Periostin, a matrix protein, is a novel biomarker for idiopathic interstitial pneumonias

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Abstract

Idiopathic interstitial pneumonias (IIPs) are histopathologically classified into several types, including usual interstitial pneumonia (UIP), non-specific interstitial pneumonia (NSIP), and cryptogenic organizing pneumonia (COP). In this study, we addressed whether periostin, a matrix protein, could be a biomarker to assess histopathological types of IIPs. To accomplish this objective, we performed immunohistochemical analyses in each histopathological type of IIP, we examined serum levels of periostin in IIPs patients and we analyzed the relationship between serum levels of periostin and the pulmonary functions in patients with idiopathic pulmonary fibrosis (IPF). Periostin was strongly expressed in lungs of UIP and fibrotic NSIP patients, whereas expression of periostin was weak in lungs of cellular NSIP and COP patients as well as in normal lungs. Serum levels of periostin in IPF were significantly higher than those of healthy subjects and COP patients. Furthermore, periostin levels in IPF patients were inversely correlated with their pulmonary functions. Thus, we have found that periostin is a novel component of fibrosis in IIPs. Periostin may be a potential biomarker to distinguish IIPs with fibrosis that may have progressive disease.

Introduction

Idiopathic interstitial pneumonias (IIPs) are acute or chronic lung disorders of unknown

etiology characterized by variable degrees of fibrosis and interstitial or intra-alveolar

inflammation [1-4]. Based on their histopathological features, IIPs are classified into several

types, including usual interstitial pneumonia (UIP), non-specific interstitial pneumonia

(NSIP), and cryptogenic organizing pneumonia (COP). These three major types account for

approximately 80% of IIPs, among which UIP, clinically diagnosed as idiopathic pulmonary

fibrosis (IPF), is the most common, whereas the rest occurs less frequently. IPF/UIP shows

fibrosis-dominant features constituted by overproduction and disorganized deposition of

extracellular matrix (ECM) proteins including type I, III, and IV collagens; fibronectin; and

tenascin-C together with abnormal proliferation of mesenchymal cells, distortion of

pulmonary architecture, and generation of subepithelial fibrotic foci (FF) [5, 6]. In contrast,

COP, characterized by excessive proliferation of granulation tissue within the alveolar ducts

leading to chronic inflammation in the surrounding alveoli, rarely shows severe fibrotic

changes in the lung. NSIP, defined as a temporally uniform interstitial pneumonia, can be

divided into cellular NSIP (cNSIP) and fibrotic NSIP (fNSIP). cNSIP, further divided into

Group I or Group II depending on the absence or presence of concomitant lung fibrosis

respectively, has prominent inflammatory components and severe lung fibrotic changes are

uncommon in both cNSIP and COP, whereas fibrosis is predominant in fNSIP (Group III) [3, 4].

It is well known that the prognoses and the responses to the corticosteroid treatment by the IIP patients can be attributed to histopathological types. IPF/UIP has a grave prognosis with 5-year mortality rates approximately 60-80% [3, 4]. In contrast, cNSIP and COP have better prognoses (5-year mortality rates are < 10% in cNSIP and < 5% in COP, and deaths are rare in COP). Most cNSIP and COP patients show a good response to corticosteroids. Mortality rates for fNSIP are intermediate between IPF/UIP and cNSIP or COP, ranging widely from 11 to 68% in various studies. The poor prognoses of IPF/UIP and fNSIP are thought to be due to resistance to corticosteroids and to impairment of pulmonary functions by fibrosis [3, 4]. Thus, it is important to diagnose the histopathological types of IIPs to predict prognoses and responses to corticosteroid treatment. However, no good biomarker has thus far been available for this purpose.

Periostin is a 90-kD ECM protein, composed of an EMI domain in its N-terminal portion, four tandemly lined fasciclin I (FAS1) domains in the middle, and the alternative splicing domain in its C-terminal portion [7, 8]. Periostin plays an important role in maintenance and development of bones, teeth, and the heart and contributes to tumor progression in several tumor cells. A lot of evidence has recently accumulated showing that

expression of periostin is involved in various pathophysiological statuses of fibrosis, including the healing process in myocardial infarction [7] and bone marrow fibrosis [9]. Furthermore, we have recently found that periostin contributes to formation of fibrosis in response to IL-4 or IL-13 [10, 11]. However, it remains unknown whether periostin is involved in the pathogenesis of IIPs.

In this study, we examined whether periostin is expressed in lung tissues in IIPs and whether serum levels of periostin can be detected in IIP patients reflecting expression of periostin in the lung tissues. In addition, the possible role of periostin in the pathogenesis of lung fibrosis is discussed.

Materials and Methods

Study subjects

Characteristics of IIPs patients enrolled in this study are shown in Table 1. Ninety-two

patients (63 males and 29 females, aged 63.5 ± 1.1 years) were diagnosed as having IIPs

including 51 with IPF, 20 with fNSIP, seven with cNSIP and 14 with COP. All IIPs patients

had been consecutively monitored from 1994 to 2008 at the Kurume University Hospital

(Kurume, Japan), the National Kyushu Medical Center (Fukuoka, Japan), the National

Omuta Hospital (Omuta, Japan), the Yame General Hospital (Yame, Japan), and/or the

Japan Social Insurance Tagawa Hospital (Tagawa, Japan). All patients were also monitored

by their primary care physicians. All IPF patients were diagnosed by at least two

respirologists in accordance with the clinical criteria established by the American Thoracic

Society (ATS)/European Respiratory Society (ERS), as previously reported [2].

