Differential proteomic analysis of respiratory failure in peripheral blood mononuclear cells using iTRAQ technology

GUOPING SUN[1*], CUIHUI CAO[1*], WENBIAO CHEN[2], YANG ZHANG[1] and YONG DAI[2]

[1]Central Laboratory of Pingshan New District People's Hospital of Shenzhen; [2]Clinical Medical Research Center, The Second Clinical Medical College of Jinan University, Shenzhen People's Hospital, Shenzhen, Guangdong 518020, P.R. China

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Abstract. Respiratory failure (RF) is a state in which the respiratory system fails by its gas exchange functions. Failure of the lung, which is caused by all types of lung diseases, leads to hypoxaemia with type I respiratory failure. Failure of the pump leads to hypercapnia or type II respiratory failure. Using isobaric tags for relative and absolute quantification (iTRAQ) technology to identify and quantify the total proteins in peripheral blood mononuclear cells (PBMCs) of RF patients and identify the differentially expressed proteome. The present study analyzed the total proteins in the PBMCs of RF patients and healthy controls using the eight-plex iTRAQ added with strong cation-exchange chromatography and liquid chromatography coupled with tandem mass spectrometry. The differentially expressed proteins were identified by MASCOT. A total of 4,795 differentially expressed proteins were identified, and 403 proteins were upregulated and 421 were downregulated. Among them, 4 proteins were significantly differentially expressed, which were upregulated KIAA1520 protein and γ fibrinogen type B (AA at 202) and downregulated chain A, crystal structure of recombinant human platelet factor 4 and myosin regulatory light polypeptide 9. iTRAQ technology is suitable for identifying and quantifying the proteome in the PBMCs of RF patients. The differentially expressed proteins of RF patients have been identified in the present study, and further research of the molecular mechanism of the differentially expressed proteins is required to clarify the pathogenesis and identify novel biomarkers of RF.

Correspondence to: Professor Yong Dai, Clinical Medical Research Center, The Second Clinical Medical College of Jinan University, Shenzhen People's Hospital, Shenzhen, Guangdong 518020, P.R. China
E-mail: daiyong2222@gmail.com

*Contributed equally

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Introduction

Respiratory failure (RF) is a state in which the respiratory system fails by its gas exchange functions. Usually, RF is defined by an arterial oxygen tension (PaO_2) of <8.0 kPa (60 mmHg) and an arterial carbon dioxide tension ($PaCO_2$) of >6.0 kPa (45 mmHg). These cut-off values of respiratory failure serve as a general guide and are coupled with the history and clinical assessment of RF patients (1). The respiratory system includes the lung and the pump that ventilates the lungs (2). Failure of the lung, which is caused by all types of lung diseases leads to hypoxemia with type I respiratory failure. Failure of the pump leads to hypercapnia or type II respiratory failure (3).

In the RF research, one of the main aims is to identify biomarkers for a better understanding of RF pathogenesis and improving diagnosis. Aberrant functions of the lymphocytic regulatory pathway were widely associated with the pathological mechanism of certain diseases and peripheral blood mononuclear cells (PBMCs) were used as feasible samples in these studies (4). In the past several years, the developments in proteomics research of numerous rheumatic diseases have been reported (4-6). Isobaric tags for relative and absolute quantification (iTRAQ) technology has increasingly been used in biomarker research for numerous diseases (7-10). However, to the best of our knowledge, there is no previous study of iTRAQ technology applied to PBMCs of RF. If the differentially expressed proteins in the PBMCs of RF patients could be identified using proteomic analysis, then these proteins could serve as a basis for the development of proteomics research of RF.

iTRAQ reagents comprise three parts: A peptide reactive group, a reporter group and a molecular mass balance. Different protein samples are noted with a corresponding iTRAQ mass group (i.e., 113, 114 and 115 Da) which could be quantified. The proteomics workflow comprises two parts: 2-dimensional orthogonal resolution of peptides by strong cation exchange (SCX) and high-performance liquid chromatography (HPLC). Subsequently, the fractions were analyzed through tandem mass spectrometry (MS/MS). The sequence information (from peptide fragments) and relative quantification (from reporter group ions) are provided from the resultant mass spectra. In the present study, the total proteins in the PBMCs of RF patients were analyzed through iTRAQ

technology. Further research of the molecular mechanism of the proteins can better clarify the pathogenesis and identify novel biomarkers of RF.

