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Prognostic factors for idiopathic pulmonary fibrosis: clinical, physiologic, pathologic, and molecular aspects

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Abstract. *Background:* Previous studies identified clinical and physiologic factors of idiopathic pulmonary fibrosis (IPF) that are related to an increased risk of mortality. But there are few studies about histologic and molecular approach. *Objective:* We investigated whether the C-reactive protein (CRP), fibroblastic foci, phosphorylated Smad2/3 (p-Smad2/3), tumor growth factor-β (TGF-β), TGF-β receptor II (TβRII), and the polymorphism of the TGF-β_1 codon 10 are associated with the progression of IPF patients. *Design:* Eighty-six IPF patients who underwent surgical lung biopsies were examined. For each patient, clinical and physiologic parameters were investigated, and we performed immunohistochemical staining for p-Smad2/3 and TβRII, and genotyping of the TGF-β_1 codon 10 polymorphism. *Results:* Age at diagnosis, gender, symptom duration, and smoking status did not show a significant association. However, the amount of smoking (p = 0.002), severe reduction in the percentages of predicted forced vital capacity (p = 0.013) and diffusion lung capacity of carbon monoxide (p = 0.023), CRP (p = 0.009) at diagnosis, and fibroblastic foci (p = 0.026) were associated with a poor prognosis. Cellularity, fibrosis, expression level of p-Smad2/3 and TβRII, and genotype of the TGF-β_1 codon 10 polymorphism did not have a statistically significant association with the prognosis. *Conclusion:* This study confirmed the amount of smoking, abrupt decrease in follow-up pulmonary function parameters, fibroblastic foci, and increased levels of CRP concentration at diagnosis were significantly associated with poor survival. Larger studies are required to confirm all prognostic factors including CRP.. *(Sarcoidosis Vasc Diffuse Lung Dis 2011; 28: 102-112)*

Key words: idiopathic pulmonary fibrosis, prognostic factor, C-reactive protein, fibrotic foci, Smad, TGF-β

Introduction

Idiopathic pulmonary fibrosis (IPF) is a diffuse, progressive parenchymal lung disease that typically affects adults over the age of 50. Its etiology is unknown, and it is associated with irreversible respiratory failure and, eventually, considerable morbidity and mortality (1, 2).

The initiating injury and subsequent pathway of IPF are not clear, but the disease is considered an epithelial fibroblastic disorder. It is characterized by epithelial injury followed by inordinate wound healing with excessive fibrosis and minimal inflammation (3). In these processes, growth factors, cytokines, and other mediators are released, excess extracellular matrix is deposited, and abnormal mesenchymal cell activation and proliferation in the lung are involved (4, 5).

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Transforming growth factor-β_1 (TGF-β_1) is a multifunctional cytokine associated with the progression of fibrosis. TGF-β_1 modulates cell growth and differentiation, extracellular matrix synthesis, remodeling, apoptosis, inhibition of collagen degradation and abnormal wound healing. It is known that, in fibrotic lung disease, TGF-β expression increases (6, 7). This TGF-β signal is transduced to target genes in the nucleus by the Smads signaling pathway. TGF-β binds to serine/threonine receptors on targeted cells. The serine/threonine kinase activity receptor triggers the phosphorylation of transcription factors Smads 2 and 3 (8).

To date, no proper treatment has proven efficacious. IPF does not respond to corticosteroids, immunosuppressive therapy, and interferon gamma-1β (9). The decision is very difficult when a patient anticipates pulmonary function decline and the start of treatment. Therefore, for a better decision, both patients and physicians need valid prognostic factors, which must be evaluated in IPF patient. Many factors have been associated with prognosis. The factors include clinical parameters (age at onset of symptoms and diagnosis (10), sex (11), duration of symptoms (12), severity of dypsnea, and history of cigarette smoking), physiologic factors (six minutes walking test) (13), radiographic findings (14), initial and decline of pulmonary function as DL_{CO} (diffusion lung capacity of carbon monoxide) and FVC (forced vital capacity) (15), histopathologic findings (fibrosis and emphysema) (16), and responsiveness to corticosteroid or immunosuppressive therapy (17).

In this study, we evaluated the above-mentioned factors and C-reactive protein (CRP). We also evaluated the degree of fibroblastic foci, phosphorylated Smad2/3 and TGF-β receptor II expression (TβRII), major signal pathway of TGF-β, and evaluated the TGF-β_1 gene polymorphisms in codon 10, in association with progression of IPF in 86 Korean patients with IPF, diagnosed from 1995 to 2009.

Methods

Patient Selection

A total of 86 patients (55 male and 31 female) with IPF were enrolled who underwent definitive surgical lung biopsies from January 1995 to December 2009 at the Severance Hospital, Yonsei University College of Medicine, in Seoul, Korea. Two pathologists (H.S. Shim and S.H. Cho) reviewed all histologic slides and confirmed the diagnosis, excluding any other forms of interstitial lung diseases, independently and without access to clinical data. Diagnoses of IPF were established according to the American Thoracic Society/European Respiratory Society Consensus Statement (18). Patients with related previous or coexistent connective tissue disease, an occupational history and/or environmental exposure that resulted in diffuse pulmonary fibrosis, or history of ingestion of a drug or an agent known to cause interstitial lung disease were excluded. Two patients had a congestive heart failure. Two patients had a history of myocardial infarction and one of these had a decreased cardiac function. Thirteen patients had a cancer history including one case of hematologic malignancy, and six of these patients had lung cancer. This study protocol was approved by the Institutional Review Board of the Severance Hospital Ethics Committee.

