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Application of Isobaric Tags for Relative and Absolute Quantification (iTRAQ) Coupled with Two-Dimensional Liquid Chromatography/ Tandem Mass Spectrometry in Quantitative Proteomic Analysis for Discovery of Serum Biomarkers for Idiopathic Pulmonary Fibrosis

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The present study was performed to explore the presence of informative protein biomarkers of human serum proteome in idiopathic pulmonary fibrosis (IPF).

Material/Methods: Serum samples were profiled using iTRAQ coupled with two-dimensional liquid chromatography/tandem mass spectrometry (2D-LC-MS/MS) technique, and ELISA was used to validate candidate biomarkers.

Results: A total of 394 proteins were identified and 97 proteins were associated with IPF. Four biomarker candidates generated from iTRAQ experiments – CRP, fibrinogen-α chain, haptoglobin, and kininogen-1 – were successfully verified using ELISA.

Conclusions: The present study demonstrates that levels of CRP and fibrinogen-α are higher and levels of haptoglobin and kininogen-1 are lower in patients with IPF compared to levels in healthy controls. We found they are useful candidate biomarkers for IPF.

MeSH Keywords: Biological Markers • Idiopathic Pulmonary Fibrosis • Proteomics

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Background

Idiopathic pulmonary fibrosis (IPF), a progressive lethal interstitial lung disease of unknown etiology, primarily affects older adults, leading to a progressive decline in lung function and quality of life [1–4]. IPF is characterized by progressive scarring of the lung parenchyma, which leads to dyspnea and eventual respiratory failure. However, there are no established drug therapies because suitable symptoms for initiating therapy, best candidates for treatment, and conceivable role for combinatory therapies are still controversial [5,6]. Despite recently approved medical therapies, IPF has a very poor prognosis, with most patients dying within 5 years of diagnosis [7–11]. Early diagnosis of IPF is difficult. High-resolution computed tomography (HRCT), surgical lung biopsy, and multi-disciplinary team assessment play important roles in the diagnosis of IPF [12]. However, patients with atypical HRCT features require a surgical lung biopsy [13]. The availability of novel effective therapies [14], coupled with the limitations of traditional therapeutic combinations [15–17], have increased the urgency of accurate diagnosis of IPF. It is thus not surprising that great effort has been made to identify methods and biomarkers for reliable prognosis, and to support appropriate treatment regimens [18].

Biomarkers for IPF are urgently needed as predictors of the progression and treatment response of the disease [19]. Molecular markers from circulating (e.g., blood) or lung-specific samples have been used to improve diagnostic and prognostic accuracy. Recent studies have identified several specific genetic variants that confer risks for the development of IPF [20,21]. Most of the studied biomarkers are used as disease prognosticators rather than disease-specific diagnostic tools [22]. Accurate and timely diagnosis is imperative.

Proteomics, which is defined as a large-scale characterization of proteins expressed by a genome [23], can be used to identify biomarkers and reveal disease-specific mechanisms [24]. Quantitative proteomics, as an important branch of proteomics, has been used to identify and quantify all the proteins expressed by a whole genome or in a complex mixture [25]. A recently developed proteomics named isobaric tags for relative and absolute quantification (iTRAQ) is a quantitative proteomic approach with relatively high throughput. It allows for simultaneous identification and quantification of peptides by measuring peak intensities of reporter ions with tandem mass spectroscopy (MS/MS), and has been developed and utilized to identify biomarkers for various disease conditions [26,27]. Following the digestion of samples with trypsin, peptides are labeled with iTRAQ reagents (4-Plex or 8-Plex), fractionated, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). iTRAQ is a technique that allows for a comprehensive, comparative, and quantitative determination of protein expression [28]. Moreover, this technique is a powerful tool with which to relatively and absolutely quantify proteins, and has been extensively applied to proteome analysis since its invention [28–32]. The iTRAQ method places tags on primary amines (NH2-terminal or ε-amino group of lysine side-chain), allowing detection of most tryptic peptides [33]. This method has been successfully applied to biomarker screening for multiple tumors and diseases in both tissue and serum samples [34]. In the present study, iTRAQ-based quantitative proteomics analysis was used to investigate the proteomic profiles and potential biomarkers for IPF.