Twenty-eight IPF patients were histopathologically diagnosed as UIP by surgical lung

biopsy (SLB). All 20 fNSIP and seven cNSIP patients were histopathologically diagnosed

by SLB. Out of 14 COP patients, five were histopathologically diagnosed by SLB and nine

by transbronchial lung biopsy (TBLB). The essential pathological feature of COP was the

presence of granulation tissue (Masson bodies) and inflammatory cells in the interstitium

with preservation of the alveolar architecture [12]. All histopathological diagnoses were

performed by at least two professional pathologists. Other diseases such as connective tissue diseases, infection, and hypersensitivity pneumonia were excluded. Clinical features, chest high-resolution computed tomography (HRCT), and bronchoalveolar lavage fluid (BALF) cell counts were consistent with COP. In addition, all of the patients showed improvement with or without corticosteroid treatment. All patients had been clinically stable with no disease exacerbations at least for three months prior to this diagnosis. Diagnosis for acute exacerbation (AE) of IPF was defined in accordance with the criteria detailed in a previous report [13]. Eight IPF patients showed AE. Importantly, oxygen partial pressure in resting arterial blood (PaO_2) was lower by more than 10 torr in all of the patients. We have analyzed serum levels of procalcitonin, β-D glucan, and cytomegalovirus antigen, and we also examined bacteria and mycobacteria in the sputa of patients to exclude infectious diseases as a cause of the symptoms. However no infections were observed in the patients. We could not perform bronchoalveolar lavage as we could not obtain written informed consent. We obtained serum samples from most patients at the time of diagnosis and also from age-matched healthy volunteers used as controls. Control normal lung tissues were obtained from thirteen non-smokers who underwent lung cancer extirpation after surgery at Kurume University Hospital. Sample collection and all procedures were approved by the ethics committees of Kurume University in accordance with the ethical standards of the

Helsinki Declaration of 1975. Informed consent was obtained from all patients and healthy

volunteers.

HRCT review

Briefly, patients were classified as having a definite UIP pattern when the HRCT

demonstrated honeycombing with predominantly peripheral and basal distribution. Patients

were classified as having a probable UIP pattern when the HRCT demonstrated a reticular

pattern with predominantly peripheral and basal distribution but only minimal in size or

with no honeycombing. In this study, two expert chest radiologists independently evaluated

the HRCT of all patients. In the cases of disagreement, the radiologists reexamined the scans

in question to reach a consensus. The radiologists were blinded to patient identification,

treatment assignment, and temporal sequence of the studies, as in previous reports [13].

BALF

BALF was obtained by washing the right middle lobe or left lingular segment three times

with 50 ml of physiological saline (total 150 ml).

Pulmonary function test

Pulmonary function tests including VC, FVC, and FEV_1 were performed using an electrical spirometer. The rates of the predicted VC values (%VC) were calculated as previously reported [14]. DLCO was measured using a single-breath technique or a rebreathing technique with adjustment to single-breath values. We performed VC in 35 patients and DLCO in 28 patients among the 37 IPF patients in whom serum levels of periostin were measured.

Establishment of anti-periostin monoclonal and polyclonal antibodies (Abs)

We newly established anti-periostin monoclonal Abs (mAbs) as previously reported [10]. Briefly, six- to ten-week-old Crj:Wistar rats (Charles River Japan, Inc., Kanagawa, Japan) were injected in footpads two or more times with 20 μg of recombinant human periostin emulsified in TiterMax Gold adjuvant (TiterMax USA, Norcross, GA). Three days after the last injection, lymphocytes from popliteal, inguinal and iliac lymph nodes were fused with a Sp2/O myeloma cell line. Rat anti-periostin mAbs (IgG) were purified from culture supernatant of the hybridomas using a protein G affinity chromatography column. Specific pathogen-free rabbits were immunized with recombinant human periostin, and antiserum was obtained. Purified rabbit anti-human periostin polyclonal Ab was generated from the antiserum as previously reported [10].

Immunohistochemical assay and immunoreactivity score

Immunohistochemical analysis was performed as reported previously [15-17]. Briefly, rat

anti-human periostin mAbs (clone no. SS19B or SS5D) were incubated with samples at 4°C

for 18 hours. Positive reactivity was identified using biotin-labeled goat–anti-rat IgG,

streptavidin-conjugated peroxidase, and 3-3'-diaminobenzidine-4HCl (DAB) employing an

LSAB2 kit (Dako, Kyoto, Japan). Semi-quantitative analysis of the immunohistochemical

assay was performed using a modified method that we previously reported [15]. Briefly, we

selected specific areas where fibroblasts, intra-alveolar macrophages, or interstitial

mononuclear cell infiltrate appeared to be most markedly observed. In each section, we

selected 3 individual square fields (0.34 × 0.27 mm) at 200x. Periostin immunoreactivity

scores for each histological feature were defined by counting the numbers of positively

stained cells in these areas at 200x magnification. Two independent examiners evaluated

these sections without prior knowledge of the patients' clinical status.

Measurements of periostin by ELISA

Serum and BALF were obtained from subjects and then stored at –80 ^{o}C until ELISA assay.

