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Vascular endothelial growth factor C and D expression correlates with lymph node metastasis and poor prognosis in patients with resected esophageal cancer

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Vascular endothelial growth factors C (VEGF-C) and D (VEGF-D) are important lymphangiogenic factors in human cancers. We studied the expression of VEGF-C and VEGF-D using immunohistochemistry in 73 resected esophageal cancer specimens, and correlated the results with patient clinicopathologic features and survival.

High expression of VEGF-C was identified in 40 (54.7%) patients, and it correlated positively with histological grade (p=0.038), tumor stage (p=0.01), depth of tumor invasion (p=0.036) and lymph node metastasis (p=0.001). In 48 of 73 (65.7%) tumors, the VEGF-D protein was also expressed at high levels. VEGF-D immunoreactivity significantly correlated with tumor location (p=0.027), size of tumor (p=0.015), histological grade (p=0.02), depth of invasion (p=0.001) and lymph node metastasis (p=0.018). In logistic multivariate analysis, high expression of VEGF-C (OR 1.941, 95% CI 1.263-7.289, p=0.024) was associated with lymph node metastasis. Calculating the prognostic relevance revealed that both VEGF-C and VEGF-D correlated with decreased overall survival (p=0.01, p=0.003), disease free survival (p=0.02, p=0.006), and cancer-specific survival (p=0.03, p=0.005).

In conclusion, our results suggest that high levels of both VEGF-C and VEGF-D proteins are associated with lymph node involvement, and that VEGF-C expression is an independent predictor of risk for lymph node metastasis in esophageal cancer. In locally advanced disease, overexpression of VEGF-C and VEGF-D may be useful in identifying patients who are more likely to have a poor prognosis even after curative resection.

Key words: esophageal cancer, VEGF-C; VEGF-D, lymph node metastasis, prognosis.

The lymphatic system serves as the primary pathway for metastasis in most human cancers, and the extent of lymph node involvement in esophageal cancer is a crucial prognostic factor for patient outcome [1]. Most patients with esophageal cancer are diagnosed in advanced stages of the disease, with subsequent poor outcome, commonly coupled with lymphatic dissemination and distant metastasis [2]. The 5-year survival rate for patients with resected esophageal cancer with lymph node metastasis is worse than those without lymph nodes metastasis [3].

During the past several years, tumor-induced lymphangiogenesis, as it is driven by lymphangiogenic growth factors, has been firmly established as a novel mechanism for cancer progression. Vascular endothelial growth factors (VEGF-A, -B,-C,-D and –E) and their receptors (VEGFR-1, VEGFR-2 and VEGFR-3) play an important role in the formation of the vascular network [4]. Among them, VEGF-C and VEGF-D are structurally closely related to one another, and are considered to be lymphangiogenic and angiogenic in both tissues and tumors, as they activate the endothelial cell surface receptors – tyrosine kinase, and VEGF receptors VEGFR-2 and VEGFR-3 [5,6]. Vascular endothelial growth factor C (VEGF-C) was initially identified to be a factor stimulating tyrosine kinase receptor Flt4 (VEGFR-3), which was purified from PC-3 prostatic adenocarcinoma cells [7]. The VEGF-C gene is localized on chromosome 4q34 and has a high degree of homology to VEGF-A [8,9]. The open reading frame of VEGF-C c-DNA encodes a protein of 419 amino acid residues, with a predicted molecular mass of 46.9kDa. Its mRNA is 2.4 and 2.0 kb, which is expressed in human adult tissues [7,9]. The expression and serum levels of VEGF-C were also detected in several types of malignant tumors, and found to positively correlate with lymphatic involvement [10-12]. Stacker et al. [13] subsequently demonstrated in a mouse model that lymphatic spread was also associated with the expression of (VEGF-D) by the tumor. Kopfstein et al. [14] further showed in a transgenic mouse model that VEGF-D, as it was expressed by the tumor cells,

induced tumor lymphangiogenesis, lymph node metastasis and promoted metastasis to the lungs. Moreover, in a variety of human cancers, including gastric, pancreatic, lung, colorectal, breast and ovary, elevated tumoral VEGF-C and VEGF-D expression correlates with an increased incidence of regional lymph node metastasis and poor prognosis [15-20].

In the present study, we evaluated the expression levels of VEGF-C and VEGF-D using immunohistochemistry to clarify their significance in relation to nodal metastasis and prognosis in esophageal cancer patients.