Material and Methods

Patients

Serum samples were collected from healthy volunteers and patients at the Department of Respiratory Medicine of the Second Hospital of Shandong University between October 2013 and October 2014. The study included a total of 20 healthy volunteers and 20 patients with IPF. The diagnosis of IPF was made according to the standards accepted by ATS/ERS/JRS/ALAT [1]. Healthy subjects were matched with patients for age and sex. Demographic details of the participants were recorded (Table 1). Peripheral blood was collected, allowed to coagulate for 30

Table 1. Characteristics of the IPF patients and healthy controls.

Groups	Samples	No.	Gender	Mean age (years)	Age range (years)	Predicted FVC (%)	Predicted DLCO (%)
1	Controls	7	Male	57.86±3.700	40–65	100	100
	IPF patients	7	Male	60.00±2.498	46–64	66.88±17.82	54.42±19.58
2	Controls	6	Female	62.00±6.250	41–77	100	100
	IPF patients	6	Female	63.00±8.202	37–87	66.92±17.76	54.38±19.62
3	Controls	7	Male	57.43±2.599	50–68	100	100
	IPF patients	7	Male	61.71±1.507	56–67	66.87±17.83	54.41±19.59

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min, and centrifuged at 1200×g for 10 min at 4°C to separate serum, which was subsequently stored at –80°C in 100-µL aliquots. All serum samples were collected before any treatment. The present study was approved by the Ethics Committee of Shandong University. Signed informed consent was obtained from all participants.

Protein extraction, quantification, and digestion

Serum protein was extracted using the ProteoPrep Blue Albumin and IgG Depletion Kit (Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations were determined using a Bradford protein assay kit (Pierce; Thermo-Fisher Scientific, Waltham, MA, USA) and measured by a spectrophotometer (765Pc; Spectrum Shanghai, Shanghai, China). All measurements were performed in duplicate.

The proteins were typically digested by sequence-grade modified trypsin (Promega, Fitchburg, WI, USA), and then the resultant peptides mixture was further labeled using chemicals from the iTRAQ reagent kit (AB Sciex, Concord, Ontario, Canada).

iTRAQ analysis

Proteins from each cell line were subjected to iTRAQ labeling according to the manufacturer's protocol (Applied Biosystems; Thermo-Fisher Scientific, Waltham, MA, USA). Briefly, 200 µg of protein was reduced using 4 µL of reducing reagent at 60°C for 1 h. Subsequently, 2 µL of cysteine-blocking reagent was added for cysteine blocking before incubation at room temperature for 10 min. The reduced and alkylated protein samples were transferred to ultrafiltration tubes and centrifuged at 12 000×g for 20 min at room temperature. Protein from each sample was mixed with 4 µg trypsin (1: 50, w/w) and incubated overnight for digestion at 37°C. After completion of trypsin digestion, protein peptide samples were collected for iTRAQ labeling. Isopropanol was used to dissolve each iTRAQ reagent, and the content in 1 sample tube was transferred to 1 iTRAQ reagent vial. Then, 100 µg digestion products were incubated with iTRAQ reagents for 2 h at room temperature. H_2O (100 µL) was used as the stop solution to terminate the reaction. After iTRAQ labeling, the samples were mixed together and lyophilized via centrifugal evaporation.

Liquid chromatography(LC)-MS/MS analysis

Reversed-phase liquid chromatography (RPLC)-MS was conducted using the Dionex Ultimate 3000 RSLCnano system coupled to a Q Exactive mass spectrometer (Thermo-Fisher Scientific, Waltham, MA, USA). The peptide mixtures were loaded onto an Acclaim PepMap RSLC C18 column (2 µm particle size, 15 cm length, 75 µm diameter, 100 Å pore size) and separated using buffer A (0.1% formic acid) for 15 min at a flow rate of 2 µL/min. Peptides were eluted at 350 nL/min. The elution process was done with buffer B' (80% acetonitrile, 0.1% formic acid) as follows: 0–5 min, 4%B; 5–45 min, 50%B; 45–50 min, 90%B; 50–65 min, 4%B. The scan range of the mass detector was set to 350–1800 m/z.