We newly established a human periostin ELISA assay. Two rat anti-human periostin mAbs

(clone no. SS18A and SS17B) were used to establish a sandwich ELISA assay. The SS18A mAb (two µg/ml) was incubated overnight at 25 ^{o}C on ELISA plates (loose MaxiSorp® Nunc-Immuno® Modules, Thermo Fisher Scientific, Rochester, NY). The ELISA plates were blocked by blocking buffer (0.5% casein, in TBS, pH 8.0) overnight at 4 ^{o}C and then washed three times with washing buffer (0.05% Tween20 in PBS). The ELISA plates were incubated with 100- to 200-diluted samples or recombinant periostin standards for 18 hours at 25 ^{o}C, followed by washing five times. The peroxidase-labeled SS17B mAb (50 ng/ml) was added followed by incubation for 90 min at 25 ^{o}C. After the ELISA plates were washed five times, reaction solution (0.8 mM 3,3',5,5'-Tetramethylbenzidine, 2.5 mM H_2O_2) was added, followed by incubation for 10 min at 25 ^{o}C and then the reaction was stopped by adding the stop solution (0.7 N HCl). The values were calculated by subtracting the absorbance at 550 nm (secondary wavelength) from the absorbance at 450 nm (primary wavelength) measured by a microplate reader (Bio Rad Laboratories, Tokyo, Japan). Periostin concentrations in the serum were calculated simultaneously using the recombinant periostin proteins. We performed the ELISA assay on duplicated samples.

Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). Differences between the two groups were analyzed by the Wilcoxon rank-sum or Fisher's exact test. Correlations between the two groups were evaluated by Spearman's rank correlation coefficient. Survival curves were obtained by the Kaplan-Meier method, and the difference in survival rates between subgroups was analyzed by using the log-rank test. $P < 0.05$ was taken to represent statistical significance. The sensitivity and specificity of serum periostin levels for detecting IPF were analyzed by using a receiver operating characteristic (ROC) curve generated by logistic regression as we previously reported [18]. Statistical analysis was performed with JMP 7.0.1 (SAS Institute Japan, Tokyo, Japan).

Results

Clinical findings of IPF, NSIP, and COP patients subjected to this study

Table 1 summarizes all subjects used in this study. It is of note that eight of 51 IPF patients

later showed episodes of acute exacerbation. VC was significantly lower in cNSIP patients

than in IPF, fNSIP, or COP patients. The mean serum levels of KL-6 greatly and

significantly ($P < 0.01$) increased in IPF (n=37, 1346.0 ± 145.6 U/mL), fNSIP (n=7, 913.3 ±

197.9 U/mL) and COP (n=9, 905.2 ± 619.1 U/mL) patients compared to those in healthy

control subjects (n=66, 104.5 ± 11.9 U/mL). The levels in IPF and fNSIP patients were

slightly higher that those of COP patients ($P < 0.01$ and $P < 0.05$, respectively).

Periostin is a novel component of fibrosis predominant in UIP and fNSIP, but not in cNSIP and COP

We first evaluated 14 rat anti-periostin mAbs for whether these mAbs could be used for

immunohistochemical analysis. We found that two anti-human periostin mAbs (clones

SS19B and SS5D) can be used for the immunohistochemical analysis. Moreover, the

positive reactivities (periostin proteins) in lungs of UIP patients detected by these two mAbs

were exactly the same as those detected by rabbit anti-human periostin polyclonal Ab (data

not shown). Therefore, in this study, we used SS19B and SS5D to perform

immunohistochemical analysis.

We investigated the expression of periostin in lung tissues obtained from 25 UIP,

20 fNSIP, seven cNSIP and five COP patients. Normal lung tissues were obtained from five

non-smokers who underwent lung cancer extirpation. Representative periostin expressions

in the lung tissues obtained from a representative control subject (69-year-old female), a UIP

patient (64-year-old male), a fNSIP patient (62-year-old female), cNSIP (56-year-old

female) and a COP patient (76-year-old female) are shown in Fig. 1A and 1B.

Periostin was not observed in pulmonary cells including epithelial cells, alveolar

macrophages, and most bronchiolar basement membrane of normal lung tissues, but was

very weakly deposited in a part of bronchiolar basement membrane (data not shown). In

contrast, the lung tissues of UIP showed strong expression of periostin in fibroblasts,

especially in FF areas, but not in regenerative alveolar epithelium or macrophages, the areas

showing established fibrosis, or inflammatory cells (left panel in Fig. 1B). Moreover, in

UIP, priostin was strongly expressed in the interstitium of alveoli, which seemed to be

normal (right panel in Fig. 1B). In fNSIP, periostin was also strongly expressed in

fibroblasts, but barely in regenerative alveolar epithelium and macrophages, or

inflammatory cells. In cNSIP and COP, periostin was scarcely observed in alveolar septa

and inflammatory cells. However, periostin was expressed in the intra-luminal fibrosis of alveolar ducts in COP patients. It has been well recognized that the intra-luminal fibrosis of alveolar ducts in COP patients is potentially reversible. These results demonstrate that periostin is a novel component for ongoing fibroproliferation especially in UIP, fNSIP, and COP patients. Semi-quantitative analysis showed that periostin expressing cells in the lungs of UIP (n=25), fNSIP (n=20), cNSIP (n=6), and COP (n=4) were significantly ($P < 0.05$) increased when compared to normal lung tissues (n=15). In addition, periostin expressing cells in the lungs of UIP were significantly increased as compared to fNSIP, cNSIP and COP (Fig. 1C).

