Gene Expression Profiles of Acute Exacerbations of Idiopathic Pulmonary Fibrosis

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Rationale: The molecular mechanisms underlying acute exacerbations of idiopathic pulmonary fibrosis (IPF) are poorly understood. We studied the global gene expression signature of acute exacerbations of IPF.

Objectives: To understand the gene expression patterns of acute exacerbations of IPF.

Methods: RNA was extracted from 23 stable IPF lungs, 8 IPF lungs with acute exacerbation (IPF-AEx), and 15 control lungs and used for hybridization on Agilent gene expression microarrays. Functional analysis of genes was performed with Spotfire and Genomica. Gene validations for MMP1, MMP7, AGER, DEFA1–3, COL1A2, and CCNA2 were performed by real-time quantitative reverse transcription-polymerase chain reaction. Immunohistochemistry and *in situ* terminal deoxynucleotidyltransferase dUTP nick end-labeling assays were performed on the same tissues used for the microarray. ELISA for α-defensins was performed on plasma from control subjects, patients with stable IPF, and patients with IPF-AEx.

Measurements and Main Results: Gene expression patterns in IPF-AEx and IPF samples were similar for the genes that distinguish IPF from control lungs. Five hundred and seventy-nine genes were differentially expressed (false discovery rate < 5%) between stable IPF and IPF-AEx. Functional analysis of these genes did not indicate any evidence of an infectious or overwhelming inflammatory etiology. CCNA2 and α-defensins were among the most up-regulated genes. CCNA2 and α-defensin protein levels were also higher and localized to the epithelium of IPF-AEx, where widespread apoptosis was also detected. α-Defensin protein levels were increased in the peripheral blood of patients with IPF-AEx.

Conclusions: Our results indicate that IPF-AEx is characterized by enhanced epithelial injury and proliferation, as reflected by increases in CCNA2 and α-defensins and apoptosis of epithelium. The concomitant increase in α-defensins in the peripheral blood and lungs may suggest their use as biomarkers for this disorder.

Keywords: CCNA2; α-defensins; microarray; apoptosis; viral infection

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic interstitial lung disease with a median survival of 2.5–3 years (1), and is largely unaffected by currently available medical therapies (2). Although most patients experience a gradual disease course characterized by steady worsening of symptoms,

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The mechanisms of acute exacerbation of idiopathic pulmonary fibrosis (IPF-AEx), a syndrome characterized by new development of pulmonary infiltrates, deterioration of lung function and hypoxemia, are unknown.

What This Study Adds to the Field

This analysis of genome-scale gene expression patterns in lungs of patients with IPF-AEx identifies epithelial injury and proliferation as the key molecular genetic events in IPF-AEx, and suggests plasma defensins as new biomarkers. Therapeutic strategies that protect the epithelium should be evaluated in this syndrome.

lung function, and gas exchange, some experience rapid deteriorations that are of unknown etiology. These deteriorations have been defined as acute exacerbations of IPF (IPF-AEx) (3–10). The pathological hallmark of IPF-AEx is diffuse alveolar damage superimposed on the usual interstitial pneumonia pattern characteristic in IPF (7). IPF-AEx can occur at any time during the disease course, and the risk of an exacerbation does not appear to be linked to the level of pulmonary function derangement, age, or smoking history (11). Little is known about the pathogenesis of IPF-AEx. Along with histopathology of diffuse alveolar damage, there is evidence of loss of alveolar epithelial cell integrity (12). It has been suggested that IPF-AEx may represent a response to a clinically occult infection (4, 13) but direct evidence of an association with infections is still missing (9).

Gene expression profiling was previously applied to stable sporadic (14–20) and familial IPF (21). To generate new hypotheses regarding the molecular events that underlie IPF-AEx and to identify new potential biomarkers for this syndrome, we analyzed global gene expression patterns in the lungs of patients undergoing IPF-AEx and compared them with stable IPF and control lungs. Some of the results have been previously reported in the form of an abstract (22).

METHODS

See the online supplement for details on methods.

Study Population

Lung tissue samples for microarray analysis were obtained through the University of Pittsburgh Health Sciences Tissue Bank (Pittsburgh, PA) as previously described (15) (and see the online supplement). Those

included 23 lungs from patients with IPF, 8 lungs from patients with acute exacerbation of IPF (IPF-AEx; obtained from explanted lungs or via the warm autopsy protocol) (23), and normal lung histology samples from control subjects. Plasma samples of patients with stable IPF (n = 10), patients with IPF-AEx (n = 16), and healthy control subjects (n = 12) were obtained from Asan Medical Center (Seoul, South Korea). The diagnosis of IPF was based on the American Thoracic Society and European Respiratory Society definition (24). The definition of IPF-AEx was based on criteria provided by Collard and colleagues (3) or Akira and colleagues (25). All cases were reviewed by expert pulmonologists and pathologists. Detailed clinical information about the subjects with IPF-AEx is provided (*see* Tables E1 and E2 in the online supplement).

The mean forced vital capacity (expressed as a percentage of the normal expected value) (FVC%) and diffusing capacity for carbon monoxide (expressed as a percentage of the normal expected value) (D$_{L_{CO}}$%) of patients with stable IPF and patients with IPF-AEx are provided in Tables 1A and 1B. All studies were approved by the Institutional Review Boards at the University of Pittsburgh and Asan Medical Center.

Oligonucleotide Microarray Experiments

Total RNA extracted from snap-frozen lung tissue was used as template for the generation of labeled cRNA that was hybridized to Agilent 4 × 4 4k whole human genome microarrays and scanned with an Agilent scanner (Agilent Technologies, Santa Clara, CA) as recommended and previously described by us (16). The complete data set is available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE10667).

Real-time Quantitative Reverse Transcription-Polymerase Chain Reaction

The same RNA samples used for microarray experiments were used to run real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on TaqMan system (Applied Biosystems, Foster City, CA). PCR was performed with TaqMan universal PCR master mix (Applied Biosystems) for the following genes: CCNA2, DEFA1–3, AGER, COL1A2, MMP1, MMP7, and GUSB. The results were analyzed by the $\Delta\Delta C_t$ method and (GUSB, encoding β-glucuronidase) was used as a housekeeping gene. Fold change was calculated by taking the average over all the control samples as the baseline.

Immunohistochemistry

OCT-embedded sections of normal and IPF-AEx samples were used for fluorescence immunohistochemistry. Rabbit polyclonal antibody against cyclin A2 (CCNA2; Abcam, Cambridge, MA), prosurfactant protein C (Abcam), and mouse monoclonal antibodies for cytokeratin (Vector Laboratories, Burlingame, CA), vimentin (Vector Laboratories), Ki-67 (Abcam), and α-defensins (Hycult Biotechnology, Uden, The Netherlands) were used as described in the online supplement. Each antigen–antibody complex was labeled with biotinylated antibody against mouse or rabbit IgG, and visualized with fluorescein green or Texas red (Vector Laboratories). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO).

ELISA

Plasma concentrations of α-defensins were determined with an ELISA kit for α-defensins (DEFA1–3) (Hycult Biotechnology).

Western Blot

Total protein was denatured by adding Laemmli sample buffer (Bio-Rad, Hercules, CA) 2-mercaptoethanol and boiling. Fifteen micrograms of total protein was used in the immunoblotting process.

