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Bioinformatic identification of hub genes and key pathways in neutrophils of patients with acute respiratory distress syndrome

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Abstract

Acute respiratory distress syndrome (ARDS) is characterized as a neutrophil-dominant disorder without effective pharmacological interventions. Knowledge of neutrophils in ARDS patients at the transcriptome level is still limited. We aimed to identify the hub genes and key pathways in neutrophils of patients with ARDS. The transcriptional profiles of neutrophils from ARDS patients and healthy volunteers were obtained from the GSE76293 dataset. The differentially expressed genes (DEGs) between ARDS and healthy samples were screened using the limma R package. Subsequently, functional and pathway enrichment analyses were performed based on the database for annotation, visualization, and integrated discovery (DAVID). The construction of a protein–protein interaction network was carried out using the search tool for the retrieval of interacting genes (STRING) database and the network was visualized by Cytoscape software. The Cytoscape plugins cytoHubba and MCODE were used to identify hub genes and significant modules. Finally, 136 upregulated genes and 95 downregulated genes were identified. Gene ontology analyses revealed MHC class II plays a major role in functional annotations. *SLC11A1*, *ARG1*, *CHI3L1*, *HP*, *LCN2*, and *MMP8* were identified as hub genes, and they were all involved in the neutrophil degranulation pathway. The MAPK and neutrophil degranulation pathways in neutrophils were considered as key pathways in the pathogenesis of ARDS. This study improves our understanding of the biological characteristics of neutrophils and the mechanisms underlying ARDS, and key pathways and hub genes identified in this work can serve as targets for novel ARDS treatment strategies.

Abbreviations: ARDS = acute respiratory distress syndrome, BP = biological process, DAVID = the database for annotation, visualization, and integrated discovery, DEG = differently expressed gene, GEO = gene expression omnibus, GO = gene ontology, GSE = gene set enrichment, KEGG = Kyoto Encyclopedia of Genes and Genomes, MCODE = molecular complex detection, MF = molecular function, STRING = search tool for the retrieval of interacting genes.

Keywords: acute respiratory distress syndrome, bioinformatics, differentially expressed genes, hub genes, MAPK signaling pathway, neutrophil degranulation pathway, neutrophils

1. Introduction

Acute respiratory distress syndrome (ARDS) is a result of systemic inflammatory response syndrome in the lung, characterized by intractable hypoxemia and non-cardiogenic pulmonary edema.[1] There are 3 overlapping pathologic phases in ARDS, that is, exudation, proliferation, and fibrosis. Mainly the epithelium and endothelium of the lung are damaged in patients with the neutrophil-dominant disorder.[2] The management of ARDS involves optimizing ventilator parameters, managing judicious fluid, and treating underlying causes.[3,4] No pharmacological interventions have been proven to be effective. Although lung-protective ventilation is applied in ARDS patients in clinical practice, the mortality rate remains high.[5]

Inappropriate activation of pulmonary neutrophils is regarded as a major feature of ARDS. The accumulation of airway neutrophils leads to the release of reactive oxygen species,

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chemokines, proteases, and neutrophil extracellular traps, which sustains inflammation and tissue damage.[6,7] Increased neutrophil infiltration in ARDS patients has been reported to be associated with more severe lung damage and higher mortality rates.[8] Therefore, improving apoptosis of neutrophils to accelerate the regression of inflammation has been proposed as a therapeutic strategy for ARDS.[9]

However, the functional activity of neutrophils in ARDS has been not been studied much because of the difficulty in isolating alveolar neutrophils from clinical samples. Rodent models have been used as substitutes in ARDS research,[10] although there is a significant difference in neutrophils between rodents and humans. Recently, Juss et al[11] have conducted a study concerning the sensitivity to phosphoinositide 3-kinase inhibition of neutrophils in ARDS. The transcriptional profiles of blood and alveolar neutrophils were compared between ARDS patients and healthy volunteers in this research, but only differentially expressed genes (DEGs) were screened. Previous investigations did not reveal a comprehensive mRNA expression profile in neutrophils in ARDS.

In the present research, the mRNA microarray data provided by Juss et al were analyzed to identify DEGs between ARDS and healthy samples. Subsequently, various bioinformatic analysis methods, such as gene ontology (GO), Kyoto Encyclopedia of Genes, and Genomes (KEGG) pathway enrichment, and protein–protein interaction network analyses were performed to improve our understanding of the biological characteristics of neutrophils in ARDS and provide new treatment targets for ARDS.

