Prediction of key genes and miRNAs responsible for loss of muscle force in patients during an acute exacerbation of chronic obstructive pulmonary disease

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Abstract. The present study aimed to identify genes and microRNAs (miRNAs or miRs) that were abnormally expressed in the vastus lateralis muscle of patients with acute exacerbations of chronic obstructive pulmonary disease (AECOPD). The gene expression profile of GSE10828 was downloaded from the Gene Expression Omnibus database, and this dataset was comprised of 4 samples from patients with AECOPD and 5 samples from patients with stable COPD. Differentially expressed genes (DEGs) were screened using the Limma package in R. A protein-protein interaction (PPI) network of DEGs was built based on the STRING database. Module analysis of the PPI network was performed using the ClusterONE plugin and functional analysis of DEGs was conducted using DAVID. Additionally, key miRNAs were enriched using gene set enrichment analysis (GSEA) software and a miR-gene regulatory network was constructed using Cytoscape software. In total, 166 up- and 129 downregulated DEGs associated with muscle weakness in AECOPD were screened. Among them, *NCL*, *GOT1*, *TMOD1*, *TSPO*, *SOD2*, *NCL* and *PA2G4* were observed in the modules consisting of upregulated or downregulated genes. The upregulated DEGs in modules (including *KLF6* and *XRCC5*) were enriched in GO terms associated with immune system development, whereas the downregulated DEGs were enriched in GO terms associated with cell death and muscle contraction. Additionally, 39 key AECOPD-related miRNAs were also predicted, including miR-1, miR-9 and miR-23a, miR-16 and miR-15a. In conclusion, DEGs (*NCL*, *GOT1*, *SOD2*, *KLF6*, *XRCC5*, *TSPO* and *TMOD1*) and miRNAs (such as miR-1, miR-9 and miR-23a) may be associated with the loss of muscle force in patients during an acute exacerbation of COPD which also may act as therapeutic targets in the treatment of AECOPD.

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Key words: loss of muscle force, acute exacerbation of chronic obstructive pulmonary disease, differentially expressed genes, miRNAs, miR-gene regulatory network

Introduction

Chronic obstructive pulmonary disease (COPD), characterized by a fixed obstruction of the airway caused by emphysema, chronic bronchitis, or both, is a common, growing public health problem that is responsible for a huge economic health burden worldwide (1). Stable COPD may lead to declines in lung functions, such as airflow obstruction, airway function decline, and respiratory muscle fatigue, which impair patient quality of life; although acute exacerbations of COPD (AECOPD) have various definitions, they are commonly characterized by worsened dyspnea and increased volumes of phlegm and phlegm purulence, usually accompanied by hypoxemia and worsened hypercapnia (2,3). Small airway lesions (due to chronic bronchiolitis) and destruction of the alveolar walls (emphysema) are the two major features of AECOPD (4). Based on available data in 2010, COPD is one of the 6 leading causes of death, and AECOPD is associated with high morbidity (5). To date, there are no methods for preventing AECOPD, and current medical therapies for AECOPD mainly involve bronchial relaxation and the use of glucocorticoids and antibiotics, which, however, are always associated with side effects leading to unsatisfactory prognosis (6).

Smoking, malnutrition, depression and drug addiction are the risk factors affecting the quality of life for patients with AECOPD (7). Furthermore, skeletal muscle dysfunction is the most severe complication of COPD, and progression is associated with oxidative stress, skeletal muscle fiber types, systemic inflammation and mitochondrial dysfunction (1),

bringing great detrimental effects to patients. In fact, skeletal muscle depletion has been adopted as a predictor of mortality in patients with COPD (8). In COPD patients, a lack of antioxidant capacity and glutathione (GSH) indicated that oxidative stress is associated with skeletal muscle dysfunction (9). Furthermore, bacterial infection is another major cause of AECOPD (10). The increasing levels of systemic inflammatory factors, such as tumor necrosis factor α (TNF-α), interleukin (IL)-6 and IL-8, may inhibit muscle shrinkage and the protein degradation of skeletal muscle, thus leading to muscle atrophy (11). Mitochondrial dysfunction, in terms of sharply increased transmembrane potential and reduced mitochondrial density, is one of the factors associated with abnormal skeletal muscle in COPD (9). Thus, further studies are warranted in order to explore the mechanism underlying the pathogenesis of AECOPD.

Previous studies have identified a number of microRNAs (miRNAs or miRs) that may have significant regulatory functions in the progression of COPD, such as miR-223, miR-1274a and miR-15b in lung tissue (12), let-7c and miR-125b (13), as well as serum miR-20a, miR-28-3p, miR-34c-5p, miR-100 and miR-7 (14). Furthermore, the expression of miR-1 in quadriceps may be responsible for muscle dysfunction in COPD (15). To the best of our knowledge, however, there have been no reports on miRNAs contributing to skeletal muscle weakness in patients with AECOPD.

In order to identify the key genes and miRNAs that may be responsible for the loss of muscle force during an acute exacerbation in COPD patients, we downloaded a gene expression profile dataset GSE10828 (16) from the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/geo) (17), which were collected from muscle samples of patients with AECOPD or stable COPD. The authors submitting this dataset identified the differentially expressed genes (DEGs) that may be associated with loss of muscle force during AECOPD, and performed gene ontology (GO) enrichment analysis (16). In the present study, which is based on the identified DEGs, we constructed a protein-protein interaction (PPI) network and conducted subsequent module analysis in order to identify genes that play critical roles in the progression of AECOPD; we also built a miR-gene regulatory network. This study aimed to deepen our understanding of the molecular mechanisms underlying the loss of muscle force in AECOPD.

Materials and methods

Affymetrix microarray data. Only one microarray dataset (GSE10828) (16) was found to be associated with AECOPD in the GEO database of NCBI (17). The annotation platform of this dataset is the GPL2891 platform (GE Healthcare/Amersham Biosciences CodeLink™ UniSet Human 20K I Bioarray, Chalfont, UK). This dataset were collected from vastus lateralis samples from 4 male patients with acute COPD and 5 male patients with stable COPD. No significant differences were found in the basic characteristics between the patients with AECOPD and stable COPD with regard to age, body mass index (BMI), forced expiratory volume in the first second (FEV_1), FEV_1/forced vital capacity (FVC), arterial oxygen and carbon dioxide tension (PaO_2 and $PaCO_2$), and maximal inspiratory mouth pressure (PI_{max}), apart from C-reactive protein (CRP) levels at admission and lower quadriceps force (16).

Data preprocessing and identification of DEGs. Firstly, the microarray data in .CEL format were converted into expression measures using the GEOquery package, a package for retrieving gene expression data sets in R/Bioconductor (Bioconductor version: Release 3.1, http://www.bioconductor.org/packages/release/bioc/html/GEOquery.html) (18). The Robust Multi-array Analysis (RMA) method was used to preprocess the downloaded raw data by background adjustment, quintile normalization and summarization (19). Subsequently, the differential expression values of genes between the acute COPD samples and the stable COPD samples were calculated by t-test using the Linear Models for Microarray Data (LIMMA) package (R/Bioconductor version: Release 3.1, http://www.bioconductor.org/packages/release/bioc/html/limma.html) (20). Multiple testing correction was conducted by the Bayesian method (21). Only genes with a false discovery rate (FDR) <0.01 and $|\log_2$ Fold-Change (FC)| >1.5 were identified as DEGs. Finally, the pheatmap package of R (http://cran.r-project.org/web/packages/pheatmap/index.html) was used for hierarchical clustering (22) of DEGs based on Euclidean distance (23), and the result was visualized using heat maps.

