Clinical significance of vascular endothelial growth factor and Delta-like ligand 4 in small pulmonary adenocarcinoma

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ABSTRACT Vascular endothelial growth factor (VEGF) plays a key role in tumor angiogenesis. The notch ligand Delta-like ligand 4 (DLL4) is induced by VEGF and acts as a negative regulator of tumor angiogenesis by reducing the numbers of non-productive sprouting vessels. Several reports have shown the prognostic role of VEGF expression in non-small cell lung cancer. However, the correlation between VEGF and DLL4 expression and their clinical significance in non-small cell lung cancer remains unclear. The aim of this study was to analyze the correlation between the expression of VEGF/DLL4 and the clinicopathological background. Fifty-eight patients with lung adenocarcinomas measuring less than 3 cm in diameter who underwent surgical resection at Kawasaki Medical School Hospital from 2008 to 2010 were enrolled in this study. The expressions of VEGF, DLL4, CD31, and Ki-67 were analyzed using immunohistochemical staining. The tumor cells were VEGF-positive in 44 patients (75.9%) and DLL4-positive in 41 patients (70.7%). No statistically significant association was observed between the patients' characteristics and VEGF/DLL4 expression. A high VEGF expression level tended to be associated with a high DLL4 expression level (P = 0.050, r = 0.258). The mean Ki-67 index was significantly lower in the patients with high VEGF expression (9.5 vs. 18.2, P = 0.011), but no significant difference was observed when patients were compared according to their DLL4 expression levels (11.8 vs. 11.0, P = 0.804). The mean Ki-67 index was higher in the $VEGF_{low}$ $DLL4_{low}$ patients than in the $VEGF_{high}$ $DLL4_{high}$ patients by a marginally significant difference (20.1 vs. 10.9 P = 0.056). The 3-year recurrence-free survival rates of the $VEGF_{high}/DLL4_{high}$ and the $VEGF_{low}/DLL4_{low}$ patients were 83.3% and 35.7%, respectively. The prognosis of the $VEGF_{high}/DLL4_{high}$ patients was significantly better than that of the $VEGF_{low}/DLL4_{low}$ patients (P = 0.032). To investigate the significance of the difference in tumor proliferation and prognosis between the $VEGF_{high}/DLL4_{high}$ and the $VEGF_{low}/DLL4_{low}$ patients, we evaluated the morphologic effect of VEGF/DLL4 expression on the intratumoral capillaries by counting the number of capillaries and calculating the luminal area (μm^2). No significant

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differences were seen between either the VEGF or DLL4 expression levels and the mean number of intratumoral capillaries or the luminal area (μm^2). In conclusion, $VEGF_{low}/DLL4_{low}$ patients with small pulmonary adenocarcinoma had a significantly poorer prognosis, although no significant difference in a morphological evaluation of the capillaries was seen between $VEGF_{high}/DLL4_{high}$ and $VEGF_{low}/DLL4_{low}$ patients.

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INTRODUCTION

Angiogenesis is required for the growth of several tumors. Vascular endothelial growth factor (VEGF) plays a major role in tumor angiogenesis[1]. The notch signaling pathway is a regulator of differentiation and cell fate during the embryonic and postnatal phases[2]. One of the notch ligands, Delta-like ligand 4 (DLL4), is induced by VEGF and acts downstream of VEGF as a brake on VEGF-induced vessel growth, forming an autoregulatory negative feedback loop that inactivates VEGF[3-5].

There were large numbers of reports showing a prognostic role for VEGF[6,7]. Ping *et al.*[8] reported a meta-analysis that suggested VEGF overexpression was an indicator of a poor prognosis for patients with adenocarcinoma or an early stage of non-small cell lung cancer. Furthermore, some reports have shown that a high VEGF expression level was associated with a high intratumoral microvessel density in non-small cell lung cancer[9-11]. On the other hand, in several tumor models, the blockade of DLL4 inhibited tumor growth by promoting nonproductive sprouting vessels[12,13].Recently, a high level of DLL4 expression has been shown to be an independent predictor of a poor prognosis in patients with several human malignancies[14-16]. Conversely, Donnem *et al.*[17] reported that a low DLL4 expression level in tumor cells was an independent negative prognostic factor in patients with lung adenocarcinoma. However, the correlation between VEGF and DLL4 expression and their clinical significance in non-small cell lung cancer remain unclear. In addition, the morphological effect of VEGF and DLL4 expression on intratumoral capillaries in vivo has never been reported.

