Prognostic value of vascular endothelial growth factor-C and podoplanin mRNA expression in esophageal cancer

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Abstract. Vascular endothelial growth factor-C (VEGF-C), VEGF-D, VEGF receptor-3 (VEGFR-3) and podoplanin (PDPN) are involved in the spread of cancer. The current study evaluated VEGF-C, VEGF-D, VEGFR-3 and PDPN mRNA expression levels in 84 esophageal cancer samples from patients who had undergone surgery according to reverse transcription-quantitative polymerase chain reaction, and correlated the results with the clinicopathological features. The effects on lymph node metastasis and survival were identified by performing univariate and multivariate analyses. VEGF-C, PDPN, VEGF-D and VEGFR-3 were overexpressed in 52.4, 52.4, 32.1 and 51.2% of esophageal cancer samples, respectively. Furthermore, the expression of VEGF-C and PDPN was significantly correlated with lymph node metastasis, depth of tumor invasion and tumor stage ($P<0.05$). Logistic regression analysis identified tumor size ($P=0.001$), depth of invasion ($P=0.002$) and PDPN mRNA expression ($P=0.022$) as significant multivariable predictors of regional lymph node metastasis. Upon univariate survival analysis, the depth of tumor invasion, lymph node metastasis, histological grade, tumor stage, tumor size, residual tumor, and VEGF-C and PDPN mRNA expression were identified to be significant independent prognostic factors for overall survival (OS) time. Additionally, multivariate analysis identified tumor size ($P=0.049$), residual tumor ($P<0.001$) and PDPN mRNA expression ($P=0.02$) as independent factors for poor OS time. Thus, it was concluded that PDPN mRNA expression may serve as predictor for regional lymph node metastasis, and that VEGF-C and PDPN may be prognostic factors in patients with resected esophageal cancer.

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Introduction

Esophageal carcinoma is the eighth most frequently diagnosed cancer and the sixth leading cause of cancer mortality worldwide, with an estimated 482,000 new cases and 407,000 mortalities in 2008. Patients with esophageal carcinoma have a poorer prognosis in comparison to patients exhibiting any other type of gastrointestinal tumor (1). Lymph node involvement is an important prognostic factor for survival in patients with esophageal carcinoma (2). Despite significant improvements in the diagnosis and available therapeutic strategies for the disease, survival rates remain low. For example, the 5-year survival rate of patients exhibiting esophageal carcinoma with lymph node metastasis who have undergone an esophagectomy and three-field lymphadenectomy is only 15-39% (3).

Five members of the vascular endothelial growth factor (VEGF) family (VEGF-A, VEGF-C, VEGF-D, VEGF-E and placental growth factor) and their receptors [VEGF receptor (VEGFR)-1, VEGF-2 and VEGF-3] are important in the formation of the vascular network (4). VEGF-C and VEGF-D have been characterized as lymphangiogenic and angiogenic growth factors, and have been demonstrated to signal through the receptors VEGFR-2 and VEGFR-3 in various physiological and pathological processes (5). A mouse model study demonstrated that lymphatic spread and lymphangiogenesis are associated with the expression of VEGF-D or VEGF-C by the tumor cells (6). Furthermore, VEGF-C and VEGF-D appear to be involved in the origin and/or progression of lymphangiogenesis in various different types of cancer, including gastric and esophageal cancer, with overexpression correlated with nodal metastasis and patient survival (7,8).

Podoplanin (PDPN) is 43-kDa mucin-type transmembrane glycoprotein that is expressed in lymphatic endothelial cells, but not in blood endothelial cells (9). PDPN has previously been used to assess lymphatic vessel density and invasion in various types of cancer, including esophageal carcinoma (10,11). Thus, it may act as a mediator of tumor cell invasion and metastasis (12).

The present study evaluated the association between VEGF-C VEGF-D, VEGFR-3 and PDPN mRNA expression

levels, and the clinicopathological factors and survival of patients with esophageal carcinoma.