Materials and methods

Main reagents. Triton X-100 and a Strata-X 33u Polymeric Reversed Phase column were separately purchased from Amersham Biosciences (Waukesha, WI, USA) and Phenomenex (Los Angeles, CA, USA). The Bicinchoninic Acid (BCA) Protein Assay Reagent kit and triethylammonium bicarbonate buffer were respectively acquired from Pierce (Thermo Fisher Scientific, Inc., Rockford, IL, USA) and Sigma-Aldrich (Supelco, Bellefonte, Pennsylvania, USA). ZipTip Pipette tips and Milli-Q water were obtained from Millipore (Billerica, MA, USA). The iTRAQ Reagent-8Plex Multiplex kit and Trypsin Gold, mass spectrometry grade were respectively acquired from Applied Biosystems (Carlsbad, CA, USA) and Promega (Madison, WI, USA). All the other reagents were obtained from commercial sources.

Patients and healthy controls. The samples included 5 patients and 5 healthy controls. The participants were from Shenzhen People's Hospital (Shenzhen, China), between April and October 2013. The 5 patients, who were diagnosed as type IIRF, included 4 women and 1 man with an average age of 35.22 years; range, 28-45 years. The age and gender of the 5 healthy controls were matched with the 5 patients. The diagnosis of RF was confirmed by pathological diagnosis and clinical evidence.

The healthy controls were confirmed to have no clinical evidence of RF. All the subjects provided informed consents. The study was approved by the regional ethics committee and abided by the Helsinki Declaration.

PBMC isolation, protein extraction and quantitation. One 5-ml fasting venous blood sample from each participant was collected into the corresponding heparinize vacutainers. According to the manufacturer's protocol (Cedarlane Laboratories, Burlington, ON, Canada), PBMCs were isolated with lymphocyte-H medium. The concentration of total protein for PBMCs, which was extracted, was measured with the BCA protein assay kit. The protein samples were kept at -80˚C for further analysis.

iTRAQ labeling and SCX fractionation. Firstly, a ratio of protein:trypsin = 30:1 was used to generate the Trypsin Gold. Subsequently, the Trypsin Gold was used to digest the equal amounts (100 μg) of protein in the PBMCs of the samples at 37˚C for 16 h. Following the digestion, peptides were dried through vacuum centrifugation. Following the manufacturer's protocol, peptides were reconstituted in 0.5 M TEAB and processed for 8-plex iTRAQ reagent. In brief, samples were thawed and reconstituted in one unit of iTRAQ reagent in 24 μl isopropanol. Samples were labeled with the iTRAQ tags (samples 114 and 115). The peptides labeled with the isobaric tags were incubated with them at room temperature for 2 h. Subsequently the labeled peptide mixtures were pooled and dried by vacuum centrifugation.

SCX chromatography was performed through an LC-20AB HPLC Pump system (Shimadzu, Kyoto, Japan). The iTRAQ-labeled peptide mixtures were reconstituted with 4 ml of buffer A [25 mM NaH_2PO_4 in 25% acetonitrile (ACN) (pH 2.7)]. Subsequently, the iTRAQ-labeled peptide mixtures were loaded onto a 4.6x250 mm Ultremex SCX column containing 5-μm particles (Phenomenex). After the loading process, the peptides were eluted at a flow rate of 1 ml/min with a gradient of 100% buffer A for 10 min. Secondly, the peptides were eluted at a flow rate of 1 ml/min with a gradient of 5-60% buffer B [25 mM NaH_2PO_4, 1 M KCl in 25% ACN (pH 2.7)] for 27 min. Thirdly, the peptides were eluted at a flow rate of 1 ml/min with a gradient of 60-100% buffer B for 1 min. Before equilibrating with buffer A for 10 min prior to the next injection, the system was maintained at 100% buffer B for 1 min. The elution was analyzed through measuring the absorbance at 214 nm, and collecting the fractions every 1 min. Following this, the eluted peptides were pooled into 20 fractions and the eluted peptides were desalted with a Strata X C18 column (Phenomenex) and vacuum-dried.

