Gene Expression Profiling of Familial and Sporadic Interstitial Pneumonia

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Rationale: Idiopathic interstitial pneumonia (IIP) and its familial variants are progressive and largely untreatable disorders with poorly understood molecular mechanisms. Both the genetics and the histologic type of IIP play a role in the etiology and pathogenesis of interstitial lung disease, but transcriptional signatures of these subtypes are unknown.

Objectives: To evaluate gene expression in the lung tissue of patients with usual interstitial pneumonia or nonspecific interstitial pneumonia that was either familial or nonfamilial in origin, and to compare it with gene expression in normal lung parenchyma.

Methods: We profiled RNA from the lungs of 16 patients with sporadic IIP, 10 with familial IIP, and 9 normal control subjects on a whole human genome oligonucleotide microarray.

Results: Significant transcriptional differences exist in familial and sporadic IIPs. The genes distinguishing the genetic subtypes belong to the same functional categories as transcripts that distinguish IIP from normal samples. Relevant categories include chemokines and growth factors and their receptors, complement components, genes associated with cell proliferation and death, and genes in the Wnt pathway. The role of the chemokine CXCL12 in disease pathogenesis was confirmed in the murine bleomycin model of lung injury, with C57BL/6^{CXCR4+/-} mice demonstrating significantly less collagen deposition than C57BL/6^{CXCR4+/+} mice. Whereas substantial differences exist between familial and sporadic IIPs, we identified only minor gene expression changes between usual interstitial pneumonia and nonspecific interstitial pneumonia.

Conclusions: Taken together, our findings indicate that differences in gene expression profiles between familial and sporadic IIPs may provide clues to the etiology and pathogenesis of IIP.

Keywords: familial interstitial pneumonia; global transcription analysis; interstitial lung disease; lung fibrosis; microarrays

Idiopathic interstitial pneumonia (IIP) is a diverse group of lung disorders of unknown etiology characterized by various degrees of chronic inflammation and progressive fibrosis of the lung parenchyma (1, 2). The most common and lethal form of IIP is idiopathic pulmonary fibrosis (IPF). IPF is histopathologically

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Idiopathic interstitial pneumonia (IIP) and its familial variants are progressive and largely untreatable disorders with poorly understood molecular mechanisms.

What This Study Adds to the Field

There are considerable gene expression changes between familial and sporadic IIPs, but the histologic disease subtypes UIP and NSIP are transcriptionally very similar.

defined by the presence of the prototypical form of pulmonary fibrosis, usual interstitial pneumonia (UIP), a fibrosing interstitial pneumonia characterized by a pattern of heterogeneous, subpleural patches of fibrotic, remodeled lung that often results in death within 3 to 5 yr of diagnosis (1, 2). Other IIPs, such as nonspecific interstitial pneumonia (NSIP), are generally associated with a more cellular interstitial pneumonia with or without accompanying fibrosis, occur earlier in life, and have a considerably lower mortality (3, 4). Although these diseases are thought to be clinically distinct (5), the transcriptional features of the different types of IIP have received little attention. In fact, two publications (6, 7) indicate that these distinct clinical-pathologic processes (UIP and NSIP) appear to be related etiologically and pathogenically.

There is emerging evidence for the role of genetic factors in the development of pulmonary fibrosis. Although a relatively uncommon disease, cases of pulmonary fibrosis have been reported in closely related family members including monozygotic twins raised in different environments (8–10), genetically related members of several families (8, 11–13), in consecutive generations in the same families (8), and in family members separated at an early age (13). Pulmonary fibrosis is also observed in genetic disorders with pleiotropic presentation including Hermansky-Pudlak syndrome (14), neurofibromatosis (15), tuberous sclerosis (16), Neimann-Pick disease (17), Gaucher disease (18), familial hypocalciuric hypercalcemia (19), and familial surfactant protein C mutation (20). Finally, variability in the development of pulmonary fibrosis in response to fibrogenic agents has been reported in workers exposed to similar concentrations of asbestos (21, 22) as well as in inbred strains of mice challenged with either asbestos (23, 24) or bleomycin (25, 26). We have reported more than 100 families with two or more cases of IIP and found that familial IIP is pleiotropic and appears to be caused by an interaction between a specific environmental exposure and a

