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Cite this article as: Yamagishi *et al.*: Histological comparison between preoperative and surgical specimens of non-small cell lung cancer for distinguishing between "squamous" and "non-squamous" cell carcinoma. *Diagnostic Pathology* 2014 **9**:103.

Kawasaki Medical Journal 40(1):23－31, 2014 doi:10.11482/KMJ-E40(1)23

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.

doi:10.11482/KMJ-E40(1)23 *(Accepted on October 22, 2013)*

Key words : **Non-small cell lung cancer, Adenocarcinoma, VEGF, DLL4, Angiogenesis**

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- μ m 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

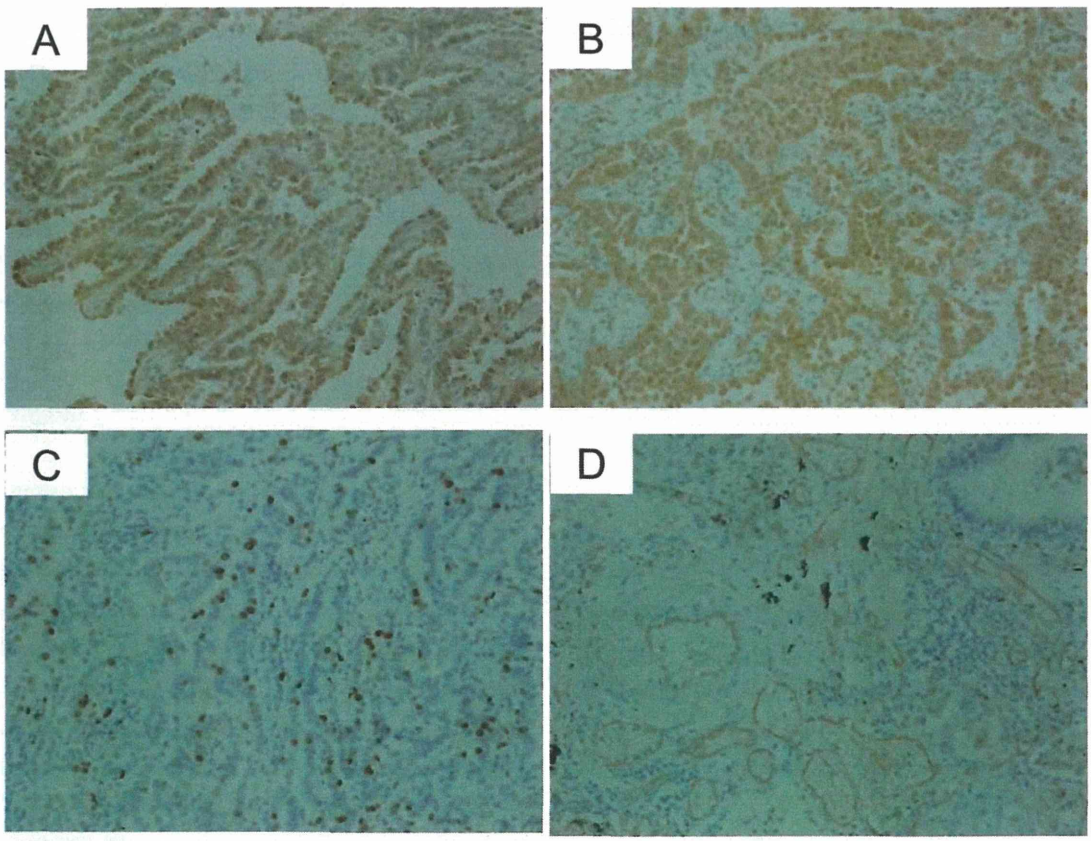

Fig. 1. Immunohistochemical staining for (A) vascular endothelial growth factor (VEGF), (B) delta-like ligand 4 (DLL4), (C) Ki-67, and (D) CD31 (x200).

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	

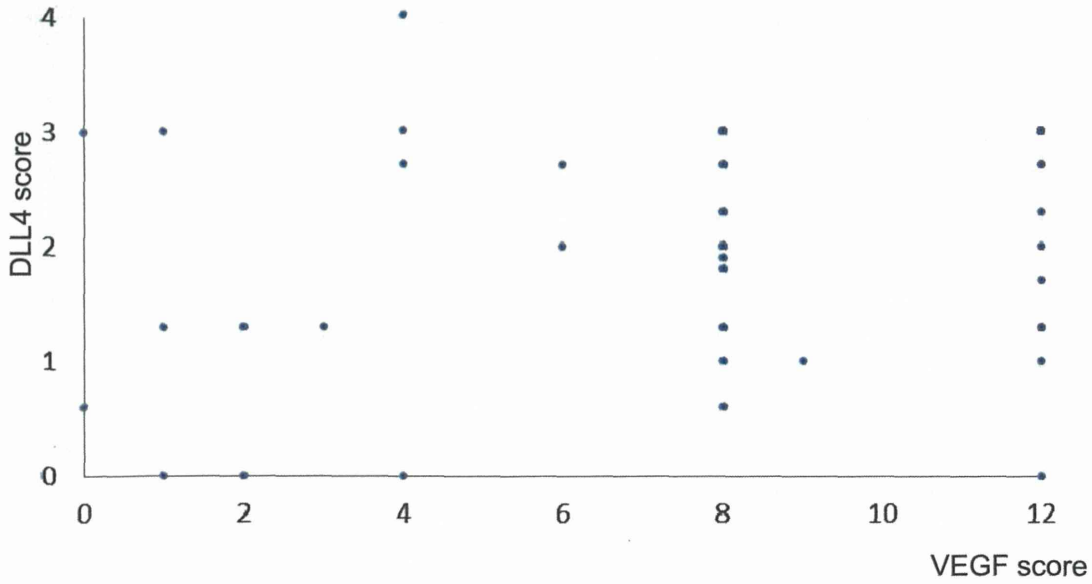

Fig. 2. Association between VEGF and DLL4 expression.