Terminal Deoxynucleotidyltransferase dUTP Nick End Labeling

Formalin-fixed, paraffin-embedded tissue samples were used for the terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assay, done with an *in situ* cell death detection kit (Roche

TABLE 1. CHARACTERISTICS OF PATIENTS WITH STABLE IDIOPATHIC PULMONARY FIBROSIS (IPF) AND PATIENTS WITH ACUTE EXACERBATION OF IPF

Variable	Stable IPF	IPF-AEx
A. Patients from University of Pittsburgh Medical Center		
Number of subjects	23	8
Average age, yr	61.71 (±5.51)	68.25 (±10.22)
Average FVC%	51.49 (±11.29)	55.73 (±15.85)*
Average D$_{L_{CO}}$%	40.26 (±16.19)	36.61 (±12.06)*
Male/female	19/4	6/2
B. Patients from Asan Medical Center		
Number of subjects	10	16
Average age, yr	63.60 (±9.94)	65.50 (±10.30)
Average FVC%	81.1 (±11.97)	55.0 (±8.3)† (n = 7)
Average D$_{L_{CO}}$%	66.40 (±12.77)	38.9 (±13.3)† (n = 7)
Male/female	10/0	9/7

Definition of abbreviations: D$_{L_{CO}}$ = diffusing capacity of carbon monoxide; IPF = idiopathic pulmonary fibrosis; IPF-AEx = acute exacerbation of idiopathic pulmonary fibrosis.

* Last before IPF-AEx.
† At the time of IPF-AEx.

Applied Sciences, Indianapolis, IN). After proteinase digestion the sections were incubated in a mixture containing terminal deoxynucleotidyltransferase and fluorescein isothiocyanate–labeled dUTP. The TUNEL conjugates were labeled with alkaline phosphatase, visualized with Vector red, and counterstained with hematoxylin. The samples were observed under a light microscope.

Statistical Analysis

Array images were processed according to the Agilent Feature Extraction protocol (26). All arrays were cyclic-LOESS normalized, using the Bioconductor package as previously described (27). For statistical analysis we applied significance analysis of microarrays (SAM) (28). A q value of 5, which corresponds to a 5% false discovery rate, was used as a cutoff of statistical significance in microarray data. Data visualization and clustering were performed with Genomica (28), Scoregene (29), and Spotfire DecisionSite 9 (TIBCO, Palo Alto, CA). For qRT-PCR, the Student *t* test was used and significance was defined as $P < 0.05$.

RESULTS

Compared with Control Samples, Global Gene Expression Patterns of IPF-AEx and IPF Are Similar

When compared with control samples, the global gene expression patterns of IPF-AEx are almost identical to those of stable IPF (Figure 1A). To better characterize this similarity we looked at genes that characterized IPF lungs in previous studies (Figure 1B) and compared their expression in IPF-AEx with that in stable IPF (Figure 1C). Impressively, all the highlighted genes were not significantly different between IPF and IPF-AEx (Figure 1C; *see also* Table 2). These results indicate that compared with control samples, IPF-AEx exhibits a fibrosis signature that is identical to that of stable IPF.

To confirm this observation we performed qRT-PCR for MMP1, MMP7, COL1A2, and AGER, which are among the genes that consistently distinguish patients with IPF from control subjects (16, 20) (Figure 2). As expected, COL1A2, MMP1, and MMP7 were significantly higher and AGER was significantly lower in IPF and IPF-AEx compared with control samples, but there was no significant difference in their expression between IPF and IPF-AEx.

Direct Comparison of IPF-AEx and Stable IPF

To identify the subtler gene expression changes that distinguish IPF-AEx from stable IPF, and to focus on clusters of genes that
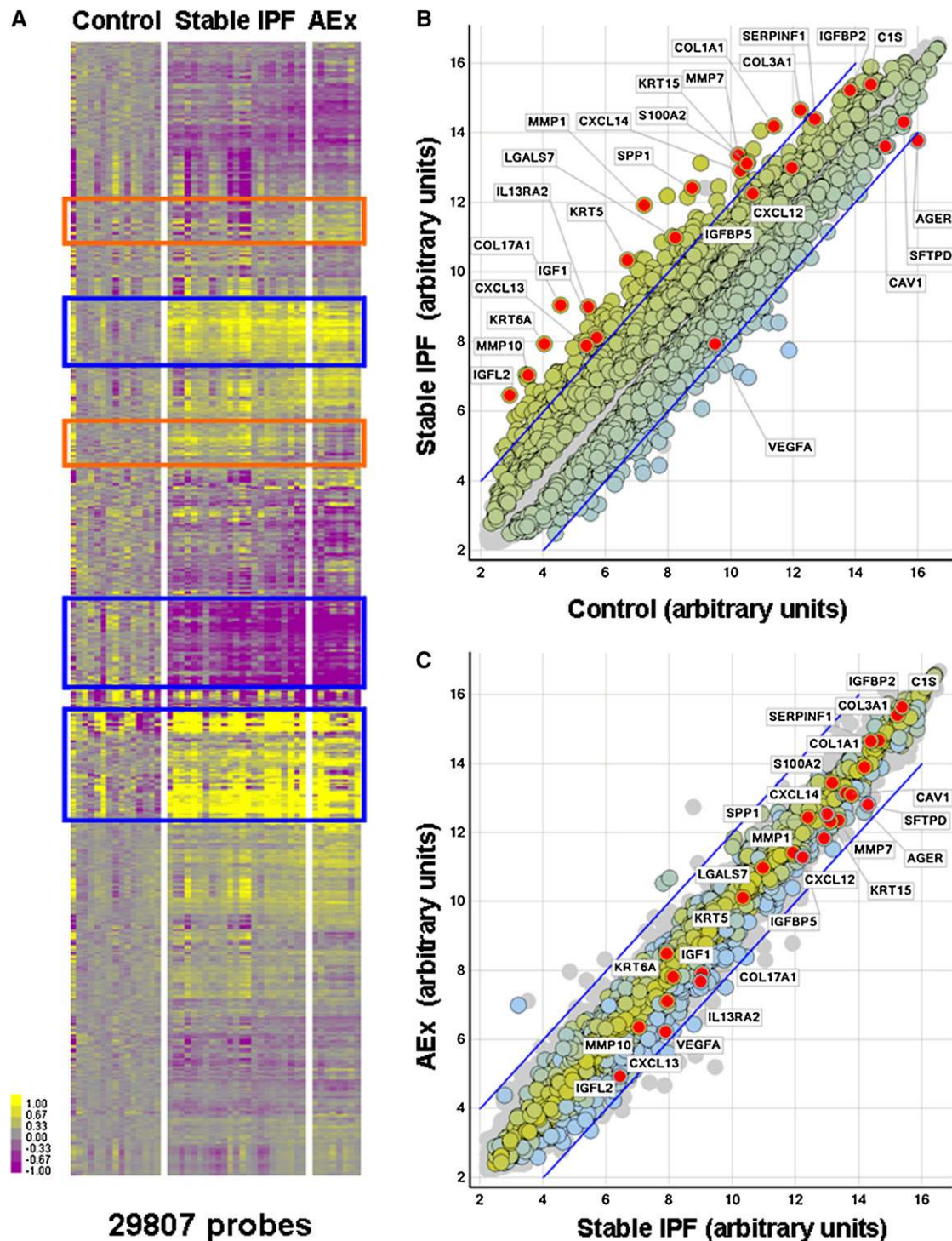
Figure 1. (*A*) When compared with control samples, gene expression signatures of acute exacerbation of idiopathic pulmonary fibrosis (IPF-AEx) and IPF are similar, as demonstrated in the heatmap. Every *row* represents a gene and every *column* a patient. *Blue rectangles* = genes expressing similar patterns in IPF and IPF-AEx; *orange rectangles* = genes expressing different patterns in IPF and IPF-AEx. (*B*) Direct comparison of gene expression between IPF and control samples. *x* axis = average gene expression in control samples; *y* axis = average gene expression in IPF. *Blue lines* indicate a twofold change between the groups. *Red circles* indicate fibrosis-related genes (all significantly different between IPF and control samples). (*C*) Direct comparison of gene expression between IPF and IPF-AEx. *x* axis = average gene expression in IPF; *y* axis = average gene expression in IPF-AEx. *Blue lines* indicate a twofold change difference between the groups. *Red circles* indicate fibrosis-related genes (all significantly different between IPF and control samples). Note that they all fall between the *blue lines* and are not differentially expressed between IPF and IPF-AEx.

