Clinical significance of serum S100 calcium binding protein A4 in idiopathic pulmonary fibrosis

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SUMMARY AT A GLANCE:

S100 calcium binding protein A4 is a promising serum biomarker that may predict disease progression and mortality in idiopathic pulmonary fibrosis patients. Insights from this study may facilitate establishment of treatment strategies for idiopathic pulmonary fibrosis.

ABSTRACT

Background and objective: Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease with a poor prognosis. There are no established serum biomarkers for predicting the outcomes of IPF. S100 calcium binding protein A4 (S100A4) is considered a marker of fibroblasts; however, its clinical application remains to be investigated. We evaluated the clinical relevance of S100A4 in IPF patients.

Methods: Serum S100A4 levels in 95 consecutive IPF patients and 50 healthy controls (HCs) were measured using enzyme-linked immunosorbent assay. S100A4 expression in lung tissues was determined using immunohistochemistry/immunofluorescence and its association with disease progression (defined as deterioration in lung function or death) and mortality was assessed using Kaplan–Meier method and Cox hazards analysis.

Results: Serum S100A4 levels were undetectable in all HCs but were detectable in 26 (27.3%) of the 95 IPF patients ($P < 0.01$). Immunostaining of lung tissues from IPF patients showed aggregation of numerous S100A4-expressing cells around the fibroblastic foci and mature fibrotic regions. IPF patients with higher serum S100A4 levels had a significantly worse prognosis than those with low serum levels (2-year cumulative survival rate, 41.7% vs. 77.0%, respectively, $P < 0.01$). On multivariate analyses, baseline serum S100A4 levels (per 10 ng/mL increase) were independently associated with higher disease progression rate (odds ratio 1.06, $P = 0.01$) and higher mortality (hazard ratio 1.18, $P =$

0.03).

Conclusions: S100A4 is a promising serum biomarker that may help predict disease progression/mortality. Our findings may help establish treatment strategies for IPF.

Key words:

Biomarker; fibroblast; idiopathic pulmonary fibrosis; S100A4

Short title:

S100A4 in IPF

Abbreviations:

AE, acute exacerbation; CI, Confidence Interval; DLCO, diffusing capacity of lung for carbon monoxide; $FEV_{1.0}$, forced expiratory volume 1.0 (sec); FVC, forced vital capacity; GAP, Gender–Age–Physiology; HC, healthy control; HRCT, high-resolution computed tomography; IPF, idiopathic pulmonary fibrosis; IQR, interquartile range; KL-6, Krebs von den Lungen-6; OR, odds ratio; SD, standard deviation; SP-D, surfactant protein D; S100A4, S100 calcium binding protein A4; UIP, usual interstitial pneumonia.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease characterized by abnormal proliferation of epithelial/mesenchymal cells, disorganized deposition of extracellular matrix, and fibroblastic foci.[1-5] IPF is associated with high mortality (average survival: 3–5 years); however, its clinical course is highly variable.[6] Current treatment options for IPF include anti-fibrotic agents and lung transplantation.[4] Early intervention may help improve clinical outcomes.[7] Therefore, identification of non-invasive biomarkers that can predict disease progression is a key imperative.

S100 calcium binding protein A4 (S100A4, also termed fibroblast-specific protein-1) belongs to the S100 family of proteins containing calcium-binding motifs. S100A4 is a marker of fibroblasts used to study the mechanism of tissue fibrosis.[8-14] Reportedly, S100A4 promotes lung fibrosis via proliferation and activation of fibroblasts.[15-17] Therefore, we hypothesized that serum S100A4 levels in IPF patients may reflect pulmonary fibroblastic activity and may serve as a useful prognostic biomarker. The clinical relevance of serum S100A4 in IPF patients remains to be investigated. Therefore, this study examined the S100A4 expression in lung specimens and evaluated the association of serum S100A4 level with clinical parameters and mortality in IPF patients.

METHODS

Patients and diagnostic criteria

We retrospectively screened consecutive IPF patients diagnosed between 2002 and 2016 at the Hamamatsu University Hospital (Hamamatsu, Japan) and for whom serum samples collected at the time of diagnosis were available. No patient had received anti-fibrotic or anti-inflammatory therapy before the diagnosis of IPF. The diagnosis of IPF was based on multidisciplinary discussion, according to the international consensus criteria.[1-3, 5] All patients were followed up for >1 year or until death. Acute exacerbation (AE) of IPF was diagnosed on the basis of 2016 International Working Group report.[18] The exclusion criteria were: acute exacerbation of IPF (AE-IPF) or presence of severe comorbidity at the time of diagnosis (advanced malignancy, liver cirrhosis or renal failure requiring haemodialysis). Consequently, 95 IPF patients were enrolled in this study. Serum samples were also collected from 50 age- and sex-matched healthy controls (HCs). This retrospective study was conducted according to the Declaration of Helsinki. Signed consent forms were obtained from all subjects with the exception of those who died before 2016. The institutional review board of the Hamamatsu University School of Medicine waived the informed consent requirement for deceased patients and approved this study (approval number 17-164).

Data collection

Data pertaining to the following variables were collected from medical records: sex; age; smoking history; laboratory data; results of pulmonary function tests, Gender–Age–Physiology (GAP) stage,[19] high-resolution computed tomography (HRCT) and histopathological examination of lung biopsy; treatment details; survival outcomes.

Measurement of serum S100A4 levels

Baseline serum samples were collected at the time of diagnosis and stored at −80°C until further analysis. Serum S100A4 levels were retrospectively measured using commercially available enzyme-linked immunosorbent assay (CircuLex S100A4 ELISA Kit Ver.2; MBL CO, LTD., Nagoya, Japan) according to the manufacturer's instructions.

S100A4 immunohistochemistry and immunofluorescence

Formalin-fixed, paraffin-embedded sections (5 μm thick) of surgically-resected lung biopsy specimens from IPF patients were analysed. Peritumoural normal lung tissues from lung cancer patients without IPF were used as control. Detailed methods are presented in Supplementary Method S1.