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

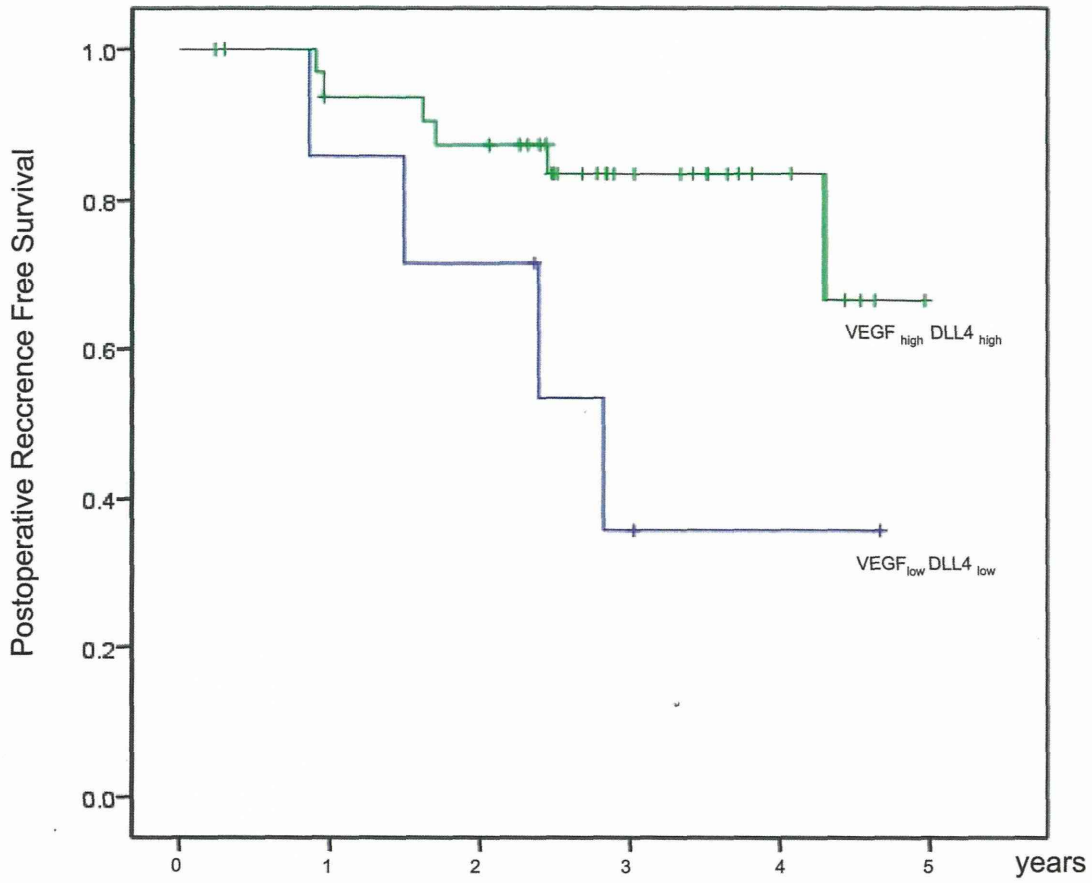

Fig. 3. Recurrence-free survival curves for $VEGF_{high}/DLL4_{high}$ and $VEGF_{low}/DLL4_{low}$ patients.

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

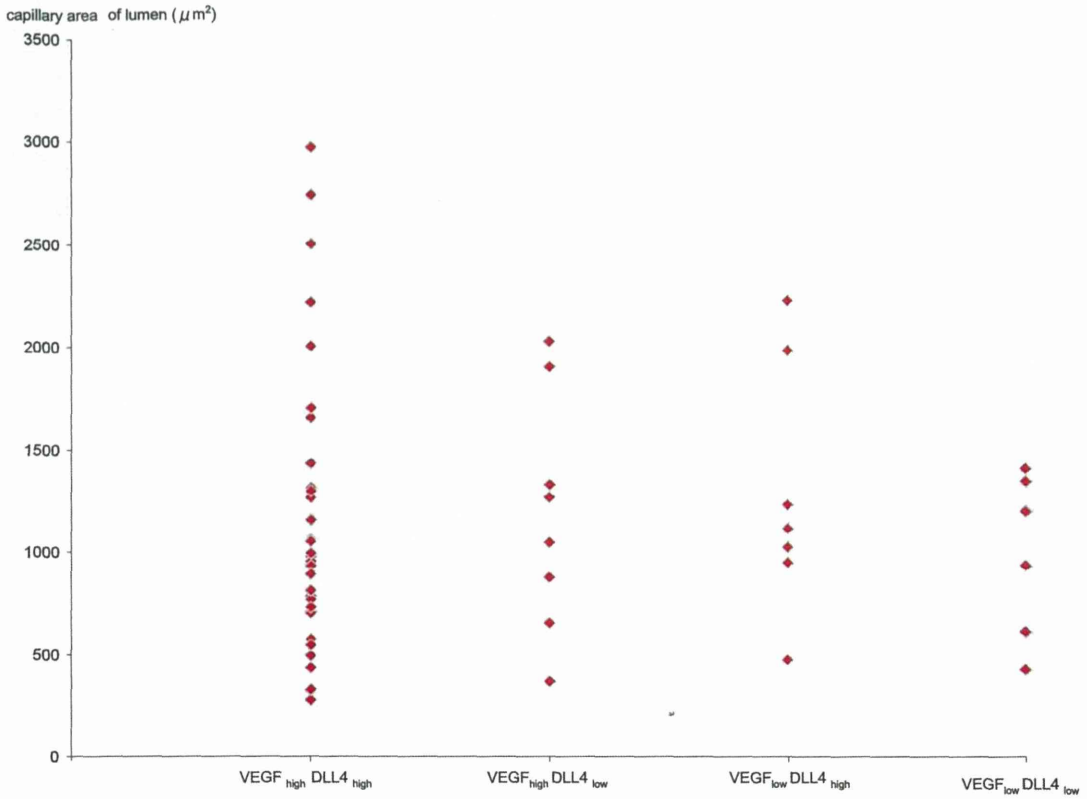

Fig. 4. Mean capillary area (μm^2) according to the co-expression of VEGF/DLL4.