2. Materials and methods

Ethical approval or patient consent was not required because the data for the present research were obtained from a public database, and the data are available without personal identifiers.

2.1. Microarray data

The gene expression dataset was downloaded from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo).[12] The probes were converted into the corresponding gene symbols according to the annotation information using the Affymetrix GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array). The transcriptional profiles of neutrophils from 12 ARDS patients and 12 healthy volunteers were obtained from the GSE76293 dataset.[11]

2.2. Identification of DEGs

The DEGs between ARDS and healthy samples were screened using GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r), an online analysis tool to identify DEGs by comparing 2 or more datasets in a GEO series based on the limma R package.[13] Probes without corresponding gene symbols or with more than one gene symbol were removed. If $|\log_2(\text{fold change})| > 1.5$ and adjusted P value $< .05$, results were considered to be statistically significant.

2.3. GO functional and KEGG pathway enrichment analyses

GO functional and KEGG pathway enrichment analyses of DEGs were performed using an online biological information database, the Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.ncifcrf.gov) (version 6.8).[14,15] GO function included cell composition (CC), biological processes (BPs), and molecular function (MF). If $P < .05$, results were considered to be statistically significant.

2.4. PPI network construction

The PPI network of DEGs was constructed using the Search Tool for the Retrieval of Interacting Genes (STRING; http://string-db.org) (version 11.0).[16] The interactions of DEGs were selected with a combination score > 0.4. Molecular interaction networks were visualized with Cytoscape (version 3.7.1),[17] an open source bioinformatics software platform.

2.5. Hub gene selection and module analysis

The hub genes of the PPI network of DEGs were evaluated using 5 topological algorithms (Maximal Clique Centrality, Density of Maximum Neighborhood Component, Edge Percolated Component, Radiality centrality, and Stress centrality) in the plugin cytoHubba (version 0.1)[18] in Cytoscape. The plugin Molecular Complex Detection (MCODE) (version 1.5.1)[19] in Cytoscape was used to cluster the PPI network to find densely connected regions based on topology. The significant modules in the PPI network were identified using MCODE with the following criteria: score > 5, degree cut-off = 2, node density cut-off = 0.1, node score cut-off = 0.2, K-core = 2, max depth from seed = 100. Further analysis for the most significant modules was performed based on the Reactome Pathway Database (http://reactome.ncpsb.org).[20,21]

3. Results

3.1. Identification and assessment of the expression levels of DEGs

A total of 231 DEGs, that is, 136 upregulated and 95 downregulated genes, were identified between ARDS patients and healthy volunteers. The relative expression levels of DEGs are illustrated in the volcano plot and the heat map in Figure 1, where obvious differences can be observed between the 2 groups.

3.2. GO functional and KEGG pathway analyses of DEGs

Both GO functional and KEGG pathway analyses of DEGs were performed using the DAVID database (Fig. 2). The GO analysis showed that the DEGs were significantly involved in CCs, such as the plasma membrane, the major histocompatibility complex (MHC) class II protein complex, the cell surface, and integral membrane components. For GO BP analysis, the DEGs were mainly enriched in antigen processing and presentation of peptide or polysaccharide antigens via MHC class II, peptide antigen assembly with the MHC class II protein complex, the response to viruses, and the inflammatory response. For GO MF analysis, the DEGs were mainly enriched in magnesium ion binding, MHC class II protein complex binding, MHC class II receptor activity, and selenium binding. In terms of KEGG pathways, the DEGs were mainly enriched in pathways involved in inflammatory bowel disease, asthma, leishmaniasis, and the intestinal immune network for IgA production.