Materials and methods

Patients and tissues. Tumor specimens were obtained from 84 patients with primary esophageal cancer who underwent an esophagectomy at the Department of Thoracic Surgery, Medical University of Białystok (Białystok, Poland). No patients had received pre-operative chemotherapy or radiotherapy. The study population consisted of 76 men (90.5%) and 8 women (9.5%), and the mean age at the time of diagnosis was 63 years (range, 42-82 years). Pathological stage was determined using the seventh edition of the American Joint Committee on Cancer tumor-node-metastasis classification system (13). Following surgery, all patients underwent clinical follow-up evaluations every 3-6 months, including a clinical history, physical examination, laboratory analysis, fiberoptic esophagoscopy, ultrasound examination of the neck and abdomen, barium esophagram, computed tomography (CT) scan, endoscopic ultrasound, positron emission tomography-CT scan, and endobronchial ultrasound if necessary. The mean follow-up time was 25 months (range, 3-101 months). Survival analysis was performed at the termination of follow-up, including an overall survival (OS) analysis. Non-malignant esophageal tissue samples were collected from the same patients at a distance of 3-5 cm from the tumor (3-8 samples, per patient).

The present study was conducted in accordance with the Declaration of Helsinki, the study protocol was approved by the local Ethics Committee (approval no. R-1-002/28/2010) and written informed consent was obtained from all participants prior to analysis.

RNA extraction and complementary (c)DNA synthesis. Tissue samples were collected intraoperatively. Following macroscopic visual assessment, the samples of tumor tissue and non-malignant esophageal tissue were frozen in liquid nitrogen and stored at -80˚C. Sections (4 μm) of frozen tissue specimens were cut and stained with hematoxylin and eosin (Cryotome™ FSE cryostat; Thermo Fisher Scientific, Inc., Hemel Hempstead, UK). The presence of carcinoma cells was confirmed by experienced pathologists. Only tumor samples composed of ≥50% tumor cells upon microscopic analysis were used for subsequent processing.

Total RNA was isolated and purified from the tissue specimens using a mirVana™ miRNA Isolation kit (Ambion Life Technologies, Austin, TX, USA), according to the manufacturer's instructions. The resulting RNA extracts were stored at -80˚C until required. RNA quantity was assessed using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). RNA quality, including 28S/18S ratio and RNA integrity number, was measured using the 2100 Bioanalyzer (serial no. DE72905449) and an RNA 6000 Nano Assay kit (Agilent Technologies Inc., Santa Clara, CA, USA), according the manufacturer's instructions. Total RNA (1 μg) was transcribed into cDNA using High Capacity RNA-to-cDNA Master Mix with No-RT Control (Applied Biosystems Life Technologies, Foster City, CA, USA) in a Labcycler (model no. 1120240193; Sensoquest GmbH, Göttingen, Germany), according to the manufacturers' instructions.

Determining mRNA expression levels. The mRNA expression levels of VEGF-C, VEGF-D, VEGFR-3 and PDPN were evaluated in the tumor and paired non-malignant esophageal tissues by performing comparative reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using commercially available TaqMan® Gene Expression assays (Applied Biosystems Life Technologies) (Table I). Amplification was performed in a 20-μl reaction mixture containing 10 μl TaqMan Gene Expression Master Mix (Applied Biosystems Life Technologies), 1 μl appropriate TaqMan Gene Expression assay solution and 2 μl cDNA solution. The PCR cycle conditions were as follows: 50˚C for 2 min and a hold at 95˚C for 10 min, followed by 40 cycles of 95˚C at 15 sec and 60˚C for 1 min. Each sample was analyzed in triplicate. The reaction was conducted on an ABI PRISM® 7900HT Sequence Detection System (SDS; Applied Biosystems Life Technologies) equipped with SDS software (version 2.4) for performing baseline and cycle threshold (Ct) calculations. Gene transcript expression levels were quantified as Ct values normalized to a reference control gene (18S rRNA), using the following equation: $\Delta Ct = Ct_{gene} - Ct_{ref}$. Gene expression levels were inversely proportional to the ΔCt values and were based on a $\log_2$ scale. The reaction mixture and cycle conditions for 18S rRNA cDNA amplification were the same as those described for VEGF-C, VEGF-D, VEGFR-3 and PDPN cDNA amplification.

Tumor-associated fold-change (FC) in mRNA expression level was calculated using the following equation: $FC = 2^{-\Delta\Delta Ct}$, where ΔCt equals the difference between the normalized expression of the gene in the tumor samples ($Ct_{gene\ T}$) and its normalized expression in the corresponding non-malignant esophageal tissue ($Ct_{gene\ N}$) (14). Logarithmically transformed FC values [$\log_2$(FC)] were used for statistical analysis. A $\log_2$(FC) value of 1.0 was used as the threshold to categorize samples into low [$\log_2$(FC)<1.0] and high [$\log_2$(FC)>1.0] gene expression groups.