Materials and methods

Patients and tissues. Tumor specimens were obtained from 73 patients with primary esophageal cancer who underwent an esophagectomy at the Department of Thoracic Surgery, Medical University of Bialystok. None of them had received preoperational chemotherapy or radiotherapy treatment. The study population consisted of 59 men (80.8%) and 14 women (19.2%). The average age at the time of diagnosis was 64.0 years (ranged from 42 to 78 years). The histological stage was based on UICC TNM classification [21]. Other clinical features are summarized in Table 1. All patients were followed up clinically after surgery. None of the patients in this series suffered major perioperative complications, and all were discharged from the hospital. There was 100% follow-up on all patients after their discharge. They were evaluated every 3-6 months by means of clinical history, physical examination, laboratory analysis, barium esophagram, computed tomography, ultrasound examination of the neck and abdomen, fiberoptic esophagoscopy if necessary, and PET-CT over the last two years. The average follow-up time was 25 months (ranged from 3 to 101 months). Survival analysis was performed, including overall survival, disease free survival, survival of distant recurrence and cancer-specific survival. Overall survival, disease free survival, survival of distant recurrence and cancer-specific survival were all calculated in several intervals, starting from the date of the surgery itself to the last contact made with all living patients, followed by the date of the last follow-up for disease free patients, to the date of the last follow-up for distant recurrence-free patients, and to the date of esophageal–cancer-induced death, respectively. Patients who died from causes unrelated to the esophageal carcinoma, with no evidence of the disease, were death-censored. The sites of recurrent tumors were documented in 45 patients, as they were determined by clinical and/or radiographic procedures. None were identified at autopsy or by reoperation of asymptomatic patients. Recurrence categories were subsequently expressed as the first site of recurrence rather than the cumulative (total) incidence of recurrence. Local recurrence was defined as the reappearance of the cancer in the tumor bed and/or at the site of anastomosis. There were 2 patients who were found with combined local and distant recurrence on the first re-examination and the recurrence site was categorized as distant or local. Sixteen patients had mediastinal, neck and abdomen lymph node metastasis, 10 pulmonary metastasis, 7 liver metastasis, and 5 a combination of pulmonary and liver metastasis. Three cases dealt with a relapse at the site of anastomosis, and 4 found recurrence in the tumor bed itself. In total, thirty six patients died of cancer. In total, thirty nine patients received chemotherapy, radiotherapy, or both postoperatively and during follow-up. Normal esophageal tissues were collected as control specimens.

Table 1. Characteristics of esophageal cancer patients.

Parameters		Number of patients
Esophageal cancer patients		73
Age	<64 years	25
	>=64 years	48
Sex	Female (F)	14
	Male (M)	59
Location	Upper (U)	4
	Midthoracic (M)	27
	Lower (L)	42
Tumor size	<4cm	35
	>=4cm	38
Histological type	Squamous cell carcinoma	34
	Adenocarcinoma	39
Histological grade	Well-G1	8
	Moderate -G2	31
	Poor-G3	34
Tumor depth	T1	6
	T2	15
	T3	47
	T4	5
Stage	I	6
	IIA	21
	IIB	5
	III	41
Lymph node metastasis	N0	24
	N1	49
Residual tumor	R0	59
	R1	11
	R2	3

Analysis of protein expression by immunohistochemistry (IHC). The surgical specimens were fixed in a 10% buffered formalin solution for 24 h, embedded in paraffin and handled in the Department of Pathology, at the Medical University of Bialystok for further processing. The tissue samples were obtained from the peripheral invasion front of the tumor. Immunohistochemistry was then performed using the avidin-biotin-peroxidase complex technique (ABC-technique). 4μm-thick paraffin-embedded slides were cut from each study block. For antigen retrieval, the slides were heated in a microwave oven containing 0.01 mmol/L sodium citrate (pH 6.0). The sections were treated with 0.3 % H_2O_2 for 10 min at room temperature. The slides were incubated for one hour at room temperature in a humidity tray with primary antibodies, VEGF-C (goat monoclonal antibody, 1:7, R&D Systems), and VEGF-D (mouse monoclonal, 1:50, R&D Systems), respectively. Slides were rinsed twice in 0.1mmol/L PBS (pH ~ 7.4) for 5 min, and incubated for 30 min at room temperature with an anti-goat biotinylated secondary

antibody (Vectastain ABC Kit,Vector), and an anti-mouse biotinylated secondary antibody (Peroxidase Detection System, Novocastra) to identify the target. The sections were stained by 3' 3-diaminobenzidine (DAB) to visualize the antigen-antibody complex. The nuclei were then stained with Mayer hematoxylin. Positive controls were made using tissue samples, proposed by the antibody manufacturer, which showed a high expression of the proteins. Negative controls were made with the same tissue without the antibody.