Physiologic Parameters (Pulmonary Function Test and Arterial Blood Gas Analysis Results)

Each patient performed a pulmonary function test at diagnosis. Spirometry (Vmax22; SensorMedics, Yorba Linda, CA, USA), plethysmographic lung volumes (6200 plethysmograph; SensorMedics), and DL_{CO} (Vmax229D; SensorMedics) were used to assess lung function. Seventy-one patients had a subsequent pulmonary function test. Disease progression was assessed by evaluating changes in pulmonary function parameters. Changes in lung function were defined as percentages of changes in absolute or percentage values with respect to the initial assessment. The change in pulmonary function parameters divided by follow-up duration (in months) was used to express accurate estimates. Arterial blood gases were analyzed at initial presentation in 57 patients.

Clinical Parameters (C-Reactive Protein and Treatment Response)

CRP was assessed in fifty-seven patients with IPF at diagnosis. The treatment of patients with IPF was initially 0.5 mg/kg of prednisolone, followed by

slowly tapering with or without immunosuppressive agents (up to 2 mg/kg of azathioprine).

Pathologic and Molecular Parameters

To evaluate the grade of fibrosis and cellularity of each biopsy specimen, a scoring system on a scale of zero to five was used, as a previous study demonstrated (19). We expressed the score correlating with the percentages of fibrosis and cellularity (absent, occasional, < 25%, 25 to 49, 50 to 75, and > 75%). For each patient, a semiquantitative assessment was undertaken using a scale of 0-6 to evaluate fibroblastic foci, as previously described (20).

The grade of p-Smad2/3 and TβRII were analyzed. Immunoreactivities were graded as negative (less than 10% of counted positive cells), mild (1+, 10~25%), moderate (2+, 25~50%), and strong (3+, more than 50% expressed positive cells) expression. Additionally, each genotype of codon 10 polymorphisms was analyzed and expressed by Leu/Leu (TT genotype), Leu/Pro (TC), and Pro/Pro (CC).

Immnohistochemical Staining and Antibodies

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections, which were cut to a thickness of 4 μm. Slides were deparaffinized and pretreated by 120˚C microwave epitope retrieval (750 W during 5 min in 10 mmol citrate buffer; pH 6.0). The sections were then immersed for 15 min in distilled water containing 3% hydrogen peroxidase in order to block any endogenous peroxidase activity. Sections were incubated for 4˚C overnight with primary antibody at a dilution of 1:100. The primary goat polyclonal p-Smad2/3 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TβRII antibody was from Upstate (Lake Placid, NY, USA). LSAB®2 from DACO Corp (CA, USA), a streptavidin-peroxidase conjugate, was used for the secondary antibody. Routinely processed tissue sections of breast cancer were used as positive staining controls and were stained with the primary antibody omitted in order to verify staining specificity (Fig. 1 and Fig. 2).

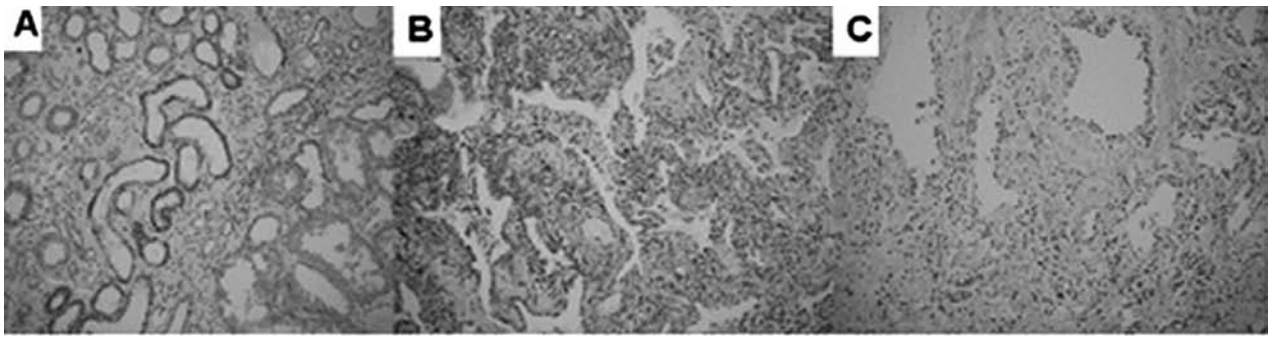

Fig. 1. Immunohistochemical staining of TGF-β_1 receptor II expression in lung tissues from idiopathic pulmonary fibrosis patients. (A) Positive control of breast cancer, ×200, (B) Positive expression in IPF tissue, ×200, (C) Negative expression in IPF tissue, ×200

