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Differential proteomic analysis of bronchoalveolar lavage fluid from lung transplant patients with and without chronic graft dysfunction

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ABSTRACT

Background: Biomarkers are urgently needed for diagnosis, prognosis and monitoring of lung transplant chronic graft dysfunction. Bronchoalveolar lavage fluid (BAL) has been used in the past as proximal fluid for biomarker discovery in various lung diseases including chronic graft dysfunction (CGD). The current study describes the proteomic analysis of BAL fluids collected from 4 asymptomatic post-transplant patients and 3 patients with symptoms of CGD.

Methods: BAL proteome was fractionated by size-exclusion chromatography at protein level and reverse-phase-chromatography at peptide level followed by Orbitrap® mass spectrometry detection.

Results: Our in-depth proteomic analysis identified 531 proteins, the largest catalog of BAL proteins reported to date in the context of CGD. A total of 30 and 39 proteins detected exclusively in CGD and non-CGD samples, respectively, are potential candidates for verification phase.

Conclusions: A new protocol was developed to enhance the sensitivity of detecting less abundant proteins in BAL.

Introduction

Lung transplantation is a well-established therapeutic option for various end-stage lung diseases such as chronic obstructive pulmonary disease, emphysema, idiopathic pulmonary fibrosis, cystic fibrosis, and idiopathic pulmonary hypertension [1–5]. The success rate of lung transplantation has improved significantly due to advances in surgical procedures and post-transplantation care. Despite these advances, the 5-year survival rate is relatively low at 45%, and only 20% of patients survive beyond 10 years [6]. Lung transplant survival rate is considerably inferior to other solid organ transplants. Chronic graft dysfunction (CGD), especially bronchiolitis obliterans (OB), is the major cause of morbidity and mortality in post-transplant patients. OB is manifested by inflammation, progressive fibrosis of small airways and irreversible airway obstruction [7,8]. Bronchiolitis obliterans syndrome (BOS), is diagnosed by a fall in forced expiratory volume in one second (FEV_1), which is frequently used as a non-invasive surrogate marker [9,10]. The progressive airway obstruction observed in OB correlates with lowering of pulmonary function in BOS. However, the true relationship between the two is yet to be established. Intensification of immunosuppressive regimen is the only available treatment option for OB, which can only slow disease progression [10]. CGD is a heterogeneous condition; BOS and restrictive allograft syndrome (RAS) are the two different subtypes; RAS is diagnosed by irreversible decline in total lung capacity (TLC) [11]. To date there are no reliable noninvasive diagnostic procedures available to forecast CGD and to clinically demarcate BOS and RAS. Identification of biomarkers that can foresee the onset of CGD and demarcation of BOS and RAS at the cellular and sub-cellular levels could facilitate alterations in therapy and, more importantly, could lead to further insights into the disease mechanism and open-up new possibilities of therapeutic intervention.

Abbreviations: BAL, bronchoalveolar lavage; MS, mass spectrometry; MALDI, matrix-assisted laser desorption ionization; HPLC, high performance liquid chromatography; LC–MS/MS, liquid chromatography tandem mass spectrometry; TOF, time-of-flight; SEC, size exclusion chromatography; RP, reverse phase; BOS, bronchiolitis obliterans syndrome; OB, bronchiolitis obliterans; RAS, restricted allograft syndrome; NO, Nitric oxide; MMP, matrix metalloproteinase; MPO, myeloperoxidase; FEV_1, forced expiratory volume in one second; ELF, epithelial lining fluid; COPD, chronic obstructive pulmonary disease; FA, formic acid; HNP, human neutrophil defensins; IPA, Ingenuity pathway analysis; EEP, exclusively expressed proteins; MRM, multiple reaction monitoring; FPR, false positive rate; GI, gastrointestinal; CCP, Clara cell protein; CGD, chronic graft dysfunction.

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Bronchoalveolar lavage (BAL) fluid is the most widely used matrix for sampling the components of the pulmonary airways [12]. BAL proteome was previously mined for several indications of lung diseases such as asthma [13,14], COPD [15], cystic fibrosis [16], idiopathic pulmonary fibrosis, acute respiratory distress syndrome, pneumonia [17,18], asbestos-induced malignant pleural mesothelioma [19] and inflammatory diseases [20].

Nelsestuen et al. were among the first to report on biomarkers of chronic lung allograft rejection using mass spectrometry-based proteomic analysis of BAL [21]. The authors reported three unusually intense peaks observed in the MALDI-TOF–MS profiles of individuals who developed BOS. These three peaks were identified as human neutrophil defensins (HNP) by tandem mass spectrometry (MS/MS). Quantitative analysis was performed using mass spectrometry and ELISA. The authors concluded that elevated levels of HNP increases the relative risk of developing BOS. In another study reported by the same group, MALDI-TOF–MS profiles of control and transplant-rejection BAL samples were compared [22]. Intensity ratios of the peaks within the same MALDI-TOF–MS profile were used to quantify the results. The study suggested the lowered ratio of Clara cell protein (CCP) to lysozyme is a better marker than HNP. In both studies, the authors arrived at the proposed biomarker candidates based on clues provided by differential mass peaks observed in the MALDI-TOF mass spectra. A caveat of this approach is that, with the limited potential of MALDI-TOF–MS in terms of mass accuracy and resolution, and proteomic analysis without prior chromatographic fractionation procedures, it may not be possible to detect less abundant but clinically significant proteins. In general, more proteins are detected in complex matrices such as BAL, using LC–MS/MS methods in comparison to MALDI-TOF–MS. Advanced protein separation and identification technologies have made it possible to detect more proteins in complex proteomes, thereby facilitating the discovery of novel biomarkers.