Evaluation of immunoreactivity. The results of the immunohistochemical staining were evaluated by a pathologist blinded to all clinical data. The staining intensity was graded on a scale of 0 to 3 (0-negative; 1- weak; 2-moderate; 3-strong). The quantity of positive tumor cells was scored as: (+) >10% of the neoplastic cells were stained; (+/-) <10% of the neoplastic cells were stained; (-) the neoplastic cells were not completely stained. In this study for statistical analysis, 0, 1, (-) and (+/-) were classified as low expression tumors, and 2, 3 and (+) as high expression tumors, respectively.

Statistical analysis. Distribution was analyzed by the Shapiro-Wilk test. Categorical data was compared by using χ^2 or Fishers' exact probability test. Logistic regression analysis was further utilized to identify the univariable predictors of lymph node disease. Variables that were significant in the univariable analysis at $p<0.05$, and that made clinical sense to include in a model that predicted lymph node involvement, were considered in a stepwise logistic regression model. The results of the final multivariable model are summarized as the p value, odds ratio, and 95% confidence interval for the odds ratio. The Kaplan-Meier method was then used to estimate the probability of survival as a function of time. The differences in survival of the differing subgroups of patients were compared using the log-rank test. The prognostic value of VEGF-C and VEGF-D was examined in univariate and multivariate analysis, using Cox's proportional hazard model. All p values were based on a two-tailed statistical analysis, and a p value of less than 0.05 was considered significant. Statistical analyses were carried out using the Statistica 8.0 PL program (StatSoft Inc., Tulsa, OK, USA) and the GraphPad Prism 5.01 program (GraphPad Software, San Diego, CA, USA).

In accordance with the Declaration of Helsinki, the study protocol was approved by the local Ethics Committee (No R-1-002/184/2008) and written informed consent was obtained from all participants prior to analysis.

Table 2. Comparison between protein expression of VEGF-C and VEGF-D with clinicopathologic features for patients undergoing esophagectomy for esophageal cancer.

Parameters	VEGF-C expression		P	VEGF-D expression		P
	Low	High		Low	High	
Overall	33(45.3%)	40(54.7%)		25(34.3%)	48(65.7%)	
Age						
<64	12	13	0.806[a]	11	14	0.298[a]
>=64	21	27		14	34	
Sex						
F	7	7	0.769[a]	7	7	0.213[a]
M	26	33		18	41	
Location						
U	1	3	0.764[b]	2	2	0.027[b]
M	12	15		14	13	
L	20	22		9	33	
Tumor size						
<4cm	18	17	0.352[a]	17	18	0.015[a]
>=4cm	15	23		6	30	
Histological grade						
G1	7	1	0.038[b]	6	2	0.02[b]
G2	12	19		7	24	
G3	14	20		12	22	
Stage						
I+II	20	12	0.01[a]	15	17	0.05[a]
III	13	28		10	31	
T1+T2	14	7	0.036[a]	14	7	0.001[a]
T3+T4	19	33		11	41	
N0	18	6	0.001[a]	13	11	0.018[a]
N1	15	34		12	37	
R0	28	31	0.554[a]	20	39	0.998[a]
R1+R2	5	9		6	8	

[a] Fisher's exact test, [b] χ^2 test

a) b)

