.54	0.79	0.98
	0-1 *vs.* 2	0.40	0.90	0.74	0.66

reveal any significant relationship between the level of VEGF-C or VEGF-D expression and the clinical properties of breast cancer. Considering the fact, that the relatively high VEGF-C expression was observed in breast cancer tumor tissue of more than 80 percent (83.7%) of the 98 specimens examined, it could be hypothesized that the increased VEGF-C expression level is an early event during development of this tumor. The confirmation of this hypothesis was given recently by Bando *et al.* (27) who examined 193 cases of primary breast carcinoma and found that the highest levels of VEGF-C protein were present in low-grade, small size tumors. Their multivariate analysis indicated an independent prognostic value of VEGF-C.

In conclusion, our study did not reveal any prognostic value of VEGF-C or VEGF-D expression in a group of patients with stage II, G2 or G3 ductal breast carcinoma and these proteins are not useful for separating a subgroup of patients with a poor prognosis who could benefit from more aggressive treatment. Accordingly, further studies, on other selected, homogenous populations, with different tumor parameters (probably very early stage breast cancers would have more promising results) are necessary in order to reveal any prognostically significant molecules.

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