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Clinical Significance of VEGF and dThdPase Expressions in Squamous Cell Carcinoma of the Esophagus

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Summary. **Objectives: The purposes of this study were to clarify the relation between the vascular endothelial growth factor (VEGF) and thymidine phosphorylase (dThdPase) expression in tumor progression, and to evaluate the prognostic influence of these angiogenic factors in patients with esophageal cancer.**

Methods: VEGF and dThdPase expression and microvessel density (MVD) were immunohistochemically studied in esophageal cancer specimens resected from 103 patients by extended radical esophagectomy. The expression status of VEGF and dThdPase was compared with intratumoral MVD, clinicopathologic parameters of tumor progression, and patient survival after esophagectomy.

Results: dThdPase expression was significantly correlated with depth of invasion ($P=0.0249$) and the size ($P=0.0004$) of primary tumors. VEGF expression significantly correlated with the depth ($P=0.0030$) and size ($P=0.0345$) of the primary tumor. There was no relation between dThdPase and VEGF expression status. dThdPase expression had a significant association with MVD ($P=0.0203$), whereas VEGF expression had no such association. The survival curves of patients did not differ according to the expression status of dThdPase and/or VEGF.

Conclusions: The expression of VEGF and dThdPase was associated with the progression of esophageal cancer by different tumor angiogenic mechanisms. However, the expression of these angiogenic factors had no significant influence upon the survival of patients who underwent extended radical esophagectomy for esophageal cancer.

Key words—tumor angiogenesis, VEGF, dThdPase, esophageal carcinoma, surgical oncology, esophagectomy.

INTRODUCTION

It has been reported that angiogenesis is essential for the growth of solid tumors[1]. Furthermore, it has been demonstrated that angiogenesis is an early event in tumorigenesis and may facilitate tumor progression and metastasis[2]. Recent studies have revealed a significant correlation between tumor angiogenesis and clinicopathologic factors regarding tumor progression in esophageal squamous cell carcinoma (SCC)[3-9], the prognosis of which is poor, even after extended radical esophagectomy[10]. Several angiogenic factors have been reported to have prognostic importance after surgical resection for esophageal SCC. Among these, vascular endothelial growth factor (VEGF) and thymidine phosphorylase (dThdPase) -- which has been revealed to be identical to platelet-derived endothelial cell growth factor (PD-ECGF), are known to play particularly important roles in the neovascularization of esophageal SCC. Although intratumoral microvessel density (MVD) as an integrated parameter of tumor angiogenesis has been repeatedly reported as a significant indicator of relatively poor prognosis after esophagectomy for esophageal SCC, the prognostic significance of VEGF and dThdPase expression in esophageal SCC is con-

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Abbreviations—dThdPase, thymidine phosphorylase, F-VIII, factor VIII; MVD, microvessel density, PD-ECGF, platelet-derived endothelial cell growth factor; SAB, streptavidin-biotins; SCC, squamous cell carcinoma, VEGF, vascular endothelial growth factor; UICC, International Union Against Cancer.

troversial. Furthermore, the relation between VEGF and dThdPase expression in tumor angiogenesis and the role of the two parameters in tumor progression also remain unclear.

Therefore, the purposes of the present study were: 1) to assess whether the intratumoral expression of VEGF and/or dThdPase correlates with MVD as a net result of tumor angiogenesis; 2) to determine which clinicopathologic parameters regarding tumor progression have a significant association with VEGF and dThdPase expression; and 3) to evaluate whether VEGF and/or dThdPase expression exerts a significant impact on postoperative survival in patients undergoing curative esophagectomy for esophageal SCC.

MATERIALS AND METHODS

Patients

During the period between August 1985 and December 1993, 103 patients with invasive SCC of the thoracic esophagus underwent extended radical esophagectomy combined with systematic lymphadenectomies of the bilateral cervical, mediastinal, and abdominal lymph nodes, i.e., 3-field lymphadenectomy, in the Division of Digestive and General Surgery, Niigata University. Our technique of extended radical esophagectomy with 3-field lymphadenectomy has been reported in detail elsewhere[11]. None of the 103 patients received preoperative chemotherapy or radiotherapy. The median age of these patients was 60.7 years (range, 40–70 years). In all cases, follow-up data after esophagectomy were available and the follow-up period ranged from 2 to 144 months (median, 41 months) after esophagectomy or until the date of death.