GO analysis. GO enrichment analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) online tool (version 6.7, http://david.abcc.ncifcrf.gov/) (24) with a p-value <0.05 as threshold.

Construction of PPI network and functional analysis of significant modules. The Search Tool for the Retrieval of Interacting Genes (STRING) (version 10, http://string-db.org/) database was used to predict the interactions between the encoded proteins of these up- and downregulated DEGs, respectively (25). Only PPI pairs with a combination score >0.4 were included. Cytoscape software (version 3.2.1, http://cytoscape.org/), an open source software platform for integrating bimolecular interaction networks with high-throughput expression data and other molecular states into a unified conceptual framework (26), was used to visualize the resulting PPIs. The top five genes with the highest connection degrees were considered to be most closely associated with AECOPD. In addition, Cluster ONE plugin (27) was used to select the significant modules of the up- and downregulated genes in the PPI network, respectively. The top three modules according to p-values were selected for further analysis.

In addition, the biological processes of DEGs in the resulting networks were functionally analyzed using DAVID (version 6.7, http://david.abcc.ncifcrf.gov/) (24) with a p-value <0.05.

Enrichment analysis of key miRNAs and regulatory network construction of key miRNAs. We used the gene set enrichment analysis (GSEA) software (version 2.0.14, http://www.broadinstitute.org/gsea/index.jsp), based on the whole genome expression profile (28), to predict the miRNAs associated with AECOPD based on the microarray data. A p-value <0.01 was selected as threshold.

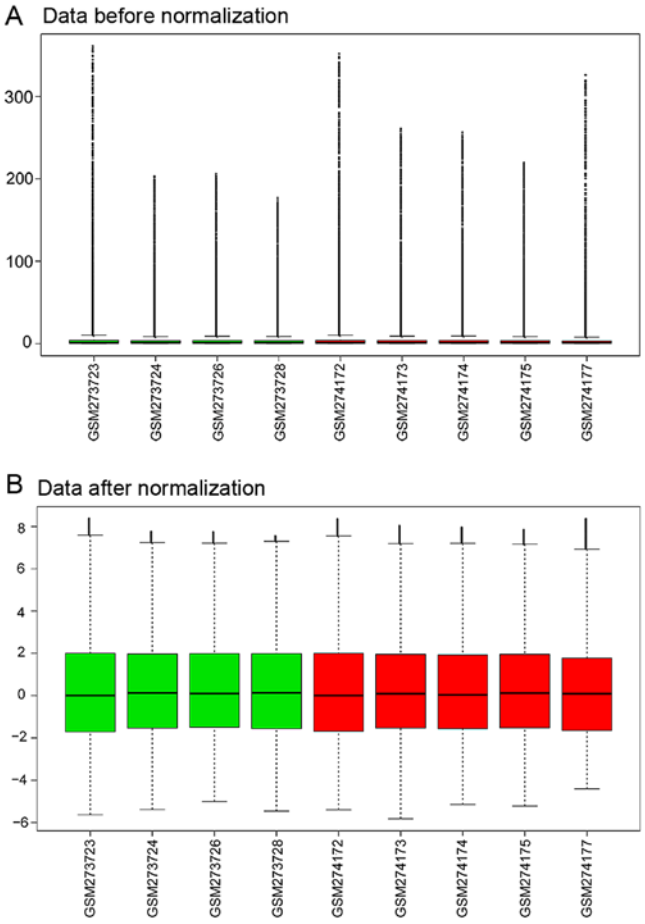

Figure 1. Cascade figure of the normalization expression value. (A) Data before normalization. (B) Data after normalization. Green represents samples from patients with acute exacerbations of chronic obstructive pulmonary disease (AECOPD); red represents samples from patients with stable COPD.

In addition, we used Cytoscape software to construct a miRNA-gene regulatory network with the miRNAs and their target DEGs. A miR-DEG regulatory network is a complicated biological system in which an miRNA functions in a variety of intracellular biological processes, such as gene regulation, cell signaling transduction, protein-protein interactions and metabolism, by regulating molecules at a transcriptional level. This provides a deeper understanding of the biological mechanisms involved (29).

Results

Screening of DEGs and hierarchical clustering analysis. The expression profiling data were preprocessed using the R GEOquery package and were normalized by the RMA method in R (Fig. 1).

With the cut-offs of FDR <0.01 and $|\log_2 FC| > 1.5$, a total of 295 DEGs were identified between the human AECOPD and stable COPD controls, including 166 upregulated genes and 129 downregulated genes (Fig. 2).

Functional annotation analysis of DEGs. According to the GO functional annotation, the top 10 enriched biological process terms included cellular respiration, generation of precursor metabolites and energy, and respiratory electron transport chain; the top 10 molecular function terms included cytoskeletal protein binding, serine-type endopeptidase inhibitor activity, protein complex binding and NADH dehydrogenase activity. Translocator protein (*TSPO*), superoxide dismutase 2 (*SOD2*) and proligeration associated protein (*PA2G4*) were the genes enriched in the significant functions (Table I).

