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Identification of microRNAs in acute respiratory distress syndrome based on microRNA expression profile in rats

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Abstract. Acute respiratory distress syndrome (ARDS) remains a severe disease associated with an ~40% mortality rate and as many as 200,000 new cases annually. MicroRNAs (miRNAs) have important roles in gene regulation and cancer development. The present study aimed to identify the potential roles of miRNAs in the pathogenesis and progression of ARDS. The miRNA expression profile of the GSE57223 dataset was downloaded from the Gene Expression Omnibus database. Following data normalization, differentially expressed miRNAs were identified using the t-test method. The miRWalk database was searched to predict target genes of the identified miRNAs and then a miRNA-miRNA network with co-regulated target genes was constructed. Additionally, Gene Ontology (GO) analysis was performed for the target genes and a miRNA-miRNA functional synergistic network (MFSN) was established. GO and pathway analyses were performed for the co-regulated target genes of significant miRNAs in MFSN. Additionally, a protein-protein-interaction network was constructed for these target genes. A total of 19 miRNAs were differentially expressed between ARDS and normal lung tissue were identified. The four downregulated rno-let-7 family members were detected to have numerous co-regulated target genes and synergistic functions. Additionally, the target genes of the four miRNAs were significantly enriched the biological processes of wounding and inflammatory response. Additionally, interleukin (IL)-6 was identified as a hub protein with a high degree. The four downregulated rno-let-7 miRNAs may be involved in the inflammatory process in the pathogenesis and progression of ARDS, via the synergistic regulation of their target genes, such as IL-6. However, additional experimental validation is required.

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Introduction

Acute respiratory distress syndrome (ARDS) is an extreme manifestation of acute injury to the lung, characterized by extensive lung inflammation, profound hypoxemia and non-cardiogenic pulmonary edema formation (1,2). In addition, ARDS is a serious complication resulting from sepsis and associated with multiple organ failure, and mortality and morbidity (3). It has been estimated that ~200,000 cases of ARDS occurred annually, with a high mortality rate of ~40% in the United States (4). The outcome in ARDS is complicated as the syndrome is influenced by multiple factors, such as the nature of the precipitating factors and the extent of the subsequent multiorgan failure (5).

The pathogenesis of ARDS involves inflammatory injury to the lung endothelium and epithelium (6), which is accompanied by an influx of neutrophils into the pulmonary interstitium and bronchoalveolar space (7). The activated neutrophils may damage endothelial and epithelial cells and are considered to be a crucial factor in pathogenesis and progression of ARDS (8). Endothelial injury may result in the effusion of protein-rich fluid into alveolar airspace and the damage to alveolar epithelium may increase the entry of fluid into the alveolar lumen (2).

Previous studies have performed proteomic and genomic analyses to determine the pathogenesis of lung diseases (9-11), an increasing number of studies have focused on the role of small molecules, such as microRNAs (miRNAs) in the pathogenesis of the diseases (12-14).

miRNAs are a class of non-coding small RNAs ~22 nucleotides in length. miRNAs negatively regulate gene expression through translational repression or mRNA degradation by binding to 3'-untranslated region (3'-UTR) of the target mRNA transcripts (14,15). miRNAs have been reported to be extensively involved in many biological processes and associated with various diseases (16,17). A considerable number of miRNAs have been observed to participate in the regulation of physiological lung function, including miRNA (miR)-17, miR-92a and miR-127 (18-20). However, how miRNAs are involved in the pathogenesis and progression of ARDS remains to be elucidated.

The present study performed miRNA microarray analysis of the GSE57223 dataset downloaded from Gene Expression Omnibus (GEO) database, and aimed to identify the potential

regulatory roles of miRNAs in the pathogenesis and progression of ARDS in order to provide novel insights into the regulatory mechanisms of the ARDS etiology.

Materials and methods

miRNA microarray data. The datasets of GSE57223 (2) deposited in the GEO database (https://www.ncbi.nlm.nih.gov/geo) were downloaded. The data set contains miRNA expression profiles of 12 samples from 6 control normal lung tissues and 6 ARDS lung tissue from a rat model, which has previously been described (2). The platform was GPL18654 (Rat microRNA array).

Data preprocessing. The probe numbers were converted into the corresponding gene symbols. The average probe value was calculated as the expression value of the gene when multiple probes corresponded to one gene. For probes with a missing value, the k-nearest neighbor (KNN) averaging method from the impute package in R (21) was applied to fill the vacancy, with k in the default parameter of 10. Subsequently, quantile normalization was conducted using the preprocessCore package in R (22).