seemed differentially expressed in IPF and IPF-AEx (orange quadrangles; Figure 1A), we performed a direct comparison of IPF-AEx and stable IPF. We identified 579 significantly differentially expressed genes ($q < 5$) (Figure 3A). Among them were genes related to stress response such as heat shock proteins and α-defensins and mitosis-related genes including histones and CCNA2 (Figure 3B and Table 3).

Impressively, the gene expression signature of IPF-AEx did not exhibit an increase in inflammatory response compared with stable IPF. Genes known to be associated with the general inflammatory response, adaptive or innate immunity, were not significantly enriched in genes that characterize IPF-

AEx. Similarly, only 2 (the α-defensins DEFA3 and DEFA4) of the 100 genes on the array that belong to gene ontology (GO) annotations associated with response to viral infection were significantly changed (*see* Figure E1 in the online supplement).

CCNA2 Is Overexpressed in IPF-AEx

When compared with stable IPF, CCNA2 was one of the top 20 up-regulated genes, with a *q* value of 0 and a 2.27-fold increase (Table 3). Considering that this gene is a regulator of the cell cycle we chose to validate and localize its expression. qRT-PCR confirmed the microarray data (Figures 3B and 3C), and Western

TABLE 2. GENES THAT DISTINGUISH STABLE IDIOPATHIC PULMONARY FIBROSIS (IPF) FROM CONTROL SAMPLES DO NOT SIGNIFICANTLY DISTINGUISH IPF FROM ACUTE EXACERBATION OF IPF

Probe ID	Gene Symbol	q Value Stable IPF/Control	q Value IPF-AEx/Stable	Direction Stable IPF/Control	Direction IPF-AEx/Stable
A_23_P2492	C1S	0	50.81235685	Up	Up
A_23_P119943	IGFBP2	0	65.54015605	Up	Up
A_23_P100660	SERPINF1	0	51.91200838	Up	Up
A_23_P142533	COL3A1	0	76.39075568	Up	Down
A_23_P207520	COL1A1	0	63.33144534	Up	Down
A_23_P52761	MMP7	0	22.96815416	Up	Down
A_23_P27133	KRT15	0	42.91521282	Up	Down
A_23_P201706	S100A2	0	68.1759329	Up	Up
A_23_P213745	CXCL14	0	11.47868805	Up	Down
A_23_P7313	SPP1	0	75.66331514	Up	Up
A_23_P1691	MMP1	0	63.33144534	Up	Down
A_23_P108062	LGALS7	0	70.79949555	Up	Down
A_23_P218047	KRT5	0	74.65137647	Up	Down
A_23_P85209	IL13RA2	0	4.219172327	Up	Down
A_23_P13907	IGF1	0	61.75314131	Up	Down
A_23_P501010	COL17A1	0	15.70941508	Up	Down
A_23_P87653	KRT6A	0	61.75314131	Up	Up
A_23_P13094	MMP10	0	55.98632176	Up	Up
A_23_P153571	IGFL2	0	7.018853253	Up	Down
A_23_P93360	AGER	0	42.91521282	Down	Down
A_24_P12626	CAV1	0	24.69547444	Down	Down
A_23_P70398	VEGFA	0	31.68574174	Down	Down

blots indicated an increase in CCNA2 protein (Figure 3D). To localize CCNA2 overexpression we performed double-fluorescence labeling for CCNA2 with either cytokeratin or vimentin. The double labeling demonstrated coexpression of CCNA2 with cytokeratin (Figures 4A–4C), but not with vimentin (Figures 4D–4F), indicating that the increase in CCNA2 was
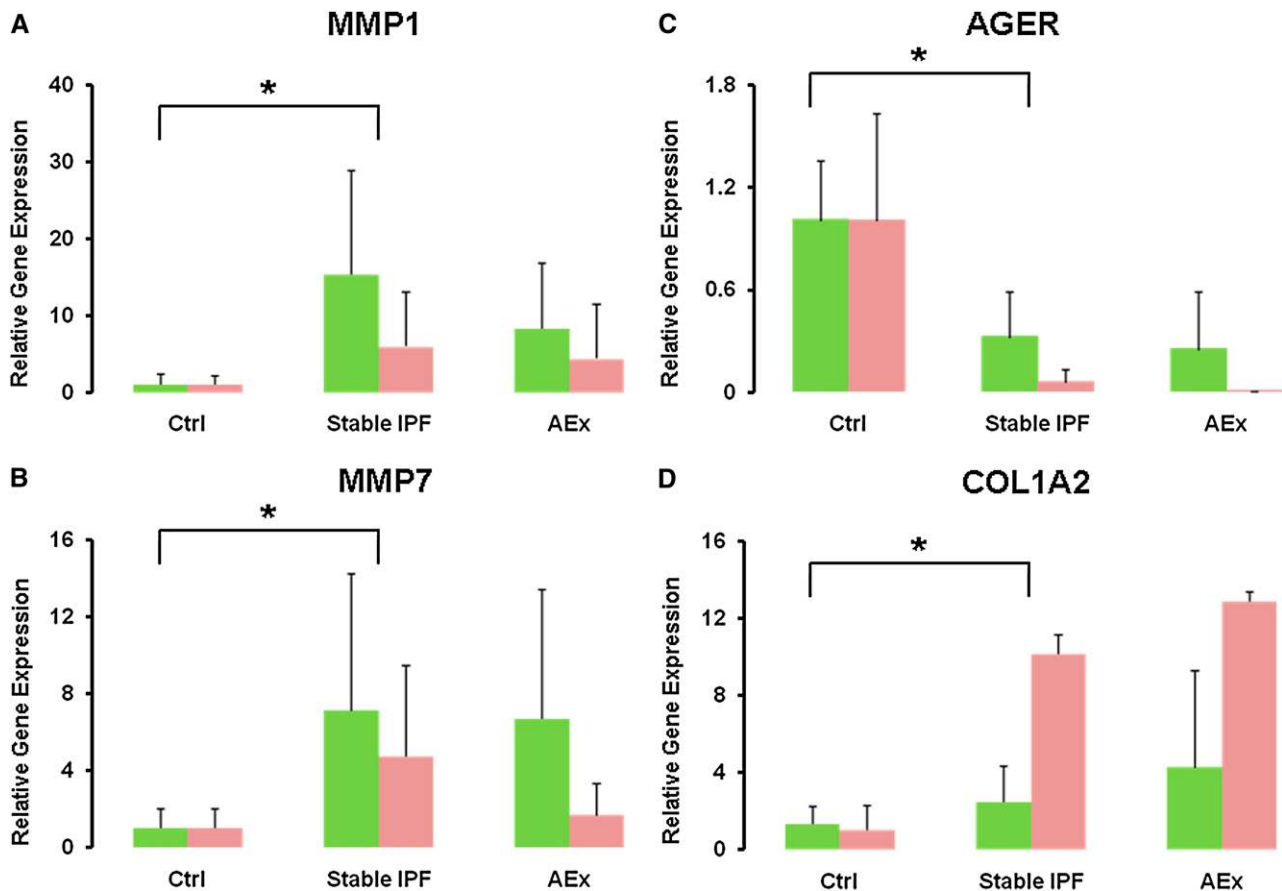