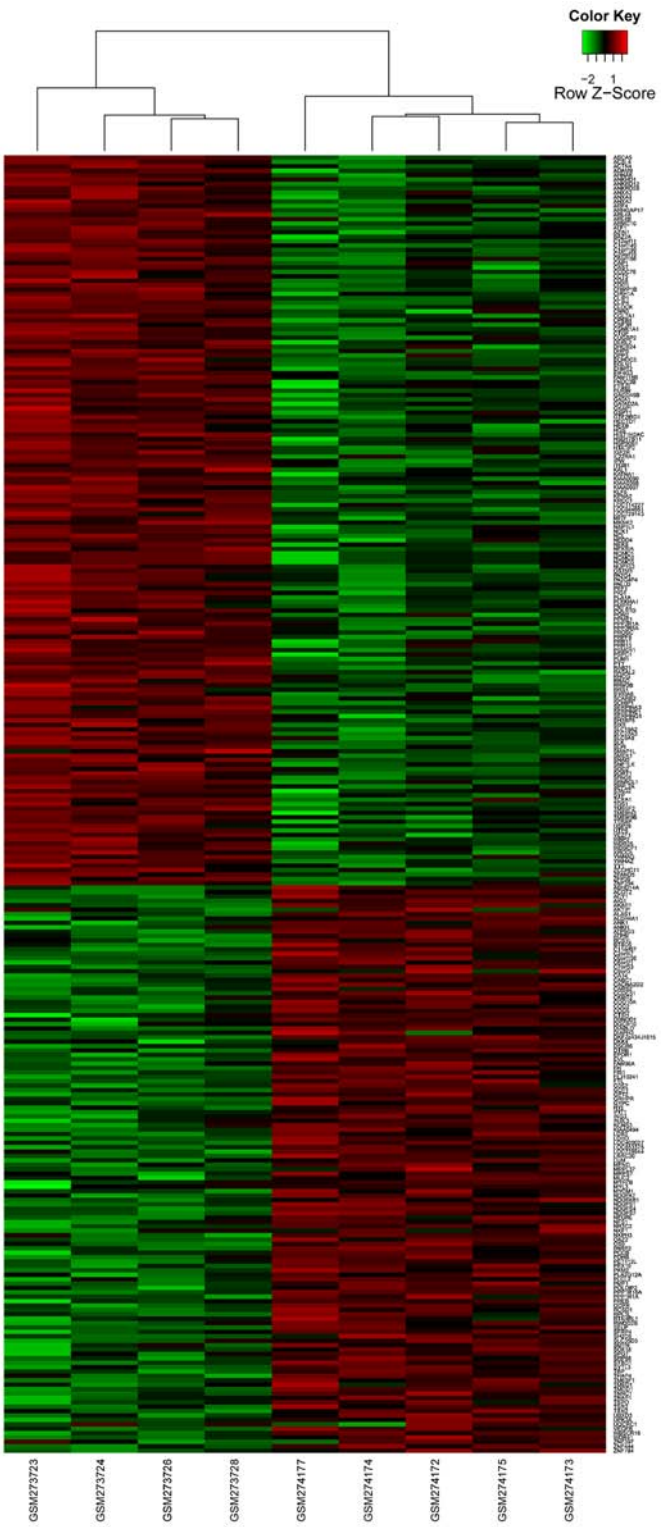

Figure 2. Hierarchical clustering heat maps of differentially expressed genes (DEGs). The gradient color from green to red represents the expression level [acute chronic obstructive pulmonary disease (COPD) sample and stable COPD samples changes from upregulation to downregulation].

Table I. The top 10 enriched GO terms in BP and MF categories.

Name	Term	Count	p-value	Genes
BP	GO:0015980 - energy derivation by oxidation of organic compounds	11	7.94E-05	*NDUFS7, NDUFS4, GOT1, UQCRC1, PPP1R1A, NDUFA7, NDUFAB1, NDUFS1, PDHB, FH, SOD2*
BP	GO:0045333 - cellular respiration	9	1.26E-04	*NDUFS7, NDUFS4, UQCRC1, NDUFA7, NDUFAB1, NDUFS1, PDHB, FH, SOD2*
BP	GO:0006091 - generation of precursor metabolites and energy	15	3.67E-04	*UQCRC1, TXN2, NDUFA7, NDUFAB1, ATP5G3, PDHB, SOD2, NDUFS7, GOT1, NDUFS4, PKM2, PPP1R1A, ATP5I, NDUFS1, FH*
BP	GO:0022904 - respiratory electron transport chain	7	4.50E-04	*NDUFS7, NDUFS4, UQCRC1, NDUFA7, NDUFAB1, NDUFS1, SOD2*
BP	GO:0006119 - oxidative phosphorylation	8	8.01E-04	*NDUFS7, NDUFS4, UQCRC1, NDUFA7, NDUFAB1, ATP5I, ATP5G3, NDUFS1*
BP	GO:0007005 - mitochondrion organization	9	0.001355	*NDUFS7, FIS1, SPG7, TSPO, YWHAZ, NDUFS4, BCS1L, RRM2B, SOD2*
BP	GO:0042773 - ATP synthesis coupled electron transport	6	0.001691	*NDUFS7, NDUFS4, UQCRC1, NDUFA7, NDUFAB1, NDUFS1*
BP	GO:0042775 - mitochondrial ATP synthesis coupled electron transport	6	0.001691	*NDUFS7, NDUFS4, UQCRC1, NDUFA7, NDUFAB1, NDUFS1*
BP	GO:0022900 - electron transport chain	8	0.001937	*NDUFS7, NDUFS4, UQCRC1, TXN2, NDUFA7, NDUFAB1, NDUFS1, SOD2*
BP	GO:0006120 - mitochondrial electron transport, NADH to ubiquinone	5	0.003914	*NDUFS7, NDUFS4, NDUFA7, NDUFAB1, NDUFS1*
MF	GO:0004857 - enzyme inhibitor activity	16	1.89E-05	*CAST, PPME1, SERPING1, AZIN1, FURIN, ANXA4, ANXA2, OAZ2, SH3BP5, YWHAG, COL7A1, PPP1R1A, KAL1, SERPINE1, SERPINA3, SLPI*
MF	GO:0008092 - cytoskeletal protein binding	19	7.97E-04	*ACTN4, CNN3, TNNC1, MYL3, DIAPH3, EVL, PALLD, ANXA2, LOC729143, SPAG9, ANK1, SMTN,KATNA1, NCK1, CLIP1, ARL8B, CAP1, MYH7B, TMOD1*
MF	GO:0004867 - serine-type endopeptidase inhibitor activity	7	0.002949	*COL7A1, KAL1, SERPINE1, SERPINA3, SLPI, SERPING1, FURIN*
MF	GO:0019899 - enzyme binding	18	0.003008	*PPME1, DIAPH3, NFKBIA, FURIN, ANXA2, SPAG9, YWHAG, PA2G4, ANK1, NEDD4, IGF2R, SERPINE1, SORT1, SYTL3, AKAP1, KPNA2, PA2G4P4, DHCR24, ADAM9*
MF	GO:0032403 - protein complex binding	10	0.003358	*SYP, INSL3, YWHAZ, UQCRC1, ACTN4, TXN2, CTGF, NFKBIA, ITGB1, ADAM9*
MF	GO:0050136 - NADH dehydrogenase (quinone) activity	5	0.004217	*NDUFS7, NDUFS4, NDUFA7, NDUFAB1, NDUFS1*
MF	GO:0008137 - NADH dehydrogenase (ubiquinone) activity	5	0.004217	*NDUFS7, NDUFS4, NDUFA7, NDUFAB1, NDUFS1*
MF	GO:0003954 - NADH dehydrogenase activity	5	0.004217	*NDUFS7, NDUFS4, NDUFA7, NDUFAB1, NDUFS1*
MF	GO:0016655 - oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	5	0.006741	*NDUFS7, NDUFS4, NDUFA7, NDUFAB1, NDUFS1*
MF	GO:0004866 - endopeptidase inhibitor activity	8	0.007169	*CAST, COL7A1, KAL1, SERPINE1, SERPINA3, SLPI, SERPING1, FURIN*

GO, gene ontology; BP, biological process; MF, molecular function.

