Life Sciences 230 (2019) 1–9

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Identification of proteomic signatures associated with COPD frequent exacerbators

Pengbo Sun, Rui Ye, Cuihong Wang, Shuang Bai, Li Zhao*

Department of Respiratory Medicine, Shengjing Hospital of China Medical University, Shenyang, Liaoning 110004, PR China

ARTICLE INFO

Keywords:
COPD
Frequent exacerbators
Proteomic profile
Human lung tissue
Immune response

ABSTRACT

Aims: Acute exacerbation is a major event that alters the natural course of chronic obstructive pulmonary disease (COPD), and recurrent exacerbation results in worse clinical outcomes and greater economic consequences. While some patients suffer frequent exacerbations, others experience no exacerbations; this study was designed to detect proteins that were differentially abundant in COPD frequent exacerbators and assess whether those expression profiles are unique among COPD patients.

Main methods: Tandem mass tag labeled quantitative proteomics combined with two-dimensional liquid chromatography-tandem mass spectrometry was used to detect the changes in the lung proteome in COPD frequent exacerbators and infrequent exacerbators. A series of bioinformatics analyses were performed to screen potential signatures of COPD frequent exacerbations. The accuracy of proteomic results was further verified by western blot studies.

Key findings: Compared with infrequent exacerbators, 23 proteins in the lung tissues from frequent exacerbators showed significant degrees of differential expression; combined bioinformatics analyses of proteome indicated that the immune network for IgA production and the phenylalanine metabolism pathway were associated with frequent exacerbations. The Western blot analysis confirmed the expression pattern of three significantly regulated proteins (HLA-DQA1, pIgR and biglycan).

Significance: These findings indicate that immune response might play a key role in the pathophysiological mechanisms of COPD frequent exacerbations. Our results make a crucial contribution to the search for a comprehensive understanding of potential pathophysiological mechanisms associated with the frequent exacerbations of COPD, and might provide guidance for treating frequent exacerbations.

1. Introduction

Acute exacerbation is a crucial event that alters the natural course of chronic obstructive pulmonary disease (COPD), and is characterized by a worsening of respiratory symptoms that is beyond normal day-to-day variations, and necessitates the use of additional therapies according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [1]. These exacerbations appear to accelerate the rate of decline in lung function [2,3], which results in a deteriorating quality of life, as well as increased mortality rates and healthcare expenditures [4–6]. Investigators who participated in the Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) study suggested that individuals who experience two or more exacerbations within a given year represent a distinct frequent exacerbator phenotype that is independent of disease severity [7]. Moreover, this phenotype is now widely accepted [8] and defined as 2 or more events occurring within in a given year [1], as mentioned in GOLD guidelines.

Despite the importance of exacerbations, the cellular and molecular mechanisms responsible for the increased susceptibility to exacerbation in the frequent exacerbators phenotype have been poorly understood until now. After considering that the cascade of inflammatory events that results in the clinical development of an exacerbation episode is centered in the lungs, we hypothesized that there might be specific alteration in the protein profiles of lung tissue in frequent and infrequent exacerbators of COPD, and the unique proteins might be a target for preventing frequent exacerbations of COPD. To test our hypothesis, we collected specimens of human lung tissue from well characterized frequent and infrequent exacerbators of COPD. Tandem mass tag (TMT) labeled quantitative proteomics analysis was adopted in this research to display alteration in proteome in frequent and

* Corresponding author at: Department of Respiratory Medicine, Shengjing Hospital of China Medical University, 36 Sanhao Street, Shenyang, Liaoning 110004, PR China.
E-mail address: zhaol@sj-hospital.org (L. Zhao).

https://doi.org/10.1016/j.lfs.2019.05.047
Received 26 March 2019; Accepted 18 May 2019
Available online 20 May 2019
0024-3205/

infrequent exacerbators. This was followed by a series of bioinformatics analyses in an attempt to reveal the underlying pathological mechanism of frequent exacerbations at the protein level. To further investigate the protein expression profile associated with the frequent exacerbators phenotype in lung tissue, we used lung tissue samples of COPD patients and immunoblotting methods to verify any promising protein candidates, and provide greater sensitivity for detecting the molecular signature of frequently exacerbated COPD. We expect that this data set will provide the foundation for revealing the pathological mechanism of frequent exacerbations of COPD and developing new therapies to treat them in the clinic.

2. Materials and methods

2.1. Specimen collection

The protocol for this study was approved by the Ethics Committee of Shengjing Hospital Affiliated with the China Medical University (Shenyang, China, ethical ttno.2016PS342K). Lung tissue samples were retrieved from patients of the Shengjing Hospital. Samples of smokers and COPD were obtained from tumor-free peripheral lung in lung lobectomy surgery for local tumors. The study inclusion criteria for COPD patients were: 1) an age of 40 to 80 years old; 2) a history of 10 or more pack-years of smoking; 3) a ratio of forced expiratory volume in 1 s (FEV1) to forced vital capacity (FVC) of 70% or less after bronchodilator use [1]; 4) the clinical pulmonary condition of the COPD subjects was stable, with no acute exacerbations for at least 4 weeks prior to enrollment. The severity of airway limitation was graded based on FEV1post%, as described in the GOLD guidelines [1]. And the COPD patients were assigned to two groups based on their medical history: frequent exacerbators (FE group) and infrequent exacerbators (IE group). COPD patients in the FE group must have experienced at least two or more exacerbations per year that required treatment with additional antibiotics or a systemic steroid, or hospitalization [1]. All the other COPD patients were included in the IE group. Each exacerbation must have occurred at least 4 weeks after the end of treatment for the previous exacerbation or 6 weeks after its onset [9,10]. This allowed us to differentiate a new exacerbation from a “treatment failure” [9]. In addition, subjects with normal lung function and a smoking history of > 10 pack years were enrolled as a control group. Patients with other respiratory diseases (e.g., asthma, pulmonary fibrosis, and hilar lung cancer) or other chronic diseases such as rheumatoid arthritis and chronic colitis, were excluded from the study. Two professional pulmonologists assembled a detailed clinical history for each patient and conducted comprehensive lung function tests using standard spirometric techniques. From September 2016 to March of 2018, 49 specimens (15 control subjects, 23 IE subjects and 11 FE subjects) were collected.

Specimens of macroscopically tumor-free lung tissue were obtained from the patients described above while they were undergoing lung lobectomy surgery. Specimens were retrieved from Biobank, Shengjing Hospital of China Medical University. The specimens of lung tissue were taken as far as possible from the tumor (at least 5 cm from the tumor's edge) and appeared to be normal when inspected as previously described [11]. Additionally, the samples of lung tissue were closely paired according to tumor type and grade. Considerable effort was made to obtain approximately equal amounts of small airways, lung parenchyma and vessels. Thus, the location and amount (about 1 cm^3) of each sample were standardized [12]. The resected samples were then washed in cold normal sterile saline to any eliminate blood and dried with sterile gauze. The tissue samples were immediately frozen in liquid nitrogen and then stored at −80 °C until use.