Statistical analysis. Due to asymmetrical data distribution (as determined by Shapiro-Wilk tests), non-parametric tests were used for all statistical analyses. Categorical data were compared using the χ^2 or Fisher's exact probability test. Logistic regression analysis was performed to identify univariable predictors of lymph node metastasis. Significant univariable predictors (and those that were clinically appropriate for inclusion in a model to predict lymph node involvement) were considered in a stepwise logistic regression model. OS times were calculated from the date of surgery to the date of mortality or the most recent follow-up. The Kaplan-Meier method was applied to estimate the probability of survival as a function of time. Differences in the survival of the subgroups of patients were compared using the log-rank test. In addition, the prognostic value of lymphatic vessel invasion was examined by performing univariate and multivariate Cox's proportional hazard models. Statistical analyses were performed using the Statistica (version 10.0; StatSoft Inc., Tulsa, OK, USA) and Stata/IC (version 12.1; StataCorp LP, College Station, TX, USA) software. $P<0.05$ was considered to indicate a statistically significant difference.

Results

In the cancerous tissues, a high level of mRNA expression for VEGF-C was observed in 44 patients (52.4%), for PDPN in

Table I. Assays analyzed in the present study.

Gene symbol	Official gene product name	Gene ID[a]	Assay ID[b]
VEGF-C	Vascular endothelial growth factor C	12682	HS01099203_m1
VEGF-D	Vascular endothelial growth factor D	3708	Hs01047677_m1
PDPN	Podoplanin	29602	Hs00366766_m1
VEGFR-3	Vascular endothelial growth factor receptor 3	3767	Hs01128659_m1

According to the [a]HUGO gene nomenclature committee and [b]Applied Biosystems Life Technologies. VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; PDPN, podoplanin.

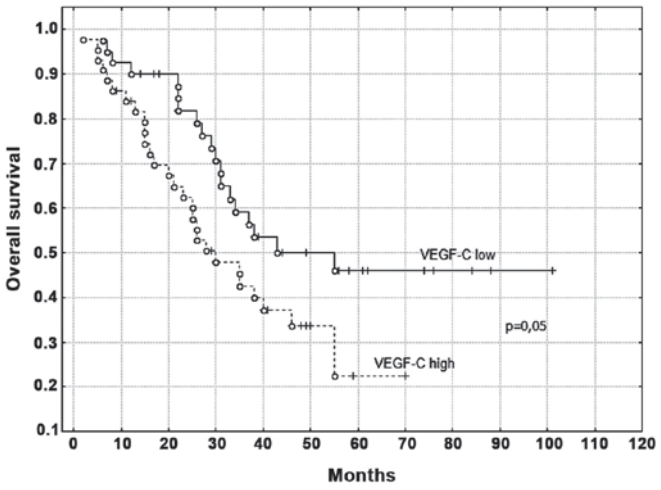

Figure 1. Kaplan-Meier analysis of overall survival according to VEGF-C mRNA expression in patients with esophageal cancer. VEGF-C, vascular endothelial growth factor-C.

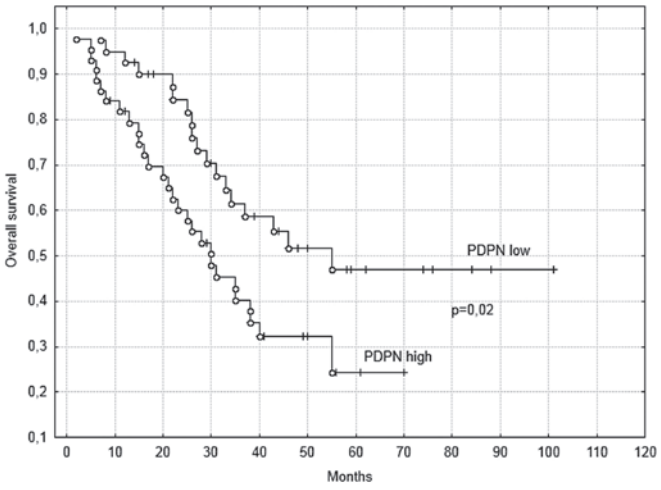

Figure 2. Kaplan-Meier analysis of overall survival according to PDPN mRNA expression in patients with esophageal cancer. PDPN, podoplanin.

