S100A9 in BALF is a candidate biomarker of idiopathic pulmonary fibrosis

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Received 4 October 2011; accepted 13 December 2011
Available online 30 December 2011

KEYWORDS
Bronchoalveolar lavage fluid; Calgranulin B; Myeloid-related protein-14; Neutrophils; Proteomics

Summary
Background: Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown cause, and the prognosis remains poor. On the other hand, other fibrotic interstitial pneumonias such as idiopathic nonspecific interstitial pneumonia (I-NSIP) and collagen vascular disease-associated interstitial pneumonia (CVD-IP) resemble IPF, but they respond to therapy and the prognosis is better. We searched for biomarkers to distinguish IPF from other fibrotic interstitial pneumonias and investigated whether S100A9 could be useful for discriminating types of fibrotic interstitial pneumonia based on our preliminary proteomic findings.

Methods: We measured S100A9 levels in serum and bronchoalveolar lavage fluid (BALF) from 28 patients with IPF, 15 with I-NSIP, 20 with cryptogenic organizing pneumonia (COP), 35 with CVD-IP and 23 healthy individuals (controls) using enzyme-linked immunosorbent assays. S100A9 in the lung was also immunohistochemically localized.

Results: S100A9 levels in BALF, but not in serum, were significantly elevated in patients with IPF compared with I-NSIP, COP, CVD-IP and healthy individuals. S100A9 immunoreactivity was localized mainly in macrophages and neutrophils in lung specimens from patients with IPF. The results of receiver operating characteristic (ROC) curve analysis showed that BALF S100A9 levels had sufficient specificity and sensitivity to distinguish IPF from I-NSIP and CVD-IP.
Introduction

Idiopathic pulmonary fibrosis (IPF) is the most prevalent form of idiopathic interstitial pneumonias (IIPs). It is defined as a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown cause that occurs primarily in older adults and it is limited to the lungs. Although recent clinical trials of some agents have suggested a benefit for IPF, medical therapy has not enhanced survival and the prognosis remains poor. The major forms of IIPs are cryptogenic organizing pneumonia (COP) and nonspecific interstitial pneumonia (NSIP) (I-NSIP). Thus, to clinically distinguish IPF from I-NSIP and interstitial pneumonia associated with CVD (CVD-IP) is often difficult. However, the prognosis is better for I-NSIP and CVD-IP than for IPF, and patients also respond well to immunosuppressive therapy. Therefore, to differentiate between IPF and other fibrotic interstitial pneumonias such as I-NSIP and CVD-IP is important. High-resolution computed tomography (HRCT) is considered useful for distinguishing I-NSIP and CVD-IP from IPF. However, patients with IPF often have atypical CT features that preclude a definitive diagnosis even when using HRCT. On the other hand, the value of histological findings that were once considered the diagnostic gold standard is in fact limited by sampling bias, interobserver variation and the risk of acute exacerbation. Therefore, biomarkers are needed to predict the activity, prognosis and etiology of IPF and distinguish it from other fibrotic interstitial pneumonias such as I-NSIP and CVD-IP. Based on this background, proteomic studies of interstitial lung disease have detected several proteins that are differentially expressed in patients with IPF.

Calcium-binding S100 proteins comprise a family of over 20 members that is characterized by two calcium binding EF-hand motifs connected by a central hinge region. Among them, S100A8 (also named calgranulin A; myeloid-related protein 8, MR8), and S100A9 (calgranulin B; MRP14) are expressed in granulocytes, monocytes and macrophages at early differentiation stages, and they can be induced in keratinocytes and epithelial cells under inflammatory conditions. S100A8 and S100A9 generally regulate gene expression in parallel, and the preferred form for human S100A8 and S100A9 is the S100A8/S100A9 heterodimer. The S100A8/S100A9 complex has proven useful as a diagnostic marker of inflammation especially in non-infectious inflammatory diseases. S100A8 and S100A9 also form homodimers, and differential S100A8 and S100A9 expression indicates chronic inflammation. Proteomics studies and sandwich enzyme-linked immunosorbent assays (ELISA) have shown upregulated and elevated S100A9 protein levels, respectively, in bronchoalveolar lavage fluid (BALF) from patients with IPF, and that these activities are associated with clinical parameters. With respect to CVD-IP, levels of S100A9 are significantly lower in BALF from patients with pulmonary fibrosis associated with systemic sclerosis than with IPF. These findings suggest that S100A9 could be a useful marker of IPF and play a pivotal role in its pathogenesis. However, S100A9 levels have not been compared among IIPs or in patients with CVD-IP other than systemic sclerosis, and serum S100A9 levels have not been measured in patients with IIPs.