Database search and proteomic data analysis

ProteinPilot Software 5.0 (AB Sciex, Concord, Ontario, Canada) was used to search the database HUMAN_uniProt to identify the differentially expressed proteins. Gene Ontology (GO) annotations (*http://www.geneontology.org*) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (*http://www.kegg.jp/kegg/pathway.html*) were conducted to analyze differentially abundant proteins, functional classifications, or signal transduction pathways.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to verify the result of iTRAQ. For the detection of serum proteins, ELISA kits were used following the manufacturer's protocols. Human C-reactive protein (ab99995), fibrinogen (ab108841), haptoglobin (ab108856), and kininogen-1 (ab108875) ELISA kits were purchased from Abcam Company (Cambridge, MA, USA).

Statistical analysis

Statistical analysis was performed by nonparametric Kruskal-Wallis test in the 3 groups. Statistical significance was evaluated using the *t* test in GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). False discovery rate (FDR) was assessed in relation to *p*-values from the Kruskal-Wallis test and the average number of spots (1000) was matched in every gel. *P*-values of less than 0.05 were considered statistically significant differences. All the tests were conducted in triplicate.

Results

The iTRAQ proteomic analysis and ELISA concurrently show that 97 serum proteins are differentially expressed in IPF patients compared with healthy controls

To understand physiological differences between IPF patients and healthy controls, protein was extracted from serum samples and detected through iTRAQ. The iTRAQ analysis of IPF samples was summarized in a flow chart (Figure 1). A protein species was considered differentially expressed if its fold change was ≥1.5 or ≤0.67 and $P<0.05$. According to iTRAQ report, a total of 97 species of serum proteins were differentially expressed in the serum of IPF patients compared with that of normal controls, including 38 proteins that were up-regulated and 59

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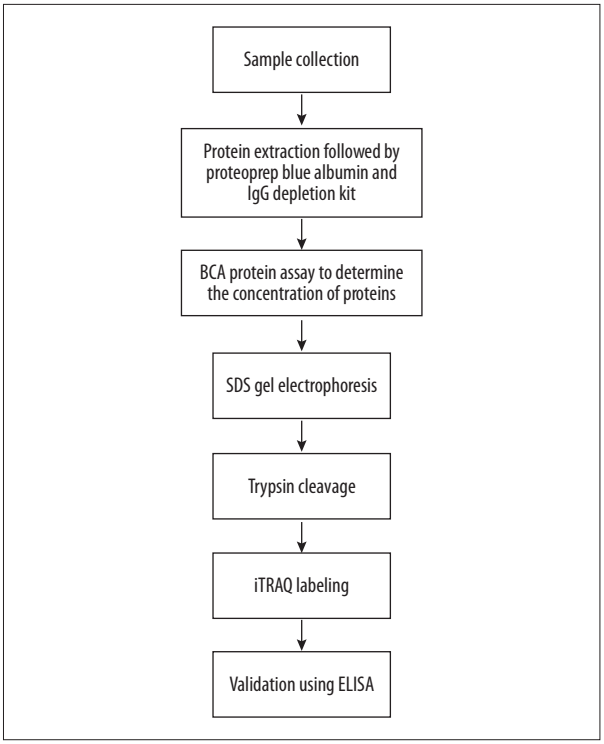

Figure 1. Flow chart of iTRAQ experimental approach

proteins that were down-regulated (Figure 2). Subsequently, 4 species of proteins were chosen to confirm the iTRAQ results by ELISA. The ELISA data show that the expression of these proteins, such as C-reactive protein (CRP), fibrinogen alpha chain (FIBA), haptoglobin, and kininogen-1, were consistent with the report conclusions (Figure 3). The results suggest that iTRAQ proteomic analysis shows that 97 serum proteins are differentially expressed in IPF patients compared with healthy controls.