In the present study, we evaluated the expressions of VEGF and DLL4 using immunochemistry in small pulmonary adenocarcinomas and compared the findings with the patients' clinical factors as well as the Ki-67 index as a tumor proliferative marker. Furthermore, we examined the morphological effect of VEGF and DLL4 expression on intratumoral capillaries.

MATERIALS AND METHODS

Patients

Fifty-eight patients with lung adenocarcinomas measuring less than 3 cm in diameter who underwent surgical resection at Kawasaki Medical School Hospital from 2008 to 2010 were enrolled in this study. Because squamous cell carcinoma or large non-small cell lung cancers often have intratumoral necrosis, we considered small adenocarcinomas were the most adequate to evaluate the impacts of VEGF/DLL4 in the similar background. None of the patients had received either radiotherapy or chemotherapy prior to undergoing surgery. The histologic tumor diagnoses were based on the criteria of the World Health Organization, and the TMN stage was determined according to the criteria published in 2009. Written informed consent was obtained from each patient for the study of excised tissue samples from the surgical specimens. This study was conducted with the approval of the Institutional Review Board of the Kawasaki Medical School (No. 589-4).

Immunohistochemical staining

The VEGF, DLL4, CD31 and Ki-67 expression levels were evaluated using resected, paraffin-embedded lung cancer tissues. After microtome sectioning (4- μm thick), tissue slides were processed using an automated immunostainer (NexES; Ventana Medical Systems, Tucson, AZ, USA) or manual methods. Streptavidin-biotin-peroxidase detection was performed, with diaminobenzidine used as the chromogen. The following primary antibodies were used according to the manufacturer's instructions: VEGF (rabbit polyclonal; sc-152; 1:300 dilution; Santa Cruze Biotechnology, Inc., Santa Cruz, CA, USA), DLL4 (rabitt polyclonal; ab7280; 1:50 dilution; Abcam, Cambridge, MA, USA), Ki-67(mouse monoclonal; MIB-1 1:50 dilution; Dako, Carpenteria, CA, USA) and CD31 (mouse monoclonal; 1:50 dilution; Dako, Carpenteria, CA, USA). The immunohistochemical results were examined by two investigators who were blinded to the corresponding clinicopathological data. The expression of each protein marker was examined and evaluated according to previously reported protocols.

VEGF expression was evaluated using the VEGF score[18],which was calculated by multiplying the staining proportion by the intensity of staining. The staining proportion was graded according to the percentage of stained cells as follows[19]: 0 for no stained cells, 1 for 1% to 25%, 2 for 26% to 50%, 3 for 51% to 75%, and 4 for greater than 75% of the tumor cells stained. The staining intensity was also

