Identification of RPSA as a Potential Biomarker in Bronchoalveolar Lavage Fluid for Acute Respiratory Distress Syndrome

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Abstract

Background

Acute respiratory distress syndrome (ARDS) is a life-threatening condition leading to severe pulmonary injuries, and proteomic analysis of bronchoalveolar lavage fluid (BALF) might elucidate potential biomarkers for diagnosis and targets for treatment of ARDS.

Methods

Through iTRAQ analysis, we investigated paired BALF samples from three ARDS patients in the acute and recovery phases. The proteins sharing the same expression patterns between the two ARDS phases among different patients were determined as co-upregulated and co-downregulated proteins (CUDPs), and differentially expressed proteins (DEPs), whose fold change > 1.2 and P value < 0.05 , were selected from CUDPs. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were applied to determine the enriched functions and pathways of the CUDPs. Protein-protein interaction (PPI) network was generated at STRING database, and hub genes were identified by the Cytoscape software. A549 cells were treated by lipopolysaccharide (LPS) to simulate **alveolar** epithelial cells in ARDS.

Results

We identified 374 CUDPs and 53 DEPs. The GO analysis indicated that the most significantly enriched function was neutrophil mediated immunity response, and the KEGG analysis revealed that the 374 CUDPs were most significantly enriched in Coronavirus disease COVID-19 interaction. RPSA was discovered as the most top hub gene among DEPs, and was downregulated at protein levels during ARDS recovery. Moreover, we further confirmed that both RNA and protein level of RPSA increased upon inflammatory stimulation in vitro.

Conclusion

Our results proposed RPSA as a candidate for biomarker and therapeutic target of ARDS.

Introduction

Acute respiratory distress syndrome (ARDS) involves diffuse alveolar damage (DAD) caused by acute and uncontrolled inflammation under the action of multiple internal and external lung injury factors such as severe infection, shock, and trauma[1]. It is a heterogeneous syndrome with complex pathophysiologic mechanisms that endangers human health and with a global incidence of about 3 million people per year, accounting for more than 10% of the number of patients treated in the ICU[2, 3]. Although the diagnosis and treatment concept and respiratory support strategies of ARDS have been continuously updated and optimized in recent years, there has been no substantial breakthrough or improvement in the overall prognosis of ARDS patients, and the case fatality rate is still $>40\%$ [4], and even as high as 60% in one

study[5]. Therefore, ARDS remains a hot issue in global clinical research. The underlying cellular and molecular mechanisms are unclear, and no targeted drug is available for ARDS treatment.

Proteomics is a powerful approach in life science research to detect protein changes and identify new targets[6, 7]. Isobaric tags for relative and absolute quantitation (iTRAQ) is one of the new techniques used in modern proteomics, coupling stable isotopes labeling and tandem mass spectrometry to compare the relative amount of proteins from paired samples in a single experiment[8]. The combination of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and iTRAQ is effective and widely used in quantitative proteomics research, with high sensitivity and repeatability[9-11].

The literature suggests that ARDS represents a heterogeneous assemblage of pathophysiological and partially overlapping processes leading to lung injury[12-14]. The 37/67-kDa high-affinity laminin receptor (LAMR1, also known as RPSA) is a non-integrin cell surface receptor for laminin and the main component of the basement membrane. Brassart et al.[15] reported that RPSA was associated with neurodegenerative diseases. Givant-Horwitz et al.[16] found that RPSA is involved in regulating the MAPK signaling pathway. Vania et al.[17] showed that RPSA is involved in maintaining cell viability through apoptotic evasion, allowing tumor progression. RPSA is a complex protein with diverse functions and plays a major role in an inflammatory response, mediating cell adhesion, migration, proliferation, and survival[18-20].

Overall, the analysis of the proteomic changes behind ARDS and RPSA has not been reported. Therefore, this study aimed to examine the proteomics of ARDS using iTRAQ. This work might lead to a more comprehensive understanding of the mechanisms underlying ARDS and the discovery of new pathways and molecules that can be used clinically.