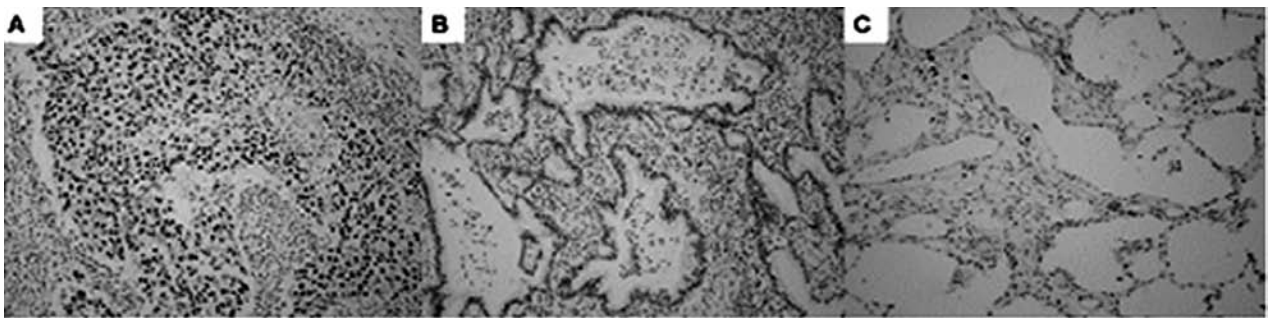

Fig. 2. Immunohistochemical staining of phosphorylated Smad2/3 expression in lung tissues from idiopathic pulmonary fibrosis patients. (A) Positive control of breast cancer, ×200, (B) Strong positive expression in IPF tissue, ×200, (C) Mild positive expression in IPF tissue, ×200

All slides were independently evaluated and scored for percentage of positive tumor cells by lung special pathologists.

DNA Extraction, PCR Amplification, and Restriction Enzyme Digestion

Dissected tissue blocks were digested in 200 μl 50 mM Tris-HCl (pH 8.0) containing 1% SDS-proteinase K (Boehringer Mannheim, Lewes, UK), then incubated at 42°C for 12 h. Stock DNA was stored at a concentration of 300 ng/μl at 20°C and polymerase chain reaction (PCR) were prepared using working DNA stocks of 100 ng/μl. Reaction mixtures of 12.5 μl were prepared, each containing 10x PCR buffer (containing 10 mM Tris HCl, pH 8.3, 50 mM KCl), DMSO, 2 mM dNTPs, 1.5 mM $MgCl_2$, 100 ng DNA, 1 U Taq polymerase (Invitrogen, Carlsbad, CA, USA), and 0.25 μM of each primer (GenoTech, Daejeon, Korea). The forward primer sequence was: 5'-CTC CTA CCT TTT GCC GGG AGA C-3'; antisense, 5'-GCC AGG CGT CAG CAC CAG TA-3'. Using a temperature cycler (Thermo Hybaid; Omnigene, Woodbridge, NJ, USA), PCR was performed with initial denaturation at 95°C for 2 min. This was followed by an additional 39 cycles, with denaturation at 94°C for 30 seconds, annealing at 60°C for 1 min, and elongation at 72°C for 1 min. A final elongation of 5 min at 72°C completed the process. An amplification check was performed by electrophoresis using a 2% agarose gel (SIGMA, Steinheim, Germany) in 0.5% TBE buffer (27 g trizma base, 13.75 g boric acid, and 1.46 g EDTA in distilled water) containing 2 μl ethidium bromide. Electrophoresis was performed in 200 ml 0.5% TBE buffer, with a voltage of 100 mV for approximately 80% of the length of the gel (Mupid-21; Seoulin Bioscience, Seoul, Korea). Gel products were collected using a gel documentation-photo system (Bio-Profil Vilber Lourmat, Marne La Vallee, France). Four μl amplification product was digested with 4 U MspA1I (New England BioLabs, Beverly, MA, USA) restriction endonuclease enzymes in a 5 μl mixture volume containing 10x NEB buffer. Restriction enzyme-digested PCR products were subjected to electrophoresis in a 2% agarose gel (SIGMA, Steinheim, Germany) at 100 mV for 30 min in 200 ml TBE buffer. Using ultraviolet transillumination after ethidium bromide staining, the products were visualized, and the size of the product was determined using a 100 bp ladder (Invitrogen, Carlsbad, CA). These samples were comfirmed via DNA sequencing, as Leu/Leu (TT genotype), Leu/Pro (TC), and Pro/Pro (CC), respectively.

Statistical Analysis

The primary endpoint at 5-year survival was analyzed to evaluate prognosis factors. The Cox proportional hazards model was used, and results were expressed as the relative hazard for death among those who had or were exposed to a factor of interest, compared with those who did not have or were not exposed to the factor. To find an optimal cut-off level, receiver operating characteristics (ROC) curve was used in CRP level. Survival curves were computed using the Kaplan-Meier method. Data were expressed as means ± SD. A $p < 0.05$ was considered to be statistically significant. All calculations were performed using SAS procedures (SAS, version 9.1.3, SAS Institute Inc., Cary, NC, USA).