Recently, Meloni et al., employed 2D gel electrophoresis coupled with MALDI-TOF–MS and LC–MS/MS and identified 11 proteins that are differentially expressed in BAL of BOS patients [23]. The authors reported that peroxiredoxin II is specifically expressed in BOS and the expression of surfactant protein A (SP-A) is significantly lowered in BOS. Other investigators proposed thioredoxin [24], Clara cell secretory protein [25] and matrix metalloproteinase-9 [26] as biomarker candidates. These reports made use of targeted immunoassays such as ELISA. But none of these studies carried out a much needed comprehensive proteomic analysis.

The complexity of BAL proteome necessitates a comprehensive differential proteomic analysis, coupled with multidimensional chromatography and high resolution mass spectrometry. This could probably deliver an inventory of differentially expressed proteins from which a set of clinically relevant biomarker candidates can be found for further verification and validation studies. With the advent of high resolution mass spectrometry technologies, the current outlook of discovering novel biomarkers appears to be promising [27]. Towards this goal, we developed a protocol with multiple chromatographic separation and in-depth proteomic analysis of BAL fluids collected from lung transplant patients with or without CGD.

Methods

Sample collection and processing

BAL samples were collected by the Toronto lung transplant group at Toronto General Hospital, University Health Network, under Institutional Review Board approval and patient consent. BAL samples were centrifuged at 13,000×*g* for 15 min to remove cellular debris. The supernatant was aliquoted into 1.5 mL Eppendorf™ tubes and stored at −80 °C until further analysis. Total protein concentration was measured using the Coomassie blue assay and ranged between 0.1 and 0.5 mg/mL. Seven BAL samples were processed for proteomic analysis; out of which four were collected from asymptomatic lung transplant patients (from now on referred to as "Control") and three from patients with signs of CGD (from now on referred to as "CGD" samples). All the CGD samples were collected from patients diagnosed with RAS. We analyzed three CGD and three control samples, of which one control sample was a pool of two control samples due to low protein concentrations.

Size exclusion chromatography

The BAL proteome was initially fractionated with a size exclusion chromatography (SEC) column (TSK GEL G3000 SW; 5 μm, 60 cm×7.8 mm; Tosoh Bioscience LLC, Montgomeryville, PA, USA) using 0.1 M NaH_2PO_4/0.1 M Na_2HPO_4, 150 mM NaCl, pH 6.8, as mobile phase at a flow rate of 0.5 mL/min, for 60 min. An Agilent 1100 series HPLC system (Santa Clara, CA, USA) equipped with a diode array detector was used. The elution of proteins was monitored at 280 nm. A total of 6 fractions were collected per sample. All fractions were desalted and concentrated to 0.5 mL using Millipore Amicon ultra centrifugal filters MWCO 3000. All the samples were subjected overnight trypsin digestion.

LC–MS/MS analysis on LTQ-Orbitrap XL

The trypsin-digested SEC fractions were desalted using the Omix C18MB tips (Varian Inc., Palo Alto, CA, USA). The desalted peptides were injected into a trap column (IntegraFrit capillary; 3 cm×150 μm, New Objective, Woburn, MA, USA) using the EASY-nLC system (Proxeon Biosystems, Odense, Denmark) connected online to LTQ-Orbitrap XL (Thermo Fisher Scientific, San Jose, CA, USA) mass spectrometer. The peptides were resolved on a C_{18} capillary column (5 cm×75 μm PicoTip Emitter, New Objective) using a 60 min linear gradient (Buffer A and B; 0.1% FA in water and 0.1% FA in acetonitrile) at a flow rate of 400 nL/min. The capillary temperature was 160 °C and spray voltage was 2 kV. The mass spectra were acquired in data-dependent mode. Collision dissociation energy for MS/MS was set at 30%. Dynamic exclusion, monoisotopic precursor selection and charge state screening were enabled. Unassigned charge states as well as charges +1 and ≥+4 were rejected from MS^2 fragmentation.