Serum periostin levels are significantly elevated in IPF patients, reflecting expression of periostin in lung tissues

We then examined whether serum levels of periostin are elevated in IIPs patients reflecting upregulated expression of periostin in lung tissues. We newly established a sandwich ELISA assay to detect periostin using two rat anti-human periostin mAbs (clone no. SS18A and SS17B). We confirmed that the limiting level of periostin for detection in this ELISA system is 100 pg/ml (data not shown). We applied serum samples from patients with IPF, fNSIP, and COP, and from control subjects to this ELISA system, but we were not able to obtain

serum samples from cNSIP patients (Fig. 2). Serum periostin levels in IPF patients were significantly elevated (n=37; 107.1 ± 11.9 ng/ml) compared to those in COP patients (n=9; 58.9 ± 8.2 ng/ml) and control subjects (n=66; 39.1 ± 3.0 ng/ml), statistically different from COP patients ($P < 0.01$) and control subjects ($P < 0.0001$). Serum periostin levels of fNSIP was moderate (n=7; 77.9 ± 15.7 ng/ml), statistically different with control subjects ($P < 0.01$), but not with COP patients. In contrast, serum periostin levels in COP patients were only slightly elevated compared to control subjects, statistically different ($P < 0.05$). Furthermore, periostin in the BALF was also detectable by ELISA in five of 11 IPF patients (data not shown). Gender, smoking status, and KL-6 levels were not associated with serum periostin levels (data not shown). These results show that serum periostin levels are elevated significantly in IPF patients, moderately in fNSIP patients, and only slightly in COP patients compared to healthy control subjects, reflecting the expression of periostin in their lung tissues.

IPF is distinguished from the control or COP by measurement of serum periostin levels with high specificities

Because serum periostin levels in IPF patients were significantly elevated compared to control subjects and COP patients, we next evaluated the sensitivities and specificities to

distinguish IPF from the control or COP by serum periostin levels using a ROC curve (Fig. 3). The area under the ROC curve (AUC) between IPF patients and control subjects was 0.905. The ROC curve analysis showed that serum periostin levels, with an optimal cut-off point of 95 ng/ml, had a good specificity (0.985) and a fair sensitivity (0.514). Nineteen of 37 IPF patients (51.4%) and one of 66 (1.5%) healthy subjects were over the cut-off value. This cut-off value also gave a good specificity (1.0) and a fair sensitivity (0.514) in the comparison study of IPF and COP patients. None of COP patients showed more than the cut-off point of serum periostin level. Furthermore, this cut-off value gave a relatively less sensitivity (0.286) in the comparison study of fNSIP and healthy subjects. These results demonstrate that IPF can be distinguished from the control or COP by measurement of serum periostin levels using the ELISA system that we established with high specificities.

Serum periostin levels are inversely correlated with the pulmonary functions in IPF patients

Serum periostin levels in IPF patients were valuable within or above the normal range, indicating the heterogeneous backgrounds of IPF patients. Therefore, we then examined the association between serum periostin levels and various clinical parameters in IPF/UIP patients. There was no correlation between serum periostin levels and gender, smoking

status, overall survival rate, BALF cell counts, or serum KL-6 levels (data not shown). In

contrast, the 6-month changes in VC (n=26) and in D_{LCO} (n=21) showed significant

negative correlations with serum periostin levels (Fig.4, ΔVC; Spearman r=-0.498, $P <$

0.01, ΔD_{LCO}; Spearman r=-0.467, $P < 0.05$). These results show that serum periostin levels

are inversely correlated with the pulmonary functions in IPF patients. In this study, there

was no significant correlation between serum KL-6 levels and pulmonary function tests

(initial levels and 6-month changes in VC and D_{LCO}). There was no significant

differentiation of serum periostin levels between in IPF patients with (n=8, 112.9 ± 35.0

ng/ml) and without (n=29, 105.6 ± 12.2 ng/ml) acute exacerbation ($P = 0.4948$). Periostin

was not associated with an increased risk of exacerbation in eight IPF patients.

Discussion

The importance of diagnosing the histopathological types of IIPs has been established as a basis for the prediction of prognoses and responses to treatment. This is due to the fact that IPF/UIP has a poorer prognosis and resists corticosteroid treatment, whereas cNSIP and COP have better prognoses and respond well to such treatment [1-4]. IIP is diagnosed based on the combined clinical, radiological, and pathological data of the patients, among which the lung biopsy is the most definitive marker of disease [2-4]. However, the lung biopsy carries a risk of death, with mortality rates ranging from 1 to 6% [19, 20]. Therefore, it would be of great value if a biomarker to assess histopathological types of IIPs could be identified. In this study, we demonstrate that measurement of serum levels of periostin may serve as an important biomarker for characterization and classification of this condition. ROC curve analysis rendered an optimal cut-off point of 95 ng/ml for the serum periostin level using our ELISA system. Only one of 66 healthy subjects (1.5%) was over the cut-off value, providing good specificity for this assay (0.985). In contrast, 19 of 37 IPF patients (51.4%) and none of COP patients (0%) showed values over 95 ng/ml. Our small-scale survey for fNSIP patients showed that two of seven fNSIP patients (28.6%) were over the cut-off value, providing less sensitivity (0.286) than that of IPF. Based on these collective results, we propose that measurement of serum