Clinicopathologic parameters

Esophagectomy specimens were both grossly and histologically examined to determine the location of the primary tumor, histologic grading, depth of tumor invasion, and pathologic stage; classification was based on criteria set by the International Union Against Cancer (UICC)[12]. In addition, the presence or absence of lymphatic and vascular invasion, intramural metastasis, and lymph node metastasis was assessed. Intramural metastasis was defined as a metastatic lesion from a primary tumor of the thoracic esophagus to the adjacent gastric and esophageal wall; criteria for these classifications have been reported previously[13].

Immunohistochemical staining of VEGF, dThdPase, and F-VIII

We used a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1 : 200 dilution for VEGF, a mouse monoclonal antibody obtained from Nippon Roche Research Center at a 1 : 200 dilution for dThdPase, and a rabbit polyclonal antibody (DAKO, Denmark) at a 1 : 200 dilution for factor VIII (F-VIII). F-VIII was used to demonstrate intratumoral microvessels. Immunostaining was performed by the streptavidin-biotins (SAB) method using Histofine SAB-PO (M) and SAB-PO (R) kits (Nichirei, Tokyo, Japan). The staining was visualized with 3,3'-diaminobenzidine tetrahydrochloride. The slides were counterstained with haematoxylin and permanently coverslipped.

Staining analysis

Microvessel counting

Before the MVD of each tumor sample was determined, immunostained slides were scanned at low power (×40) in order to identify the area of greatest vascular density. The five most vascularly dense areas in the tumors were selected for each slide, and a microvessel count was performed at a magnification of ×200. The mean value for a total of 5 high-power (×200) fields was regarded as the MVD for each tumor. In order to eliminate interobserver variation, all counts were performed by a single investigator who had no knowledge of the clinical or pathologic variables.

VEGF and dThdPase staining analysis

For the evaluation of VEGF and dThdPase expression, a scoring system was used. The scoring system was designed so that the expression corresponded to the sum of: 1) the staining intensity (0=negative; 1=weak; 2=intermediate; 3=strong), and 2) the percentage of positive cells (0=0% positive cells; 1=<25% positive cells; 2=26–50% positive cells; 3=>50% positive cells), according to the report by Matlern et al[14]. With this scoring system, a maximum score of 6 could be given to any tissue with high levels of VEGF and dThdPase. A score of greater than 3 was considered as the criterion for an elevated positive expression of VEGF and dThdPase.

Statistical methods

The chi-square test, Fisher's exact probability test, and the Mann-Whitney U test were used to evaluate

Table 1. Relation between clinicopathologic features and VEGF and dThdPase expressions in SCC of the thoracic esophagus

Variable	VEGF			dThdPase		
No. of Patients	Negative	Positive	P value	Negative	Positive	P value
	50	47		27	74	
Location*			0.3087			0.1791
U	2	5		2	5	
M	22	23		17	32	
L	26	19		8	37	
Size of tumor			0.0345			0.0004
≦5.0 cm	15	24		19	23	
>5.0 cm	35	23		8	51	
Histologic grading**			0.1955			0.1366
G1	19	24		9	37	
G2+G3	31	23		18	37	
Primary tumor***			0.0030			0.0249
T1	16	3		10	12	
T2	4	10		5	10	
T3+T4	30	34		12	52	
Lymphatic invasion			0.2596			0.6210
(−)	23	27		15	37	
(+)	27	20		12	37	
Blood vessel invasion			0.5797			0.1120
(−)	27	28		19	39	
(+)	23	19		8	35	
Intramural metastasis			0.0538			0.2569
(−)	40	43		25	62	
(+)	10	3		2	12	
Lymph node metastasis			0.1476			0.7909
(−)	22	14		11	28	
(+)	28	33		16	46	
Microvessel density			0.1386			0.0906
low	26	18		16	31	
high	20	26		9	39	
Recurrence			0.7900			0.3095
(−)	29	26		18	41	
(+)	21	21		9	33	
TNM Stage			0.5902			0.4337
0, I and II	24	20		14	31	
III and IV	26	27		13	41	

*U, upper thoracic esophagus; M, mid-thoracic esophagus; L, lower thoracic esophagus; **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.

differences among clinicopathologic features. Estimation of survival was calculated by the Kaplan-Meier method, and the statistical differences were analyzed using the log rank test. The Cox proportional hazards model was used for multivariate survival analysis. A P-value of less than 0.05 was considered