Database searching and bioinformatics

The resulting spectra from each SEC fraction were searched against the non-redundant IPI human database (version 3.71) containing both forward and reverse protein sequences, using two search engines separately; Mascot, version 2.1.03 (Matrix Science) and the Global Proteome Machine manager version 2006.06.01 (GPM X! Tandem; Beavis Informatics Ltd., Canada). The following parameters were used: (I) enzyme: trypsin; (II) one missed cleavage allowed; (III) fixed modification: carbamidomethylation of cysteines; (IV) variable modifications: oxidation of methionines; (V) MS^1 tolerance, 7 ppm; and (VI) MS^2 tolerance, 0.4 Da. The resulting Mascot DAT and X! Tandem XML files were loaded into Scaffold® (version 3.0, Proteome Software Inc., Portland, Oregon). The data files DAT and XML for all the SEC fractions were merged and cross-validated to create 6 "biological samples" in Scaffold®; 3 control and 3 CGD samples. Scaffold result data was filtered using the X! Tandem LogE and Mascot ion-score filters in order to obtain a protein false-positive rate (FPR) of ≤1%. FPR = 2×(number of proteins identified by searching the reverse sequences)/(the total number of identified proteins). Ingenuity pathway analysis (IPA) (Ingenuity Systems, Redwood City, CA, USA) was used to depict signal pathway networks and canonical pathways from comparative proteomic data. A Fisher exact test with a significance level of 0.05 was used to test the statistical value of canonical pathways and networks.

Results

Size exclusion chromatography shows different patterns between control and CGD samples

Fig. 1 shows the overlaid chromatograms of control and CGD BAL samples. The control BAL chromatogram shows the major absorbance in the elution zone of molecular mass between 44 and 158 kDa. By contrast, the CGD BAL chromatogram shows the major absorbance in the >670 kDa elution zone. The molecular masses of elution zones were established with molecular mass markers. The distinction in these elution patterns was consistently observed across all three control and three CGD samples, irrespective of total protein concentration.

BAL proteomes show differential expression of proteins between control and CGD samples

A total of 531 proteins were identified in the BAL proteome, with a false positive rate of ≤1%. Among 531 proteins, 282 proteins were identified with 2 or more peptide hits. All 531 proteins and the number peptide hits for each protein are listed in Supplementary Table 1. Serum albumin, serotransferrin, complement C3 and alpha 2-macrogloblin were the top four most abundant proteins identified in BAL, as determined by the number of identified peptides. Out of 531 proteins, 183 were only detected in control samples; 140 were only detected in CGD and 208 proteins were identified in both (Fig. 2A). In control samples 86 proteins were detected in all three samples (Fig. 2C), whereas in CGD samples 95 proteins were found in all three samples (Fig. 2D). Fig. 2B shows comparisons of proteins reproducibly detected in all three control and all three CGD samples. Using this stringent selection criterion, 30 proteins were only seen in control group and 39 were only in CGD group. Interestingly, in the control group, there are 7 trypsin-related proteins, 2 carboxypeptidase, 2 lipase-related proteins, 5 complement-related proteins and 2 ribosomal proteins, and 2 UV excision-repair proteins (Table 1). These proteins may represent normal cellular metabolism in the alveoli and small airways. Lack of expression may suggest the disruption of normal cellular function. In contrast, in the CGD group, there are 6 mucin proteins, 4 S100 family proteins, 4 neutrophil enzymes, 2 histone proteins, and 2 heat shock proteins (Table 2). Abundant mucin proteins found in CGD group may indicate the "activation" of airway epithelium. S100 proteins are related to inflammatory responses; especially, S100A9, together with S100-A8 have broad regulatory effects on vascular inflammation and promote leukocyte recruitment and macrophage cytokine production [28,29]. These differentially expressed proteins may have diagnostic value and merit further investigation.

Comparison with the plasma proteome and the gastric/biliary proteome

In order to assess the possible role of serum protein leak into alveolar spaces in the CGD group, the BAL proteome from the current study was compared to the human plasma protein database (HIP2), containing 11,866 proteins [30]. Out of 531 BAL proteins, 349 were found in the plasma protein database. Similar number of plasma proteins were detected in the CGD (238 proteins) and the control groups (260 proteins). Comparison of the BAL proteome with the gastric and biliary proteomes is of clinical interest because gastro-esophageal reflux is prevalent in acute lung transplant rejections. A literature-based database of gastrointestinal and biliary proteomes [31,32] containing 110 proteins was compared with the BAL proteome. Forty three proteins detected in CGD samples were also found in biliary and gastric proteome, whereas 47 proteins from control samples were matched with GI proteins.

Comparison with literature-reported biomarkers

To the best of our knowledge, there are only a few publications on proteomic analysis of BAL in the context of lung transplant patients. Table 3 shows the number of proteins and peptides detected in control and CGD samples that corresponds to previously published makers. Patel et al. proposed thioredoxin as a biomarker for lung transplant patients with graft rejection [24], and Hubner et al. reported that matrix metalloproteinase-9 was up-regulated in transplant patients with BOS [26]. These two proteins were almost undetectable in control group, but found consistently in the CGD group. Meloni et al. identified 11 proteins which are differentially expressed between control post-transplant and BOS patients [23]. Ten of the 11 proteins were also identified in the current study. Using the number of identified peptides as an indicator, we compared the abundance of these proteins between the two groups. Interestingly, among 6 proteins reported to be increased in BOS, 5 of them showed lower numbers of peptides in the CGD group and one is almost undetectable in both groups. Among 4 proteins reported to be reduced in BOS, pulmonary surfactant-associated protein A1 and α1-anti-chymotrypsin showed reduced numbers of peptides in the CGD group in our study. Nelsestuen et al. reported increased levels of human neutrophil defensins and Clara cell protein in transplant patients with CGD [21]. In the current study, HNPs were identified in both control and CGD BAL samples with low abundance.