2.2. Proteomics

2.2.1. Sample preparation

200 μg of each lung tissue sample was lysed and homogenized in SDT buffer (4% [w/v] SDS, 100 mM Tris/HCl pH 7.6, 0.1 M DTT) with a MP homogenizer. The homogenate was sonicated, boiled for 15 min, and then centrifuged at 14,000*g* for 40 min. Next, the supernatant filtrate was quantified with a BCA Protein Assay Kit (Bio-Rad, Hercules, CA, USA) and then stored at −80 °C. To introduce more sample information, all 15 raw samples in the IE group were equally mixed together by combining five samples to form three pooled samples. The same procedure was used for the control group while every two samples were equally combined to form three pooled samples in the FE group due to relative small sample size. Eventually three biological repeats were created. Subsequently, filter-aided sample preparation (FASP) digestion was processed as described previously [13]. A 100 μg mixture of peptides obtained from each sample was labeled with ten plex TMT reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Finally, a Pierce high pH reversed-phase fractionation kit (Thermo Scientific) was used to divide the TMT-labeled samples into 15 fractions via step-gradient elution with increasing concentrations of acetonitrile. The entire workflow is shown in Fig. 1.

2.2.2. LC-MS/MS analysis

A nano LC–MS/MS analysis was performed on each prepared fraction as previously described [14]. LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific) that was combined with a with Easy nLC chromatography system (Thermo Fisher Scientific Proxeon Biosystems) for 90 min. The mass spectrometer was operated in positive ion mode and MS data were dynamically obtained by using a superior data-dependent top 10 method for selecting the most abundant precursor ions from the survey scan (300–1800 *m/z*) for HCD (Higher energy collision dissociation) fragmentation. The automatic gain control (AGC) target was set to 1e6, and the maximum injection time to 50 ms. The dynamic exclusion duration was 60.0 s. Survey scans were acquired at a resolution of 70,000 at *m/z* 200, and the resolution for HCD spectra was set to 35,000 at *m/z* 200; the isolation width was 2 *m/z*. The normal energy caused by collision was 30 eV, and the underfill ratio was set at 0.1%.

2.2.3. Protein identification and quantification

The resulting spectra were searched against the Uniprot database (uniprot_human_161105_20171211.fasta) [15] and embedded into Proteome Discoverer 1.4. by using the MASCOT engine (Matrix Science, London, UK; version 2.2). The peptide and fragment mass tolerances used for protein identification were ± 20 ppm and 0.1 Da, respectively. Two missed cleavages were allowed, and full cleavage by trypsin was used. At least one unique peptide was required for protein quantification and a FDR < 1% was deemed acceptable for both the peptide and protein level. The protein ratios were calculated as the median of only unique peptides of the protein. Proteins were defined as significant regulated when the results showed a TMT ratio of > 1.2 or < 0.83 and a statistically confirmed P-value ≤0.05. Proteins with TMT ratios of > 1.2 were considered to be up-regulated, and those with TMT ratios < 0.83 were considered to be down-regulated.

2.3. Bioinformatics tools

A hierarchical clustering analysis was performed by using Pearson correlation coefficients as distance metrics and the Complexheatmap R software package (R Version 3.4). Blast2GO (Version3.3.5) [16] was used to extract GO terms associated with all significantly regulated as previously described [14], and GO enrichment was based on three aspects: molecular function, biological process, and cellular components. A KEGG pathway analysis was performed using KAAS (KEGG Automatic Annotation Server) software [14]. Fisher's exact test was used to

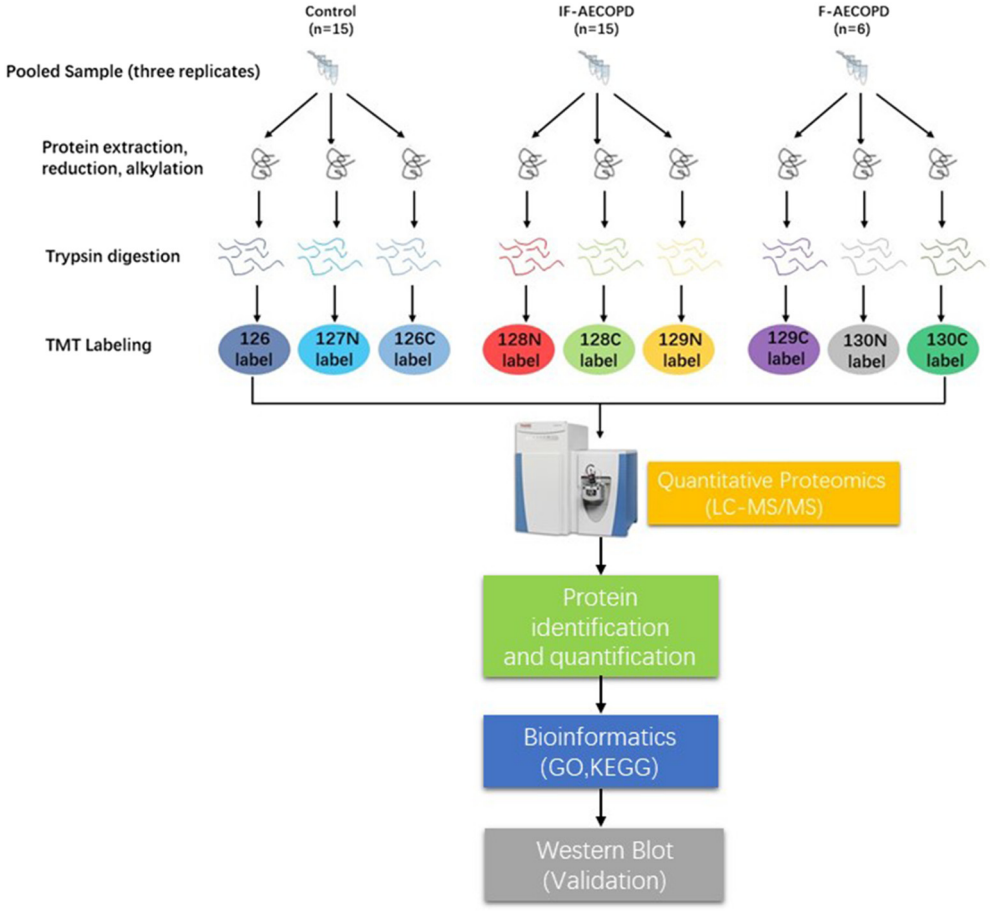

Fig. 1. The workflow for our proteome study.
IE group: infrequent exacerbators group; FE group: frequent exacerbators group.

identify pathways that were significantly enriched, and the resulting P-values were adjusted by using the Benjamini correction for multiple testing. Only the functional categories and pathways with P-values < 0.05 were considered relevant.

