

Original Article

Potential biomarkers of idiopathic pulmonary fibrosis discovered in serum by proteomic array analysis

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Abstract: Idiopathic pulmonary fibrosis (IPF) is a serious interstitial pneumonia leading to considerable morbidity and mortality. The unavailability of prompt biomarkers for IPF has hampered our ability to uncover preventive and therapeutic measures for this disease in a well-timed manner. The objective of this study was to identify valuable IPF associated blood biomarkers for stratifying patients and predicting outcome. By analyzing the expression of proteins in sera of 50 IPF patients and 10 healthy individuals using Biotin Label-based Antibody Array, we identified a signature of 46 differentially expressed proteins including 15 up-regulated and 31 down-regulated proteins as potential IPF biomarkers. The PPI network showed strong and complex interactions between identified biomarkers while functional enrichment analysis revealed their implications in 589 biological processes and 40 KEGG metabolic pathways. Western blotting and RT-PCR validation results corroborated with the microarray data. Our research unearthed candidate biomarkers with great potential for diagnosis of IPF and suggested that recombinant thrombopoietin and anti-CCL18 antibodies, as well as other identified biomarkers may represent a novel advance in the medical treatment of patients with IPF.

Keywords: Idiopathic pulmonary fibrosis, biomarkers, proteomic array

Introduction

Diverse responses and reactions that occur in the lung, including granulomatous disorders, exposure to environmental dusts and toxins, autoimmune diseases and drugs, generally lead to pulmonary fibrosis. The most common and severe form of pulmonary fibrosis is idiopathic pulmonary fibrosis (IPF) which is characterized by a progressive distortion of the alveolar architecture and the replacement by fibrotic tissues, resulting in a progressive dyspnea and a loss of the respiratory function [1]. On average, half of patients with IPF deacease within 3 years after diagnosis as a result of respiratory or heart failure [2, 3]. The high incidence of IPF has conducted to the search for biomarkers as an essential asset for diagnosis and prognosis of IPF patients and, more importantly, for the assessment of therapeutic interventions.

Proteomics tools allow the large-scale study of gene expression at the protein level, and thereby enables the identification of gene regulation

that are responsible for the development of specific diseases [4]. Compared to RNA-seq or microarray-based profiling of gene expression, which have been previously applied to IPF, proteomics analysis offers various advantages [5, 6]. In particular, proteomics indicates the effective existence of functional proteins in studied samples. In addition, high transcriptional level producing abundant quantity of mRNA does not imply the high amount of the corresponding protein or its effectual activity [7]. Therefore, proteomics is significantly advantageous in facilitating the discovery of specific diagnostic biomarkers and new therapeutic targets. Proteome profiler antibody arrays are suitable for screening biomarkers involved in a specific disease because they allow high-throughput and rapid detection of multiple proteins with low sample requirement [8-10]. In laboratories and clinics, antibody arrays have been successfully applied to uncover biomarkers of a variety of diseases such as cancers [11-13]. Nevertheless, to the best of our knowledge, up to date, the applica-

tion of antibody arrays to the identification of biological markers in sera of IPF patients has not been deeply assessed.

In this study, we used a RayBio® Human Biotin-labeled Antibody Array I to concurrently identify and determine concentrations of 507 serum proteins in a cohort of IPF patients and healthy individuals. The study allowed the discovery of a panel of 46 serum proteins as IPF biomarkers and indicated their promising application in personalized medicine for discriminating patients with IPF and healthy subjects.

Materials and methods

Study population

This study included 50 IPF patients recruited at Second Hospital (20 cases) and Qilu Hospital (10 cases) of Shandong University, Shandong Province Hospital (10 cases) and Qianfoshan Hospital of Shandong (10 cases). The diagnosis of IPF was based on published consensus guidelines [14] and the “Guidelines for Diagnosis and Management of Idiopathic Pulmonary Fibrosis” established by the Chinese Medicine Association and determined on the basis of high-resolution computed tomography (HRCT) or surgical lung biopsy showing a definite usual interstitial pneumonia pattern. The most common symptoms at the onset of the disease were irritating cough, shortness of breath, dyspnea, loss of endurance activities and other symptoms of dyspnea. The anomalies of pulmonary function included restriction of ventilation function and impairment of lung diffusion. The X film of the chest showed a reticular shadow in the surrounding lung area, which occurred mainly in the basal part of the lung. The main HRCT results showed double lung bottom patch or mesh like changes, with varying degrees of grinding glass-like shadow. The healthy controls (10 cases) were selected after a rigorous physical examination at the Physical Examination Center of Second Hospital of Shandong University. Written informed consent was obtained from all subjects according to the declaration of Helsinki and the study was approved by the institutional review board of Second Hospital and Qilu Hospital of Shandong University, Shandong Province Hospital, and Qianfoshan Hospital of Shandong prior to patients' enrollment.

Collection and storage of blood

From each fasting subjects, 5 mL of peripheral blood were collected into a BD Vacutainer tube without anticoagulant using standardized phlebotomy procedures. After centrifugation at 10,000 rpm for 10 minutes at 4°C, all samples were rapidly aliquoted and stored at -80°C until analysis. Only samples obtained before treatment of IPF patients were employed for proteomic analysis. All serum samples from IPF patients and healthy individual groups were randomized, and the researcher was blinded to their characteristics.