Identification of differentially expressed miRNAs. Following normalization of the data, the limma package in R (23) was used to identify differentially expressed miRNAs between the ARDS lung tissues and the control tissues, using the t-test method. The P-value was adjusted using the Benjamini-Hochberg method (24). Significant differentially expressed miRNAs with $P<0.05$ and $|\log2FC|>1$ were identified.

Construction of miRNA-miRNA network with co-regulated target genes. A gene that may be targeted by more than one miRNA was termed the co-regulated target gene of these miRNAs. The miRWalk database (25), which provides 10 different types of prediction algorithms, including DIANAmT, miRanda, miRDB, miRWalk, RNAhybrid, PICTAR4, PICTAR5, PITA, RNA22 and TargetScan, was used to construct the regulatory network of miRNAs and their target genes. The database was searched to construct the regulatory network of miRNAs and their target genes. Each method may produce a prediction result. In the present study, the target genes were predicted by at least 4 different algorithms to construct the miRNA-miRNA network with co-regulated target genes, which was visualized utilizing Cytoscape software version 3.4.0 (26).

Synergistic function analysis of miRNAs and biological function analysis of their target genes. The gene symbol of target genes was transformed into gene ID and clusterProfiler package in R (27) was used to perform Gene Ontology (GO) enrichment analysis, particularly for the Biological Process (BP) term. A functional synergistic miRNA-miRNA interaction pair was defined when their co-regulated target genes were enriched in at least one BP term. The threshold criteria were $P<0.05$ and $q<0.05$ for significant BP terms. The miRNA interaction pairs having common target genes were filtered out to establish the miRNA-miRNA functional synergistic network (MFSN) (15).

Table I. Predicted target genes of differentially expressed miRNAs based on the mirWalk database.

miRNA	Target genes	Regulation
rno-miR-126	28	Down
rno-miR-129	674	Up
rno-let-7a	409	Down
rno-let-7b	409	Down
rno-miR-135b	495	Down
rno-let-7c	409	Down
rno-miR-103	757	Up
rno-let-7f	409	Down

miR, microRNA.

In the MFSN, the co-regulated target genes of the significant miRNA-miRNA interaction pairs were screened for further analysis. GO and pathway analyses were conducted using the Database for Annotation, Visualization and Integrated Discovery online software (28) and the threshold for significant GO and pathway terms were P-<0.05. Additionally, the interacted protein-protein pairs with combined score >0.04 were selected to establish the protein-protein-interaction (PPI) network for the co-regulated target genes using STRING version 9.1 (29), which was visualized with Cytoscape software (26). In addition, hub proteins were screened out through the analysis of topological characteristics of the PPI network.

Results

miRNAs differentially expressed between ARDS lung tissues and normal tissues. Normalization of the microarray data was performed and the medians of miRNA expression values are presented in Fig. 1. According to the defined threshold criteria, a total of 19 significant differentially expressed miRNAs were identified between ARDS lung tissues and normal tissues, with 6 upregulated miRNAs, including rno-miR-30e, rno-mir-129, rno-miR-199a, rno-miR-34b and rno-miR-103, and 13 downregulated miRNAs, namely rno-miR-122a, rno-miR-290, rno-miR-324-5p, rno-miR-35, rno-miR-223, rno-let-7f, rno-miR-26a, rno-miR-24, rno-miR-143, rno-let-7b, rno-let-7c, rno-let-7a and rno-miR-126.

miRNA-miRNA network with co-regulated target genes. Based on the prediction of mirWalk databases and the selection criterion, the target genes of eight miRNAs were screened out. Notably, rno-miR-103 had the highest number (n=757) of target genes and the 4 rno-let-7 miRNAs also had high numbers (n=409) of target genes (Table I). To detect the associations of the miRNAs and their target genes, the regulatory network for the 8 miRNAs with their target genes was constructed, comprising of 3590 edges and 2152 nodes (Fig. 2). Furthermore, a miRNA-miRNA network with co-regulated target genes was established, consisting of 27 miRNA-miRNA interaction pairs. As presented in Fig. 3 and Table I, the four downregulated miRNAs from the rno-let-7 family had high numbers (n=409) of target genes.