Statistical methods

Statistical analyses were performed using EZR (Jichi Medical University, Saitama, Japan),

which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). Data are expressed as median [interquartile range (IQR) or range] or as frequency (%). Fisher's exact test was used for comparing proportions among groups. Between-group differences were assessed using the Wilcoxon/Kruskal–Wallis test. Correlation between different parameters was evaluated using the Spearman's correlation test. Disease progression was defined as deterioration in lung function [≥10% relative decline in %predicted forced vital capacity (%FVC)] or death. Progression-free survival (PFS) was defined as the time from the date of diagnosis until the date of first 'disease progression', death or the most recent follow-up. The observation period lasted from the date of diagnosis until the most recent follow-up or the date of death. Patients were censored if they remained alive until 31 August 2018. Receiver-operating characteristic (ROC) curve analysis was performed to identify an optimal cut-off value, which was decided as the point with the highest value of sensitivity + specificity – 1 (Youden's index). Survival rates and between-group differences were calculated using the Kaplan–Meier analysis and log-rank test, respectively. Cox proportional hazards regression analyses with time-dependent covariates were performed to identify factors associated with disease progression and mortality in IPF patients; age, sex, anti-fibrotic treatment, and all variables that showed a significant association in univariate analysis were included in the multivariate analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Baseline characteristics

Baseline characteristics are summarized in Table 1. No significant differences were observed between IPF patients and HCs with respect to age or sex. Among the 95 IPF patients, 71 (74.7%) experienced disease progression, 26 (27.4%) developed AE, and 51 (53.7%) died during the observation period. Forty-six deaths were due to respiratory failure, 3 were attributed to lung cancer that developed after IPF diagnosis, and 2 were attributed to a coronary event.

Treatment details pertaining to the observation period are presented in Supplementary Table S1. Among the 95 patients, 65 (68.4%) received specific treatment for IPF. Fifty (52.6%) patients were treated with ant-fibrotic agents (pirfenidone and nintedanib). Thirty-two (33.7%) patients were treated with corticosteroids with/without immunosuppressants during the observation period due to AE-IPF that developed after IPF diagnosis in most cases.

Serum S100A4 levels

Baseline serum S100A4 levels are presented in Figure 1. Serum S100A4 were undetectable in all the HCs (nearly 0 ng/mL), but were detectable in 26 of the 95 (27.3%)

IPF patients ($P < 0.01$).

S100A4 expression in lung tissues

Representative results of immunostaining are presented in Figure 2 and Supplementary Figure S1-S2. Immunohistochemistry of control lungs (n = 4) demonstrated expression of S100A4 in intra-alveolar macrophages; however, S100A4 was sparsely expressed in normal alveolar tissue (Figure 2A). Conversely, lung tissues of IPF patients exhibited diffuse and partially strong expression of S100A4 (n = 8; Figure 2B). In particular, abundant S100A4-expressing cells were observed in the areas between the periphery of fibroblastic foci and adjacent, nearly intact alveolar structures as well as adjacent to areas of mature fibrosis (Figures 2B and 2C). Immunofluorescence also showed abundant S100A4-expressing cells around the periphery of fibroblastic foci that were constituted by αSMA-positive but S100A4-negative myofibroblasts (Supplementary Figure S1A–D). Although there were a small number of S100A4-expressing cells among the fibrotic foci, co-expression of S100A4 and αSMA was not observed in these cells (Supplementary Figure S1D).

S100A4 levels and clinical parameters

Serum S100A4 levels in IPF patients showed neither a significant correlation with any

clinical parameter (age, laboratory data or results of pulmonary function tests; Supplementary Table S2) nor an association with smoking habit (Supplementary Figure S3).

Subgroup analysis based on S100A4 levels

ROC curve analysis was performed to identify the optimal cut-off values of serum S100A4 for predicting disease progression (Supplementary Figure S4). Using 22.3 ng/mL as the cut-off value of serum S100A4, the sensitivity and specificity were 42.9% and 83.3%, respectively. Considering this result, patients with serum S100A4 level < 22.3 ng/mL and those with serum S100A4 level ≥ 22.3 ng/mL were categorized as serum $S100A4^{low}$ and serum $S100A4^{high}$ subgroups, respectively.

The baseline characteristics of IPF patients disaggregated into $S100A4^{high}$ and $S100A4^{low}$ subgroups and are presented in Supplementary Table S3. The median PaO_2 level in the $S100A4^{high}$ subgroup was significantly higher than that in the $S100A4^{low}$ subgroup (80 Torr vs. 75 Torr, respectively; $P = 0.04$). A significant between-group difference was observed in baseline GAP stage ($P < 0.01$). However, no significant between-group differences were observed with respect to other baseline characteristics.

Kaplan–Meier survival curves of the $S100A4^{high}$ and $S100A4^{low}$ subgroups are shown in Figure 3. PFS rate in the $S100A4^{high}$ subgroup was significantly lower than that in the

S100A4^{low} subgroup (1-year cumulative PFS rate, 58.4% vs. 77.8%, respectively; *P* = 0.01; Figure 3A). Survival rate in the S100A4^{high} subgroup was significantly lower than that in the S100A4^{low} subgroup (2-year cumulative survival rate, 41.7% vs. 77.0%, respectively; $P < 0.01$; Figure 3B).

Analyses regarding the combination of serum S100A4 and GAP staging system are presented in Supplementary Figure S5. GAP system solely showed a good prognostic separation (Figure S5A). Regarding cumulative survival rates in the GAP stage III group, the S100A4^{high} and S100A4^{low} subgroups showed no between-group differences (log-rank, P = 0.93; Figure S5B). In the GAP stage I group, the S100A4^{high} subgroup tended to have a lower cumulative survival rate compared with that in the S100A4^{low} subgroup (log-rank, *P* = 0.09; Figure S5C).

Prognostic significance of serum S100A4

The results of Cox proportional hazards regression analysis showing correlates of disease progression are presented in Table 2. On univariate analysis, higher PaO_2 levels, higher %DLCO, and higher %FVC were associated with a lower disease progression rate. Conversely, serum S100A4^{high} (vs. S100A4^{low}) and higher serum S100A4 levels (per 10 ng/mL increase) were associated with a higher disease progression rate. We adjusted for each of 'serum S100A4^{high}' and 'serum S100A4 level' in separate multivariate models. In

both models, higher %FVC was independently associated with a lower disease progression rate, whereas higher serum S100A4 levels [hazard ratio (HR) 1.06 per 10 ng/mL increase, $P = 0.01$], but not serum S100A4^{high} (vs. serum S100A4^{low}; HR 1.65, $P = 0.07$), was an independent predictor of a higher disease progression rate.

Results of the Cox proportional hazards regression analysis of mortality are presented in Table 3. On univariate analysis, higher PaO_2 levels, higher %DLCO, and higher %FVC were associated with significantly lower mortality rate. Conversely, higher age, serum S100A4^{high} (vs. S100A4^{low}), and higher serum S100A4 levels were associated with a higher mortality rate. We also adjusted for both 'serum S100A4^{high}' and 'serum S100A4 level' in separate multivariate models. In both models, higher PaO_2 levels and higher %FVC showed an independent association with a lower mortality rate, whereas higher serum S100A4 levels (HR 1.18 per 10 ng/mL increase, $P = 0.03$), but not serum S100A4^{high} (vs. serum S100A4^{low}; HR 1.69, $P = 0.10$), was an independent predictor of a higher mortality rate.