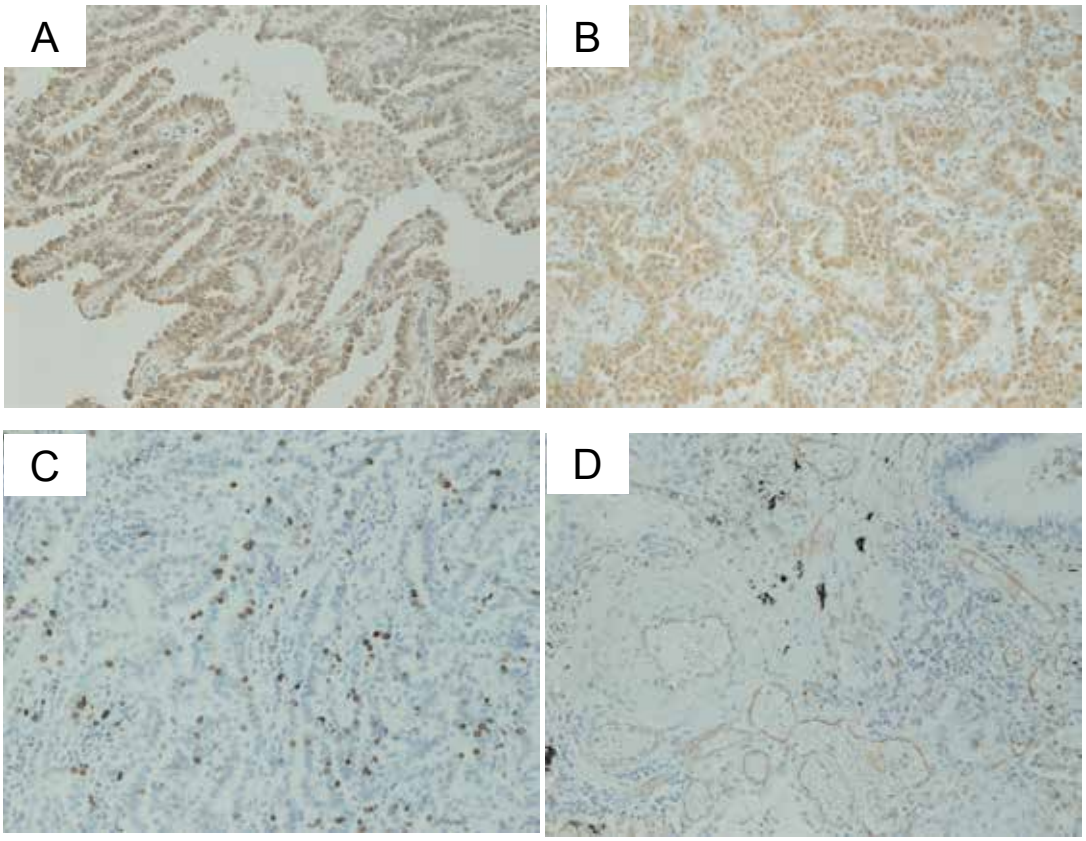

Fig. 1. Immunohistochemical staining for (A) vascular endothelial growth factor (VEGF), (B) delta-like ligand 4 (DLL4), (C) Ki-67, and (D) CD31 (x200).

divided into 4 grades. High VEGF expression was defined as a score of greater than 8, which was the overall median (Fig. 1A).

To evaluate DLL4 staining in the tumor cells, the intensity of expression was scored using a semiquantitative scale in three x200 magnification fields. Negative cores were scored as 0, weak expression was scored as 1, moderate expression was scored as 2, and strong expression was scored as 3. High DLL4 expression was defined as a score of greater than 1.5[17] (Fig. 1B).

To evaluate the proliferation potential of tumor cells, we used the labeling index of Ki-67. The labeling index of Ki-67 was measured by determining the percentage of cells with positively stained nuclei (Fig. 1C).

Evaluation of intratumoral capillaries

To evaluate the intratumoral capillaries, we counted the whole numbers of CD31-positive capillaries and calculated the luminal area (μm^2) of CD31-positive capillaries in three x200 magnification fields using Adobe Photoshop CS3, Extended (Adobe Systems Inc., San Jose, CA) (Fig. 1D).

Statistical analysis

The statistical analysis was performed using the Fisher exact test or the chi square (x2) test, as appropriate. An unpaired t-test was used to compare continuous data. A Kaplan-Meier survival analysis was performed to explore the association between VEGF/DLL4 expression and postoperative recurrence-free survival. All the analyses were performed using SPSS software, version 17.0 (SPSS Inc., Chicago, IL). All the statistical tests were two-sided, and a probability value <0.05 was regarded as being statistically significant.

RESULTS

Relationship between clinicopathological

Table 1 Clinical characteristics and VEGF/DLL4 expression.