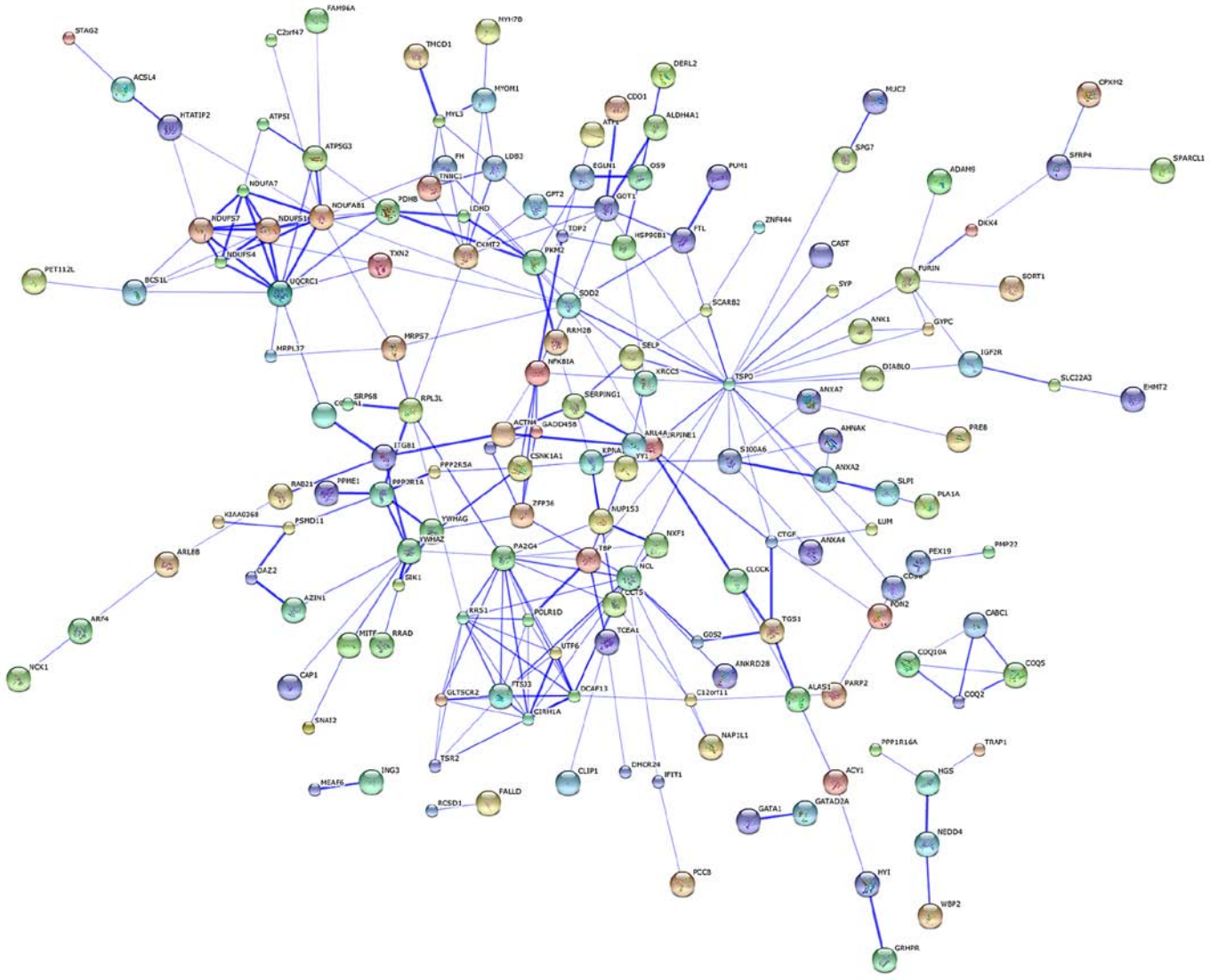

Figure 3. Protein-protein interaction (PPI) network of differentially expressed genes (DEGs).

PPI network. The protein-protein network of the identified DEGs was mapped using the software STRING in order to predict the protein interactions. By integrating these correlations, interaction networks between the target genes and their interactive genes were constructed (Fig. 3). In the core of the PPI network, genes belonged to more than one module. *TSPO*, nucleolin (*NCL*), NADH dehydrogenase (ubiquinone) 1, α/β subcomplex (*NDUFAB1*), *PA2G4* and *SOD2* were the top 5 protein nodes with the highest connection degrees (Table II and Fig. 3).

Module analysis and functional analysis of DEGs in PPI network. For the upregulated DEGs, three significant modules were obtained: module 1 consisting of 11 nodes (including NCL) and 33 edges (p-value=3.017E-5), module 2 of 14 nodes and 16 edges (p-value=3.115E-5), and module 3 of 13 nodes and 17 edges (p-value=3.194E-5) (Fig. 4A). Moreover, 3 significant modules were also obtained for the downregulated DEGs: module 1 consisting of 16 nodes and 35 edges (p-value=1.418E-6), module 2 of 18 nodes and 21 edges (p-value=4.592E-6), and module 3 of 10 nodes and 15 edges (p-value=3.642E-4). Pyruvate dehydrogenase (lipoamide) beta (*PDHB*) was commonly detected in module 1 and 2; glutamic pyruvate transaminase (alanine aminotransferase) 2 (*GPT2*), glutamic-oxaloacetic transaminase 1 (*GOT1*) and aldehyde dehydrogenase 4 family member A1 (*ALDH4A1*) were common in module 2 and 3 (Fig. 4B).

Table II. Genes with the top 5 node degrees in PPI network.

Gene	Degree	Log2FC	p-value
TSPO	25	-2.73239	0.007907
NCL	14	2.021786	0.001702
NDUFAB1	13	-27.2233	0.006066
PA2G4	12	3.607543	0.006317
SOD2	11	21.28352	0.005172

Degree, degree of node in PPI network; Log2FC, logarithms of amplitude changes for differentially expressed gene expression values; PPI, protein-protein interaction