DISCUSSION

In this study, serum S100A4 levels were undetectable in all HCs but were detectable in approximately 27% of IPF patients. Immunostaining demonstrated infiltration of abundant S100A4-expressing cells in lung tissues of IPF patients. Multivariate analyses revealed an

independent association of higher serum S100A4 levels with both a higher disease progression rate and a higher mortality rate. To our knowledge, this is the first study that identified the clinical significance of serum S100A4 in IPF patients.

Activated fibroblasts and myofibroblasts play a key role in fibrogenesis in IPF.[20-23] S100A4 reportedly promotes the transition of fibroblasts to myofibroblasts, inducing αSMA and collagen 1 expression.[8] In this context, the role of S100A4 in lung fibrosis has been investigated in a mouse model of bleomycin-induced lung fibrosis.[9, 11, 15-17] Tanjore et al. found that S100A4-positive lung fibroblasts were derived from both lung epithelial cells undergoing epithelial–mesenchymal transition and bone marrow progenitor cells.[9] Xia et al. reported that administration of mesenchymal progenitor cells derived from IPF patients converts the model of self-limited lung fibrosis to model of persistent fibrosis in an S100A4-dependent manner.[15] Li et al. reported that while the deficiency of S100A4 attenuated pulmonary fibrosis, adoptive transfer of $S100A4^{+}$ macrophages induced lung injury/fibrosis in $S100A4^{-/-}$ mice.[16] Collectively, these studies indicated a pro-fibrotic/pathogenic role of tissue S100A4 in pulmonary fibrosis.

Two studies have documented increased expression of S100A4 in lung tissues of IPF patients by immunohistochemistry.[11, 15] Consistently, we also found prominent expression of S100A4-positive cells in IPF lungs. Considering that S100A4 is a protein that can be secreted into extracellular space,[8] fibrotic lung tissue is a potential source of serum

S100A4 in IPF patients. Interestingly, a previous study found numerous S100A4-expressing cells primarily in the interface between relatively unaffected alveolar structures and the periphery of fibroblastic foci that were constituted by αSMA-positive but S100A4-negative myofibroblasts; these findings suggested that these regions are an active fibrotic front-line and that S100A4-expresing cells induce fibrosis in the adjacent unaffected alveolar structures in IPF patients.[15] This is consistent with our findings. Moreover, we found infiltration of abundant S100A4-expressing cells in areas adjoining the area of mature fibrosis. These findings also suggest that greater expression of S100A4 in lung tissues represents higher pro-fibrotic activity, which participates in the progression of IPF.

In this study, serum S100A4 level was an independent prognostic factor in IPF patients. Higher serum S100A4 levels were independently associated with a higher disease progression rate. Therefore, the increased mortality risk in the $S100A4^{high}$ subgroup is likely attributable to the higher disease progression rate. These results suggest that serum S100A4 level as well as tissue S100A4 expression reflects the activity of lung fibrosis. Interestingly, the combination of S100A4 and GAP system suggested that serum S100A4 facilitates further prognostic stratification in the GAP I group of this study. However, the sample size may have been too small to detect a significant difference. A prospective larger study is required to validate this result. Collectively, the serum S100A4 level can be a

useful prognostic biomarker that may help identify patients who may benefit from early therapeutic intervention (e.g., pirfenidone or nintedanib therapy).

Several studies have evaluated the potential of S100A4 protein as a therapeutic target. In studies by Li et al. and Zhang et al., blockade with neutralizing antibodies against S100A4 and pharmacologic inhibition of S100A4, respectively, attenuated pulmonary fibrosis in the bleomycin mouse model.[16, 17] Our results suggest that IPF patients with elevated serum S100A4 levels represent a phenotype associated with poor prognosis. Therefore, these patients may possibly benefit from anti-S100A4 therapy. Stratification of IPF patients based on S100A4 expression level seems a plausible strategy under the paradigm of precision medicine that warrants further investigation.

The present study had several limitations. First, the retrospective design of the study renders it vulnerable to several biases. Our institution is a regional ILD referral centre, which may have introduced an element of selection bias. Second, baseline serum S100A4 levels were detectable in approximately 27% of IPF patients. It is possible that its clinical usefulness is applicable only to a subgroup of IPF patients. Additionally, a larger study is required to identify the prevalence of IPF patients with high serum S100A4 levels and its optimal cut-off value for predicting outcome. Third, we did not evaluate serial changes in serum S100A4 levels. Further investigation is required to determine whether serum S100A4 levels in $S100A4^{low}$ IPF patients increase over their clinical course, thereby

clarifying the association between its changes and their clinical outcome. Future studies should perform repeated measurements of serum S100A4 levels to unravel predictors of the risk of disease progression or mortality. Finally, the different treatment regimens for IPF may have affected the outcomes in our study population.

In conclusion, the present study indicated that serum S100A4 is a promising non-invasive biomarker that may help predict disease progression/mortality in IPF patients. This biomarker may facilitate risk stratification of patients. A prospective, multicentre study is required to validate the clinical and pathophysiological utility of serum S100A4 in IPF.

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Author contributions:

N.A., H.H., N.E., T.F., N.I., Y.N., and T.S. designed the research; N.A., H.H., T.I., J.O., K.S., H.Y., Y.S., M.K., M.K., K.F., N.E., T.F., N.I., Y.N., and T.S. contributed to the acquisition or analysis of the data; N, A. and H.H. wrote the initial and final drafts of the manuscript; N.A., H.H., T.I., J.O., K.S., H.Y., Y.S., M.K., M.K., K.F., N.E., T.F., N.I., Y.N., and T.S. revised the drafts of the manuscript; and all authors approved the final version of the manuscript.

Guarantor statement:

H.H. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest:

This study was conducted as a collaboration between Hamamatsu University School of Medicine and Medical and Biological Laboratories. Takuya Isayama, Jun Okada, and Katsunori Sugiura are employees of Medical and Biological Laboratories. The remaining authors declare no conflict of interests, including personal or financial support, and no

involvement with organization(s) that have financial interest in the subject matter.

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Table 1 Baseline characteristics

	IPF n = 95	**HC n = 50**	***P*-value**
Age, years	71 (64–77)	69 (66–73)	0.77
Male/female	83 (87.4)/12 (12.6)	42 (84)/8 (16)	0.62
Smoking habit, never/former/current	19 (20.0) / 61 (64.2)/15 (15.8)		
Diagnosis, clinical/pathologically proven	72 (75.8)/23 (24.2)		
Laboratory data			
KL-6, U/mL	924 (596–1390)		
PaO_2, Torr	76 (69–85)		
Pulmonary function test			
%FVC, %	74 (58–89)		
$FEV_{1.0}$/FVC, %	82 (79–89)		
%DLCO, %*	61.5 (44.8–86)		
GAP stage, I/II/III§	32 (47.8)/20 (29.9)/15 (22.4)		
Observation period, months	33.5 (15.1–50.1)		
Disease progression‡	71 (74.7)		
Progression-free survival, months	22.3 (11.3–44.4)		
Deterioration in lung function	41 (43.1)		
Death	30 (31.6)		
AE‡	26 (27.4)		
All-cause death‡	51 (53.7)		
Death from respiratory failure‡	46 (48.4)		

Data are presented as median (IQR), or number (%).