(Received in original form January 15, 2006; accepted in final form September 10, 2006)

Supported by the Department of Veterans Affairs (Merit Review), the National Institute of Environmental Health Sciences (ES11375 and ES011961), the National Heart, Lung, and Blood Institute (HL67467), and the Intramural Research Program of the NIH, National Institute of the Environmental Health Sciences.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 175, pp 45–54, 2007

Originally Published in Press as DOI: 10.1164/rccm.200601-062OC on September 22, 2006
Internet address: www.atsjournals.org

gene (or genes) that predisposes to the development of several subtypes of IIP (7). However, the transcriptional features that distinguish familial from nonfamilial (or sporadic) interstitial lung disease have not been established.

Expression profiling studies of the bleomycin mouse model of fibrosis (27) and five patients with IPF (28) revealed differential expression of several gene families expressed in the fibrotic lung specimens. Most of the genes upregulated in lung fibrosis encode proteins involved in extracellular matrix formation, degradation, and signaling. In addition, smooth muscle markers and genes encoding immunoglobulins, complement, and some chemokines were also found to be overexpressed in fibrotic lungs. These findings support the hypothesis that pulmonary fibrosis is a disease of constant matrix deposition and removal that is associated with modest chronic inflammation. In another study, the IPF gene expression signature was defined by the expression of tissue-remodeling, epithelial, and myofibroblast genes when compared with hypersensitivity pneumonitis (HP), in which genes associated with inflammation, T-cell activation, and immune responses predominated (29). On the basis of these signatures, the authors classified some NSIPs as more IPF-like and some as more HP-like but also identified a subset of NSIP cases that were not similar in expression to either HP or IPF.

Given the importance of genetic susceptibility and the histologic type of IIP in understanding the etiology and pathogenesis of interstitial lung disease, we profiled gene expression in patients with UIP or NSIP that was either sporadic or familial (two or more cases of IIP in the same nuclear family), and compared these results with gene expression from control specimens of normal lung parenchyma. We had hypothesized that the histologic subtype of IIP (UIP vs. NSIP) would result in more extensive changes in gene expression than the genetic subtype of IIP (sporadic vs. familial). Whereas we found that sporadic and familial forms of the disease are transcriptionally distinct, the histologic subtypes UIP and NSIP were surprisingly quite similar (30).

METHODS

Study Population and Specimen Collection

We profiled lung tissue from 35 individuals: 9 nondiseased normal control subjects, 16 patients with sporadic pulmonary fibrosis (14 with

UIP and 2 with cellular NSIP; 1 surgical biopsy, 6 transplants, and 9 autopsies), and 10 patients with familial pulmonary fibrosis (6 with UIP, 1 with fibrotic NSIP, and 3 with cellular NSIP; 5 biopsies, 1 transplant, and 4 autopsies). Thirty-five snap-frozen lung specimens were collected under institutional review board-approved protocols. Diseased lung tissue was obtained at the time of diagnostic surgical lung biopsy, from explanted lungs at the time of lung transplantation, or at the time of autopsy through the Duke University Medical Center (Durham, NC) and National Jewish Medical and Research Center (Denver, CO). Nondiseased (normal) lung was obtained from Tissue Transformation Technologies (Edison, NJ). Criteria for inclusion of subjects in the normal group are described in more detail in the online supplement. Each patient with IIP (sporadic or familial) was examined by one of the pulmonologists (M.P.S., K.K.B., or D.A.S.) involved in this investigation. The diagnosis was made after complete clinical evaluation (history, examination, radiology, and physiology) and surgical lung biopsy at a tertiary referral center. The pathologic subtype of the lung tissue was confirmed after examination by an expert pulmonary pathologist (T.A.S.). We used criteria established by the American Thoracic Society and European Respiratory Society to guide the classification of patients with interstitial lung disease (5, 31). Selected demographic and clinical data (age, sex, smoking status, and immunosuppressive drug treatment) on the subjects included in the study are reported in Table 1, with the exception of cases for which the information was not available to us.