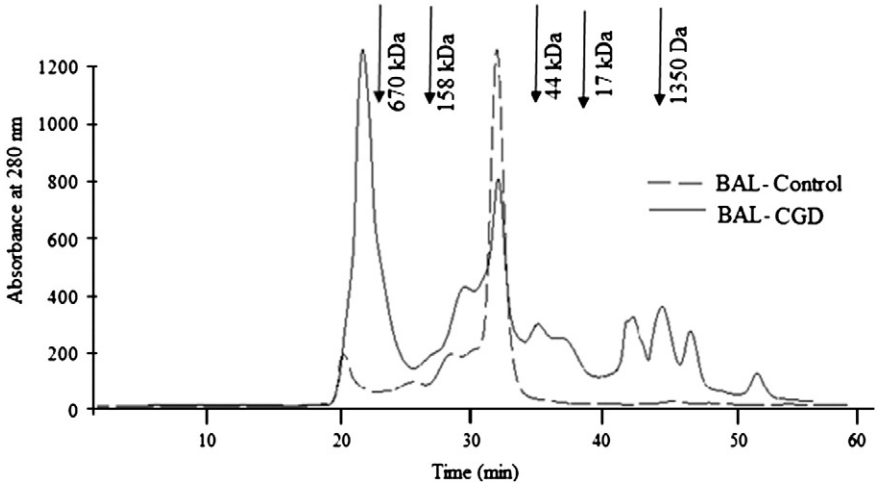

Fig. 1. Comparison of size exclusion chromatograms of BAL between control and chronic graft dysfunction samples. The arrows show the approximate elution times of molecular mass standards. BAL samples from CGD patients exhibit high absorbance at >670 kDa molecular mass, whereas BAL samples from control patients show major absorbance peaks between 44 and 158 kDa. A representative sample from each group is shown.

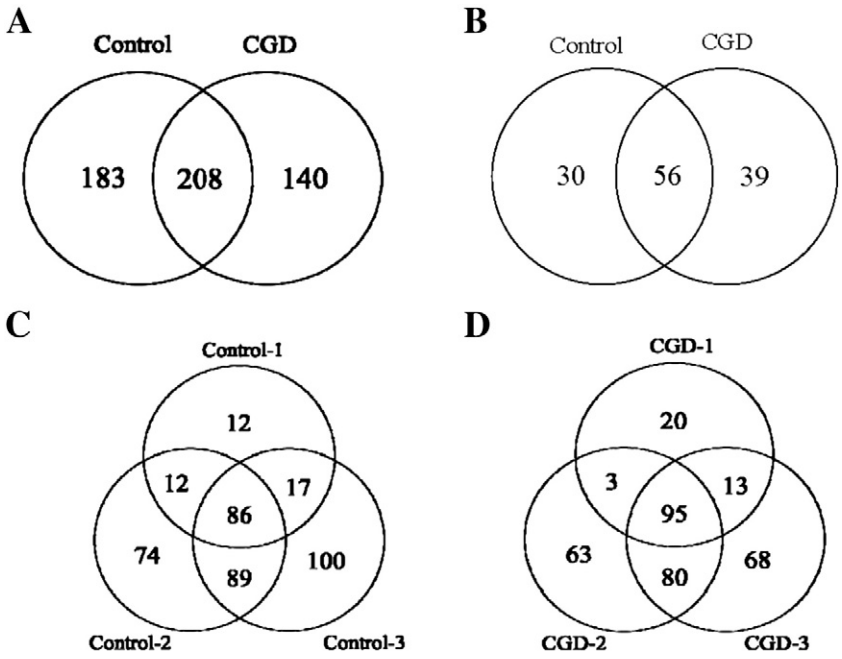

Fig. 2. Number of proteins identified by LC–MS/MS in BAL samples from patients with or without CGD. (A) Overlap and differentiation of the numbers of proteins between control and CGD groups; (B) Number of proteins detected in all the 3 CGD and control samples and their overlap. (C) Number of proteins detected in all 3 control samples. (D) Number of proteins detected in all 3 CGD samples.

Ingenuity pathway analysis

Ingenuity pathway analysis of exclusively expressed proteins (EEPs) either in control or in CGD samples identified statistically significant pathway networks in the control and CGD samples. The top three statistically significant networks and their functions are shown in Supplementary Table 2. EEPs in control samples did not show networks related to any disorders. Tissue development, cell-to-cell signaling interaction and metabolism are the major networks. In contrast, oxidative stress, inflammation, immune cell trafficking are major networks seen in CGD. CGD EEPs proteins were associated with cancer (61 proteins), respiratory disease (27 proteins), infectious diseases (19 proteins) and inflammatory response (22 proteins). Fig. 3 shows the inflammation network in the CGD group. TNFα and NFκB are found in the center of this pathway.

