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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.