Protein expression profiling using antibody chip technology

We employed the RayBio® Human Biotin-labeled Antibody Array I (AAH-BLG-1, RayBiotech, Norcross, GA, USA), constituted of 507 different human proteins, to determine proteins differentially expressed between IPF patients and control subjects. The first step consisted in the biotinylation of the primary amine of the proteins in serum samples. Thereafter, the glass chip arrays were blocked and 400 µl biotin-labeled samples were added onto the glass chip which was preprinted with capture antibodies, and incubated at room temperature for 2 h. Subsequently, the chips were washed to remove free components. Protein chips were subsequently incubated at room temperature for 1 h with streptavidin-conjugated fluorescent dye, HiLytePlus™ 532 (Cy3 equivalent) that was purchased from AnaSpec (Freemont, CA, USA). After that, the excess of streptavidin was removed and the glass chip dried. Finally, the signals were scanned using a GenePix™ 4000B laser scanner (Axon Instruments, Sunnyvale, CA 94089, USA) coupled with GenePix® ProMicroarray Image Analysis Software which was used for image analysis.

Protein array data analysis

The data resulted from RayBio® Human Biotin-labeled Antibody Array I assay of serum samples stemming from IPF patients and healthy controls were normalized based on the positive control signal, consisting of biotin-labeled antibodies printed on each array, compared to a common reference array. Signals of adjacent spots were measured as background signals. After withdrawing background signals and nor-

IPF biomarkers

Table 1. Clinical and demographic IPF variables categorized by forced vital capacity (FVC, (%)) and diffusing capacity for carbon monoxide (D_LCO (%))

Variable	Characteristics	IPF (N=50)	Healthy control (N=10)
Predicted FVC (%)		80±10%	100%
Predicted DLCO (%)		72±8%	100%
Age (years)	Mean ± SD	64.3±10.56	66.98±8.48
	Range	41-71	49-80
Sex	Male	34	6
	Female	16	4
Smoking status	Current smokers	0	0
	Former smokers	11	0
	Never smoked	30	0
	Not reported	9	0

Table 2. Primers used in this study

Gene name	Primer
THPO	F: 5'-ATGGAGCTGACTGAATTGCTCCTCG-3'
	R: 3'-CGAGGAGCAATTCAGTCAGCTCCAT-5'
IL-17B	F: 5'-ATGGACTGGCCTCACAACCTGCTGT-3'
	R: 3'-ACAGCAGGTTGTGAGGCCAGTCCAT-5'
uPA	F: 5'-ATGGTCTCCATTTGAGAAGTAGAT-3'
	R: 3'-ATCTAGTTCTCAAATGGAAGACCAT-5'
BIK	F: 5'-ATGTCTGAAGTAAGACCCTCTCCA-3'
	R: 3'-TGGAGAGGGTCTTACTTCAGACAT-5'
TNFRSF1B	F: 5'-ATGGCGCCCGTCGCCGCTGGGCCG-3'
	R: 3'-CGGCCAGACGGCGACGGGCGCCAT-5'
CCL25	F: 5'-ATGAACCTGTGGCTCCTGGCCTGCC-3'
	R: 3'-GGCAGGCCAGGAGCCACAGGTTTCAT-5'
TGF-β3	F: 5'-ATGAAGATGCACTTGCAAAGGGCTC-3'
	R: 3'-GAGCCCTTTGCAAGTGCATTTTCAT-5'
CCL18	F: 5'-ATGAAGGGCCTTGACAGCTGCCCTCC-3'
	R: 3'-GGAGGGCAGCTGCAAGGCCCTTCAT-5'

malizing to positive controls, comparison of final signal intensities among array images was used to determine relative difference in expression levels of each protein between samples or groups. The expression levels of proteins with fold increase ≥ 1.5 -fold or fold decrease ≤ 0.65 -fold in signal intensity were considered as measurable and presenting significant difference among patients and healthy subjects.

Construction of IPF regulatory network and functional enrichment analyses

The set of differentially expressed proteins was mapped by the online Search Tool for the

Retrieval of Interacting Genes (STRING) database (<http://string-db.org>) to disclose possible connections among proteins and visualize the PPI (protein-protein interaction) network. The PPI network was constructed using the cut-off value of a confidence score >0.15 . The GO and KEGG pathway enrichment analyses of genes in the PPI network were realized to pinpoint their biological functions and pathways, based on the hypergeometric distribution algorithm. $P < 0.05$ was chosen as the cut-off value for significantly enriched functions and pathways.