Expression Profiling

RNA extraction and expression profiling were performed according to standard protocols and are described in more detail in the online supplement. All primary data have been deposited in the Gene Expression Omnibus database (accession GSE5774).

Microarray Analysis

Three separate analyses were performed on the microarray data set to identify three sets of transcripts: genes/expressed sequence tags (ESTs) that are differentially expressed in all diseased samples compared with normal control subjects, those that best differentiate familial from sporadic IIPs, and transcripts that are differentially expressed between histologically defined disease subtypes UIP and NSIP. Genes that are differentially expressed in familial IIP were identified by taking an intersection of the three-class (normal, sporadic, and familial IIP) and two-class (sporadic and familial IIP) analyses. An analogous approach was taken for identification of genes that differentiate UIP and NSIP. Details of the analysis protocol are described in the online supplement.

TABLE 1. SELECTED DEMOGRAPHIC AND CLINICAL DATA ON SUBJECTS INCLUDED IN STUDY

	Normal Control Subjects (n = 9)	Sporadic IIP (n = 16)	Familial IIP (n = 10)
Mean age, yr $\pm$ SD.	45.6 $\pm$ 25.9	54.4 $\pm$ 11.8	61.1 $\pm$ 11.1
Sex, male/female/unknown	5/3/1	11/3/2	7/1/2
Sample type			
Autopsy	0	9	4
Transplant	9	6	1
Surgical lung biopsy	0	1	5
Histology, UIP/NSIP	—	14/2	6/4
Smoking status			
Current	2	2	0
Former	1	7	4
Never	5	5	1
Unknown	1	2	5
Mean years smoking	*	26.1 $\pm$ 9.5	20.8 $\pm$ 12.7
Mean packs per day	*	47 $\pm$ 32	27 $\pm$ 23
Previous immunosuppressant treatment, yes/no/unknown	1/7/1	8/6/2	4/4/2

Definition of abbreviations: IIP = idiopathic interstitial pneumonia; NSIP = nonspecific interstitial pneumonia; UIP = usual interstitial pneumonia.

* Data not available.

Quantitative Reverse Transcription–Polymerase Chain Reaction

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) assays were used to confirm the differential expression of eight genes. The complete list of primers used is given in the online supplement (*see* Table E1) and the methodology is described in more detail in the online supplement.

Immunohistochemistry

Paraffin-embedded whole lung samples were analyzed for immunohistochemical localization of CXCL12, using standard methodology (described in the online supplement). Anti-human/mouse CXCL12 antibody (MAB350; R&D Systems, Minneapolis, MN) was used as the primary antibody at a concentration of 8 µg/ml.

Bleomycin Exposure

To determine whether genes that were differentially expressed and found to be associated with interstitial lung disease were indeed relevant to the etiology of fibroproliferation, we used a murine model of bleomycin-induced lung disease in a CXCR4-deficient mouse (CXCR4 is the receptor for CXCL12, which was found to be strongly associated with IIP). Six-week-old male C57BL/6^{CXCR4+/-} heterozygous mice (homozygosity is lethal) (stock number 4341; backcrossed on C57BL/6J background for at least eight generations) and C57BL/6J control mice (stock number 664) were obtained from the Jackson Laboratory (Bar Harbor, ME) and used under a Duke University Institutional Animal Care and Use Committee-approved protocol. Details pertaining to the exposure, whole lung lavage, bronchoalveolar lavage (BAL) processing, soluble collagen assay, and histologic evaluation are given in the online supplement.

RESULTS

Identification of Genes Involved in the Pathogenesis of IIP

We profiled RNA from the lungs of 26 patients with IIP and 9 normal control subjects on a customized whole human genome microarray from Agilent Technologies (Palo Alto, CA) containing probes for about 41,000 human genes and transcripts. Significance analysis of microarrays (SAM) revealed that there were 558 differentially expressed transcripts in the disease group relative to normal control subjects at a 5% false discovery rate (FDR), with 135 of the significant genes/ESTs being up- or down-regulated more than 1.8-fold in the lungs of patients with pulmonary fibrosis (*see* Table E2). A fold change cutoff of at least 1.8 was chosen to reduce the list of significant genes to about the top 100 transcripts that exhibited the most differential expression. When hierarchical clustering was applied to this set of 135 transcripts, all except two samples clustered according to the presence or absence of IIP (Figure 1). The fact that this simple unsupervised approach places the majority of samples into correct clusters (disease vs. no disease) supports the notion that transcripts that were selected appear to be capable of distinguishing fibrotic from normal lungs and are likely to be involved in either the pathogenesis or pathogenic sequelae of IIP.