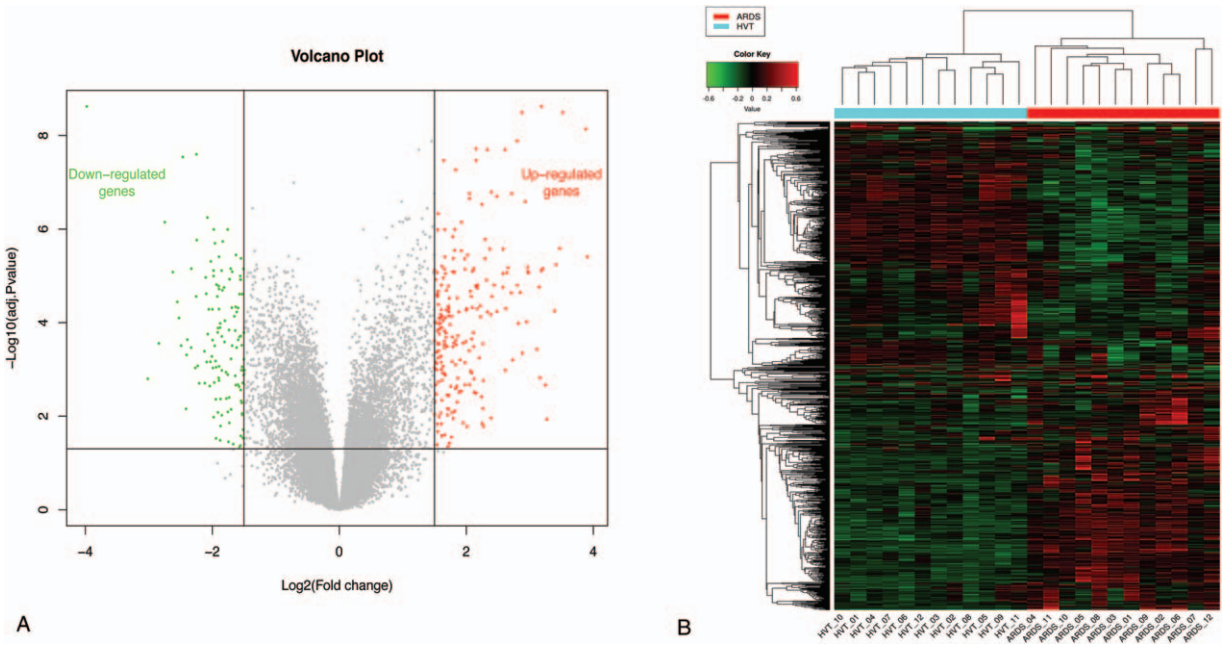

Figure 1. Volcano plot and heat map of the DEGs. (A) Green represents significant downregulated genes, gray represents non-differentially expressed genes, red represents upregulated genes (based on $|\log_2(\text{fold change})| > 1.5$ and adjusted $P < .05$). (B) The gradual color changes from green to red represent the changing process from upregulation to downregulation. The hierarchical clustering tree is shown on top, with the light blue part representing the healthy group and the light red part representing the ARDS group. ARDS = acute respiratory distress syndrome, DEG = differently expressed gene.