Results

Relationship Between Clinical Parameters and Prognosis in IPF Patients (Age, Gender, Smoking, Treatment Response, and Initial CRP Level)

The mean age of eligible patients at diagnosis was 61.3 ± 8.9 years. The average follow-up period of these patients was 37.2 months. During the follow-up period, thirty patients died, and the attrition count was two patients. Respiratory failure was the cause of death in 17 patients, infection in 8 patients, lung cancer in 3 patients, and other cause in 2 patients. Univariate analysis was used to estimate the relative risk of death associated with each clinical parameter (Table 1). Kaplan-Meier survival plots of IPF patients are shown in Figure 3.

Patients less than 60 years of age at diagnosis tended to have better survival than older patients, but the difference was not statistically significant. There was no significant association in gender and duration of symptoms prior to IPF diagnosis. The smoking status also did not appear to be associated with the risk of death. Among smokers, the greater amount of cigarette smoking was associated with worse survival (Table 1 and Fig. 3).

Table 1. Analysis of survival in patients with idiopathic pulmonary fibrosis (IPF) for clinical parameters

Variables	No. of patients (%)	Relative hazard rate	95% CI	p-value
Age (years)				
Mean ± SD	61.3 ± 8.9			
< 60	35 (40.7)	1.00		
≥ 60	51 (59.3)	2.37	0.85 to 6.59	0.098
Gender				
Male	55 (64.0)	1.65	0.73 to 3.70	0.227
Female	31 (36.0)	1.00		
Duration of symptoms at diagnosis (months)				
< 12	52 (60.5)	1.00		
≥ 12	34 (39.5)	1.06	0.51 to 2.19	0.885
Smoking status				
Never smoker	33 (39.5)	1.00		
Ex-smoker	34 (40.7)	1.19	0.51 to 2.76	0.685
Current smoker	17 (19.8)	1.90	0.75 to 4.82	0.178
Amount of smoking (park-year)		1.04	1.01 to 1.06	0.002
< 30	21 (41.2)	1.00		
30 - 60	25 (49.0)	1.71	0.58 to 5.08	0.332
≥ 60	5 (9.8)	13.6	3.44 to 53.94	< 0.001
Treatment or conservative care				
Treated*	73 (84.9)	1.00		
Not treated	13 (15.1)	2.89	1.28 to 6.55	0.011
CRP (mg/dL)		1.03	1.01 to 1.05	0.009
< 6.3	34 (59.6)	1.00		
≥ 6.3	23 (40.4)	2.38	0.90 to 6.26	0.080

CI, confidence interval; AZA, azathioprine; CRP, C-reactive protein.
* steroid only 26 (30.2%), steroid with azathioprine 46 (53.5%), and lung transplant 1 (1.1%)

Fifty-seven patients' data were available for CRP at diagnosis. The patients separated by clinical diagnosis of infection. The mean CRP of IPF patients at diagnosis was 11.25 ± 19.38 mg/dL. The patients were divided into two groups by serum CRP levels (as above or below the cut-off level of 6.3 mg/dL). Patients with increased levels of CRP presented a worse survival in this analysis (Table 1 and Fig. 3).

Most patients (n = 71) were treated with corticosteroids. The use of corticosteroids had an effect on the relative survival. Only one patient had a lung transplant. The observed survival rate and relative survival at five years after diagnosis was lower in the non-treatment group (Fig. 3).

Physiologic Parameters (Pulmonary Function Test and Arterial Blood Gas Analysis)

The risk of death was significantly higher for patients with less than 60% of initial FVC (% pred) ($p = 0.013$). The survival distribution trend of FEV_1 (% pred) and DL_{CO} (% pred) at diagnosis were similar to those for FVC (% pred) (Table 2 and Fig. 4). But, PaO_2, $PaCO_2$ and alveolar-arterial O2 difference did not appear to be associated with survival (Table 2).

The changes in pulmonary function parameters during the follow-up period were measured in 71 patients. In order to adequately evaluate these changes during the follow-up period, the changes in pulmonary function parameters of each patient were divided by the follow-up duration (in months), and the results are shown in Table 3. This showed that the abrupt decrease in changes of pulmonary function parameters resulting during the follow-up periods was significantly associated with risk of death ($p < 0.05$).

Pathologic Parameters (Fibrosis, Cellularity, Total Pathologic Score and Fibroblastic foci)

In this analysis, neither cellularity ($p = 0.730$) nor fibrosis ($p = 0.342$) revealed an influence on the