LC-ESI-MS/MS analysis based on Q-Exactive. Each fraction was resuspended in buffer A [2% ACN, 0.1% formic acid (FA)] and centrifuged at 20,000 x g for 10 min. The final concentration of the peptide was ~0.5 μg/μl. In total, 10 μl supernatant was loaded on an LC-20AD nano-HPLC (Shimadzu) through the autosampler onto a 2-cm C18 trap column. The peptides were eluted onto a 10-cm analytical C18 column (inner diameter 75 μm) packed in-house. The samples were loaded at 8 μl/min for 4 min. The 44 min gradient was run at 300 nl/min from 2 to 35% B (98% ACN, 0.1% FA), followed by a 2-min linear gradient to 80%, and maintained at 80% B for 4 min, prior to reverting to 5% for 1 min.

The peptides were subjected to nanoelectrospray ionization through MS/MS in an Q-Exactive (Thermo Fisher Scientific, San Jose, CA, USA), which was online to the HPLC and detected the intact peptides in the orbitrap at a resolution of 70,000. The peptides for MS/MS were selected through high-energy collision dissociation. A data-dependent procedure was used, which alternated between one MS scan through 15 MS/MS scans. The data-dependent procedure was applied to the 15 most abundant precursor ions above a threshold ion count of 20,000 in the MS survey scan with a following dynamic exclusion duration of 15 sec. The operating electrospray voltage was 1.6 kV. Automatic gain control (AGC) was used to optimize the spectra generated through the orbitrap. The AGC target for a full MS was 3e6. The m/z scan range was 350-2,000 Da for the MS scans.

Data analysis. MASCOT version 2.3.02 (Matrix Science, Ltd., London, UK) was used to analyze the identification and quantification of the proteins. The peptide sequences were searched in the nonredundant NCBI database. The search criteria were set to permit a maximum of 1 missed cleavage. Certain peptide modifications were permitted: For example, Gln->pyro-Glu, iTRAQ 8plex, Phospho. The values supplied with the Applied Biosystems reagents were used to carry out automatic isotope correction through both software packages. Subsequently, Gene Ontology (GO) (http://www.geneontology.org) was used to elucidate the molecular

Table I. Identification results.

Source	Total spectra	Spectra	Unique spectra	Peptide	Unique peptide	Protein
Homosapien	342,324	81,690	67,339	21,788	19,771	4,795

Table II. Differential proteins.

Participants	Upregulation proteins	Downregulation proteins	Total differentially expressed proteins
Healthy versus respiratory failure	403	421	824

Table III. Proteins identified as upregulated from the isobaric tags for relative and absolute quantification experiment, and an indication of the molecular function and biological processes of these proteins.

No.	Accession	Protein name	Molecular function	Biological process	Max multiple
1	gil58257696	KIAA1520 protein	TAP2 binding	Positive regulation of T-cell mediated cytotoxicity	34.504
2	gil182443	5 fibrinogen type B (AA at 202)	Protein binding, bridging	platelet activation	27.155
3	gil193244949	β-globin	Peroxidase activity	Hydrogen peroxide catabolic process	26.911
4	gil62088878	Protein 4.1 variant	Calmodulin binding	Positive regulation of protein binding	20.145
5	gil229959	β-globin (fragment)	Peroxidase activity	Hydrogen peroxide catabolic process	19.487
6	gil284521122	A-γ globin Osilo variant	Oxygen transporter activity	Oxygen transport	19.257
7	gil28332	cDNA FLJ35730 fis, clone TESTI2003131, highly similar to α-1-antichymotrypsin	Serine-type endopeptidase inhibitor activity	Negative regulation of endopeptidase activity	18.890

function, biological process and cellular component associated with each individual protein.

Results

Identification of proteins. A default significance threshold of <0.05 for individual variation was used as the cut-off. There were 19,711 iTRAQ-labeled unique peptides that mapped to a total of 4,795 proteins identified and quantified from PBMCs (Table I). The overlap or commonality of up- and downregulated proteins were subtracted, and 403 proteins were upregulated and 421 proteins were downregulated (Table II). Of these, a difference of a multiple of ≥0.5 points identified 604, a difference of upregulated >18 points included 7 proteins (Table III) and a difference of downregulated <0.13 points included 5 proteins (Table IV).