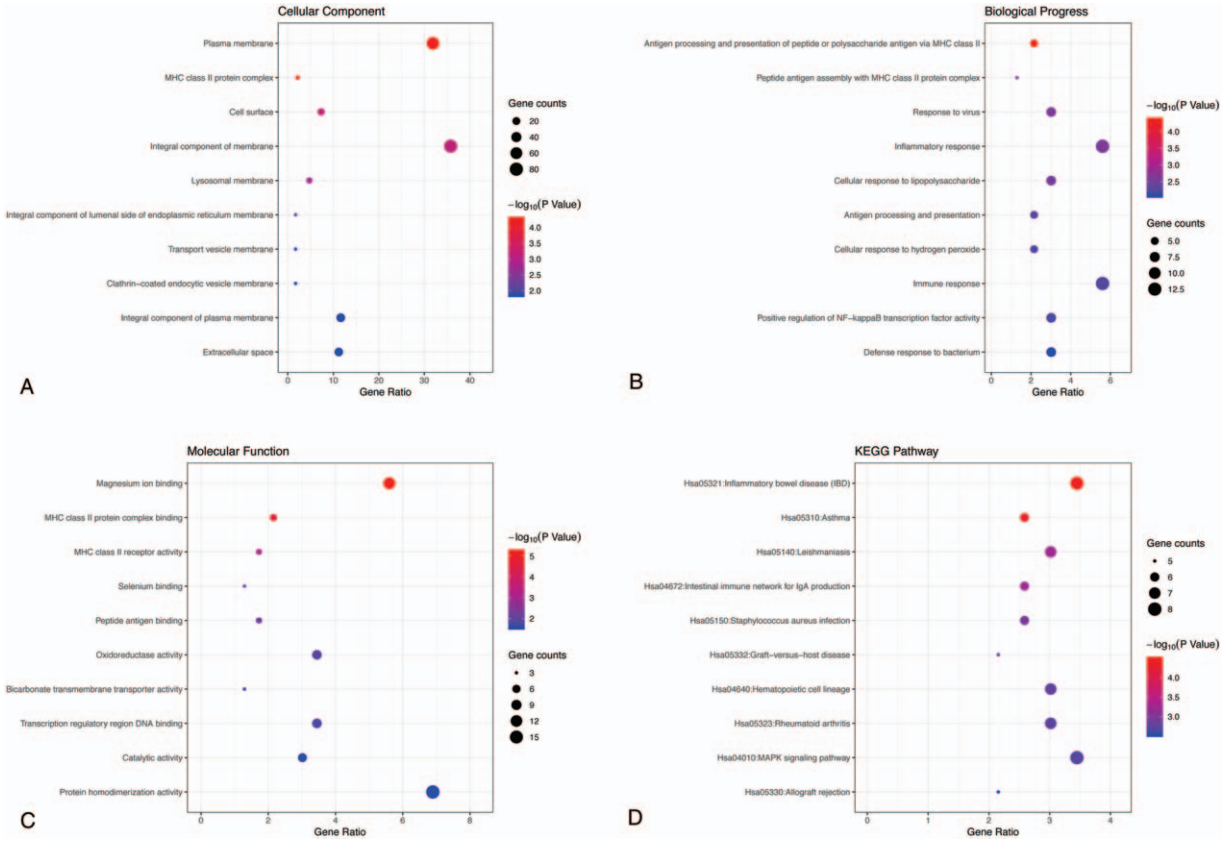

Figure 2. Top 10 significant GO enrichment and KEGG pathway terms of DEGs. Functional and pathway enrichment analyses of DEGs. (A) Cellular components of GO enrichment analysis. (B) Biological processes of GO enrichment analysis. (C) Molecular functions of GO enrichment analysis. (D) KEGG pathway analysis.

