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Molecular Markers of Telomere Dysfunction and Senescence are Common Findings in the Usual Interstitial Pneumonia Pattern of Lung Fibrosis

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Abstract

Background: Idiopathic pulmonary fibrosis (IPF) is a genetically-mediated, age-associated, progressive form of pulmonary fibrosis characterized pathologically by a usual interstitial pneumonia (UIP) pattern of fibrosis. The UIP pattern is also found in pulmonary fibrosis attributable to clinical diagnoses other than IPF (Non-IPF UIP) whose clinical course is similarly poor, suggesting common molecular drivers.

Methods: To test whether patients with IPF and non-IPF UIP share molecular drivers, lung tissue from 169 IPF patients and 57 non-IPF UIP patients were histopathologically and molecularly compared.

Results: Histopathologic changes in both IPF and non-IPF UIP patients included temporal heterogeneity, microscopic honeycombing, fibroblast foci and dense collagen fibrosis. Non-IPF UIP lungs were more likely to have lymphocytic infiltration, noncaseating granulomas, airway centered inflammation or small airways disease. Telomeres were shorter in alveolar type II (AECII) cells of both IPF and non-IPF UIP lungs compared to age-similar, unused donor, controls. Molecular markers of senescence (p16, p21) were elevated in lysates of IPF and non-IPF UIP lungs. Immunostaining localized expression of these proteins to AECII cells. The *MUC5B* promoter variant minor allele frequency was similar between IPF and non-IPF UIP patients and MUC5B protein expression was similar in IPF and non-IPF UIP lungs.

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Conclusions: Molecular markers of telomere dysfunction and senescence are pathologically expressed in both IPF and non-IPF UIP lungs. These findings suggest that common molecular drivers may contribute to the pathogenesis of UIP-associated pulmonary fibrosis, regardless of the clinical diagnosis.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a genetically-mediated, age-associated form of pulmonary fibrosis that has an overall prevalence of 14 to 43 per 100,000 in the United States (1). IPF is characterized histopathologically by subpleural accentuation of fibrosis, microscopic honeycombing, and fibroblast foci (2). This pattern of pulmonary fibrosis is termed usual interstitial pneumonia (UIP). Studies have shown that molecular changes commonly associated with aging are found in IPF patients (3). Consistent with molecular aging contributing to the pathogenesis of IPF, IPF patients have short telomeres (4, 5) and express markers of senescence (p16 and p21, p53) in alveolar type II cells (AECII cells) (6, 7) where they have been shown to be sufficient to cause lung remodeling and fibrosis (8). IPF patients with short telomeres in peripheral blood leukocytes have more rapid progression and worse survival than those IPF patients with longer telomeres (9-11). These data suggest that molecular mediators of aging may contribute directly to the pathogenesis of the disease.

The histopathologic pattern of UIP is found in types of pulmonary fibrosis attributable to a clinical diagnosis other than IPF (e.g. rheumatoid arthritis, scleroderma, hypersensitivity pneumonitis) (12-16). In these cases, the fibrosis tends to be progressive with a survival pattern similar to IPF

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patients (12-16), suggesting shared molecular drivers. Further, patients

with UIP attributable to rheumatoid arthritis also share a similar genetic

risk profile compared to patients with IPF, including a higher prevalence of

the minor allele of the *MUC5B* promoter variant and telomerase mutations

compared to controls (17, 18) . Recently we reported that short peripheral

blood leukocyte telomere length in patients with hypersensitivity

pneumonitis is associated with subpleural fibrosis, fibroblast foci, and

microscopic honeycombing (19); features of lung remodeling commonly

associated with UIP/IPF, but not hypersensitivity pneumonitis. Considering

these findings, it suggests that genetic predisposition, short telomere

length and cellular senescence may be key molecular drivers of lung

remodeling and fibrosis in any clinical context in which they are found

and, when present, engender features of remodeling currently attributed

to UIP/IPF. To address this possibility, we compared lung histopathology,

AECII cell telomere lengths, expression of molecular markers of cellular

senescence, and MUC5B in patients with UIP/IPF and UIP in clinical

contexts other than IPF.