The present study measures serum and BALF levels of S100A9 in IIPs and CVD-IP and determines associations between these values and the clinical features of IPF.

Materials and methods

Study population

We enrolled 28 patients (23 males and 5 females; age, 63.2 ± 10.2 years; mean ± SD) with IPF, 15 (8 males and 7 females; age, 56.7 ± 13.0 years) with I-NSIP, 20 (12 males and 8 females; age, 67.0 ± 12.9 years) with COP, 35 (11 males and 24 females; age, 62.2 ± 11.8 years) with CVD-IP, and 23 healthy volunteers (17 males and 6 females; age, 28.0 ± 8.6 years) from Nagasaki University Hospital between 1992 and 2010. The IIPs were diagnosed according to the consensus criteria of the American Thoracic Society/European Respiratory Society. The associated diagnoses in the patients with CVD-IP were rheumatoid arthritis (RA; n = 8), polymyositis (PM)/dermatomyositis (DM) (n = 7), Sjögren’s syndrome (SjS) (n = 6), systemic sclerosis (SSc; n = 12), PM + RA (n = 1) and SSc + DM (n = 1).

Of these, 41 (IPF, n = 16; I-NSIP, n = 15, CVD-IP, n = 10) were pathologically confirmed by analyzing surgical lung biopsy specimens obtained from at least two sites. All patients with NSIP were pathologically diagnosed with fibrotic NSIP. Patients with CVD-IP were pathologically diagnosed with usual interstitial pneumonia (UIP) or fibrotic NSIP. Other patients with CVD-IP that were not confirmed by surgical lung biopsy were clinically diagnosed based on HRCT and BALF findings of fibrotic interstitial pneumonia. Among patients with CVD, those with organizing pneumonia, airway involvement and infections were excluded. None of the included patients had received steroids or other immunosuppressive therapy at the time of sample collection. Data from pulmonary function tests, arterial blood gas analyses, markers of interstitial pneumonia including lactic dehydrogenase (LDH), Krebs von den Lungen 6 (KL-6), surfactant proteins (SP)-A and SP-D, and survival rates were obtained from medical records.

Conclusion: S100A9 in BALF might serve as a candidate biomarker to discriminate between IPF and other fibrotic interstitial pneumonias.
Patients with cancer were excluded. All healthy volunteers had normal chest radiographs, were asymptomatic and not under any medication. The Human Ethics Review Committee of Nagasaki University School of Medicine approved the study protocol and all participants provided written, informed consent prior to enrollment in the study.

**Bronchoalveolar lavage and cell preparation**

Bronchoscopy and bronchoalveolar lavage proceeded as described.²⁰ Three instillations of sterile physiological saline (50 mL) were delivered at body temperature through a bronchoscope (BF P200, Olympus, Tokyo, Japan). Lavage fluid was immediately retrieved by gentle suction using a sterile syringe, passed through two sheets of gauze, and then the supernatant separated by centrifugation at 400 × g for 10 min at 4 °C was stored at −80 °C. Sedimented cells were washed twice with phosphate buffered saline (PBS), suspended in 10% heat-inactivated fetal calf serum (FCS) and incubated in plastic dishes for 60 min at 37 °C in a humidified 5% CO₂-air environment. Trypan blue exclusion showed that the viability of the non-adherent cells collected for flow cytometry was >90%. The borderline of cells stained or unstained with phycoerythrin (PE)-conjugated CD4 and CD8 antibodies was determined by flow cytometry using mouse anti-human S100A9 monoclonal antibody (Becton Dickinson, San Jose, CA, USA).

**Protein extraction and two-dimensional gel electrophoresis**

The protein concentration in BAL samples from five patients with IPF and from healthy individuals (controls) was determined using a Protein Assay kit (Bio-Rad Japan, Tokyo, Japan). Albumin and IgG from BAL samples containing 100 μg of protein were removed using ProteoPrep® Blue Albumin and an IgG Depletion kit according to the manufacturer’s protocol (Sigma, St. Louis, MO, USA). Five samples from patients with the same disease were mixed and precipitated in acetone at −30 °C for 2 h and then the precipitated proteins were collected by centrifugation at 20,000 × g for 20 min. The acetone was discarded and the pellet was dried for a few minutes at room temperature. The protein precipitate was resuspended in 250 μL of isolectric focusing (IEF) lysis buffer (6 M urea, 2 M thiourea, 3% 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulphonate, 1% Triton X-100 and DeStreak reagent (GE Healthcare Biosciences, Tokyo, Japan), gently vortexed for 45 min at room temperature and then the lysate was separated by centrifugation at 20,000 × g for 15 min.