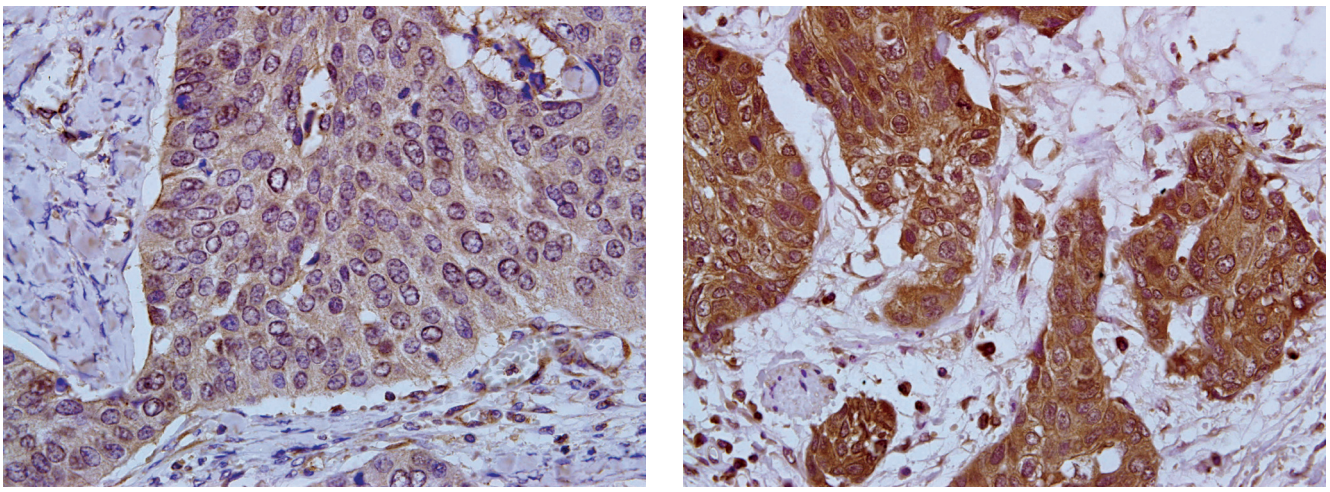

Figure 1. Immunohistochemical study of VEGF-C protein expression in esophageal carcinoma tissue. Fig.1a. Squamous cell carcinoma, low expression. Fig. 1b. Squamous cell carcinoma, high expression. Magnification x400.

a) b)

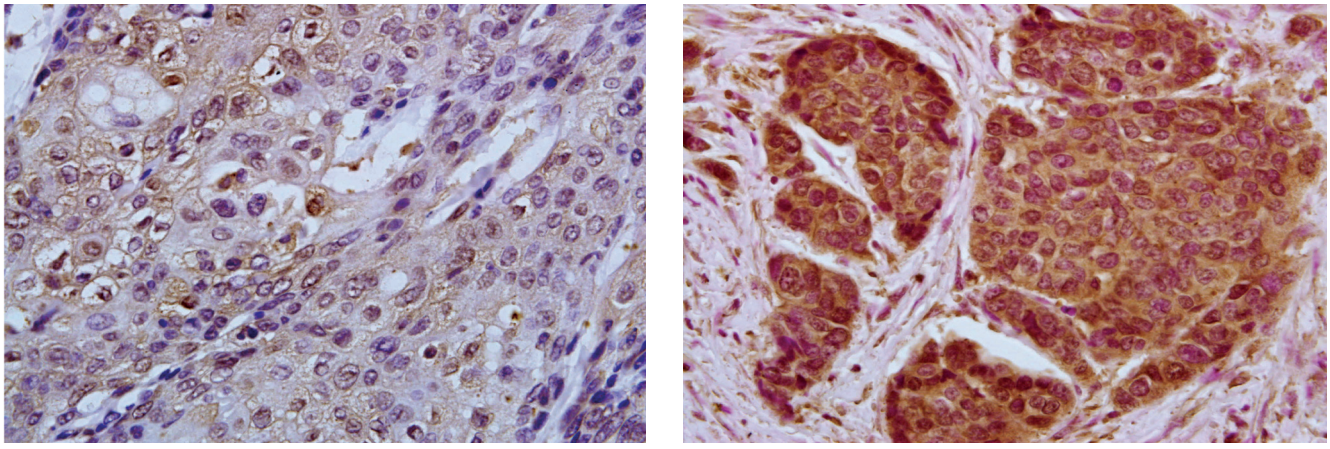

Figure 2. Immunohistochemical study of VEGF-D protein expression in esophageal carcinoma tissue. Fig.2a. Squamous cell carcinoma, low expression. Fig.2b. Squamous cell carcinoma, high expression. Magnification x400.

Results

Among the 73 examined tumors, 67 showed heterogeneous intensity of VEGF-C and 69 showed heterogeneous intensity of the VEGF-D protein expression in the cytoplasm and predominately at the invasive edge. No expression was observed in the nucleus area however. In stromal tissue, vessels, some macrophages and some esophageal glands exhibited weak and moderate expression of VEGF-C and VEGF-D, respectively. In 6 of the 73 esophageal cancer specimens, VEGF-C was not expressed at all. In contrast, normal and dysplastic esophageal epithelium cells exhibited a lack of, or only faint cytoplasmic staining of both VEGF-C and VEGF-D. In 4 of the 73 tumors, VEGF-D was not expressed [Fig. 1a,b, Fig. 2a,b].