Methods

Patient cohort. Patients were enrolled from the ongoing, longitudinal, prospective cohort of patients with interstitial lung disease (ILD) at the University of California San Francisco (UCSF). Patients were enrolled into the ILD cohort from 2001- 2012 and had baseline clinical information collected at the time of enrollment. The diagnosis of IPF was made by an in-person multidisciplinary team discussion (MDD) according to published guidelines (20, 21). Patients were included in the study if they had a UIP pattern of pulmonary fibrosis on surgical lung biopsy. There were no additional exclusion criteria. The institutional review board at UCSF approved the parent databases and patients provided written informed consent (IRB #s 10-00198 and 10-01592).

Lung Histopathology and immunohistochemistry. Lung tissue samples, obtained from either a surgical lung biopsy or harvested at explant, were reviewed and scored by an expert lung pathologist (K.J.D.) using a structured pathology data collection form (16). Using this form, an acute lung injury pattern was diagnosed when there were histologic changes that included hyaline membrane formation, type 2 pneumocyte hyperplasia, alveolar septal edema, and airspace organization or edema. Organizing pneumonia was diagnosed when rounded plugs of granulation tissue-like fibrosis were noted in airspaces. When organizing pneumonia

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was observed with acute lung injury, both were coded as present. When organizing pneumonia was observed in isolation, it was coded only as organizing pneumonia.

For immunostaining, endogenous peroxidase was inhibited in 5-μm sections of explanted tissue by incubating the sections in 3% hydrogen peroxide for 30 minutes. After washing with PBS, the sections were incubated for one hour in PBS containing 5% normal goat serum and 1% bovine serum albumin (BSA), then incubated with antibodies against p16 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p21 (Santa Cruz Biotech) or surfactant protein C (MilliporeSigma, Burlington, MA, USA) overnight at 4°C. The sections were washed in 0.1% PBS-Tween 20, then incubated for 40 minutes with conjugated secondary antibodies and washed again. The immunostained sections were then counterstained with Meyer's hematoxylin (MilliporeSigma). For each tissue, an adjacent section was stained only with secondary antibody alone to address nonspecific binding of the secondary antibody.

For MUC5B staining, deparaffinized sections of explanted lung tissue, rehydrated with series of ethanol solutions, heated in citrate buffer for antigen retrieval (20 min boiling), and then incubated overnight in rabbit primary antibodies against MUC5B (1:1000, Santa Cruz). Secondary anti-rabbit antibody diluted 1:500 tagged with AlexaFluor488 (ThermoFisher) were applied for 1 hour at room temperature. Slides were

counterstained 1:20,000 DAPI (BioLegends, San Diego, CA, USA) for analysis. Stained slides were scanned with Aperio Versa pathological slide scanner (Leica). Whole tissue scans were analyzed for MUC5B protein expression and distribution using ImageScope software (Leica Biosystems, Buffalo Grove, IL, USA).

Immunoblots. Fragments of lung tissue were snap frozen at the time of lung transplant or donor harvest, then lysed in RIPA buffer.   Equal amounts of lung lysate protein were subjected to SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane (Life Sciences Products, Boston, MA, USA). The membrane was washed with 50 mM Tris-HCl containing 0.5 M NaCl, 0.01% Tween-20, (TBS; pH 7.5) and incubated overnight in 5% milk containing primary antibody. The membrane was then washed with TBS, incubated in TBS for 30 min containing a 1:2,000 dilution of horse radish peroxidase-conjugated secondary antibody (New England Biolabs, Beverly, MA) and washed again. Immunoreactivity was detected using the phototope-HRP-detection kit (New England Biolabs) and band intensity quantified by densitometry.