2.4. Western blot analysis

To further verify the results of MS, three representative proteins of interest (HLA-DQA1, pIgR, and Biglycan), among all the differentially abundant proteins identified, were selected to perform as previously described [17]. The optimized screening criteria were formulated based on the following principles: 1) proteins with potential biological function and significance; 2) proteins with a peptide fragment > 2, as identified by LC–MS/MS; 3) for those proteins wherein differences could be seen between the FE/IE groups. The primary antibodies used in the western blot studies were polyclonal rat anti-human GAPDH antibody (1:5000, Proteintech, Chicago, IL, USA), polyclonal rat anti-human β-actin antibody (1:4000, Proteintech, USA), polyclonal rabbit anti-human HLA-DQA1 antibody (1:500, Proteintech, USA), polyclonal rabbit anti-human pIgR antibody (1:500, Abcam, Cambridge, MA, US), and polyclonal rabbit anti-human biglycan antibody (1:800, Abcam, USA). Quantification of immunostaining was performed by using Image J software. Three assays were performed for each experimental sample.

3. Statistical analysis

All statistical analyses were performed using SPSS Statistics for Windows, Version 17.0 (SPSS Inc., Chicago, IL, USA). Measurement data are expressed as the mean ± standard deviation (SD) of results. The independent Student's *t*-test was used to compare values obtained for continuous variables in two different groups and one-way ANOVA was used for comparing values in three different groups. Data with a non-normal distribution were analyzed using the Mann-Whitney *U* test or Kruskal-Wallis H test. Categorical variables were compared using the Pearson's Chi-square test or Fisher's exact test. A P-value < 0.05 was considered statistically significant.

4. Results

4.1. Demographics of the clinical subjects

From September 2016 to March of 2018, we enrolled 49 subjects (15 control subjects, 23 IE subjects and 11 FE subjects). There were no significant differences in the age and gender of the subjects in the discovery and validation cohort; however, there was a clear male predominance. There are 15 control subjects, 15 IE subjects and 6 FE subjects in the discovery cohort. 8 IE subjects and 5 FE subjects were additionally added in the validation cohort. The demographic and clinical data for subjects in the discovery and validation cohort are presented in Tables 1 and 2. Lung tissue samples of subjects enrolled in the validation cohort were included in the western blot studies.

4.2. Differentially abundant proteins discovered in lung tissue

Proteins that were differentially abundant in the IE and FE patients were identified and analyzed. Our MS results identified 25,927 peptide fragments (Supplementary Table S1) that were derived from 4506 corresponding proteins (Supplementary Table S2). The fold-changes of

Table 1
Demographic description of the Discovery cohort.

	IE group (n = 15)	FE group (n = 6)	Control (n = 15)	P-value*
Gender M (M%)	12 (80%)	5 (83.3%)	12 (80%)	1.0
Age (mean ± SD) years	63.80 (± 5.91)	67.3 (± 4.08)	63.3 (± 5.64)	0.321
Smoke history (pack-years)	41.60 (± 23.30)	38.33 (± 13.29)	45 (± 22.60)	0.802
GOLD Stage I (%)	6	0	N.A	0.147
GOLD Stage II (%)	7	5	N.A	
GOLD Stage III (%)	2	1	N.A	

M: male. N.A: not applicable. SD: standard deviation.
* P-value: Fisher's test for categorical variables and one-way ANOVA for continuous variables.

Table 2
Demographic description of the validation cohort.

	IE group (n = 23)	FE group (n = 11)	P-value*
Gender M (M%)	20 (87.0%)	9 (81.8%)	1.0
Age (mean ± SD) years	63.69 (± 5.93)	66.18 (± 4.47)	0.279
Smoke history (pack-years)	38.87 (± 21.54)	36.36 (± 14.33)	0.093
GOLD Stage I (%)	10	1	0.079
GOLD Stage II (%)	11	7	
GOLD Stage III (%)	2	3	

M: male. SD: Standard deviation.
* P-value: Fisher's test for categorical variables and Mann-Whitney-Wilcoxon rank sum test for continuous variables.

significantly regulated in the control, IE and FE groups were determined based on the intergroup ratios reported in the TMT results. Proteins that showed expression changes consistent with the following screening criteria were regarded as differentially abundant: a > 1.2-fold or < 0.83-fold change in expression (up or down regulated, respectively) and a P-value < 0.05. Comparisons made between the FE/IE, IE/Control, and FE/Control groups identified 23 differentially abundant proteins (including 9 that were up-regulated and 14 that were down-regulated), 39 differentially abundant proteins (19 that were up-regulated and 20 that were down-regulated) and 28 differentially abundant proteins (21 that were up-regulated and 7 that were down-regulated), respectively (Supplementary Table S3). The DAPs (differentially abundant proteins) identified in the FE and IE group are shown in Table 3.

4.3. Bioinformatics analysis

Bioinformatics analysis was conducted for all the differentially abundant proteins identified by MS.

4.3.1. Cluster analysis

The cluster analysis was performed for the preselected set of differentially abundant proteins. A tree heat map was constructed showing results of the hierarchical cluster analysis (Fig. 2), with red representing upregulation and blue indicating down-regulation. The X- and Y-coordinates represented the sample and differentially abundant proteins, respectively. A horizontal comparison showed that the samples could be classified into two groups: FE and IE group, respectively. This classification indicated that the selected differentially abundant proteins could effectively be used to distinguish samples with high accuracy. In addition, a vertical comparison suggested that the proteins could be classified into two categories with opposite directional variation, which revealed the expression patterns of significantly regulated proteins in the two groups, and indicated that the selected differentially abundant proteins were reasonable. Thus, the cluster analysis supported the accuracy and rationality of the significantly regulated proteins selection process used in this study.

4.3.2. GO annotations and pathway analysis

In our study, the total DAPs in the IE and FE groups corresponded to 880 functional annotations (Supplementary Table S4). The GO annotations could be classified into three major categories, which included biological process, molecular function, and cell component, and each protein might be noted with more than one functional annotation. The results showed that in terms of biological process, 20 proteins were annotated as cellular process, with the other three major categories being biological regulation (16 proteins), response to stimulus (14 proteins), and immune system process (8 proteins) (Fig. 3A). The top three molecular functions were binding (19 proteins), catalytic activity (3 proteins), and molecular transducer activity (4 proteins) (Fig. 3B). In the cell components category, the DAPs were concentrated in cell part (20 proteins), organelle (20 proteins), and cell part (20 proteins)

Table 3
Differentially abundant proteins in frequent exacerbators and infrequent exacerbators of COPD.