Characteristic	No. of Patients	VEGF			DLL4		
	(n=58)	high	low	*P*	high	low	*P*
		44	14		41	17	
Age, years							
Mean	69.2	68.1	72.9	*0.083*	69.8	67.9	*0.471*
Sex							
Female	29	22	7	*0.999*	20	9	*0.773*
Male	29	22	7		21	8	
Pathological stage							
I	55	42	13		39	16	
II	2	2	0	*0.151*	2	0	*0.198*
III	1	0	1		0	1	
Lymph node metastasis							
(−)	55	43	12	*0.142*	40	15	*0.203*
(+)	3	1	2		1	2	
Lymphatic invasion							
(−)	51	40	11	*0.215*	35	16	*0.329*
(+)	7	4	3		6	1	
Vessel invasion							
(−)	46	34	12	*0.397*	30	16	*0.069*
(+)	12	10	2		11	1	
Tumor diffrentiation							
well	37	30	7		27	10	
moderate	15	10	5	*0.468*	9	6	*0.501*
low/poor	6	4	2		5	1	

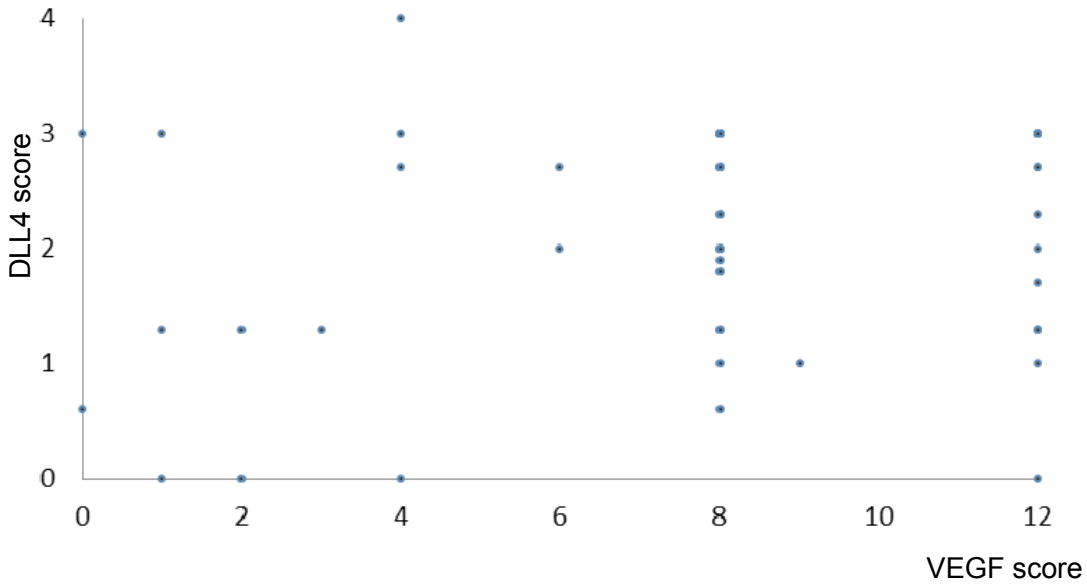

Fig. 2. Association between VEGF and DLL4 expression.

characteristics and VEGF/DLL4 expression

The characteristics of the patients are summarized in Table 1. The patients ranged in age from 50 to 89 years (mean, 69.2 years), and 29 were men. There were 44 VEGF-positive patients (75.9%) and 41 DLL4-positive patients (70.7%). No statistically significant association was observed between the patients' characteristics and the VEGF/DLL4 expression levels. A high VEGF expression level tended to be associated with a high DLL4 expression level (Fig. 2, $P = 0.050$, $r = 0.258$).

Relationship between Ki-67 index and VEGF/DLL4 expression

We evaluated the relationship between VEGF/DLL4 expression and the labeling index of Ki-67 (Table 2). The mean Ki-67 index was significantly lower in patients with high VEGF expression levels than in patients with low VEGF expression levels (9.5 vs. 18.2, $P = 0.011$). However, no significant association was observed between the DLL4 expression level and the Ki-67 index (11.8 vs. 11.0, $P = 0.804$). The mean Ki-67 index was higher in the $VEGF_{low}/DLL4_{low}$ patients than in the $VEGF_{high}/DLL4_{high}$ patients by a marginally significant difference (20.1 vs. 10.9, $P = 0.056$) (Table 3).