Validation of the proteomic array data

Protein isolation and western blot analysis

Prior to western blotting analysis, total proteins were extracted from blood samples. Equivalent quantities of extracts (10 μ g) were electrophoresed on 10% SDS-PAGE gels followed by transfer to PVDF-Plus membranes (GE Osmonics, Trevose, PA). Western blotting was achieved with primary antibodies purchased from ABCAM including anti-thrombopoietin antibody (ab115679), anti-IL17B antibody (ab106272), Anti-uPA antibody [U-16] (ab131433), anti-Bik antibody (ab52182), anti-TNFRSF1B antibody (ab117543), anti-TECK antibody [EPR12388(2)] (CCL25) (ab2003-43) and anti-TGF-β3 antibody (ab15537) and Anti-Macrophage Inflammatory Protein 4 antibody (CCL18) [MMO142-7N58] (ab89338). Subsequently to the incubation with secondary antibodies, specific bands were visualized by using an improved chemiluminescence system (PerkinElmer Life Sciences, Boston, MA) according to the vendor's guidelines while the shareware software, ImageJ (<http://rsbweb.nih.gov/ij/>) was employed for densitometry. GAPDH was used as endogenous control.

RNA extraction and real time PCR

Total RNA was extracted from blood samples using the PAX gene Blood RNA kit (PreAnalytiX, 762164) according to the protocol provided with the kit. After quantification of total RNA by Nanodrop ND-1000 spectrophotometer (Na-

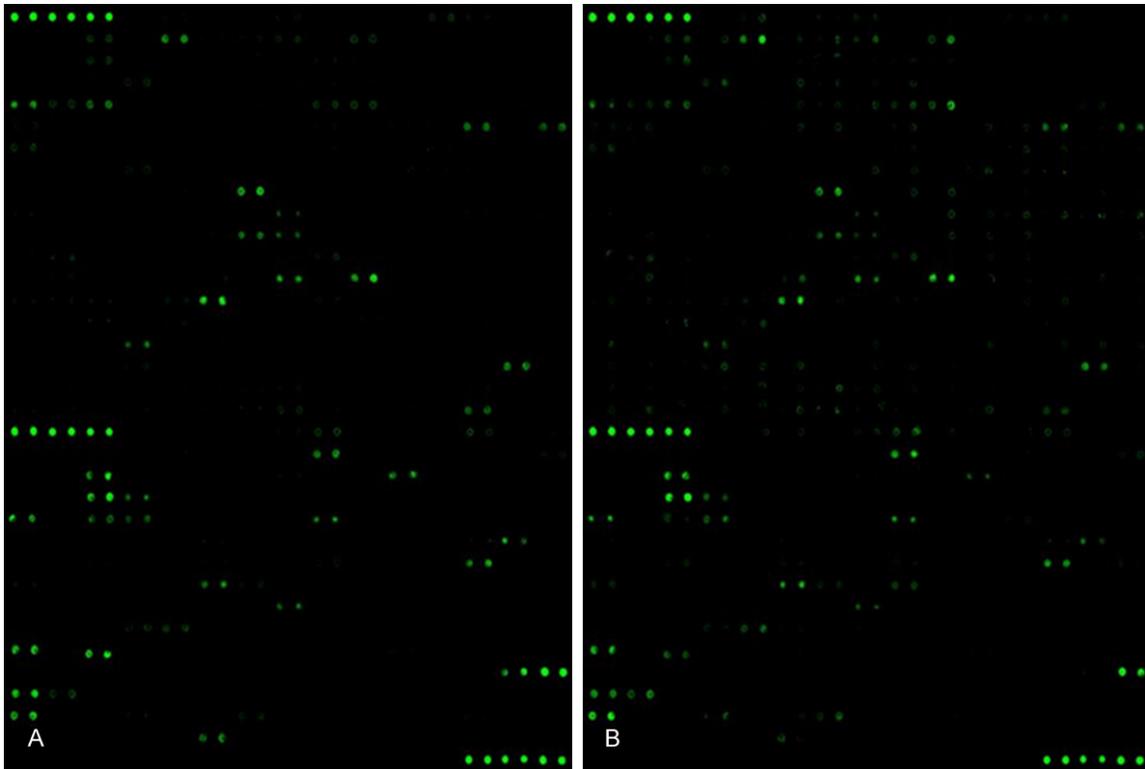


Figure 1. Representative results for 507-marker antibody arrays. Spectrum of proteins was profiled using RayBio® Human Biotin-labeled Antibody Array I from serum of normal case (A) and IPF patient (B).

noDrop Technologies, Wilmington, DE), the quality of the RNA was evaluated using the 2100 Bioanalyzer (Agilent, Palo Alto, CA). PCR was carried out using a 7900TH Fast Real-Time PCR model (Applied Biosystems, Foster City, CA, USA). PCR reactions were done in triplicate, and the threshold cycle numbers were averaged. ACTB (actin, beta) was employed as an endogenous control. Calculations of gene expression levels were performed using the $2^{-\Delta\Delta Ct}$ method. PCR primers for THPO, IL-17B, uPA, BIK, TNFRSF1B, CCL25, TGF- β 3 and β -actin were designed using the Primer Premier software version 5 and are summarized in **Table 2**. purchased from Weitonglihu procedure and the pulmonary fibrosis instillation phosphate-buffered eobnn perni-osuiiytem k histopathological examination fibrosis.

Statistical analysis

One-way ANOVA statistical analyses incorporated in GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) was used to evaluate the significance of protein expression dif-

ferences between IPF patients and healthy controls.