Table 2. Correlation between VEGF and dThdPase expression

dThdPase expression	VEGF expression		P value
	Positive	Negative	
Positive	34 (35%)	37 (38%)	0.7235
Negative	13 (14%)	12 (13%)	

Table 3. Correlation between status of VEGF/dThdPase expression and microvessel density

VEGF/dThdPase expression	Microvessel density
VEGF status	
Positive (n=47)	64.6±22.1 (27.8-113.6)
Negative (n=50)	59.3±23.7 (28.8-117.6)
dThdPase status	
Positive (n=74)	64.5[1)]±23.2 (30.2-117.6)
Negative (n=27)	59.3[2)]±19.0 (26.8-90.6)
VEGF/dThdPase status	
Positive/positive (n=34)	67.5[3)]±22.2 (38.2-113.6)
Positive/negative or negative/positive (n=50)	60.9±23.9 (27.8-117.6)
Negative/negative (n=12)	49.9[4)]±17.6 (28.8-85.8)

[1)] vs [2)]; P=0.0203, [3)] vs [4)]; P=0.0170 (all analyzed by Man-Whitney U test).

Table 4. Multivariate analysis

Prognostic factors	Multivariate analysis	
	Chi-square	P-value
Tumor size (≦5.0 cm vs. >5.0 cm)	0.007	0.9320
Location (U, M vs. L)	1.410	0.2350
T-factor (T1, T2 vs. T3, T4)	1.084	0.2979
Histologic grade (G1 vs. G2, G3)	1.725	0.1890
Intramural metastsis	26.527	<0.0001
Lymph node metastasis	4.191	0.0406
VEGF (negative vs. positive)	1.234	0.2665
dThdPase (negative vs. positive)	0.004	0.9505
MVD (low vs. high)	3.083	0.0791

significant. All analyses were performed using Statview 4.5 (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

VEGF expression

In most cases, normal stratified squamous epithelium was not stained by anti-VEGF antibody. However, occasional weak VEGF staining was observed in some vascular endothelial cells. VEGF expression could not be evaluated in six tumors because of insufficient tumor material. Positive staining for VEGF expression was detected in 47 (48.4%) of the remaining 97 tumors. (Fig. 1) The correlations between the clinicopathologic parameters and VEGF

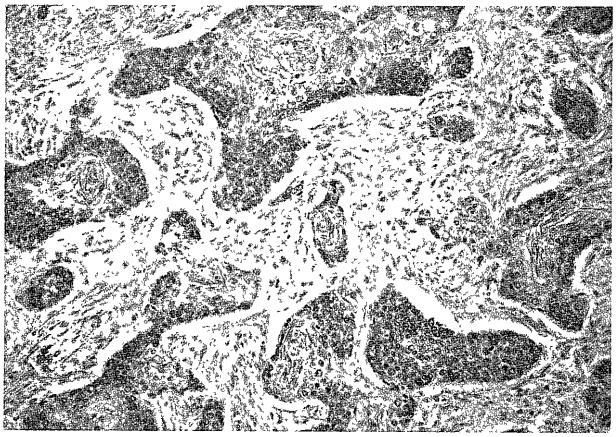

Fig. 1. VEGF expression is evident in both the cytoplasm and the nuclear compartments of cancer cells (original magnification, ×200).

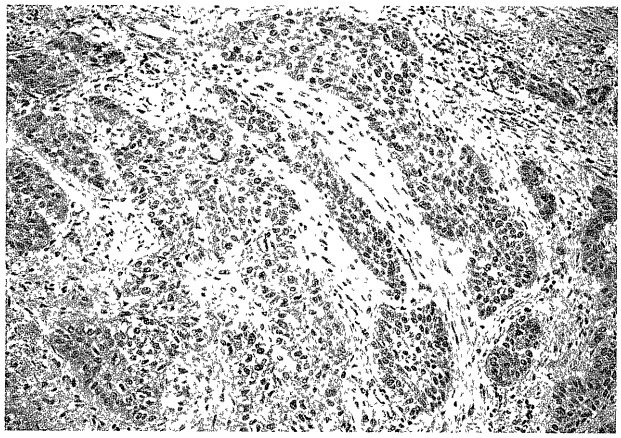

Fig. 2. dThdPase expression is evident in both cytoplasm and the nuclear compartments of cancer cells (original magnifcation, ×200).