Table 2 Relationship between Ki-67 index and VEGF/DLL4 expression.

		Ki-67 index	*P*
VEGF	high	9.5	*0.011*
	low	18.2	
DLL4	high	11.8	*0.804*
	low	11.0	

Table 3 Relationship between Ki-67 index and co-expression of VEGF/DLL4.

	VEGF	
	high	low
DLL4		
high	10.9*	16.3
low	4.6	20.1*

*P=0.056

Prognostic significance of co-expression of VEGF/DLL4

Postoperative recurrence-free survival was evaluated using a median follow-up period of 1077

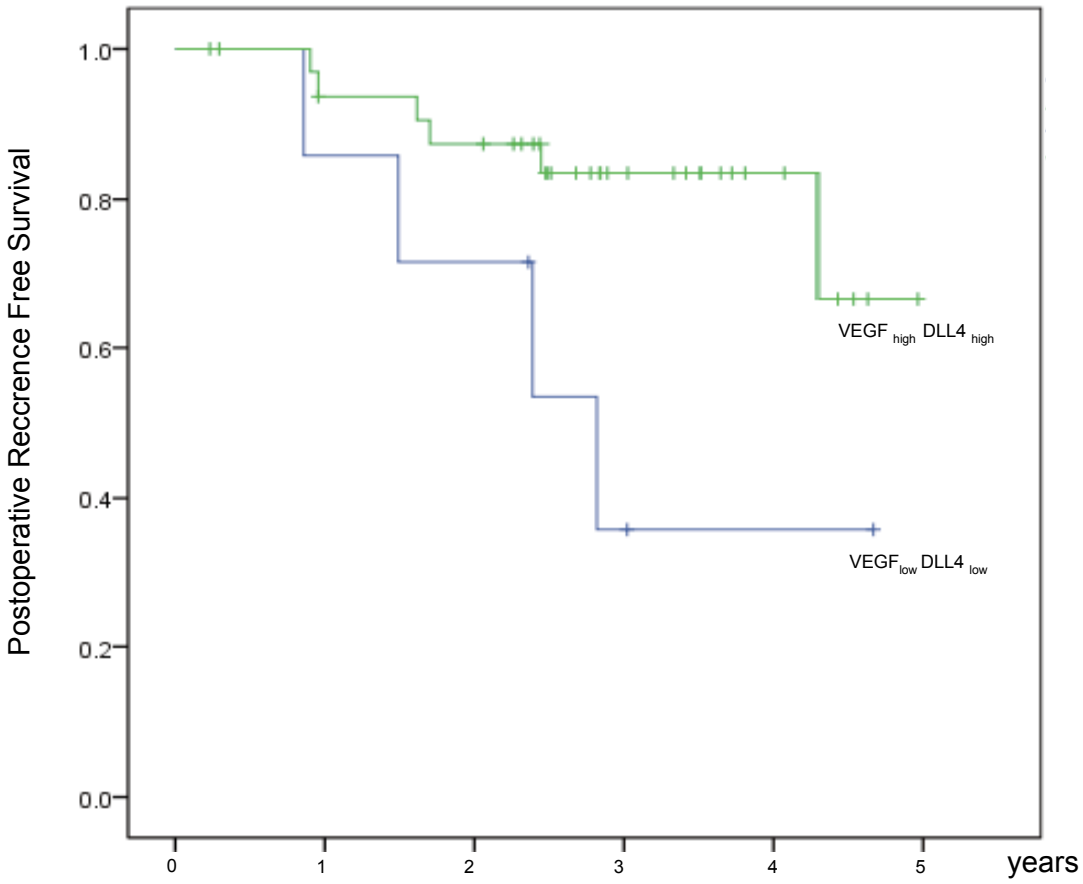

Fig. 3. Recurrence-free survival curves for $VEGF_{high}/DLL4_{high}$ and $VEGF_{low}/DLL4_{low}$ patients.

days. The 3-year recurrence-free survival rates of $VEGF_{high}/DLL4_{high}$ and $VEGF_{low}/DLL4_{low}$ patients were 83.3% and 35.7%, respectively (Fig. 3). The prognosis of $VEGF_{high}/DLL4_{high}$ patients was significantly better than that of $VEGF_{low}/DLL4_{low}$ patients ($P = 0.032$).