Samples were rehydrated in Immobiline DryStrip gels (pH 3–10 non-linear, 13 cm; GE Healthcare Biosciences) for 12 h and then equilibrated in strip equilibration buffer [6 M urea, 20% glycerol, 2% dithiothreitol (DTT), 2% sodium dodecyl sulfate and 375 mM Tris–HCl pH 8.8] for 45 min before being isoelectrically focused at 150 V for 1 h, 5000 V ramping for 2.5 h and IEF at 5000 V for 15 h. Two-dimensional gel electrophoresis (2DE) proceeded as described²¹ at a constant current of 25 mA for ~4 h (10–18% polyacrylamide gradient gel; 16 × 16 cm). Gels were placed in SYPRO Ruby Protein Gel Stain (Bio-Rad, Hercules, CA, USA) for 16 h and then destained in 10% methanol and 7% acetic acid. Images were acquired using a MOLECULAR IMAGER FX (Bio-Rad) and the images were exported to the image analysis software program, PDQuest (Bio-Rad).

**Mass spectrometry and database analysis**

Spots from SYPRO Ruby Protein Gel Stain 2D gels were excised, reduced in-gel, alkylated, trypsinized and analyzed by liquid chromatography-mass spectrometry (LC-MS) using a DiNa-2A nanoLC system (KYA Technologies, Tokyo, Japan) coupled online to a LCMS-IT-TOF mass spectrometer (Shimadzu, Kyoto, Japan). The LC separation proceeded using a PicoFrit column BetaBasic C18 (New Objective, Woburn, MA, USA) at a constant flow rate of 300 nL/min. Peptides were eluted from 10 μL samples using gradients of 5–40% solvent B (0.1% formic acid in 80% ACN)/0–30 min, 40–100% solvent B/30–40 min, and 100–100% solvent B/40–60 min. LCMS-IT-TOF was operated in the data-dependent MS/MS mode. The capillary temperature and electrospray voltage were set at 200 °C and 2.2 kV, respectively. Data were collected at scan ranges of 400–1500 for MS and 50–1500 for MS/MS. Proteins were identified by Mascot searches (Matrix Science, http://www.matrixscience.com/).

**Measurement of S100A9 by ELISA**

Serum and BALF concentrations of S100A9 were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (BMA Biomedicals, Augst, Switzerland) according to the manufacturer’s protocols. The lower limit of detection was 0.31 ng/mL. Albumin concentrations in BALF were determined by a Human Albumin ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX, USA).

**Immunohistochemical staining**

Specimens were immunohistochemically analyzed by conventional avidin-biotin-peroxidase staining using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Briefly, sequential 4-μm paraffin sections were deparaffinized with toluene and rinsed thoroughly with absolute ethanol. Antigen was retrieved by incubating sections with proteinase K for 15 min. The sections were soaked in absolute methanol containing 0.3% H₂O₂ with for 20 min at room temperature to inactivate endogenous peroxidase and then incubated with serum for 20 min followed by a mouse anti-human S100A9 monoclonal antibody.
overnight at 4°C (BMA Biomedicals). The sections were washed in PBS, processed according to the manufacturer’s instructions provided with the kits, developed with 3,3’-diaminobenzidine and H2O2 and then stained with Mayer’s hematoxylin. Control lung tissues were obtained from normal areas of lungs that were surgically removed to treat cancer. Negative control studies included non-immunized immunoglobulin G with the same subclass of first antibody instead of primary antibody.

Statistical analysis

All values are expressed as means ± SD. Differences between multiple groups were compared using the Kruskal–Wallis test and the post hoc Scheffé test. Correlations between parameters were determined by Pearson’s correlation coefficient. Survival data were analyzed using Kaplan–Meier curves and survival between two groups was compared using the log-rank test. The concentrations of serum S100A9, KL-6, SP-D and SP-A, and of S100A9 in BALF were analyzed using receiver operating characteristic (ROC) curves to determine cut-off values for optimal discriminative accuracy. p Values of <0.05 were regarded as significant. Data without ROC curves were analyzed using statistical software (Stat ViewJ-5.0, SAS Institute, Cary, NC, USA) and ROC curves were analyzed using Eksuer-Toukei 2010 (Social Survey Research Information Co. Ltd., Tokyo, Japan).

