

Periostin Expression in Non-Small Cell Lung Cancer: Clinical Significance

DAIGO MURAKAMI, SHINZO TAKAMORI, AKIHIKO KAWAHARA*, MASAHIRO MITSUOKA,
MASAKI KASHIHARA, KOICHI YOSHIYAMA, RYOICHI MATSUMOTO,
SHINTARO YOKOYAMA, KIMINORI FUJIMOTO**, ATSUSHI KAWAGUCHI†,
KENJI IZUHARA‡ AND YOSHITO AKAGI

*Department of Surgery, Kurume University School of Medicine, *Department of Diagnostic Pathology, Kurume University Hospital,*

***Department of Radiology, Kurume University School of Medicine, Kurume, 830-0011 Japan*

*†Center for Comprehensive Community Medicine, ‡Division of Medical Biochemistry, Department of Biomolecular Sciences,
Faculty of Medicine, Saga University, Saga, 849-8501 Japan.*

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Summary: Periostin is an extracellular matrix N-glycoprotein that is a major constituent of the desmoplastic stroma around solid tumors. Periostin promotes tumor invasion and metastasis via epithelial-mesenchymal transition. The aims of this study were to evaluate periostin expression immunohistochemically and quantitatively in patients with non-small cell lung cancer (NSCLC) and to assess any associations with clinical features and prognosis. A total of 184 specimens of NSCLC tissue were investigated, including 134 adenocarcinomas, 39 squamous cell carcinomas, and 11 other histologic subtypes. The intra-tumoral periostin expression area in each captured field was calculated using the image processing integration software WinROOF. The mean periostin expression score was classified as high or low by the median value of its expression area. Univariate analysis demonstrated that gender, tumor size, T status, N status, stage, histologic type, smoking habits, percent vital capacity, 1% forced expiratory volume, and pleural invasion were each significantly associated with periostin scores. Multivariate analysis revealed that high periostin expression score was an independent prognostic factor significantly associated with decreased cancer-specific survival (HR, 3.65; 95% CI, 1.04–12.84; P=0.0439). We concluded that intra-tumoral periostin expression was an independent prognostic factor for NSCLC.

Key words periostin, non-small cell lung cancer, survival, prognostic factor, multivariate analysis

INTRODUCTION

Periostin, also known as osteoblast-specific factor 2, is a 93.3-kDa extracellular matrix (ECM) protein, cloned from a murine osteoblast-like cell line in 1993 [1]. Periostin has been found to play an important role in tumor growth and to up-regulate various cancers [2]. Periostin is a ligand for integrin such as $\alpha V\beta 1$, $\alpha V\beta 3$, $\alpha V\beta 5$, and $\alpha 6\beta 4$, and activates the Akt/PKB and FAK-mediated signaling pathways. Periostin has

been reported to increase the adhesion, angiogenesis, invasion and metastasis of cancer cells [3-5]. It also induces neovascularization and supports tumor growth by inducing vascular endothelial growth factor receptor expression in vascular endothelial cells, while also promoting the survival of vascular endothelial cells through the Akt pathway [6-8]. Periostin supports oncogenesis not only by activating intracellular pathways but also through its effect on ECM desmoplasia. The desmoplastic stroma of a malignant neoplasm consti-

Corresponding author: Shinzo Takamori M.D., Ph.D., Department of Surgery, Kurume University School of Medicine, 67 Asahi-machi, Kurume-city, Fukuoka, 830-0011, Japan. Tel: +81-942-31-7566, Fax: +81-942-34-0709, E-mail: stakam@med.kurume-u.ac.jp

Abbreviations: CSS, cancer-specific survival; ECM, extracellular matrix; FEV, forced expiratory volume; HE, hematoxylin-eosin; NSCLC, non-small cell lung cancer; WHO, World Health Organization.

tutes a tumor microenvironment that supports tumor growth and invasion. Periostin forms a plexus of ECM by binding to tenascin-C and fibronectin [9, 10]. The excessive production of ECM components such as periostin may be involved in generating a microenvironment that supports the tumor [11]. Importantly, periostin is involved in the epithelium-mesenchymal transition of carcinoma cells [12-14]. Periostin was found to be over-expressed in various types of human cancers, including neuroblastoma, head and neck cancers, nasopharyngeal carcinoma, thyroid carcinoma, ovarian cancer, breast cancer, pancreatic ductal adenocarcinoma, and non-small cell lung carcinoma (NSCLC) [2,15,16]. NSCLC is the most common type of lung cancer, and its development and progression is a complex process in which the tumor microenvironment plays an important role. In this study, we immunohistochemically and quantitatively investigated periostin expression in NSCLC, and evaluated its clinical significance.