expression status are summarized in Table 1. Since the median value of microvessel counts in the 95 tumors was 56.6 (the mean value; 61.3, range 26.8–117.6), this median value of 56.6 was taken as the cutoff point. Tumors were thus classified as having a high (≧56.6) or low (<56.6) count in the analysis. VEGF expression was significantly associated with the depth (P=0.0030) and size (P=0.0345) of primary tumors. Although VEGF expression correlated with the depth of tumor invasion, VEGF expression was most frequent among tumors smaller than 5 cm.

dThdPase expression

In most cases, normal stratified squamous epithelium was not stained by the anti-dThdPase antibody. In contrast, dThdPase expression was evident in both the cytoplasm and the nuclear compartments of cancer cells. However, in a few cases, dThdPase expression was found in infiltrating cells. dThdPase expression could not be evaluated in two of the tumors due to an insufficient amount of tumor material. Positive staining for dThdPase expression was detected in 74 (73.3%) of the remaining 101 tumors (Fig. 2). The correlations between the clinicopathologic parameters and dThdPase expression status are summarized in Table 1. There was a statistically significant association between dThdPase expression and the size (P=0.0004) and depth (P=0.0249) of primary tumors.

Relation between VEGF and dThdPase expression

The respective relationships between VEGF and dThdPase expression in esophageal SCC are summarized in Table 2. There was no significant association between VEGF and dThdPase expression (P=0.7235).

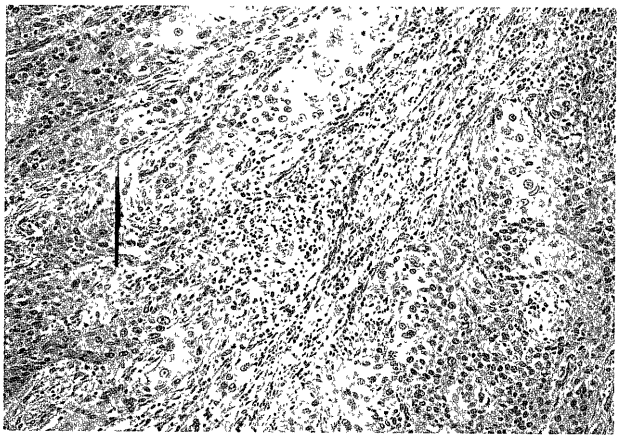

Fig. 3. Microvessels are stained with the F-VIII antibody and represented by either brown capillaries or small clusters (original magnification, ×200).

Correlation between VEGF/dThdPase expression and MVD

All endothelial cells were stained with the F-VIII antibody (Fig. 3). Microvessels were represented by either brown capillaries or small clusters. The greatest vascularization was most frequently observed at the peripheral regions of the tumors, although the distribution of vascular spots was heterogenous in each tumor. Due to the weak intensity of the stain or insufficient tumor material, 8 cases could not be evaluated for MVD. Therefore, MVD was assessed in

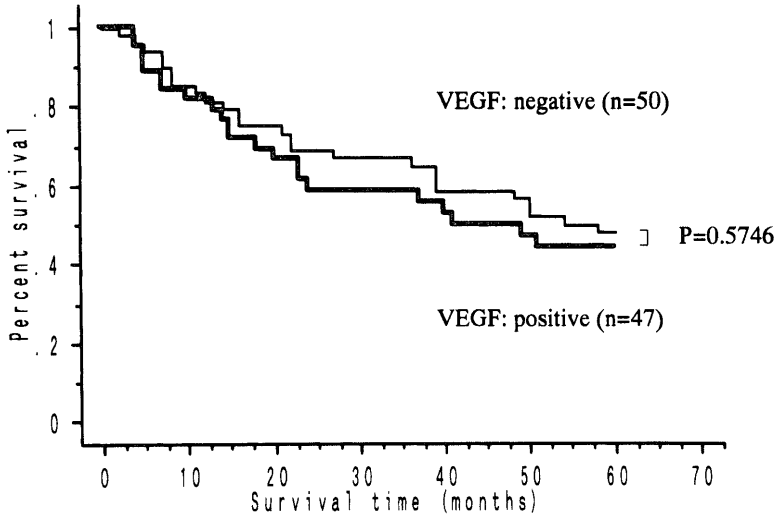

Fig. 4. Survival curves according to VEGF expression status. There was no significant difference between the survival curve of patients with VEGF-positive expression and the survival curve of those with VEGF-negative expression.

the remaining 95 tumors.