Morphological evaluation of intratumoral capillaries

To investigate the cause of the significant difference in tumor proliferation and prognosis between the $VEGF_{high}/DLL4_{high}$ and the $VEGF_{low}/DLL4_{low}$ patients, we evaluated the morphological effect of VEGF/DLL4 expression in the intratumoral capillaries by counting the number of capillaries and calculating the luminal area (μm^2). A total of 2783 capillaries were analyzed in 58 patients. The mean number of capillaries per field was 48 (6-118), and the mean luminal area of the capillaries was 1237.7 μm^2 (279.8-2965.6 μm^2). Regardless of the VEGF or DLL4 expression levels, there was no significant difference in the mean number of intratumoral capillaries or the luminal area of the capillaries (μm^2) (Table 4). Furthermore,

Table 4 Mean number of capillaries and capillary area (μm^2) according to VEGF/DLL4 expression.

		number of capillaries	capillary area (μm^2)
VEGF	high	51.2	1147.1
	low	44.5	1154.5
DLL4	high	50.7	1163.7
	low	46.5	1108.6

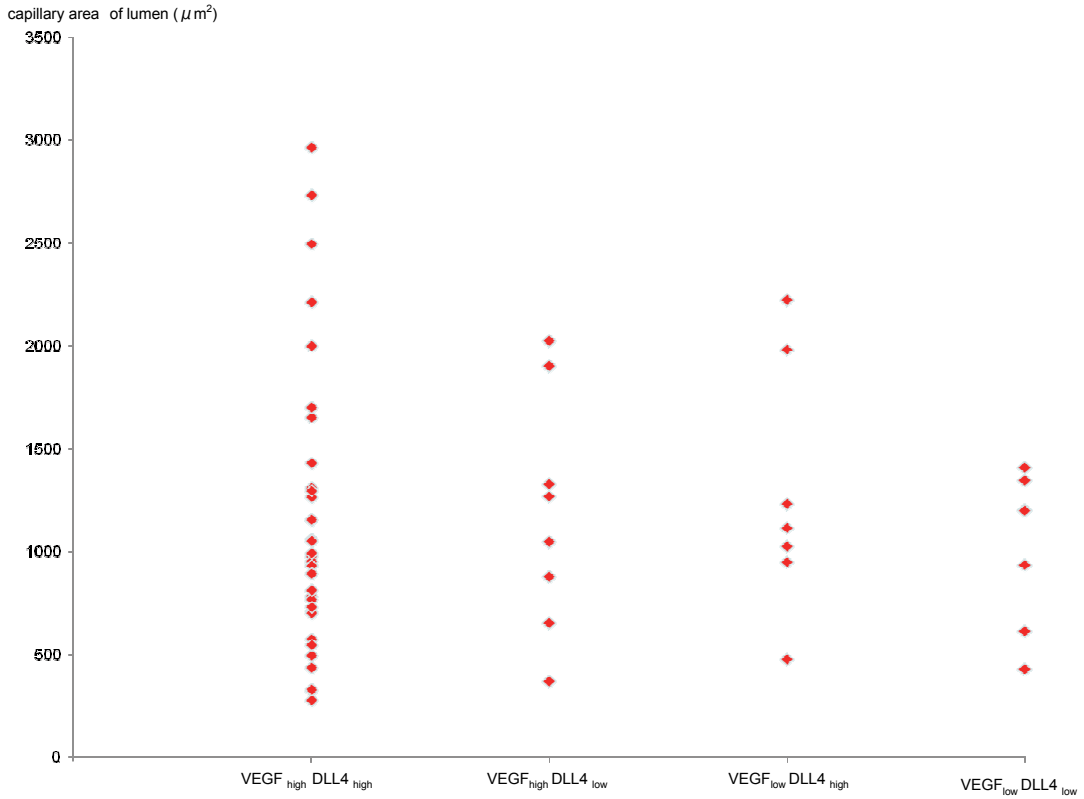

Fig. 4. Mean capillary area (μm^2) according to the co-expression of VEGF/DLL4.