The relationship between VEGF-C and VEGF-D expression and clinicopathological features is presented in Table 2. High expression of VEGF-C was observed in 40 (54.7%) patients. Expression of VEGF-C correlated positively with the histological grade (p=0.038), tumor stage (p=0.01), depth of tumor invasion (p=0.036) and lymph node metastasis (p=0.001). No association was seen with age, sex, location, tumor size, and residual tumor. In 48 of the 73 (65.7%) tumors, VEGF-D protein was also expressed

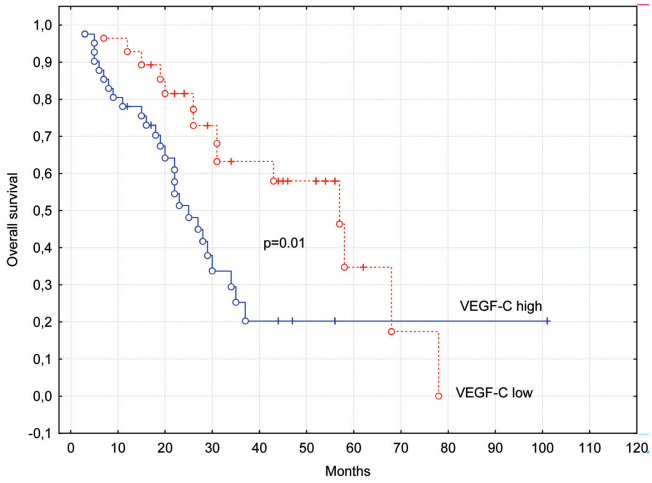

Figure 3. Kaplan-Meier analysis of overall survival according to VEGF-C expression in patients with esophageal cancer.

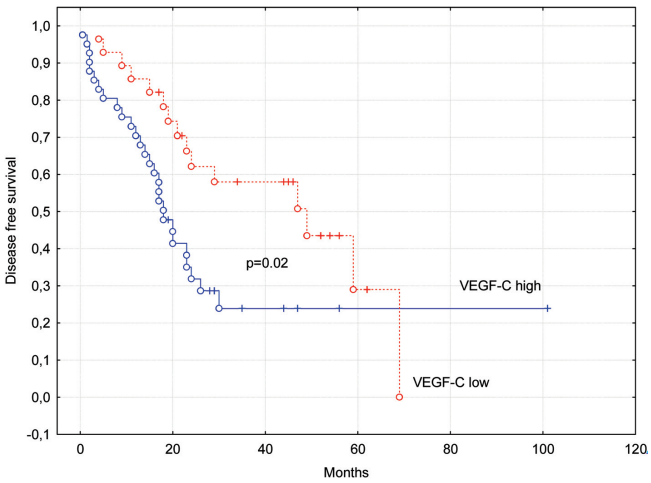

Figure 4. Kaplan-Meier analysis of disease free survival according to VEGF-C expression in patients with esophageal cancer.

at high levels. VEGF-D immunoreactivity in the cancer cells was further found to significantly correlate with tumor location (p=0.027), the size of the tumor (p=0.015), histological grade (p=0.02), depth of invasion (p=0.001) and lymph node metastasis (p=0.018). However, there was no correlation between VEGF-D overexpression and age, sex, stage, and residual tumor.

To investigate the risk factors for lymph node metastasis, univariate and multivariate regression analyses were conducted to include tumor size, histological grade, depth of invasion, residual tumor, VEGF-C expression and VEGF-D expression. Logistic univariate analysis revealed that VEGF-C expression, depth of invasion, tumor size, VEGF-D expression, residual tumor and histological grade were all related to lymph node metastasis. Among these factors, VEGF-C expression (p=0.024), increasing depth of tumor invasion (p=0.012) and increasing tumor size (p=0.005) were further deemed as significant independent risk factors for lymph node metastasis, when used in logistic multivariate analysis. Other factors were not statistically predictive for lymph node metastasis (Table 3).

Univariate survival analysis revealed that high expression of VEGF-C was associated with poor overall survival

Table 3. Logistic regression analysis in relation to lymph node metastasis.