Figure 2. Relative gene expression levels of representative genes known to distinguish idiopathic pulmonary fibrosis (IPF) from control samples, and their behavior in acute exacerbation of idiopathic pulmonary fibrosis (IPF-AEx) is shown. Gene expression levels of (*A*) MMP1, (*B*) MMP7, (*C*) AGER, and (*D*) COL1A2 were measured by microarray (*green columns*) and real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (*pink columns*) (*$q < 5$ by microarray and $P < 0.05$ by qRT-PCR).
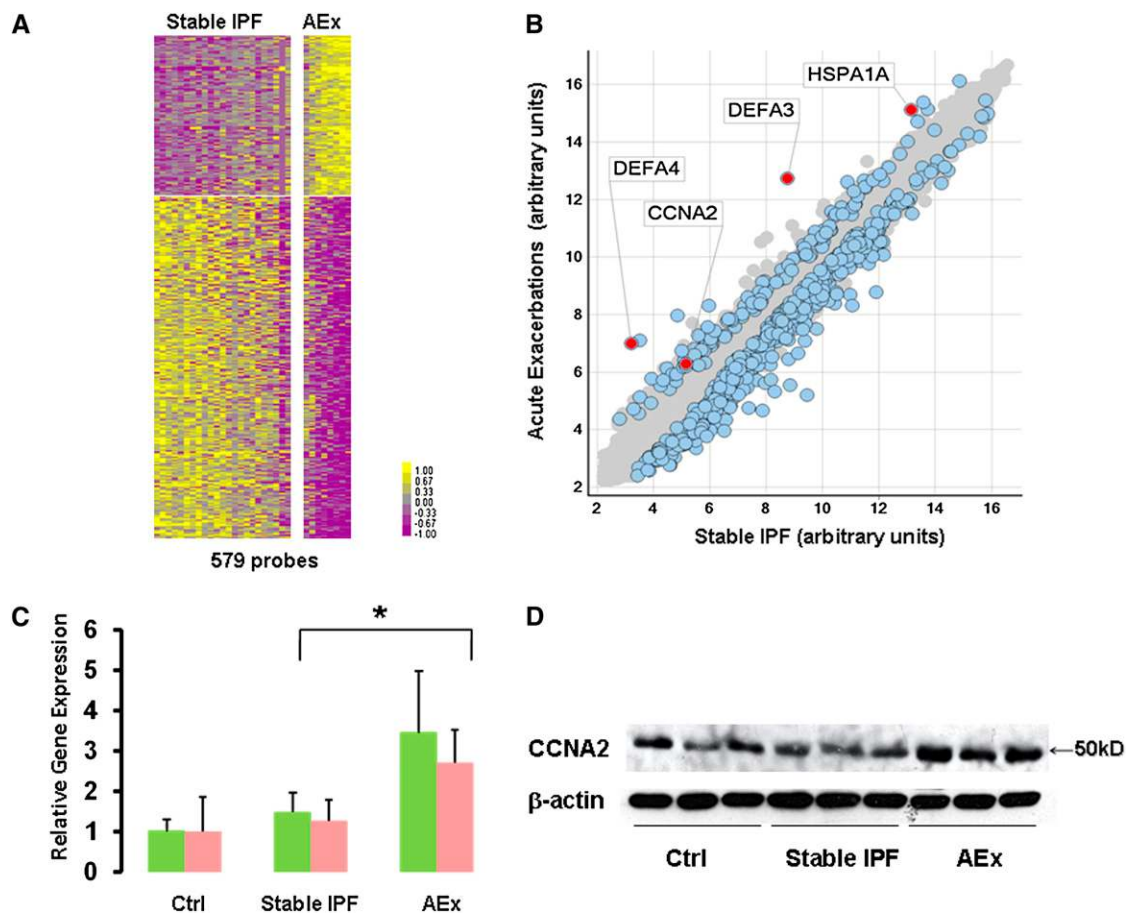
Figure 3. Signature of acute exacerbation of idiopathic pulmonary fibrosis (IPF-AEx) and validation of upregulation of CCNA2. (*A*) Direct comparison of stable IPF with IPF-AEx reveals 579 differentially expressed genes between these two groups, as shown in the heatmap. (*B*) Among them CCNA2 was one of the most up-regulated genes in the IPF-AEx group. (*C*) CCNA2 gene expression as determined by (*green columns*) microarray was confirmed by (*pink columns*) real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and (*D*) CCNA2 protein expression was determined by Western blot (*$q < 5$ for microarray and $P < 0.05$ for qRT-PCR).

localized to epithelial cells and not fibroblasts. Confocal microscopy revealed localization of CCNA2 in the alveolar epithelium as well as the basal portion of the bronchial epithelium (Figures 4G and 4H). To determine whether increases in CCNA2 were associated with cellular proliferation, we double-labeled IPF-AEx tissues with CCNA2 and Ki-67. CCNA2 and Ki-67 colocalized to the pulmonary epithelium (Figure 4I), suggesting accelerated epithelial cell proliferation, potentially as a compensatory response of the injured epithelium.

IPF-AEx Lungs Exhibit Widespread Epithelial Apoptosis

To determine whether increased epithelial proliferation was associated with epithelial cell death in IPF-AEx, we studied apoptosis in IPF-AEx tissues by *in situ* TUNEL assay. We observed significant and widespread positive epithelial TUNEL staining in IPF-AEx tissues (Figure 4K). This pattern was consistent with previous observations in IPF (30). In addition, positive TUNEL stains were also observed in the hyaline membranes typical of diffuse alveolar damage, a pathological hallmark of IPF-AEx (Figure 4J). In control lungs, rare TUNEL-positive structures were predominantly observed in apoptotic bodies engulfed by alveolar macrophages (Figure 4L).

α-Defensin Expression Is Increased in Lungs and Peripheral Blood of Patients with IPF-AEx

Gene expression of α-defensins (DEFA3 and DEFA4) was significantly increased in IPF-AEx lungs compared with stable IPF in the microarray data (Figure 3B), and the increase was confirmed by qRT-PCR (Figure 5A). To determine whether α-defensins may serve as peripheral blood markers for IPF-AEx, we analyzed their levels in the plasma of patients with IPF-AEx

from Asan Medical Center. Plasma defensin concentrations were significantly higher in patients with IPF-AEx compared with control subjects ($P = 0.0007$) or patients with stable IPF ($P = 0.025$) (Figure 5B). To determine the cellular origins of α-defensins in IPF-AEx, we performed double labeling on IPF-AEx lung sections with antibodies against α-defensins and against the alveolar type II cell marker surfactant protein C (SFTPC). These experiments identified alveolar type II cells as the source of α-defensins in IPF-AEx (Figures 5C–5E).