44 patients (52.4%), for VEGFR-3 in 43 patients (51.2%) and for VEGF-D in 27 patients (32.1%). The expression of PDPN was significantly correlated with the histological type, tumor stage, lymph node metastasis, depth of tumor invasion and tumor location (P<0.05). However, there was no significant association between PDPN mRNA expression and age, gender, tumor size, histological grade or residual tumor (P>0.05). VEGF-C overexpression was significantly associated with tumor depth, tumor stage and lymph node metastasis (P<0.05). Furthermore, the expression of VEGF-D was significantly associated with histological grade, tumor stage and lymph node metastasis, and VEGFR-3 expression was significantly correlated with tumor size (P<0.05) (Table II).

To investigate the risk factors associated with lymph node metastasis, univariate and multivariate regression analyses of gender, tumor location, tumor size, depth of invasion, and PDPN, VEGFR-3, VEGF-C and VEGF-D mRNA expression were conducted. Logistic univariate analysis identified that tumor size, depth of invasion, and VEGF-C, VEGF-D and PDPN mRNA expression were all significantly associated with lymph node metastasis (P<0.05). Among these factors, PDPN mRNA expression (P=0.022), increasing tumor size (P=0.001) and increasing depth of invasion (P=0.002) were significant independent risk factors for lymph node metastasis. The other factors were not predictive for lymph node metastasis (Table III).

The median patient follow-up period was 31 months (range, 2-101 months). For VEGF-C mRNA expression, the median OS time of the patients was 37 months in the low expression group [95% confidence interval (CI), 29-44 months] and 27 months in the high expression group (95% CI, 20-38 months; Fig. 1). For VEGF-D mRNA expression, the median OS time of the patients was 31 months in the low expression group (95% CI, 26-38 months) and 30 months in the high expression group (95% CI, 14-50). For PDPN mRNA expression, the median OS time was 37 months in the low expression group (95% CI, 27-46 months) and 28.5 months in the high expression group (95% CI, 20-38 months) (Fig. 2). Furthermore, for VEGFR-3 mRNA expression, the median OS time of the patients was 34 months in the low expression group (95% CI, 26-40 months) and 29 months in the high expression group (95% CI, 21-41 months).

The patients in the high VEGF-C expression group were associated with a significantly shorter OS time following surgery compared with the patients in the low expression group (P=0.05; Fig. 1). Furthermore, the OS time of the patients in the high PDPN expression group was significantly shorter than that in the low expression level (P=0.02; Fig. 2). By contrast, no association was identified between VEGFR-3 and VEGF-D expression levels and OS time.

In univariate analysis, the following parameters significantly affected OS: Tumor size, depth of tumor invasion, lymph node metastasis, histological grade, tumor stage,

Table II. Association between PDPN, VEGF-C, VEGF-D and VEGFR-3 mRNA expression levels, and clinicopathological criteria.