MATERIALS AND METHODS

Patients and Samples

The study was approved by the Ethics Committee of Kurume University (Research No. 11018). Specimens from a total of 184 consecutive patients with stage I–III NSCLC who underwent complete resection without preoperative therapy at Kurume University Hospital between 2007 and 2010 were reviewed. The patients consisted of 101 men and 83 women, with a median age of 68 years (age range: from 15 to 87 years). Tumor samples were classified according to the 2004 World Health Organization (WHO) histological classification and the 2010 7th TNM edition of the UICC. There were 134 adenocarcinomas, 39 squamous cell carcinomas, and 11 other histologic types (including 4 pleomorphic carcinomas, 2 typical carcinoids, 1 carcinosarcoma, 1 mucoepidermoid carcinoma, and 3 unclassified tumors). There were 79 patients at stage IA, 44 at stage IB, 21 at stage IIA, 13 at stage IIB, 20 at stage IIIA, and 7 patients at stage IIIB disease (Table 1).

Inpatient, outpatient and surgical medical records were reviewed for each patient. The gender, age, tumor size, TNM staging, WHO histological classification, pleural invasion, smoking status, pulmonary function, including percent vital capacity (%VC) and forced expiratory volume (FEV) 1%, and survival period were each investigated for all patients. Preoperative therapy was not performed in any patient. Postoperative adjuvant therapy including chemotherapy, radiation, or other therapy was performed in conformity

TABLE 1.
Patients Characteristics

Characteristic	Number (n = 184)	%
Sex		
Male	101	54.9%
Female	83	45.1%
Age (years)		
Median [range]	68 [25-87]	
< 65	77	41.8%
≥ 65	107	58.2%
Tumor status		
T1a	59	32.1%
T1b	29	15.8%
T2a	62	33.7%
T2b	12	6.5%
T3	20	10.9%
T4	2	1.0%
Lymph node status		
N0	144	78.3%
N1/N2/N3	17/18/5	21.7%
Stage		
IA/ IB	79/44	66.8%
IIA/ IIB	21/13	18.5%
IIIA/ IIIB	20/7	14.7%
Histology		
Adenocarcinoma	134	72.8%
Squamous cell carcinoma	39	21.2%
Other histologic type	11	6.0%
(Pleomorphic carcinoma 4, Typical carcinoid 2, Carcinosarcoma 1, Mucoepidermoid carcinoma 1, Unclassified 3)		
Pleural invasion		
p10	125	67.9%
p11/ p12/ p13	35/14/10	32.1%

with established criteria. Review of the postoperative clinical course was performed using the outpatient medical records and by inquiries by telephone or letter. A follow-up survey was completed in full by patients with a return rate of 100%. Primary tumor specimens were immediately fixed in 10% formalin and embedded in paraffin. Serial 5- μ m sections were prepared from each specimen, and used for routine hematoxylin-eosin (HE) staining and immunohistochemistry.

Immunohistochemistry for periostin

The monoclonal antibody used and the method of

immunohistochemistry have been reported previously [17,18]. Paraffin-embedded tissue samples were cut into 5 μm sections that were captured on a coated glass slide and labeled with anti-periostin antibody using the ChemMate ENVISION method (Dako Cytomation, Glostrup, Denmark). Intrinsic peroxidase activity was blocked by treating the sections with peroxidase-blocking reagent for 5 min. After washing with Tris-buffered saline for 10 min, the rat anti-human periostin monoclonal antibody of immunoglobulin G (clone no. SS19B or SS5D, produced in the laboratory of Biomolecular Sciences, Saga Medical School, Saga, Japan) was diluted 1:100 and applied to the sections. The histological specimens were incubated at room temperature for 30 min, washed with phosphate-buffered saline (PBS) for 15 min, and then incubated with labeled polymer-HRP secondary antibody for 30 min at room temperature. After washing with PBS for 10 min, the slides were visualized using 3, 3'-diaminobenzidine. Immunohistochemical results were evaluated by the authors without knowledge of the patients' clinical data.