Discussion

Proteomic approaches for searching for biomarkers in BAL fluids, collected from lung transplant patients, have been attempted by several research groups. Enhancing the sensitivity of detecting low abundance proteins is the essential step for large database informatics research. In this study, we conducted a differential proteomic analysis of BAL fluid from lung transplant patients with or without signs of CGD. Multidimensional chromatography, coupled with high-resolution Orbitrap mass spectrometry was used. A large dataset (531 proteins) was created, that allowed us to delineate the BAL proteome with various bioinformatics techniques. Several potential candidate biomarkers and functional pathways were identified, which demonstrate the feasibility of this approach for future studies.

Complex biological fluids such as BAL may contain hundreds of proteins, ranging from low abundance to high abundance. BAL was previously identified as an albumin-rich medium [14]. Hence, appropriate depletion and fractionation procedures must be employed to enhance the detection of low abundance proteins. Previous attempts to delineate the BAL proteome did not include any fractionation methods. In the present study, chromatographic separation was utilized at the protein and peptide level, respectively. The combination of these chromatographic methods, together with nanospray ionization/high resolution Orbitrap mass spectrometry, has led to the detection of 531 proteins, one of the largest datasets of BAL proteins in lung transplant patients to date. In this feasibility study, only three samples were tested per patient group. To increase our stringency, we paid special attention to proteins which were found in all three samples from each group. This strategy, revealed about 30–40 protein signatures per patient group. With increased samples size, the list of differentially expressed protein may increase further.

The size exclusion chromatogram of CGD BAL samples showed relatively high protein elution at >670 kDa molecular mass, compared to the second most intense peak at around 70–100 kDa molecular mass (Fig. 1). Mass spectrometric analysis of the early fraction, after trypsin digestion, revealed high abundance of mucin-related proteins (MW 500–3000 kDa) while the second fraction mainly represented serum albumin. This chromatographic elution pattern was reproducible in all three CGD BAL samples and it was not observed in any of the three control samples. This result suggests that the chromatographic elution pattern itself may help to differentiate control from CGD patients. It will be necessary to compare this pattern with samples collected from other patients with lung diseases, especially from lung transplant acute rejection, chronic rejection, BOS, infection and other clinical conditions, to determine whether this chromatographic pattern is unique to CGD or is common in damaged lungs.

Serum proteins can leak into the alveolar space during the early post-transplant reperfusion period and inhibit the biophysical activity of pulmonary surfactant, which, in turn, impairs the graft function and contributes to graft dysfunction [33,34]. To this extent, we compared our BAL proteome with serum proteins. Approximately, 65% of identified BAL proteins were found in the plasma protein database. The number of serum proteins found in the control and the CGD groups was very similar. However, plasma proteins such as myeloperoxidase, actinin alpha 1 and enolase-1 were found specifically in CGD samples. With a larger number of samples, the levels of serum proteins in different groups should be compared in detail. It was also reported that gastrointestinal reflux and bile acid aspiration contributes to allograft dysfunction and particularly to the development of bronchiolitis obliterans [35,36].

Table 1
Compilation of 30 BAL proteins exclusively expressed in control sample and the number of peptides detected for each protein.

Protein description	IPI accession	MW kDa	Control[a]			CGD[a]		
Trypsin related proteins								
Chymotrypsinogen B2	IPI00742763 (+1)	28	4	6	9	0	0	0
Chymotrypsin-like elastase family member 3A	IPI00295663	29	1	4	10	0	0	0
Chymotrypsin-like elastase family member 2A	IPI00027722	29	1	3	4	0	0	0
Chymotrypsinogen B	IPI00015133	28	1	1	1	0	0	0
Chymotrypsin-like elastase family member 3B	IPI00307485	29	1	1	2	0	0	0
Inter-alpha-trypsin inhibitor heavy chain H1	IPI00292530	101	8	4	3	0	0	0
Trypsin-1	IPI00011694 (+1)	27	1	6	6	0	0	0
Carboxypeptidases								
Carboxypeptidase A1	IPI00009823	47	1	10	19	1	0	0
Carboxypeptidase B	IPI00009826	47	4	8	13	0	0	0
Lipase related proteins								
Pancreatic triacylglycerol lipase	IPI00027720	51	4	6	20	0	0	0
Carboxyl ester lipase precursor	IPI00099670 (+1)	80	4	6	13	0	0	0
Complement proteins								
Complement component C9	IPI00022395	63	7	6	6	0	0	0
Complement C5	IPI00032291	188	8	1	3	0	0	0
Complement component C8 alpha chain	IPI00011252	65	3	3	2	0	0	0
Complement component C8 beta chain	IPI00294395	67	4	2	0	0	0	0
Complement component C7	IPI00296608	94	2	3	1	0	0	0
Ribosomal proteins								
40S ribosomal protein S7	IPI00013415 (+4)	22	1	1	1	0	0	0
60S acidic ribosomal protein P0	IPI00008530 (+3)	34	1	1	1	0	0	0
UV excision repair proteins								
UV excision repair protein RAD23 homolog B	IPI00008223	43	1	1	1	0	0	0
UV excision repair protein RAD23 homolog A	IPI00008219	40	1	1	1	0	0	0
Other proteins								
Ribonuclease pancreatic	IPI00014048	18	1	5	3	0	0	0
Serum amyloid A protein	IPI00552578	14	2	2	4	0	0	0
Putative uncharacterized protein AMY2A	IPI00939512	58	1	1	4	0	0	0
Isoform 1 of N-acetylmuramoyl-L-alanine amidase	IPI00163207 (+1)	62	1	1	3	0	0	0
Lithostathine-1-alpha	IPI00009027	19	1	1	3	0	0	0
Moesin	IPI00219365 (+1)	68	1	3	2	0	0	0
Isoform B of Fibulin-1	IPI00218803 (+4)	77	1	2	1	0	0	0
Proteasome subunit beta type-7	IPI00003217	30	1	1	1	0	0	0
Isoform Alpha of signal transducer and activator of transcription 1-alpha/beta	IPI00030781 (+1)	87	1	1	1	0	0	0
Inter-alpha (Globulin) inhibitor H2, isoform CRA_a	IPI00305461 (+1)	107	8	3	1	0	0	0
Protease serine 2 isoform B	IPI00011695 (+1)	28	1	2	6	0	0	0