Clinicopathological criteria	n	PDPN			VEGFR-3			VEGF-D			VEGF-C		
		Low n (%)	High n (%)	P-value	Low n (%)	High n (%)	P-value	Low n (%)	High n (%)	P-value	Low n (%)	High n (%)	P-value
Overall	84	40 (47.6)	44 (52.4)		41 (48.8)	43 (51.2)		57 (67.9)	27 (32.1)		40 (47.6)	44 (52.4)	
Age, years				ns[a]			0.064[a]			ns[a]			ns[a]
<64	41	21 (51.2)	20 (48.8)		24 (58.5)	17 (41.5)		25 (61.0)	16 (39.0)		23 (56.1)	18 (43.9)	
≥64	43	19 (44.2)	24 (55.8)		17 (39.5)	26 (60.5		32 (74.4)	11 (25.6)		17 (39.5)	26 (60.5)	
Gender				ns[a]			ns[a]			ns[a]			ns[a]
Female	8	4 (50.0)	4 (50.0)		3 (37.5)	5 (62.5)		6 (75.0)	2 (25.0)		3 (37.5)	5 (62.5)	
Male	76	36 (47.4)	40 (52.6)		38 (50.0)	38 (50.0)		51 (67.1)	25 (32.9)		37 (48.7)	39 (51.3)	
Histological type				0.023[a]			ns[a]			ns[a]			ns[a]
Sqcc	40	14 (35.0)	26 (65.0)		19 (47.5)	21 (52.5)		28 (70.0)	12 (30.0)		18 (45.0)	22 (55.0)	
Adc	44	26 (59.1)	18 (40.9)		22 (50.0)	22 (50.0)		29 (65.9)	15 (34.1)		22 (50.0)	22 (50.0)	
Location				0.037[b]			ns[b]			ns[b]			ns[b]
Upper	2	0 (0.0)	2 (100.0)		1 (50.0)	1 (50.0)		2 (100.0)	0 (0.0)		1 (50.0)	1 (50.0)	
Midthoracic	54	31 (57.4)	23 (42.6)		31 (57.4)	23 (42.6)		37 (68.5)	17 (31.5)		30 (55.5)	24 (44.5)	
Lower	28	9 (32.1)	19 (67.9)		9 (32.1)	19 (67.9)		18 (64.3)	10 (35.7)		9 (32.1)	19 (67.9)	
Tumor size, cm				ns[a]			0.013[a]			ns[a]			ns[a]
<4	38	14 (36.8)	24 (63.2)		13 (34.2)	25 (65.8)		24 (63.1)	14 (36.9)		18 (47.4)	20 (52.6)	
≥4	46	26 (56.5)	20 (43.5)		28 (60.9)	18 (39.1)		33 (71.7)	13 (28.3)		22 (47.8)	24 (52.2)	
Histological grade				ns[b]			ns[b]			0.005[b]			ns[b]
G1	9	5 (55.5)	4 (44.5)		4 (44.4)	5 (55.6)		2 (22.2)	7 (77.8)		7 (77.8)	2 (22.2)	
G2	37	20 (54.0)	17 (46.0)		19 (51.3)	18 (48.7)		29 (78.4)	8 (21.6)		16 (43.2)	21 (56.8)	
G3	38	15 (39.5)	23 (60.5)		18 (47.4)	20 (52.6)		26 (68.4)	12 (31.6)		17 (44.7)	21 (55.3)	
Tumor stage													
I+II	38	23 (60.5)	15 (39.5)	0.026[a]	19 (50.0)	19 (50.0)	ns[a]	21 (55.3)	17 (44.7)	0.022[a]	23 (60.5)	15 (39.5)	0.026[a]
III	46	17 (37.0)	29 (63.0)		22 (47.8)	24 (52.2)		36 (78.3)	10 (21.7)		17 (37.0)	29 (63.0)	
Depth of tumor invasion													
T1+T2	28	18 (64.3)	10 (35.7)	0.026[a]	15 (53.6)	13 (46.4)	ns[a]	16 (57.1)	12 (42.9)	ns[a]	19 (67.8)	9 (32.2)	0.008[a]
T3+T4	56	22 (39.3)	34 (60.7)		26 (46.4)	30 (53.6)		41(73.2)	15 (26.8)		21 (37.5)	35 (62.5)	
Lymph node metastasis													
N0	30	19 (63.3)	11 (36.7)	0.027[a]	14 (46.7)	16 (53.3)	ns[a]	16 (53.3)	14 (46.7)	0.031[a]	19 (63.3)	11 (36.7)	0.027[a]
N1	54	21 (38.9)	33 (61.1)		27 (50.0)	27 (50.0)		41(75.9)	13 (24.1)		21 (38.9)	33 (61.1)	

Table II. Continued.

