

Urinary levels of prostaglandin E₂ are positively correlated with intratumoral infiltration of Foxp3⁺ 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 one of the factors influencing the prognosis of patients with cancer. The tumor-infiltrating regulatory T cell (Treg) count has previously been revealed to be positively correlated with intratumoral cyclooxygenase-2 (Cox-2) expression, and was also associated with poor survival among patients with non-small cell lung cancer (NSCLC). In addition, the urinary levels of a prostaglandin E₂ (PGE₂) metabolite (PGE-M) were used as a biomarker in clinical trials of the Cox-2 inhibitor celecoxib. In the current prospective study, the association of urinary PGE₂ and PGE-M levels with intratumoral Cox-2 expression and Treg count was examined in patients with NSCLC. A total of 21 patients with NSCLC who underwent complete resection of the tumor at Kawasaki Medical School Hospital (Kurashiki, Japan) were enrolled. Urine specimens were obtained prior to surgery in order to examine urinary PGE₂ and PGE-M levels. A significant positive association was observed between urinary PGE₂ levels and the intratumoral Treg count ($P=0.023$), but not the intratumoral Cox-2 expression levels. No significant associations were identified between urinary PGE₂ levels and any of the other clinicopathological characteristics examined, including age, sex, smoking history, histology, tumor size, nodal status and disease stage. However, no significant association was observed between urinary PGE-M levels and the intratumoral Treg count ($P=0.069$) or Cox-2 expression. In conclusion, urinary PGE₂ levels were positively correlated with intratumoral Treg counts in patients with NSCLC in the current study. This indicates that urinary PGE₂ may be an improved biomarker, compared with PGE-M, for the prediction of intratumoral Treg numbers.

Introduction

Lung cancer is a major cause of mortality in developed countries (1). Surgical resection is the prominent curative treatment option for this type of disease, particularly during the early stages of non-small cell lung cancer (NSCLC) (1). However, the 5-year survival rate for patients with NSCLC who undergo surgery remains ~70% (1,2). Several biomarkers have been reported as predictors of survival and recurrence in patients with NSCLC, including tumor-infiltrating regulatory T cells (Tregs) (3). A number of previous studies have demonstrated that the immune microenvironment of the primary tumor is a significant prognostic factor. Immunological biomarkers in the tumor microenvironment are useful prognostic predictors, in addition to promising targets for novel therapeutic approaches (4-9). In particular, a promising immunological biomarker may be Tregs; the potential mechanism underlying the induction of Tregs is the expression of cyclooxygenase-2 (Cox-2) in tumor cells (10). These findings may facilitate the development of individualized immunomodulatory therapies to deplete the tumor microenvironment from Tregs.

A major limitation to individualized immunomodulatory therapies is the requirement for adequate tumor specimens, which frequently necessitates an invasive procedure (11). Furthermore, in patients with recurrent disease, further tissue specimens are required; however, rebiopsies are difficult to perform in certain cases, including those with brain metastasis (11). Urinary prostaglandin E₂ (PGE₂) metabolite (PGE-M) is a major urinary metabolite of PGE₂ and may be used as an index of systemic PGE₂ production (11). Cox-2-derived PGE₂ serves important roles in cancer progression. PGE₂ is an unstable compound that is rapidly metabolized to stable PGE-M *in vivo* by the enzyme 15-hydroxyprostaglandin dehydrogenase (12). Furthermore, the direct quantification of PGE₂ levels has been revealed to be an unreliable indicator of a biomarker of inflammation caused by infection or malignancy (12); therefore, several previous studies have used measurements of urinary PGE-M instead (13,14).

In the present study, urinary PGE₂ levels were directly quantified using a highly sensitive PGE₂ ELISA kit, to investigate whether urinary PGE₂ levels were associated with the expression of Cox-2 protein or levels of Tregs in patients with NSCLC.