periostin levels is useful to distinguish IPF/UIP from COP or normal lung function. It has been reported that IPF/UIP and fNSIP share a similar presentation with respect to clinical/radiological/pathological parameters; however the prognosis of fNSIP is much better than IPF/UIP [1-6]. Semi-quantitative immunohistochemical analysis showed that periostin expressing cells (mainly fibroblasts) in the lungs of UIP were significantly increased as compared to fNSIP. Although there was not a significant difference between IPF and fNSIP, the mean levels of serum periostin in IPF patients were higher (n=37; 107.1 ± 11.9 ng/ml) than observed in fNSIP patients (n=7; 77.9 ± 15.7 ng/ml). Moreover, 51.4% (19/37) of IPF and 28.6% (2/7) of fNSIP patients were over the cut-off value 95 ng/ml. Therefore, further analysis is needed to determine whether measurement of serum periostin levels will serve as a higly sensitive marker to distinguish IPF/UIP from fNSIP. In addition, we performed preliminary analysis of serum levels of periostin in patients with interstitial lung disease associated with rheumatoid arthritis (RA-ILD). The serum levels of periostin in RA-ILD patients were significantly increased when compared with control subjects (data not shown). Thus periostin may have an important role in secondary interstitial pneumonia such as collagen tissue disease-associated ILD. This hypothesis is currently under investigation.

It has been demonstrated that several biomarkers such as KL-6, surfactant protein

(SP)-A, and SP-D, are elevated in serum of IIPs patients, thus providing potential biomarkers

for the diagnosis of IIPs [21-23]. Particularly, KL-6 has been shown to increase in all of the

major types of IIPs including IPF, NSIP, and COP. In agreement with these reports, the

serum levels of KL-6 were elevated in all of the IPF, fNSIP, and COP groups, although in our

study, the levels of COP were slightly less than those of IPF and fNSIP. Periostin is distinct

from KL-6 in that IPF/UIP can be distinguished from COP by measurement of serum levels

of periostin, but not KL-6. Furthermore, we did not observe a correlation between serum

levels of periostin and KL-6. These results may be due to the different mechanisms

controlling expression of KL-6 and periostin. KL-6, a high molecular glycoprotein (also

called mucin 1, MUC1), is produced by regenerating type II pneumocytes, and is released

from the cell surface by shedding [21, 24], whereas periostin is secreted by activated

fibroblasts [10]. Thus, periostin may directly reflect lung fibrotic changes in IIPs more than

KL-6. It is assumed that combining measurements of periostin with other biomarkers,

including KL-6, could further improve the accuracy of diagnosing IIPs.

We analyzed serum levels of periostin and various clinical parameters in IPF patients

based on the observation that the serum levels of periostin in IPF patients are variable,

ranging within the normal range to very high values. Consequently, we found that serum

levels of periostin in IPF patients correlate with 6-month changes in VC and in DLCO. It is well known that the change in VC (or FVC) is highly correlated with survival rate and that the change in DLCO has a similar but lesser prognostic value [3] [25, 26]. For example, patients who decline more than 10% of FVC show a poor prognosis with a 5-year survival rate of 20%, whereas patients who improve more than 10% of FVC have a 5-year survival rate of 65%. The inverse association between the serum levels of periostin and the pulmonary functions in our study indicates the possible application of serum periostin levels as a prognostic parameter, in addition to the assessment of the histopathological types of IIPs. The use of this marker is also supported by the present finding that periostin was strongly expressed in FF areas in the lungs of UIP patients. These areas consist of aggregating myofibroblasts that play an important role in ongoing regeneration of fibroblasts in UIP and have been reported to be relevant for predicting the prognosis of UIP patients [3]. Further studies will be needed that focus on measuring the serum levels of periostin in individual patients and a determination if levels correlate with prognoses.

It is known that several ECM proteins — including type I, III, IV collagens; fibronectin; and tenascin-C — are involved in the process of fibrosis in UIP patients [5, 6]. Here we demonstrate that periostin is a novel component of fibrosis in UIP and fNSIP,that negatively correlates with the pulmonary functions in IPF/UIP. Thus far, the exact role of

periostin in the pathogenesis of these diseases remains unknown. We and others have demonstrated that periostin can interact with other ECM proteins including collagen, tenascin C, and fibronectin, and plays an important role in collagen fibrillogenesis or organization of an extracellular meshwork architecture [10, 27, 28]. Accordingly, a genetic defect of periostin causes impaired amelogenesis in teeth [29], valve abnormalities in the heart [30], and a propensity for cardiac rupture in the healing stage of myocardial infarction [7]. These results raise the possibility that periostin participates in the formation of fibrotic structure in IIPs. As we expected, we have found that administration of bleomycin induces less pulmonary fibrotic changes in periostin-deficient mice (Uchida M, unpublished data). These results suggest the possibility that periostin plays an important role in generation of fibrosis in IIPs and that periostin can be a therapeutic target for IIPs.

We found that serum levels of periostin were significantly high in IPF patients, moderate in fNSIP patients, and slightly elevated in COP patients, compared to normal control subjects and in parallel with expression of periostin in lung tissues. Furthermore, serum periostin levels were inversely correlated with pulmonary functions in IPF/UIP patients. The distribution of periostin in immunohistochemical studies suggests that it is expressed in areas of fibroblast proliferation. Our results, taken together, show that periostin is a novel component of fibrosis in IIPs. Thus periostin may be a potential biomarker to

distinguish IIPs with fibrosis that may have progressive disease.