Factors	Univariate P	Multivariate		
		Odds ratio	95% CI	P
Tumor size	0.001	1.722	0.864-6.934	0.005
Histological grade	0.048	0.735	0.324-1.672	0.342
Depth of invasion	<0.001	1.842	1.242-8.212	0.012
Residual tumor	0.036	0.894	0.594-3.435	0.136
VEGF-C expression	<0.001	1.941	1.263-7.289	0.024
VEGF-D expression	0.007	0.845	0.520-2.841	0.426

CI – confidence interval

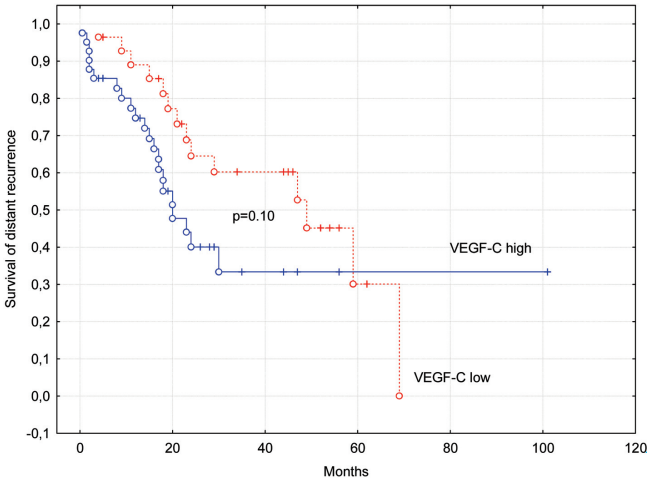

Figure 5. Kaplan-Meier analysis of survival of distant recurrence according to VEGF-C expression in patients with esophageal cancer.

(Fig. 3, p=0.01), disease free survival (Fig. 4, p=0.02), and cancer-specific survival (Fig. 6, p=0.03). Similarly, VEGF-D immunoreactivity in the cancer cells significantly correlated with poor overall survival (Fig. 7, p=0.003), disease free survival (Fig. 8, p=0.006), survival of distant recurrence (Fig. 9, p=0.02) and cancer-specific survival (Fig. 10, p=0.005). No association was found between VEGF-C expression and survival of distant recurrence (Fig. 5, p=0.1).

Discussion

In esophageal cancer, the presence of metastases in regional lymph nodes is a known predictor of poor prognosis, even

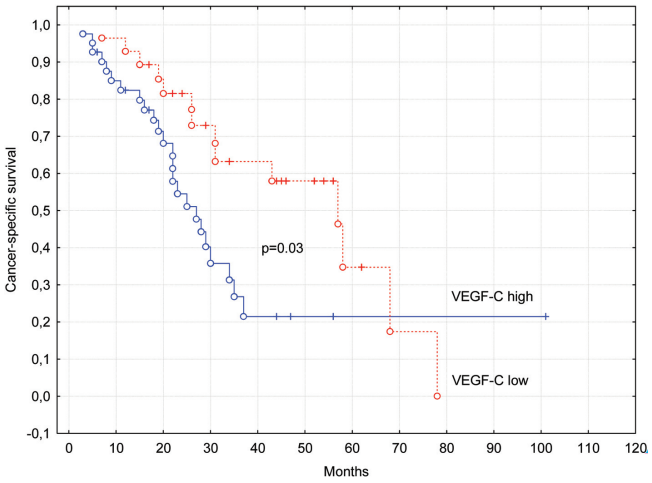

Figure 6. Kaplan-Meier analysis of cancer specific survival according to VEGF-C expression in patients with esophageal cancer.

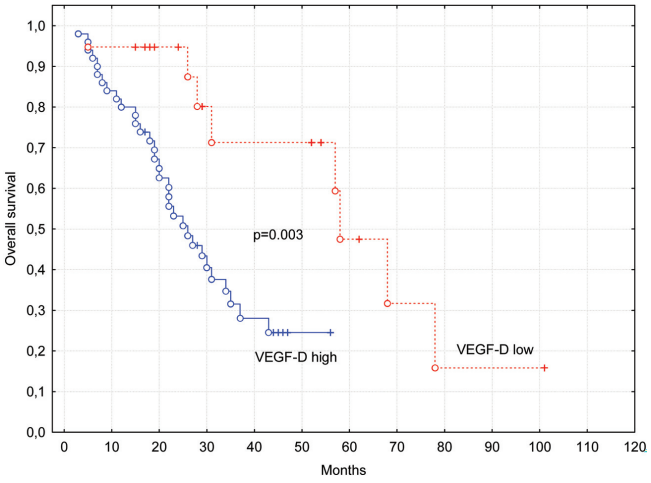

Figure 7. Kaplan-Meier analysis of overall survival according to VEGF-D expression in patients with esophageal cancer.

when there is no evidence of systemic metastases. The overall 5-year survival rate of patients with lymph node metastasis after surgical resection is only about 20% [22], and most of these patients die from tumor recurrence [23]. Despite new imaging techniques like computed tomography (CT) and positron emission tomography (PET), the sensitivity and specificity of clinical evaluation of lymph node involvement remains unsatisfactory [24].