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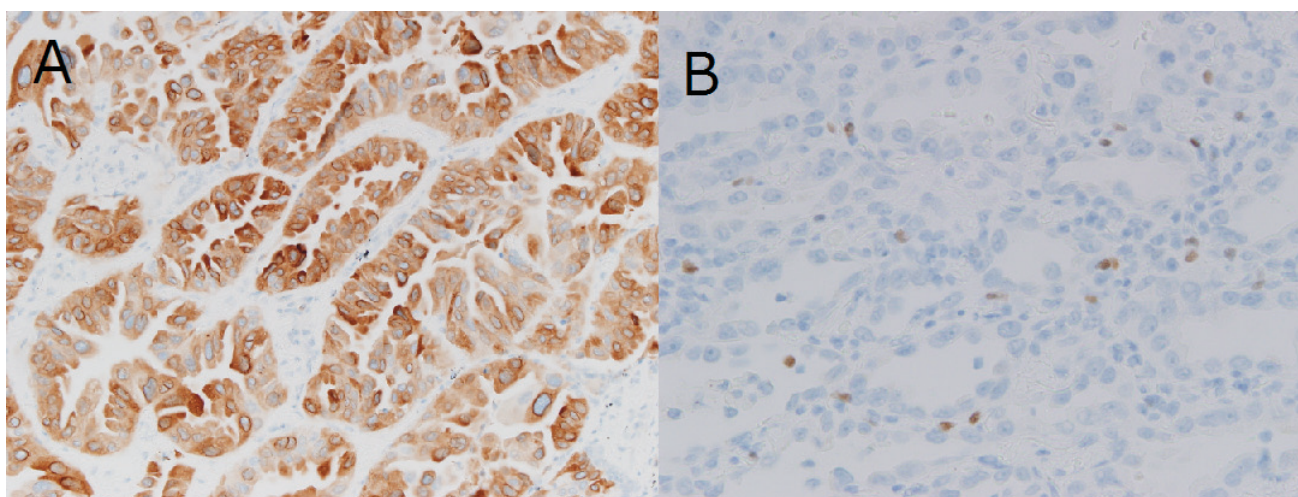


Figure 1. Immunohistochemical staining revealed high levels of (A) cyclooxygenase-2 expression and (B) tumor-infiltrating forkhead box P3⁺ regulatory T-cells. Magnification, x200.

Patients and methods

Study population. Urinary and paraffin-embedded tumor samples were obtained from 21 consecutive patients with NSCLC who underwent surgical resection at Kawasaki Medical School Hospital (Kurashiki, Japan) between September 2014 and March 2015. None of the patients had received radiotherapy or chemotherapy prior to surgery. This prospective study was conducted with the approval of the Institutional Ethics Committee of Kawasaki Medical School, and informed consent for the use of urine and tumor specimens was obtained from all patients. The histological diagnosis of the tumors was based on the criteria of the World Health Organization, and the tumor-node-metastasis (TNM) stage was determined according to the criteria established in 2009 (15). Fluorodeoxyglucose (¹⁸FDG)-positron emission tomography-computed tomography scanning was used to calculate the maximal standardized uptake value (SUV_{max}). Scanning was performed 60 min following intravenous injection of 150-220 MBq of ¹⁸FDG. The regions of interest were placed three-dimensionally over the lung cancer nodules.

Patients were excluded from enrollment if they were taking, or had a history of regularly taking, aspirin or other nonsteroidal anti-inflammatory drugs (NSAID). Patients were also ineligible if they had concurrent severe or uncontrolled medical diseases, including active systemic infection, diabetes or renal failure.

Measurement of urinary PGE₂ and PGE-M via ELISA. The urine samples were obtained prior to surgery and stored at -20°C following centrifugation at 500 x g for 5 min at room temperature. The urinary PGE₂ level was determined using a Correlate-EIA™ PGE₂ Enzyme Immunoassay kit (Assay Designs; Enzo Life Sciences, Inc., Farmingdale, NY, USA) according to the manufacturer's instructions. Plates were read at an absorbance wavelength of 450 nm (Varioskan® Flash Spectral Scanning Multimode Reader; Thermo Scientific, Inc., Waltham, MA, USA). The urinary PGE₂ level was calculated in pg/ml, according to the protocol provided by the manufacturer of the assay kit. In addition, measurement of urinary

PGE-M levels were performed at SRL, Inc. (Tokyo, Japan), and expressed in pg/ml.