Telomere Q-FISH assay. Telomere lengths were measured on paraffin-embedded sections of lung tissue by Quantitative Fluorescence in Situ Hybridization (Q-FISH). Briefly, after deparaffinixation, tissues were

suspended in 10mM sodium citrate buffer, pH 6.5, heated in a microwave, then incubated for 15 min in 0.01M HCL containing 1% pepsin (Thermofisher Scientific, South San Francisco, CA). The tissues were washed then treated with 10mg/ml RNase solution (Qiagen, Hilden, German). After washing, the tissues were incubated with 0.3 μg/ml PNA probe TelC-Cy3 (Panagene, Daejeon, Korea) suspended in formamide buffer (70% formamide, 10 mmol/L Tris, pH 7.5), heated to 78ºC for 10 min then incubated overnight at 20ºC. The tissues were then washed sequentially with formamide buffer then PBS containing 0.1% Tween, blocked with 3% BSA (Sigma, St. Louis, MO), 10% donkey serum and incubated overnight at 4ºC with rabbit-anti SPC antibody (MilliporeSigma). Tissues were washed with PBS containing 0.1% Tween and incubated secondary antibody at 20ºC for 1 h, washed, and mounted using prolong gold anti-fade mounting medium with DAPI (Life Technologies). Images were acquired using a Zeiss Axio Imager 2 microscope (Zeiss, Oberkochen, Germany) and telomere signal intensity was quantified in a blinded manner using MetaMorph imaging analysis software (Molecular Devices, Sunnyvale, CA).

MUC5B Genotyping. MUC5B rs35705950 single-nucleotide polymorphism (SNP) was measured using a Taqman SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA).

Statistics: All data analyses were carried out with Statistical Package for the Social Science, version 17.0 for windows (SPSS Inc., Chicago, IL, USA) and STATA 12.0 (Stata Corp LP). Continuous data were compared using ANOVA or Kruskal-Wallis test as appropriate. Categorical variables were compared using the Chi-squared test.

Results

Patient cohort

The cohort consisted of 226 patients with a UIP pattern of pulmonary fibrosis on surgical lung biopsy and/or lung explant, of which 169 were diagnosed with IPF, and 57 with non-IPF UIP. Baseline characteristics for the IPF and non-IPF UIP groups are shown in table 1 (**Table 1**). Mean age was 67.1 years and 65.2 years, respectively. There were significantly more males in the IPF cohort (**Table 1**). There were no significant differences in age, smoking history, or lung function as measured by forced vital capacity (FVC) or diffusion capacity of carbon monoxide (DLco).

Histopathologic comparison of UIP-IPF and Non-IPF UIP.

Histopathologic changes, including those commonly found in IPF patients (fibroblast foci and dense collagen fibrosis) were quantified in IPF lungs using a standardized scoring system. Histopathologic changes typically found in a UIP pattern of fibrosis, including temporal heterogeneity (97.0% vs. 96.4%), microscopic honeycombing (94.5% vs. 89.3%), fibroblast foci (98.8% vs. 100%) and dense collagen fibrosis (98.8% vs. 100%) were similarly present in the IPF and non-IPF UIP groups respectively. However, when quantified, the density of fibroblast foci differed between IPF and non-IPF UIP patients (p= 0.025). In contrast, non-IPF UIP patients tended

to have findings not typically associated with a UIP/IPF pattern of fibrosis including: lymphocytic infiltration (p < 0.001), noncaseating granulomas (P< 0.001), airway centered inflammation (p < 0.001) small airways disease (p < 0.001) and acute lung injury (p= 0.022). These results show that the fundamental changes attributable to a UIP pattern of disease are similar in IPF and non-IPF UIP patients, but that non-IPF UIP patients have additional elements of lung remodeling, which led to clinical diagnoses other than IPF.

AECII cell telomere measurements

Given the shared histopathologic changes between the lungs of IPF and non-IPF UIP patients, we next sought to determine whether they also similarly expressed proteins recognized to be molecular drivers of IPF. To examine whether telomeres are short in AECII cells of IPF patients and similarly shortened in AECII cells of non-IPF UIP patients, teloFISH was performed on sections of lung tissue (Figure 1 A, B). The analysis confirmed prior reports (4, 5) that within IPF AECII cells, telomere staining was significantly less intense than in age-similar control lungs (Figure 1 C). Similarly, telomere staining was significantly less intense in non-IPF UIP lungs. Telomere signal intensity was similar between IPF and non-IPF UIP lungs (Figure 1 C). These data suggest that telomeres are selectively

shortened in AECII cells of IPF and non-IPF UIP lungs compared to age-similar unused donor controls.