Results

Performance of biotin-labeled-based antibody arrays

To discover biomarkers for IPF, serum samples from 50 IPF patients and 10 healthy controls were assayed for differential expression analysis using the antibody microarray constituted of 507 proteins. Serum samples were provided by our colleagues and were collected from individuals of different age and sex (**Table 1**). After random division into 3 groups, sera from IPF patients or healthy controls within a group were mixed and respectively used to perform expression analysis. Representative antibody microarray images of sera from IPF patients and healthy controls are shown in **Figure 1**. The result demonstrated that active signal intensities, regular and different spot morphologies and high signal to noise ratios were achieved. The signal intensity reflected the expression level of specific proteins in each sample. The

IPF biomarkers

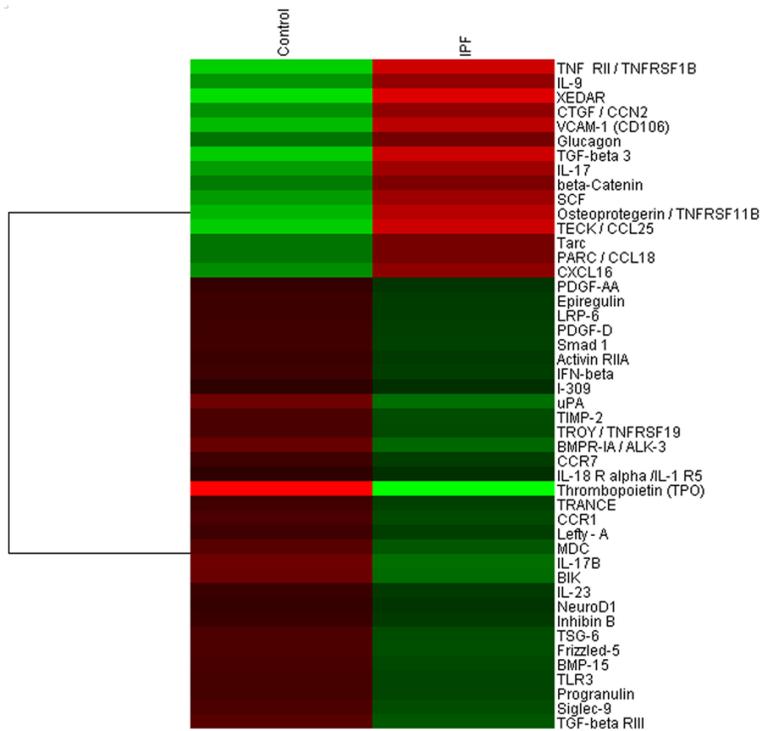


Figure 2. Heatmap showing the expression profile of the differentially expressed serum proteins.

array data was normalized to the average of positive control signal intensity of each array.

Protein expressions analysis and discovery of biomarkers in serum of IPF patients

The expression levels of all the proteins in healthy and IPF individuals were obtained after the antibody microarray analysis. After normalization and eliminating of proteins showing fold increase <2 and those with fold decrease >0.5 , we obtained proteins presenting potential interest as IPF biomarkers. **Figure 2** depicts the heatmap of the expression profiles of these proteins in IPF patients and healthy individuals. After comparing the relative protein concentrations among the two groups, we found that 15 proteins were up-regulated in the serum of IPF patients compared to that of healthy individuals. As shown in **Figure 2**, the 15 up-regulated biomarkers included CXCL16, CCL18, CCL17, CCL25, TNFRSF11B, TNFRSF1B, XEDAR, CTGF/CCN2, Glucagon, IL-9, IL-17, SCF, TGF- β 3 and VCAM-1 and β -catenin. Among these proteins, chemokines and tumor necrosis factor receptor (TNFR) superfamily members were the most predominant. Interestingly enough, values of fold-change of TNFR superfamily members

(TNFRSF11B, TNFRSF1B and XEDAR) between IPF patients and healthy controls were largely higher compared to other proteins. Proteins with high fold increase (3.53-4.93 folds) included XEDAR, TNFRSF1B, CCL25, TGF- β 3, VCAM-1 and TNFRSF11B in this order. IL-17, SCF and IL-9 displayed average fold increase values. In addition to up-regulated proteins, we equally identified 31 down-regulated proteins in the assay (**Figure 2**). Among these proteins, the expression of thrombopoietin was decreased about 12.5 fold compared to healthy subjects. The expression levels of the remaining proteins were decreased to almost the same extent.

Characteristic IPF regulatory network based on identified proteins

To further our understanding on the regulatory mechanisms of screened differentially expressed proteins and find out their potential role in IPF, we assembled down-regulated and up-regulated proteins for building a regulatory network using String software. The constructed regulatory network was depicted on **Figure 3**. The result demonstrated that the screened differentially expressed proteins form a complex regulatory network characterized by protein-protein interactions. Strong protein-protein interactions were symbolized by thick lines while tiny line denoted low interactions (**Figure 3**). As shown in **Figure 3**, four cores of robust interactions were identified. The first one was recorded among chemokines and was orchestrated by CCR1 and CCR7 while the second one was governed by IL-17A, the third one by TGF- β 3 and the fourth one predominantly controlled by CTNNB1.