Most of the cancers spread primarily by means of lymphatic vessels. In esophageal cancer, recent work in tumor lymphangiogenesis research has been focused on the VEGF family, especially VEGF-C [25-27], leaving limited reports on VEGF-D [11,28]. Employing an animal model, VEGF-C and VEGF-D have been shown to induce lymphangiogenesis that is associated with lymph node metastasis [13,29].

In the current study, high expression of VEGF-C was observed in 54.7% of the esophageal cancer patients. Our findings are in accordance with the findings of Okazawa et al. [30], Kimura et al.[31] and Liu et al. [27], who showed by immunohistochemistry that esophageal tumors express VEGF-C. Previous studies have demonstrated that increased expression of VEGF-C in primary tumors correlates with increased dissemination of tumor cells to regional lymph nodes, in a variety of human carcinomas, including esophageal cancer [10,11]. We found that high VEGF-C expression correlated with the factors of tumor progression, like depth of tumor invasion, pathological stage, lymph node metastasis and histological grade. These results are mostly consistent with those reported by Kitadai et al. [25], Kimura et al. [31] and Matsumoto et al. [32]. In contrast, Noguchi et al. [33] failed to show a positive correlation of VEGF-C expression with pathological stage, depth of tumor invasion and lymph node metastasis.

Several reports demonstrate that the presence of the VEGF-D protein is also associated with lymph node involvement in colorectal, breast and ovarian cancer [18-20]. In the present study, high expression of VEGF-D was found in 65.7% of the tumors, correlating significantly with tumor location, size, histological grade, depth of invasion and lymph node metastasis. Our findings are thus in agreement with those obtained by Jiang et al. [34] and Tzao et al. [28], who showed a significant correlation of VEGF-D tumor overexpression with lymph node metastasis and the pathological stage of esophageal cancer.

We demonstrated that VEGF-C expression, VEGF-D expression, depth of invasion, tumor size, residual tumor and histological grade were related to lymph node metastasis in univariate regression analysis. Multivariate logistic regression analysis also revealed the presence of the VEGF-C protein, increasing depth of tumor invasion and increasing tumor size to be independent factors influencing lymph metastasis. These findings suggest that VEGF-C and VEGF-D were associated more significantly with lymphatic rather than hematogenous metastasis, indicating their possible usefulness in predicting the nodal status of esophageal cancer patients.

According to survival analysis in the present series, poor overall survival, disease free survival, and cancer-specific survival were all positively correlated with high expression of VEGF-C in the esophageal cancer tissue. As shown in Figures 7, 8, 9 and 10, the overall survival, disease free survival, survival of distant recurrence, and cancer-specific survival of patients with high expression of VEGF-D tumor were discovered to be significantly shorter than that with low expression of VEGF-D tumor. However, in a multivariate analysis, the overexpression of VEGF-C and VEGF-D was not found to be a significant independent prognostic factor (data not show). Kimura et al. [31] found that the prognosis of patients with VEGF-C positive tumors was significantly worse than that of patients with VEGF-C negative tumors. However, VEGF-C was not an

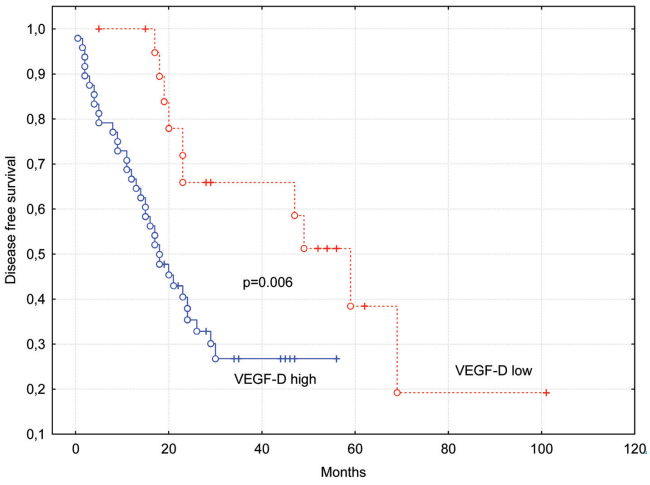

Figure 8. Kaplan-Meier analysis of disease free survival according to VEGF-D expression in patients with esophageal cancer.