Clinicopathological criteria	n	PDPN Low n (%)	PDPN High n (%)	PDPN P-value	VEGFR-3 Low n (%)	VEGFR-3 High n (%)	VEGFR-3 P-value	VEGF-D Low n (%)	VEGF-D High n (%)	VEGF-D P-value	VEGF-C Low n (%)	VEGF-C High n (%)	VEGF-C P-value
Residual tumor													
R0	72	37 (51.4)	35 (48.6)	ns[a]	36 (50.0)	36 (50.0)	ns[a]	51 (70.8)	21 (29.2)	ns[a]	36 (50.0)	36 (50.0)	ns[a]
R1+R2	12	3 (25.0)	9 (75.0)		5 (41.7)	7 (58.3)		6 (50.0)	6 (50.0)		4 (33.3)	8 (66.7)	

[a]Fisher's exact test; [b]χ^2 test. PDPN, podoplanin; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; Sqcc, squamous cell carcinoma; Adc, adenocarcinoma; G1, well-differentiated; G2, moderately-differentiated; G3, poorly-differentiated; T1, tumor invades lamina propria or submucosa; T2, tumor invades muscularis propria; T3, tumor invades adventitia; T4, tumor invades adjacent structures; N0, no regional lymph node metastases; N1, regional lymph node metastases, R0, no residual tumor; R1, microscopic residual tumor; R2, macroscopic residual tumor.

residual tumor, and VEGF-C and PDPN mRNA expression. In multivariate analysis, tumor size, residual tumor and PDPN mRNA expression were identified as independent prognostic factors for a poor OS time in esophageal cancer (Table IV).

Discussion

Metastasis is directly or indirectly responsible for >90% of all cancer mortalities (15). In numerous types of carcinoma, the presence of tumor cells in the lymph nodes is the initial manifestation of metastasis and one of the most important factors of a poor prognosis. The rapid growth and invasive character of esophageal tumors has often been associated with the lymphatic spread of the disease at diagnosis (3).

The induction of lymphangiogenesis by tumors is mediated by growth factors. The most widely investigated factors are members of the VEGF family (VEGF-C, VEGF-D and VEGF-A) and their receptors (16,17). Furthermore, previous studies have indicated that PDPN may be associated with lymphatic dissemination and prognosis in patients with esophageal cancer (18).

The aim of the present study was to analyze the association between the mRNA expression of VEGF-C, VEGF-D, VEGFR-3 and PDPN, and the clinicopathological factors and outcomes of patients with esophageal cancer. Overexpression of VEGF-C, VEGF-D, VEGFR-3 and PDPN was observed in the cancerous tissue samples. These findings are in accordance with the results of studies by Kimura *et al* (19) and Okazawa *et al* (20), and our previous study (8), which used immunohistochemistry to demonstrate that esophageal tumors express VEGF-C and VEGF-D. In addition, Tanaka *et al* (21) used RT-qPCR to demonstrate that esophageal cancer cells express VEGF-C, and Tong *et al* (22) and Rahadiani *et al* (23) identified PDPN overexpression in esophageal carcinoma.

Previous studies have demonstrated that increased VEGF-C and VEGF-D expression is correlated with increased tumor cell dissemination to the regional lymph nodes in a range of primary human carcinomas, including esophageal cancer (8,20,21). The present study identified that VEGF-D mRNA overexpression was associated with histological grade, tumor stage and lymph node metastasis. The study by Tzao *et al* (24) and our previous study (8) obtained similar results. In the current study, a high expression level of VEGF-C was significantly correlated with tumor stage, depth of tumor invasion and lymph node metastasis. This is in accordance with previous studies by Okazawa *et al* (20), Tanaka *et al* (21) and Kitadai *et al* (25), which identified a close correlation between VEGF-C expression and depth of tumor invasion, tumor stage and lymph node metastasis. In the patients with esophageal cancer, VEGFR-3 expression was only correlated with tumor size.

The current study identified that PDPN overexpression was correlated with tumor stage, depth of tumor invasion, lymph node metastasis, tumor location and histological type. The current findings are in agreement with those obtained by Nakayama *et al* (18), Rahadiani *et al* (23) and Tong *et al* (22), which demonstrated a significant correlation between PDPN tumor expression and pathological stage, depth of tumor invasion and lymph node metastasis in esophageal carcinoma.

Furthermore, the present study demonstrated that VEGF-C, VEGF-D and PDPN mRNA expression, tumor

Table III. Univariate and multivariate analysis of risk factors for lymph node metastases.