Accession	Description	Gene name	FE/IE ratio	*t*-Test P value
A0A142L067	MHC class II antigen	HLA-DQB1	0.20818458	0.001280582
A0A024F8S6	HLA-DRB5 protein	HLA-DRB5	0.299674906	0.006890417
Q59GC2	Erythrocyte membrane protein band 4.1 like 4B variant		0.508722442	0.025633709
H0YMP5	Arpin	ARPIN	0.628794653	0.009535997
O43818	U3 small nucleolar RNA-interacting protein 2	RRP9	0.663507158	0.004151627
B4DM05	cDNA FLJ51241, highly similar to Nidogen-1		0.702485841	0.046147777
P30838	Aldehyde dehydrogenase, dimeric NADP-preferring	ALDH3A1	0.708331065	0.010460676
P08729	Keratin, type II cytoskeletal 7	KRT7	0.767582076	0.009179271
B4DT61	Syndecan		0.772083061	0.031293239
A8K3J8	cDNA FLJ75332, highly similar to *Homo sapiens* Ras-related associated with diabetes (RRAD)		0.800896161	0.011878838
Q8TD06	Anterior gradient protein 3	AGR3	0.823000019	0.018797085
Q59FZ8	Nebulette non-muscle isoform variant		0.82401559	0.01742316
Q0P607	Splicing factor, arginine/serine-rich 15	SFRS15	0.828375086	0.000824778
A0A087WU03	Heterogeneous nuclear ribonucleoprotein D-like	HNRNPDL	0.829590169	0.049352112
E7EMN6	Protein phosphatase inhibitor 2	PPP1R2	1.202531263	0.01534761
P81605	Dermcidin DCD	DCD	1.253916255	0.029421513
P01833	Polymeric immunoglobulin receptor	PIGR	1.265718204	0.027232195
A6NLG9	Biglycan	BGN	1.295683153	0.047350059
Q0ZCF6	Immunglobulin heavy chain variable region		1.311548136	0.043743403
Q05BJ5	STIM2 protein	STIM2	1.314525493	0.041870712
C9JKG1	Biglycan	BGN	1.464843561	0.038073177
O78117	MHC class II antigen	HLA-DR	1.524844878	0.034962222
A0A0F6R8Y9	MHC class II antigen	DQA1	1.929537714	0.009955157

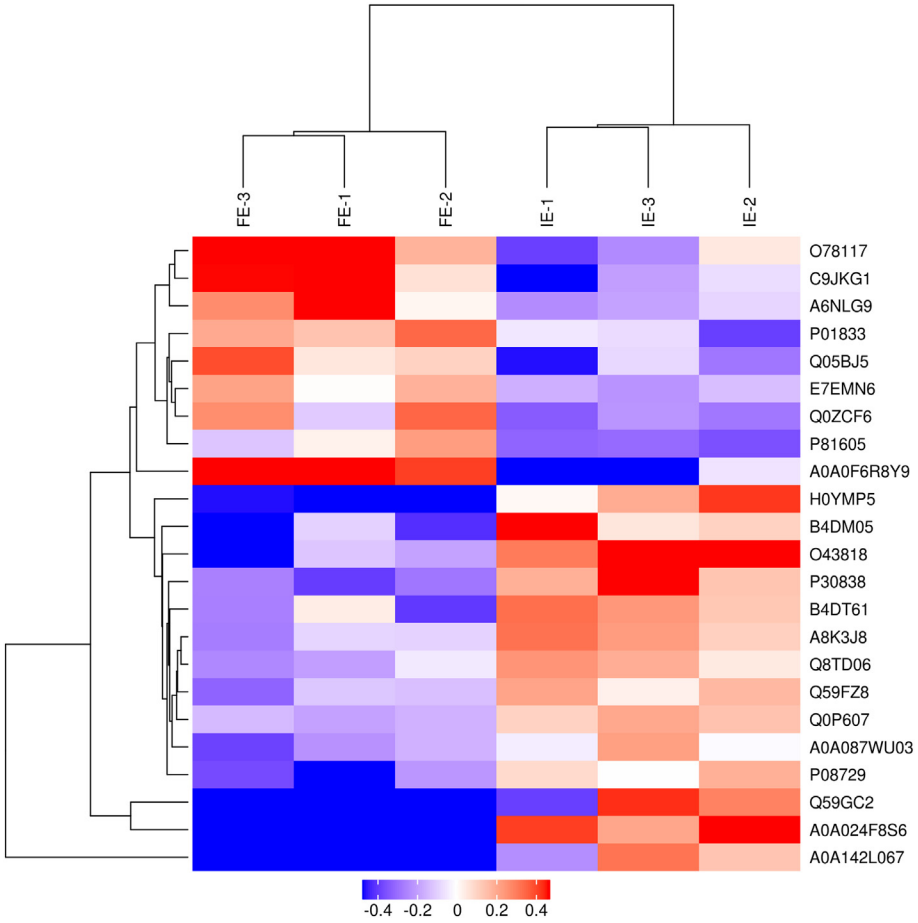

Fig. 2. Cluster Analysis. Cluster analysis indicated that differentially abundant proteins screened out were reasonable and accurate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3C). As for GO terms enrichment, the top 20 most represented terms for the total GO category, which included biological process, molecular function, and cellular component, are shown in Fig. 4. The biological process classifications for those DAPs were enriched in the areas of proteoglycan biosynthetic process, aminoglycan biosynthetic process, and chondrotin sulfate proteoglycan metabolic process. Additionally, based on the cell component classifications, the DAPs were enriched in the areas of lytic vacuole, lysosome, vacuole, and clathrin-coated endocytic vesicle membrane. Enriched molecular function classifications of the DAPs included store-operated calcium channel activity, polymeric immunoglobulin receptor activity, and immunoglobulin receptor activity, which suggests that the immune response might play a vital role in the pathogenesis of frequent exacerbations. In addition, to further understand the major biological pathways and relevant regulatory processes that involved DAPs in infrequent exacerbators and frequent exacerbators, KEGG pathway and enrichment analyses were performed by using the Kyoto Encyclopedia of Genes and Genomes database. The results indicated that the intestinal immune network for IgA production and the phenylalanine metabolism signaling pathway had the highest enrichment of DAPs in FE/IE (Supplementary Table S5). Two upregulated proteins (the polymeric immunoglobulin receptor and MHC class II antigen fragment) were identified as important signaling molecules in the immune network for IgA production pathway (Table 4). These two proteins were closely related to immune system processes as annotated in the GO analysis.