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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Cyclooxygenase-2 genetic variants influence intratumoral infiltration of Foxp3-positive regulatory T cells in non-small cell lung cancer

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Received June 17, 2014; Accepted July 28, 2014

DOI: 10.3892/or.2014.3561

Abstract. The immune microenvironment of primary tumors has been reported to be a prognostic factor. We previously reported that the tumor-infiltrating regulatory T cell (Treg) count was positively correlated with the intratumoral cyclooxygenase-2 (COX-2) expression level and was associated with a poor survival among patients with non-small cell lung cancer (NSCLC). Recently, numerous single nucleotide polymorphisms (SNPs) in the COX-2 gene have been identified, and these SNPs may contribute to differential gene expression and enzyme activity levels. However, whether COX-2 genetic variants influence the functions of COX-2 in NSCLC remains unclear. Eighty NSCLC patients who underwent a complete resection at our institute were enrolled. We extracted DNA from the peripheral blood and identified five different *COX-2* SNPs. The correlations between the *COX-2* SNPs and the expression levels of COX-2, Tregs and Ki-67 were studied. The prognostic significance of the *COX-2* SNPs was also evaluated. *COX-2* SNPs were not correlated with the expression of COX-2. However, for the *COX-2* -1195G/A polymorphism, the AA genotype group had a significantly higher Treg score. Furthermore, the AA group had a significantly higher Treg score regardless of the COX-2 expression level. The *COX-2* -1195AA genotype group tended to have a shorter disease-free survival period than the GA/GG group. In conclusion, the *COX-2* -1195G/A polymorphism influences the infiltration of Tregs into NSCLC, and the *COX-2* SNP factor may be a prognostic factor reflecting Treg infiltration in NSCLC.

Introduction

Cyclooxygenase (COX) is the key enzyme required for the conversion of arachidonic acid to prostaglandins (PGs). Two COX isoforms have been identified and are referred to as constitutive COX (COX-1) and inducible COX (COX-2). COX-1 is constitutively expressed in many tissues and plays important roles in the control of homeostasis (1). On the other hand, COX-2 is an inducible enzyme that is activated in response to extracellular stimuli, such as growth factors and pro-inflammatory cytokines (2). Some investigators have demonstrated that COX-2 is constitutively overexpressed in a variety of epithelial malignancies, such as lung, breast, pancreas, colon, esophagus, and head and neck cancers, and COX-2 overexpression is usually associated with a poor prognosis (3-6).

Regulatory T cells (Tregs) were initially characterized as having a $CD4^+CD25^+$ phenotype, and these cells are thought to modulate the antitumor immune response (7). Tregs can suppress the activity of cytotoxic T cells through direct cell-to-cell contact or via the release of cytokines. The most specific Treg cell marker identified to date is a nuclear transcription factor known as Foxp3 (8,9). A high density of tumor-infiltrating $Foxp3^+$ Tregs is reportedly associated with a higher risk of recurrence and a poor overall survival among patients with non-small cell lung cancer (NSCLC) (10). In 2010, we demonstrated that the tumor-infiltrating $Foxp3^+$ Treg count (Foxp3 score) was positively correlated with the intratumoral COX-2 expression level and was associated with a poor recurrence-free survival period, particularly among patients with node-negative NSCLC (11).

Recently, numerous single nucleotide polymorphisms (SNPs) in the *COX-2* gene have been identified, and these SNPs may contribute to differential gene expression and enzyme activities (12,13). In NSCLC, Bi *et al* (14) reported that a certain *COX-2* SNP was a potential predictor of survival in patients with locally advanced NSCLC who were treated with chemoradiotherapy or radiotherapy alone. However, whether COX-2 genetic variants influence the function of COX-2 in NSCLC remains unclear. In the present study, we analyzed five types of *COX-2* SNPs and evaluated whether the *COX-2* SNPs were correlated with the intratumoral expression levels of COX-2, $Foxp3^+$ Tregs and Ki-67 in NSCLC.

Patients and methods

Study population. Blood and tumor samples were obtained from 80 consecutive patients with NCSLC who underwent

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Key words: non-small cell lung cancer, regulatory T cells, single nucleotide polymorphism, cyclooxygenase-2

a complete resection with systematic lymph node dissection at Kawasaki Medical School Hospital between August 2011 and March 2013. None of the patients had received either radiotherapy or chemotherapy prior to surgery. This study was conducted with the approval of the institutional Ethics Committee of Kawasaki Medical School, and informed consent for the use of blood and tumor specimens was obtained from each of the patients. The histological diagnosis of the tumors was based on the criteria of the World Health Organization, and the TNM stage was determined according to the criteria established in 2009.

Genotyping of COX-2 SNPs. Blood samples were collected at the time of pre-operation. Genomic DNA was isolated from whole peripheral blood and was subjected to DNA amplification using a DNA Extractor WB-Rapid kit. The genomic DNA region containing the SNP was amplified using a polymerase chain reaction (PCR) performed using an Ampdirect Plus kit. The PCR primers used for the detection of the *COX-2* -1195G/A, -1290A/G, -765G/C, 1759G/A and 8473T/C SNPs were as follows: -1195F, 5'-TCCACTTCTTTTCTGGTGTGTG-3' and -1195R, 5'-CTGGGCTTATTGGGGCTAA-3'; -1290F, 5'-CCA CTTCTTTTCTGGTGTGTG-3' and -1290R; 5'-GGGAGATT TTGACAGTTGGAA-3'; -765F, 5'-CCAAAATAATCCACG CATCA-3' and -765R; 5'-TACCTTCACCCCCTCCTTG-3'; 1759F, 5'-GGGCTGTCCCTTTACTTCATT-3' and 1759R, 5'-GACTCCTTTCTCCGCAACA-3'; 8473F, 5'-TGTCACAA GATGGCAAAATGC-3' and 8473R, 5'-GCTTTTACAGGTG ATTCTACCCTATGA-3', respectively.

DNA sequencing. The polymorphisms were analyzed using the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The results were analyzed using GeneMapper Software, ver. 4.0 (Applied Biosystems).

Immunohistochemical study. Immunohistochemical analyses were performed using resected paraffin-embedded lung cancer tissues. After microtome sectioning, the slides were processed for COX-2, Foxp3 and Ki-67 staining using an automated immunostainer (NexES; Ventana, Tucson, AZ, USA). The streptavidin-biotin-peroxidase detection technique using diaminobenzidine as a chromogen was applied. The primary antibodies were used according to the manufacturer's instructions (COX-2, clone CX-294, 1/50 dilution; DakoCytomation; Foxp3, clone 22510, 1/100 dilution; Abcam; Ki-67, MIB-1, 1/100 dilution; DakoCytomation). The expression of each marker protein was examined and evaluated according to a previously reported original protocol. The slides were examined by an investigator who had no knowledge of the corresponding clinicopathological data.