Materials And Methods

Patients and sample collection

From the perspective of the clinical outcomes, this study examined three pairs of BALF samples from patients diagnosed with ARDS type I in the acute and recovery phases (Table 1). The BALF samples of acute inflammation-phase and the hyperplasia recovery-phase ARDS were obtained from clinically indicated bronchoscopies with excess material made available for the study. BAL (100 ml of normal saline) was performed using a standard protocol in either the right middle lobe or left upper lobe (lingual). For this study, the patients were grouped based on the timing of the bronchoscopy: conducted in either the acute inflammation phase of ARDS (days 1-3) or the recovery phase (days 4-8), referenced to the initiation of mechanical ventilation (designated day 1). The APACHE-II scores were calculated to assess the severity of illness on the day of bronchoscopy for patients with ARDS, as previously described[21].

Protein extraction and quality control

Figure 1 shows the framework of this study. The high abundance proteins were removed using the Proteominer kit (Bio-Rad, Hercules, CA, USA). The proteins were reduced and alkylated with 10 mM dithiothreitol (DTT) and 55 mM iodoacetamide (IAM) followed by precipitation with five volumes of cold acetone at -20°C for ≥ 2 h. After sonication, the samples were centrifuged at 25,000 $\times g$ for 15 min at 4°C to remove debris. The protein concentration was determined using the Bradford assay. Proteins (10 μg) were mixed with loading buffer, heated at 95°C for 5 min, centrifuged at 25,000 $\times g$ for 5 min, and were separated by 12% SDS polyacrylamide gel. After electrophoresis, the gel was stained and de-stained by a protein staining instrument for 10 min. The images were scanned. Dyeing and stripping instrument: L00657C, Genscript, China; Gluesweeper: 2100XL, UMAX, China; Electrophoresis: POWERPAC BASIC, BIO-RAD, USA

Protein digestion, iTRAQ labeling, and peptide fractionation

Total protein (100 μg) was added to a 1.5-mL centrifuge tube. Trypsin enzyme (Hualishi Scientific, Shanghai, China) was added 1:20, vortexed, centrifuged at a low speed 12,000 $\times g$ for 1 min, and incubated at 37°C for 4 h. The peptide solution was frozen-dried after salt removal. The tryptic peptides for each pooled sample were labeled using one of the 8-plex iTRAQ reagents (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. In the 8-plex labeling, reporters 113, 115, and 117 were used for duplicates of the pooled acute ARDS samples, and reporters 114, 116, and 118 were used for duplicates of the pooled recovery ARDS samples. The Shimadzu LC-20AB liquid phase system (Prominence, Shimadzu, Japan) was used, and the separation column was a 5 μm 4.6 $\times$ 250 mm Gemini C18 column (150 μm inner diameter, 1.8 μm column particle size, 35 cm column length) for liquid phase separation of the sample. The elution peak was monitored at a wavelength of 214 nm, and one component was collected per minute. The samples were combined according to the chromatographic elution peak map to obtain 10 fractions, which were then frozen-dried.

HPLC, LC-MS/MS identification, and quantification of the labeled peptides

The dried peptide samples were reconstituted with mobile phase A (2% ACN, 0.1% FA), centrifuged at 20,000 $\times g$ for 10 min, and the supernatant was taken for injection. Separation was performed using an Easy-nLC 1200 (Thermo Fisher Scientific, San Jose, CA, USA) and tandem self-packed C18 column (75 μm internal diameter, 1.9 μm column size, 25 cm column length) and separated at a flow rate of 200 nL/min by the effective gradient. The nanoliter liquid phase separation end was directly connected to a mass spectrometer. The peptides separated by liquid-phase chromatography were ionized by a nanoESI source and then passed to a tandem mass spectrometer Orbitrap Exploris 480 (Thermo Fisher Scientific, San Jose, CA, USA) for data-dependent acquisition (DDA) mode detection.

Identification of proteins by quantitative proteomics analysis

The resulting data were searched in the UniProt database (<https://www.uniprot.org/>). The data were analyzed using the IQuant (BGI) software[22].

Gene ontology enrichment and pathway enrichment analyses

Gene ontology (GO) (<http://www.geneontology.org>) is a commonly used bioinformatic tool that provides comprehensive information on the gene function of individual genomic products based on defined features. The Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg>) is an easy-to-use automated database resource for understanding high-level biological functions and utilities. GO and KEGG pathway analyses of co-upregulated and co-downregulated proteins were performed via The Database for Annotation, Visualization, and Integrated Discovery. The GO analysis consisted of biological processes (BP), cellular components (CC), and molecular functions (MF).