GO functional enrichment analysis

GO enrichment analysis revealed that differentially down- and up-regulated proteins were involved in 589 significant functional terms (p -value <0.05) in the category of "biological process", 18 in the category of "molecular fun-

IPF biomarkers

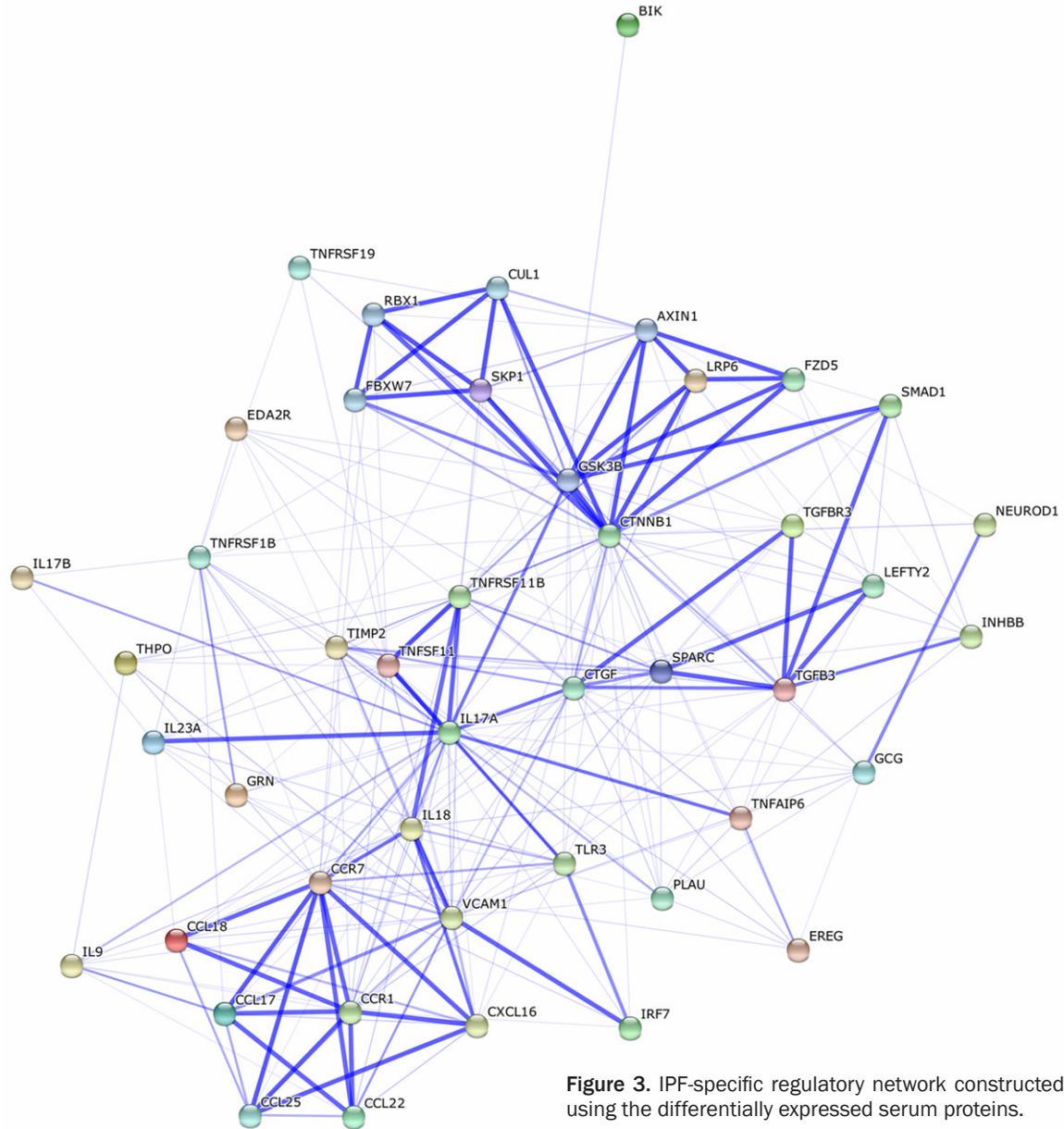


Figure 3. IPF-specific regulatory network constructed using the differentially expressed serum proteins.

ction” and 25 in the category of “cellular component”. The functional terms in each category, ranked by statistical significance, were summarized in [Supplementary Table 1](#). As presented in **Figure 4**, the top significantly enriched GO terms in the category of “biological process” included positive regulation of multicellular organismal process (14 proteins), inflammatory response (10 proteins), positive regulation of cytokine production (9 proteins), regulation of cytokine production (10 proteins), cell-cell signaling (12 proteins) and regulation of MAPK cascade (10 proteins). For “molecular function”, growth factor activity (7 proteins), cytokine acti-

city (7 proteins), receptor binding (2 proteins), C-C chemokine binding (2 proteins), Wnt-activated receptor activity (2 proteins), chemokine binding (2 proteins) and CCR chemokine receptor binding (2 proteins) were the most enriched terms. Extracellular space [15] and extracellular region part [14] were the most significantly enriched terms in the category of “cellular components”.