DISCUSSION

In this study, we used gene expression microarrays to characterize acute exacerbations of IPF. Compared with control samples, IPF and IPF-AEx lungs exhibited similar gene expression signatures. However, on direct comparison of IPF and IPF-AEx we identified differentially expressed genes and chose to focus our validation on CCNA2 and α-defensins. CCNA2, a general regulator of the cell cycle, was among the most up-regulated genes in IPF-AEx. Increased CCNA2 protein expression was localized to proliferating epithelial cells but not to mesenchymal cells. TUNEL staining was also positive and localized to the epithelium in IPF-AEx. Gene expression levels of α-defensins were up-regulated in IPF-AEx and their protein expression was localized to the alveolar epithelium in IPF-AEx. Plasma α-defensin concentrations were higher in patients with IPF-AEx compared with those with stable IPF or control subjects. Taken together, these results indicate the central role of the pulmonary epithelium in IPF-AEx and suggest a potential role for α-defensins as peripheral blood biomarkers in IPF-AEx.

TABLE 3. TOP 20 UP-REGULATED PROBES THAT DISTINGUISH ACUTE EXACERBATION OF IDIOPATHIC PULMONARY FIBROSIS (IPF-AEX) FROM STABLE IPF

Probe ID	Gene Symbol	q Value IPF-AEx/Stable	Fold Ratio AEx/Stable
A_23_P31816	DEFA3	0	15.452
A_23_P326080	DEFA4	0	13.436
A_23_P219045	HIST1H3D	0	5.0072
A_32_P221799	HIST1H2AM	0	4.1309
A_23_P20022	HIG2	0	3.3581
A_23_P74059	NPPA	0	3.3542
A_24_P123616	HSPA1A	0	3.3534
A_23_P93258	HIST1H3B	0	3.0033
A_23_P329593	SEC24A	0	2.604
A_24_P933565	PGAP1	0	2.4868
A_23_P431734	SLC25A37	0	2.4823
A_23_P368740	HDAC10	0	2.4006
A_23_P381431	NPL	0	2.3955
A_23_P3643	DNASE1L2	0	2.3867
A_23_P48803	TMOD2	0	2.3068
A_23_P428184	HIST1H2AD	0	2.3003
A_23_P111132	HSPA1A	0	2.29
A_23_P58321	CCNA2	0	2.271
A_23_P333484	HIST1H3H	0	2.1118
A_23_P30799	HIST1H3F	0	2.0654

One impressive feature of our results is the relative similarity of the gene expression patterns that distinguish IPF or IPF-AEx from control lungs. We have previously reported the up-regulation of matrix metalloproteinase-7 (MMP7), matrix metalloproteinase-1 (MMP1), collagens I and III, and osteopontin (14–21), as well as down-regulation of caveolin-1 (31) and advanced glycosylation end products-specific receptor (AGER)

(32), in IPF. All these genes behaved similarly in stable IPF and IPF-AEx, as did the majority of all other genes that distinguished IPF from control samples. We did not detect any dramatic shift in gene expression that would indicate a new process or a dramatic shift in lung cellular phenotype or content. Although we found increases in some genes associated with response to stress (HMOX1 and HSP1A1), we did not find any changes in known inflammation-related genes, such as IL-1, IL-6, tumor necrosis factor-α, or NF-κB target genes in the comparison of IPF and IPF-AEx. Interestingly, other genes increased in acute lung injury such as AGER, a known marker of generalized inflammation (33), were not increased in IPF-AEx lungs. In fact, AGER was significantly decreased in IPF-AEx compared with control samples, potentially reflecting loss of type I alveolar epithelial cells (16, 32). Taken together, these results do not support an overwhelming lung inflammatory response as a potential mechanism for acute exacerbation. We also did not find any gene expression patterns indicative of a response of the lung to viral or bacterial infections, a mechanism observed in animals (34) but not yet confirmed in human IPF-AEx (9, 35). Although our results do not rule out an occult viral infection or a previous viral infection as the triggering mechanism for IPF-AEx, neither do they support a role for an active infection during the last phase of the syndrome.

Naturally, our analysis is limited by our dependence on tissue harvested at explant or warm autopsy. It is entirely possible that by the time the patients experienced the final deterioration all evidence of response to an infection or infected tissue was destroyed. In this context the finding of increased α-defensin levels and the evidence of epithelial injury may be interpreted as remnants of an infectious process that triggered the acute lung injury but was cleared by the time the lungs were harvested.
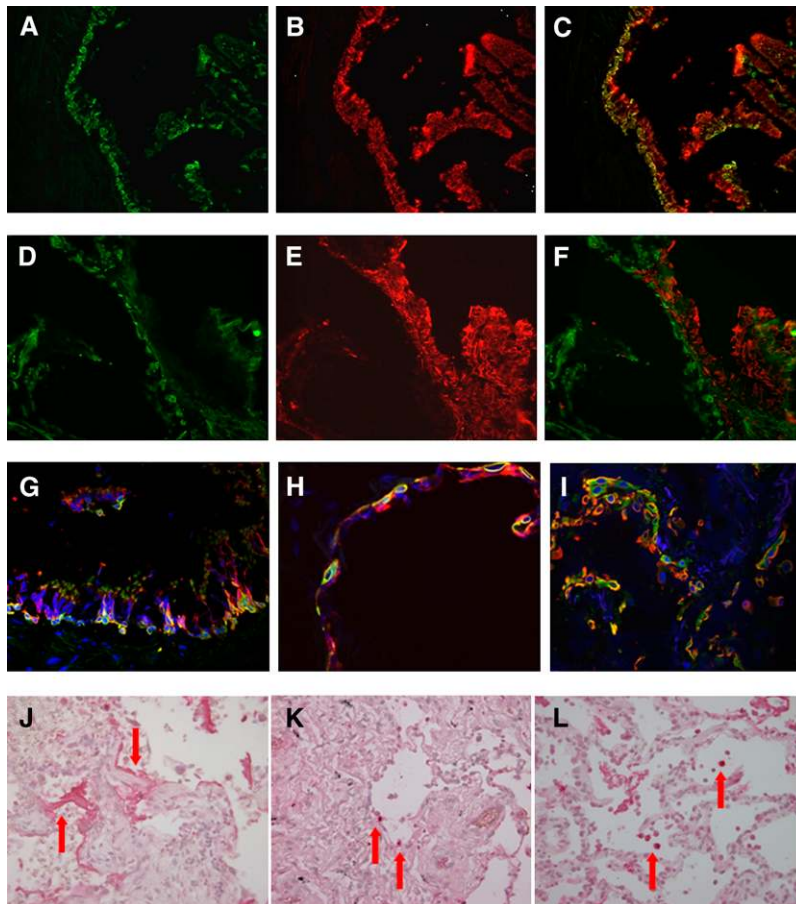