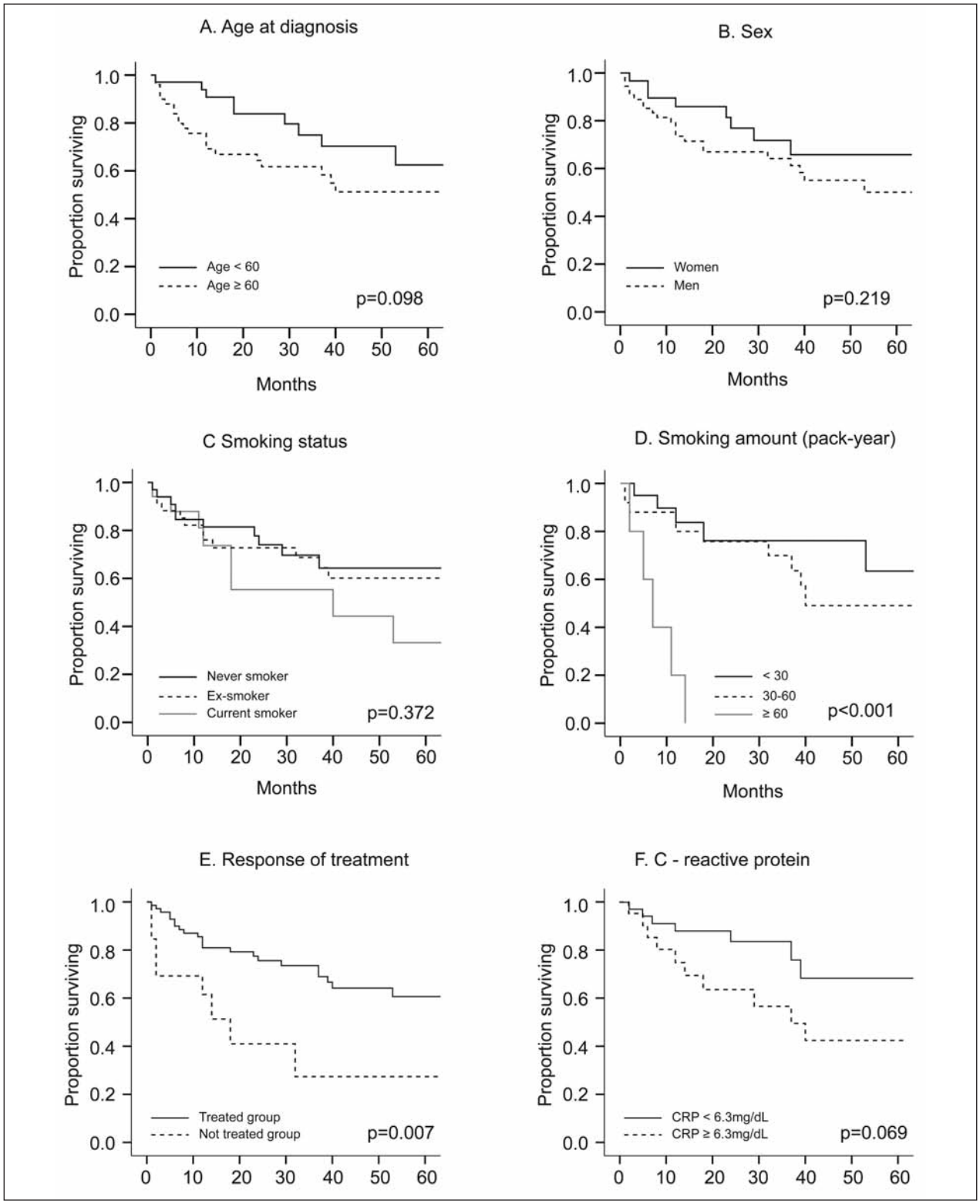

Fig. 3. Kaplan-Meier estimates of survival for patients with idiopathic pulmonary fibrosis (IPF). (A) age (in years), (B) sex, (C) smoking status, (D) smoking amount (pack-year), (E) treatment response, and (F) C – reactive protein (mg/dL) by log rank test

Table 2. Analysis of survival in IPF patients for physiologic parameters

Factor	No. of patients (%)	Mean ± SD	Relative hazard rate	95% CI	p-value
FVC (L) at diagnosis	86 (100)	2.46 ± 0.80	0.78	0.49 to 1.25	0.305
FVC (% pred) at diagnosis	86 (100)	72.3 ± 18.0	0.99	0.97 to 1.01	0.137
≥ 60 (%)	67 (77.9)		1.00		
< 60 (%)	19 (22.1)		2.58	1.22 to 5.43	0.013
FEV_1 (L) at diagnosis	86 (100)	2.06 ± 0.66	0.69	0.38 to 1.24	0.211
FEV_1 (% pred) at diagnosis	86 (100)	82.1 ± 20.8	0.16	0.97 to 1.01	0.164
≥ 60 (%)	75 (87.2)		1.00		
< 60 (%)	11 (12.8)		1.66	0.58 to 4.77	0.347
DL_{CO} at diagnosis	73 (100)	75.6 ± 22.5	0.97	0.95 to 1.00	0.023
≥ 60 (%)	56 (76.7)		1.00		
< 60 (%)	17 (23.3)		2.30	0.94 to 5.63	0.068
Arterial blood gas analysis at diagnosis					
PaO_2 (mmHg)*	57 (100)	76.8 ± 16.6			
PaO_2 ≥ 60	49 (86.0)		1.00		
PaO_2 < 60	8 (14.0)		1.56	0.52 to 4.71	0.430
$D(A\text{-}a)O_2$	57 (100)	27.2 ± 14.4	1.02	0.99 to 1.05	0.203

CI = confidence interval; FVC = forced vital capacity; FEV_1 = forced expiratory volume in one second; % pred = percentage of the predicted value; DL_{CO} = diffusing capacity of the lung for carbon monoxide; $D(A\text{-}a)O_2$ = alveolar-arterial O_2 difference.
* Excluded with O2 supply

risk of death. Total pathologic score, which is the fibrosis score plus cellularity score, was also not associated with survival. Only fibrotic foci score (p = 0.026) was a significant pathologic parameter in IPF patients. Table 4 shows the overall assessments of fibrosis, cellularity and fibrotic foci. The Kaplan-Meier survival analysis for fibroblastic foci score is shown in Figure 5.