Quantification of aging markers in fibrotic human lungs.

Telomere shortening leads to activation of senescence programming and senescence markers p16 and p21 have been shown to be elevated in the lungs of IPF patients (6, 22, 23). Immunoblots were used to examine whether p16 and p21 expression is also increased non-IPF UIP lungs. IPF and non-IPF UIP lung lysates had significantly higher levels of p16 (Figure 2) , and p21 (Figure 3) compared to lung lysates from age-similar donor controls. Although both IPF and non-IPF UIP lungs had elevated levels of p16 and p21, the levels were higher in IPF lung lysates.

Immunostaining of senescence markers in IPF and non-IPF lungs.

To verify AECII cells are the source of p16 and p21, sections of lung tissue were immunostained for p16 and p21. Whereas control lungs had no immunoreactive cells (Figure 4), type II AECs in tissue sections from IPF and non-IPF UIP patients were immunoreactive for p16, p21.

MUC5B genotyping and expression in non-IPF patients.

Presence of the MUC5B minor allele rs35705950 is a major risk factor for IPF (24, 25) . The prevalence of the MUC5B minor allele was examined in

the cohort of non-IPF UIP patients and IPF patients. In patients with non-IPF UIP, the MUC5B rs35705950 minor allele frequency (MAF) was 34% and was similar to the 32% MAF in the IPF cohort (reported MAF in normal non-Hispanic Whites is 9%; https://www.ncbi.nlm.nih.gov/snp/rs35705950). Because the MUC5B rs35705950 variant affects MUC5B expression (26), IPF and non-IPF UIP lungs were immunostained for MUC5B. Notably, both IPF and non-IPF UIP lungs had a similar distribution of MUC5B immunoreactivity, with regions of immunostaining in airway epithelial cells, epithelial cells lining honeycomb cysts, and secreted mucin in the lumens of honeycomb cysts. This is distinct from staining in normal lungs, which was present only in proximal airway epithelial cells (Figure 5).

Discussion

Idiopathic pulmonary fibrosis is a progressive age-associated lung disease characterized histopathologically by the UIP pattern of lung fibrosis. A UIP pattern of fibrosis is also found in other clinical contexts.   This study reports that both IPF and non-IPF UIP similarly contain the core histopathologic changes attributable to UIP, but the density of fibroblast foci differed between the groups, and non-IPF UIP patients tended to have additional pathologic changes such as non-caseating granulomas, lymphocytic inflammation or small airways disease, consistent with the ATS/ERS/JRS/ALAT clinical practice guidelines on diagnosis of IPF (27). Telomeres were short in AECII cells of both IPF and non-IPF UIP patients, both expressed markers of cellular senescence in their AECII cells, and both had similar prevalence of the minor allele of MUC5B and MUC5B protein expression. These findings suggest that both IPF and non-IPF UIP patients may share genetic risk and molecular drivers of disease pathogenesis.

Patients with non-IPF UIP, in particular those with rheumatoid arthritis associated ILD (RA-ILD), have a clinical phenotype that is similar to patients with IPF. Most RA-ILD patients have a similar age, gender distribution, and smoking history compared to IPF patients (13, 28, 29). Further, clinical predictors of mortality and the development of acute

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exacerbations are also similar between RA-ILD and IPF patients (30). A

UIP pattern of fibrosis also predicts poor prognosis when found in patients

with a clinical diagnosis of scleroderma or hypersensitivity pneumonitis

(12, 16). These demographic observations suggest that common cellular

or molecular pathways may contribute to the pathogenesis of UIP.