[a] Numbers represent number of peptides identified per protein.

To this end, we have compared our BAL proteome with literature-based gastric [31] and biliary proteomes [32]. Although a significant proportion of the gastrointestinal and biliary proteomes overlaps with BAL, there is no clear distinction between control and CGD overlaps. It seems that the differential protein populations found in BAL from control and CGD patients cannot be simply explained by serum protein leakage or GI reflux. However, these data should be viewed with caution since the plasma, gastrointestinal and biliary proteome databases used for comparison do not represent complete proteomes.

Table 2
Compilation of 39 BAL proteins exclusively expressed in CGD sample and the number of peptides detected for each protein.

Protein description	IPI accession	MW kDa	Control[a]			CGD[a]		
Mucin related								
Mucin-5AC (fragment)	IPI00103397	527	0	0	0	6	34	57
Mucin-16	IPI00103552	2353	0	0	0	6	21	25
Gastric mucin (fragment)	IPI00816128	148	0	0	0	1	1	1
Isoform 1 of Mucin-1	IPI00013955 (+13)	122	0	0	0	1	3	2
Isoform 12 of Mucin-4	IPI00178316 (+3)	125	0	0	0	1	10	10
Mucin 5AC, oligomeric mucus/gel-forming	IPI00918002	649	0	0	0	3	26	47
S100 family								
Protein S100-A9	IPI00027462	13	0	0	0	1	7	6
Protein S100-A8	IPI00007047	11	0	0	0	1	5	5
Protein S100-A12	IPI00218131	11	0	0	0	4	3	4
Protein S100-P	IPI00017526	10	0	0	0	1	3	3
Neutrophil enzymes								
Isoform H17 of myeloperoxidase	IPI00007244 (+2)	84	0	0	0	13	20	19
Neutrophil collagenase	IPI00027846	53	0	0	0	1	1	4
Neutrophil gelatinase-associated lipocalin	IPI00299547 (+1)	23	0	0	0	6	7	10
Neutrophil elastase	IPI00027769	29	0	0	0	1	1	5
Histone proteins								
Histone H2B type 1-C/E/F/G/I	IPI00020101 (+3)	14	0	0	0	1	3	1
Histone H4	IPI00453473	11	0	0	0	3	6	5
Heat shock proteins								
Heat shock 70 kDa protein 1A/1B	IPI00304925 (+1)	70	0	0	0	3	2	1
Isoform 1 of Heat shock cognate 71 kDa protein	IPI00003865	71	0	0	0	3	1	2
Others								
Glucose-6-phosphate isomerase	IPI00027497 (+1)	63	0	0	0	1	10	5
Matrix metalloproteinase-9	IPI00027509	78	0	0	0	1	7	11
Annexin A3	IPI00024095 (+1)	36	0	0	0	10	8	6
Myeloblastin	IPI00027409	28	0	0	0	1	2	2
Prominin-1	IPI00012540 (+6)	97	0	0	0	1	5	8
Olfactomedin-4	IPI00022255	57	0	0	0	3	1	5
Calmodulin	IPI00075248 (+4)	17	0	0	0	2	2	4
Prostate stem cell antigen	IPI00013446	13	0	0	0	1	1	1
IgGFc-binding protein	IPI00242956	572	0	0	0	1	6	25
Alpha-actinin-1	IPI00013508 (+3)	103	0	0	0	6	6	2
Catalase	IPI00465436	60	0	0	0	2	8	5
Thioredoxin	IPI00216298	12	0	0	0	3	1	3
Transcobalamin-1	IPI00299729	48	0	0	0	1	3	5
Dipeptidyl peptidase 1	IPI00022810	52	0	0	0	1	2	5
Carbonic anhydrase 1	IPI00215983	29	0	0	0	3	1	1
Cathepsin B	IPI00295741 (+1)	38	0	0	0	1	1	1
Tetraspanin-1	IPI00030936	26	0	0	0	1	1	1
Peptidoglycan recognition protein 1	IPI00021085	22	0	0	0	1	1	1
Glutathione S-transferase A1	IPI00657682 (+2)	26	0	0	0	1	2	2
Coronin-1A	IPI00010133 (+1)	51	0	0	0	1	1	2
Desmoglein-1	IPI00025753	114	0	0	0	1	1	1

[a] Numbers represent number of peptides identified per protein.