4.4. Western blot analysis to validate the results of proteomics

To validate the accuracy of our TMT results, western blot analyses were performed to examine the relative abundance of three candidate proteins (HLA-DQA1, pIgR, and Biglycan) found in human lung tissues of validation cohort. In the validatioh cohort, our results showed that the 3 proteins were differentially abundant in the lung tissues from F-E and IE patients (Fig. 5). HLA-DQA1, pIgR, and Biglycan were significantly up-regulated in the FE group ($P < 0.05$) when compared with their expression in the IE group, which is consistent with our MALDI-TOF MS data. These results verified the accuracy of our MALDI-TOF MS data.

5. Discussion

COPD is a heterogenous disease that is characterized by different susceptibility to acute exacerbation. Exacerbations accelerate the decline in lung function ([2,3], resulting in reduced physical activity, poorer quality of life, an increased risk of death, and a large proportion of the health care costs ([4–6]. Although exacerbations become more frequent and severe as COPD progresses, the rate at which they occur appears to reflect an independent susceptible phenotype [7]. Furthermore, it has been postulated that there is a frequent-exacerbation phenotype of COPD that is independent of disease severity. Considering that the cellular and molecular mechanisms responsible for increased susceptibility to exacerbations in the frequent exacerbators phenotype have been poorly understood, we investigated the proteomics of lung tissue specimens obtained from infrequent exacerbators and frequent exacerbators of COPD for evidence of significantly regulated proteins.

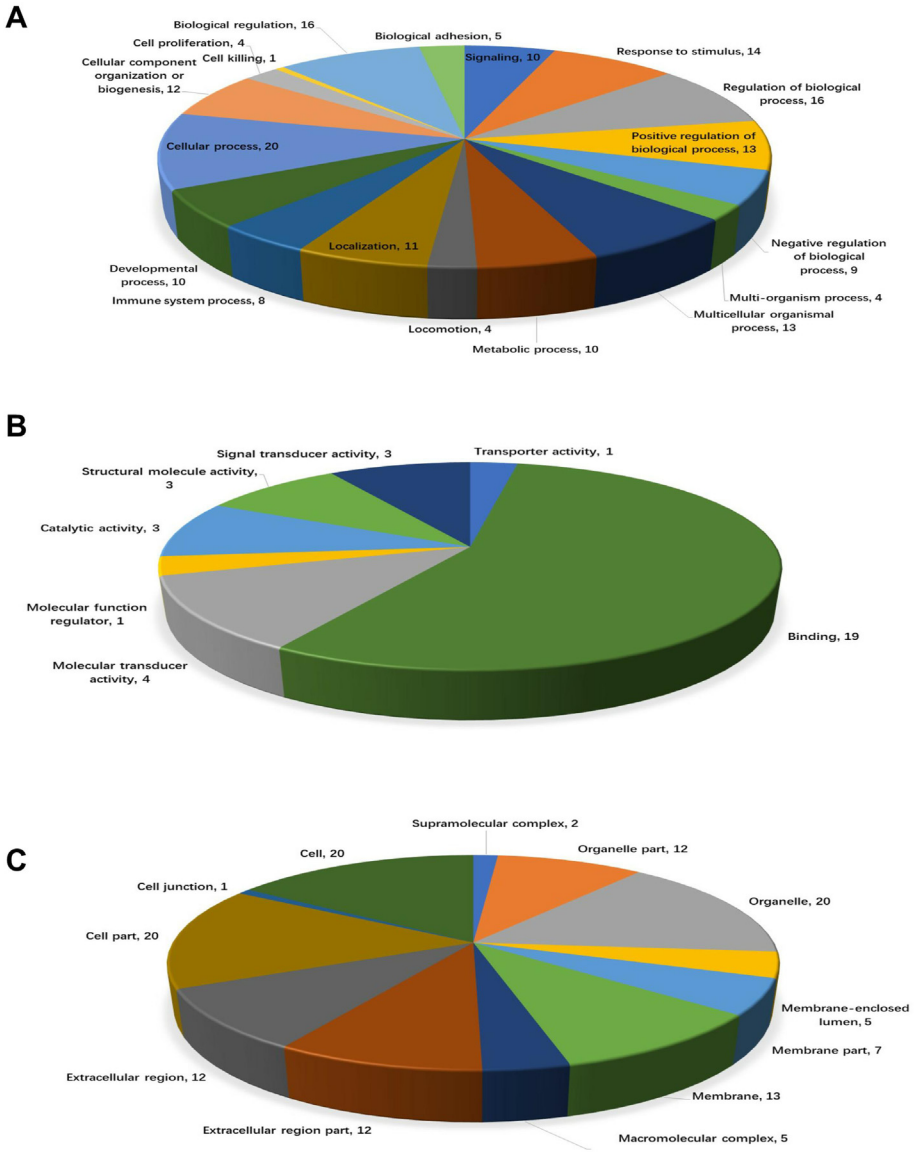

Fig. 3. Gene ontology annotation. The differentially abundant proteins are mainly annotated as cellular process, protein binding and cell part in terms of biological process, molecular function and cell component, respectively. (A) Biological process; (B) molecular function; (C) cell component.

We hypothesized that such proteins may represent one mechanism involved in frequent exacerbations and further provide a basis for treating COPD frequent exacerbations. To the best of our knowledge, the results of our present study constitute the first comparison of differnetially abundant lung tissue proteins in FE and IE patients. Pooled lung tissue samples labeled with stable isotopes (TMT10-plex) were analyzed by mass spectrometry, and comparisons between FE and IE group were carried out. Those comparisons identified 23 unique proteins that were differentially abundant; among which, 9 were upregulated and 14 were down-regulated. The significantly regulated proteins detected by TMT analysis included proteins classically involved in the immune response, cell adhesion, and other biological functions. Furthermore, to guarantee the accuracy of our results, three significantly regulated proteins (HLA-DQA1, pIgR and Biglycan) were selected to be validated by western blot methodology in both discovery and validation cohort. Based on significant up- or down-regulation in the TMT experiments, identification and quantification with two or more statistically significant peptides, and a biological function that might be implicated in the onset mechanism of COPD frequent exacerbations.

Previous studies have mainly focused on COPD proteomics [18–21], and ignored the fact that COPD is a heterogeneous disease with different phenotypes [22–24]. In contrast, we took this factor into consideration and divided the enrolled COPD subjects into FE and IE groups, and then compared those two groups in our study. Interestingly, Dave Singh et al. [25] performed microarray gene expression and PCR studies that investigated gene expression profiles in sputum and blood cells that might be associated with frequent exacerbations. They identified 3 genes (B3GNT, LAF4 and ARHGEF10) that predicted frequent exacerbations, and several of the highly regulated genes identified in their study, such as HLA-DQA1 and HLA-DQB1, coincide with the corresponding significantly regulated proteins in our study. This finding suggests that those previously identified genes might be involved in the physiological mechanism of COPD frequent exacerbations.

