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

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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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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'-CCACTTCTTTTCTGGTGTGTG-3' and -1290R; 5'-GGGAGATTTTGACAGTTGGAA-3'; -765F, 5'-CCAAAATAATCCACGCATCA-3' and -765R; 5'-TACCTTCACCCCCTCCTTG-3'; 1759F, 5'-GGGCTGTCCCTTTACTTCATT-3' and 1759R, 5'-GACTCCTTTCTCCGCAACA-3'; 8473F, 5'-TGTCACAAGATGGCAAAATGC-3' and 8473R, 5'-GCTTTTACAGGTGATTCTACCCTATGA-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).

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.

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

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

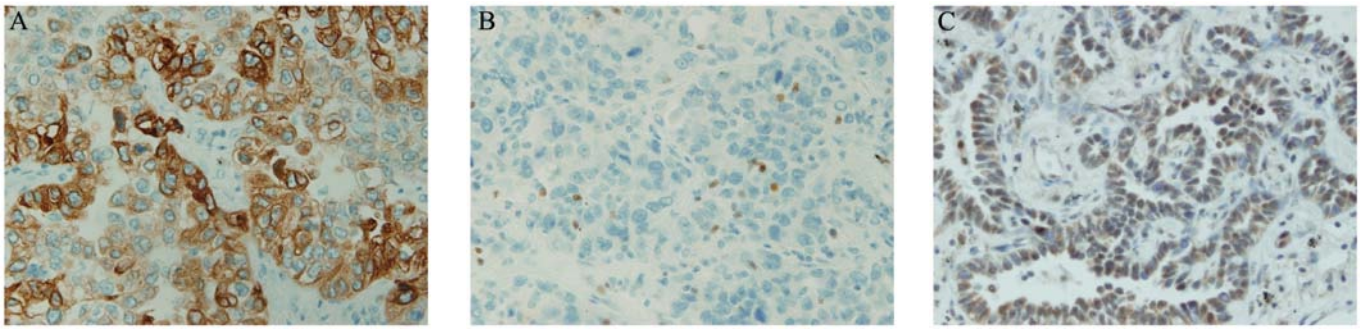

Figure 1. Immunohistochemical staining of (A) cyclooxygenase-2 (COX-2), (B) regulatory T cells (Tregs) and (C) Ki-67. Magnification, x200.

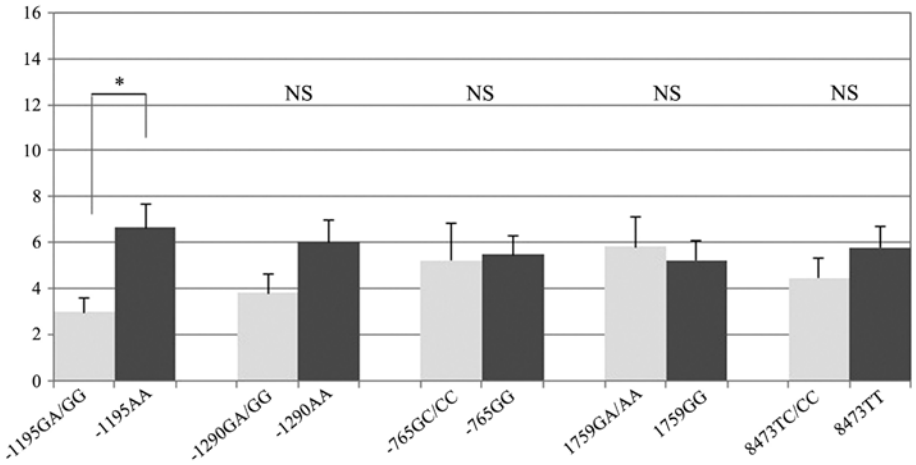

Figure 2. Association between cyclooxygenase-2 (COX-2) single nucleotide polymorphisms (SNPs) and regulatory T cell (Treg) score. *P=0.003.

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.