Computer-assisted measurements of periostin expression areas

The greatest dimensions of the extracted specimens were evaluated. We photographed five fields of vision, and the periostin expression area in each field was examined microscopically at $\times 100$ magnification. The image processing integration software WinROOF Version 5.7 (Mitani Corp., Tokyo, Japan) was used to objectively quantify the areas of periostin expression in each field. This analysis of the periostin expression using WINROOF used a semi-quantitative method. The results were digitized and calculated as units $\mu\text{m}^2/\text{field}$. The average of the five areas of periostin expression was analyzed for periostin expression score in each individual case, and the mean value of periostin expression in the five fields was calculated as the overall periostin expression value.

Statistical analysis

All data regarding the clinical and histopathological variables were grouped using standard thresholds for laboratory parameters. Continuous data were presented as median and range, and category data were presented as counts and percentages. These data included gender, age, tumor size, tumor status, lymph node status, stage, histology, smoking status, %VC, FEV1%, and pleural invasion. The correlation between groups of patients and their periostin expression scores were analyzed using the Wilcoxon test for trends. Univariate and multivariate analyses with calculated

hazard ratios (HRs) and 95% confidence intervals (CIs) were performed using Cox's proportional hazards regression model to determine the association between each prognostic factor and clinical parameter. In the multivariate analyses, the survival analysis, tumor size, tumor status, lymph node status and pleural invasion were excluded because they were highly correlated with stage. Kaplan–Meier curves were used to plot cancer-specific survival (CSS), and these were compared using the log-rank test. To define the cut-off of periostin expression for the Harrell's concordance index, receiver operating characteristic analysis for survival data was computed. The CSS was defined as time duration until death due to lung cancer from the date of surgery, or until the most recent follow-up date for surviving patients. All analyses were performed using SAS 9.3 software (SAS Institute, Cary, NC, USA). *P* values below 0.05 were considered statistically significant.

RESULTS

In normal lung tissue, periostin was weakly expressed in parts of the bronchiolar basement membrane but not in pulmonary cells or alveolar macrophages. In lung cancer tissue, periostin was expressed in the mesenchymal areas, but not in the cancer cells. Lung adenocarcinoma sections were HE stained (Fig. 1A, 1B) and immunohistochemically stained for periostin (Fig. 1C, 1D). Periostin expression was found in the intratumoral stroma, while weak or negative staining was found in the peri-tumoral stroma and in the tumor epithelium. Similar results were observed in the squamous cell carcinoma sections (HE: Fig. 2A, 2B; periostin: Fig. 2C, 2D). The image processing integration software WinROOF showed similar areas of periostin expression in the two tumor types (Fig. 1E, Fig. 2E). The periostin expression score ranged from 2.1 to 311,350.7, and the median value was 43,265.5 (Fig. 3A, 3B). Periostin expression scores were classified as high or low using this median value as a cut-off.

Univariate analysis showed significant associations between periostin expression levels and male gender, tumor size, high T status, positive N status, high stage, squamous cell carcinoma, smoking, low %VC, low FEV1%, or positive pleural invasion (Table 2).

Cumulative CSS for the 184 patients according to periostin expression scores are shown in Figure 3. The 5-year CSS rate in patients with low periostin expression was 94.4%, but fell significantly to 77.6% in patients with high periostin scores (Fig. 4).