For COX-2, the slides were scored according to the intensity of staining (0-3), and the percentages of cells with scores of 0 (0%), 1 (1-9%), 2 (10-49%), and 3 (50-100%) were determined. The immunohistochemistry (IHC) score (0-9) was defined as the product of the intensity and the percentage of stained cells. COX-2 expression was judged as positive when the IHC score was ≥4 (groups 3 and 4) (Fig. 1A) (15).

To evaluate Treg immunostaining, 10 high-power field (HPF) digital images of the tumor areas were selected, and the absolute number of Foxp3-positive lymphocytes in these 10 HPF digital images was determined. The number of immunostained Foxp3 cells was then determined by averaging the 10 HPF digital image cell counts, resulting in the Treg score (Fig. 1B) (16).

The labeling index of Ki-67 was measured by determining the percentage of cells with positively stained nuclei. Ki-67 expression was judged as positive when >10% of the cancer cell nuclei showed positive staining (Fig. 1C) (17).

Table I. Patient characteristics.

Characteristics	No. of patients	Percentage
Gender		
Male	50	62.5
Female	30	37.5
Age, mean ± SD	69.9±9.6	
Histology		
Adenocarcinoma	61	76.3
Squamous cell carcinoma	17	21.3
Large cell carcinoma	1	1.2
Pleomorphic carcinoma	1	1.2
Pathological stage		
IA	41	51.2
IB	19	23.8
IIA+IIB	12	15.0
IIIA+IIIB	8	10.0
Adjuvant chemotherapy		
(+)	20	25.0
(-)	60	75.0

Statistical analysis. All the statistical analyses were performed using the SPSS statistical package (version 17.0; SPSS, Chicago, IL, USA). The Chi-square test and the Fisher's exact test were used to examine the association between *COX-2* SNPs and various clinicopathological parameters and protein expression levels evaluated using IHC. The vascular score was presented as the mean ± SD, and the difference between groups was analyzed using an unpaired Student's t-test. The significance level was $P<0.05$. A prognostic evaluation was performed using the disease-free survival (DFS) period. The DFS was defined as the time from surgical resection until lung cancer recurrence or non-lung cancer-related death. To explore the association between DFS and *COX-2* SNPs, a Kaplan-Meier survival analysis was performed by stratifying significant predictor variables that had been identified using the COX proportional hazards model. Two-sided P-values of <0.05 were considered to be statistically significant.

Results

Patient characteristics. The patient characteristics are documented in Table I. The mean age of the 80 patients was 69.9 years, and 50 of the patients were male. The histological type was adenocarcinoma in 61 cases, squamous cell

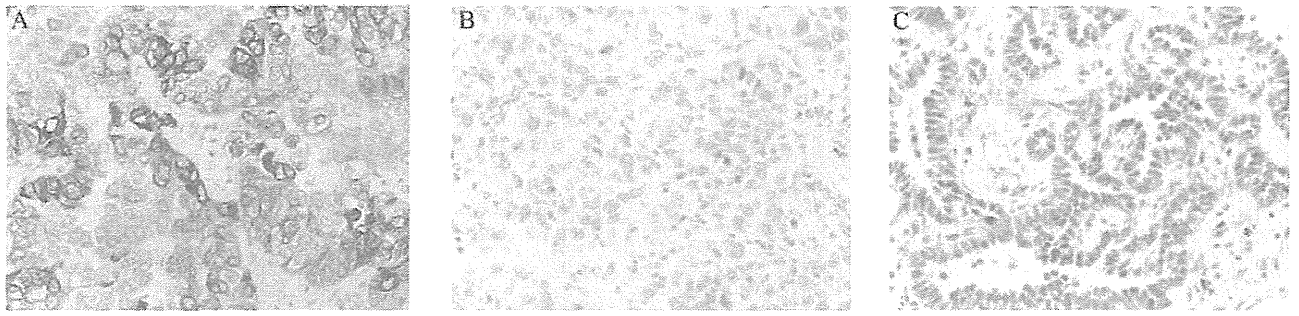

Figure 1. Immunohistochemical staining of (A) cyclooxygenase-2 (COX-2), (B) regulatory T cells (Tregs) and (C) Ki-67. Magnification, x200.

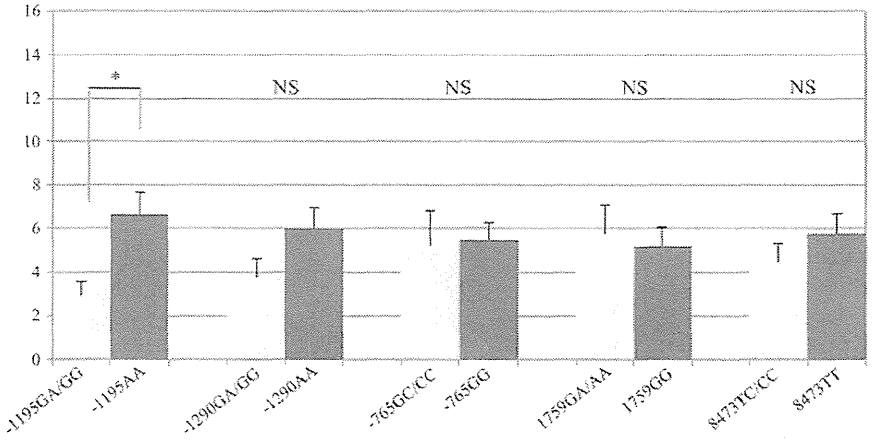

Figure 2. Association between cyclooxygenase-2 (COX-2) single nucleotide polymorphisms (SNPs) and regulatory T cell (Treg) score. *P=0.003.

carcinoma in 17 cases, and other types in 2 cases. The median follow-up period was 24 months (range, 9-30 months).

Relationship between the expression status of COX-2 and the Foxp3-positive lymphocyte count. An immunohistochemical study showed that COX-2 was positive in 27 cases and negative in 53 cases. In the COX-2-positive group, the mean Treg score was 9.22. Conversely, in the COX-2-negative group, the mean Treg score was 3.47. The Treg score was significantly and positively correlated with the COX-2 expression level (P<0.001).

Associations between genotypes and clinicopathological findings. The associations between the *COX-2* genotypes and the clinicopathological findings are shown in Table II. For the -1195G/A polymorphism, the AA genotype was observed in 53 cases and the GA/GG genotype was observed in 27 cases. Pleural invasion was significantly higher in the AA group than that in the GA/GG group (P=0.040). For the 1759G/A polymorphism, the GG genotype was observed in 56 cases and the GA/AA genotype was observed in 24 cases. The GA/AA group contained more patients who were over 70 years of age than the GG group. For the other genotypes, however, no significant correlations were found between the *COX-2* genotypes and the clinicopathological findings.