KEGG pathway enrichment analysis

In order to identify metabolic pathways in which participate the differentially expressed pro-

IPF biomarkers

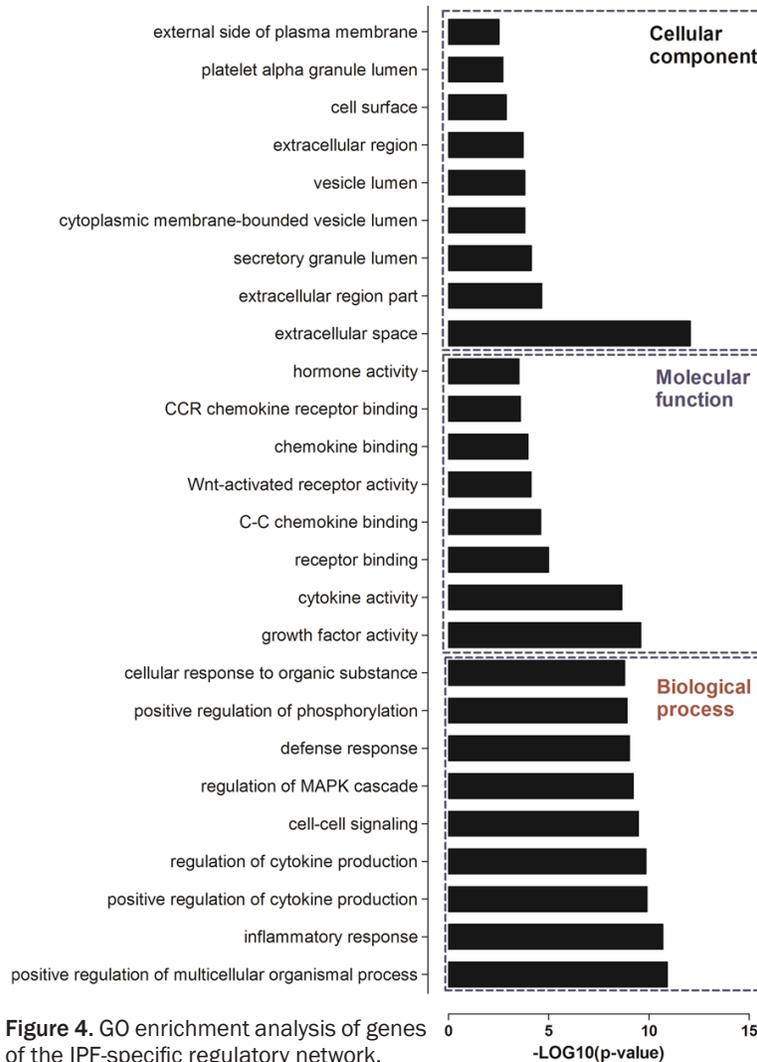


Figure 4. GO enrichment analysis of genes of the IPF-specific regulatory network.

teins, we proceeded to the KEGG enrichment analysis using the String database. The whole set of proteins involved in the regulatory network was significantly (p -value < 0.05) enriched for 40 metabolic pathways (Supplementary Table 2). The 21 most significant pathways were presented in Figure 5. The result showed that the cytokine-cytokine receptor

interaction (19 proteins) was the topmost enriched pathway. In addition, TGF- β signaling pathway (7 proteins), Wnt signaling pathway (8 proteins), chemokine signaling pathway (8 proteins) and Hippo signaling pathway (7 proteins) were found as significant pathways following cytokine-cytokine receptor interaction. Toll-like receptor signaling pathway, TNF signaling pathway and Jak-STAT signaling pathway were also significantly enriched. Up-regulated proteins

were mostly partners of cytokine-cytokine receptor interaction and Chemokine signaling pathway.