Subpleural fibrosis, microscopic honeycombing, fibroblast foci and

temporal heterogeneity characterize the UIP pattern of lung fibrosis. All of

these features were present in IPF and non-IPF UIP patients. The two

groups could be distinguished by slightly more fibroblast foci in IPF

patients and additional elements of lung remodeling such as lymphocytic

inflammation, non-caseating granulomas or small airways disease. These

additional features non-IPF UIP patients are consistent with the clinical

context where the cases were found. Patients with autoimmune diseases

such as rheumatoid arthritis or scleroderma tend to have more

lymphocytic inflammation or small airways disease, and hypersensitivity

pneumonitis patients have non-caseating granulomas or small airways

disease as histopathologic features of their disease. These findings

suggest that if patients have a UIP pattern of fibrosis, plus additional

histopathologic changes (UIP-plus pattern) not commonly attributable to

UIP/IPF, then a clinical condition other than IPF should be considered.

Variants in genes regulating telomere length have been identified as susceptibility loci for development of sporadic IPF (*TERT*, *TERC*, *OBFC1*) (31, 32). Mutations in *TERT*, *TERC,* regulator of telomere elongation helicase 1 (*RTEL1*), and poly(A)-specific ribonuclease (*PARN*), have been identified in kindreds of familial pulmonary fibrosis and IPF patients (33-36). Consistent with linkage of telomere-associated genes to pulmonary fibrosis, telomeres were confirmed (4, 5) to be short in AECII cells of IPF patients where they have been shown to be sufficient to cause lung remodeling and fibrosis (8). Similarly, in this study, telomeres were short in AECII cells of non-IPF UIP patients. A subset of patients with rheumatoid arthritis associated ILD (17) and hypersensitivity pneumonitis (37) have also been found to have mutations in telomere related genes. Whether short AECII cell telomeres could in part be due to genetic variants was not investigated in this study and will require additional work in a broader cohort of non-IPF UIP patients.

Cellular senescence is a condition of stable cell-cycle arrest that is involved in multiple pathologic processes including tumor suppression, (38) tissue repair, (39, 40), aging, and IPF (6, 23). In IPF, cells reported to express senescence markers include fibroblasts (41, 42), and more prominently AECII cells (6, 23). Senescent cells may have dual contributions to lung fibrosis, with senescent fibroblasts limiting fibrosis

(43, 44) and senescent AECII cells promoting lung fibrosis (8, 23). Herein we confirmed that IPF AECII cells express the senescent markers p16 and p21. Further, AECII cells in non-IPF UIP patients also expressed p16 and p21. Because telomere shortening leads to cellular senescence it is possible expression of senescence markers in IPF and non-IPF UIP patients is due to critically short telomeres in these cells. These findings suggest that senescent AECII cells contribute to the pathogenesis of all cases of UIP irrespective of the clinical context where they are found.

The common gain-of-function variant rs3570595016 in the promoter of MUC5B, encoding mucin 5B, is the strongest genetic risk factor for IPF (24, 25). The variant is present in at least 50% of patients with IPF and accounts for nearly 30% of the risk of developing IPF. More recently, the rs3570595016 variant has also been associated with the UIP pattern of fibrosis in RA-ILD patients (18), and elements of remodeling attributable to UIP in patients with hypersensitivity pneumonitis (19). Consistent with these findings, this study found the MAF of the MUC5B minor allele variant rs3570595016 to be similar in IPF and non-IPF UIP patients. In addition, the pathologic pattern of MUC5B protein expression was similar in IPF and non-IPF UIP patients. Collectively, these findings suggest that the MUC5B minor allele may be a risk factor for all forms of UIP regardless of clinical context in which the pattern of fibrosis is found.

Patients with a UIP pattern of lung fibrosis have common histopathologic changes and rates of clinical progression. The progression may in part be due to pathologic telomere shortening or activation of senescence programming in lung epithelial cells, which are found in all cases of UIP fibrosis.   These findings provide additional evidence that genetic predisposition and molecular commonalities in UIP patients extend beyond current clinical classification systems and support the perspective that histopathologic, genetic and molecular determinants could be considered for reclassifying how lung fibrosis patients are categorized.