Associations between genotypes and COX-2, Treg and Ki-67 expression levels. The associations between the *COX-2* genotypes and the expression levels of COX-2, Tregs and Ki-67 are shown in Table III. No significant correlations were found between the *COX-2* genotypes and the COX-2 score or the Ki-67 labeling index. For the -1195G/A polymorphism, however, the mean Treg score was 6.6 in the AA group and 3.0 in the GA/GG group. The mean Treg score was significantly higher in the AA group (P=0.003). Other polymorphisms showed no significant associations with the Treg score (Fig. 2).

Associations between COX-2 genotypes and Treg score according to the COX-2 expression level. Next, we evaluated whether the influence of the COX-2 genotype on the Treg score differed according to the COX-2 expression level (Table IV and Fig. 3). In the COX-2-positive expression group, a significant difference in the Treg scores was observed between the genotypes with the -1195G/A and -1290G/A polymorphisms. For the -1195G/A polymorphism, the mean Treg score was 11.2 in the AA group and 5.3 in the GA/GG group (Fig. 3A). The Treg score of the AA group was significantly higher than that of the GA/GG group (P=0.03). For the -1290G/A polymorphism, the mean Treg score was 11.4 in the AA group and 5.6 in the GA/GG group (Fig. 3A). The Treg score for the AA group was significantly higher than that for the GA/GG group (P=0.033). On the other hand, in the COX-2-negative expression group, a significant difference in the Treg scores was only observed for the -1195G/A polymorphism. The mean Treg score was 4.3 in the AA group and 1.8 in the

Table II. Association between *COX-2* genotypes and clinicopathological findings.

	-1195G/A			-1290A/G			-765G/C		
Factor	AA	GG+GA	P-value	AA	GG+GA	P-value	GG	CC+GC	P-value
Age (years)			0.943			0.651			0.459
<70	24	12		27	9		28	8	
≥70	29	15		31	13		31	13	
Gender			0.360			0.698			0.646
Male	35	15		37	13		36	14	
Female	18	12		21	9		23	7	
Histology			0.378			0.895			0.994
Adenocarcinoma	42	19		44	17		45	16	
Squamous cell carcinoma	9	8		12	5		12	5	
Others	2	0		2	0		2	0	
Pleural invasion			**0.040**			0.822			0.624
Negative	31	22		38	15		40	13	
Positive	22	5		20	7		19	8	
Vascular invasion			0.686			0.291			0.779
Negative	30	14		34	10		33	11	
Positive	23	13		24	12		26	10	
Nodal status			0.116			0.985			0.934
N0	48	21		50	19		51	18	
N1/N2	5	6		8	3		8	3	

	1759G/A			8473T/C		
Factor	GG	AA+GA	P-value	TT	CC+TC	P-value
Age (years)			**0.019**			0.503
<70	29	6		27	9	
≥70	27	18		30	14	
Gender			0.614			0.848
Male	34	16		36	14	
Female	22	8		21	9	
Histology			0.863			0.788
Adenocarcinoma	43	18		45	16	
Squamous cell carcinoma	11	6		12	5	
Others	2	0		2	0	
Pleural invasion			0.642			0.242
Negative	38	15		40	13	
Positive	18	9		17	10	
Vascular invasion			0.281			0.862
Negative	33	11		31	13	
Positive	23	13		26	10	
Nodal status			0.620			0.907
N0	49	20		49	20	
N1/N2	7	4		8	3	

COX-2, cyclooxygenase-2.

Table III. Association between *COX-2* genotypes and COX-2, Treg and Ki-67 expression.

	-1195G/A			-1290A/G			-765G/C		
Factor	AA	GG+GA	P-value	AA	GG+GA	P-value	GG	CC+GC	P-value
COX-2 score	2.9	2.9	0.932	3.3	2.8	0.205	2.8	3.1	0.947
Treg score	6.6	3.0	**0.003**	6.0	3.8	0.063	5.5	5.2	0.382
Ki-67 labeling index	28.7	30.0	0.792	28.3	31.0	0.571	29.5	28.4	0.832

	1759G/A			8473T/C		
Factor	GG	AA+GA	P-value	TT	CC+TC	P-value
COX-2 score	2.7	3.3	0.730	2.8	3.0	0.150
Treg score	5.2	5.8	0.653	5.8	4.5	0.108
Ki-67 labeling index	27.0	33.7	0.198	29.6	28.2	0.786

COX-2, cyclooxygenase-2; Tregs, regulatory T cells.