PPI network creation and hub gene identification

The PPI network of differentially expressed proteins (DEPs) was constructed using the Search Tool for the Retrieval of Interacting Genes (STRING; <https://string-db.org/>)[23], with a combined score >0.4 as the cut-off point. Hub genes were identified using Cytohubba, a plug-in of the Cytoscape software (Cytoscape, 3.9.0)[24]. Significant modules in the PPI network were identified by molecular complex detection (MCODE)[25], another plug-in of Cytoscape software.

Cell culture and stimulation

A549 cells were purchased from CELLCOOK (<http://www.cellcook.com/>). A549 cells are human alveolar basal epithelial cells that grow adherently as a monolayer in vitro. The A549 cell line is widely used as a model of lung adenocarcinoma, as well as an in vitro model for type II pulmonary epithelial cells. The cells were cultured in 1640 medium with 10% fetal bovine serum (FBS). LPS (10 µg/ml, Sigma, St, Louis, MO, USA) was added to the culture medium to stimulate the cells for 48 h.

RNA extraction, reverse transcription, and quantitative real-time PCR

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed with 1 µg of total RNA and the 5× HiScript $\square$ qRT SuperMix (Vazyme Biotech, Co., Ltd., Nanjing, China), according to the manufacturer's instructions. Endogenous mRNA levels were measured by real-time PCR analysis based on AceQ qPCR SYBR Green Master Mix (Vazyme Biotech, Co., Ltd., Nanjing, China) detection with a Roche real-time PCR machine. In order to confirm the expression of RNAs, qPCR was performed under the following sequential conditions: 1) 95°C for 5 min, 2) 40 cycles at 95°C for 10 s and 60°C for 34 s. The primers are shown in Table 2. Samples were assayed in duplicate. The $2^{-\Delta\Delta CT}$ methodology was to obtain the ratios after normalizing to endogenous β -actin.

Western blotting and antibodies

Protein extracts were analyzed by western blotting according to standard protocols. Cells were harvested and boiled in a 4× loading buffer. Equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane, blocked with 8% skimmed milk. The membranes were incubated with primary antibodies against RPSA (1:1000 dilution, eBioscience, San

Diego, CA, USA), and anti-GAPDH (#5174S, 1:1000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA) followed by a secondary antibody (#PMS302, 1:1000 dilution, Proteinbiotechnology, Shanghai, China) bearing an oligonucleotide. After two washes, each membrane was read using a chemiluminescence developing solution (#180-506, Tanon™ Femto-sig ECL).

Enzyme-linked immunosorbent assay (ELISA) detection process

RPSA was detected in A549 cell supernatant using ELISA, according to the manufacturer's instructions. 1) Adding standards and samples in duplicate to the microtiter plate. 2) Adding 50µl of standard or sample to the appropriate wells. 3) Adding 100µl of enzyme conjugate to standard wells and sample wells except the blank well, covered with an adhesive strip and incubate for 60 minutes at 37°C. 4) Washing the microtiter plate 4 times, remove incubation mixture by aspirating contents of the plate into a sink or proper waste container. Using a squirt bottle, fill each well completely with wash solution (1X), then aspirate contents of the plate into a sink or proper waste container. Repeat this procedure for a total of four times. After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. 5) Aspirating all wells, then wash plates four times using wash buffer (1X). Always adjust your washer to aspirate as much liquid as possible and set fill volume at 350 ul/well/wash. After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. 6) Adding substrate A 50µl and substrate B 50µl to each well. Gently mix and incubate for 15 minutes at 37°C. 7) Adding 50µl stop solution to each well. The color in the wells should change from blue to yellow. 8) Reading the Optical Density (O.D.) at 450 nm using a microtiter plate reader within 15 minutes.

Statistical analysis

The identification and verification results were based upon statistical evaluation and were expressed as means ± SDs. Single sample t-tests were used to compare the two groups. P-values <0.05 were considered statistically significant. *P<0.05, **P<0.01, ***P<0.001.