Table 3
Comparison of the number of peptide hits between control and CGD groups for BAL proteins that have been previously proposed as biomarker of CGD or BOS.

Protein	Control			CGD			Ref/expression in CGD or BOS
Matrix metalloproteinase-9	0	0	0	1	7	11	[26] ↑
Thioredoxin	0	0	0	3	1	3	[24] ↑
Complement C3 (fragment)	63	30	54	23	33	38	[23] ↑
Apolipoprotein A-I	19	19	18	10	12	12	[23] ↑
α2-HS-glycoprotein	7	10	10	2	0	3	[23] ↑
Leucine-rich α2-glycoprotein	1	5	11	0	3	3	[23] ↑
α1-B-glycoprotein	8	17	18	6	4	8	[23] ↑
Peroxiredoxin-II	0	0	0	0	0	2	[23] ↑
Pulmonary surfactant-associated protein A1	4	11	10	0	1	0	[23] ↓
α1-Anti-chymotrypsin	3	7	12	0	1	9	[23] ↓
Haptoglobin	7	14	17	13	10	13	[23] ↓
α1-Acid glycoprotein 2	2	4	3	1	0	5	[23] ↓
Human neutrophil defensin 1	0	1	2	1	2	2	[21] ↑

The elevation (↑) or decrease (↓) of these markers in BAL, in the previous published reports is indicated.

Protein expression data obtained from control and CGD samples were analyzed with IPA software to construct statistically significant cell signaling and metabolic pathway networks. IPA can identify signaling pathway networks by analyzing the proteomic data in a large-scale knowledge repository containing ~2.2 million scientific findings and ~250 canonical pathways [37]. Knowledge of signaling pathway networks provides a better understanding of disease mechanisms that could facilitate biomarker discovery and development of therapeutic agents. Network#1 in CGD samples is associated with oxidative stress, which has been implicated in the pathophysiology of lung transplantation. Network#2 functions in inflammatory response and out of 35 proteins associated with this network, 15 were detected in CGD samples. TNF and NFκB are found in the center of this network. TNF is a cytokine that enhances inflammation and alters the expression of Class I and Class II histocompatibility antigens [38]. NFκB is a transcription factor of inflammatory cytokines. Previously known inflammatory markers such as ELANE, LTF, PRTN3, S100A9, S100A8 and TXN were also detected in CGD samples.

Pathologically, RAS (all CGD patients in the current study were diagnosed with RAS) is characterized by diffused alveolar damage and extensive fibrosis in the alveolar interstitium, visceral pleura and interlobular septa. Radiological evaluation of majority of RAS (Chest X-ray and CT-scans) patients showed upper-lobe dominant fibrosis, a similar characteristic of several interstitial lung diseases [11]. The current proteomic analysis revealed many proteins that are indicative of severe pulmonary fibrosis; surfactant, mucin and matrix metalloprotease family of proteins exclusively detected in CGD samples are known markers of pulmonary fibrosis; it has been reported that increased secretion (4–5× more than normal) of mucins (MUC5AC, MUC5B and MUC2) may lead to bronchiolar plugging producing a chronic inflammatory and toxic burden on the alveolar surface. In addition, MUC 5AC is a major component of airway mucus and has been shown to be elevated during episodes of airway inflammation [39]. The large molecular size of mucin proteins may help explain the shift of the chromatogram towards