In the present study, we focused on rather specialized interaction molecules: the MHC class II complex (HLA-DQA1), pIgR, and Biglycan. As specialized recognition molecules, the MHC class II complex (HLA-DQA1) was identified as cell surface proteins that are loaded with antigenic peptides and recognized by the T-cell receptor (TCR) on T-cells. They initiate or propagate acquired immunity [26], and thereby dictate the ability of an organism to respond to specific antigenic stimuli. While

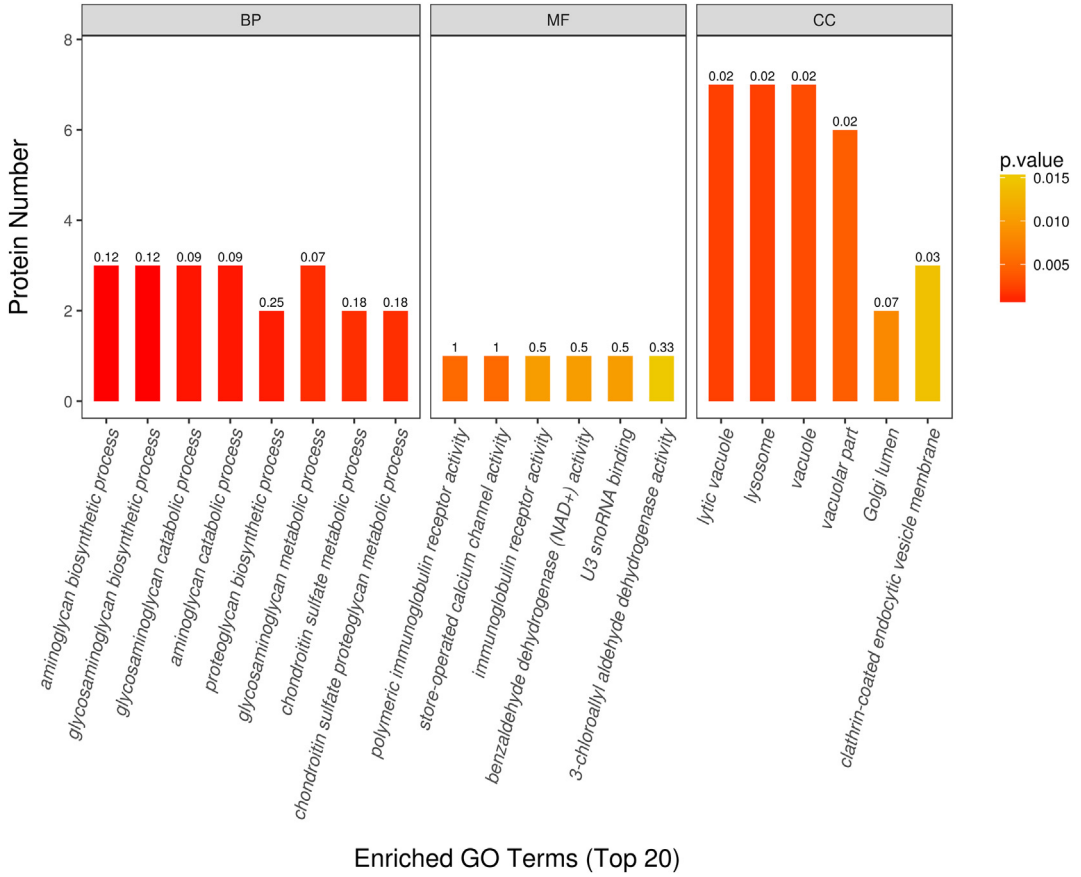

Fig. 4. Gene Ontology (GO) enrichment analysis of the significantly up and down-regulated proteins in FE/IE. Complete enrichment analysis and the proteins associated with these terms are provided in Supplement Table S4.

Table 4
Differentially abundant proteins in intestinal immune network for IgA production pathway.

Protein ID	Protein	Fold change	P value
A0A0F6R8Y9	MHC class II antigen	1.929537714	0.009955
P01833	Polymeric immunoglobulin receptor	1.265718204	0.027232

Dave Singh et al. [25] found decreased expression of HLA-DQA1 in the sputum and blood of frequent exacerbators when compared to infrequent exacerbators, we hypothesize that the proinflammatory microenvironment induces MHC class II expression on the airway epithelium; which in turn, might participate in a localized immune reaction that contributes to the progression of frequent COPD exacerbations. This issue will be the subject of further analyses. This being so, we can assume that MHC class II expression in lung tissue is associated with inflammatory processes. Human leukocyte antigen (HLA-DQA1) expression might be a predictor of frequent COPD exacerbation. And the polymeric immunoglobulin receptor (pIgR) plays a major role in reacting with antigens distributed over the huge area of mucosal surfaces that comprise the digestive, respiratory, and urogenital tracts [27,28], and participates in the generation of protective secretory IgA. IgA is the most abundant immunoglobulin found in mucosal secretions, and participates in frontline defense mechanisms found in the respiratory tract, along with mucociliary clearance [29]. Biglycan is a stationary component of the ECM, and can be found in most tissues. It is a member of the family of small proteoglycans, has LRR motifs similar to TLRs and NLRs [30,31], and interacts with TLR2/4 on macrophages. Soluble biglycan induces the NLRP3/ASC inflammasome; thereby activating caspase-1 and releasing mature IL-1β without the participation of additional costimulatory factors [32]. Among all the candidates identified, three significantly regulated proteins were chosen to be validated by western blot analysis. Their similar trends of expression validated the accuracy of the proteomics in this study.

We developed a set of strict criteria for selecting subjects who were eligible for enrollment in our study. Considering that human lung tissue has greater sensitivity for detecting the molecular signature of COPD frequent exacerbators than serum, which might be affected by complex systemic factors, we chose human lung tissue for quantitative proteomics analysis. Due to the difficulty of acquiring human lung tissue and the fact that it was a monocentric study, we've painstakingly recruit a rational number of subjects for proteomics analysis compared with previous studies [12,20,33]. To eliminate individual differences within the group and accurately reflect the differences between groups, and in order to be able to analyze more different individual samples instead of repeating the only samples in the group, a mixed sample was selected. In addition, our data set of our proteomics research will be shared online freely, available to more researchers majoring in COPD exacerbation. Despite our promising results, we acknowledge that our study has several limitations that should be mentioned. First of all, the sample size of validation cohort is relatively small, and larger number of samples might be more convincible. Second, comparison between exacerbation stage and stable stage for frequent exacerbators is absent in our present study due to that lung tissue sample during exacerbation stage is not accessible.