MVD values are shown in Table 3, according to the expression status of VEGF and dThdPase. The mean value of the MVD of the 74 tumors showing positive dThdPase expression was significantly higher than that of the 27 tumors that were negative for dThdPase staining ($P=0.0203$). Furthermore, the mean MVD value of 34 tumors positive for both VEGF and dThdPase staining was significantly higher than that of 12 tumors negative for both types of staining ($P=0.0170$). However, no significant association between VEGF expression and MVD was detected.

Patient outcome

Postoperative survival of the patients according to VEGF and/or dThdPase expression status is shown in Figs. 4 and 5, respectively. No significant differences could be detected between the survival curves of the patients with tumors showing VEGF-positive expression and those showing VEGF-negative expression. Likewise, no significant differences could be detected between the survival curves of the patients with tumors showing dThdPase-positive expression and those showing dThdPase-negative expression. Furthermore, there were no significant differences between the survival curves of the patients with tumors showing both positive VEGF and dThdPase expression and those showing both negative VEGF and dThdPase expression ($P=0.1930$) (Fig. 6).

To specify significant factors influencing survival, 9 factors regarding tumor status were examined, as listed in Table 4. A multivariate analysis showed lymph node metastasis ($P=0.0406$) and intramural metastasis ($P<0.0001$) as significant independent predictors of long-term survival (Table 4). However, VEGF, dThdPase, and MVD were not found to be independent prognostic factors.

DISCUSSION

Previously reported experimental findings have indicated that tumor growth is dependent on angiogenesis[1]. When tumors reach a few millimeters in diameter, capillaries penetrate, permitting rapid tumor growth. These new vessels facilitate the entry of tumor cells into the vasculature and their subsequent metastasis, and angiogenesis is therefore associated with an increased probability of metastasis[15]. Several angiogenic factors have been reported to play an important role in the progression of esophageal SCC[3-9]. VEGF and dThdPase are two such angiogenic factors, and both of them have been well characterized. Although these two factors were initially isolated as endothelial growth factors, their characteristics appear to differ. VEGF is a selective mitogen for endothelial cells and acts on endothelial

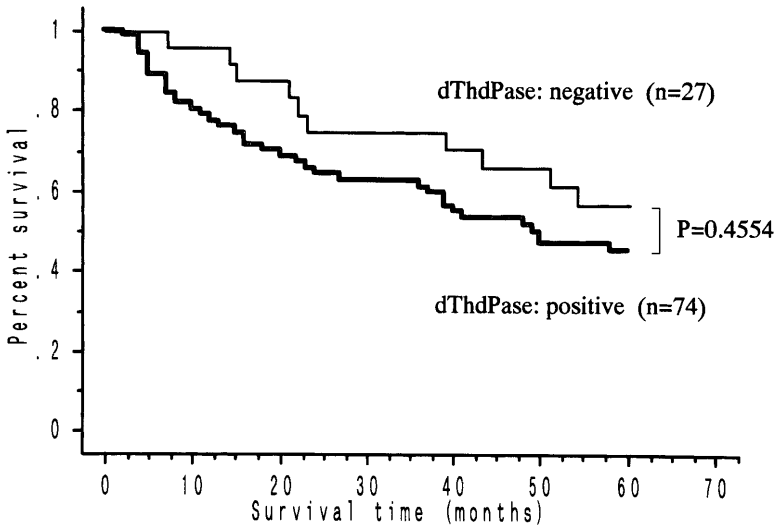

Fig. 5. Survival curves according to dThdPase expression ststus. There was no significant difference between the survival curve of patients with dThdPase-positive expression and the survival curve of those with dThdPase-negative expression.