A number of genes known to be involved in IIP are overrepresented among our cases of IIP. These include genes involved in extracellular matrix (ECM) turnover, ECM structural constituents (collagens, keratins, and proteoglycan), proteins involved in ECM degradation (fibroblast activation protein- α and matrix metalloproteinase-1 and -11), and cell adhesion molecules (vascular cell adhesion molecule-1, tenascin C, and integrin β -like 1). Hyaluronoglucosaminidase-2 (another ECM degradation protein) is, on the other hand, down-regulated in fibrotic lungs. Our gene expression profiling study identified several chemokines (CCL13, CXCL12, and CXCL14) and growth factors and their receptors (insulin-like growth factor-I [IGF-I], IGF-I-binding protein-5, and platelet-derived growth factor receptor-like) to be overexpressed

in the lung specimens obtained from patients with IIP. Among other potentially relevant differentially expressed transcripts are complement components (B factor, H factor-1, and complement factor H-related 3), members of the coagulation cascade (tissue factor pathway inhibitors 1 and 2), genes associated with cell proliferation and death (tumor necrosis factor receptor superfamily, member 10b, and death-associated kinase-2), and genes in the Wnt signaling pathway (β -catenin-interacting protein-1, secreted frizzled-related protein-2, and frizzled homologs 4 and 5). Two genes identified as highly overexpressed in pulmonary fibrosis in previous studies, osteopontin and matrilysin or matrix metalloproteinase (MMP-7) (28, 32), are not found in our list of top differentially genes; they were removed from further analysis by our data-filtering strategy (*see* METHODS for details) because of the limited number of data points across all samples that we examined. However, if they are included in the analysis, osteopontin and MMP-7 are up-regulated in IIP by 5.7- and 4.3-fold, respectively. Both observations are significant at a 5% false discovery rate (FDR) if one uses the subset of samples with reliable osteopontin and MMP-7 expression levels in the statistical analysis.

Identification of Genes That Are Differentially Expressed in Familial IIP

Of the 26 patients with IIP in our study, 10 individuals have a family history of the disease. Closer inspection of the sample tree in Figure 1 reveals that most samples of familial IIP cluster together and that gene expression changes seem to be more pronounced in these individuals than in those with the sporadic form of the disease. We sought to distinguish genes that are differentially expressed in the lungs of familial cases of IIP from those with sporadic IIP. To accomplish this, we first performed three-class significance analysis of microarrays (familial IIP, sporadic IIP, and normal) and identified 701 transcripts as differentially expressed at 1% FDR among the three groups. To further identify genes that differentiate the familial from the sporadic form of the disease, two-class SAM was used (normal control subjects were ignored in this analysis) and an overlap between the two analyses was taken. Of the 676 significant genes/ESTs identified in the two-class comparison of sporadic IIP and familial IIP, 332 are in common to the three-class analysis at the same significance level (*see* Venn diagram in Figure 2). Of these 332 transcripts, 142 are up- or downregulated more than 1.8-fold (listed in Table E3) and 62 of these are genes with known functions. Shown in Figure 3 are the 62 genes with known functions and their mean expression levels in the three groups of individuals. The majority of the genes that are differentially expressed in the familial form of the disease belong to the same functional categories as transcripts that distinguish IIP (regardless of origin) from normal samples (*see* Table E1), but they are over- or under-expressed to a greater extent in familial IIP than in all cases of IIP. This is also true for previously identified candidates osteopontin (2.3-fold up-regulated in familial relative to sporadic cases) and matrilysin (3.5-fold overexpressed in familial IIPs). To validate findings from the microarray study, we measured expression levels of seven transcripts in the same patient samples, using real-time quantitative RT-PCR. CCL13, CXCL12, CXCL14, MMP-1, and secreted frizzled-related protein (SFRP)-2 are significantly differentially expressed in both disease-versus-normal and familial-versus-sporadic comparisons (*see* Tables E2 and E3 and Figure 3). PLEKHK1 (pleckstrin homology domain containing, family K member 1) is the most down-regulated gene in the familial-versus-sporadic IIP comparison (*see* Table E3). CYP1B1 is significantly differentially expressed in familial versus sporadic IIP, but is not listed in Table E3 because its up-regulation in familial IIP represents a less than 1.8-fold change. These genes