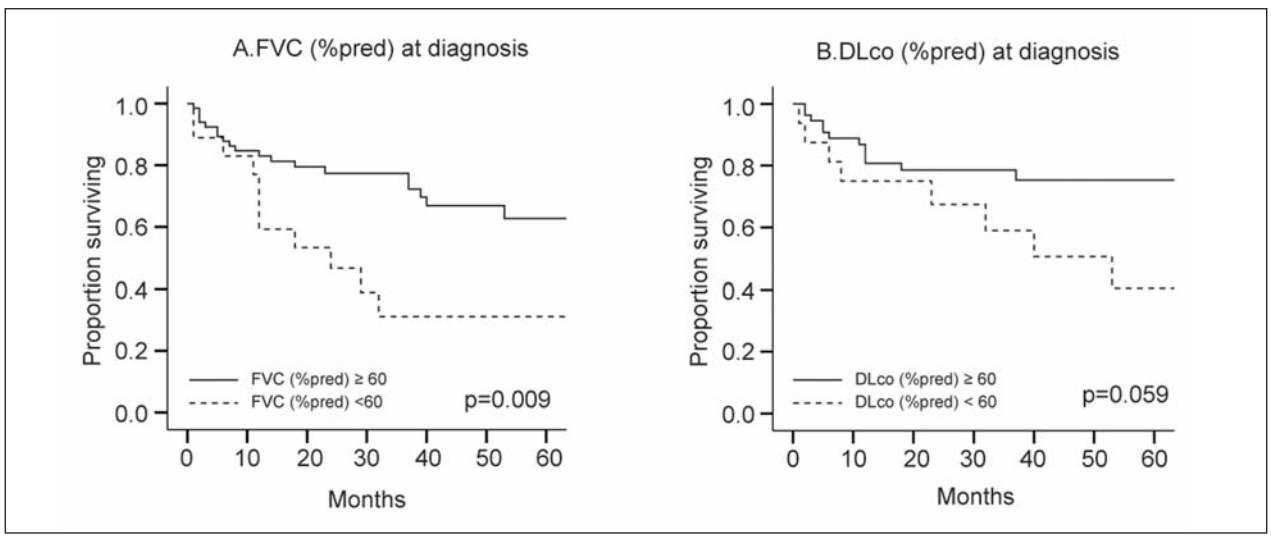

Fig. 4. Kaplan-Meier estimates of survival for IPF patients. (A) forced vital capacity (FVC) (% pred) at diagnosis, and (B) diffusion capacity of the lung for carbon monoxide (DL_{CO}) (% pred) at diagnosis by log rank test.

Table 3. Analysis of survival in IPF patients for pulmonary function deterioration (N=71)

Factor	Mean ± SD	Relative hazard ratio	95% CI	p-value
f/u pulmonary function parameters				
ΔFVC (mL)*	−6.73 ± 26.37	0.971	0.96 to 0.99	0.001
ΔFVC (% pred)†	−0.25 ± 1.47	0.650	0.50 to 0.84	0.001
FEV_1 (mL)*	−12.98 ± 53.52	0.966	0.94 to 0.99	0.007
ΔFEV_1 (% pred)†	−0.36 ± 2.00	0.565	0.38 to 0.85	0.006
ΔDL_{CO} (% pred)†	−0.52 ± 1.59	0.286	0.15 to 0.55	< 0.001

f/u = follow-up; CI = confidence interval; FVC = forced vital capacity; FEV_1 = forced expiratory volume in one second; DL_{CO} = diffusing capacity of the lung for carbon monoxide; % pred = percentage of the predicted value.
* Percentage of change in absolute values with respect to initial assessment, divided by the follow-up time in months.
† Percentage of change in percentage values with respect to initial assessment, divided by the follow-up time in months.