Univariate analysis of CSS demonstrated that age,

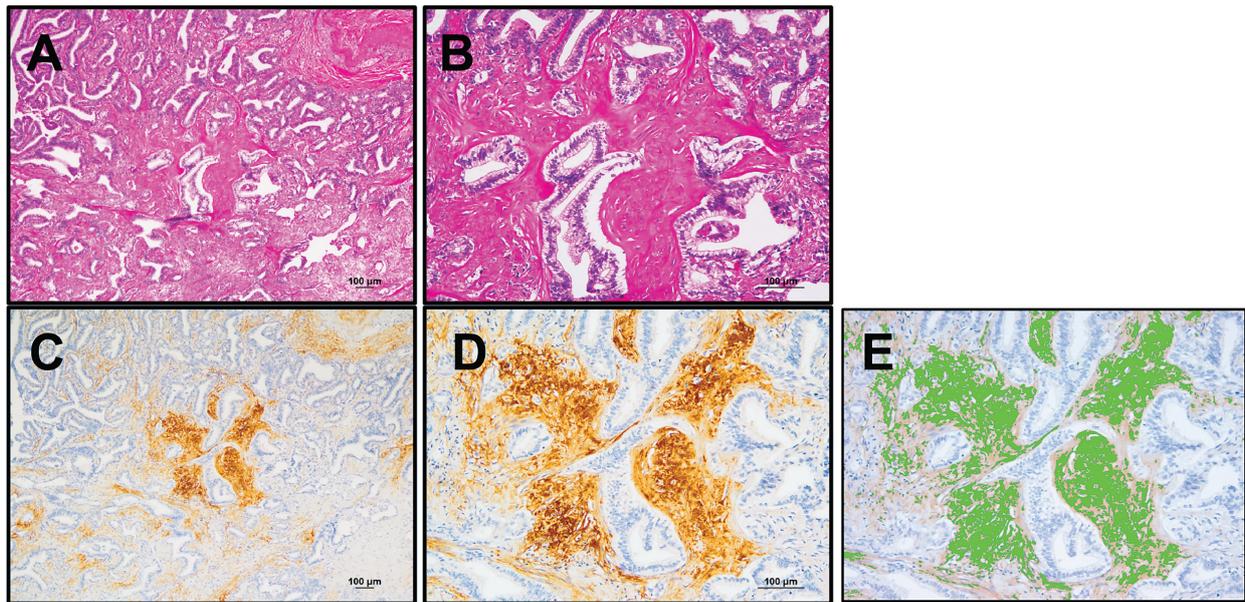


Fig. 1. Representative images of lung adenocarcinomas. A. Hematoxylin and eosin stained section ($\times 40$). B. Hematoxylin and eosin stained section ($\times 100$). C. Periostin immunohistochemistry ($\times 40$). D. Periostin immunohistochemistry ($\times 100$). E. Periostin expression labeled by WinROOF. The WinROOF labeled areas show similar periostin expression.