Immunohistochemical (IHC) analysis. IHC analyses were performed using resected, paraffin-embedded lung cancer tissues. Following microtome sectioning, the tissue slides (4-μm-thick) were stained for Cox-2 and forkhead box P3 (Foxp3), a marker of Tregs, using an automated immunostainer (NexES Special Stainer; Ventana Medical Systems, Inc., Tucson, AZ, USA) according to the manufacturer's instructions. Slides were de-paraffinized using EZprep solution (Ventana Medical Systems, Inc.) for 30 min at 75°C. Epitope retrieval was accomplished on the automated stainer with cell conditioning 1 solution (Ventana Medical Systems, Inc.) for 60 min at 95°C. The antibodies were transferred with diluent to user-fillable dispensers for use on the automated stainer. Slides were developed using the Optiview DAB IHC detection kit (Ventana Medical Systems, Inc.). Briefly, the slides were treated with the inhibitor included was for 4 min, the multimer for 12 min, DAB/peroxide for 8 min and copper solution for 4 min at 37°C. Slides were subsequently counterstained with hematoxylin II (Ventana Medical Systems, Inc.) for 4 min at 37°C.

Primary antibodies directed against Cox-2 (dilution, 1:50; catalog no., CX-294; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) and Foxp3 (dilution, 1:100; catalog no., 22510; Abcam, Cambridge, UK) were used at 37°C according to the manufacturer's protocol. Secondary antibody (Discovery Universal Secondary Antibody, Ventana Medical Systems, Inc.; catalog no., 760-4205) was used at 37°C according to the manufacturer's protocol. The expression levels of each marker protein were examined and evaluated according to a previously reported original protocol (16,17). For Cox-2, the slides were scored according to the intensity of staining (0-3) and the percentage of positively stained cells (0, 0%; 1, 1-9%; 2, 10-49%; and 3, 50-100%). The IHC score (0-9) was calculated as the product of multiplying the intensity and percentage scores. Cox-2 expression was considered positive when the IHC score was ≥4 (16). To evaluate the immunostaining of the Tregs, digital high-power field (HPF) images of the tumor area

Table I. Clinicopathological characteristics of the patients (n=21).

Clinicopathological characteristic	No. of patients (%)
Age	
<70	11 (52.4)
≥70	10 (47.6)
Sex	
Male	8 (38.0)
Female	13 (62.0)
Tumor histology	
Adenocarcinoma	18 (85.7)
Squamous cell carcinoma	3 (14.3)
Tumor stage	
T1	11 (52.2)
T2	9 (43.0)
T3	1 (4.8)
Pathological lymph node status	
N0	17 (80.9)
N1	1 (4.8)
N2	3 (14.3)
Pathological tumor stage	
IA	10 (47.7)
IB	5 (9.5)
II (A+B)	2 (23.8)
IIIA	4 (19.0)
Surgical procedure undergone	
Lobectomy	20 (95.2)
Wedge resection	1 (4.8)

SD, standard deviation; T, tumor; N, node.

were taken using a light microscope (Axiophot microscope; Carl Zeiss AG, Oberkochen, Germany), of which 10 were selected and the absolute number of Foxp3⁺ lymphocytes in these images determined (17). The number of immunostained Foxp3 cells was then determined as the mean count from the images and used to obtain the tumor-infiltrating Foxp3⁺ Treg count (Treg score; 0-24). IHC staining demonstrated high levels of Cox-2 expression (Fig. 1A) and Foxp3⁺ Tregs (Fig. 1B).

Statistical analysis. All statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). The χ^2 test and Fisher's exact test were used to examine the association between urinary PGE₂ or PGE-M levels and various clinicopathological parameters of the patients. P<0.05 was considered to indicate a statistically significant difference.

Results

Patient clinicopathological characteristics. Clinicopathological characteristics of the patients are summarized in Table I. The patients ranged in age from 40-83 years old (mean, 69.1 years), and included 8 males and 13 females. Adenocarcinoma was

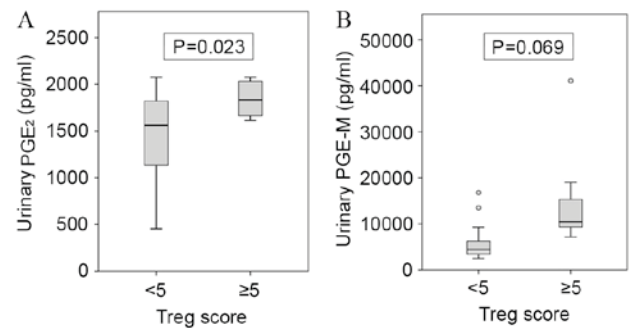


Figure 2. Association of (A) urinary PGE₂ and (B) PGE-M levels with the tumoral Treg count. The results are presented as the mean ± standard deviation. Treg, regulatory T cell; PGE₂, prostaglandin E₂; PGE-M, PGE₂ metabolite.

detected in 18 patients (85.7%) and squamous cell carcinoma was observed in 3 patients (14.3%). Pathological lymph node N0 disease was detected in 17 patients (80.9%), and N1 or N2 disease in 4 patients (19.1%). Pathological stage I disease was observed in 15 patients (71.5%), and stage II or stage IIIA disease was detected in 6 patients (28.5%).