The functional distribution of these proteins is shown in Fig. 1. The GO was used to perform an analysis of 4,795 proteins and to divide the proteins into respective classes based on their molecular function. It found that two of the major groups involved binding (48.71%) and catalytic activity (28.68%) that were apparently changed in RF patients versus healthy controls.

Discussion

In the past few years, protein quantification has become a critical component of modern MS-based proteomic research (11,12). In addition, numerous quantification strategies have been improved. Almost all of them depend on the incorporation of steady isotopes for subsequent mass spectrometric sorting and relative quantification (13,14).

Through measuring the peak intensities of reporter ions, which are released from iTRAQ-tagged peptides, iTRAQ can compare the protein abundance. iTRAQ could be a potential tool for quantitative proteomic study. In the present study, the proteomics of PBMCs were analyzed in RF patients and healthy controls quantitatively through iTRAQ technology. As a result, 824 proteins, which are involved with different biological functions and cellular locations, were identified, and a proteome database was built for the RF proteome, which to the best of our knowledge has not been reported previously.

Table IV. Proteins identified as downregulated from the isobaric tags for relative and absolute quantification experiment, and an indication of the molecular function and biological processes of these proteins.

No.	Accession	Protein name	Molecular function	Biological process	Max multiple
1	gil809369	Chain A, crystal structure of recombinant human platelet factor 4	Heparin binding	Negative regulation of angiogenesis	0.091
2	gil431896311	Myosin regulatory light polypeptide 9	Calcium ion binding	Regulation of cell shape	0.092
3	gil406717976	MHC class II antigen	Glutamate receptor binding	T-cell costimulation	0.101
4	gil206581665	Chain A, human Ll-37 structure	Protein binding	Protein localization to microtubule	0.124
5	gil122939159	Protein-arginine deiminase type-2	Protein-arginine deiminase activity	Peptidyl-citrulline biosynthetic process from peptidyl-arginine	0.128