Figure 1. Hierarchical clustering of 35 samples based on 135 transcripts that best differentiated patients with pulmonary fibrosis from normal control subjects. Samples are color-coded according to disease inheritance status (orange = normal; blue = sporadic idiopathic interstitial pneumonia [IIP]; magenta = familial IIP) or histologic features (orange = normal; green = nonspecific interstitial pneumonia [NSIP]; red = usual interstitial pneumonia [UIP]); the asterisk (*) denotes one case of fibrotic NSIP. Average linkage clustering with euclidian distance metric was used.

were found to follow a pattern similar to that identified using microarrays of higher over- or underexpression in patients with a family history of the disease as compared with sporadic IIP (see Figure E1).

Identification of Genes That Are Differentially Expressed in UIP and NSIP

We applied the same strategy that we used to identify genes that differentiate familial IIP from sporadic IIP to identify transcripts that are differentially expressed in histologic forms of IIP (UIP vs. NSIP), regardless of whether they were sporadic or familial. One hundred and twenty-eight transcripts were significant at a 1% FDR in three-class SAM analysis (normal, NSIP, and UIP), and 39 were differentially expressed at the same significance level between UIP ($n = 20$) and NSIP ($n = 6$) samples (Figure 4). Of the 33 genes/ESTs that were in common to the two analyses, only 8 were differentially regulated more than 1.8-fold (see Table E4). We confirmed down-regulation of C3 in UIP as compared with NSIP by RT-PCR. Almost identical fold changes were observed by the two techniques (2.3-fold down-regulated on the array and 2.2-fold down-regulated by RT-PCR). Although we acknowledge the limitations of this analysis because of the small

sample size for NSIP ($n = 6$), these data suggest that there are consistent, but few, differences between the two types of IIP at the transcriptional level.

Increased Expression of CXCL12 Is Confirmed by Immunohistochemistry

The chemokine CXCL12, or stromal cell-derived factor 1 (SDF-1), is an important regulator of hematopoietic cell development, migration, and proliferation, and is the only known ligand for the CXCR4 receptor. CXCL12 is up-regulated 2.6-fold in fibrotic lungs compared with normal lung tissue and 2.4-fold in familial IIP compared with sporadic IIP (both observations are significant at a 5% FDR). The CXCR4 receptor is not differentially expressed in sporadic IIP but is slightly up-regulated (1.5-fold; not significant by SAM) in familial IIP. Neither CXCL12 nor CXCR4 is differentially expressed between UIP and NSIP. To confirm increased expression of CXCL12 in the fibrotic lung, we performed immunohistochemistry on lungs of subjects with pulmonary fibrosis and normal control subjects. CXCL12 expression is localized primarily in macrophages and airway epithelial cells and to a much smaller extent in endothelial cells. As shown

Figure 2. Venn diagram depicting the approach to identification of pulmonary fibrosis susceptibility genes on the basis of gene expression profiling. Genes (701) were identified as significantly differentially expressed (1% false discovery rate [FDR]) in three groups of patients (normal, sporadic, and familial IIP) by significance analysis of microarrays (SAM). Similarly, 676 genes were identified as significant at 1% FDR between sporadic and familial groups. Three hundred and thirty-two genes were common to the two analyses.

in the representative section in Figure 5, elevated concentration of the CXCL12 protein in the fibrotic lung (Figure 5b) compared with normal tissue (Figure 5a) is attributed to the presence of a significantly larger number of macrophages expressing

Figure 4. Venn diagram illustrating the approach taken to identify genes that differentiate UIP and NSIP. Genes (128) were identified as significantly differentially expressed (1% false discovery rate [FDR]) in three groups of patients (normal [N], UIP, and NSIP) by SAM. Similarly, 39 genes were identified as significant at a 1% FDR between UIP and NSIP groups. Thirty-three genes were in common to the two analyses.