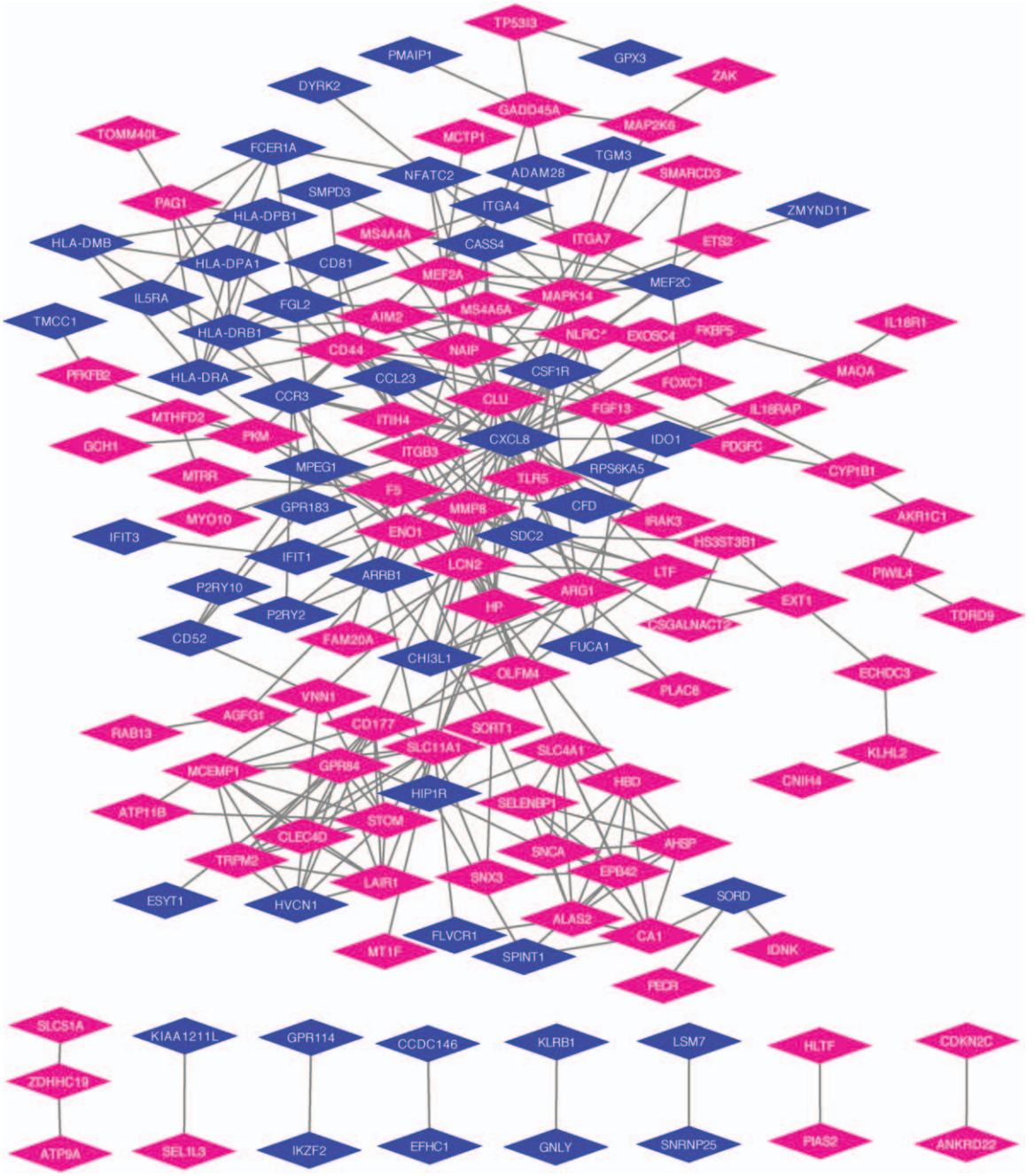

Figure 3. Protein–protein interaction network constructed with the up- and downregulated DEGs. Red nodes represent upregulated genes, and blue nodes represent downregulated genes. DEG = differently expressed gene, GO = gene ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes.

3.3. PPI network construction

The PPI network of DEGs was constructed with the STRING database with parameters including a minimum required interaction score > 0.4 (medium confidence). There were 220 nodes and 319 edges in the network, which was visualized using Cytoscape (Fig. 3).

3.4. Identification of hub genes

CytoHubba of Cytoscope was used to identify and select hub genes with 5 ranked algorithms. The ranks and names for these top 20 hub genes of each method are shown in Table 1. All 5 methods in cytoHubba identified solute carrier family 11 member 1 (*SLC11A1*) among the top 20 hub genes. Five hub genes, that is, Arginase1 (*ARG1*), Chitinase 3 Like 1 (*CHI3L1*), Haptoglobin (*HP*), Lipocalin 2 (*LCN2*), and Matrix Metallopeptidase 8 (*MMP8*), were identified by 4 of the 5 algorithms (Fig. 4).

3.5. Module analysis

A total of 3 significant modules were defined from the PPI network using the plugin MCODE (Fig. 5). The pathway analysis results for the most significant module based on the Reactome Pathway Database are shown in Table 2. Neutrophil degranu-

Table 1

Top 20 hub genes identified by different topological algorithms in cytoHubba.

Rank	MCC	DMNC	EPC	Radiality	Stress
1	*STOM*	*GPR84*	*CXCL8*	*CXCL8*	*CXCL8*
2	*GPR84*	*CD177*	*ARG1**	*CD44*	*CD44*
3	*CD177*	*LAIR1*	*LCN2**	*TLR5*	*MAPK14*
4	*CLEC4D*	*TRPM2*	*CD44*	*MAPK14*	*TLR5*
5	*LAIR1*	*MCEMP1*	*HP**	*HP**	*SDC2*
6	*TRPM2*	*SLC11A1**	*MMP8**	*CSF1R*	*SLC11A1*
7	*MCEMP1*	*HVCN1*	*TLR5*	*ARG1**	*HP**
8	*SLC11A1**	*CHI3L1**	*CSF1R*	*MMP8**	*ITGB3*
9	*HVCN1*	*CLEC4D*	*SLC11A1*	*ITGB3*	*CSF1R*
10	*LCN2**	*HBD*	*CHI3L1**	*LCN2**	*ARG1**
11	*ARG1**	*SLC4A1*	*MAPK14*	*IDO1*	*MMP8**
12	*HP**	*ALAS2*	*OLFM4*	*ARRB1*	*ARRB1*
13	*MMP8**	*AHSP*	*CD177*	*SDC2*	*SLC4A1*
14	*CHI3L1**	*SELENBP1*	*LTF*	*ITIH4*	*LCN2**
15	*LTF*	*SNCA*	*STOM*	*CHI3L1**	*IDO1*
16	*OLFM4*	*LTF*	*GPR84*	*SLC11A1*	*PKM*
17	*EPB42*	*OLFM4*	*CLU*	*NFATC2*	*HLA-DRB1*
18	*SLC4A1*	*STOM*	*LAIR1*	*FGF13*	*CYP1B1*
19	*ALAS2*	*HLA-DPB1*	*CLEC4D*	*CCR3*	*CA1*
20	*AHSP*	*HLA-DMB*	*TRPM2*	*CLU*	*EXT1*