* n = 58

§ n = 67

‡ During observation period

IPF, idiopathic pulmonary fibrosis; HC, healthy control; KL-6, Krebs von den Lungen-6; PaO_2, arterial oxygen pressure; %FVC, percent predicted forced vital capacity; $FEV_{1.0}$, forced expiratory volume 1.0 (sec); %DLCO, percent predicted diffusing capacity of the lung carbon monoxide; GAP, Gender–Age–Physiology index; AE, acute exacerbation.

Table 2 Results of the Cox proportional hazards regression analysis of disease progression

Variable	HR	95%CI	*P*-value
Univariate analysis			
Male (vs. female)	0.65	0.34–1.42	0.26
Age, years	1.03	0.99–1.06	0.10
UIP pattern on HRCT, yes (vs. no)	0.98	0.55–1.66	0.94
PaO_2, per 1Torr increase	0.97	0.95–0.99	0.03*
%DLCO, per 1% increase §	0.97	0.96–0.99	<0.01*
%FVC, per 1% increase	0.96	0.95–0.97	< 0.01*
KL-6, per 100 U/mL increase	1.01	0.99–1.03	0.36
Anti-fibrotic treatment, yes (vs. no)	0.79	0.42–1.49	0.47
Serum $S100A4^{high}$ (vs. $S100A4^{low}$)	1.85	1.11–3.02	0.02*
Serum S100A4, per 10 ng/mL increase	1.05	1.01–1.08	0.01*
Multivariate analysis model 1			
Male (vs. female)	0.84	0.42–1.90	0.66
Age, years	1.02	0.98–1.05	0.33
PaO_2, per 1Torr increase	0.98	0.96–1.004	0.12
%DLCO, per 1% increase §	0.98	0.96–0.99	0.02*
%FVC, per 1% increase	0.97	0.95–0.98	< 0.01*
Anti-fibrotic treatment, yes (vs. no)	0.97	0.59–1.56	0.89
$S100A4^{high}$ (vs. $S100A4^{low}$)	1.65	0.96–2.78	0.07
Multivariate analysis model 2			
Male (vs. female)	0.80	0.40–1.78	0.56
Age, years	1.02	0.98–1.05	0.30
PaO_2, per 1Torr increase	0.98	0.96–1.003	0.11
%DLCO, per 1% increase §	0.98	0.96–1.002	0.08
%FVC, per 1% increase	0.97	0.95–0.98	<0.01*
Anti-fibrotic treatment, yes (vs. no)	0.95	0.58–1.53	0.82
Serum S100A4, per 10 ng/mL increase	1.06	1.02–1.10	0.01*

*$P < 0.05$.

§ n = 58

HR, hazard ratio; 95%CI, 95% confidence interval; UIP, usual interstitial pneumonia; HRCT, high-resolution computed tomography; PaO_2, arterial oxygen pressure; %FVC, predicted forced vital capacity; $FEV_{1.0}$, forced expiratory volume 1.0(sec); %DLCO, predicted diffusing capacity of the lung carbon monoxide; KL-6, Krebs von den Lungen-6; SP-D, surfactant protein D.

Table 3 Results of the Cox proportional hazards regression analysis of mortality

Variable	HR	95%CI	*P*-value
Univariate analysis			
Male (vs. female)	0.65	0.30–1.71	0.35
Age, years	1.04	1.01–1.09	0.02*
UIP pattern on HRCT, yes (vs. no)	0.98	0.53–1.91	0.95
PaO_2, per 1 Torr increase	0.94	0.91–0.97	<0.01*
%DLCO, per 1% increase §	0.97	0.95–0.99	<0.01*
%FVC, per 1 % increase	0.95	0.93–0.96	<0.01*
KL-6, per 100 U/mL increase	1.02	0.99–1.04	0.15
Anti-fibrotic treatment, yes (vs. no)	0.68	0.39–1.20	0.19
Serum $S100A4^{high}$ (vs. $S100A4^{low}$)	2.10	1.18–3.69	0.01*
Serum S100A4, per 10 ng/mL increase	1.07	1.03–1.10	<0.01*
Multivariate analysis model 1			
Male (vs. female)	0.65	0.04–3.38	0.67
Age, years	1.03	0.97–1.09	0.29
PaO_2, per 1Torr increase	0.92	0.87–0.96	<0.01*
%DLCO, per 1% increase §	0.98	0.95–1.002	0.06
%FVC, per 1% increase	0.96	0.94–0.99	<0.01*
Anti-fibrotic treatment, yes (vs. no)	0.28	0.09–0.87	0.03*
Serum $S100A4^{high}$ (vs. $S100A4^{low}$)	1.69	0.88–3.20	0.10
Multivariate analysis model 2			
Male (vs. female)	0.72	0.04–3.73	0.74
Age, years	1.06	0.99–1.13	0.08
PaO_2, per 1Torr increase	0.91	0.87–0.96	<0.01*
%DLCO, per 1% increase §	0.98	0.95–1.001	0.06
%FVC, per 1% increase	0.96	0.94–0.99	<0.01*
Anti-fibrotic treatment, yes (vs. no)	0.39	0.12–1.30	0.12
Serum S100A4, per 10 ng/mL increase	1.18	1.01–1.37	0.03*

*$P < 0.05$.

§ n = 58

HR, hazard ratio; 95%CI, 95% confidence interval; UIP, usual interstitial pneumonia; HRCT, high-resolution computed tomography; PaO_2, arterial oxygen pressure; %FVC, predicted forced vital capacity; $FEV_{1.0}$, forced expiratory volume 1.0(sec); %DLCO, predicted diffusing capacity of the lung carbon monoxide; KL-6, Krebs von den Lungen-6.

476 **Figure legends**

477 **Figure 1.** Baseline serum S100A4 levels

478 Serum S100A4 levels were undetectable in all HCs (approximately 0 ng/mL) but were
479 detectable in 26 (27.3%) of the 95 IPF patients ($P < 0.01$). The median serum S100A4
480 level (range) in IPF patients was 0 (0–450) ng/mL.

481 IPF, idiopathic pulmonary fibrosis; HC, healthy control

482

483 **Figure 2.** S100A4 immunostaining in lung tissues from controls and IPF patients

484 A: A representative image of control lung tissues (×100 magnification, scale bar: 50 μm).
485 S100A4 is expressed in intra-alveolar macrophages (arrowhead); normal alveolar
486 structures show sparse expression of S100A4 (arrow).