Results

Identification of proteins related to ARDS

In order to estimate the efficiency of high-abundance protein removal, we performed 12% SDS-PAGE followed by Coomassie blue staining (Supplementary Figure S1). In order to identify the proteins between the acute inflammation and recovery phases, we extracted the intersection 1459 proteins from the three sets of mass spectrometry data, as shown in the venn diagram (Figure 2A) and heatmap (Figure 2B). In this iTRAQ quantification project, based on the discovery analysis, 398,924 spectra were generated, and 4036 peptides and 1475 proteins were controlled below 1% FDR. At least one unique peptide was required to qualify a protein. Among all identified proteins, the molecular weight (kDa) of the 55% of the identified proteins was approximately 10-50 kDa, and that of the 10% of the proteins was more than 100 kDa (Supplementary Figure S2A). The length of about 50% peptides was 5-10 amino acids (Supplementary

Figure S2B). Besides, about 70% of the proteins included no more than two unique peptides. The proteins were identified with low sequence coverage, about 97% of proteins with less than 30% sequence coverage (Supplementary Figure S2C).

Comparison of the CUDPs

As shown in Figure 3A and Figure 3B, we took three pairs of samples that were all detected by LC-MS/MS and used the upset diagram to show the number and ratio of proteins. The volcano plots (Figure 3) displayed the CUDPs of recovery/acute inflammation, respectively. The red dots indicate significantly upregulated proteins that passed the screening threshold (P-value <0.05 and fold-change >1.2 or Log2FC >0.263). The blue dots indicate significantly downregulated proteins that passed the screening threshold (P-value <0.05 and fold-change <0.83 or Log2FC <-0.263). The abundance of 19 proteins was significantly increased in the recovery phase (decreased in the acute phase), while the abundance of 34 proteins was significantly decreased in the recovery phase (increased in the acute phase). The detailed results of the differentially abundant proteins are summarized in Table 3.

GO enrichment analysis of 374 CUDPs

In order to determine the biological features of the CUDPs, GO analysis was performed. Changes in BP of CUDPs were significantly enriched in neutrophil mediated immunity, neutrophil activation involved in immune response and neutrophil degranulation (Figure 4A). The CC analysis showed that CUDPs were enriched in vesicle lumen and cytoplasmic vesicle lumen (Figure 4B). The MF analysis revealed that the CUDPs were enriched in cell adhesion molecule binding, cadherin binding and structural constituent of ribosome (Figure 4C).

KEGG enrichment analysis of CUDPs

KEGG enrichment analysis of CUDPs was performed to explore the potential mechanism of these CUDPs. CUDPs were mainly involved in Coronavirus disease-COVID-19, Ribosome, Prion disease, and Endocytosis (Figure 5).

Protein-protein interaction analysis

Fifty-three proteins were differentially expressed in the iTRAQ LC-MS/MS analysis and PPI network analysis. These proteins represent different functions in cell regulation and association with an inflammatory response. In order to identify the most significant clusters of the DEPs, PPI network of DEPs was constituted by STRING. All DEPs were pasted into the STRING website and then analyzed with the Cytoscape software. The 53 proteins formed 34 nodes with 70 edges were involved in constructing the PPI networks. Five proteins did not enter the PPI networks. The protein selection was based on significance and high frequency between recovery and acute by cytoscape analysis (Figure 6 A).

Hub genes recognition

In order to identify the hub gene among the DEPs, Cytohubba, and MCODE, a plug-in Cytoscape was performed. All the gene codes and edges were calculated (Table 4). The most significant module was identified by MCODE (score = 5.33) (Figure 6B, Table 5). Finally, the top six genes were identified as hub genes by the intersection of 12 algorithms and MCODE (Table 6). The analysis showed that RPSA was discovered as the most top hub gene among DEPs accordingly.

Hub gene RPSA verification in A549 cells

We searched the hsa05171 pathway diagram and selected some inflammation-related factors for testing to ensure the successful construction of the cell model. RPSA, IL-6, IL-8, and COX2 mRNAs were detected by qPCR. The mRNA levels of all of them were upregulated in A549 cells (Figure 7A). Western blot also confirmed the above statement for RPSA (Figure 7B). The ELISA used to test the cell cultures proved that the inflammatory response induced by LPS also supports the increases of RPSA (Figure 7C).