DMNC = density of maximum neighborhood component, EPC = edge percolated component, MCC = maximal clique centrality.
* the hub genes identified by at least four ranked methods in cytoHubba.

lation was considered as the most important pathway. Interestingly, all 6 hub genes (*SLC11A1*, *ARG1*, *CHI3L1*, *HP*, *LCN2*, and *MMP8*) obtained from the cytoHubba analysis were included in the most significant module (Fig. 6).

4. Discussion

Neutrophils are the first line of cell biological defense against pathogens, and they mainly exert their protective effects through respiratory burst and degranulation.[22] Inappropriate activation or accumulation of neutrophils is considered as a major feature of ARDS. In the present study, we carried out several classical bioinformatics analysis methods to identify DEGs in neutrophils between ARDS patients and healthy volunteers to obtain a better understanding of the biological characteristics of neutrophils in ARDS.

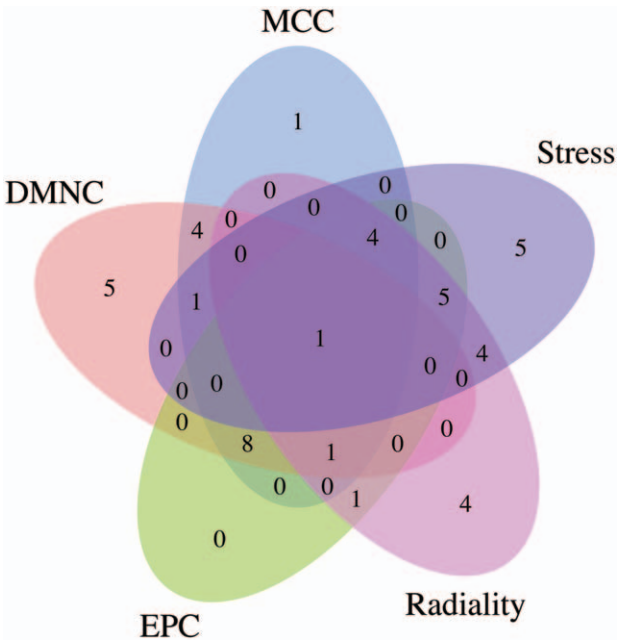

Figure 4. Venn diagram of the top 20 hub genes of the 5 ranked methods.

A total of 231 DEGs, that is, 136 upregulated genes and 95 downregulated genes, were identified in our study. GO analysis revealed MHC class II plays a major role in functional annotations. MHC class II is located on antigen presenting cells and starts the immune response by activating T helper cells, and evidence has shown that MHC class II is expressed in neutrophils.[23] The MHC class II protein complex in GO cellular component analysis, antigen processing and presentation of peptide or polysaccharide antigens via MHC class II and peptide antigen assembly with the MHC class II protein complex in GO BP analysis, and MHC class II protein complex binding and MHC class II receptor activity in GO MF analysis were all included in the significant GO enrichment. This finding suggests MHC class II plays an important role in ARDS, a correlation that has been reported in few studies. In our KEGG pathway analysis of DEGs, the MAPK signaling pathway was included in the most important signaling pathways. Many studies have confirmed that the MAPK signaling pathway is activated in ARDS animal models, and lung injury could be alleviated by downregulating this pathway.[24–26] Our study suggests that MAPK could be an important target for ARDS therapy.