Table 5 Mean number of capillaries and capillary area(μm^2) according to the co-expression of VEGF/DLL4.

number of capillaries	VEGF	
	high	low
DLL4		
high	52.6	41.3
low	45.4	47.7

capillary area of lumen (μm^2)	VEGF	
	high	low
DLL4		
high	1138.1	1288.3
low	1185.5	1020.6

no statistically significant differences in the mean number of intratumoral capillaries and the luminal area (μm^2) were seen between the $VEGF_{high}/DLL4_{high}$ and the $VEGF_{low}/DLL4_{low}$ patients (Table 5, Fig. 4).

DISCUSSION

Our data showed that the Ki-67 index, which reflects tumor proliferation, was higher in $VEGF_{low}/DLL4_{low}$ patients than in $VEGF_{high}/VEGF_{high}$ patients by a marginally significant difference, and the $VEGF_{low}/DLL4_{low}$ patients had a significantly poorer prognosis than the $VEGF_{high}/DLL4_{high}$ patients in terms of the 3-year recurrence-free survival rate in 58 patients of adenocarcinoma less than 3cm. To our knowledge, this is the first report to show that $VEGF_{low}/DLL4_{low}$ patients with non-small cell lung cancer have a relatively poor prognosis.

To investigate the cause of the significant difference in tumor proliferation and prognosis between $VEGF_{high}/DLL4_{high}$ and $VEGF_{low}/DLL4_{low}$ patients, we evaluated the morphologic effect of VEGF/DLL4 expression in intratumoral capillaries by counting the number of capillaries and calculating the luminal area (μm^2). VEGF/DLL4 regulates angiogenetic sprouting and promotes the

formation of well-differentiated vascular networks[3]. We hypothesized that there might be a significant morphological difference between the $VEGF_{high}$/$DLL4_{high}$ and the $VEGF_{low}DLL4_{low}$ capillaries of tumors. However, our data showed that there was no statistically significant difference in either the intratumoral number of capillaries or the luminal area of the capillaries between the $VEGF_{high}$/$DLL4_{high}$ and the $VEGF_{low}$/$DLL4_{low}$ patients. We suspect that the in vivo network of intratumoral sprouting vessels might have been too fine to evaluate using light microscopy. In addition, the distribution of the capillary area was uneven because of cellular variations and tumor heterogeneity. An alternative method for evaluating tiny vascular network in vivo might need to be examined in a larger case series.

In addition, our data had some limitations. First, even small lung adenocarcinomas have been reported to exhibit varying malignant behaviors[20,21]. This observation makes the present results more difficult to interpret. Second, we used immunohistochemical staining to analyze the VEGF and DLL4 expression levels. However, the evaluation of immunohistochemical staining might not be objective. Third, the present series contained only 58 patients. A larger number of cases is needed to analyze the prognostic role of VEGF and notch signals using clinical data from a matched cohort.

In conclusion, $VEGF_{low}$/$DLL4_{low}$ patients with adenocarcinomas less than 3 cm in size had a significantly poorer prognosis than $VEGF_{high}$/$DLL4_{high}$ patients. However, no statistically significant difference in the number of intratumoral capillaries or the luminal area was seen between patients grouped according to their VEGF/DLL4 expression levels. A larger number of cases is needed to analyze the prognostic role of VEGF/DLL4 expression, and an alternative system is needed for performing in vivo evaluations of the tiny vascular network regulated by VEGF/DLL4.

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