487 B: A representative image of lung tissues of an IPF patient (×12.5 magnification, scale bar
488 500 μm). Numerous S100A4-expressing cells are present largely adjacent to areas of
489 mature fibrosis.

490 C: A representative image of lung tissues from an IPF patient (×100 magnification, scale
491 bar: 50 μm). Abundant S100A4-expressing cells (arrow) are present in the areas between
492 the periphery of fibroblastic foci (arrowhead) and adjacent nearly intact alveolar structures.
493 The S100A4-expressing cell mostly exhibit spindle-like shape, a finding consistent with
494 fibroblasts.

Figure 3. Kaplan–Meier survival curve

A: PFS rate of IPF patients in the S100A4^{high} subgroup was significantly lower than that in the S100A4^{low} subgroup (1-year cumulative PFS rate, 58.4% vs. 77.8%, respectively; P = 0.01).

B: Survival rate of IPF patients in the S100A4^{high} subgroup was significantly lower than that in the S100A4^{low} subgroup (2-year cumulative survival rate, 41.7% vs. 77.0%, respectively; $P < 0.01$).

PFS, progression-free survival; IPF, idiopathic pulmonary fibrosis. S100A4^{high} subgroup had serum S100A4 level ≥ 22.3 ng/mL; S100A4^{low} subgroup had serum S100A4 level < 22.3 ng/mL.

Figure.1

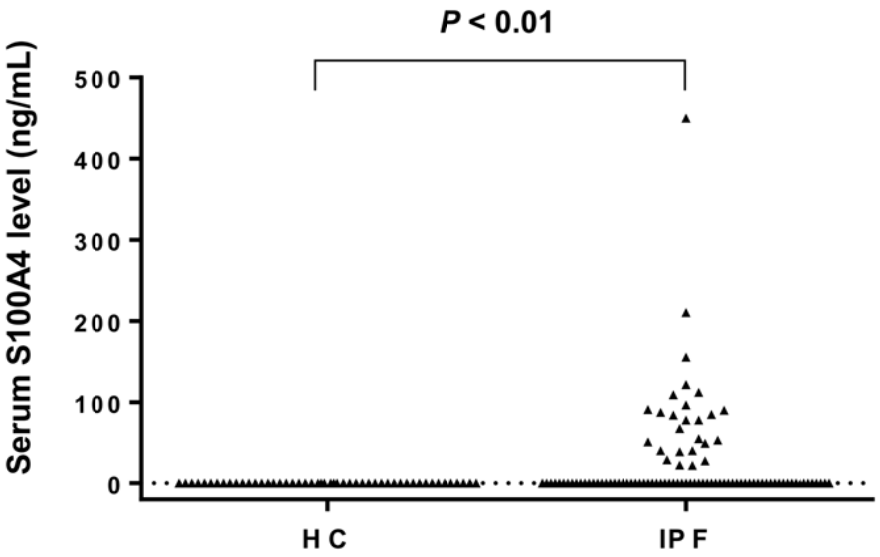


Figure.2

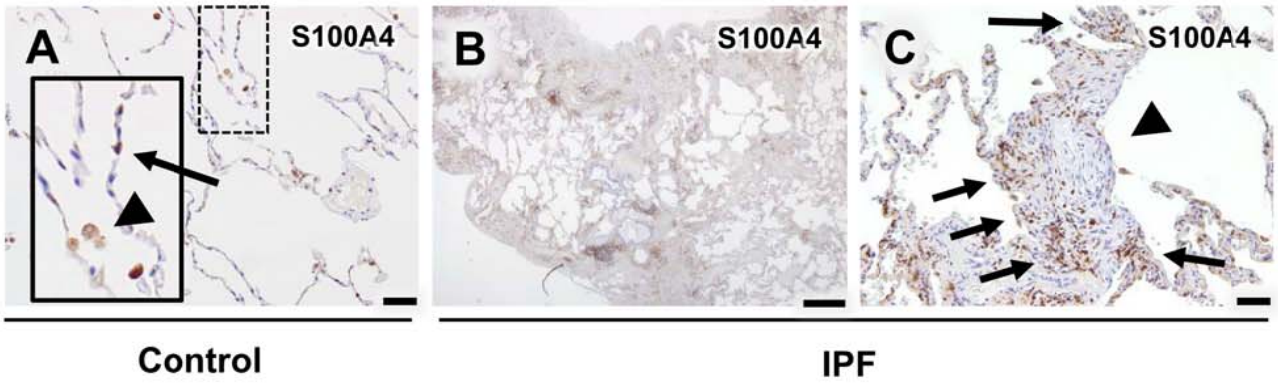
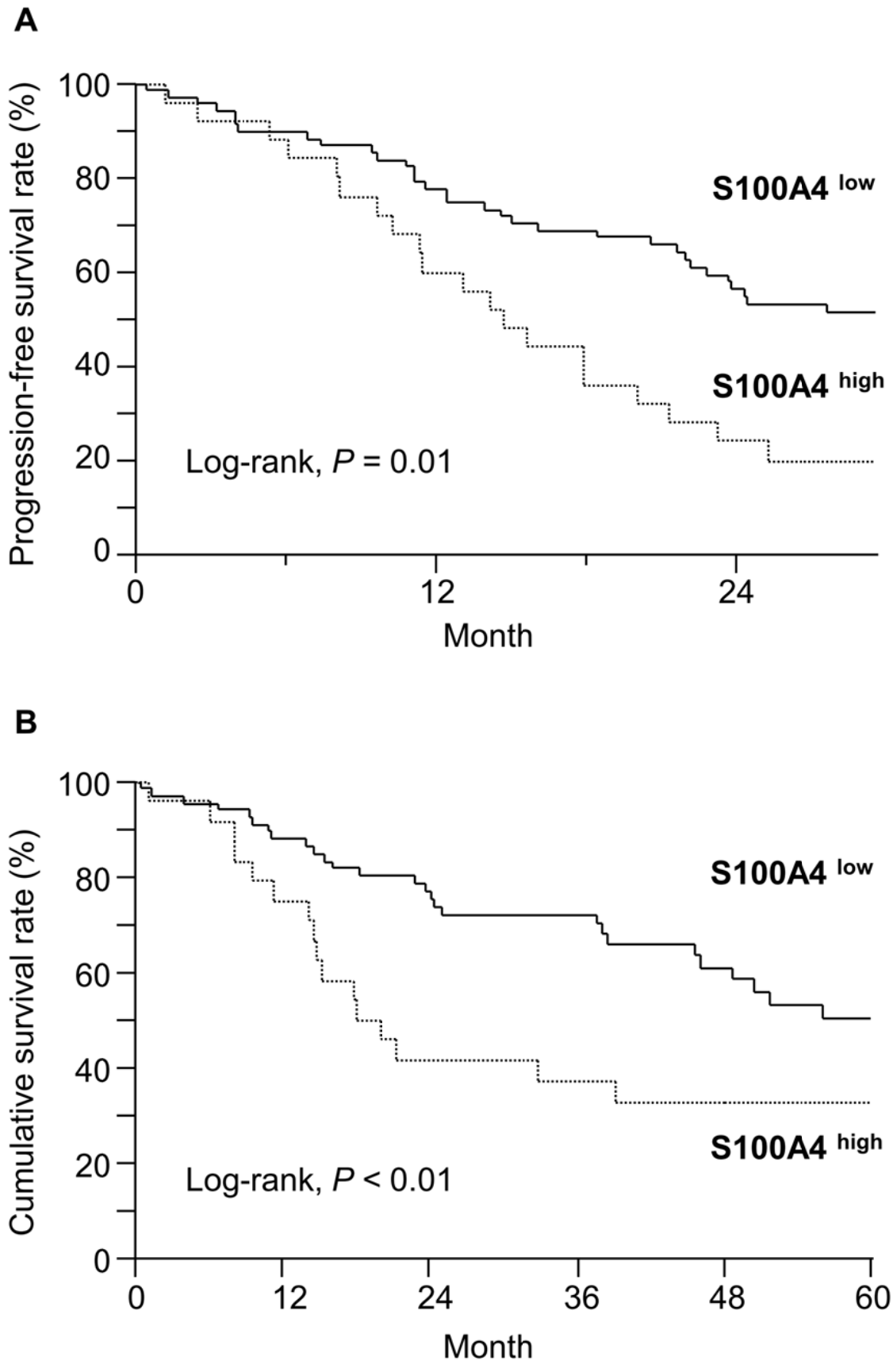