Western blotting and real-time PCR validation

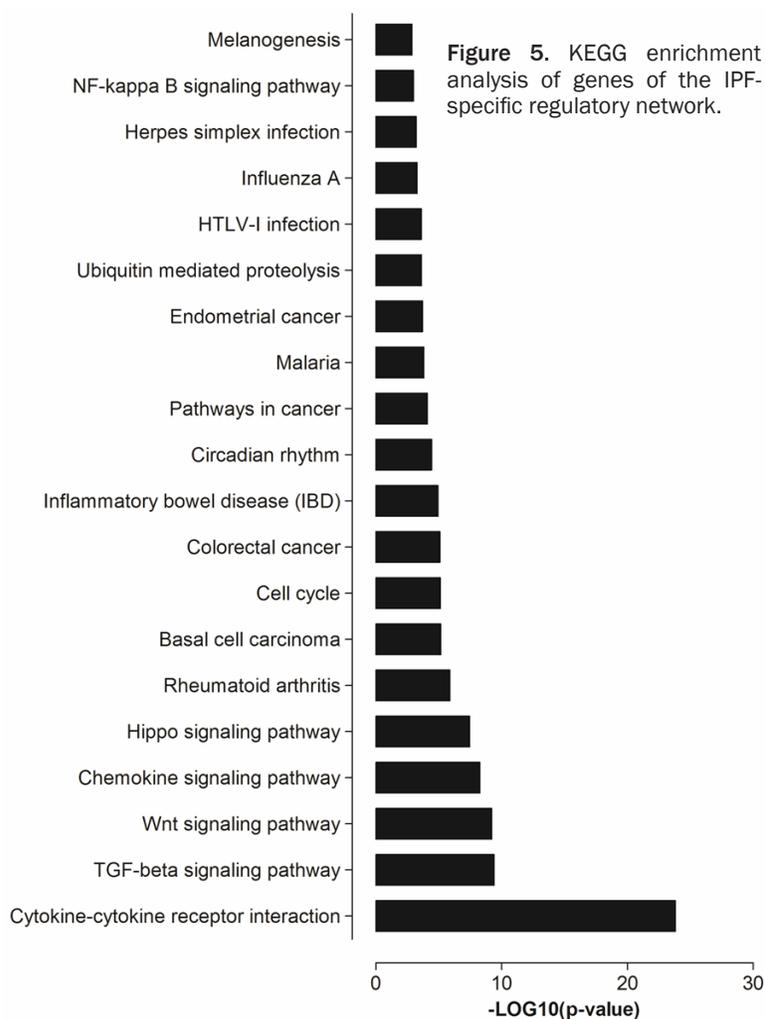
Western blotting and Real-Time PCR validation were performed to validate the antibody microarray profiling results using serum samples collected from IPF patients and healthy controls. All the proteins tested were validated by Real-Time PCR and Western blotting validation as depicted in Figure 6 and presented the same trends as in the microarray data.

Discussion

IPF is a progressive and irreversible fibrosing lung disease with unknown etiology and unfavorable outcome, leading ultimately to death due to respiratory failure [1, 3]. Although the pathophysiology underlying this disease has not been fully elucidated, animal models of pulmonary fibrosis have tremendously contributed in dissecting the

molecular basis of this disease with a particular emphasis on cytokines as important pathogenic mediators of lung fibrosis [15-17]. In this study, the comparative analysis of expression levels of proteins in sera of IPF patients and healthy controls using antibody-based microarrays allowed the identification of forty-six differentially expressed proteins. Remarkably, chemokines and members of the TNFR superfamily were the most abundant in terms of number and expression levels. This result was in accordance with previous findings which reported the role of these molecules in the development of fibrosis [18-20]. Chemokines constitute a crucial group of cytokines that has the potential to regulate cell recruitment, the amplification and polarization of the immune response and vascular remodeling [21, 22]. Chemokines, by

IPF biomarkers



mobilizing mononuclear cells which can impact on the epithelial-myofibroblast axis, play a key role in IPF pathogenesis. Therefore, the disequilibrium of the expression of chemokines seems to play important functions in fibroproliferative disorders in the lung. Recently, it has been reported that CXCL16 is induced in renal tubular epithelial cells in response to angiotensin II and plays a pivotal role in the pathogenesis of angiotensin II-induced renal injury and fibrosis by controlling the intrusion of macrophage and T cell accumulation in bone marrow-derived fibroblast [23]. CCL17 has been found in the epithelium of both the bleomycin model and human IPF lung tissues, suggesting that CCL17 plays an important function in the epithelium during the pathogenesis of pulmonary fibrosis [24]. Similarly, CCL18, one of the most promising biomarkers for IPF (independent predictor of outcome, overexpressed in serum and

BAL of IPF patients) was found up-regulated in this study and was consistent with the previous published studies [25-27]. CCL25 is chemotactic for lymphocytes, dendritic cells (DCs), and activated macrophages [28]. It was found to contribute to liver fibrosis by directly targeting HSCs in the injured liver and recruiting CCR9 positive macrophages [29]. In our array, the expression of CCL25 increased highly in IPF patient (fold change =4.228) indicating its potential application in IPF diagnosis.

The beta-Catenin pathway is down-regulated in emphysema but up-regulated in IPF, as confirmed by several studies [30, 31]. In the present study, β -catenin was up-regulated in IPF and further confirmed its implication in the pathogenesis of this disease.

As expected, numerous proteins involved in the extracellular space and extracellular region part were the most significantly represented in the

category of “cellular components” as revealed by GO functional enrichment, showing deregulations of genes implicated in ECM. The pathophysiology of fibrosis remains an enigma, but considerable researches have highlighted inflammatory response as the key driver of unrestrained wound healing following fibrosis in numerous organ systems [32, 33]. The common paradigm hypothesizes that an initial alveolar injury causes an inflammatory response to subsequently cause fibrosis [34]. The GO enrichment of differentially expressed proteins was in accordance with the above statement since inflammatory response, positive regulation of cytokine production and regulation of cytokine production were among the most represented proteins.