CXCL12 in the parenchyma of individuals with IPF. CXCL12 levels in airway epithelial cells are comparable in diseased and control tissue (data not shown).

CXCR4^{+/-} Mice Develop Less Bleomycin-induced Fibrosis but Show Increased Inflammatory Response

To determine whether CXCL12 is involved in the fibrotic process, we examined C57BL/6^{CXCR4^{+/-}} heterozygous mice (homozygous null mice die perinatally) in a bleomycin murine model of lung injury (33). Fourteen days after intratracheal instillation

Figure 3. Mean expression ratios in normal, sporadic IIP, and familial IIP groups of 62 genes with known function that best differentiate familial IIP from sporadic IIP. Genes are grouped according to their function (CC = coagulation cascade; nuclear factor [NF]-κB = regulation of I-κB/NF-κB cascade). Genes are labeled by gene symbol followed by familial/sporadic IIP fold change in parentheses.

Figure 5. CXCL12 protein levels are significantly higher in (b) diseased lungs than in (a) normal control subjects because of the presence of a larger number of macrophages expressing CXCL12 in the parenchyma of subjects with pulmonary fibrosis.

of bleomycin (2 U/kg), the lungs of C57BL/6^{CXCR4+/-} mice and of C57BL/6^{CXCR4+/+} wild-type control mice were analyzed for the extent of fibrosis. The amount of soluble collagen in the lung increased considerably after bleomycin treatment as compared with saline-administered control animals in the C57BL/6^{CXCR4+/+} group, whereas C57BL/6^{CXCR4+/-} animals showed no significant increase in collagen deposition in the lung in response to bleomycin (Figure 6a). Similarly, histologic evaluation of the left lung by Masson trichrome staining revealed extensive and widespread fibrosis and collagen deposition in the lungs of C57BL/6^{CXCR4+/+} mice treated with bleomycin (Figure 6b). In contrast, C57BL/6^{CXCR4+/-} mice developed substantially less fibrosis that was localized peribronchially and did not extend toward the lung pleura (Figure 6c). Mice that were treated with phosphate-buffered saline alone did not develop any lung injury, regardless of the status of the CXCR4 gene (Figures 6D and 6E). We also measured the concentration of inflammatory cells in the BAL fluid as a way of quantifying inflammatory response to bleomycin in the two murine strains. Bleomycin treatment elicits a much more pronounced inflammatory response in C57BL/6^{CXCR4+/-} mice compared with wild-type control mice (Figure 7a). Macrophages and lymphocytes together account for 80 to 90% of recruited cells in the BAL fluid in both strains of animals (Figure 7b). Although there is no difference in the number of macrophages in the BAL fluid of C57BL/6^{CXCR4+/-} and C57BL/6^{CXCR4+/+} mice, there are twice as many lymphocytes in C57BL/6^{CXCR4+/-} mice than in wild-type control mice ($p < 0.005$). Taken together, differences in the extent of lung fibrosis and inflammation provide further support for a biological role of CXCR4 and its ligand CXCL12 in the pathogenesis of pulmonary fibrosis.

DISCUSSION

Our results demonstrate that IIP, regardless of type, involves disordered homeostatic control of genes that regulate the extracellular matrix and chemokine activity in the lung. Moreover, our findings demonstrate that although familial and nonfamilial forms of IIP appear to be transcriptionally distinct, the histopathologic categories of UIP and NSIP appear to be remarkably similar. In aggregate, our findings have identified several categories of genes, as well as specific genes, that appear to be important in the development and progression of IIP.