Figure. 3


A
Progression-free survival rate (%)
100
80
60
40
20
0
S100A4 low
S100A4 high
Log-rank, P = 0.01
0
12
24
Month
B
Cumulative survival rate (%)
100
80
60
40
20
0
S100A4 low
S100A4 high
Log-rank, P < 0.01
0
12
24
36
48
60
Month

Supplementary Information template:

SUPPLEMENTARY INFORMATION

TITLE: Clinical significance of serum S100 calcium binding protein A4 in idiopathic pulmonary fibrosis

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Supplementary Method S1- S100A4 immunohistochemistry and immunofluorescence

Immunohistochemistry

The sections were deparaffinized and were then preheated for 30 min in 0.05% citrate buffer pH 6.0. After blocking endogenous peroxidase activity with 3% H_2O_2 for 15 min, the sections were incubated with a primary rabbit anti-human S100A4 monoclonal antibody (1:250; Anti-S100A4 antibody; Abcam, Cambridge, UK) or isotype control rabbit IgG for 1 hour. Subsequently, the sections were incubated with visualization reagent (Histofine simple stain MAX-PO [M]; Nichirei Co., Tokyo, Japan) for 30 min. The immunoreaction was visualized using 3, 3-diaminobenzidine chromogen and the sections were counterstained with hematoxylin.

Immunofluorescence

The sections were deparaffinized and subsequently preheated for 30 min in 0.05% citrate buffer pH 6.0. The sections were incubated with a blocking solution (Blocking One Histo; nacalai tesque., Kyoto, Japan) for 10 minutes and subsequently incubated with the primary antibody mixture of the anti-human S100A4 antibody (1:200) and a mouse anti-human alpha smooth muscle actin (αSMA) monoclonal antibody (1:200; anti-αSMA antibody; Abcam, Cambridge, UK) or the isotype control mixture of rabbit IgG and mouse IgG2a for 1 hour. Subsequently, the sections were incubated with DAPI (1:1000; sigma, New York, USA) with Alexa Fluor conjugated secondary antibodies (1:1000; Goat Anti-Rabbit IgG H&L Alexa Fluor® 555 and Goat Anti-Mouse IgG H&L Alexa Fluor® 647; Abcam, Cambridge, UK) for 1 hour. To reduce auto-fluorescence in the sections, an auto-fluorescence quenching kit was used (TrueVIEW™ Autofluorescence Quenching Kit; Vector Laboratories, Inc., California, USA). The sections were visualized using a confocal microscope (Leica, Wetzlar, Germany).

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Table S1- Treatment for IPF during the observation period

	IPF (n = 95)
Treatment for IPF, n (%)	
Any	65 (68.4)
Anti-fibrotic	50 (52.6)
Pirfenidone	44 (46.3)
Nintedanib	12 (12.6)
Anti-inflammatory	
Corticosteroids	32 (33.7)
with immunosuppressant	13 (13.7)
Cyclophosphamide	6
Cyclosporin A	6
Azathioprine	1

Data are presented as n (%)

IPF, idiopathic pulmonary fibrosis

Table S2- Correlation between serum S100A4 levels and clinical parameters

Characteristics	**Correlation coefficient**	***P*-value**
Age, years	−0.09	0.52
Laboratory data		
KL-6, U/mL	0.02	0.84
PaO_2, Torr	0.15	0.15
Pulmonary function test		
%FVC, %	−0.07	0.88
FEV_1/FVC, %	0.02	0.88
%DLCO, % *	0.15	0.24

KL-6, Krebs von den Lungen-6; PaO_2, arterial oxygen pressure; %FVC, predicted forced vital capacity; $FEV_{1.0}$, forced expiratory volume 1.0 (sec); %DLCO, predicted diffusing capacity of the lung carbon monoxide.

* n = 58

Table S3- Characteristics of IPF patients disaggregated by S100A4^{high} and S100A4^{low} subgroups

Characteristics	**S100A4^{high} n = 25**	**S100A4^{low} n = 70**	***P*-value**
Age, years	71 (64–77)	71 (65–77)	0.51
Male / female	20 (80)/5 (20)	63 (90)/7 (10)	0.29
Smoking habit, never / former or current	8 (32)/17 (68)	11 (16)/59 (84)	0.09
Diagnosis, clinical / biopsy proven	17 (68)/8 (32)	55 (79)/15 (21)	0.29
Laboratory test			
KL-6, U/mL	1030 (551–1605)	922 (606–1292)	0.51
PaO_2, Torr	80 (73–92)	75 (69–82)	0.04*
Pulmonary function test			
% FVC, %	63 (52–85)	77 (63–90)	0.10
$FEV_{1.0}$ / FVC, %	86 (81–91)	82 (79–87)	0.09
%DLCO, % ‡	71 (59–86)	61 (44–86)	0.33
GAP stage, I / II / III ¶	6 (50)/0 (0)/6 (50)	26 (47.3)/20 (36.4)/9 (16.4)	<0.01*
Observation period, months	19.7 (12.6–55.5)	35.9 (16.1–53.7)	0.29
Disease progression §	25 (100)	46 (65.7)	<0.01*
Progression-free survival, months	14.8 (9.1–24.7)	24.8 (11.7–47.4)	0.10
Deterioration in lung function	15	26	
Death	10	20	
Acute exacerbation §	10 (40.0)	16 (22.9)	0.12
All-cause death §	21 (84.0)	30 (42.9)	< 0.01*
Death from respiratory failure §	20 (80.0)	26 (37.1)	< 0.01*

Data are presented as median (IQR) or number (%).