Discussion

ARDS is a critical condition that poses a serious threat to human health due to its high mortality rate. Although numerous studies investigated the mechanisms underlying ARDS, effective biomarkers for the diagnosis, prognosis, and therapeutic targeting of ARDS remain scarce, and the mechanisms governing ARDS have not been fully elucidated. Quantitative proteomics has been widely applied to detect differential proteins and pathways in many disease models, including ARDS. In this study, an 8-plex iTRAQ analysis enabled the investigation of large-scale protein alterations. The paired BALF samples from three ARDS patients in the acute and recovery phases were selected to identify the hub genes associated with ARDS, as well as their associated biological pathways by integrated bioinformatics analysis. In total, 1475 proteins were identified, including 98 co-upregulated proteins, and 278 co-downregulated proteins were identified. Fifty-three proteins were found to be differentially expressed. Finally, the 374 CUDPs were enriched in cell adhesion molecule binding, cytoplasmic vesicle lumen, and neutrophils.

Activated neutrophils are involved in many pathologies, especially infectious and lung diseases, including the ARDS[26]. The most enriched gene set of CUDPs in the BP of GO were neutrophil activation involved in immune response and neutrophil degranulation. According to Robb et al.[27], COVID-19 causes a spectrum of respiratory conditions differing in severity, including mild upper respiratory tract complications, ARDS, and pneumonia (due to secondary infection) in more deleterious cases. Chiumello et al.[28] showed that COVID-19-ARDS is a subset of ARDS. The primary enriched pathways were the Coronavirus disease COVID-19 interaction (hsa05171) and Ribosome (hsa03010). Some of 53 DEPs have been discovered to be related to pulmonary inflammation. For instance, HSP90AB1 was increased in the acute phase in this study, agreeing with a previous study by Ding Ma et al, and he identified that HSP90AB1 obviously up-regulated in ACE2-positive bronchial epithelial cells of patients with mild disease[29]. The COX-2/PGE2 pathway is a classic pro-inflammatory pathway. COX-2 is a pivotal enzyme in the production of prostaglandins, and evidence suggests that COX-2 plays an important role in the

pathogenesis of ARDS[30]. Kovalchuk et al.[31] showed that COX2, TNF α , IL-6, CCL2, and other cytokines and pathways related to inflammation and fibrosis are significantly upregulated in ARDS. The top six genes were identified as hub genes based on the degree of connectivity in the PPI network and through Cytohubba and MCODE analysis. The results revealed that RPSA is the most core gene, which was ranked first many times by the algorithms.

Alveolar epithelial-type cells are the main target cells being damaged and releasing the inflammatory mediators during ARDS [14]. LPS-induced A549 cells are a common model used to research pulmonary inflammation and injury. In this study, we evaluated the role of the RPSA protein in the regulation of inflammation in A549 cells treated with LPS. In order to verify our hypothesis, we used 10 ug/ml LPS to induce inflammation in A549 cells. After 48 h of stimulation, we firstly detect their mRNA expression level. Surprisingly, the RNA expression levels of RPSA showed elevated expression in induced A549 cells compared with the control group. Consistent with previous results, the protein expression of RPSA was upregulated by western blot. Given that RPSA is detected in the BALF, we used ELISA to detect RPSA in the cell culture supernatant of the processed samples compared with the control group. Consistent with the mRNA and protein expression, ELISA also showed that RPSA was highly expressed in the supernatant of A549 induced by LPS.

This study had limitations. The pairs of samples consisted of one sample in the acute phase and one sample in the recovery phase. Still, no sample without ARDS was included, and there is a possibility that some proteins were still affected in the recovery phase. This study only identified proteins and supposed mechanisms based on bioinformatics, but these mechanisms were not validated in vitro. A future study should specifically examine the value of BALF RPSA for the screening for ARDS.

In conclusion, based on iTRAQ proteomic profiling, a dataset to summarize protein alterations in the BALF of patients with ARDS was generated. The results might suggest novel biomarker candidates and potential therapeutic targets for ARDS. RPSA might be a potential biomarker or therapeutic target in ARDS.

Declarations

ACKNOWLEDGEMENTS

Not applicable.

AUTHOR CONTRIBUTIONS

L.Z., G.S., H.Z., and H.G. conceived and designed the project. Y.X., L.G. and L.Guo. collected the clinical samples, performed most of the experiments and analyzed the data. H.L., M.S., Q.Y., N.L., M.F., H.X. and J.L. provided technical assistance. Y.X. and L.Z. wrote the manuscript.

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AVAILABILITY OF DATA AND MATERIALS

All of the datasets in this study can be obtained by reasonable request to the corresponding authors.