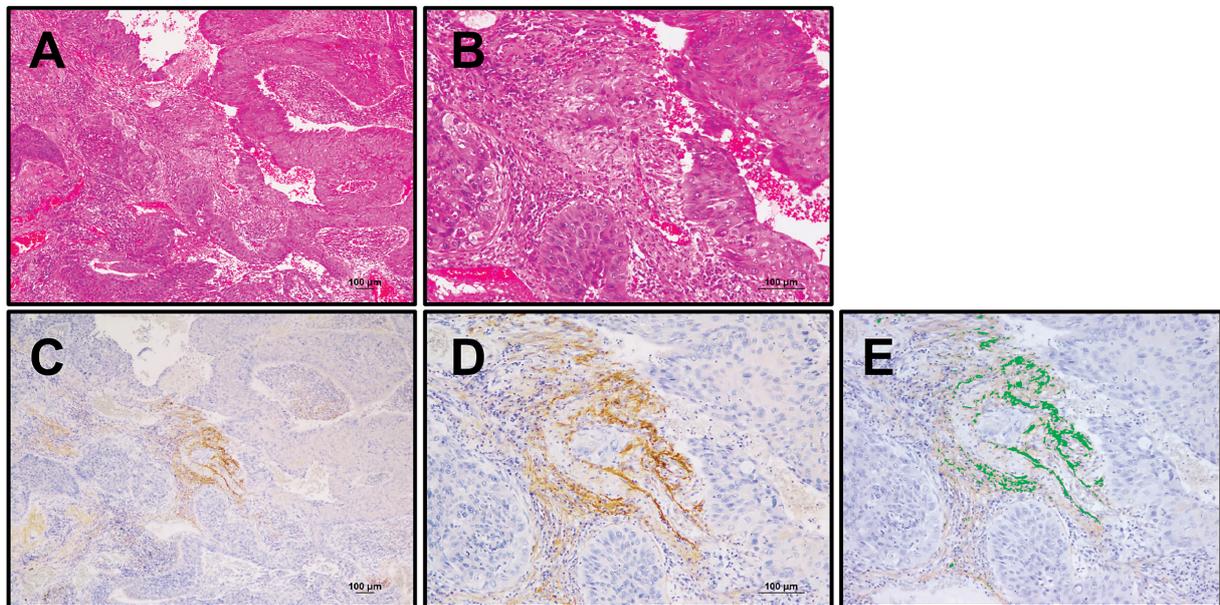


Fig. 2. Representative images of lung squamous cell carcinomas. A. Hematoxylin and eosin stained section ($\times 40$). B. Hematoxylin and eosin stained section ($\times 100$). C. Periostin immunohistochemistry ($\times 40$). D. Periostin immunohistochemistry ($\times 100$). E. Periostin expression labeled by WinROOF. The WinROOF labeled areas show similar periostin expression.

tumor size, T status, N status, stage, histology, %VC, positive pleural invasion or periostin expression score were each significantly associated with CSS. Multivariate analysis revealed that high periostin expres-

sion score, age, and stage were independent prognostic factors associated with CSS (HR, 3.65; 95% CI, 1.04–12.84; $p=0.0439$) (Table 3).

TABLE 2.
Univariate Analysis between Clinicopathological Features and Periostin Expression

Variables	Number	Median Periostin Expression Score	P value
Sex			
Male	101	68176.24	0.0001*
Female	83	22910.08	
Age (years)			
< 65	77	37496.60	0.4023
≥ 65	107	51841.20	
Tumor size			
< 2.5 cm	90	25622.11	0.0003*
≥ 2.5 cm	94	71171.85	
Tumor status			
T1/T2	162	37596.74	0.0035*
T3/T4	22	96187.39	
Lymph node status			
N0	144	33486.56	0.0049*
N1/N2/N3	40	81785.24	
Stage			
I	123	29306.78	0.0006*
II/III	61	76481.50	
Histology			
Adenocarcinoma	134	36660.67	0.0251*
Squamous cell carcinoma	39	72357.32	
Smoking Status			
Never-smoker	84	25622.11	0.0005*
Ever-smoker	100	65296.62	
%VC			
< 80	4	138187.40	0.0290*
≥ 80	180	39482.10	
FEV1.0%			
< 70	61	69986.38	0.0448*
≥ 70	123	37507.74	
Pleural invasion			
p10	125	29306.78	0.0045*
p11/ p12/ p13	59	72357.32	

Abbreviations: VC, vital capacity; FEV1.0%, forced expiratory volume
* $P < 0.05$

DISCUSSION

In this study, we used the image processing integration software WinROOF to objectively and quantitatively investigate periostin expression. The immunostaining results were captured as digital images, and then analyzed using WinROOF with macroinstruc-

tions for analyzing each captured area either immunolabeled with chromogen or counterstained with hematoxylin. The immunostained carcinoma cells were evaluated based on staining intensity and revealed periostin labeling index. The labeling index correlated highly to the immunohistochemical results for NSCLC. Periostin expression was observed in intra-tumoral stro-