Association between clinicopathological characteristics and urinary PGE₂/PGE-M levels. No significant correlation was observed between the urinary PGE₂ and PGE-M levels ($r=0.372$; $P=0.097$; data not shown). However, urinary PGE₂ levels ($P=0.023$), but not the urinary PGE-M levels ($P=0.069$), were significantly positively correlated with Treg score (Fig. 2 and Table II). The mean value of the urinary PGE₂ level was 1467 ± 478 pg/ml in the group with a Treg score <5 ($n=13$) and 1844 ± 204 pg/ml in the group with a Treg score ≥ 5 ($n=8$) (Table II). No significant association was observed between the urinary PGE₂ levels and the Cox-2 IHC score ($P=0.986$; Table II). In addition, no significant associations were identified between urinary PGE₂ and any of the other clinicopathological characteristics examined, including age ($P=0.863$), sex ($P=0.265$), smoking history ($P=0.465$), histology ($P=0.094$), tumor size ($P=0.524$), nodal status ($P=0.395$), disease stage ($P=0.680$) and the SUVmax ($P=0.308$) (Table II).

The mean urinary PGE-M level was $6,162 \pm 4,468$ pg/ml in the group with a Treg score <5 ($n=13$), and $14,799 \pm 11,192$ pg/ml in the group with a Treg score ≥ 5 ($n=8$) (Table II). No significant association was observed between the urinary PGE-M levels and the Cox-2 IHC scores ($P=0.657$) or any of the other clinicopathological characteristics examined (Table II).

Discussion

In 2010, it was demonstrated that the tumor-infiltrating Foxp3⁺ Treg count (Treg score) was positively correlated with intratumoral Cox-2 expression, and was also associated with recurrence-free survival, particularly in patients with lymph node-negative NSCLC (10). In the present study, the association of urinary PGE₂ levels with the Cox-2 IHC score and Treg score were examined in 21 consecutive patients with NSCLC who underwent surgical tumor resection at Kawasaki Medical School Hospital. The results revealed a significant association between the urinary PGE₂ levels

Table II. Association of urinary PGE₂ and PGE-M levels with the clinicopathological characteristics of patients with non-small cell lung cancer.

Clinicopathological characteristic	No. of patients	Urinary PGE ₂ (pg/ml)	P-value	Urinary PGE-M (pg/ml)	P-value
Age			0.863		0.177
<70	11	1,594±384		6,818±3,225	
≥70	10	1,629±501		12,349±11,651	
Sex			0.265		0.423
Female	13	1,529±451		8,400±10,278	
Male	8	1,744±392		11,161±5,076	
Smoking history			0.465		0.785
Never smoked	14	1,561±450		9,157±10,274	
Smoker	7	1,709±409		10,041±4,285	
Histology			0.094		0.854
Adenocarcinoma	18	1,581± 77		9,334±9,095	
Squamous cell carcinoma	3	1,788±392		10,160±6,247	
Tumor size			0.524		0.741
T1	11	1,672±295		10,052±10,920	
T2-3	10	1,543±557		8,792±5,628	
Pathological nodal status			0.395		0.271
N0	17	1,679±335		10,329±9,132	
N1+2	4	1,321±715		5,725±5,260	
Pathological stage			0.680		0.332
I	15	1,645±342		10,354±9,757	
II+IIIA	6	1,525±640		7,197±4,701	
SUVmax			0.308		0.693
<5	10	1,506±458		8,607±1,1652	
≥5	11	1,705±406		10,220±5,004	
Cox-2 score			0.986		0.657
<4	14	1,609±416		9,924±10,312	
≥4	7	1,613±499		8,509±3,963	
Treg score			0.023		0.069
<5	13	1,467±478		6,162±4,468	
≥5	8	1,844±204		14,799±11,192	

Treg, regulatory T cell; PGE₂, prostaglandin E₂; PGE-M, PGE₂ metabolite; Cox-2, cyclooxygenase-2; SUVmax, maximal standardized uptake value from fluorodeoxyglucose-positron emission tomography.

and Treg score. In addition, to the best of our knowledge, the current study was the first to use the urinary PGE₂ level, and not the PGE-M level, for the assessment of prognosis in patients with NSCLC.