ETHICS APPROVAL

Three cases of ARDS patients were selected in the First Affiliated Hospital of Anhui Medical University from October 2020 to November 2020. This study was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University. Informed consent was obtained from patients or their closest relatives in accordance with the 2013 (7th Edition) Declaration of Helsinki.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare no competing financial interests.

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Tables

Table 1. Demographic characteristics of ARDS patients for iTRAQ analysis.

Case	Sex	Age	Etiology	Minimum P/F (mmHg)	APACHEII score	SOFA score	Prognosis
1	Female	27	Severe pneumonia	68	20	10	Recovered
2	Male	62	Severe pneumonia(inhalation)	55.7	18	10	Improved
3	Female	73	Severe sepsis	91	14	8	Improved

Table 2. Primer sequences and lengths of amplified templates for real-time quantitative polymerase chain reaction (qPCR).

Target Gene	Sequence
IL-6	5'-GTGTTGCCTGCTGCCTTC-3' 5'-AGTGCCTCTTTGCTGCTTTC-3'
IL-8	5'-GACATACTCCAAACCTTTCCAC-3' 5'- CTTCTCCACAACCCTCTGC-3'
COX-2	5'- CCCTTCTGCCTGACACCTTT-3' 5'- TTCTGTACTGCGGGTGGAAC-3'
β -actin	5'-GTGGCCGAGGACTTTGATTG-3' 5'-CCTGTAACAACGCATCTCATATT-3'
RPSA	5'-TTTGTGGAACAGTGAGGGCT-3' 5'-GCTGCCTGGATCTGGTTAGT-3'

Table 3. The 53 differentially expressed proteins (DEPs) between the RG and AIG

Uniprot ID	Log2FC	Pvalue	Gene symbol	Description
HBB_HUMAN	2.627773944	0.017116429	HBB	Hemoglobin subunit beta
HBA_HUMAN	2.65529676	0.015447252	HBA1	Hemoglobin subunit alpha
HBD_HUMAN	2.210485866	0.018627454	HBD	Hemoglobin subunit delta
CAH1_HUMAN	2.521450132	0.025770378	CA1	Carbonic anhydrase 1
LEG1H_HUMAN	1.147659333	0.021488374	LEG1	Protein LEG1 homolog
SPTB1_HUMAN	2.087092251	0.046460874	SPTB	Spectrin beta chain
RBMX_HUMAN	0.881975836	0.020501773	RBMX	RNA-binding motif protein, X chromosome
GALT_HUMAN	0.393981481	0.020570424	GALT	Galactose-1-phosphate uridylyltransferase
PRDX2_HUMAN	2.457006621	0.048327571	PRDX2	Peroxiredoxin-2
HBG1_HUMAN	2.46538042	0.032869509	HBG1	Hemoglobin subunit gamma-1
PSB1_HUMAN	0.379463091	0.006043111	PSMB1	Proteasome subunit beta type-1
KLK10_HUMAN	0.749255172	0.033499861	KLK10	Kallikrein-10
HV313_HUMAN	0.58344586	0.007459594	IGHV3-13	Immunoglobulin heavy variable 3-13
SPTA1_HUMAN	2.440895078	0.019976544	SPTA1	Spectrin alpha chain, erythrocytic 1
PMGE_HUMAN	0.812211578	0.044945408	BPGM	Bisphosphoglycerate mutase
PCKGM_HUMAN	0.299326639	0.049074472	PCK2	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial
HS90B_HUMAN	-0.480596476	0.007619525	HSP90AB1	Heat shock protein HSP 90-beta
RSSA_HUMAN	-0.472690289	0.025227593	RPSA	40S ribosomal protein SA
EIF3E_HUMAN	-0.390708644	0.032985467	EIF3E	Eukaryotic translation initiation factor 3 subunit E
K2C4_HUMAN	-0.492516261	0.045628768	KRT4	Keratin, type II cytoskeletal 4
PSB5_HUMAN	-0.565192039	0.046053559	PSMB5	Proteasome subunit beta type-5
IPO4_HUMAN	-0.413558863	0.039795041	IPO4	Importin-4
PDC10_HUMAN	-0.920770717	0.022947081	PDCD10	Programmed cell death protein 10
USO1_HUMAN	-0.946038187	0.02370787	USO1	General vesicular transport