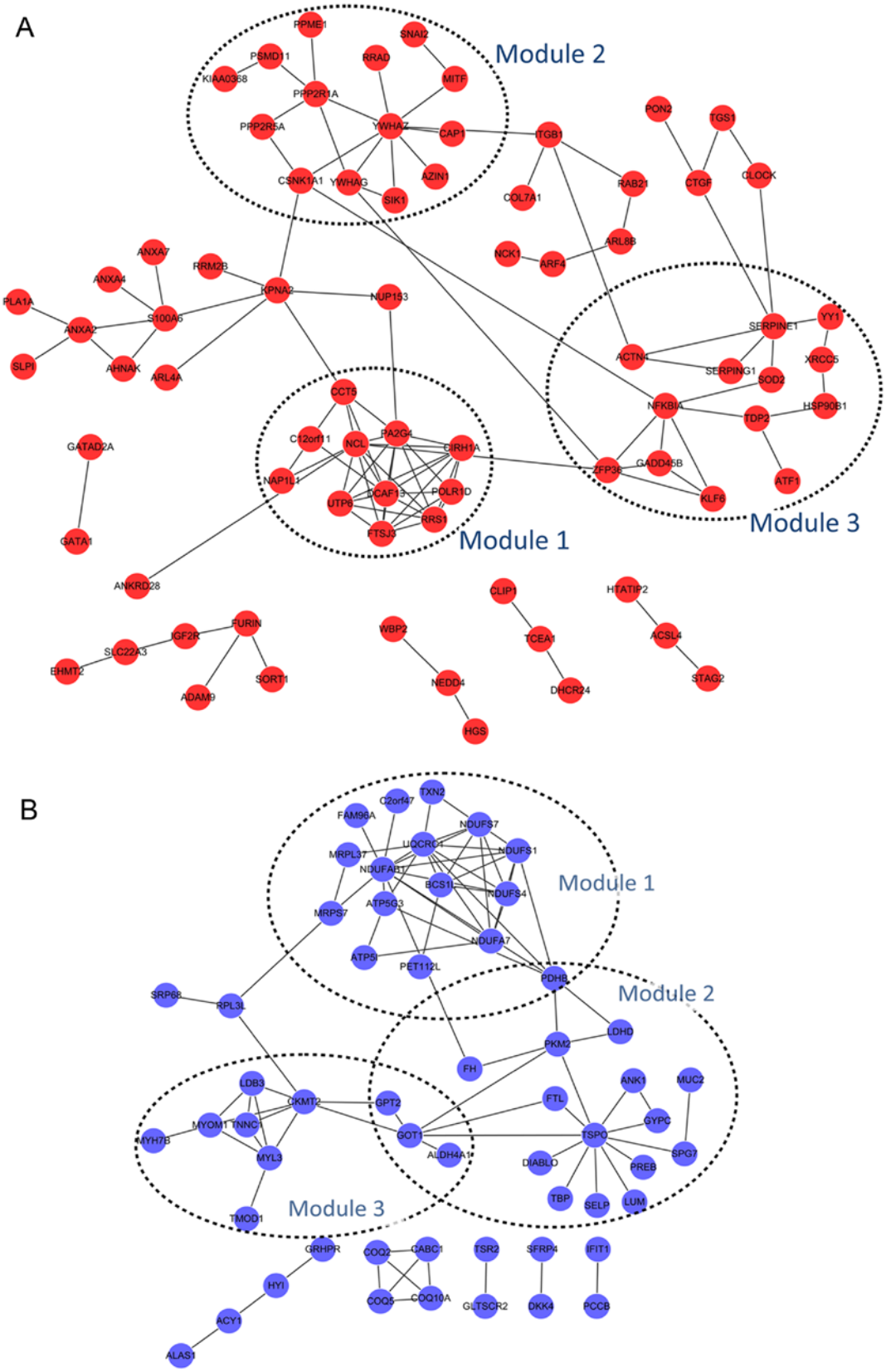

Figure 4. Significant modules of differentially expressed genes (DEGs) in the protein-protein interaction (PPI) network. (A) Three modules for the upregulated DEGs, (B) three modules for the downregulated DEGs. Red nodes stand for the upregulated DEGs while blue nodes represent downregulated DEGs. Edges stand for the protein interaction and dot circle stand for the modules.

In addition, GO terms of the DEGs in the significant modules are presented in Table III. Functions of upregulated DEGs in module 1 were in the ribosome biogenesis. No GO term of the upregulated DEGs in module 2 was detected. Upregulated genes in module 3, such as X-ray repair complementing defective repair in Chinese hamster cells 5 (*XRCC5*)

Table III. GO terms of the DEGs in significant modules.

Module	Term	Count	p-value	Genes
A, GO terms of the upregulated DEGs in the significant modules				
Module 1	Cluster 1 enrichment score: 4.308352643161865			
	GO:0042254 - ribosome biogenesis	5	7.66E-07	*DCAF13*, *PA2G4*, *UTP6*, *RRS1*, *FTSJ3*
	GO:0022613 - ribonucleoprotein complex biogenesis	5	3.63E-06	*DCAF13*, *PA2G4*, *UTP6*, *RRS1*, *FTSJ3*
	GO:0006364 - rRNA processing	4	2.48E-05	*DCAF13*, *PA2G4*, *UTP6*, *FTSJ3*
	GO:0016072 - rRNA metabolic process	4	2.82E-05	*DCAF13*, *PA2G4*, *UTP6*, *FTSJ3*
	GO:0034470 - ncRNA processing	4	2.05E-04	*DCAF13*, *PA2G4*, *UTP6*, *FTSJ3*
	GO:0034660 - ncRNA metabolic process	4	3.78E-04	*DCAF13*, *PA2G4*, *UTP6*, *FTSJ3*
	GO:0006396 - RNA processing	4	4.60E-03	*DCAF13*, *PA2G4*, *UTP6*, *FTSJ3*
Module 2	None			
Module 3	Cluster 1 enrichment score: 2.0090957996722376			
	GO:0043066 - negative regulation of apoptosis	4	2.51E-03	*XRCC5*, *HSP90B1*, *NFKBIA*, *SOD2*
	GO:0043069 - negative regulation of programmed cell death	4	2.61E-03	*XRCC5*, *HSP90B1*, *NFKBIA*, *SOD2*
	GO:0060548 - negative regulation of cell death	4	2.63E-03	*XRCC5*, *HSP90B1*, *NFKBIA*, *SOD2*
	GO:0042981 - regulation of apoptosis	5	2.92E-03	*XRCC5*, *HSP90B1*, *ACTN4*, *NFKBIA*, *SOD2*
	GO:0043067 - regulation of programmed cell death	5	3.03E-03	*XRCC5*, *HSP90B1*, *ACTN4*, *NFKBIA*, *SOD2*
	GO:0010941 - regulation of cell death	5	3.07E-03	*XRCC5*, *HSP90B1*, *ACTN4*, *NFKBIA*, *SOD2*
	GO:0001666 - response to hypoxia	3	5.05E-03	*HSP90B1*, *ACTN4*, *SOD2*
	GO:0070482 - response to oxygen levels	3	5.58E-03	*HSP90B1*, *ACTN4*, *SOD2*
	GO:0006916 - anti-apoptosis	3	1.16E-02	*HSP90B1*, *NFKBIA*, *SOD2*
	GO:0042592 - homeostatic process	4	2.01E-02	*XRCC5*, *HSP90B1*, *SERPINE1*, *SOD2*
	Cluster 2 enrichment score: 1.7537673375612617			
	GO:0030097 - hemopoiesis	3	1.50E-02	*XRCC5*, *KLF6*, *SOD2*
	GO:0048534 - hemopoietic or lymphoid organ development	3	1.81E-02	*XRCC5*, *KLF6*, *SOD2*
	GO:0002520 - immune system development	3	2.02E-02	*XRCC5*, *KLF6*, *SOD2*
	Cluster 3 enrichment score: 1.1482146347041753			
	GO:0051252 - regulation of RNA metabolic process	6	9.85E-03	*ZFP36*, *KLF6*, *YY1*, *NFKBIA*, *ATF1*, *SOD2*
	GO:0006357 - regulation of transcription from RNA polymerase II promoter	4	1.84E-02	*KLF6*, *YY1*, *NFKBIA*, *SOD2*
	GO:0006355 - regulation of transcription, DNA-dependent	5	4.53E-02	*KLF6*, *YY1*, *NFKBIA*, *ATF1*, *SOD2*
B, GO terms of the downregulated DEGs in the significant modules				
Module 1	Cluster 1 enrichment score: 8.207305207832107			
	GO:0006091 - generation of precursor metabolites and energy	10	1.12E-12	*NDUFS7*, *NDUFS4*, *UQCRC1*, *TXN2*, *NDUFA7*, *NDUFAB1*, *ATP5I*, *ATP5G3*, *NDUFS1*, *PDHB*
	GO:0006119 - oxidative phosphorylation	8	1.39E-12	*NDUFS7*, *NDUFS4*, *UQCRC1*, *NDUFA7*, *NDUFAB1*, *ATP5I*, *ATP5G3*, *NDUFS1*
	GO:0045333 - cellular respiration	7	1.92E-10	*NDUFS7*, *NDUFS4*, *UQCRC1*, *NDUFA7*, *NDUFAB1*, *NDUFS1*, *PDHB*

Table III. Continued.