6. Conclusion

Collectively, this study describes the first use of TMT tenplex™ in the context of COPD frequent exacerbations. This technology permitted the quantification of 4506 proteins. In addition, we were able to identify 23

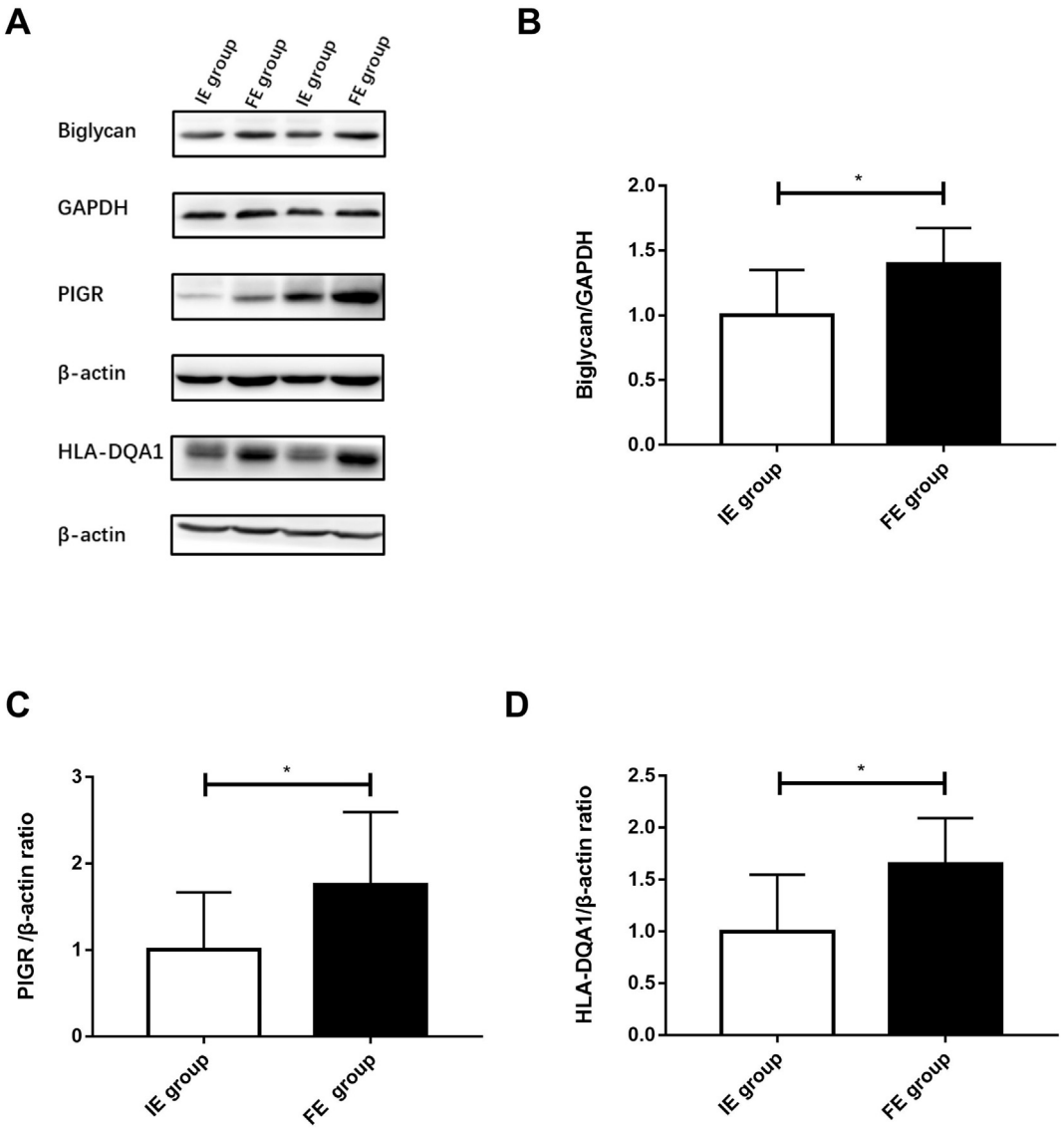

Fig. 5. Verification of the candidates by Western Blot analysis in the validation cohort (A) Representative western blotting images. (B) Quantitative analysis of Biglycan. (C) Quantitative analysis of PIGR. (D) Quantitative analysis of HLA-DQA1. GAPDH or β-actin was run as an internal standard. Values are mean ± SD , *P < 0.05,compared with IE group, ***P < 0.001, compared with IE group. Independent *t*-test, Welch's *t*-test, or Mann-Whitney *U* test was used for data analysis.

proteins that were differentially abundant in the lung tissues of FE and IE patients. We validated three of the significantly regulated proteins (HLA-DQA1, PIGR, and Biglycan) by western blot analysis, and found that there is significant difference of the innate and acquired immunity response level between infrequent and frequent COPD exacerbators. Application of this process would permit identification of differences in population characteristics and clinical outcomes between different exacerbation subpopulations and provides new insights into the mechanism of frequent COPD exacerbations, and enables physicians to better diagnose and manage patients suffering from this pathology. However, the association between significantly regulated proteins and frequent COPD exacerbations, as well as the precise molecular mechanisms involved in those exacerbations, requires our additional verification through further studies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.05.047.

Authors' contributions

Design and conduct of the study: Pengbo Sun, Rui Ye, Cuihong Wang, Li Zhao. Data collection and analysis: Shuang Bai, Cuihong Wang, Rui Ye, Pengbo Sun. Data interpretation: Pengbo Sun, Shuang Bai. Manuscript writing: Pengbo Sun, Li Zhao. All authors have read and approved the final version of the article.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

Funding: This work was supported by the Nature Science Foundation of Liaoning Province [2015020548].

References

[1] C.F. Vogelmeier, G.J. Criner, F.J. Martinez, A. Anzueto, P.J. Barnes, J. Bourbeau, et al., Global strategy for the diagnosis, management, and prevention of chronic obstructive lung disease 2017 report. GOLD executive summary, Am. J. Respir. Crit. Care Med. 195 (2017) 557–582.