Table 4. Analysis of survival in IPF patients for pathologic and molecular parameters

Characteristic, Grade (score)	No. of Patients (%)	Relative hazard rate	95% CI	p-value
Cellularity*				
Low [1-2]	54 (62.8)	1.00		
High [3-5]	29 (33.7)	1.14	0.54 to 2.42	0.730
Fibrosis grade*				
Low [1-3]	44 (51.2)	1.00		
High [4-5]	39 (45.3)	1.42	0.69 to 2.95	0.342
Total pathologic score*				
Low [1-5]	36 (41.9)	1.00		
High [6-10]	47 (54.7)	1.16	0.81 to 1.67	0.416
Fibroblastic foci frequency*				
Low [0-1]	27 (31.4)	1.00		
High [2-6]	56 (65.1)	2.99	1.14 to 7.85	0.026
p-Smad2/3 staining				
Weak [1-2]	39 (45.3)	1.00		
Strong [3]	47 (54.7)	1.20	0.84 to 1.72	0.316
TGF-β receptor staining†				
Weak [0-1]	31 (36.0)	1.00		
Strong [2-3]	55 (64.0)	1.05	0.72 to 1.52	0.817
$TGF\text{-}\beta_1$ polymorphism†				
CC and TC	48 (55.8)	1.00		
TT	38 (44.2)	1.15	0.56 to 2.36	0.706

CI = confidence interval.
* Data are unavailable for three patients.
† $TGF\text{-}\beta_1$, Transforming growth factor- β_1 ($TGF\text{-}\beta_1$)

Molecular Parameters (Grading of Immunohistochemical Staining of T RII and p-Smad2/3, and Genotypic Distributions of TGF- 1 Gene Polymorphism in Lung Tissues from IPF Patients)

TβRII and p-Smad2/3, major signal molecules of $TGF\text{-}\beta_1$, were strongly expressed in lung tissues from IPF patients. The expression of TβRII was negative (0), mild (+1), moderate (+2), and strong (+3) in 9 (10.5%), 22 (25.6%), 21 (24.4%), and 34 (39.5%) cases, respectively. Similar to TβRII, p-Smad2/3 was prominently expressed in honeycombing areas and especially fibroblastic foci. The expression of p-Smad2/3 was negative (0), mild (+1), moderate (+2), and strong (+3) in 0, 12 (14.0%), 27 (31.4%), and 47 (54.7%) cases, respectively. However, there was no significance in expression of p-Smad2/3 and TβRII expression in this study (Table 4).

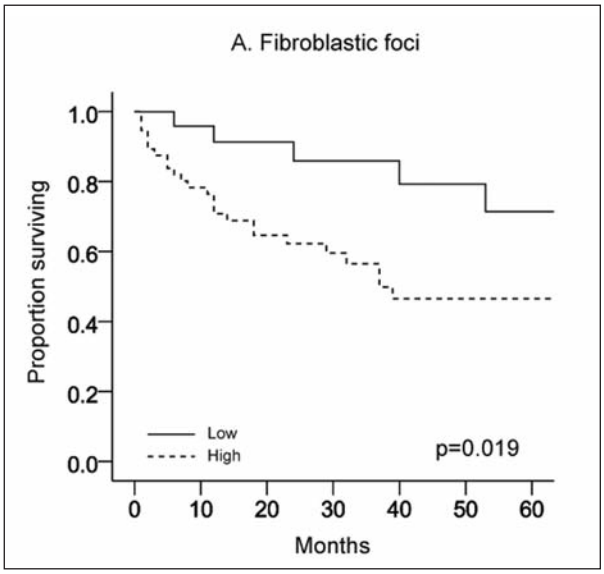

Fig. 5. Kaplan-Meier estimates of survival for IPF patients. (A) fibroblastic foci score (Low: range of score, 0 to 1; High : range 2 to 6 score) by log rank test

The genotypes of patients consisted of CC 16 (19.0%) cases, TC 31 (36.9%) cases, and TT 37 (43.0%) cases. There was no statistically significant difference between genotypes.

Discussion

This study analyzed the factors associated with IPF in Korean patients. Like previous studies, the amount of smoking, initial pulmonary function parameters, an abrupt decrease of pulmonary function during follow-up, and fibroblastic foci were associated with patient survival (21-23). In addition, higher levels of CRP were associated with worse prognosis.

In smokers, oxidative stress from cigarette smoking might contribute IPF pathogenesis and progression. However, in our study, smoking status did not appear to be associated with the risk of death, which may be a reason that symptomatic patients with severely deteriorated disease may be more likely to stop smoking than asymptomatic patients. Thus, there might be a "healthy smoker" effect (24). More studies, adjusting for key severity variables, including smoking status, are required (25). We confirmed that clinical and physiological parameters were more important predictors of survival than the pathological, molecular parameters such as cellularity, fibrosis, immunostaining grade of p-Smad2/3 and TβRII, and TGF-β_1 polymorphism in patients with IPF (23). We recommend that physicians check serial pulmonary function parameters to estimate prognosis of IPF patients.

In IPF patients, there is no proven response to steroids. In our study, the treatment group had a significantly better prognosis, a feature which has been reported previously (26). Our study excludes patients with severe disease who could not undergo surgery, and most patients (n = 73, 84.9%) were treated, the estimate for five-year survival for these patients with use of corticosteroid therapy may be imprecise.