**Support Statement**

Acknowledgments

Figure legends

Figure 1 Expression of periostin in lungs of UIP, fNSIP, cNSIP, and COP

(A) The expressions of periostin were examined by immunohistochemical analysis in control subjects (non-smokers), UIP, NSIP and COP patients. Representative periostin expressions in the lung tissues obtained from a representative control subject (69-year-old female), a UIP patient (64-year-old male), a fNSIP patient (62-year-old female), a cNSIP (56-year-old female) patient, and a COP patient (76-year-old female) are shown.

(B) Immunohistochemical analysis of periostin in a UIP patient (64-year-old male). Magnification at 200X.

(C) Semi-quantitative analysis of immunohistochemical analysis of the lungs of UIP, fNSIP, cNSIP, COP, and control subjects. Semi-quantitative analysis of the immunohistochemical assay was performed as described in the methods section. Periostin immunoreactivity scores for each histological feature were defined by counting the numbers of positively stained cells in these areas at 200x magnification. *: $P < 0.05$.

| | HE (40X) | HE (100X) | Periostin (100X) |
Non-smoker			
UIP			
fNSIP			
cNSIP			
COP			
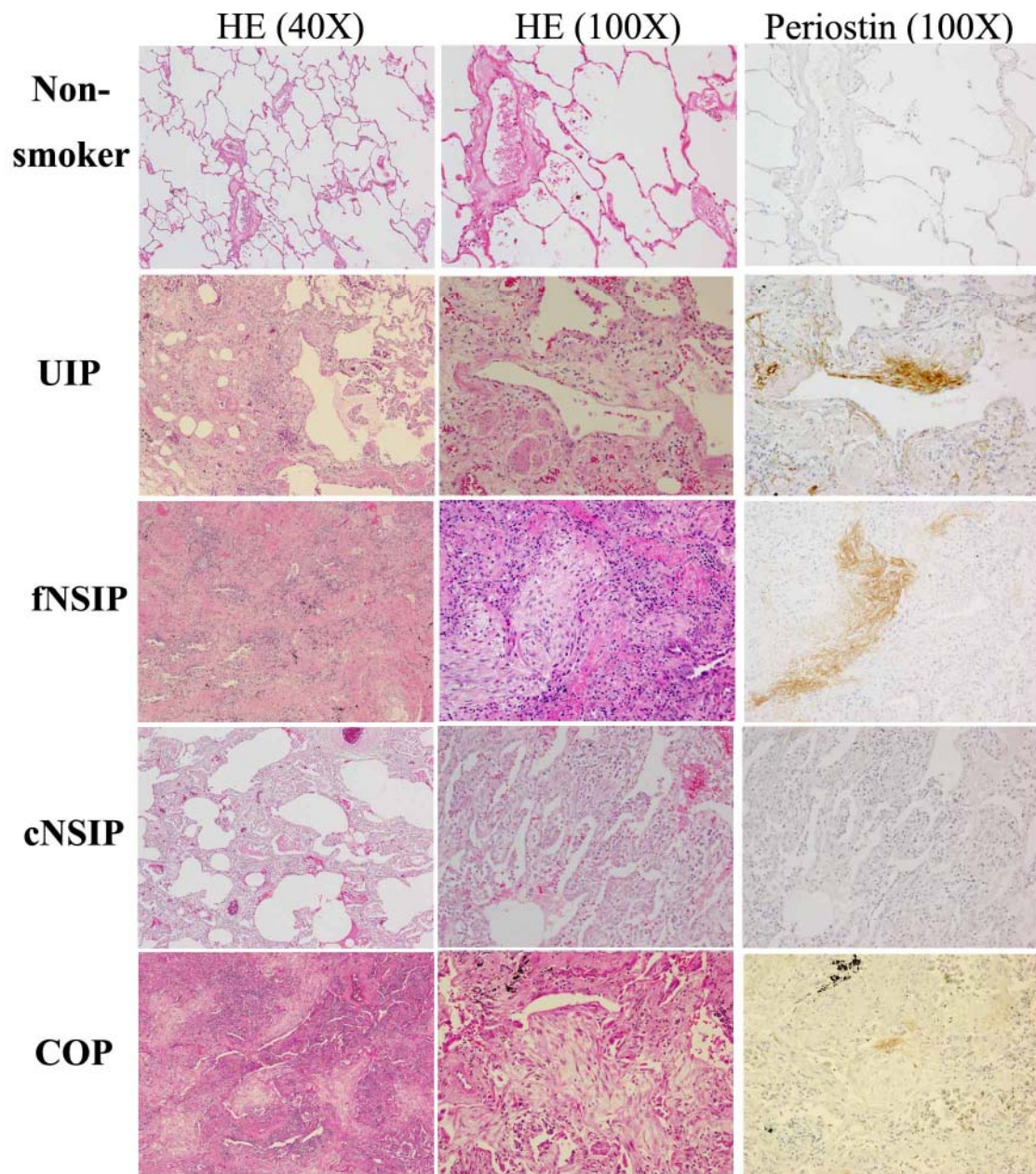
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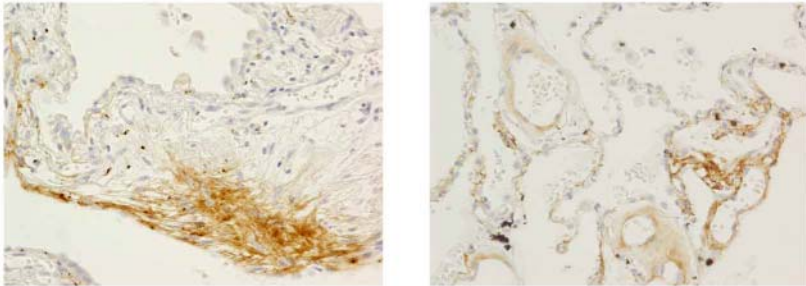
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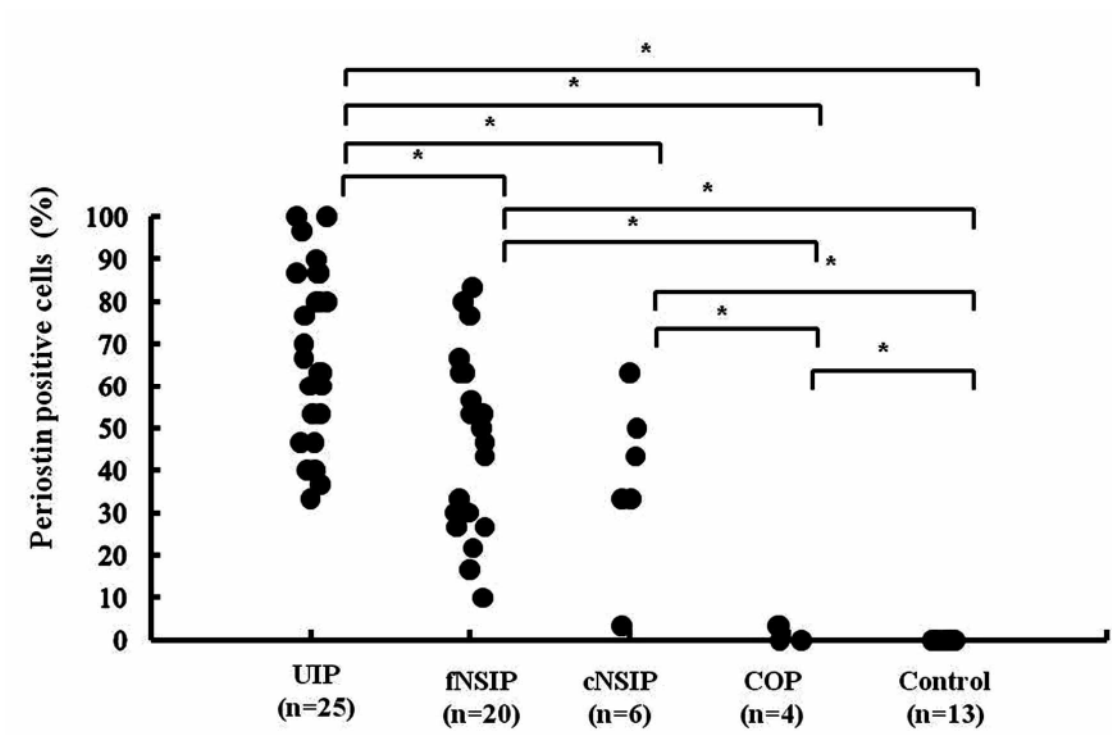
Figure 2 Serum periostin levels in IPF, fNSIP, and COP patients and in healthy control subjects