Our findings suggest that familial IIP is simply a more extreme biological variant of IIP. Genes that distinguish the inherited form of the disease from sporadic cases of IIP belong to the same functional categories as genes that are differentially expressed among all patients with the disease relative to normal control subjects (28). In fact, our results suggest that the expression changes are simply more extreme among familial cases of IIP

when compared with sporadic cases. These findings suggest that identifying susceptibility genes in families with a propensity for this disease will also be informative in understanding the pathogenesis of sporadic IIP.

Although IPF/UIP and NSIP are thought to be clinically distinct disorders of the interstitium, our results indicate that these diseases are transcriptionally similar. Thus, despite the differences in clinical and morphologic features of IPF/UIP and NSIP, gene expression profiles suggest that these are similar disease processes. It is tempting to speculate that NSIP occurring earlier in life and having a better prognosis has the potential to progress to IPF/UIP, and that IPF/UIP simply represents that late presentation of untreated (or poorly responsive) NSIP. This speculation is supported by independent observations. First, it is well known that a substantial number of patients have histologic evidence of both UIP and NSIP in the same lung (6). Moreover, we have found that a substantial portion of the families with familial IIP had several radiographic or histologic patterns of IIP (most often including UIP and NSIP), suggesting that the different histologic types of IIP may be related etiologically and even pathogenically (7). Although more research is needed, multiple lines of evidence suggest that UIP and NSIP do not always represent distinct forms of IIP. However, different subtypes of NSIP were identified on the basis of gene expression profiles (similar to IPF, similar to HP, or not similar to either) (29). Our study identified only a few differences between transcription profiles of whole lungs despite differences in cell types (i.e., the presence of lymphocytes in NSIP) and biological processes (formation of fibroblastic foci, and aberrant epithelial and vascular remodeling in UIP) present in the two disease subtypes. One limitation of both the previous and present studies is the small sample size for NSIP, suggesting that future studies with more cases will be needed to validate our initial findings. In addition, gene expression profiling of different cell types isolated from NSIP and UIP lungs may provide further insight into biological processes underlying these two disease subtypes.

Our results indicate that chemokines of the CXCL family play a role in the fibroproliferative response of the lung. Although this has been suggested by others (34–39), our findings are specifically supported by a study (37) demonstrating that circulating fibrocytes are recruited to bleomycin-treated lung in response to CXCL12. Phillips and coworkers also found that fibrocyte trafficking to the lung could be attenuated by using a specific neutralizing anti-CXCL12 antibody. Hashimoto and coworkers (35) used chimeric mice whose bone marrow-derived cells were labeled with the green fluorescent protein to show that lung fibroblasts in pulmonary fibrosis can be derived from bone marrow

Figure 6. C57BL/6^{CXCR4+/-} mice develop less fibrosis than do wild-type control mice after bleomycin-induced lung injury. (a) Soluble collagen content in the right lungs of C57BL/6^{CXCR4+/-} and C57BL/6^{CXCR4+/+} mice 14 d after administration of bleomycin or phosphate-buffered saline (PBS) was measured in a Sircol collagen assay (Biorcolor, Newtownabbey, UK). **p* < 0.05 by two-tailed Student *t* test. (b–e) Masson trichrome staining of left lung sections indicates dense and widespread fibrosis as revealed by collagen deposition in bleomycin-treated C57BL/6^{CXCR4+/+} mice and much less fibrosis in C57BL/6^{CXCR4+/-} animals. No fibrosis is seen in animals that were administered PBS alone.

progenitor cells and that CXCL12 and CXCR4 are overexpressed in the lungs of bleomycin-treated chimeric mice. Although the role of the circulating fibrocytes once in the lung is at present uncertain, it is becoming clear that the CXCL chemokines play an important role in fibroblast recruitment and fibroproliferation.