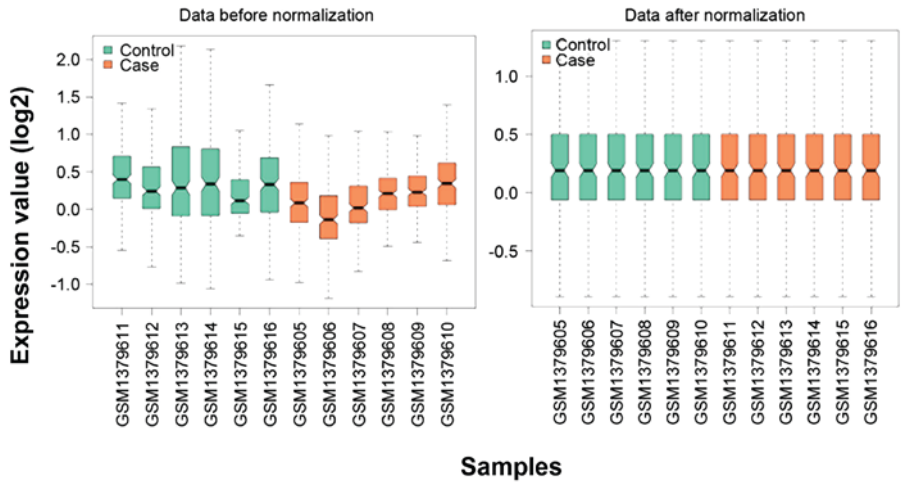

Figure 1. Normalization of the gene expression profiling. Box plots displaying the intensity log ratio distribution before and after normalization procedures. The x-axis indicates the name of the sample in the dataset and the y-axis indicates the value of the expression following a $\log_2$ transformation. Thick black lines, median.

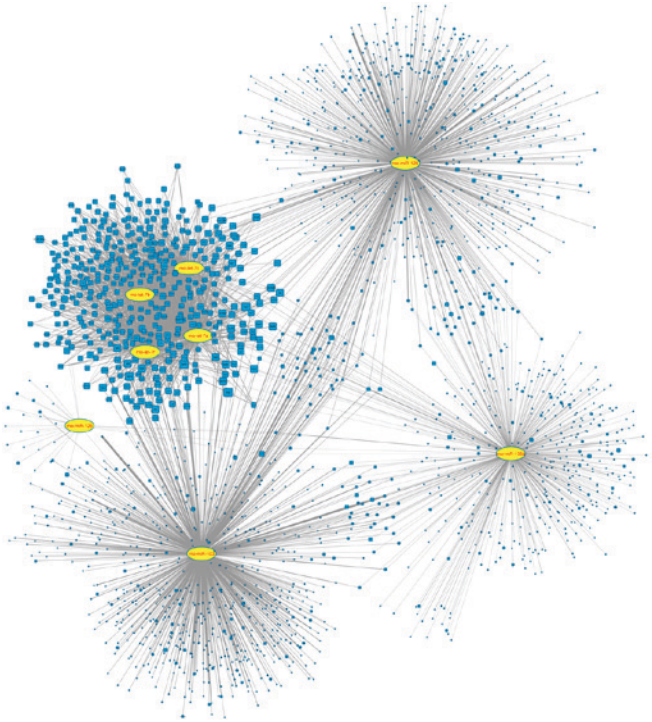

Figure 2. Regulatory network of miRNAs and their target genes. Nodes in yellow nodes represent miRNAs and nodes in blue represent target genes. miRNA, microRNA.

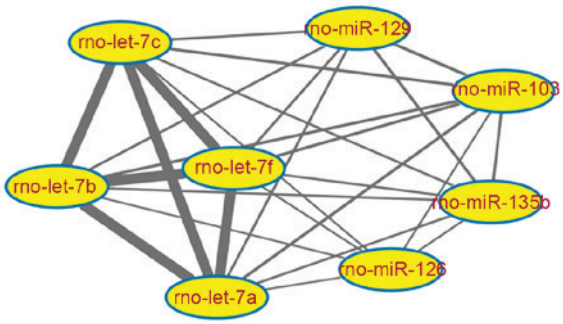

Figure 3. miRNA-miRNA network of differentially expressed miRNAs with co-regulated target genes. Yellow nodes denote differentially expressed miRNAs, and edges denote the interaction between two miRNAs. The thickness of the edge represents the number of co-regulated target genes. miRNA, microRNA.