SC31A_HUMAN	-0.411900273	0.043970593	SEC31A	Protein transport protein Sec31A
LA_HUMAN	-0.313811738	0.008750025	SSB	Lupus La protein
FUMH_HUMAN	-0.536132104	0.048848794	FH	Fumarate hydratase, mitochondrial
SNRPA_HUMAN	-1.494449366	0.03138552	SNRPA	U1 small nuclear ribonucleoprotein A
LMNB1_HUMAN	-2.497840747	0.034597297	LMNB1	Lamin-B1 OS=Homo sapiens
AL3B1_HUMAN	-0.600144637	0.04066091	ALDH3B1	Aldehyde dehydrogenase family 3 member B1
RS5_HUMAN	-0.510399958	0.016076005	RPS5	40S ribosomal protein S5
PSMD8_HUMAN	-0.391098914	0.030352322	PSMD8	26S proteasome non-ATPase regulatory subunit 8
NASP_HUMAN	-0.505129065	0.031247282	NASP	Nuclear autoantigenic sperm protein
RS25_HUMAN	-1.031973977	0.048764712	RPS25	40S ribosomal protein S25
PFKAP_HUMAN	-0.474427166	0.036422564	PFKP	ATP-dependent 6-phosphofructokinase, platelet type
PSMD6_HUMAN	-0.918268455	0.042373214	PSMD6	26S proteasome non-ATPase regulatory subunit 6
DEN11_HUMAN	-3.06247017	0.007101412	DENND11	DENN domain-containing protein 11
S35U4_HUMAN	-2.374981406	0.013025699	SLC35A4	SLC35A4 upstream open reading frame protein
HNRPQ_HUMAN	-0.538422185	0.002871159	SYNCRIP	Heterogeneous nuclear ribonucleoprotein Q
DJC13_HUMAN	-0.326652616	0.018838668	DNAJC13	DnaJ homolog subfamily C member 13
COX2_HUMAN	-2.584175982	0.024334787	MT-CO2	Cytochrome c oxidase subunit 2
FABP4_HUMAN	-0.891392428	0.029084499	FABP4	Fatty acid-binding protein, adipocyte
EF1D_HUMAN	-0.708277213	0.007120905	EEF1D	Elongation factor 1-delta
FCERG_HUMAN	-1.306211684	0.023721845	FCER1G	High affinity immunoglobulin epsilon receptor subunit gamma
SKP1_HUMAN	-0.673831792	0.040215231	SKP1	S-phase kinase-associated protein 1

CTBP1_HUMAN	-1.239318276	0.022408252	CTBP1	C-terminal-binding protein 1
DC1I2_HUMAN	-0.438790689	0.010799153	DYNC1I2	Cytoplasmic dynein 1 intermediate chain 2
GLPK3_HUMAN	-0.873261218	0.001988595	GK3P	Glycerol kinase 3
PDIA6_HUMAN	-0.294379172	0.021287533	PDIA6	Protein disulfide-isomerase A6
H2A3_HUMAN	-1.019243294	0.030188071	H2AW	Histone H2A type 3
WBP2_HUMAN	-0.673205102	0.004978431	WBP2	WW domain-binding protein 2
CNTP4_HUMAN	-2.890928445	0.021512285	CNTNAP4	Contactin-associated protein-like 4

Table 4. The top-10 proteins of each algorithm (12) and gene frequency by Cytohubba.

MCC	DMNC	MNC	Degree	EPC	BottleNeck
RPSA	SKP1	RPSA	RPSA	RPSA	RPSA
RPS5	PSMB5	RPS5	HBA1	RPS5	HBA1
RPS25	EEF1D	RPS25	RPS5	RPS25	HSP90AB1
PSMD8	RPS5	PSMD8	PSMD8	EIF3E	SYNCRIP
EIF3E	RPS25	EIF3E	RPS25	PSMD8	SPTB
PSMB1	SPTB	SYNCRIP	EIF3E	SYNCRIP	DYNC1I2
PSMD6	HGB1	PSMB1	SYNCRIP	HBA1	FH
SKP1	USO1	PSMD6	HSP90AB1	PSMB1	SKP1
PSMB5	SPTA1	HBA1	PSMB1	EEF1D	RPS25
EEF1D	DYNC1I2	SKP1	PSMD6	HSP90AB1	PSMB1