Module	Term	Count	p-value	Genes
B, GO terms of the downregulated DEGs in the significant modules				
	GO:0022900 - electron transport chain	7	5.13E-10	*NDUFS7*, *NDUFS4*, *UQCRC1*, *TXN2*, *NDUFA7*, *NDUFAB1*, *NDUFS1*
	GO:0042773 - ATP synthesis coupled electron transport	6	1.27E-09	*NDUFS7*, *NDUFS4*, *UQCRC1*, *NDUFA7*, *NDUFAB1*, *NDUFS1*
	GO:0042775 - mitochondrial ATP synthesis coupled electron transport	6	1.27E-09	*NDUFS7*, *NDUFS4*, *UQCRC1*, *NDUFA7*, *NDUFAB1*, *NDUFS1*
	GO:0015980 - energy derivation by oxidation of organic compounds	7	2.11E-09	*NDUFS7*, *NDUFS4*, *UQCRC1*, *NDUFA7*, *NDUFAB1*, *NDUFS1*, *PDHB*
	GO:0022904 - respiratory electron transport chain	6	2.53E-09	*NDUFS7*, *NDUFS4*, *UQCRC1*, *NDUFA7*, *NDUFAB1*, *NDUFS1*
	GO:0006120 - mitochondrial electron transport, NADH to ubiquinone	5	5.62E-08	*NDUFS7*, *NDUFS4*, *NDUFA7*, *NDUFAB1*, *NDUFS1*
	GO:0055114 - oxidation reduction	8	6.79E-07	*NDUFS7*, *NDUFS4*, *UQCRC1*, *TXN2*, *NDUFA7*, *NDUFAB1*, *NDUFS1*, *PDHB*
	GO:0016310 - phosphorylation	8	3.09E-06	*NDUFS7*, *NDUFS4*, *UQCRC1*, *NDUFA7*, *NDUFAB1*, *ATP5I*, *ATP5G3*, *NDUFS1*
	GO:0006793 - phosphorus metabolic process	8	1.14E-05	*NDUFS7*, *NDUFS4*, *UQCRC1*, *NDUFA7*, *NDUFAB1*, *ATP5I*, *ATP5G3*, *NDUFS1*
	GO:0006796 - phosphate metabolic process	8	1.14E-05	*NDUFS7*, *NDUFS4*, *UQCRC1*, *NDUFA7*, *NDUFAB1*, *ATP5I*, *ATP5G3*, *NDUFS1*
	Cluster 2 enrichment score: 2.1705698016603754			
	GO:0010257 - NADH dehydrogenase complex assembly	3	3.82E-05	*NDUFS7*, *NDUFS4*, *BCS1L*
	GO:0032981 - mitochondrial respiratory chain complex I assembly	3	3.82E-05	*NDUFS7*, *NDUFS4*, *BCS1L*
	GO:0033108 - mitochondrial respiratory chain complex assembly	3	5.60E-05	*NDUFS7*, *NDUFS4*, *BCS1L*
	GO:0007005 - mitochondrion organization	3	7.49E-03	*NDUFS7*, *NDUFS4*, *BCS1L*
	GO:0043623 - cellular protein complex assembly	3	1.02E-02	*NDUFS7*, *NDUFS4*, *BCS1L*
	GO:0034622 - cellular macromolecular complex assembly	3	3.62E-02	*NDUFS7*, *NDUFS4*, *BCS1L*
	GO:0034621 - cellular macromolecular complex subunit organization	3	4.47E-02	*NDUFS7*, *NDUFS4*, *BCS1L*
	Cluster 3 enrichment score: 2.16913552816325			
	GO:0046034 - ATP metabolic process	3	4.40E-03	*ATP5I*, *ATP5G3*, *NDUFS1*
	GO:0009205 - purine ribonucleoside triphosphate metabolic process	3	5.44E-03	*ATP5I*, *ATP5G3*, *NDUFS1*
	GO:0009199 - ribonucleoside triphosphate metabolic process	3	5.53E-03	*ATP5I*, *ATP5G3*, *NDUFS1*
	GO:0009144 - purine nucleoside triphosphate metabolic process	3	5.90E-03	*ATP5I*, *ATP5G3*, *NDUFS1*
	GO:0009141 - nucleoside triphosphate metabolic process	3	6.77E-03	*ATP5I*, *ATP5G3*, *NDUFS1*
	GO:0009150 - purine ribonucleotide metabolic process	3	7.49E-03	*ATP5I*, *ATP5G3*, *NDUFS1*
	GO:0009259 - ribonucleotide metabolic process	3	8.46E-03	*ATP5I*, *ATP5G3*, *NDUFS1*
	GO:0006163 - purine nucleotide metabolic process	3	1.33E-02	*ATP5I*, *ATP5G3*, *NDUFS1*

Table III. Continued.

Module	Term	Count	p-value	Genes
B, GO terms of the downregulated DEGs in the significant modules				
Module 2	Cluster 1 enrichment score: 2.0431078400255807			
	GO:0008219 - cell death	6	1.12E-03	*MUC2*, *SPG7*, *TSPO*, *PKM2*, *DIABLO*, *TBP*
	GO:0016265 - death	6	1.15E-03	*MUC2*, *SPG7*, *TSPO*, *PKM2*, *DIABLO*, *TBP*
	GO:0012501 - programmed cell death	4	3.31E-02	*MUC2*, *TSPO*, *PKM2*, *DIABLO*
	GO:0006915 - apoptosis	3	1.58E-01	*MUC2*, *TSPO*, *DIABLO*
Module 3	Cluster 1 enrichment score: 4.256164391089315			
	GO:0006936 - muscle contraction	4	2.77E-05	*MYL3*, *TNNC1*, *CKMT2*, *MYOM1*
	GO:0003012 - muscle system process	4	3.66E-05	*MYL3*, *TNNC1*, *CKMT2*, *MYOM1*
	GO:0006941 - striated muscle contraction	3	1.68E-04	*MYL3*, *TNNC1*, *MYOM1*

GO, gene ontology; DEGs, differentially expressed genes.

and Kruppel-like factor 6 (*KLF6)*, were enriched in GO terms including hemopoietic or lymphoid organ development and immune system development (Table III-A). GO functions of downregulated DEGs in module 1 were in generation of precursor metabolites and energy, whereas that in module 2 was in cell death, and that in module 3 were in muscle contraction and muscle system process (Table III-B).

Enrichment analysis of key miRNAs. In total, we identified 39 AECOPD-associated miRNAs using GSEA software at a p-value <0.01 (Table IV).

miRNA regulatory network. Thirty-nine predicted miRNAs and their target DEGS were constructed in an miR-DEG regulatory network (Fig. 5). miR-9, miR-524, miR-23a, miR-15a and miR-16 were the top 5 miRNAs with the most target DEGs (Table V), and their target DEGs included tropomodulin 1 (*TMOD1*), *GOT1*, *NR3C2* and *CPEB4* among others.