Results

Characteristics of study population

Table 1 shows the characteristics of the patients with IPF, I-NSIP, COP and CVD-IP. Serum concentrations of C-reactive protein (CRP) in patients with COP were increased compared with IPF and I-NSIP (p < 0.05), and those of KL-6 and LDH in patients with IPF were higher than those with COP (p < 0.05). Serum SP-D levels in patients with I-NSIP were higher than in those with COP and CVD-IP (p < 0.05). Table 2 shows the components of BALF from patients with IPF, I-NSIP, COP and CVD-IP. The ratio of lymphocytes was significantly higher in patients with I-NSIP and COP than with IPF (p < 0.05) and that of neutrophils was higher in patients with CVD-IP than with I-NSIP (p < 0.05).

Two-dimensional gel electrophoresis of BALF

We analyzed protein profiles in BALF from healthy controls and from patients with IPF (Fig. 1a and b, respectively). Among a total of 170 and 206 respectively resolved protein spots, one was selected for differential expression analysis based on at least a fivefold increase in the optical density in samples from patients with IPF compared with values from controls. Mass spectrometry and Mascot searches revealed that the intense spot in BALF from IPF patients was S100A9.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of patients with IPF, I-NSIP, COP and CVD-IP.</th>
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<tbody>
<tr>
<td></td>
<td>IPF (N = 28)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>63.2 ± 10.2</td>
</tr>
<tr>
<td>Gender</td>
<td>23/5</td>
</tr>
<tr>
<td>Pulmonary function tests</td>
<td></td>
</tr>
<tr>
<td>%VC (%)</td>
<td>82.8 ± 20.1</td>
</tr>
<tr>
<td>FEV1.0 (%)</td>
<td>81.2 ± 7.9</td>
</tr>
<tr>
<td>%DLCO (%)</td>
<td>50.7 ± 14.8</td>
</tr>
<tr>
<td>Laboratory data</td>
<td></td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>0.46 ± 0.49</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>300 ± 127</td>
</tr>
<tr>
<td>KL-6 (U/mL)</td>
<td>1448 ± 1020</td>
</tr>
<tr>
<td>SP-A (ng/mL)</td>
<td>120 ± 62</td>
</tr>
<tr>
<td>SP-D (ng/mL)</td>
<td>297 ± 175</td>
</tr>
<tr>
<td>PaO2 (Torr)</td>
<td>81.0 ± 12.0</td>
</tr>
</tbody>
</table>

%DLCO, ratio of diffusing capacity for carbon monoxide; CRP, C-reactive protein; LDH, lactic dehydrogenase; KL-6, Krebs von den Lungen 6; SP-A, surfactant protein A; SP-D, surfactant protein D; Data are means ± SD. *p < 0.05 vs. IPF; †p < 0.05 vs. I-NSIP.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Cell findings in BALF from patients with IPF, I-NSIP, COP and CVD-IP.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>IPF (N = 28)</td>
</tr>
<tr>
<td>TCC (×10^6/mL)</td>
<td>4.1 ± 1.5</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>70.7 ± 15.4</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>16.5 ± 12.9</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>6.5 ± 6.6</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>6.1 ± 7.2</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td>1.5 ± 1.1</td>
</tr>
</tbody>
</table>

TCC, total cell count; Data are means ± SD. *p < 0.05 vs. IPF, †p < 0.05 vs. I-NSIP.
We quantified S100A9 levels in serum and BALF from healthy controls and from patients with IPF, I-NSIP, COP and CVD-IP using an ELISA. Serum levels of S100A9 (Fig. 2a) in IPF patients tended to be higher than in patients with I-NSIP, COP and in controls, but the difference did not reach statistical significance. Levels of S100A9 in BALF (Fig. 2b) were higher in patients with IPF than with I-NSIP, COP and CVD-IP, and in controls ($p < 0.05$). BALF S100A9 levels normalized for albumin levels in BALF showed similar results including ROC curve analysis and association with clinical parameters (data not shown).

**Figure 1** Analytical two-dimensional gel electrophoresis of BALF from healthy control (a) and IPF (b). Non-linear 10–18% gradient gels (pH 3–0–10–0) were stained with SYPRO Ruby Protein Gel Stain. Spot indicated by arrow is more intense in (b) than in (a).