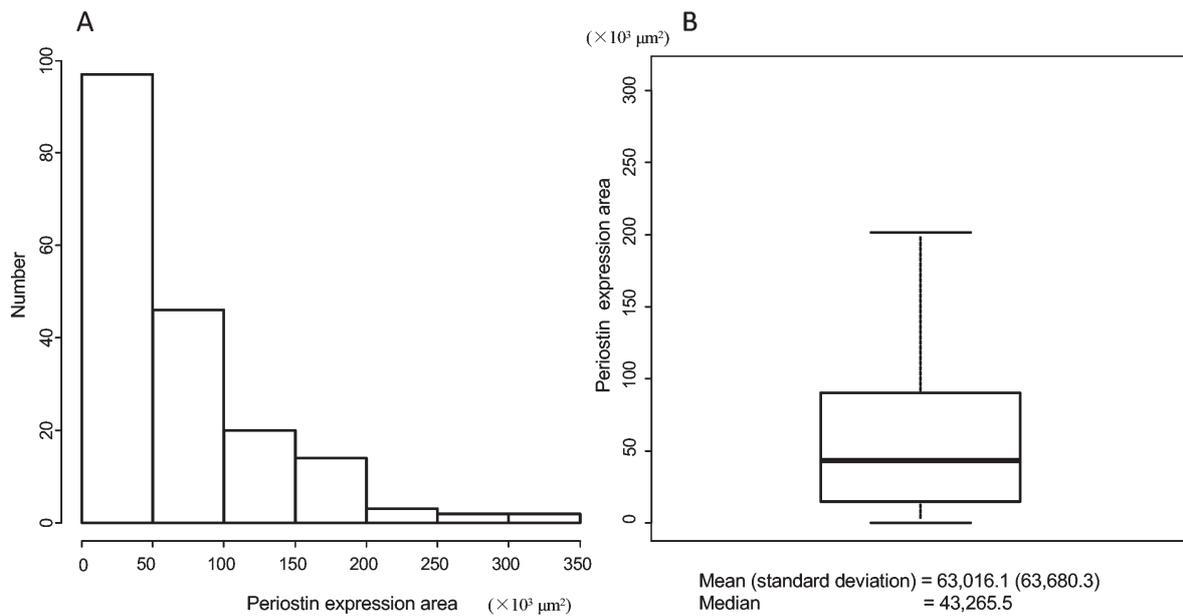


Fig. 3. Calculated periostin expression in resected lung tumors. A. Histogram of periostin expression scores. B. Five-number summary in box plot indicates minimum, lower quartile, median, upper quartile, and maximum. Periostin expression scores were divided into high or low by the median value.

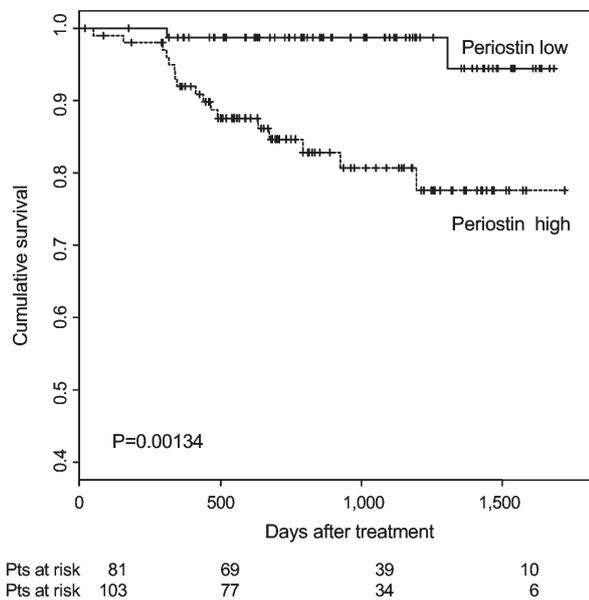


Fig. 4. Kaplan-Meier survival curves according to high or low periostin expression. For cancer-specific survival, patients with high periostin scores showed significantly lower survival rates than patients with low periostin scores.

periostin expression areas were similar in both adenocarcinomas and squamous cell carcinomas, showing that cytometric image analysis using WinROOF was applicable for immunohistochemical determination of periostin status in NSCLC.

Univariate analysis in the present study showed that male gender, tumor size, high T status, positive N status, high stage, squamous cell carcinoma, smoking status, low %VC, low FEV1%, and positive pleural invasion were each significantly associated with periostin expression scores. These findings are consistent with a previous report by Takanami et al., who showed that periostin expression was significantly correlated with tumor size, lymph node status, stage, lymphatic invasion, microvessel density, and with lymphatic microvessel density [5], and with a second study which found that periostin expression was significantly correlated with male gender, high stage, high pleural invasion, tumor size, and with tumor relapse [14]. However, in the present study, we found for the first time that histological type and pulmonary function, including %VC and FEV1%, were also correlated with periostin expression scores. This suggests that periostin expression could potentially be used to predict disease progression in respiratory diseases. Our univariate analysis revealed that gender was associated with periostin expression. Other studies have found no significant correlation between periostin expression score and

ma, while weak or negative staining was found in the peri-tumoral stroma and in the tumor epithelium. The