* $P < 0.05$, ‡ n = 58, ¶ n= 67, § During observation period

S100A4^{high} subgroup had serum S100A4 level ≥ 22.3 ng/mL; S100A4^{low} subgroup had serum S100A4 level < 22.3 ng/mL. IPF, idiopathic pulmonary fibrosis; KL-6, Krebs von den Lungen-6; SP-D, surfactant protein D; PaO_2, arterial oxygen pressure; %FVC, %predicted forced vital capacity; $FEV_{1.0}$, forced expiratory volume 1.0 (sec); %DLCO, %predicted diffusing capacity of the lung carbon monoxide; GAP, Gender–Age–Physiology.

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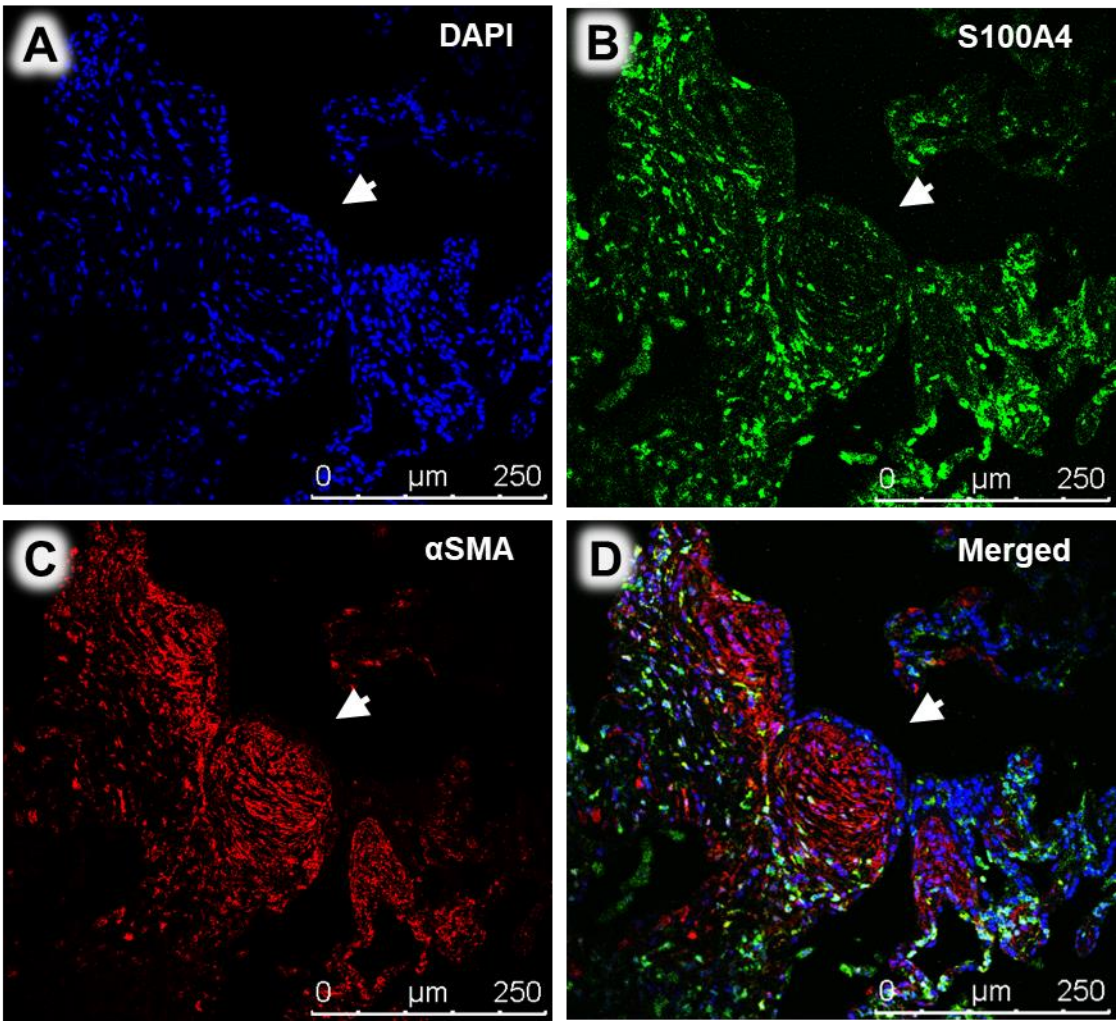
Figure S1- Immunofluorescence images of lung



A, B, C, D: Immunofluorescence images of lung tissues from a patient with IPF (×200 magnification, scale bar: 250 µm), stained by DAPI (blue), anti-S100A4 antibody (green), and anti-αSMA antibody (red) and merged image, respectively. Abundant S100A4-expressing cells (arrowhead) have infiltrated around the periphery of fibroblastic foci that are constituted by αSMA-positive but S100A4-negative myofibroblasts. Although there are a small number of S100A4-expressing cells among fibrotic foci, co-expression of S100A4 and αSMA is not observed in these cells.

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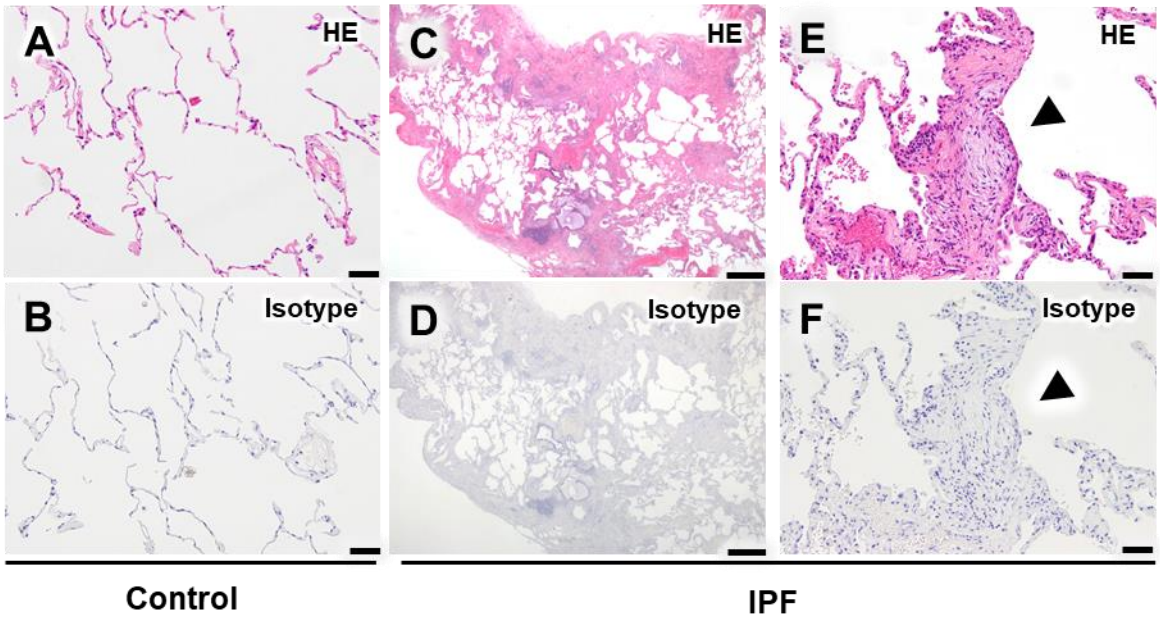
Figure S2- Histological images of lung