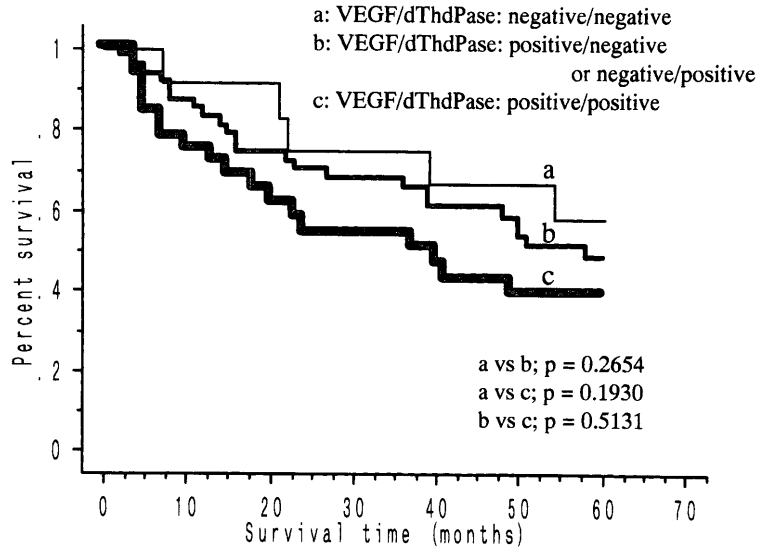

Fig. 6. Survival curves according to the combination of VEGF and dThdPase expression status. There was no significant difference between the survival curve of patients with a simultaneously positive expression of VEGF and dThdPase and the survival curve of those with a simultaneously negative expression of VEGF and dThdPase.

cells to increase microvascular permeability[16-20]. On the other hand, dThdPase stimulates the chemotaxis of endothelial cells in vitro and exhibits angiogenic activity in vivo. Although the frequent expression of VEGF or dThdPase has been reported in esophageal SCC, the relationship between the two angiogenic factors in cases involving tumor progression as well as the prognostic significance of these factors in patients with esophageal SCC is not fully understood.

In the present study, both VEGF and dThdPase

expression significantly correlated with the depth of primary tumor invasion. These results are consistent with most previous studies[3,4,5,7]; however, Ikeguchi et al. found no association between dThdPase levels in cases of esophageal tumor and histopathologic parameters of tumor progression[9]. Furthermore, in the present study, the positive rate of VEGF expression following increases in tumor size in cases of esophageal SCC did not rise, in contrast with dThdPase expression. Moreover, VEGF expression did not correlate with dThdPase expression. In accordance with our findings, Kimura et al. have found no association in staining status between VEGF and dThdPase antigens in cases of gastric carcinoma[21]. Taken together, these findings suggest that VEGF and dThdPase act independently in the angiogenesis of esophageal SCC. The present results are consistent with the different biological functions of VEGF and dThdPase reported in previous experimental studies[16–20].

Both VEGF and dThdPase expression have been reported to correlate with MVD as an integrated parameter of tumor angiogenesis in cases of esophageal SCC. Moreover, Inoue et al. have found a close correlation between MVD and VEGF positivity[4]. In addition, Igarashi et al. have reported a significant correlation between MVD and dThdPase expression scores[3]. In the present study, dThdPase expression of esophageal SCC correlated with intratumoral MVD. However, VEGF expression alone had no significant association with MVD. Our findings suggest that dThdPase plays a more important role in angiogenesis than does VEGF in cases of esophageal SCC. However, it should be noted that Ikeda et al. have reported that VEGF gene expression was associated with an increase in MVD; no significant relationship was found between platelet-derived endothelial cell growth factor, i.e., dThdPase, gene expression, and MVD in their cases of pancreatic cancer[22].

The prognostic significance of VEGF or dThdPase expression of esophageal SCC remains controversial although MVD as a net result of tumor angiogenesis has been repeatedly reported to be associated with poorer survival rates in patients having undergone an esophagectomy for esophageal SCC[3–9]. Inoue et al. have reported that high VEGF levels were predictive of poor patient survival after an esophagectomy[4]. Likewise, Takebayashi et al. demonstrated an adverse influence of dThdPase expression upon postoperative survival in patients with esophageal SCC[7]. However, in the present study, neither VEGF nor dThdPase expression had any impact on patient survival. The fact that all of the patients included in the present study underwent extended radical esophagectomy with 3-field lymphadenectomy may explain such contradictory results, as the extent of esophagectomy has been reported to be a strong prognostic factor in patients with esophageal SCC[10].

In conclusion, both VEGF and dThdPase expression in tumor cells are associated with the progression of esophageal SCC; this occurs by potentially different angiogenic mechanisms. However, a positive expression of these angiogenic factors has no significant influence upon survival in patients undergoing extended radical esophagectomy for esophageal SCC.

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