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Table 1. Patient characteristics

Variable	Non-IPF UIP (n=57)	IPF (n=169)	P-value
Age, mean (SD)	65.2 (11.8)	67.1 (7.7)	0.16
Male, n (%)	29 (51.7)	119 (70.4)	0.014
Ever Smoker, n (%)	34 (60.7)	120 (71.0)	0.11
FVC (% pred), mean (SD)	72.0 (14.7)	75.0 (18.5)	0.27
Dlco (% pred), mean (SD)	53.6 (20.1)	52.9 (17.0)	0.92
Clinical Dx			
IPF, n (%)	0 (0)	169 (100)	< 0.001
HP, n (%)	14 (25.0)	0	< 0.001
CTD-ILD, n (%)	16 (28.5)	0	< 0.001
Unclassifiable, n (%)	21 (37.5)	0	< 0.001
Other, n (%)	5 (8.9)	0	< 0.001

Age, FVC, and DLCO are reported as mean % predicted (standard deviation).

Abbreviations: FVC, forced vital capacity; DLco, diffusion capacity of carbon monoxide; IPF, idiopathic pulmonary fibrosis; HP, hypersensitivity pneumonitis; CTD-ILD, connective tissue disease-associated interstitial lung disease. There were no current smokers in the cohort. Other includes drug induced and asbestos mediated lung fibrosis.

Table 2. Histopathologic scoring of lung tissue obtained from IPF and non-IPF UIP patients.

Pathologic grade	IPF (N = 169)				Non-IPF UIP (N = 57)				*p* value
	0	1	2	3	0	1	2	3	
Specific findings	N (%)				N (%)				
Fibroblast foci	2 (1.2)	72 (43.1)	78 (46.7)	15 (9.0)	0 (0)	2 (3.5)	36 (63.2)	2 (3.5)	0.03
Organizing pneumonia	136 (81.4)	26 (15.6)	4 (2.4)	1 (0.6)	40 (70.2)	12 (21.1)	4 (7.0)	1 (1.8)	0.20
Lymphocytic interstitial infiltration	111 (66.5)	52 (31.1)	4 (2.4)	0 (0)	22 (38.6)	26 (45.6)	7 (12.3)	2 (3.5)	< 0.001
Alveolar macrophages	53 (31.7)	100 (59.9)	10 (6.0)	4 (2.4)	12 (21.1)	42 (73.7)	2 (3.5)	1 (1.8)	0.32
Dense collagen fibrosis	2 (1.2)	1 (0.6)	24 (14.4)	140 (83.8)	0 (0)	2 (3.5)	8 (14.0)	47 (82.5)	0.34
Granuloma/giant cells	147 (88.0)	18 (10.8)	2 (1.2)	0 (0)	41 (71.9)	6 (10.5)	7 (12.3)	3 (5.3)	< 0.001
Germinal centers	154 (92.2)	10 (6.0)	2 (1.2)	1 (0.6)	49 (86.0)	4 (7.0)	1 (1.8)	3 (5.3)	0.14
Hemosiderin laden macrophages	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.17

Eosinophils	163 (97.6)	4 (2.4)	0 (0)	0 (0)	56 (98.2)	1 (1.8)	0 (0)	0 (0)	0.78
Airway centered inflammation	120 (71.9)	47 (28.1)	0 (0)	0 (0)	26 (45.6)	24 (42.1)	6 (10.5)	1 (1.8)	< 0.001
Small airways disease	100 (59.9)	51 (30.5)	14 (8.4)	2 (1.2)	22 (38.6)	18 (31.6)	14 (24.6)	3 (5.3)	<0.001
Emphysema	135 (80.8)	17 (10.2)	13 (7.8)	2 (1.2)	54 (94.7)	3 (5.3)	0 (0)	0 (0)	0.07
Acute lung injury	166 (99.4)	1 (0.6)	0 (0)	4 (50)	54 (94.7)	3 (5.3)	0 (0)	0 (0)	0.02