[2] G.C. Donaldson, T.A. Seemungal, A. Bhowmik, J.A. Wedzicha, Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease, Thorax 57 (2002) 847–852.
[3] R.E. K, N.R. A, J.E. C, Lower respiratory illnesses promote FEV(1) decline in current smokers but not ex-smokers with mild chronic obstructive pulmonary disease: results from the lung health study, Am. J. Respir. Crit. Care Med. 164 (2001) 358–364.
[4] Seemungal T, Donaldson G, Ea, Bestall J, Jeffries D, Wedzicha J. Effect of exacerbation on quality of life in patients with chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med.. 1998;157:1418–22.
[5] J.J. Soler-Cataluna, M.A. Martinez-Garcia, P.R. Sanchez, E. Salcedo, M. Navarro, R. Ochando, Severe acute exacerbations and mortality in patients with chronic obstructive pulmonary disease, Thorax 60 (2005) 925–931 (Respiratory Medicine Copd Update. 2006;1:104-).
[6] S.D. Sullivan, S.D. Ramsey, T.A. Lee, The economic burden of COPD, Chest 117 (2000) 5S.
[7] J.R. Hurst, J. Vestbo, A. Anzueto, N. Locantore, H. Mullerova, R. Tal-Singer, et al., Susceptibility to exacerbation in chronic obstructive pulmonary disease, N. Engl. J. Med. 363 (2010) 1128–1138.
[8] O. Keene, J. Roger, Temporal clustering of exacerbations in chronic obstructive pulmonary disease, Am. J. Respir. Crit. Care Med. 182 (2010) 984–985 (author reply 5).
[9] J.J. Soler-Cataluna, R. Rodriguez-Roisin, Frequent chronic obstructive pulmonary disease exacerbators: how much real, how much fictitious? Copd 7 (2010) 276–284.
[10] S. Spencer, P.W. Jones, Time course of recovery of health status following an infective exacerbation of chronic bronchitis, Thorax 58 (2003) 589–593.
[11] H. Bi, J. Zhou, D. Wu, W. Gao, L. Li, L. Yu, et al., Microarray analysis of long noncoding RNAs in COPD lung tissue, Inflamm. Res. 64 (2015) 119–126.
[12] S.G. Kelsen, X. Duan, R. Ji, O. Perez, C. Liu, S. Merali, Cigarette smoke induces an unfolded protein response in the human lung: a proteomic approach, Am. J. Respir. Cell Mol. Biol. 38 (2008) 541.
[13] J.R. Wisniewski, A. Zougman, N. Nagaraj, M. Mann, Universal sample preparation method for proteome analysis, Nat. Methods 6 (2009) 359–362.
[14] H. Jiang, W. Bai, W. Wang, K. Wang, J. Jia, J. Zhang, et al., Proteomics in plasma of ovariectomized rats and those exposed to estradiol valerate, J. Steroid Biochem. Mol. Biol. 178 (2018) 1–12.
[15] S. Pundir, M. Magrane, M.J. Martin, C. O'Donovan, Searching and navigating UniProt databases, Curr. Protoc. Bioinformatics 50 (2015) 1.27.1–10.
[16] S. Götz, J.M. García gómez, J. Terol, T.D. Williams, S.H. Nagaraj, M.J. Nueda, et al., High-throughput functional annotation and data mining with the Blast2GO suite, Nucleic Acids Res. 36 (2008) 3420–3435.
[17] C. Yang, L. Li, J. Guo, W. Zhang, W. Zhu, X. Rao, et al., Up-regulation of Pim-3 in Chronic Obstructive Pulmonary Disease (COPD) patients and its potential therapeutic role in COPD rat modeling, Pathol. Res. Pract. 213 (2017) 322–326.
[18] A. Baralla, A.G. Fois, E. Sotgiu, E. Zinellu, A.A. Mangoni, S. Sotgia, et al., Plasma proteomic signatures in early chronic obstructive pulmonary disease, Proteomics Clin. Appl. 12 (2018) e1700088.
[19] A.L.T. Loi, S. Hoonhorst, C. van Aalst, J. Langereis, V. Kamp, S. Sluis-Eising, et al., Proteomic Profiling of Peripheral Blood Neutrophils Identifies Two Inflammatory Phenotypes in Stable COPD Patients, vol. 18, (2017), p. 100.
[20] S. Ohlmeier, P. Nieminen, J. Gao, T. Kanerva, M. Ronty, T. Toljamo, et al., Lung tissue proteomics identifies elevated transglutaminase 2 levels in stable chronic obstructive pulmonary disease, Am. J. Physiol. Lung Cell. Mol. Physiol. 310 (2016) L1155–L1165.
[21] B. Titz, A. Sewer, T. Schneider, A. Elamin, F. Martin, S. Dijon, et al., Alterations in the sputum proteome and transcriptome in smokers and early-stage COPD subjects, J. Proteome 128 (2015) 306–320.
[22] Y.H. Chen, W.Z. Yao, Chronic obstructive pulmonary disease phenotypes, Zhonghua Jie He He Hu Xi Za Zhi 34 (2011) 619.
[23] A. Kania, R. Krenke, K. Kuziemski, M. Czajkowska-Malinowska, N. Celejewska-Wojcik, B. Kuznar-Kaminska, et al., Distribution and characteristics of COPD phenotypes - results from the Polish sub-cohort of the POPE study, Int. J. Chron. Obstruct. Pulmon. Dis. 13 (2018) 1613–1621.
[24] S.I. Rennard, COPD heterogeneity: what this will mean in practice, Respir. Care 56 (2011) 1181–1187.
[25] D. Singh, S.M. Fox, R. Talsinger, S. Bates, J.H. Riley, B. Celli, Altered gene expression in blood and sputum in COPD frequent exacerbators in the ECLIPSE cohort, PLoS One 9 (1932) e107381-e.
[26] E.S. Trombetta, I. Mellman, Cell biology of antigen processing in vitro and in vivo, Annu. Rev. Immunol. 23 (2005) 975–1028.
[27] P. Brandtzaeg, F.E. Johansen, Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties, Immunol. Rev. 206 (2005) 32–63.
[28] C.S. Kaetzel, The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces, Immunol. Rev. 206 (2005) 83–99.
[29] C. Pilette, S.R. Durham, J.P. Vaerman, Y. Sibille, Mucosal immunity in asthma and chronic obstructive pulmonary disease: a role for immunoglobulin A? Proc. Am. Thorac. Soc. 1 (2004) 125–135.
[30] R.V. Iozzo, The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins, J. Biol. Chem. 274 (1999) 18843–18846.
[31] L. Schaefer, R.V. Iozzo, Biological functions of the small leucine-rich proteoglycans: from genetics to signal transduction, J. Biol. Chem. 283 (2008) 21305–21309.
[32] A. Babelova, K. Moreth, W. Tsalastra-Greul, J. Zeng-Brouwers, O. Eickelberg, M.F. Young, et al., Biglycan, a danger signal that activates the NLRP3 inflammasome via toll-like and P2X receptors, J. Biol. Chem. 284 (2009) 24035–24048.
[33] M. Yang, M. Kohler, T. Heyder, H. Forsslund, H.K. Garberg, R. Karimi, et al., Long-term smoking alters abundance of over half of the proteome in bronchoalveolar lavage cell in smokers with normal spirometry, with effects on molecular pathways associated with COPD, Respir. Res. 19 (2018) 40.