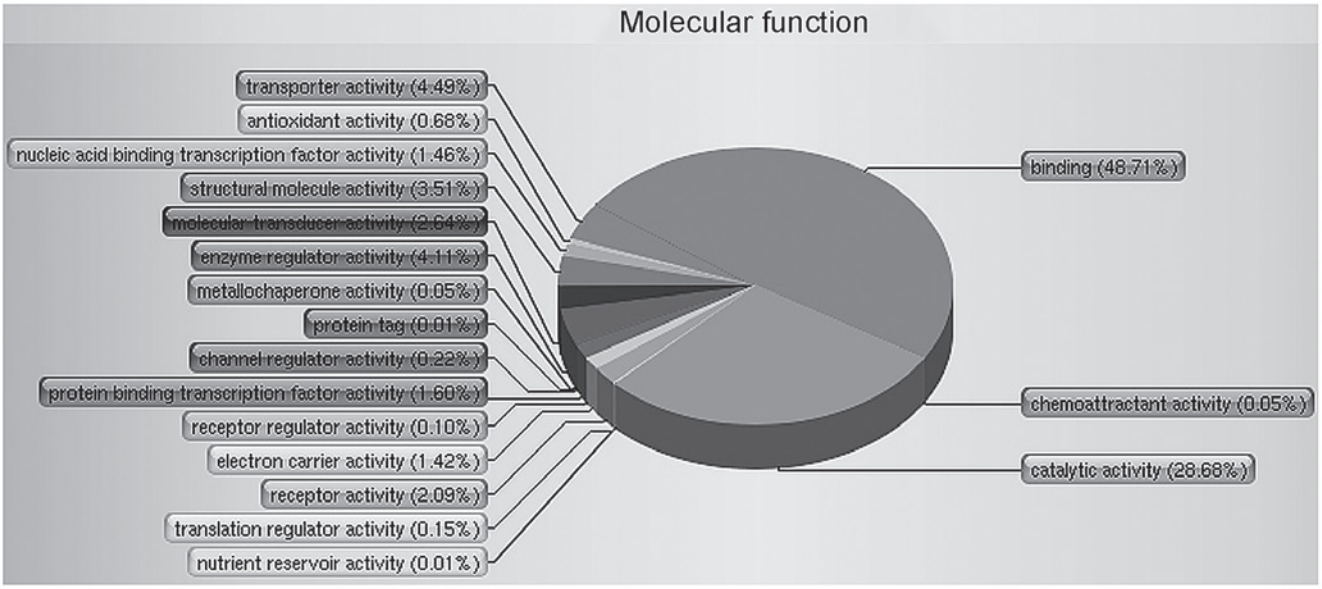

Figure 1. Number of peripheral blood mononuclear cell proteins identified using isobaric tags for relative and absolute quantification reagent and their classification based on molecular function. In total, 4,795 proteins were identified.

The up- and downregulated proteins for the RF and healthy controls are shown in Tables I and II. Among them, there was a significant difference of 4 proteins [upregulated KIAA1520 protein, γ fibrinogen type B (AA at 202) and downregulated chain A, crystal structure of recombinant human platelet factor 4 and myosin regulatory light polypeptide 9] in the present iTRAQ study. This provided additional certification that the iTRAQ technique could quantify relative changes in the proteins of PBMC accurately. In addition, the iTRAQ technique has been used for detecting pathological stages or prognosis in certain diseases (12-14). While iTRAQ-based biomarker profiles from tissue have been used for analyzing the pathological stages or prognosis of certain diseases (15,16), the analysis of PBMC in diagnosing the pathological result or progression of RF is only beginning to be explored.

Nagase *et al* (17) reported the entire sequences of 100 cDNA clones of KIAA1444 to KIAA1543 human genes from cDNA libraries. They found that open reading frames (ORFs) in 10 clones (KIAA1513, KIAA1515, KIAA1520-KIAA1522, KIAA1524, KIAA1525, KIAA1529, KIAA1531 and KIAA1538) carried single or multiple deletions; however, certain ORFs in 23 clones (KIAA1509-KIAA1512, KIAA1514-KIAA1517, KIAA1519-KIAA1521, KIAA1523, KIAA1524, KIAA1526-KIAA1528, KIAA1530 and KIAA1532-KIAA1537) carried single or multiple insertions. The study also reported that 48 gene products have functions correlated with nucleic acid management, cell structure/motility or cell signaling/communication.

The γA and γB were the two forms of the γ chain of human fibrinogen. The differences between them are only in their carboxyl termini. The protein sequence of γ-fibrinogen in rats and humans is generally highly conserved (18). The unique γB sequence, which is coded by human fibrinogen, contained 1 basic residue and 7 acidic (19). Song *et al* (20) found that

the levels of plasma viscosity, blood viscosity, hematocrit, fibrinogen and D-dimer were significantly higher in acute exacerbations of chronic obstructive pulmonary disease (AECOPD) patients. The levels of fibrinogen and D-dimer had significantly positively associated with the $PaCO_2$ and negatively associated with the PaO) in AECOPD patients combined with RF.

Myosin regulatory light polypeptide 9 is a type of myosin regulatory subunit. It exhibited a critical role in regulating the activities of the smooth muscle and non-muscle cell contractile. In addition, it was involved with cell locomotion, receptor capping and cytokinesis. In lipopolysaccharide-induced lung inflammatory injury, which is the chief cause of the acute respiratory distress syndrome, non-muscle myosin light-chain kinase mediates increased lung vascular endothelial permeability (21).

The functions of certain other novel candidates, such as the chain A, crystal structure of human platelet factor 4 (downregulated in RF), remain to be elucidated. The novel candidates would be more worthy for further investigation.

In the present study, every candidate protein was not discussed in detail. The aim of this preparatory investigation was centered on illustrating the primary comparative protein profiles of RF patients and healthy controls using iTRAQ technology. Furthermore, future studies require the collection of more patient samples to identify the beneficial biomarker candidates of the pathogenesis in RF. In conclusion, the present study showed the potential application of iTRAQ-based quantitative proteomics for the identification of protein changes and potential biomarker candidates in certain diseases.

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