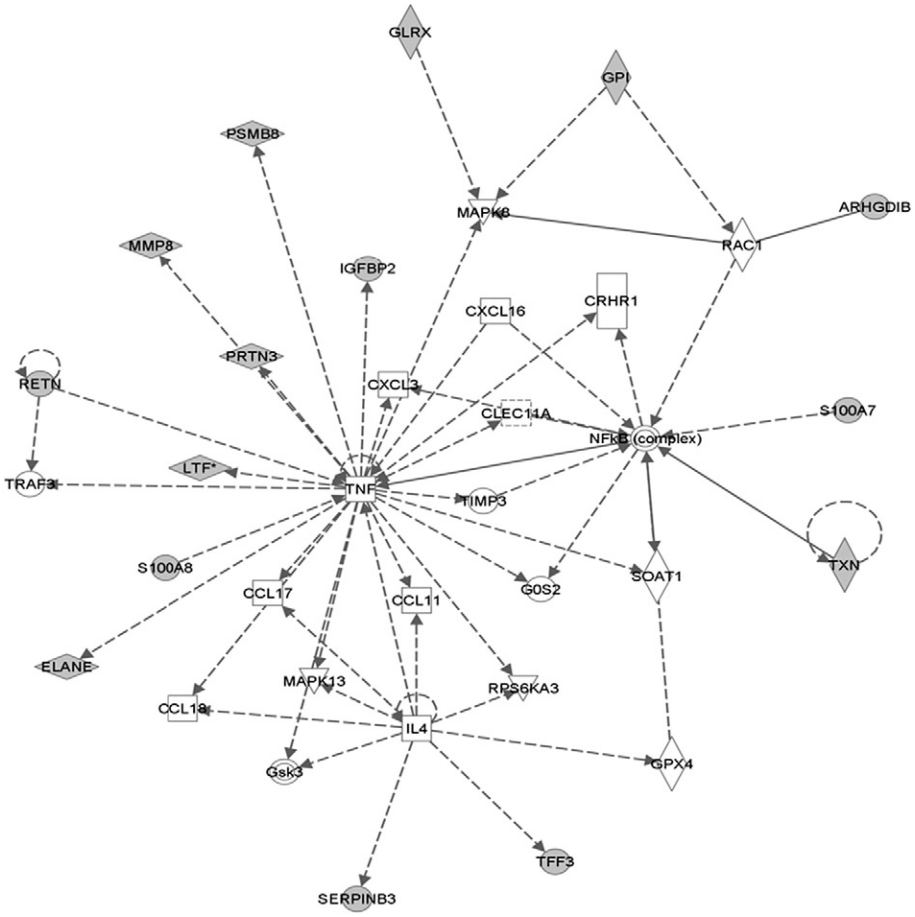

Fig. 3. A network identified by Ingenuity pathway analysis in the context of CGD is involved in inflammation with TNF and NFκB at the centers of the network. Black solid lines denote a direct relationship between two molecules. A dotted line indicates an indirect relationship. Gray node denotes identified proteins in the current study.

high molecular masses, in CGD samples (Fig. 1). The S100 family of proteins is low molecular weight calcium-binding proteins involved in the regulation of inflammation. Myeloperoxidase (MPO) and matrix metalloproteinase-9 (MMP-9) were also predominantly expressed in CGD. MPO is a neutrophil oxidative enzyme detected at higher levels in BOS patients as an indicator of increased oxidative stress [40]. Over expression of matrix metalloproteases (MMP9) was found to attenuate the pulmonary fibrosis induced by bleomycin. [41]. Detection of all these proteins specifically in CGD (not detected in controls) samples corroborates radiological and pathological observations. Ingenuity pathway analysis points to TNFα and NFκB centered inflammatory pathways. TNFα is a cytokine secreted by mononuclear cells that amplifies inflammation by modulating expression of Class I and Class II histocompatibility antigens. The role of TNFα in the defense and rejection of the transplanted lung is well documented [38]. NFκB is a transcriptional factor for inflammatory cytokines and nitric oxide synthases (NOS) and triggers elevated production of nitric oxide (NO), a known marker of lung transplant rejection. Ohmori et al. showed that an NFκB decoy containing specific p50 and p65κB binding sites, binds to NFκB transcriptional factor, which in turn inhibits NOS and alleviates lung injury in rat models [42].

We did not perform quantitative studies (such as ELISA) to validate our findings, because our objective was to develop a sensitive protocol to detect less abundant proteins for bioinformatics studies. However, we used the number of peptide hits for each protein as an indirect measure of concentration. It has been shown that pulmonary surfactant-associated protein A1 (SP-A) is down-regulated in BOS after lung transplantation [23]. The current study is the first to report that SP-D is also reduced in CGD. Since both SP-A and SP-D are hydrophilic proteins in pulmonary surfactant for host defense, decrease of these proteins may reflect the damage of type II pneumocytes in the alveoli.

Our findings confirm some of the previously reported BOS biomarkers discovered with different analytical methods and we report some differentially expressed proteins in RAS, a new subtype of CGD. Interestingly, in comparison with biomarkers proposed for BOS in the literature, some of our results did not match with the proposed changes (Table 3). For example, complement C3, apolipoprotein A-1, α2-HS-glycoprotein, leucine-rich α2-glycoprotein and α1-B-glycoprotein were reported to be increased in BOS samples [23], but were decreased in CGD (RAS) samples in the current study. The patients enrolled in this study had excessive inflammatory responses in the lung than those normally seen in BOS patients. The discrepancy between our results and the literature may indicate that it is possible to differentiate different types of chronic injury to the lungs after transplantation with sensitive proteomic approaches.

In summary, in the present study, using a small sample size, we developed a protocol to detect less abundant proteins from BAL of patients with CGD after lung transplantation. The large number of proteins detected allowed us to perform meaningful bioinformatic analysis. We have further identified many promising proteins as biomarker candidates. In addition to diagnosis, our compendium of the BAL proteome could help to better understand CGD mechanisms, monitor disease progression and in identifying novel drug targets. Our future directions involve verification and validation of biomarker candidates in a larger population of patients by utilizing multiple reaction monitoring (MRM) based LC–MS/MS assays and ELISA.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clinbiochem.2011.11.015.

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