TABLE 3.
Univariate and Multivariate Analysis on Cancer-Specific Survival for Periostin Expression

Variables	Univariate Analysis		P value	Multivariate Analysis		P value
	HR	95% CI		HR	95% CI	
Sex (female vs. male)	2.04	0.77-5.37	0.1489			
Age (< 65 vs. ≥ 65 years)	4.14	1.21-14.21	0.0240	3.67	1.06-12.64	0.0394*
Tumor size (< 2.5 vs. ≥ 2.5 cm)	8.38	1.93-36.26	0.0045			
Tumor status (T1/T2 vs. T3/T4)	7.22	2.89-18.04	<0.0001			
Lymph node status (N0 vs. N1/N2/N3)	2.73	1.10-6.79	0.0308			
Stage (I vs. II/III)	6.51	2.34-18.07	0.0003	4.92	1.74-13.92	0.0027*
Histology (adenoca. vs. squamous cell ca.)	2.77	1.03-7.45	0.0435			
Smoking status (never-smoker vs. ever-smoker)	1.33	0.54-3.32	0.5361			
%VC (≥ 80 vs. < 80)	8.62	1.92-38.73	0.0050			
FEV1.0% (≥ 70 vs. < 70)	1.91	0.78-4.70	0.1588			
Pleural invasion (p10 vs. p11/p12/p13)	2.95	1.19-7.33	0.0200			
Periostin expression (low vs. high)	5.96	1.73-20.47	0.0046	3.65	1.04-12.84	0.0439*

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval, VC, vital capacity; FEV1.0%, forced expiratory volume **P* < 0.05

gender [14,15]. In molecular analysis by Morra et al., periostin mRNA was up-regulated in NSCLC tissue compared with normal lung tissue, with significantly higher scores in the adenocarcinoma compared with the squamous cell carcinoma, while protein scores were correlated with squamous cell carcinoma and with larger tumor size [16]. Our results similarly showed that periostin expression scores were higher in squamous cell carcinoma than in adenocarcinoma, and higher in large tumors than small tumors. These findings suggest that intra-tumor stroma in squamous cell carcinoma and in larger tumors could lead to high periostin expression scores.

With regard to survival analysis, Sasaki et al. have reported that NSCLC patients with periostin expression had significantly poorer survival than patients without periostin expression [15]. Takanami et al. has found that the five-year survival rates were worse in patients with periostin expression than in patients without periostin expression [5]. Soltermann et al. showed that high periostin expression was a prognostic factor for decreased progression-free survival, in univariate analysis [14]. Our multivariate analysis revealed that high periostin expression score, age, and stage were independent prognostic factors associated with CSS. Taken together, these findings indicate that periostin

expression was significantly associated with survival in NSCLC patients.

Sasaki et al. reported that there was no correlation between serum periostin levels and gender, stage, bone metastasis, N status or T status, but NSCLC patients with high periostin levels had significantly poorer survival than patients with normal periostin levels. They concluded that serum periostin levels were an independent prognostic factor in NSCLC [19]. An in vitro study by Hong et al. showed that the mean value of serum periostin in NSCLC patients was significantly higher than that in normal healthy volunteers, but serum periostin levels in NSCLC patients had no association with gender, age, pathological type, TNM stage, lymph node status, tumor size, or invasiveness. They suggested that periostin played an important role during NSCLC progression and development [20]. Kudo et al. has described the roles of periostin in tumor development, and suggested the usefulness of periostin as a therapeutic and diagnostic target [21].

In the present study we investigated periostin expression in NSCLC immunohistochemically and quantitatively, and demonstrated that intra-tumoral periostin expression was an independent prognostic factor in NSCLC. We also found for the first time that histological type and pulmonary function, including %VC

and FEV1%, were also correlated with periostin expression scores. We believe that these findings may open the way to new types of therapeutic interventions as future studies clarify the mechanisms by which periostin mediates cancer cell growth and survival.

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