In published studies, the histopathologic pattern provides significant diagnostic and prognostic data. Nicholson AG et al. showed that the fibroblastic foci count was strongly associated with mortality and increasing interstitial mononuclear cell infiltrate scores were related with pulmonary function decline in 53 patients with IPF (20). King TE et al. also demonstrated that IPF patients with lesser degrees of granulation/connective tissue deposition (fibroblastic foci) showed longer survival. However, the degree of alveolar space cellularity, alveolar wall fibrosis, and cellularity did not affect survival (27). We independently evaluated the cellularity, fibrosis, fibroblastic foci, immunostaining grade of p-Smad2/3 and TβRII using both PCR and RFLP techniques, and polymorphism in the codon 10 of TGF-β_1 gene was investigated. We expected that the severity of cellularity and fibrosis was associated with a risk of death. But, our study did not show any significant association for these factors. The reason is suggested that our study excluded patients with severe disease who could not perform a surgical biopsy. Excluding severe disease might explain why cellularity and fibrosis were not associated with disease severity. Also, cellurality and fibrosis could differ greatly from one part to another within a patient. Only the fibrotic foci show a predictive value in IPF patients. This finding is in agreement with previous studies (16, 20, 27). We found that higher fibroblastic foci score is related with worse survival in patients with IPF. It means that fibroblastic foci may be a marker of active IPF progression in only surgically confirmed IPF patients.

The expression of p-Smad2/3 and TβRII was measured by immunohistochemical staining. It is

known that TβRII reflects the starting point of the TGF-β_1 signal pathway, and p-Smad2/3 detection implied major activity of TGF-β_1. However, the degree of expression did not correlate with a worse prognosis. In codon 10, there is a polymorphism at nucleotide +869 (T869C) that produces a Leu → Pro replacement. Such a change is associated with increased formation of TGF-β_1 (28). Therefore, the more common genotype (TT) of TGF-β_1 codon 10 can be associated with survival, but our results did not show a significant association. A reason for this could be explained as such: first, lung fibrotic changes are probably modulated by multiple genes and interrelations of aggravating factors. Second, Wu L et al. showed TGF-β_1 could conceivably act to prevent the degradation of elastin in chronic obstructive pulmonary disease (COPD) patients (29). This suggests the probability that TGF-β_1 may act bidirectionally, both in deterioration and protection. A third reason is that there is data demonstrating that increased TGF-β_1 levels in circulation do not always result in increased expression or activity in selected target tissues (30). We only evaluated the surgical biopsy tissue in this study. So there could be a difference in TGF-β_1 levels between target tissue and blood.

Many serum markers were tested for their use in IPF. These markers include surfactant proteins A and D (SP-A and SP-D), KL-6, lactate dehydrogenase (LDH), and CC-chemokine ligand 18 (CCL-18). But these studies were performed with a small numbers of subjects and a lack of specificity of the marker. In addition, to validate a CCL18, further studies are needed to standardize the entire procedure. So, these serum markers are not ready for clinical use to predict prognosis of IPF patients (16, 31). Plasma CRP is a marker of host response synthesized by the liver in response to infection or inflammation. Ando M et al. and Mura M et al. demonstrated that CRP did not correlate with the severity of IPF in 41 IPF patients (32, 33). Unlike their study, our study showed that higher CRP serum levels, in 57 surgically confirmed patients, were associated worse prognosis. This finding supports that serum CRP may be a prognostic biomarker of IPF patients. In a multivariate analysis controlling for smoking, initial pulmonary function, fibrotic foci and CRP, CRP remained an independent risk factor (Table 5). Because of the retrospective study, there were parameters missing, and follow-up CRP testing was not available in most patients. Therefore, we cannot reveal the relationship between serial follow-up CRP testing with mortality. Further studies are needed to reveal a role of CRP serum level as a prognostic biomarker.

Our study had some limitations: First, non-surgically confirmed subjects were excluded. Therefore, we might have excluded patients with severe disease because they were not able to tolerate surgical biopsy. It was closely related to the disease severity and progression, and, as a result, there is a patient selection bias. Through surgical lung biopsy, a specific diagnosis for IPF was made. Second, in the retrospective study design, we could not check plasma TGF-β_1 levels, even though this information may bear great importance in terms of the interpretation of relationships between genotypes of polymorphism and survival. Third, the molecular and histopathologic severity could vary greatly from one part of the patient to another. Fourth, this study had a retrospective design and some parameters, such as DL_{CO}, were not tested during the follow-up period.

In conclusion, this study examined 86 patients with IPF and confirmed that the widely known prognostic markers (amount of smoking and pulmonary function test results) were important, as previous studies found. We showed that clinicophysiologic parameters have a significant association with survival. And fibroblstic foci may be a pathologic and prognostic marker in IPF patients. CRP could

Table 5. Multivariate analysis of survival in IPF patients

Variables	Relative hazard rate	95% CI	p-value
Amount of smoking (pack-year)	1.02	0.98 to 1.06	0.273
CRP (mg/dL)	1.04	1.02 to 1.07	0.002
FVC (% pred) at diagnosis (%)	3.28	0.33 to 32.25	0.308
DL_{CO} at diagnosis (%)	0.98	0.94 to 1.02	0.363
Fibroblastic foci frequency (Score)	4.53	0.54 to 37.90	0.163

CI, confidence interval; CRP, C-reactive protein; FVC = forced vital capacity; DL_{CO} = diffusing capacity of the lung for carbon monoxide

constitute a useful parameter in evaluation of the severity of IPF patients, although serial determinations of CRP level are required to confirm an association between CRP expression and prognosis.

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