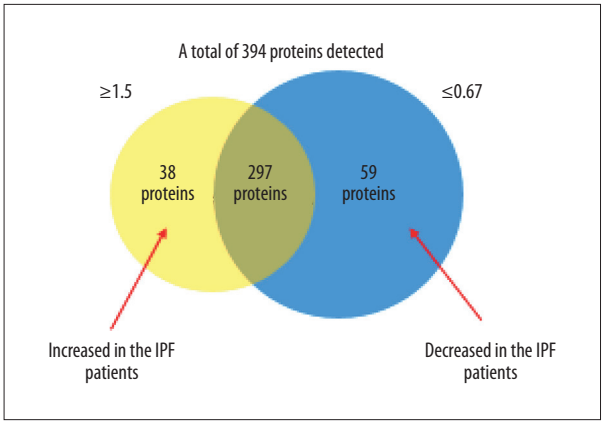

Figure 2. Number of proteins that were increased and decreased in IPF patients compared with controls.

Bioinformatics analysis and identification of differentially accumulated protein species by iTRAQ

To further examine the 97 differentially expressed proteins between IPF patients and controls, Gene Ontology (GO) annotation was carried out. The 97 proteins were classified into 38 functional groups, of which cellular components accounted for 24 GO terms (the most representative terms were "extracellular region"), biological processes accounted for 55 GO terms (the most representative terms were "response to stress"), and molecular functions accounted for 21 GO terms (the most representative terms were "ion binding"). The 10 most significantly enriched GO terms and their percentages in the enrichment results of 3 kinds of basic categories are shown in Figure 4. Metabolic pathways and biochemical signal transduction pathways in which proteins were involved were investigated by the KEGG toll. According to KEGG pathway analysis, complement and coagulation cascades were the most represented, including 19 proteins, followed by systemic lupus erythematosus and metabolic pathways.

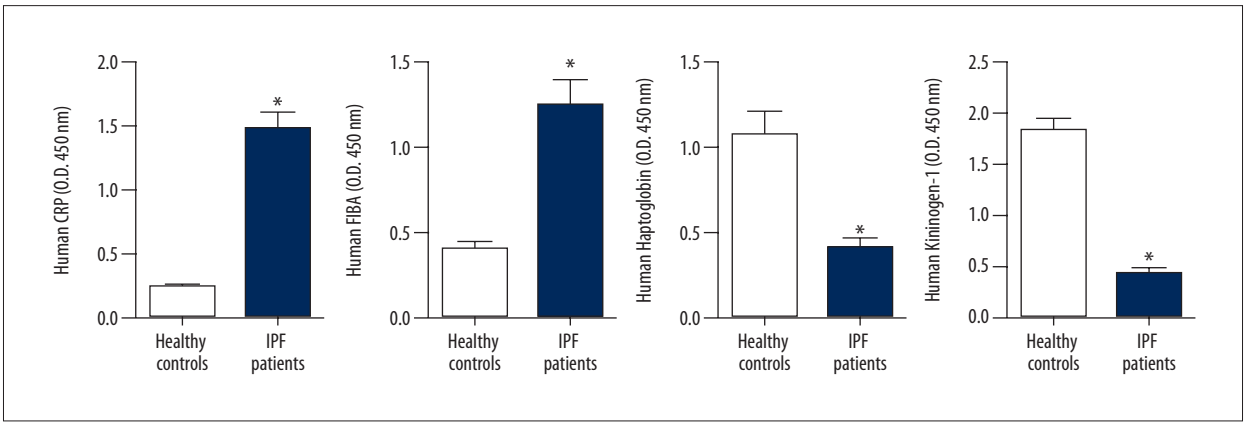

Figure 3. Validation of the iTRAQ results by ELISA. Optical density at 450 nm of CRP, FIBA, haptoglobin, and kininogen-1 in IPF patients and healthy controls. * P<0.05 compared with healthy controls.