EcCentricity	Closeness	Radiality	Betweenness	Stress	Clustering Coefficient
HBA1	RPSA	RPSA	RPSA	RPSA	EEF1D
HSP90AB1	HBA1	HBA1	HBA1	HBA1	CA1
RPSA	RPS5	HSP90AB1	HSP90AB1	HSP90AB1	PSMB5
SYNCRIP	HSP90AB1	RPS5	DYNC1I2	DYNC1I2	PDIA6
SPTB	RPS25	RPS25	SPTB	SPTB	PRDX2
DYNC1I2	PSMD8	SYNCRIP	USO1	PSMD8	PSMB1
SKP1	SYNCRIP	PSMD8	RPS5	RPS5	PSMD6
RPS25	EIF3E	EIF3E	SKP1	USO1	RPS25
RPS5	PSMB1	PSMB1	PSMD8	SYNCRIP	RBMX
HBG1	SPTB	SPTB	SYNCRIP	EIF3E	SSB

Protein symbol	Statistical frequency
RPSA, RPS5, and RPS25	10
HBA1 and SYNCRIP	9
HSP90AB1 and PSMD8	8
EIF3E, PSMB1, and SPTB	7

Table 5. The major cluster top10 hub genes identified by MCODE.

Cluster	Score	Nodes	Edges	Node IDs
1	5.333	10	24	RPS25, PSMB5, RPS5, SKP1, RPSA, PSMB1, EIF3E, EEF1D, PSMD6, PSMD8
2	3.667	7	11	DYNC1I2, HBG1, HBD, USO1, HBB, SPTB, SPTA1

Table 6. Six intersections selected by Cytohubba and MCODE as hub genes.

Gene symbol	Log2FC	frequency	Description	P value
RPSA	-0.468575285	10	40Sribosomal protein SA	0.025227593
RPS5	-0.507471255	10	40S ribosomal protein S5	0.016076005
RPS25	-0.993420327	10	40S ribosomal protein S25	0.048764712
PSMD8	-0.387680737	8	26S proteasome non-ATPase regulatory subunit 8	0.030352322
EIF3E	-0.387088397	7	Eukaryotic translation initiation factor 3 subunit E	0.032985467
PSMB1	0.380074002	7	Proteasome subunit beta type-1	0.006043111

Figures

Figure 1

The schematic outline of the experimental workflow for ARDS biomarkers discovery and verification.

Figure 2

(A) Venn diagram of patient #1, patient #2, and patient #3 showing overlap of 1459 proteins. (B) Heatmap of 1459 proteins screened by R packages "pheatmap" in Rstudio software. Red areas represent highly expressed genes, and blue areas represent lowly expressed genes in three patients with protein expression. P1: Patient 1, P2: Patient 2, P3: Patient 3.

Figure 3

(A/B) Upset graph showing the 98 upregulated and 276 down-regulated proteins. (C) Volcano plot analysis identifying the differentially expressed proteins (DEPs). The red dots represent 19 upregulated

proteins, and the blue dots represent 34 downregulated proteins.

Figure 4

Gene ontology (GO) enrichment result of CUDPs. (A) Biological processes (BP). (B) Cellular components (CC). (C) Molecular functions (MF). The X-axis label represents the protein ratio, and the Y-axis label represents GO terms. The size of the circle represents the protein count. Different colors of circles represent different adjusted P-values.

Figure 5

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially expressed proteins. Advanced bubble chart showing the enrichment of CUDPs in signaling pathways. Y-axis labels represent the pathways. X-axis labels represent rich factors. The size and color of the bubble represent the amount of CUDPs enriched in pathway and enrichment significance, respectively.

Figure 6

The PPI network and the most significant modules of differentially expressed proteins (DEPs). (A) The PPI network was analyzed by the String software. There were 34 nodes, and 70 edges in the PPI network. (B) The most significant module was identified by MCODE (score = 5.33). The red circle represents the upregulated proteins, and the green boxes represent the downregulated proteins.

Figure 7

Validation of the expression levels of RPSA between Control group and 10 μ g/ml LPS induced samples by qPCR (A), western blot (B), and ELISA (C) analysis. Control : Untreated cells. All data are presented as means $\pm$ SD.

Supplementary Files

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