Although the finding that mice lacking one copy of the CXCR4 gene develop substantially less lung fibrosis than do wild-type control mice is supported by other published studies (35, 37), the increased infiltration of lymphocytes to the lungs

of C57BL/6^{CXCR4+/-} animals compared with C57BL/6^{CXCR4+/+} control mice 2 wk after bleomycin administration is a novel observation. Inflammatory response generally precedes the development of fibrosis in the bleomycin model of lung injury and mostly resolves by 14 d after bleomycin treatment (33). The fact that more inflammation persists in the CXCR4 heterozygous animals than in homozygous control animals on Day 14 is a result that requires further investigation. It is possible that the CXCR4–CXCL12 axis is involved in the development of pulmonary fibrosis via mechanism(s) other than fibrocyte homing, especially

Figure 7. Inflammation in whole lung lavage 14 d post-treatment with bleomycin or saline. (a) Significantly more cells in total (* $p < 0.05$ by two-tailed Student *t* test) were recovered from the bronchoalveolar lavage (BAL) fluid of C57BL/6^{CXCR4+/-} animals compared with wild-type control animals in response to bleomycin. Both murine strains were unresponsive to saline. (b) No differences in macrophage recruitment were observed between the two strains of mice. Significantly more lymphocytes (** $p < 0.005$ by two-tailed Student *t* test) were recruited to the lungs of C57BL/6^{CXCR4+/-} animals than C57BL/6^{CXCR4+/+} control animals in response to bleomycin.

considering the fact that CXCR4 is expressed on a number of different hematopoietic and nonhematopoietic cell types. Another possibility is that there is a compensatory increase in expression of other chemokines/receptors in response to bleomycin in the absence of one copy of CXCR4.

Expression of CXCR4 and its ligands was examined at the RNA and protein levels in lung biopsies from patients with UIP or NSIP, and in normal margins from tumor cases (40). Choi and coworkers showed that no significant differences in expression of CXCR4 exist between UIP and normal tissue nor between UIP and NSIP, which is in agreement with our expression data. However, the finding that CXCL12 is not differentially expressed in IIP compared with normal control subjects is different from the results of our study and quite surprising, considering both our findings and those by Phillips and coworkers (37). One possible explanation for this difference is the choice of normal control subjects; gene expression in normal tissue from tumor margins can be altered due to the close proximity to tumor cells. Clearly more studies will be needed to address the expression of chemokines and their receptors in IIP.

Aberrant activation of the Wnt/ β -catenin signaling pathway has been proposed as a potential molecular event leading to dysregulated repair in the fibrotic lung (41). Our findings indicate that several components of the Wnt signaling pathway are differentially expressed in the lungs of patients with IIP; for example, SFRP2 is almost fourfold upregulated in the diseased tissue. Interestingly, SFRP1 was identified as important in the susceptibility to bleomycin-induced lung injury of mice, using a combination of quantitative trait locus mapping and gene expression analysis (42). Defects in Wnt signal transduction lead to initiation of various tumors, but the role of the pathway in the context of fibroproliferation and fibrogenesis is still being defined. One possible mechanism might involve the metalloproteinase matrilysin/MMP-7, a target of Wnt/ β -catenin *trans*-activation and a molecule found to be involved in IPF (28). There is also evidence for a role of Wnt signaling in the induction of epithelial-mesenchymal transition (43), an important process that occurs during fibroproliferative repair after lung injury. Finally, studies have implicated a role for the Wnt pathway in self-renewal of hematopoietic stem cells and at several stages of lymphocyte

development by providing proliferative and/or maintenance signals to these cell populations (44). Thus, the role of the Wnt/ β -catenin signaling pathway in lung fibrosis is in need of further investigation.

In summary, we have identified several functional categories of differentially expressed genes in the fibrotic lung compared with normal lung tissue that are associated with a familial history of IIP. We have also shown that there are considerable gene expression changes between familial and sporadic IIPs but that UIP and NSIP are transcriptionally similar. Finally, our findings highlight the importance of CXCL12/CXCR4 in the pathogenesis of IIP. In aggregate, our study has identified several categories of genes that are involved in fibroproliferation and suggests that transcriptionally regulated genes may more precisely define the type and activity of the interstitial fibroproliferative disease process.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Ed Lobenhofer, Ken Philips, Yang Qiu, and Pat Hurban (Icoria, Inc.) for assistance in microarray design and hybridization assays; Stavros Garantziotis, David Brass, and Greg Whitehead for help with bleomycin exposure; Dolly Kervitsky (NJMC) for assistance in obtaining clinical data; and Kimwa Walker (NIEHS Immunohistochemistry Core Facility) for performing CXCL12 immunohistochemistry.

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