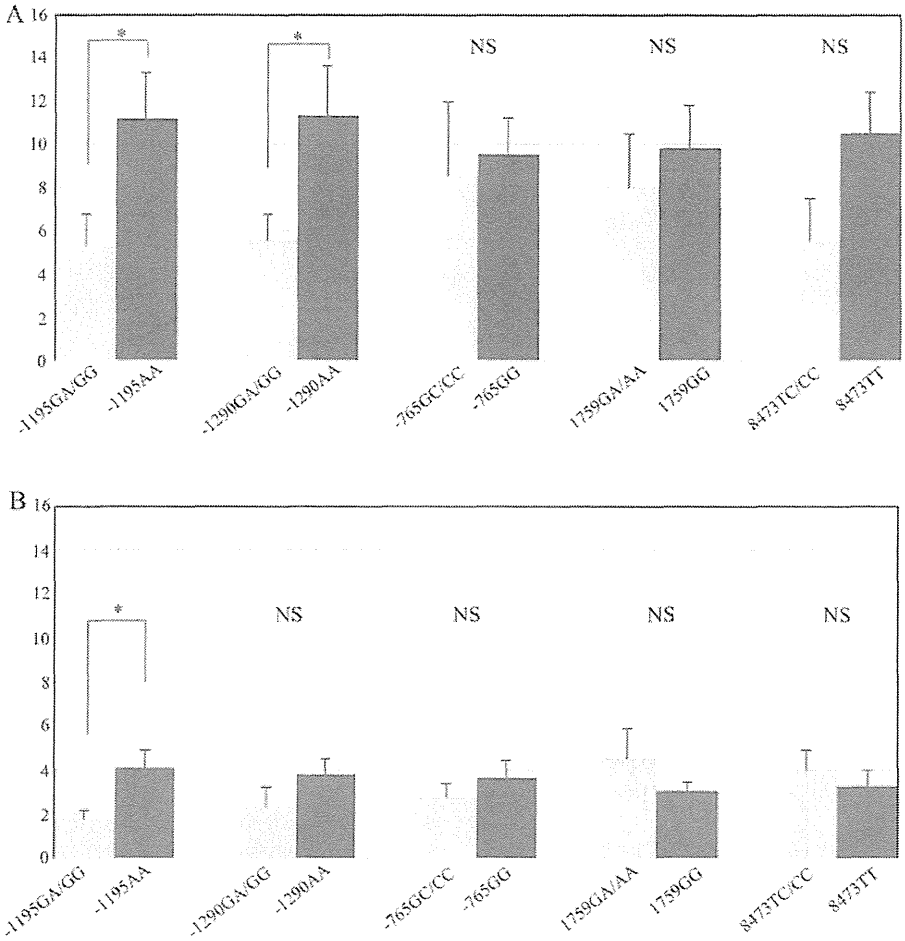

Figure 3. Association between cyclooxygenase-2 (COX-2) single nucleotide polymorphisms (SNPs) and regulatory T cell (Treg) score according to COX-2 expression. (A) COX-2-positive group, (B) COX-2-negative group. *P=0.033 in A; *P=0.011 in B.

GA/GG group (Fig. 3B). Similar to the COX-2-positive expression group, the Treg score of the AA group was significantly higher than that of the GA/GG group (P=0.011). These results showed that the -1195AA genotype group had a significantly higher Treg score than the GA/GG group, regardless of the intratumoral COX-2 expression level. For the other *COX-2*

Table IV. Associations between COX-2 genotypes and Treg score in regards to COX-2 expression.

		COX-2-negative group			COX-2-positive group		
Genotype	N	n	Treg score	P-value	n	Treg score	P-value
-1195G/A				**0.011**			**0.030**
AA	53	35	4.3±5.1		18	11.2±9.0	
GG+GA	27	18	1.8±1.6		9	5.3±4.2	
-1290A/G				0.211			0.033
AA	58	41	3.8±4.6		17	11.4±9.3	
AG+GG	22	12	2.3±3.1		10	5.6±3.6	
-765G/C				0.346			0.797
GG	59	41	3.7±4.8		18	9.6±7.1	
GC+CC	21	12	2.8±2.2		9	8.6±10.2	
1759G/A				0.340			0.576
GG	56	38	3.1±3.9		18	9.8±8.5	
GA+AA	24	15	4.5±5.3		9	8.0±7.6	
8473T/C				0.521			0.088
TT	57	37	3.2±4.7		20	10.5±8.7	
TC+CC	23	16	4.0±3.5		7	5.6±5.1	

COX-2, cyclooxygenase-2; Tregs, regulatory T cells.

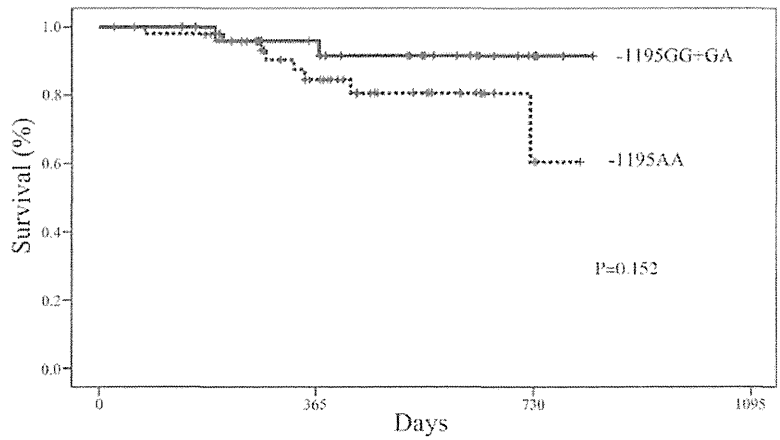

Figure 4. Disease-free survival of the patients with different cyclooxygenase-2 (COX-2) -1195G/A genotypes.

SNPs, significant differences in the Treg scores were not found when the data were examined according to genotype.

Association between genotypes and DFS. The DFS period of the -1195AA genotype group was shorter than that of the GA/GG genotype group; however, the difference was not significant (Fig. 4). For the other *COX-2* SNPs, no differences in the DFS period were observed when the data were examined according to genotype.

Discussion

In 2010, we demonstrated that the tumor-infiltrating Foxp3$^+$ Tregs count (Treg score) was positively correlated with the intratumoral COX-2 expression level and was associated with a poor recurrence-free survival period, in particular among patients with node-negative NSCLC (11). In the present study, we examined whether *COX-2* SNPs are associated with the expression of COX-2, Foxp3$^+$ Treg and Ki-67 in 80 consecutive NSCLC patients who underwent resection. Our results showed that the AA genotype of the -1195G/A SNP in the *COX-2* promoter region significantly contributed to the increased tumor-infiltrated Foxp3-positive lymphocyte count and indicated that NSCLC with an AA genotype for the -1195G/A SNP had a shorter DFS, compared with the GA/GG genotype.

A few studies have described different COX-2 SNPs and the associated clinical outcomes for several types of cancer. Li *et al* (18) reported that COX-2 SNPs were associated with

the prognosis of patients with colorectal cancer. Bi *et al* (14) showed that genetic polymorphisms in *COX-2* were associated with survival in patients with locally advanced NSCLC who had undergone chemoradiotherapy or radiotherapy alone They reported that the AA genotype of the -1195G/A SNP in the *COX-2* promoter region significantly contributed to an unfavorable overall survival and progression-free survival, compared with the other genotype. Our results were similar to their results, but this study is the first to point out that the *COX-2* polymorphism is associated with the Treg score in NSCLC.