We further constructed a PPI network and identified 6 hub genes according to 5 different ranked methods. *SLC11A1*, the most outstanding hub gene, is a member of the solute carrier family that is expressed in macrophages and neutrophils, and encodes an endosomal multichannel membrane protein which and participates in iron metabolism and host resistance to pathogens. It has been reported to promote ear tissue repair[27] and it has been correlated with the enhancement of susceptibility to pristane-induced arthritis in mice.[28] Friedman et al found

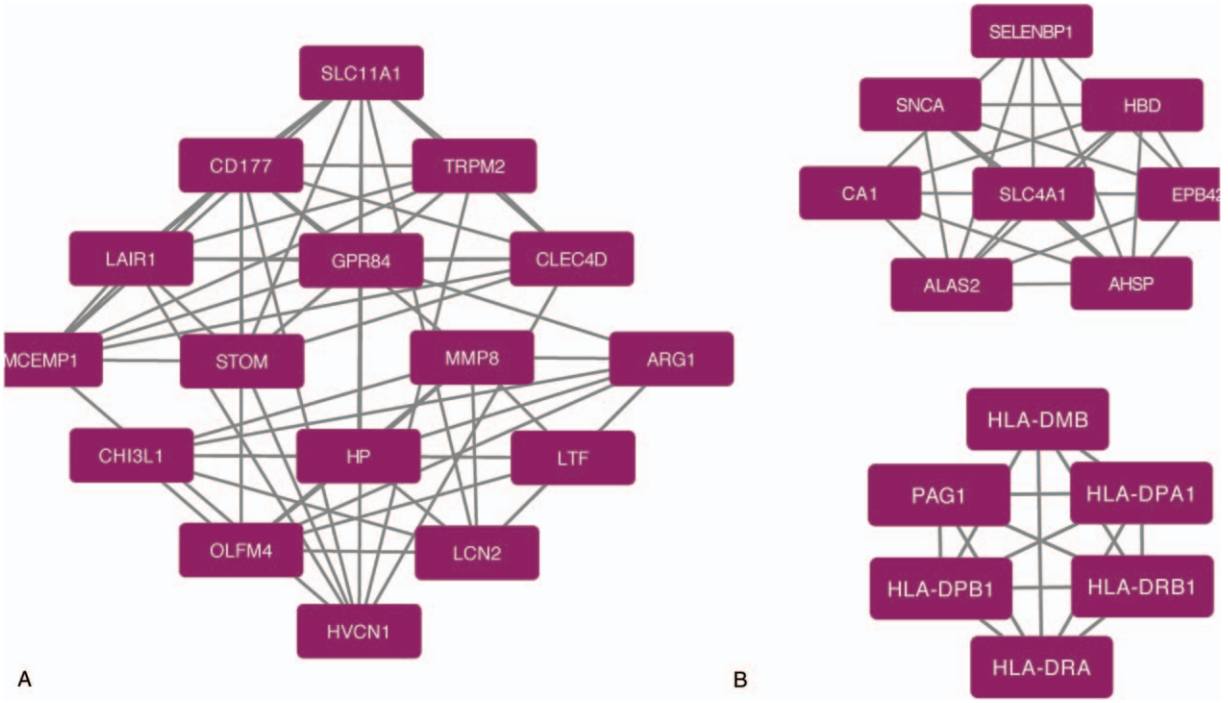

Figure 5. Three significant modules were obtained from the PPI network. (A) Score = 8.133, nodes = 16, edges = 61. (B) Score = 7.143, nodes = 8, edges = 25. (C) Score = 5.600, nodes = 6, edges = 14.

SLC11A1 plays an important role in antineutrophil cytoplasmic antibody-associated vasculitis.[29] Considering that *SLC11A1* is also an important innate immune gene, its role in ARDS requires further investigation.

HP functions to bind free plasma hemoglobin and exhibits antimicrobial activity. It is well known to be linked to inflammatory disease, behaving as an acute phase protein in hemolysis.[30,31]*CHI3L1* encodes a glycoprotein member of the glycosyl hydrolase 18 family which is mainly secreted by activated macrophages, neutrophils, and synovial cells. It plays a role in inflammation, including processes such as the T helper cell type 2-mediated inflammatory response, IL-13-induced inflammation, and the regulation of inflammatory cell apoptosis. Pulmonary inflammation, epithelial apoptosis, and injury induced by hyperoxia are also regulated by CHI3L1. Kim et al reported it plays a critical role in respiratory syncytial virus-induced airway inflammation.[32] Shao et al found a significant association between a genetic variation in *CHI3L1* and bronchial asthma in the Chinese population.[33] LCN2, a neutrophil gelatinase-associated lipocalin, is critical in innate immunity, as it limits bacterial growth and it can be upregulated in response to oxidative stress.[34] Due to its high expression in response to infections and tissue injury, the LCN2 concentration in blood and urine has already been identified as an early biomarker of acute kidney injury.[35,36] MMP8 is involved in the onset of inflammation. An animal model has been used to evaluate the role of MMP8 in acute lung injury; MMP8 was found to regulate neutrophil migration through the dense collagenous extracellular matrix of the corneal stroma.[37] It was also reported to play a

Table 2

Reactome pathway analysis of DEGs in the most significant module.