Discussion

Since AECOPD are one of the leading causes of death, there is an urgent need to investigate the mechanism underlying AECOPD and to develop an effective preventative strategy. Microarray-based studies have been performed to analyze the pathogenesis of AECOPD and to identify the AECOPD-associated genes in peripheral blood mononuclear cells (30) and skeletal muscle (16). However, no research investigating AECOPD-associated miRNAs has been reported, to the best of our knowledge. Hence, the present study was performed in order to predict AECOPD-associated mRNAs and miRNAs that may be responsible for the loss of muscle force, and to discuss the molecular mechanisms underlying the loss of muscle force during AECOPD.

NCL was observed in the Module 1 of the upregulated genes. It encodes a eukaryotic nucleolar phosphoprotein that is involved in the synthesis and maturation of ribosomes, which is mainly located in dense, fibrillar regions of the nucleolus. Nucleolin is one of the three components consisting of a D4Z4 repeat (31), in which the number variation is frequently detected in facioscapulohumeral muscular dystrophy (32). Thus, it can be inferred that *NCL* may also play a role in the loss of muscle force in AECOPD since it is associated with muscular function. Furthermore, *NCL* was also predicted to be regulated by miR-1 in the present study. miR-1 and miR-206, another miRNA also observed to regulate the differential gene expression herein, promote myotube formation (33). Another study reported the reduced expression of miR-1 in the quadriceps of patients with COPD, suggesting that miR-1 downregulation may contribute to COPD-associated skeletal muscle dysfunction (34), and they further observed an inverse correlation between miR-1 and Akt phosphorylation levels or HDAC4 protein levels in patients. Thus, it is likely that NCL may be downregulated during the AECOPD due to the downregulation of miR-1. *SOD2* was another upregulated gene observed in module 3. An imbalance of the oxidation-antioxidant system in the body represents the principal cause of AECOPD (35). SOD2 (Mn-SOD) is a key enzyme that prevents cells from damage by eliminating the endogenous free radicals in the body (36), and increased expression was found in patients with AECOPD in the present study. Considering that samples were taken from patients with an exacerbation on day 4 of hospitalization, the antioxidant system may be activated by upregulating SOD2 in patients with AECOPD. However, this hypothesis requires further careful consideration. Additionally, Togliatto *et al* have reported that unacylated ghrelin (UnAG) induced skeletal muscle regeneration following hindlimb ischemia and was mediated by SOD2 (37). SOD2 may also play similar roles in muscle dysfunction during AECOPD, which suggests that SOD2 may be used as a therapeutic target in AECOPD.

In addition, *XRCC5* and *KLF6* were also found in module 3 of the upregulated DEGs. Both genes are involved in immune system development according to GO analysis, suggesting that the two genes may be important in AECOPD. KLF6 is

Table IV. Enrichment analysis of key miRNAs.

Name	Basic groups	Size	ES	NES	NOM	FDR
miR-23a, miR-23b	AATGTGA	343	0.278676	1.438706	0	0.089588
miR-103, miR-107	ATGCTGC	177	0.335453	1.486206	0	0.088895
miR-221, miR-222	ATGTAGC	108	0.336992	1.396569	0	0.094994
miR-320	CAGCTTT	213	0.343332	1.552349	0	0.078454
miR-520f	AAGCACT	194	0.344786	1.56579	0	0.091075
miR-183	GTGCCAT	152	0.355742	1.541346	0	0.073964
miR-524	CTTTGTA	365	0.35657	1.562035	0	0.084759
miR-493	ATGTACA	266	0.384502	1.549554	0	0.074808
miR-494	ATGTTTC	128	0.386803	1.631935	0	0.135062
miR-498	GCTTGAA	92	0.397854	1.605433	0	0.115428
miR-1, miR-206	ACATTCC	253	0.398441	1.603156	0	0.108254
miR-323	TAATGTG	131	0.400318	1.56248	0	0.087979
miR-373	TTTTGAG	194	0.406584	1.664687	0	0.125706
miR-485-3p	TGTATGA	128	0.41496	1.611675	0	0.123895
miR-9	TAGCTTT	190	0.419432	1.627284	0	0.120835
miR-17-3p	ACTGCAG	87	0.421458	1.635642	0	0.150641
miR-409-3p	AACATTC	120	0.421607	1.671052	0	0.156583
miR-422b, miR-422a	AAGTCCA	56	0.428607	1.58016	0	0.109384
miR-200a	GTAAGAT	44	0.440217	1.63578	0	0.179569
miR-518a-2	TTTGCAG	169	0.441104	1.684444	0	0.204788
miR-202	ATAGGAA	84	0.445265	1.574246	0	0.100272
miR-410	GTTATAT	76	0.466728	1.711476	0	0.295102
miR-217	ATGCAGT	95	0.477771	1.610814	0.009328	0.113216
miR-15a, miR-16, miR-15a, miR-195						
miR-424, miR-497	TGCTGCT	499	0.293906	1.427648	0.009452	0.09335
miR-126	TAATAAT	179	0.379129	1.572559	0.009524	0.09167
miR-186	ATTCTTT	234	0.390894	1.593438	0.009785	0.099291
miR-182	TTGCCAA	274	0.355565	1.520188	0.00994	0.085925
miR-519e	GGCACTT	105	0.382867	1.579575	0.00994	0.104196
miR-527	CTTTGCA	192	0.316954	1.487434	0.00996	0.089759

Basic groups: the targets of miRNAs; size, the number of target genes regulated by miRNA; ES, the integral enrichment of miRNAs; NES, standardization of the enrichment integration; NOM, standardization of p-value; FDR, false discovery rate.

Table V. miRNAs with the top 5 degrees in regulatory network.

miRNA	Degree	miRNA	Degree
miR-9	20	miR-15A	11
miR-524	13	miR-15B	11
miR-23a	10	miR-16	11
miR-23b	10	miR-182	11
miR-320	10	miR-186	11
miR-373	10	miR-195	11
miR-1	9	miR-424	11
miR-206	9	miR-497	11
miR-518a-2	9		

a member of the Kruppel-like family of transcription factors that functions as a tumor suppressor (38). Mgbemena *et al* have proven that KLF6 regulated the apoptosis of lung cells through iNOS expression during respiratory syncytial virus infection (39). In addition, KLF6 may also be involved in cell atrophy during an acute exacerbation. On the other hand, XRCC5 is an ATP-dependent DNA helicase II or DNA repair protein (40). The role of XRCC5 in COPD has not been fully elucidated. However, previous findings have revealed that DNA damage or lack of DNA repair regulated the immune response to the tissue destruction in COPD (41). Therefore, XRCC5 may be a novel target for protecting against AECOPD.