Figure 4. (*A–C*) Localization of CCNA2 in tissue obtained from patients with acute exacerbation of idiopathic pulmonary fibrosis (IPF-AEx) (original magnifications: *A*, ×20; *B*, ×20; *C*, ×20 merge): CCNA2 (*green*) is colocalized with cytokeratin (*red*). (*D–F*) CCNA2 (*green*) does not colocalize with vimentin (*red*) (original magnifications: *D*, ×20; *E*, ×20; *F*, ×20 merge). (*G* and *H*) Confocal microscopy of CCNA2 and cytokeratin. CCNA2 (*green*) and cytokeratin are colocalized in the basal portion of bronchial epithelium (*G*; original magnification, ×40). Coexpression of CCNA2 and cytokeratin is also observed in the alveolar epithelium (*H*; original magnification, ×60). Coexpression of CCNA2 (*green*) and Ki-67 (*red*), a proliferation marker, is observed in IPF-AEx tissue (*I*; original magnification, ×40). *In situ* terminal deoxynucleotidyltransferase dUTP nick end-labeling (TUNEL) reveals positive stains in hyaline membranes (*arrows* in *J*; original magnification, ×40) and alveolar epithelium (arrows in *K*; original magnification, ×40) in lung tissues with diffuse alveolar damage superimposed on the usual interstitial pneumonia pattern. In lungs with normal morphology, TUNEL-positive structures are observed predominantly in the alveolar macrophages (*arrows* in *L*; original magnification, ×40).
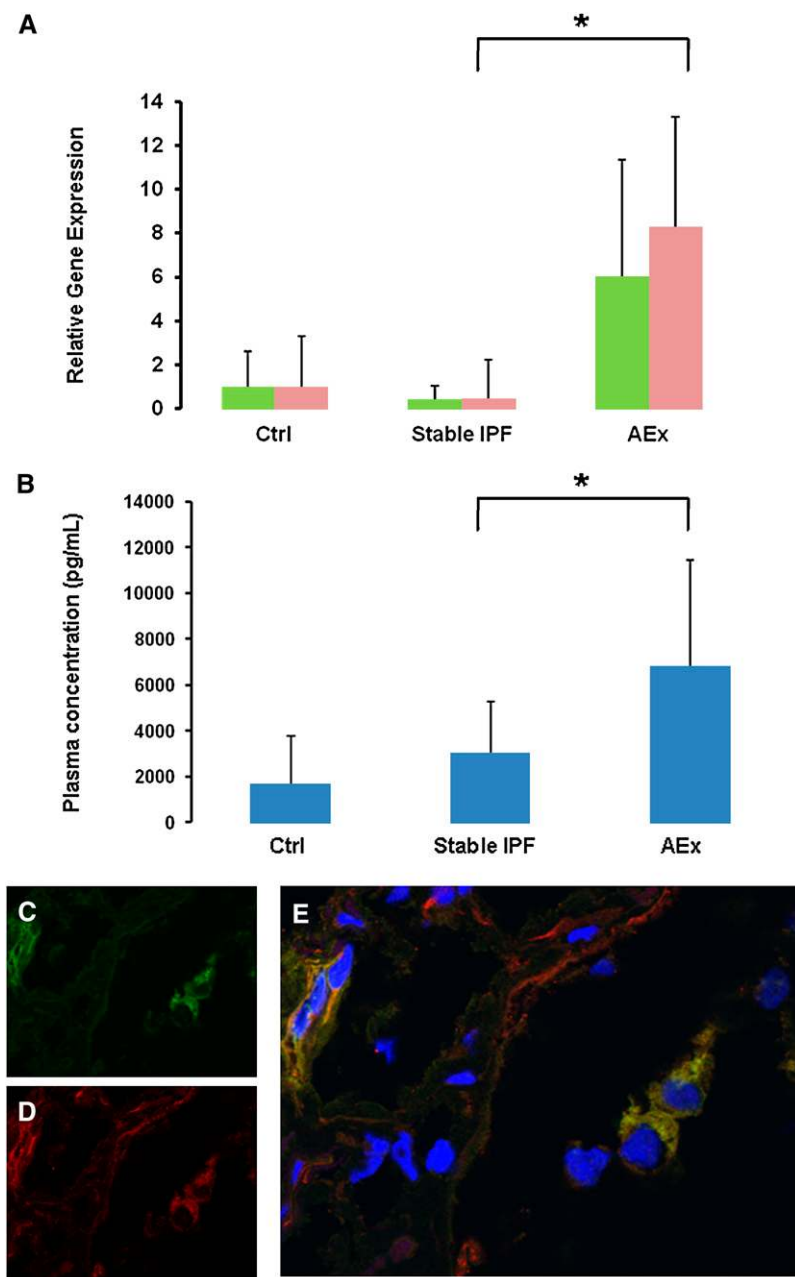
Figure 5. (*A*) Gene expression of α-defensins (DEFA1–3) observed by microarray (*green columns*) or real-time quantitative reverse transcription-polymerase chain reaction (*pink columns*). Gene expression levels of α-defensins are significantly higher in patients with acute exacerbation of idiopathic pulmonary fibrosis (IPF-AEx) compared with patients with stable IPF or control subjects. (*B*) ELISA for α-defensins in plasma obtained from control subjects, patients with stable IPF, and patients with IPF-AEx. Plasma levels of α-defensins in patients with IPF-AEx are significantly higher (*$q < 5$ for microarray and $P < 0.05$ for ELISA). (*C–E*) Histological localization of α-defensins in tissues from patients with IPF-AEx (original magnification, ×60). (*C, green*) α-Defensins are present in cells expressing surfactant protein C (*D, red* and *E*, merge), suggesting type II pneumocytes as one of the cellular sources for α-defensins.

Although we cannot disprove this interpretation, we do think that the lack of expression of viral response genes reduces the likelihood of an active infection. A definite answer regarding the role of infections will require sampling earlier at presentation and longitudinal studies of the same patient, a task impossible with lung tissue but attainable with bronchial lavage or peripheral blood samples.

One of the remarkable features of our study is the localization of increased CCNA2 expression to the alveolar epithelium, rather than to fibroblasts or myofibroblastic foci. CCNA2 is the main A-type cyclin present in somatic cells (36) and a mediator of the cell cycle. The overexpression and localization of CCNA2 to epithelial cells but not to mesenchymal cells suggests that IPF-AEx is probably an extension of the epithelial injury and dysregulation that characterizes IPF (37) and definitely is not a result of uncontrolled fibroblast proliferation. The fact that the majority of CCNA2-expressing cells were also positive for Ki-67, a proliferation marker, suggests that CCNA2 expression represents a proliferative response of the epithelium. In light of the positive TUNEL staining in the epithelium and hyaline membranes, it is tempting to hypothesize that this enhanced proliferation represents a failed compensatory response to injury, localizing the pathogenesis of IPF-AEx to the epithelium.