Pathway	Description	Count in Gene Set	False Discovery Rate
HSA-6798695	Neutrophil degranulation	16 of 471	8.06×10^{-25}
HSA-6799990	Metal sequestration by antimicrobial proteins	2 of 6	2.10×10^{-4}
HSA-6803157	Antimicrobial peptides	3 of 87	4.80×10^{-4}
HSA-382551	Transport of small molecules	4 of 706	1.76×10^{-2}
HSA-2672351	Stimuli-sensing channels	2 of 104	2.27×10^{-2}

DEG = differently expressed gene.

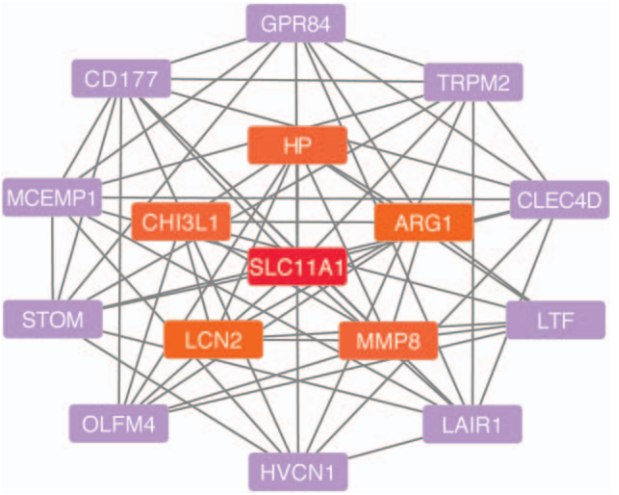

Figure 6. The defined hub genes in the network of the most significant module. Red presents the gene identified by all 5 topological algorithms, orange presents genes identified by 4 topological algorithms, and purple presents the other genes in the module.

critical role in lung injury induced by pulmonary ischemia–reperfusion[38] and ventilation.[39] The metabolism of arginine could regulate the innate and adaptive immune responses. ARG1, an M2 macrophage marker, is involved in an antimicrobial effector pathway in polymorphonuclear granulocytes.[40] These hub genes are related to the innate immune system and inflammation, and knowledge of their functional mechanisms may provide therapy targets for the treatment of ARDS.

Interestingly, all 6 hub genes identified in our study were included in the neutrophil degranulation pathway of the most significant module based on Reactome pathway analysis. Our study confirms the findings from a previous study that neutrophils and their secretory products play an important role in the early stages of ARDS.[41] Elevated neutrophil degranulation was observed within minutes after the initiation of trauma, which leads to ARDS. It implies that blocking or inhibiting neutrophil degranulation may be useful for treatment during early phases of ARDS.

5. Conclusion

In the present study, we identified marked biological changes of MHC class II in ARDS patients. The MAPK and neutrophil degranulation pathways in neutrophils were found to be of great importance in the pathogenesis of ARDS. Six hub genes (*SLC11A1*, *ARG1*, *CHI3L1*, *HP*, *LCN2*, and *MMP8*), all involved in the neutrophil degranulation pathway, were also identified. Our findings provide new clues to further investigate the biological characteristics of neutrophils and the mechanisms underlying ARDS. Key pathways and hub genes could serve as new treatment targets for ARDS.

Author contributions

Conceptualization: Lan Hu, Feng Xu.
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Formal analysis: Lan Hu, Tianxin Zhao, Yuelin Sun.
Funding acquisition: Lan Hu.
Investigation: Lan Hu, Feng Xu.
Methodology: Tianxin Zhao, Yuelin Sun.
Project administration: Lan Hu, Yingfu Chen, Ke Bai.
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Software: Lan Hu, Tianxin Zhao, Yuelin Sun.
Supervision: Feng Xu.
Validation: Yuelin Sun, Yingfu Chen, Ke Bai.
Writing – original draft: Feng Xu.

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