A, B: Control lung tissues (×100 magnification, scale bar: 50 μm)

C, D: Lung tissues from a patient with IPF (×12.5 magnification, scale bar 500 μm)

E, F: Lung tissues from a patient with IPF (×100 magnification, scale bar: 50 μm)

Arrowhead: fibroblastic foci

HE, Hematoxylin-Eosin (HE); Isotype, isotype IgG

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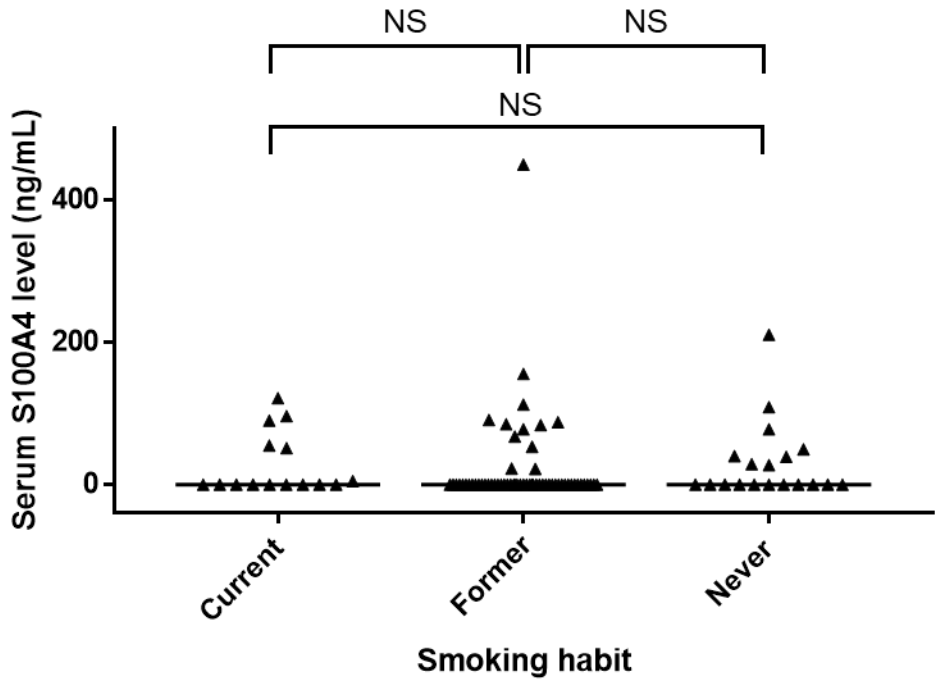
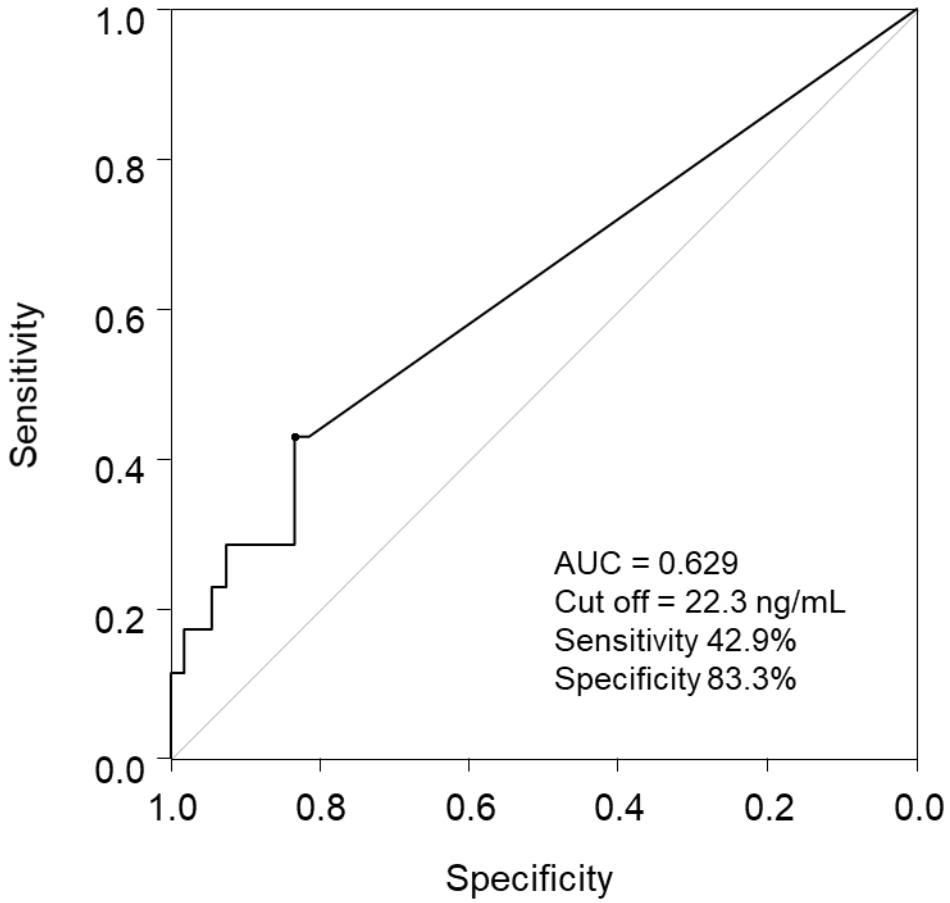
Figure S3- Association of serum S100A4 levels with smoking habit



Serum levels of S100A4 in IPF patients showed no association with smoking habit.

Figure S4- Receiver operating characteristic curve



Receiver operating characteristic curve of serum S100A4 for predicting disease progression within the first year from the date of diagnosis. The area under the curve (AUC) was 0.629. Using 22.3 ng/mL as the cut-off level of serum S100A4, the sensitivity and specificity were 42.9% and 83.3%, respectively.

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Figure S5- Analyses regarding the combination of serum S100A4 and GAP staging system