The genotype frequencies for *COX-2* -1195G/A SNPs in this study were equivalent to those in a previous study (19). Regarding the function of the -1195G/A polymorphism in *COX-2*, the -1195G to A change reportedly creates a c-MYB binding site in the *COX-2* promoter region, thereby increasing the promoter activity (12). Compared with the -1195G-containing counterparts, the -1195AA carriers showed a significantly higher COX-2 expression level (12). In the present study, no significant correlations were found between the COX-2 expression level and the genotype of *COX-2*. However, the Treg score for the AA genotype of the -1195G/A polymorphism was significantly higher than that for the GA/GG group. Furthermore, the AA genotype group showed a significantly higher Treg score than the GA/GG group, regardless of the intratumoral COX-2 expression. These results suggest that the polymorphism may influence the inducing capacity of Tregs into NSCLC, as well as the prognosis of patients with NSCLC as a result of the infiltration of Tregs. To validate our hypothesis, the quantity or biological activity of prostaglandin E_2 (PGE_2) may need to be measured in lung tumor tissue, followed by an investigation of the correlation between *COX-2* SNPs and intratumoral PGE_2, which is converted from arachidonic acid in the presence of COX-2 as a catalytic substance and is considered to stimulate the infiltration of Tregs into tumor tissue (6).

Recently, a clinical trial by Cancer and Leukemia Group B demonstrated that among patients with increased COX-2 expression levels, survival was better among those who received treatment with a COX-2 inhibitor than among those who did not receive this treatment (15). Considering the present results, it may be necessary to investigate the *COX-2* -1195 genetic polymorphism status when deciding upon a treatment strategy for NSCLC in the future.

This study has several limitations. First, the sample size may not be sufficiently large. The sample size of this study was smaller than that of a previous study (14) in which the correlation between the outcome of patients with unresectable NSCLC and the *COX-2* polymorphism status was investigated. Second, the present study included only cases of resectable, relatively early-stage NSCLC and did not include any advanced NSCLC cases. Thus, our results may not be representative of NSCLC in general. Our results should thus be validated for a range of disease stages in the future.

In conclusion, our results showed significant differences in intratumoral Treg expression among NSCLC patients with different *COX-2* -1195G/A genotypes. The tumor-infiltrating Treg count was significantly higher among the -1195AA genotype group, regardless of the COX-2 expression level. These findings suggest that the *COX-2* -1195G/A polymorphism is a potential regulator of the infiltration of Tregs into NSCLC and that it may affect patient prognosis through its influence on Treg infiltration in NSCLC.

Acknowledgements

The authors thank Keiko Isoda for the technical assistance.

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Shimizu et al. World Journal of Surgical Oncology 2014, 12:343
http://www.wjso.com/content/12/1/343

WORLD JOURNAL OF SURGICAL ONCOLOGY

RESEARCH Open Access

Difference in prognostic values of maximal standardized uptake value on fluorodeoxyglucose-positron emission tomography and cyclooxygenase-2 expression between lung adenocarcinoma and squamous cell carcinoma

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Abstract

Background: The maximal standardized uptake value (SUVmax) on fluorodeoxyglucose-positron emission tomography (FDG-PET) for primary tumors is correlated with clinicopathological and prognostic factors in patients with non-small cell lung cancer. However, previous investigations have discussed the role of SUVmax without distinguishing among the histological subtypes of lung cancer. Herein, we investigated the correlations among the SUVmax on FDG-PET, clinicopathological or prognostic factors, and the expression of tumor angiogenic biomarkers according to histological subtypes.

Methods: We conducted a retrospective review of data from 52 patients with invasive adenocarcinoma (ADC) and 32 patients with squamous cell carcinoma (SQC) measuring less than 3 cm in diameter. Immunohistochemical staining for cyclooxygenase-2 (Cox-2), Ki-67, and vascular endothelial growth factor, which might influence cancer progression, was performed and the correlations between the expressions of these biomarkers and the SUVmax were evaluated.

Results: Among ADC patients, a statistically significant correlation was observed between the SUVmax and the major clinicopathological factors; among SQC patients, however, no statistically significant association was observed. The disease-free survival (DFS) period of the ADC patients with a high SUVmax was significantly poorer than that of the patients with a low SUVmax, but the DFS of the SQC patients with a high SUVmax was not significantly poorer. In a multivariate analysis, the pathological stage and the SUVmax were independent prognostic factors of the DFS among the ADC patients. Among the SQC patients, however, only Cox-2 expression was an independent prognostic factor of DFS.

Conclusions: Some clear differences in prognostic values of the SUVmax on FDG-PET and Cox-2 expression exist between patients with ADC and those with SQC. Based on these relationships between the SUVmax and clinicopathological or biological factors that influence cancer progression, the importance of the SUVmax appears to be quite different for patients with ADC and those with SQC.

Keywords: Non-small cell lung cancer, FDG-PET, SUV, Cox-2

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Background

Fluorodeoxyglucose-positron emission tomography (FDG-PET) has become an important tool for the diagnosis and staging of non-small cell lung cancer (NSCLC) [1]. The maximal standardized uptake value (SUVmax) on FDG-PET is the ratio of the activity in the tissue per unit volume relative to the injected dose according to body weight, and this parameter is widely used because of its simplicity. The SUVmax of primary tumors has been shown to be correlated with the stage, nodal status, histological type, differentiation, and progression of tumors in patients with NSCLC [2-4]. In addition, a high SUVmax has been reported to be a powerful prognostic factor in patients with NSCLC [4-6].

Recently, several studies have reported the existence of a relationship between FDG uptake and the expressions of some molecular biomarkers. Of these, the most famous biomarker related to the SUVmax is glucose transporter 1 [7]. Several other studies have investigated the correlation between FDG uptake and the expressions of biological markers of lung cancer, such as Ki-67, p53, and vascular endothelial growth factor (VEGF) [8-10]. In 2012, we demonstrated that the expression of cyclooxygenase-2 (Cox-2) in tumors was as strongly correlated with a poor clinical outcome as an increase in FDG uptake in lung adenocarcinoma [11].

However, these investigations discussed the role of SUVmax without distinguishing among histological subtypes of lung cancer. Therefore, we investigated the correlation among the expression of selective tumor biomarkers, the SUVmax on FDG-PET, and clinicopathological or prognostic factors according to histological subtypes, specifically adenocarcinoma (ADC) and squamous cell carcinoma (SQC).