Periostin levels in sera obtained from IPF (n = 37), fNSIP (n = 7), COP (n = 9), and healthy control subjects (n = 66) using an ELISA are depicted. All IPF patients were diagnosed by at least two respirologists and at least two radiologists in accordance with the clinical criteria established by the American Thoracic Society (ATS)/European Respiratory Society (ERS, Ref. 4). All seven fNSIP and nine COP patients were histopathologically diagnosed by SLB or TBLB.
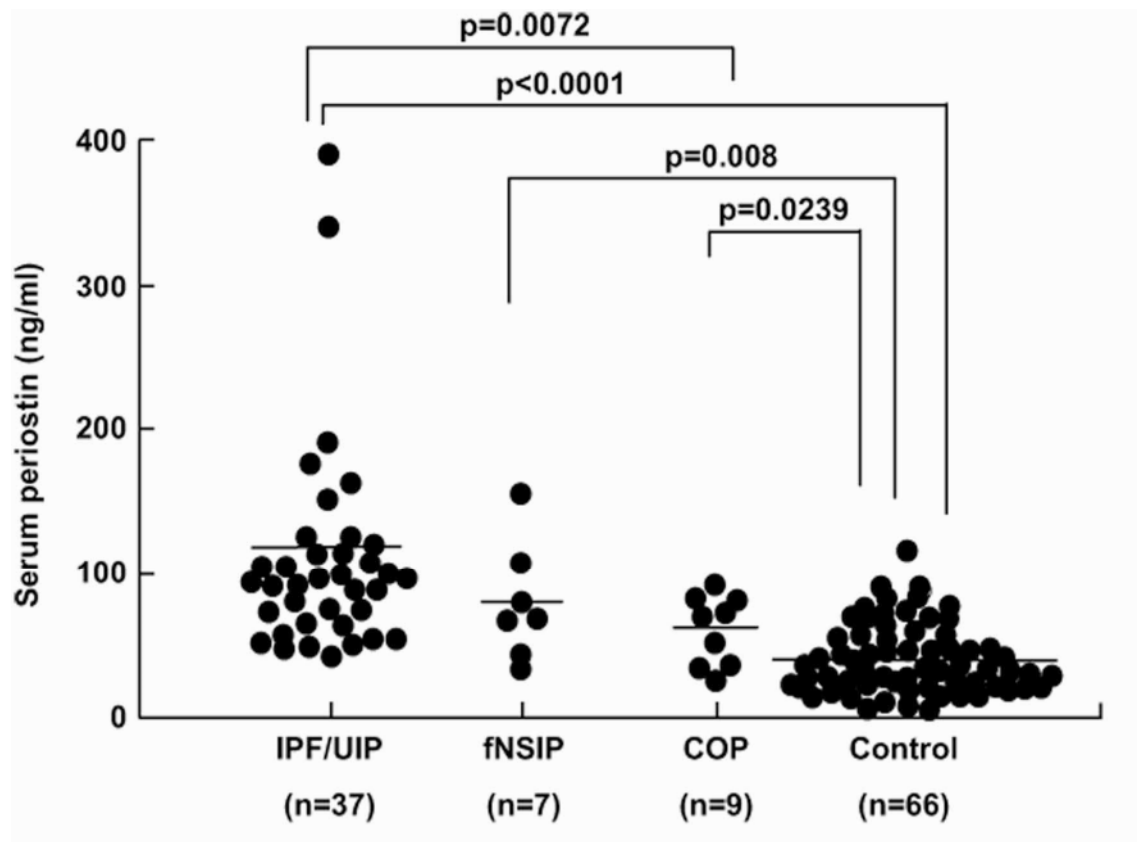
Figure 3 Specificity and sensitivity of measurement of serum periostin levels