One use of lung gene expression data is in the identification of differentially expressed genes that encode secreted proteins. Such secreted proteins may be detected in the alveolar fluid or peripheral blood and thus be useful as potential surrogate markers for disease activity (16). Previous studies suggested that peripheral blood IL-8, KL-6, and most recently circulating fibrocytes may be increased in IPF-AEx (38–40). In our study the genes encoding α-defensins were significantly increased in IPF-AEx lungs compared with stable IPF or control samples, and their protein expression was increased in the plasma of patients with IPF-AEx. α-Defensins are innate immunity antimicrobial peptides abundant in neutrophil granules and mucosal surfaces (41, 42). α-Defensins affect various immune

functions. α-Defensins are involved in activation of the classical complement pathway (43, 44). *In vitro* α-defensins induce the production of heat shock proteins and type I collagens in human lung fibroblasts (45), and stimulate cytokine production of bronchial epithelial cells (46). Elevation of α-defensins has been described in pulmonary alveolar proteinosis (47), α_1-antitrypsin deficiency (48), acute respiratory distress (49), and chronic lung allograft rejection (50) and in patients with IPF but not in the context of acute exacerbation (51). In this context, it is important to note that we observed α-defensin expression in surfactant protein C–expressing cells in IPF-AEx lungs—a finding that suggests that the plasma increases in α-defensins may be indicative of the lung microenvironment in IPF-AEx and again highlights the central role of the epithelium in IPF-AEx.

In summary, this is the first study of lung gene expression patterns in IPF-AEx lungs. Gene expression patterns indicate that IPF-AEx represents an extension of the molecular process that underlies IPF and not a new process. Although expression patterns that distinguish stable IPF and IPF-AEx lungs from normal lung are similar, we have identified genes that are differentially expressed in a direct comparison of IPF and IPF-AEx lungs. The increased expression of CCNA2 and α-defensins is localized to the epithelium of IPF-AEx lungs, where widespread proliferation and apoptosis are detected, suggesting that the central molecular events in IPF-AEx are localized to the alveolar epithelium. Taken together, our results indicate the central role of alveolar epithelial injury in IPF-AEx and thus support the study of agents that protect the epithelium as therapeutic measures in this devastating syndrome. The identification of increases in plasma concentrations of proteins originating from the pulmonary epithelium in patients with IPF-AEx suggests their use as tools for evaluating patients with IPF during the course of the disease.

References

1. Flaherty KR, Travis WD, Colby TV, Toews GB, Kazerooni EA, Gross BH, Jain A, Strawderman RL, Flint A, Lynch JP, *et al*. Histopathologic variability in usual and nonspecific interstitial pneumonias. *Am J Respir Crit Care Med* 2001;164:1722–1727.
2. Kim DS, Collard HR, King TE Jr. Classification and natural history of the idiopathic interstitial pneumonias. *Proc Am Thorac Soc* 2006;3: 285–292.
3. Collard HR, Moore BB, Flaherty KR, Brown KK, Kaner RJ, King TE Jr, Lasky JA, Loyd JE, Noth I, Olman MA, *et al*. Acute exacerbations of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2007;176: 636–643.
4. Hyzy R, Huang S, Myers J, Flaherty K, Martinez F. Acute exacerbation of idiopathic pulmonary fibrosis. *Chest* 2007;132: 1652–1658.
5. Kubo H, Nakayama K, Yanai M, Suzuki T, Yamaya M, Watanabe M, Sasaki H. Anticoagulant therapy for idiopathic pulmonary fibrosis. *Chest* 2005;128:1475–1482.
6. Noth I, Martinez FJ. Recent advances in idiopathic pulmonary fibrosis. *Chest* 2007;132:637–650.
7. Parambil JG, Myers JL, Ryu JH. Histopathologic features and outcome of patients with acute exacerbation of idiopathic pulmonary fibrosis undergoing surgical lung biopsy. *Chest* 2005;128:3310–3315.
8. Rice AJ, Wells AU, Bouros D, du Bois RM, Hansell DM, Polychronopoulos V, Vassilakis D, Kerr JR, Evans TW, Nicholson AG. Terminal diffuse alveolar damage in relation to interstitial pneumonias: an autopsy study. *Am J Clin Pathol* 2003;119:709–714.
9. Tomioka H, Sakurai T, Hashimoto K, Iwasaki H. Acute exacerbation of idiopathic pulmonary fibrosis: role of *Chlamydophila pneumoniae* infection. *Respirology* 2007;12:700–706.
10. Yasuda H, Yamaya M, Yanai M, Ohrui T, Sasaki H. Increased blood carboxyhaemoglobin concentrations in inflammatory pulmonary diseases. *Thorax* 2002;57:779–783.
11. Kim DS, Park JH, Park BK, Lee JS, Nicholson AG, Colby T. Acute exacerbation of idiopathic pulmonary fibrosis: frequency and clinical features. *Eur Respir J* 2006;27:143–150.
12. Ambrosini V, Cancellieri A, Chilosi M, Zompatori M, Trisolini R, Saragoni L, Poletti V. Acute exacerbation of idiopathic pulmonary fibrosis: report of a series. *Eur Respir J* 2003;22:821–826.
13. Vannella KM, Moore BB. Viruses as co-factors for the initiation or exacerbation of lung fibrosis. *Fibrogenesis Tissue Repair* 2008;1:2.
14. Cosgrove GP, Brown KK, Schiemann WP, Serls AE, Parr JE, Geraci MW, Schwarz MI, Cool CD, Worthen GS. Pigment epithelium–derived factor in idiopathic pulmonary fibrosis: a role in aberrant angiogenesis. *Am J Respir Crit Care Med* 2004;170:242–251.
15. Pardo A, Gibson K, Cisneros J, Richards TJ, Yang Y, Becerril C, Yousem S, Herrera I, Ruiz V, Selman M, *et al*. Up-regulation and profibrotic role of osteopontin in human idiopathic pulmonary fibrosis. *PLoS Med* 2005;2:e251.
16. Rosas IO, Richards TJ, Konishi K, Zhang Y, Gibson K, Lokshin AE, Lindell KO, Cisneros J, Macdonald SD, Pardo A, *et al*. MMP1 and MMP7 as potential peripheral blood biomarkers in idiopathic pulmonary fibrosis. *PLoS Med* 2008;5:e93.
17. Selman M, Pardo A, Barrera L, Estrada A, Watson SR, Wilson K, Aziz N, Kaminski N, Zlotnik A. Gene expression profiles distinguish idiopathic pulmonary fibrosis from hypersensitivity pneumonitis. *Am J Respir Crit Care Med* 2006;173:188–198.
18. Selman M, Carrillo G, Estrada A, Mejia M, Becerril C, Cisneros J, Gaxiola M, Perez-Padilla R, Navarro C, Richards T, *et al*. Accelerated variant of idiopathic pulmonary fibrosis: clinical behavior and gene expression pattern. *PLoS One* 2007;2:e482.
19. Kaminski N, Allard JD, Pittet JF, Zuo F, Griffiths MJ, Morris D, Huang X, Sheppard D, Heller RA. Global analysis of gene expression in pulmonary fibrosis reveals distinct programs regulating lung inflammation and fibrosis. *Proc Natl Acad Sci USA* 2000;97: 1778–1783.
20. Zuo F, Kaminski N, Eugui E, Allard J, Yakhini Z, Ben-Dor A, Lollini L, Morris D, Kim Y, DeLustro B, *et al*. Gene expression analysis reveals matrilysin as a key regulator of pulmonary fibrosis in mice and humans. *Proc Natl Acad Sci USA* 2002;99:6292–6297.
21. Yang IV, Burch LH, Steele MP, Savov JD, Hollingsworth JW, McElvania-Tekippe E, Berman KG, Speer MC, Sporn TA, Brown KK, *et al*. Gene expression profiling of familial and sporadic interstitial pneumonia. *Am J Respir Crit Care Med* 2007;175:45–54.
22. Konishi K, Richards TJ, Collard HR, Lindell KO, Chensny LJ, Yu G, Herazo JD, Gibson KF, Kaminski N. Gene expression profiles highlight role of epithelial injury in acute exacerbations of idiopathic pulmonary fibrosis [abstract]. Presented at the 15th International Colloquium on Lung and Airway Fibrosis, September 28–October 1, 2008, Sunset Beach, NC.
23. Lindell KO, Erlen JA, Kaminski N. Lessons from our patients: development of a warm autopsy program. *PLoS Med* 2006;3: e234.
24. American Thoracic Society, European Respiratory Society. Idiopathic pulmonary fibrosis: diagnosis and treatment [international consensus statement]. *Am J Respir Crit Care Med* 2000;161:646–664.
25. Akira M, Hamada H, Sakatani M, Kobayashi C, Nishioka M, Yamamoto S. C_t findings during phase of accelerated deterioration in patients with idiopathic pulmonary fibrosis. *AJR Am J Roentgenol* 1997;168: 79–83.