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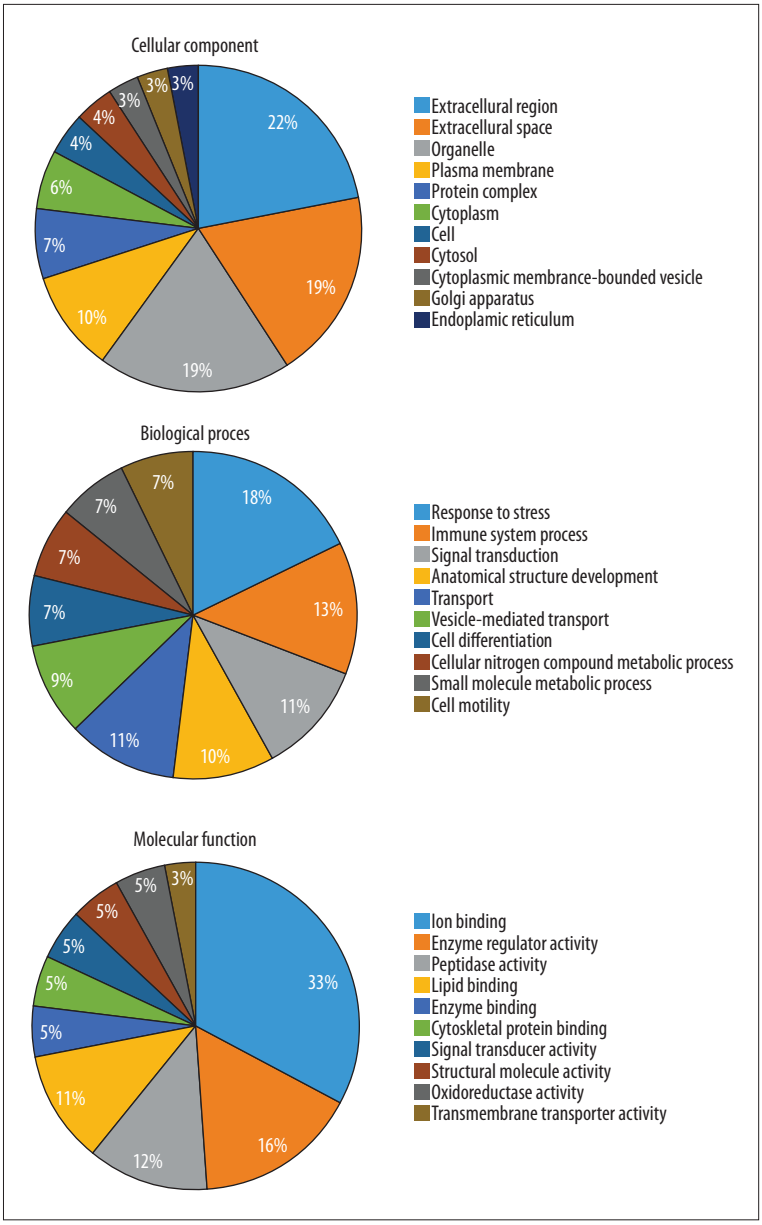

Figure 4. Gene Ontology (GO) analysis of differentially expressed proteins based on their cellular component, biological process, and molecular function. Distribution of proteins based on their (**A**) cellular component, (**B**) biological process, and (**C**) molecular function. Since the 11th GO term contained the same number of proteins as the 10th, it was also included in GO category of cellular component.

Discussion

IPF is a progressive fibrotic disorder of the lower respiratory tract, with increasing incidence rates [35]. The causes of IPF remain elusive and are not easy to identify, as patients diagnosed are typically at an advanced stage of the disease. Several associated risk factors without a clear causative role have been reported [36], including environmental and occupational exposures, infections, and genetic polymorphisms. Although IPF is considered a rare disease, its social, healthcare, and economic burdens are far from irrelevant. It is estimated that approximately 40 000 new cases are diagnosed in Europe each year, with more than 5000 in the UK alone [35]. However, there have been few epidemiology studies in Asian countries. Recently, a Japanese study [37] explored the certificates of medical benefits for IPF in Hokkaido prefecture between 2003 and 2007, reporting a prevalence and incidence of 10.0 and 2.23 per 100 000/year, respectively. In addition, the incidence, prevalence, and number of deaths from IPF may be increasing [35,38–40].