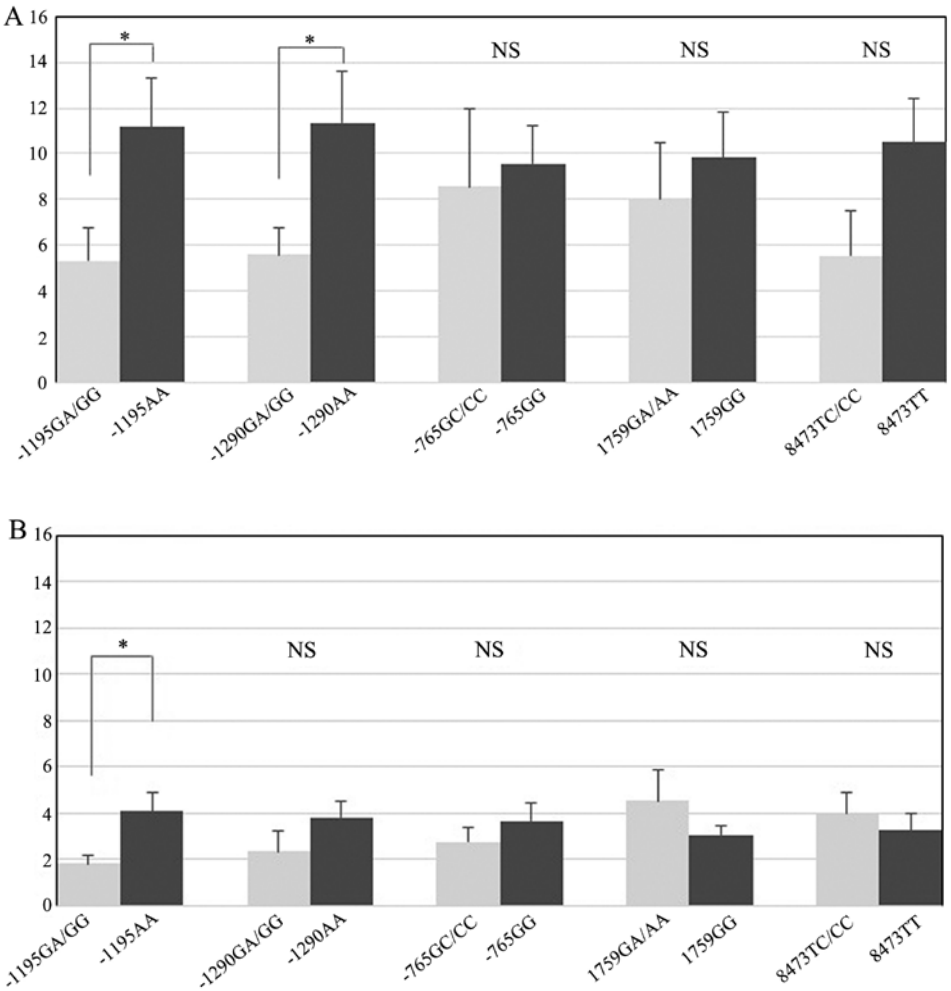

Figure 3. Association between cyclooxygenase-2 (COX-2) single nucleotide polymorphisms (SNPs) and regulatory T cell (Treg) score according to COX-2 expression. (A) COX-2-positive group, (B) COX-2-negative group. *P=0.033 in A; *P=0.011 in B.

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.

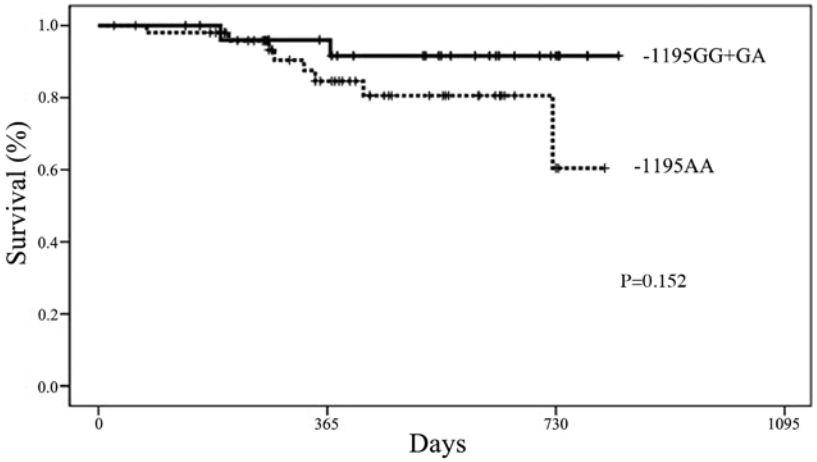

Figure 4. Disease-free survival of the patients with different cyclooxygenase-2 (COX-2) -1195G/A genotypes.

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

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