26. Zahurak M, Parmigiani G, Yu W, Scharpf RB, Berman D, Schaeffer E, Shabbeer S, Cope L. Pre-processing Agilent microarray data. *BMC Bioinformatics* 2007;8:142.

27. Wu W, Dave N, Tseng GC, Richards T, Xing EP, Kaminski N. Comparison of normalization methods for codelink bioarray data. *BMC Bioinformatics* 2005;6:309.

28. Segal E, Friedman N, Kaminski N, Regev A, Koller D. From signatures to models: understanding cancer using microarrays. *Nat Genet* 2005; 37:S38–S45.

29. Dave NB, Kaminski N. Analysis of microarray experiments for pulmonary fibrosis. *Methods Mol Med* 2005;117:333–358.

30. Plataki M, Koutsopoulos AV, Darivianaki K, Delides G, Siafakas NM, Bouros D. Expression of apoptotic and antiapoptotic markers in epithelial cells in idiopathic pulmonary fibrosis. *Chest* 2005;127:266–274.

31. Wang XM, Zhang Y, Kim HP, Zhou Z, Feghali-Bostwick CA, Liu F, Ifedigbo E, Xu X, Oury TD, Kaminski N, *et al.* Caveolin-1: a critical regulator of lung fibrosis in idiopathic pulmonary fibrosis. *J Exp Med* 2006;203:2895–2906.

32. Englert JM, Hanford LE, Kaminski N, Tobolewski JM, Tan RJ, Fattman CL, Ramsgaard L, Richards TJ, Loutaev I, Nawroth PP, *et al.* A role for the receptor for advanced glycation end products in idiopathic pulmonary fibrosis. *Am J Pathol* 2008;172:583–591.

33. Uchida T, Shirasawa M, Ware LB, Kojima K, Hata Y, Makita K, Mednick G, Matthay ZA, Matthay MA. Receptor for advanced glycation end-products is a marker of type I cell injury in acute lung injury. *Am J Respir Crit Care Med* 2006;173:1008–1015.

34. McMillan TR, Moore BB, Weinberg JB, Vannella KM, Fields WB, Christensen PJ, van Dyk LF, Toews GB. Exacerbation of established pulmonary fibrosis in a murine model by gammaherpesvirus. *Am J Respir Crit Care Med* 2008;177:771–780.

35. Saydain G, Islam A, Afessa B, Ryu JH, Scott JP, Peters SG. Outcome of patients with idiopathic pulmonary fibrosis admitted to the intensive care unit. *Am J Respir Crit Care Med* 2002;166:839–842.

36. Gong D, Pomerening JR, Myers JW, Gustavsson C, Jones JT, Hahn AT, Meyer T, Ferrell JE Jr. Cyclin A2 regulates nuclear-envelope breakdown and the nuclear accumulation of cyclin B1. *Curr Biol* 2007;17:85–91.

37. Selman M, Pardo A. Role of epithelial cells in idiopathic pulmonary fibrosis: from innocent targets to serial killers. *Proc Am Thorac Soc* 2006;3:364–372.

38. Moeller A, Gilpin SE, Ask K, Cox G, Cook D, Gauldie J, Margetts PJ, Farkas L, Dobranowski J, Boylan C, *et al.* Circulating fibrocytes are an indicator for poor prognosis in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2009;179:588–594.

39. Tajima S, Oshikawa K, Tominaga S, Sugiyama Y. The increase in serum soluble ST2 protein upon acute exacerbation of idiopathic pulmonary fibrosis. *Chest* 2003;124:1206–1214.

40. Ziegenhagen MW, Zabel P, Zissel G, Schlaak M, Muller-Quernheim J. Serum level of interleukin 8 is elevated in idiopathic pulmonary fibrosis and indicates disease activity. *Am J Respir Crit Care Med* 1998;157:762–768.

41. Boman HG. Peptide antibiotics and their role in innate immunity. *Annu Rev Immunol* 1995;13:61–92.

42. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002;415:389–395.

43. van den Berg RH, Faber-Krol MC, van Wetering S, Hiemstra PS, Daha MR. Inhibition of activation of the classical pathway of complement by human neutrophil defensins. *Blood* 1998;92:3898–3903.

44. Prohaszka Z, Nemet K, Csermely P, Hudecz F, Mezo G, Fust G. Defensins purified from human granulocytes bind C1q and activate the classical complement pathway like the transmembrane glycoprotein gp41 of HIV-1. *Mol Immunol* 1997;34:809–816.

45. Yoshioka S, Mukae H, Ishii H, Kakugawa T, Ishimoto H, Sakamoto N, Fujii T, Urata Y, Kondo T, Kubota H, *et al.* α-Defensin enhances expression of hsp47 and collagen-1 in human lung fibroblasts. *Life Sci* 2007;80:1839–1845.

46. Sakamoto N, Mukae H, Fujii T, Ishii H, Yoshioka S, Kakugawa T, Sugiyama K, Mizuta Y, Kadota J, Nakazato M, *et al.* Differential effects of α- and β-defensin on cytokine production by cultured human bronchial epithelial cells. *Am J Physiol* 2005;288:L508–L513.

47. Mukae H, Ishimoto H, Yanagi S, Ishii H, Nakayama S, Ashitani J, Nakazato M, Kohno S. Elevated BALF concentrations of α- and β-defensins in patients with pulmonary alveolar proteinosis. *Respir Med* 2007;101:715–721.

48. Spencer LT, Paone G, Krein PM, Rouhani FN, Rivera-Nieves J, Brantly ML. Role of human neutrophil peptides in lung inflammation associated with α₁-antitrypsin deficiency. *Am J Physiol* 2004;286: L514–L520.

49. Ashitani J, Mukae H, Arimura Y, Sano A, Tokojima M, Nakazato M. High concentrations of α-defensins in plasma and bronchoalveolar lavage fluid of patients with acute respiratory distress syndrome. *Life Sci* 2004;75:1123–1134.

50. Nelsestuen GL, Martinez MB, Hertz MI, Savik K, Wendt CH. Proteomic identification of human neutrophil α-defensins in chronic lung allograft rejection. *Proteomics* 2005;5:1705–1713.

51. Mukae H, Iiboshi H, Nakazato M, Hiratsuka T, Tokojima M, Abe K, Ashitani J, Kadota J, Matsukura S, Kohno S. Raised plasma concentrations of α-defensins in patients with idiopathic pulmonary fibrosis. *Thorax* 2002;57:623–628.