Biomarkers are urgently needed for IPF. Many biomarkers have already been studied both in serum and bronchoalveolar lavage from interstitial lung diseases and IPF, where inflammation seems to take part in the disease [41] and in the remodelling process [42]. For example, the levels of matrix metalloproteinase-8, pepsin, bronchoalveolar lavage fluid YKL-40, S100A9, and myeloid-related protein-14 were shown to be elevated

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in the bronchoalveolar lavage fluids of IPF patients [43–47]. Moreover, serum levels of the Krebs von den Lungen-6 (KL-6) antigen, surfactant protein A and D (SP-A and SP-D), matrix metalloproteinases-1 and -7 (MMP-1 and MMP-7), and periostin were elevated in IPF [48–58]. Proteomic techniques are increasingly being applied to investigate associations between differentially expressed proteins in bronchoalveolar lavage fluid and IPF [59,60].

In the present study, we initially used 2 pooled samples for proteomic studies to identify biomarker candidates using iTRAQ and LC-MS/MS. We identified 4 new molecules that show expression differences between patients and healthy controls. CRP is an essential acute-phase reactant that is synthesized by hepatocytes in response to cytokines following inflammatory stimulus [61]. It has been shown to be an independent predictor of stroke, myocardial infarction, atherosclerosis, peripheral vascular disease, and sudden cardiac death [62–66]. Previous studies showed that CRP is a non-specific inflammatory marker that is elevated in coronary artery disease and obstructive lung disease [67,68]. CRP production has been demonstrated in alveolar macrophages in pulmonary inflammatory disease [69]. In the present study, a significant increase in CRP expression was observed in IPF (p=0.005).

Fibrinogen, synthesized by the liver, is a major plasma protein that consists of pairs of 3 different polypeptide chains – α, β, and γ – that are joined by disulfide bonds to form a symmetric dimeric structure. Fibrinogen is directly involved in the clotting process as a clotting factor, and releases 2 fibrinopeptides (A and B) from the NH2 terminus of α and β chains cleavage by thrombin [70]. Fraction of fibrinogen α-chain or isoform was also found to be evaluated in many cancers such as ovarian cancer [71], breast cancer [72], urothelial cancer [73], gastric cancer [74], and other diseases such as urinary tract infection [75], Alzheimer's disease [76], nephritic syndrome [77], and even HCV-related liver disease [78].

Haptoglobin (Hp) is a glycoprotein synthetized by the liver sorting within α-2 globulins at serum electrophoresis. It has antioxidant and immunomodulatory properties [79] and acts as a scavenger by stably binding free serum circulating hemoglobin that is released by hemolysis or normal RBC turnover. A previous study shows that Hp is classified as an acute-phase protein, the serum expression levels of which are increased with inflammation, infection, and several cancers [80].

Kininogen-1 is a multifunctional protein that plays an important role in many pathophysiological processes [81], including fibrinolysis, thrombosis, and inflammation, and has a role in oncogenesis [82]. It was also demonstrated that kininogen-1 exhibits antiangiogenic properties and mediates inhibitory actions on the proliferation of endothelial cells [83]. Analyses of the urine proteome of patients with chronic pancreatitis [84] and interstitial cystitis [85] showed reduced KNG1 levels. It has been suggested that KNG1 is proteolyzed by neutrophils at inflammation sites. Lower levels of KNG1 are found in fibrosis/cirrhosis patients compared with non-fibrosis hepatitis C patients [86], indicating that KNG1 is directly related to the severity of impaired liver function.

Conclusions

In conclusion, the present study demonstrates that levels of CRP and fibrinogen-α are higher and levels of haptoglobin and kininogen-1 are lower in patients with IPF compared with those in healthy controls. CRP, fibrinogen-α chain, haptoglobin, and kininogen-1 may be useful candidate biomarkers for IPF. In addition, the present study provides an efficient approach for the differential analysis of IPF.

Conflict of interests

None.

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