	Univariate analysis			Multivariate analysis		
Factor	Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value
Gender	3.400	0.752-15.379	ns			ns
Tumor location	0.712	0.297-1.706	ns			ns
Tumor size	15.667	5.144-47.713	<0.001	8.286	2.355-29.158	0.001
Depth of tumor invasion	6.531	2.408-17.718	<0.001	10.272	2.277-46.331	0.002
VEGFR-3 mRNA expression	0.875	0.358-2.139	ns			ns
VEGF-C mRNA expression	2.714	1.079-6.827	0.034			ns
VEGF-D mRNA expression	0.362	0.140-0.939	0.036	0.315	0.084-1.184	ns
PDPN mRNA expression	2.714	1.079-6.827	0.034	5.980	1.301-27.481	0.022

CI, confidence interval; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; PDPN, podoplanin; ns, not significant (P>0.05).

Table IV. Cox regression analysis of independent factors affecting overall survival.

	Univariate			Multivariate		
Factor	HR	95% CI	P-value	HR	95% CI	P-value
Depth of tumor invasion	3.765	1.802-7.87	<0.001	2.191	0.957-5.014	ns
Lymph node metastasis	7.72	3.099-19.231	<0.001			ns
Histological grade	1.832	1.156-2.905	0.010			ns
Tumor stage	3.937	2.025-7.654	<0.001			ns
Tumor size	2.277	1.236-4.197	0.008	1.955	1.002-3.811	0.049
Residual tumor	4.227	2.131-8.384	<0.001	3.784	1.858-7.707	<0.001
VEGFR-3 mRNA expression	1.015	0.572-1.800	ns			ns
VEGF-C mRNA expression	1.768	0.982-3.183	0.050			ns
VEGF-D mRNA expression	0.845	0.445-1.604	ns			ns
PDPN mRNA expression	1.95	1.079-3.524	0.027	2.081	1.121-3.865	0.020

HR, hazard ratio; CI, confidence interval; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; PDPN, podoplanin; ns, not significant (P>0.05).

size, and depth of tumor invasion were associated with lymph node metastasis by performing univariate regression analysis. Multivariate logistic regression analysis also revealed PDPN mRNA expression, increasing tumor size and increasing depth of tumor invasion to be independent factors affecting lymph node metastasis. These findings indicate that PDPN, VEGF-C and VEGF-D mRNA expression were more significantly associated with lymphatic spread than hematogenous metastasis, highlighting their possible efficacy in predicting the nodal status of patients with esophageal cancer.

In the present study, a poor OS time was positively correlated with the overexpression of VEGF-C and PDPN in the esophageal cancer cells. Multivariate analysis identified tumor size, residual tumor and mRNA PDPN expression as independent factors of patient prognosis. Furthermore, the present study used the Kaplan-Meier method to determine that high VEGF-C expression was associated with a significantly shorter OS time compared with low VEGF-C expression. In agreement with this finding, Kitadai *et al* (25), Kimura *et al* (26) and our previous study (8) used immunohistochemistry to demonstrate that the prognosis of patients with VEGF-C-positive tumors was significantly worse than that of patients with VEGF-C-negative tumors. Similarly, Okazawa *et al* (20) identified a significant difference in survival rates between groups with or without VEGF-C overexpression in patients with esophageal cancer. In addition, this study performed a multivariate analysis that determined that gender, age, VEGF-C expression and lymphatic invasion were all prognostic determinants in esophageal cancer. Using univariate survival analysis, Tanaka *et al* (21) determined a significant difference in OS between high and low VEGF-C mRNA expression in patients with esophageal cancer. Tong *et al* (22) performed immunohistochemical analysis and, using univariate and multivariate analysis, identified that overexpression of PDPN was both a prognostic factor and independent prognostic factor for 5-year disease-free survival. Furthermore, Rahadiani *et al* (23) demonstrated that PDPN overexpression was a prognostic factor for OS and

disease-free survival, and an independent prognostic factor for disease-free survival.

The present results indicated that, as they are secreted by esophageal cancer cells, VEGF-C and PDPN may be able to induce and mediate tumor cell invasion, spread cancer cells beyond the primary tumor, and form a metastatic focus in the lymph nodes.

In conclusion, the current study identified that the expression of VEGF-C, VEGF-D and PDPN mRNA was significantly correlated with lymph node metastasis and tumor stage. In particular, PDPN overexpression was significantly associated with patients at a high risk of regional lymph node metastasis. Thus, VEGF-C and PDPN overexpression may be useful as possible indicators of poor prognosis, and PDPN overexpression may be applied as an independent prognostic marker in patients with esophageal cancer that have undergone potentially curative esophagectomy.

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