FIGURE LEGENDS

Figure. 1. AECII cell telomere length quantification by teloFISH. (A) Example of Cy3-labeled telomere signal (red), SPC–immunoreactive cells (green), and nuclei stained with DAPI (blue) in age similar unused donor control and (B) non-IPF UIP patient samples. (C) Telomere length was quantified by teloFISH on sections of lung harvested from age similar unused donor control, IPF and non-IPF UIP patient lung sections. Each data point represents average telomere fluorescence intensity in surfactant protein C (SPC)-immunoreactive cells for an individual patient. Magnification: ×40. Telomere fluorescence intensity was quantified with respect to DAPI area using Metamorph software. Data were collected from 11 IPF, 11non-IPF UIP (5 scleroderma, 1 rheumatoid arthritis, 4 hypersensitivity pneumonitis, 1 Sjogren's syndrome) and 12 unused donor controls , **$P < 0.01$

Figure. 2. P16 levels in IPF lung lysates. (A) Immunoblots for p16 shows significantly higher levels of p16 in lung lysates of IPF patients (n=12) and non-IPF UIP patients (n=15; 8 scleroderma, 1 rheumatoid arthritis, 4 hypersensitivity pneumonitis, 2 Sjogren's syndrome) relative to those from unused donor control subjects (n=12). (B) Relative densitometric ratios of p16 to β-actin are presented by bar graphs (* $p < 0.01$, **$p < 0.05$, *** $p < 0.01$).

Figure. 3. p21 levels in IPF lung lysates. (A) Immunoblots for p21 shows significantly higher levels of p21 in lung lysates of IPF patients (n=19) and non-IPF UIP patients (n=14; 7 scleroderma, 1 rheumatoid arthritis, 4 hypersensitivity pneumonitis, 2 Sjogren's syndrome) relative to those from unused donor control subjects (n=12). (B) Relative densitometric ratios of p21 to β-actin are presented by bar graphs (* $p < 0.01$, ** $p < 0.05$ compared to normal controls).

Figure. 4. Immunohistochemistry of IPF, non-IPF UIP, and normal lung with p16 or p21 antibodies. Normal, IPF, and non-IPF UIP lungs were immunostained for p16 or co-immunostained for p21 (green fluorescence) and surfactant protein C (red fluorescence). IPF lungs stained with secondary antibodies alone (nonimmune) served as controls. Images are representative of tissues stained from 12 patients with IPF, 8 patients with non-IPF UIP (4 scleroderma, 2 rheumatoid arthritis, and 2 hypersensitivity pneumonitis. Note: representative non-IPF UIP images are from a patient with scleroderma UIP), and 10 control subjects.

Figure. 5. Immunohistochemistry of IPF, non-IPF UIP, and normal lung with MUC5B antibodies. Lung tissue sections taken from 13 donor control (A), 14 IPF UIP (B) and 14 non IPF UIP (3 scleroderma, 4 rheumatoid arthritis, and 4 hypersensitivity pneumonitis, 1 Sjogren's disease, 2

unclassifiable. Note: representative non-IPF UIP images are from a patient with scleroderma UIP). (C) were stained and examined for MUC5B (green) protein expression and distribution. Low power magnification image shows that in control lungs the majority of MUC5B was found in proximal (region 1, high power magnification on the right) airways, with some expression present in distal (region 2, high power magnification on the right) airways and alveoli (white arrows). Overall MUC5B expression was increased in IPF and non IPF UIP lungs and had similar distribution. Number of cells and amount of MUC5B per cell was increased in proximal (regions 1, high power magnification is on the right) and distal (regions 2, high power magnification is on the right) airways, with mucus accumulation in the lumen of the airways. Honeycomb cysts, lined with MUC5B expressing cells and with mucus accumulation in the lumen of the cysts, were present in both IPF and non IPF UIPs lungs. Nuclei were stained with DAPI (blue). Bar=500um for low power magnification, and 50um for high power magnification.