Methods

Study population

A total of 52 patients with invasive ADC and 32 patients with SQC measuring less than 3 cm in diameter, who had undergone surgical resection with systematic lymph node dissection at Kawasaki Medical School Hospital between 2007 and 2010, were enrolled in this study. We restricted the tumor size to less than 3 cm in diameter because the SUVmax is known to be higher in large tumors [4,12]. Furthermore, ADC was limited to radiologic 'invasive' ADC. Invasive ADC was defined based on the radiologic criteria of a consolidation-to-tumor ratio of greater than 0.50 [13]. None of the patients had received either radiotherapy or chemotherapy prior to the surgery. The histological diagnosis of the tumors was based on the criteria of the World Health Organization, and the TNM stage was determined according to the criteria established in 2009. Written informed consent was obtained from each patient for the study of the excised tissue samples from the surgical specimens. This study was conducted with the approval of the institutional ethics committee of Kawasaki Medical School. (number: 1396, approved on 13 May 13 2013).

Fluorodeoxyglucose-positron emission tomography

In our institute, all patients with lung cancer had undergone FDG-PET before surgery. However, patients with blood glucose levels of 150 mg/dL or more were excluded from positron emission tomography/computed tomography (PET/CT) acquisition. All PET/CT examinations were performed using a dedicated PET/CT scanner (Discovery ST Elite; GE Healthcare, Tokyo, Japan). PET/CT scanning was performed at 60 minutes after the intravenous injection of 150 to 220 MBq of ^{18}FDG (FDGscan, Universal Giken, Nihon Mediphysics, Tokyo, Japan). The regions of interest (ROI) were placed three-dimensionally over the lung cancer nodules. A semi-quantitative analysis of the images was performed by measuring the SUVmax of the lesions. The SUV was calculated based on the following equation:

$$\text{Tumor activity concentration}/(\text{Injected dose}/\text{Body weight})$$

Immunohistochemical staining

Immunohistochemical analyses of resected, paraffin-embedded lung cancer tissues were performed. After microtome sectioning (4 μm thickness), the slides were processed for staining using an automated immunostainer (Nexes; Ventana, Tucson, Arizona, United States). The streptavidin-biotin-peroxidase detection technique, using 3,3'-diaminobenzidine as the chromogen, was applied. The primary antibodies were used according to the manufacturer's instructions (Cox-2: DakoCytomation, CX-294, CA, USA, 1/50 dilution; Ki-67: DakoCytomation,

Table 1 Patient characteristics

Factor	All cases	ADC	SQC	*P* value
Number	84	52	32	
Age (Mean ± SD)		66.7 ± 8.6	72.7 ± 7.8	0.002
Sex				<0.001
Male	56	24	32	
Female	28	28	0	
Tumor size (Mean ± SD)		22.4 ± 6.9	21.2 ± 6.9	0.423
Pathological stage (%)				0.595
IA	55	36(69)	19(60)	
IB	14	8(15)	6(19)	
IIA + B	6	3(6)	3(9)	
IIIA + B	9	5(10)	4(12)	
SUVmax (Mean ± SD)	7.4 ± 4.7	6.6 ± 5.2	8.8 ± 3.7	0.032

ADC: adenocarcinoma, SQC: squamous cell carcinoma.

MIB-1, CA, USA, 1/100 dilution; VEGF: Santa Cruz, sc-152, CA, USA, 1:300 dilution). The slides were examined by two investigators who had no knowledge of the clinicopathological data. The expression of each marker protein was examined and evaluated according to a previously reported original protocol. For Cox-2, the slides were scored for the intensity of staining (0 to 3) and the percentages of cells with scores of zero (0%), one (1 to 9%), two (10 to 49%), and three (50 to 100%) were determined. The immunohistochemistry (IHC) score (zero to nine) was defined as the product of the intensity and percentage of the cells. Cox-2 expression was judged as positive when the IHC score was four or more [14]. The labeling index of Ki-67 was measured by determining the percentage of cells with positively stained nuclei. Ki-67 expression was judged as positive when more than 10% of the cancer cell nuclei showed positive staining [15]. VEGF expression was judged as positive when more than 20% of the cancer cell cytoplasm showed positive staining [16].

Statistical analysis

All the statistical analyses were performed using the SPSS statistical package (version 17.0; SPSS, Chicago, Illinois, United States). Frequencies were compared using the chi-square test for categorical variables, and the Fischer exact test was applied for small samples. Mann-Whitney U tests were performed when comparing continuous variables. Receiver operating characteristic (ROC) curves of the SUVmax for the prediction of recurrence were generated to determine the cutoff value that yielded an optimal

Table 2 Relationship between the SUVmax and clinicopathological/IHC findings

Factor	ADC (n =52)	SUVmax (Mean ± SD)	*P* value	SQC (n =32)	SUVmax (Mean ± SD)	*P* value
Age			0.778			0.747
<70 years	30	6.4 ± 5.1		11	8.4 ± 4.5	
≥70 years	22	6.9 ± 5.3		21	8.9 ± 3.4	
Sex			0.230			-
Male	24	7.6 ± 5.5		32	8.8 ± 3.7	
Female	28	5.8 ± 4.8		0		
Tumor differentiation			0.008			0.565
Well	30	4.1 ± 3.3		2	12.0 ± 2.1	
Moderate	16	8.5 ± 5.4		21	8.3 ± 3.9	
Poor	6	14.1 ± 2.7		9	9.1 ± 3.6	
Pleural invasion			0.001			0.077
No	38	5.0 ± 4.0		26	8.2 ± 3.7	
Yes	14	11.1 ± 5.4		6	11.2 ± 3.2	
Vascular invasion			0.001			0.602
No	33	4.7 ± 4.0		18	9.1 ± 3.8	
Yes	19	9.9 ± 5.5		14	8.4 ± 3.7	
Nodal status			0.007			0.075
Negative	44	5.6 ± 4.6		26	8.1 ± 3.5	
Positive	8	12.0 ± 4.9		6	11.6 ± 3.7	
Cox-2 expression			<0.001			0.048
Negative	18	2.8 ± 2.4		19	7.7 ± 3.6	
Positive	34	8.7 ± 5.1		13	10.3 ± 3.5	
Ki-67 expression			0.001			0.016
Negative	26	4.3 ± 3.3		15	7.0 ± 4.0	
Positive	26	9.0 ± 5.7		17	10.3 ± 2.9	
VEGF expression			0.004			0.719
Negative	17	3.9 ± 4.1		16	9.0 ± 4.3	
Positive	35	8.0 ± 5.1		16	8.5 ± 3.3	

ADC: adenocarcinoma, Cox-2, cyclooxygenase-2; IHC, immunohistochemical; SQC: squamous cell carcinoma; SUVmax, maximal standardized uptake value; VEGF, vascular endothelial growth factor.