Tregs were initially characterized as possessing a CD4⁺CD25⁺ phenotype and are considered to modulate the antitumor immune response (18). Tregs are able to suppress the activity of cytotoxic T cells through direct cell-to-cell contact or via the release of cytokines (19). The most specific Treg cell marker currently identified is the nuclear transcription factor Foxp3 (19,20). A high density of tumor-infiltrating Foxp3⁺ Tregs has been reported to be associated with a higher risk of recurrence and a poorer overall survival in patients with NSCLC (21). Sharma *et al* (22) demonstrated that

tumor-derived Cox-2/PGE₂ induces the expression of Foxp3 and increases Treg activity in lung cancer.

Cox-2-derived PGE₂ has been demonstrated to be important in cancer progression (23). Previous studies have suggested that the majority of PGE₂ formed *in vivo* is derived from Cox-2 (24,25). Urinary PGE-M levels in healthy patients or patients with lung cancer are suppressed significantly by nonselective Cox inhibitors, including aspirin, and by Cox-2-selective inhibitors (25). As the antitumor effects of NSAIDs depend on the inhibition of Cox-2 and subsequent reduction in the quantity of PGE₂ produced, urinary PGE-M levels may serve as a valuable intermediate marker of the pharmacological activity of NSAIDs. A previous phase II clinical trial revealed that patients with NSCLC exhibiting complete and partial responses to

adjuvant therapy with carboplatin, paclitaxel and celecoxib had significantly decreased urinary PGE-M levels (26). In another phase II clinical trial of combined treatment with celecoxib and docetaxel, patients with recurrent NSCLC with the greatest proportional decline in urinary PGE-M levels exhibited a longer survival time, compared with patients with no change or an increase in urinary PGE-M levels (27). These findings indicate that urinary PGE-M is a potential biomarker for predicting the efficacy of Cox-2 inhibitors in adjuvant therapies.

Depleting Tregs via targeting C-C motif chemokine receptor 4 (CCR4) may be a potential cancer immunotherapy, as CCR4 is highly expressed on the surface of type 2 helper T cells and Tregs (28). Mogamulizumab, a humanized anti-CCR4 monoclonal antibody, has been demonstrated to reduce the numbers of CCR4⁺ malignant T cells and Tregs in cutaneous T-cell lymphoma (28). However, to the best of our knowledge, no previous studies have examined whether urinary PGE₂ may serve as a potential biomarker for predicting the efficacy of Treg-targeting therapy.

There were several limitations of the current study. Firstly, the sample size was small compared with previous studies. Secondly, urinary PGE₂ levels were directly quantified in the current study, whilst previous studies have evaluated the urinary PGE-M levels in patients with cancer (11-14). To the best of our knowledge, this is the first study to utilize the direct quantification of urinary PGE₂ levels. The direct quantification of PGE₂ levels has been revealed to be an unreliable indicator, however, the optimal method for the assessment and use of this marker remains to be established (12).

In the current study, urinary PGE₂ levels were not associated with tumor Cox-2 expression levels. Numerous single nucleotide polymorphisms (SNPs) in the Cox-2 gene have been identified, which may contribute to divergent Cox-2 expression levels and PGE₂ activities in patients with cancer (29). Compared with patients with esophageal tumors harboring the Cox-2-1195G, carriers of the Cox2-1195AA variant exhibit significantly increased Cox-2 expression levels (29). In 2012 it was reported that Cox-2 SNPs contributed significantly to increased tumor infiltration by Tregs (30). The results from a previous study revealed that the AA genotype group exhibited a significantly higher Treg score compared with the GA/GG group, independent of the intratumoral Cox-2 expression levels (30). The results of the present study revealed that urinary PGE₂ levels were positively correlated with tumor Treg expression, but not Cox-2 expression. This may be attributable to SNPs in the Cox-2 gene.

In conclusion, that present study demonstrated that urinary PGE₂ levels were positively correlated with intratumoral Treg count in patients with NSCLC. In addition, urinary PGE₂ levels may be an improved biomarker, relative to PGE-M, for the prediction of intratumoral Treg expression. Additional studies in larger patient populations are required to evaluate the efficacy of urinary PGE₂ as a biomarker in this regard.

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