**Figure 2** Serum (a) and BALF (b) levels of S100A9. Levels of S100A9 in BALF are higher in patients with IPF than with I-NSIP, COP, CVD-IP and healthy controls. *$p < 0.05$ compared with IPF.*
ROC for biomarkers of IPF versus other fibrotic interstitial pneumonias

We evaluated the sensitivity and specificity of distinguishing IPF from I-NSIP and CVD-IP based on BALF S100A9 levels using ROC curves, because the levels were significantly elevated in patients with IPF compared with those who had I-NSIP and CVD-IP, and thus, might serve as a useful marker of IPF. Fig. 3 shows the ROC curves for S100A9 obtained from serum and BALF and for markers of interstitial lung disease (KL-6, SP-D and SP-A) in patients with IPF and other fibrotic interstitial pneumonias (I-NSIP and CVD-IP). The area under the ROC curve (AUC) was the largest for S100A9 in BALF; serum S100A9, 0.773; BALF S100A9, 0.972; KL-6, 0.571; SP-D, 0.684; SP-A, 0.726. Cut-off values taken as the points closest to 100% sensitivity and 100% specificity, were 4.95 and 1 ng/mL for serum (sensitivity, 96.4%; specificity, 57.8%) and BALF (sensitivity, 96.4%; specificity, 87.8%) S100A9, respectively. Levels of S100A9 in BALF were more specific and sensitive than other markers of interstitial pneumonia in terms of distinguishing IPF from other fibrotic interstitial pneumonias.

Association between S100A9 and clinical parameters in patients with IPF

We analyzed the relationship between the clinical parameters of pulmonary function, arterial blood gases, markers of interstitial lung diseases and BALF cell findings, and levels of S100A9 in patients to determine the role of S100A9 in IPF (Table 3). Levels of S100A9 in both serum and BALF from all study participants did not significantly correlate \( r = 0.24, p = 0.22 \). Serum and BALF levels of S100A9 did not significantly correlate with pulmonary function, partial pressure of oxygen, or serum levels of CRP, LDH, SP-D and SP-A. Higher levels of BALF S100A9 were associated with lower serum levels of KL-6 \( r = -0.41, p = 0.03 \). Serum and BALF levels of S100A9 significantly correlated with the number of neutrophils in BALF (Table 3, Fig. 4) and did not correlated with survival rates for patients with IPF (data not shown). The results were similar even when the data was analyzed in patients with biopsy proven IPF (data not shown).

Immunohistochemical staining of S100A9

Fig. 5 shows representative lung specimens that were immunohistochemically stained for S100A9 and obtained from normal areas of lungs surgically removed to treat cancer and from patients with IPF and I-NSIP. S100A9 was expressed weakly in control lung specimens (Fig. 5a) but obviously and mainly in macrophages in the airspace and in inflammatory cells that were supposedly macrophages and neutrophils in lung specimens from patients with IPF (Fig. 5d). S100A9 was also overexpressed in endothelial vascular cells (Fig. 5e), weakly expressed in alveolar epithelial cells and absent in fibroblasts. Although S100A9 immunoreactivity did not significantly differ between IPF and I-NSIP, lymphocytes did not overexpress S100A9 in lung specimens from patients with I-NSIP (Fig. 5f). S100A9 immunoreactivity in CVD-IP patients did not differ between IPF and CVD-IP (data not shown).

Discussion

The IIPs are an important subset of a broader category of interstitial lung diseases. Among these, IPF and I-NSIP have provoked the most debate and discussion because they are clinically similar, but the outcome of the latter is
far more favorable. The potential of several proteins, such as KL-6, SP-A and SP-D, as biomarkers of interstitial lung disease has been examined in the clinical setting. However, no biomarkers can yet distinguish IPF from I-NSIP. In addition, CVD is accompanied by fibrotic interstitial pneumonia that resembles IPF and I-NSIP. Some patients with CVD do not have typical symptoms other than interstitial pneumonia. Thus, to distinguish IPF from I-NSIP and CVD is often difficult. The present study found elevated levels of S100A9 in BALF from patients with IPF compared with other types of IIPs, CVD-IP and from healthy controls. Furthermore, ROC curve analyses showed that BALF S100A9 levels could distinguish IPF from I-NSIP and CVD-IP with higher specificity and sensitivity than other markers of interstitial pneumonia. This suggests that S100A9 in BALF could serve as a novel biomarker, especially in terms of distinguishing IPF from I-NSIP and CVD-IP. Furthermore, others have found that S100A9 levels in BALF are increased compared with sarcoidosis, suggesting that high S100A9 expression in BALF is specific to IPF.