TSPO displayed downregulated expression in patients with AECOPD, and this was observed in module 2. This gene encodes a protein transformation-related 18-kDa protein that

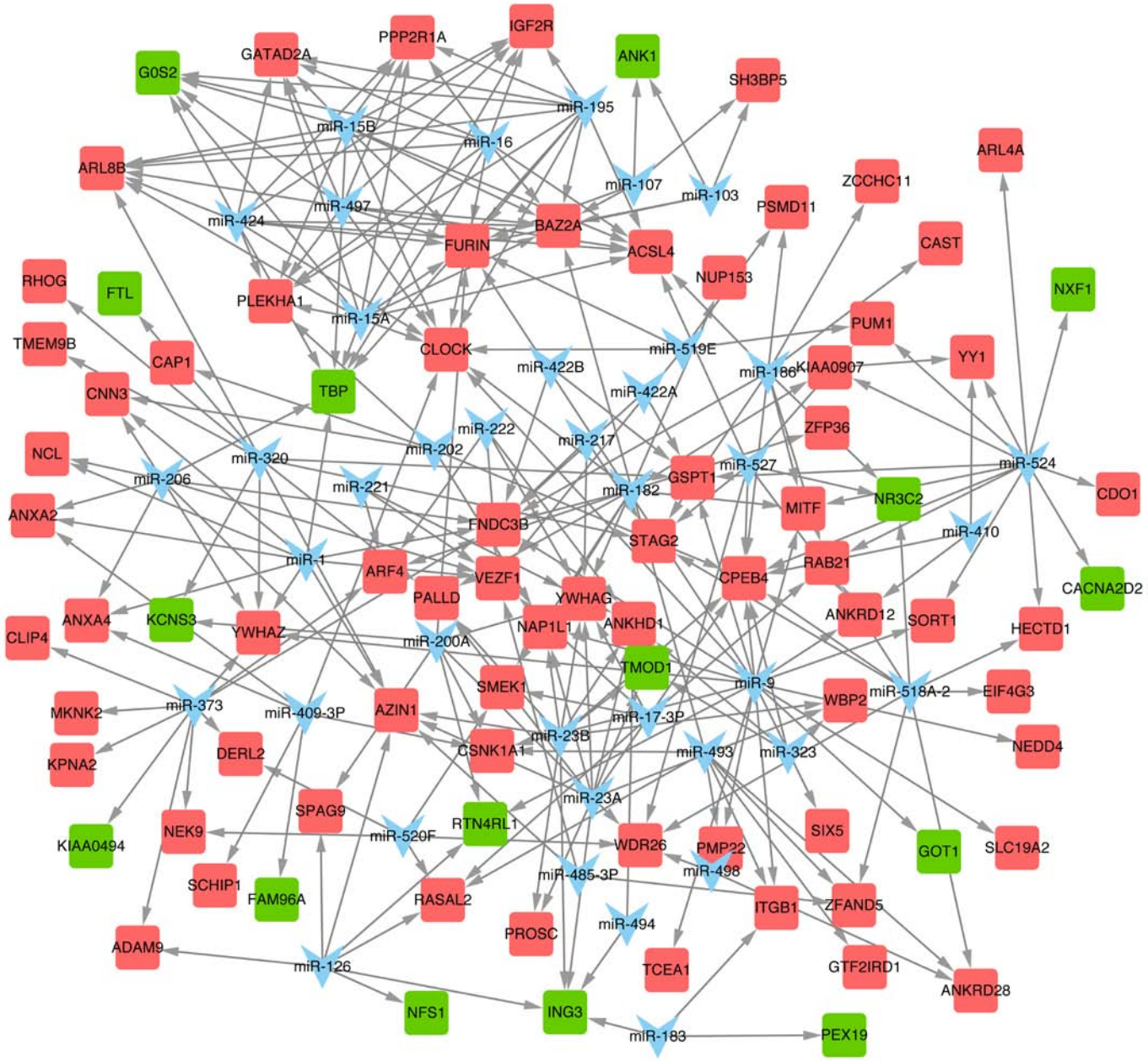

Figure 5. The miRNA regulatory network. Blue represents miRNA; red represents upregulated differentially expressed genes (DEGs); green represents downregulated DEGs.

assists in the recognition of the mitochondrial proteins prior to intracellular transportation (42). Otherwise, dysfunction of mitochondria caused by permeability transition would lead to the apoptosis of muscle cells, which plays a principal role in the progression of COPD (43). Thus, the downregulation of *TSPO* expression observed suggests that it may have an important role in the loss of muscle force occurring in AECOPD.

TMOD1 was another key downregulated gene, observed in module 3. Tropomodulin is a binding protein of tropomyosin, existing in the muscle cells, and is extracted from erythrocytes. It is necessary for many key biological functions including cell migration, differentiation and muscle contraction (44). The most important cause of the progression of AECOPD is oxygen deficiency resulting from several factors, such as the transformation of pulmonary blood vessel structures, manifested by hyperplasia and hypertrophy of pulmonary arterial muscle cells, leading to the incrassation of membranes and fibroblast proliferation (45). In the present study, *TMOD1* was predicted to be regulated by miR-23a. It has been reported that the upregulation of miR-23a inhibits the development of B cells (46).

GOT1, displaying downregulated expression in AECOPD, was found in both modules 2 and 3 of the downregulated genes. *GOT1* encodes glutamic-oxaloacetic transaminase 1, which is a cytoplasmic form of glutamic-oxaloacetic transaminase that is involved in amino acid metabolism. De Palma *et al* have also reported the decreased expression of GOT1 in patients with Ullrich congenital muscular dystrophy compared to controls (47). However, there have been no reports regarding the role of GOT1 in AECOPD, to the best of our knowledge. However, it has been reported as a putative target gene of miR-9 by Thulin *et al* (48), which is consistent with our predictions. miR-9 is known to play a key role in the activation of monocytes

and/or macrophages during inflammatory responses (49). More importantly, it is also reported to play a role in Huntington's disease, a type of motor neuron disease (50). Furthermore, the abnormal expression of miR-9 alters motor neuron subtype differentiation as well as columnar development of spinal cords in chick embryos (51). Although the involvement of miR-9 in AECOPD has not been reported to date, to the best of our knowledge, it is possible that this miRNA may play a role in the loss of muscle force in AECOPD, and *GOT1* may also be involved this process.

Finally, another two miRNAs, miR-15a and miR-16, were also found overexpressed in AECOPD patients in the present study. The pathway of Wnt signaling is known to be a promising target for mediating the development of COPD (52). A knockout of Wnt2 gene would induce lung hypoplasia and pulmonary hemorrhage caused by the abnormal muscle cells (53). Notably, miR-15a and miR-16-1 are reported to inhibit Wnt signaling (54). Thus, we hypothesized that both miR-15a and miR-16 play key roles in preventing the progression of AECOPD.

In conclusion, the present study identified some DEGs, such as *NCL*, *GOT1*, *SOD2*, *KLF6*, *XRCC5*, *TSPO* and *TMOD1*, and several miRNAs (e.g., miR-1, miR-9 and miR-23a) which may be associated with the pathomechanism of AECOPD. Among them, *SOD2*, *KLF6* and *XRCC5* may be involved in AECOPD due to infection via immune system development. The present study provides in-depth knowledge of the pathogenesis underlying the loss of muscle force during an acute exacerbation of COPD, despite using non-experimental methods. Since the public microarray data used in this study comes from a small sample size - 4 male patients with acute COPD and 5 male patients with stable COPD - it is necessary to validate our findings using experimental methods in a Chinese population with a larger sample size.

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