The ROC curve compares serum periostin levels in IPF patients and healthy control subjects. The area under the ROC curve (AUC) between IPF patients and control subjects was 0.905. The optimal cut-off value of the serum periostin level (95 ng/ml) is inserted. The numbers of IPF, fNSIP, and COP patients and of healthy control subjects over the optimal cut-off value (95 ng/ml) are shown.

Type	IPF/UIP	fNSIP	COP	Control
Number of over 95 ng/ml	19/37 (51.4%)	2/7 (28.6%)	0/9 (0%)	1/66 (1.5%)
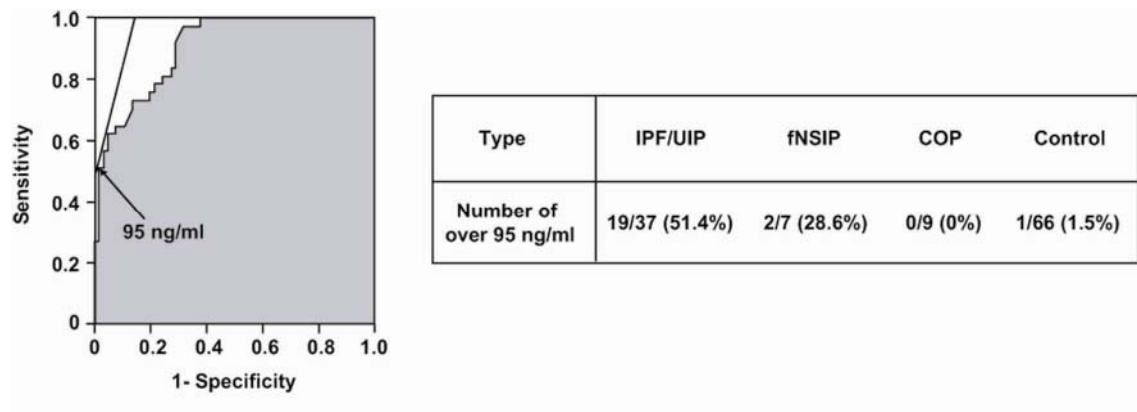
Figure 4 Correlation between serum periostin levels and pulmonary functions in IPF patients

The correlation between serum periostin levels and 6-month changes in VC (n=26) and in DLCO (n=21) in IPF patients is shown.
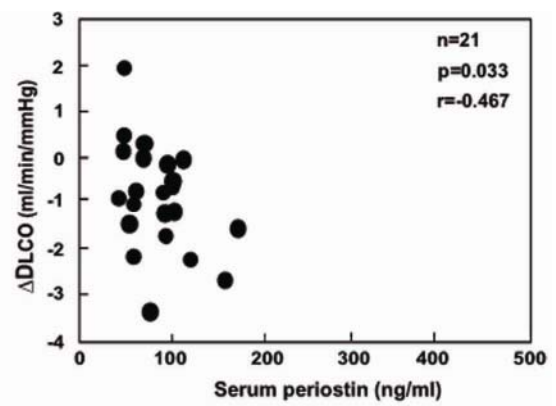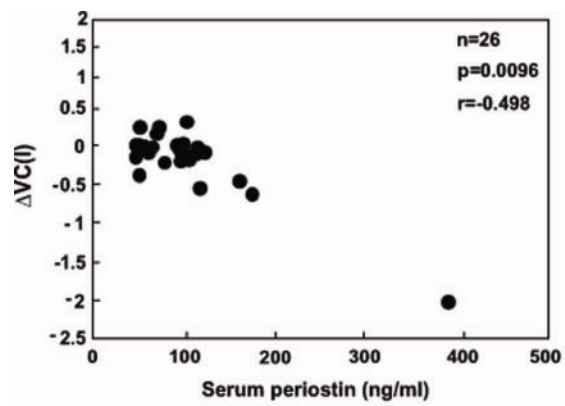
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