Serum S100A9 levels in the present study were increased not only in patients with IPF, but also in those with CVD-IP. S100A9 in serum is overexpressed under inflammatory conditions such as chronic inflammatory bowel diseases, Kawasaki disease and collagen vascular diseases (rheumatoid arthritis, systemic lupus erythematosus, Sjögren’s syndrome, systemic sclerosis, dermatomyositis and polymyositis). Even patients with CVD-IP and increased levels of S100A9 in serum did not have such an increase in BALF. These results suggest that S100A9 in serum reflects systemic inflammation, whereas S100A9 in BALF reflects local inflammatory conditions in the lung. As a result, S100A9 in BALF, and not in serum, might serve as a marker to distinguish IPF from I-NSIP and CVD-IP.

Both serum and BALF levels of S100A9 in patients with IPF were associated with the number of neutrophils in BALF, which supports previous findings. S100A9 causes neutrophil chemotaxis and neutralization with antibodies against S100A9 blocks neutrophil recruitment. Increased levels of S100A9 in BALF might result in neutrophil accumulation in the lungs of patients with IPF. Neutrophilia frequently appears in BALF from patients with IPF and is associated with the clinical features of IPF and the early mortality of such patients. Sustained neutrophil accumulation in the alveolar space and neutrophil-mediated injury to the alveolar wall are believed to play a role in interstitial fibrosis and abnormal lung repair. Furthermore, S100A9 reportedly stimulates the proliferation of fibroblasts that play a pivotal role in lung fibrosis. In contrast, S100A9 participates in wound-healing, protects against bacterial infection and oxidative damage, and exerts anti-asthmatic effects. Thus, S100A9 is considered to have anti-inflammatory roles and to mediate host protection. Although further studies are required to elucidate the role of S100A9 in pulmonary fibrosis, the present results suggest that S100A9 is involved in the pathogenesis of IPF via neutrophilic inflammation in IPF.

The immunohistochemical findings revealed S100A9 expression mainly in macrophages in the air space, in interstitial inflammatory cells such as macrophages and neutrophils, and in endothelial vascular cells of the lungs from patients with IPF. Interstitial inflammatory cells in the lungs of patients with I-NSIP and CVD-IP express similar amounts of S100A9, whereas the S100A9 levels in BALF differed between IPF and others. S100A9 is now considered as a damage-associated molecular pattern protein (DAMP), because it exists in an intracellular form that is released by activated or damaged cells under conditions of cellular stress. S100A9 is secreted by neutrophils during interactions with endothelial cells, suggesting that it plays a role in leukocyte migration. In addition, S100A9 secretion is induced in neutrophils by factors such as lipopolysaccharide and C5a, as well as in monocytes by PMA. Necrotic neutrophils also release S100A9. Although the reason for the discrepancy between BALF levels and immunohistochemical expression of S100A9 remains unclear, different mechanisms might be involved such as the release, clearance or degradation of S100A9 in the lungs of patients with IPF compared with other fibrotic interstitial pneumonias.
The present study did not identify an association between S100A9 levels and other clinical parameters such as lung function, arterial blood gases and prognosis in IPF patients. Korthagen et al. reported a similar lack of association between BALF S100A9 levels and lung functions. On the other hand, Bargagli et al. reported that BALF S100A9 levels normalized for BALF total protein concentrations inversely correlate with lung function. Further studies are required to clarify the role of S100A9 from clinical aspects and the pathogenesis of IPF.

In conclusion, this study demonstrated higher BALF levels of S100A9 in patients with IPF than in those with I-NSIP, COP and CVD-IP and in healthy controls. S100A9 in BALF might be a useful candidate biomarker with which to discriminate IPF from other fibrotic interstitial pneumonias such as I-NSIP and CVD-IP.

**Conflict of interest statement**

The authors have no conflict of interest.

**Acknowledgements**

The authors thank Mr. Atsushi Yokoyama (Nagasaki University Hospital) for excellent technical assistance, Dr. Masanori Kitaichi (Department of Laboratory Medicine and Pathology, NHO Kinki-Chuo Chest Medical Center) for valuable advice regarding pathological diagnoses, Dr. Mariko Mine (Division of Scientific Data Registry, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences) for valuable advice regarding the statistical analysis, and SHIMADZU TECHNO-RESEARCH, INC.