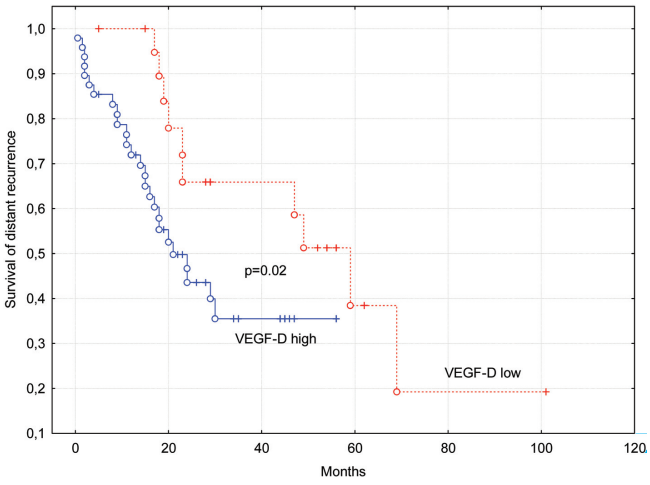

Figure 9. Kaplan-Meier analysis of survival of distant recurrence according to VEGF-D expression in patients with esophageal cancer.

independent prognostic factor in their study, possibly because most of their patients had advanced esophageal cancer with poor prognosis, just as in our series. Okazawa et al. [30] showed a significant difference in the survival rates between VEGF-C positive overexpression and negative overexpression groups of their esophageal cancer patients. In their study, multivariate analysis indicated that gender, age, VEGF-C expression and lymphatic invasion were all prognostic determinants. Tzao et al. [28] demonstrated that positive tumor overexpresion of VEGF-D was a strong independent survival predictor in resected squamous cell esophageal cancer. Our results were in general agreement with the results of a study that compared the prognostic significance of VEGF-C and VEGF-D expression in patients with colorectal cancer, showing that both of these two factors correlated positively with tumor invasion and nodal metastasis, but inversely with overall survival [18]. These results suggest that the prognostic impact of VEGF-C and VEGF-D expression may vary in different types of human cancer [15,18,20].

The present results suggest that VEGF-C and VEGF-D, as they are secreted by esophageal cancer, may be able to induce and mediate both the spread of cancer cells beyond the primary tumor, as well as the formation of a metastatic focus in the lymph nodes. These results also indicate that VEGF-C and VEGF-D protein may play a stimulatory role in the dissemination of tumor cells to the lymph nodes.

We conclude, that high levels of both VEGF-C and VEGF-D proteins are associated with lymph node involvement, and that VEGF-C expression is an independent predictor of the risk of lymph node metastasis in esophageal cancer. In locally advanced disease, both VEGF-C and VEGF-D overexpression may be useful as a possible indicator of poor prognosis in patients, that are undergoing potentially curative esophagectomy.

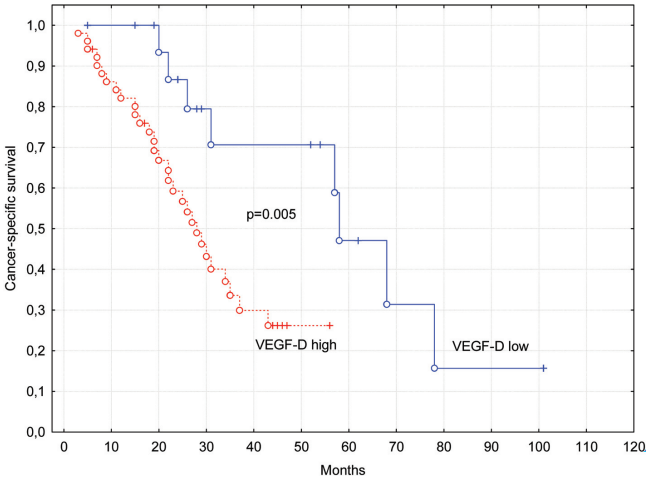

Figure 10. Kaplan-Meier analysis of cancer specific survival according to VEGF-D expression in patients with esophageal cancer.

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