Lung fibrosis is linked with abnormal expression of TGF- β receptors, increased activity of

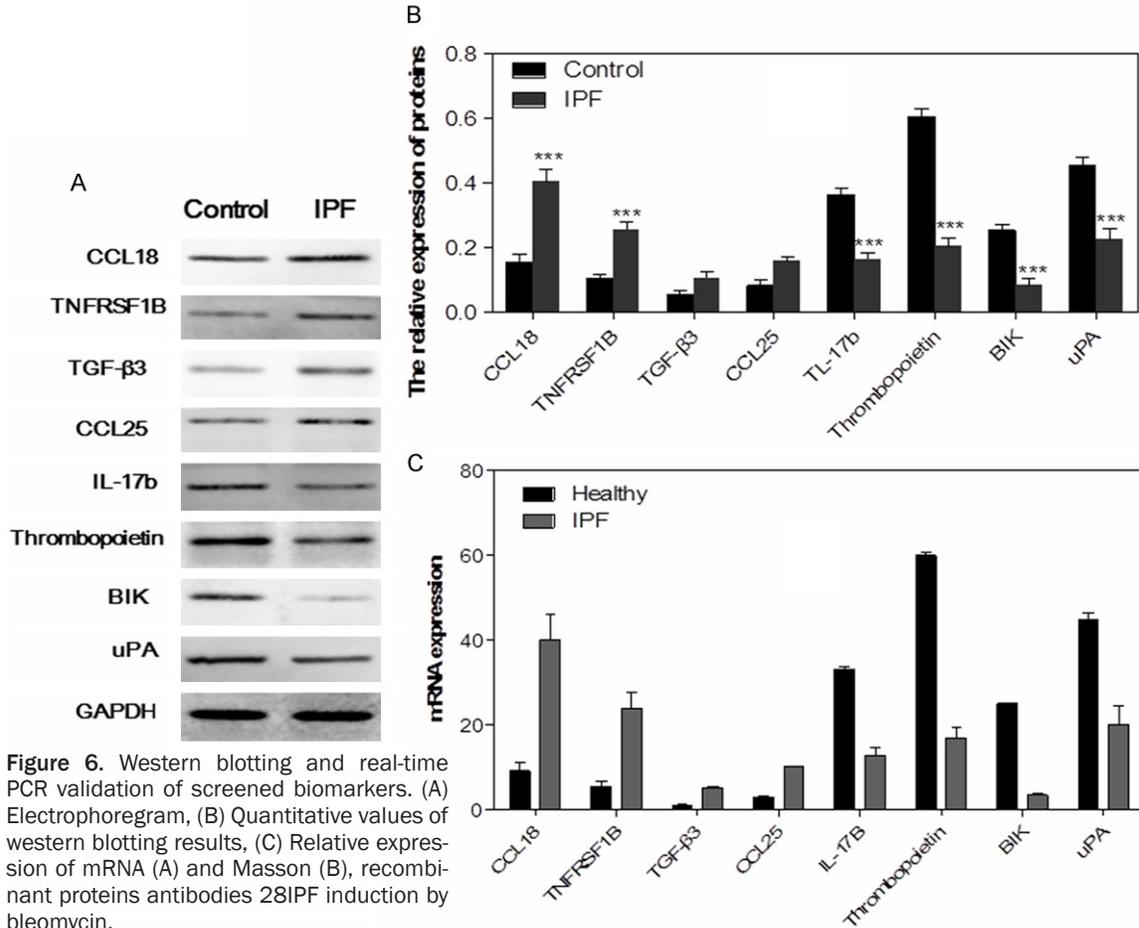


Figure 6. Western blotting and real-time PCR validation of screened biomarkers. (A) Electrophoregram, (B) Quantitative values of western blotting results, (C) Relative expression of mRNA (A) and Masson (B), recombinant proteins antibodies 28IPF induction by bleomycin.

TGF-β-regulated proteins especially TGF-β1 signaling pathway [35]. In this study, we found up-regulation of TGF-β3 and down-regulation of the type III TGF-β receptor (TGF-β RIII) showing the implication of TGF-β signaling pathway in IPF. CTGF, a major profibrotic growth factor was also found among the most downregulated proteins in patients with IPF compared to controls.

Although our antibody microarray detected some of biomarkers previously reported in IPF, the data obtained here contains a substantial number of unidentified biomarkers that could potentially contribute in the diagnosis and treatment of IPF. For example, we found that THPO was the most down-regulated protein while XEDAR was the most overexpressed. These proteins have not been reported previously as IPF biomarkers. The regulatory network constructed in the present study demonstrated robust interactions between chemokines. In addition, functional analyses revealed

enrichment for cytokine-cytokine receptor interaction and chemokine related activities. No strong interaction was recorded for the highly increased XEDAR protein or the extremely decreased thrombopoietin suggesting that these proteins might work independently or interact with other proteins that were not identified in this study. The strong interactions between some of differentially expressed protein indicated their validity as useful molecular biomarkers for IPF. This observation also suggests that IPF is a result of conjugated actions of genes or proteins working independently or in complex interaction networks.

The antibody microarray data was further validated using western blotting and RT-PCR. The validation experimental results were in conformity with the antibody microarray. In summary, in this study we identify proteomic signatures that characterize IPF using the antibody microarray. Protein-protein network and GO and KEGG enrichment analyses identified several

biological processes and metabolic pathways, yielding new insight into mechanisms involved in fibrosis development. Among the 46 differentially expressed proteins discovered, the majority can prove interesting in the future as real diagnostic and prognostic biomarkers, but further investigations are required for the evaluation and validation of their medical utility.

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Disclosure of conflict of interest

None.

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