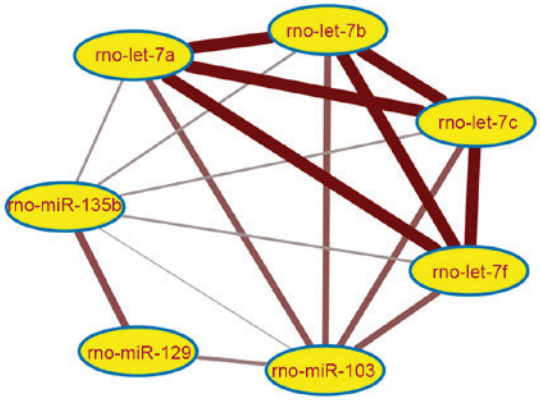

Figure 4. miRNA-miRNA functional synergistic network of differentially expressed miRNAs. Yellow nodes denote diff erentially expressed miRNAs and edges denote the synergistic relationship between two miRNAs. The thickness of the edge represents the number of biological process terms of co-regulated target genes. miRNA, microRNA.

MFSN and biological functions of the target genes. In order to investigate the synergic functions between miRNAs, the MFSN was established, consisting of 17 miRNA-miRNA functional synergistic pairs, of the 27 identified interaction pairs with co-regulated target genes, involving a total of 7 miRNAs. As presented in Fig. 4, the 4 rno-let-7 family members were detected with synergic functions. Further functional analysis indicated that 409 target genes of the 4 rno-let-7 family miRNAs were significantly enriched in BP terms, such as response to wounding and inflammatory response and cellular component terms, including cell fraction and insoluble fraction, and molecular function terms, such as manganese ion binding and activin receptor activity. In addition, these target genes were significantly enriched in the chronic myeloid leukemia and Graft-vs.-host disease pathways (Table II). The

Table II. Top 5 significant GO and KEGG pathway terms for the co-regulated target genes of 4 rno-let-7 microRNAs.

A, Biological processes

Term	Description	Count	P-value
GO:0010033	Response to organic substance	29	1.66×10^{-5}
GO:0009611	Response to wounding	17	1.08×10^{-4}
GO:0006954	Inflammatory response	12	1.09×10^{-4}
GO:0032496	Response to lipopolysaccharide	9	1.27×10^{-4}
GO:0009991	Response to extracellular stimulus	14	1.36×10^{-4}

B, Cellular component

Term	Description	Count	P-value
GO:0000267	Cell fraction	33	6.66×10^{-6}
GO:0005626	Insoluble fraction	28	9.99×10^{-6}
GO:0005624	Membrane fraction	27	1.14×10^{-5}
GO:0031090	Organelle membrane	31	2.81×10^{-5}
GO:0043235	Receptor complex	9	1.37×10^{-4}

C, Molecular function

Term	Description	Count	P-value
GO:0048037	Cofactor binding	13	4.29×10^{-4}
GO:0030145	Manganese ion binding	7	0.001334536
GO:0017002	Activin receptor activity	3	0.001543724
GO:0050662	Coenzyme binding	10	0.002201207
GO:0008289	Lipid binding	13	0.002212067

D, KEGG pathway

Term	Description	Count	P-value
rno05220	Chronic myeloid leukemia	6	0.010215393
rno05332	Graft-versus-host disease	5	0.01224159
rno04142	Lysosome	7	0.017086633
rno00260	Glycine, serine and threonine metabolism	4	0.018428521
rno04060	Cytokine-cytokine receptor interaction	9	0.022832763

GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

PPI network for these target genes was constructed and had 176 edges and 153 nodes (Fig. 5). Based on the topological characteristics of the PPI network, the hub proteins with high degrees were identified, including interleukin (IL)-6 (degree, 17), RNA polymerase I subunit A (degree, 11), Fas cell surface death receptor (degree, 10), IL-10 and cyclin-dependent kinase-inhibitor 1 (degree, both 9).

Discussion

ARDS is a severe syndrome of acute respiratory failure associated with multiple symptoms and influenced by numerous factors (2,6). The expression of miRNAs is critically associated with cancer initiation, development and metastasis (30). The present study identified a total of 19 significant differentially expressed miRNAs between ARDS rat lung tissues and normal rat lung tissues. Notably, 4 downregulated let-7 miRNA family members (rno-let-7a, rno-let-7b, rno-let-7c and rno-let-7f) were observed to have a high number of co-regulated target genes and were recognized as miRNA-miRNA interactions with synergistic functions. Furthermore, the target genes of the miRNAs were significantly involved in the wounding and inflammatory response BP functions. In addition, IL-6 was identified as a vital hub protein based on the PPI network.

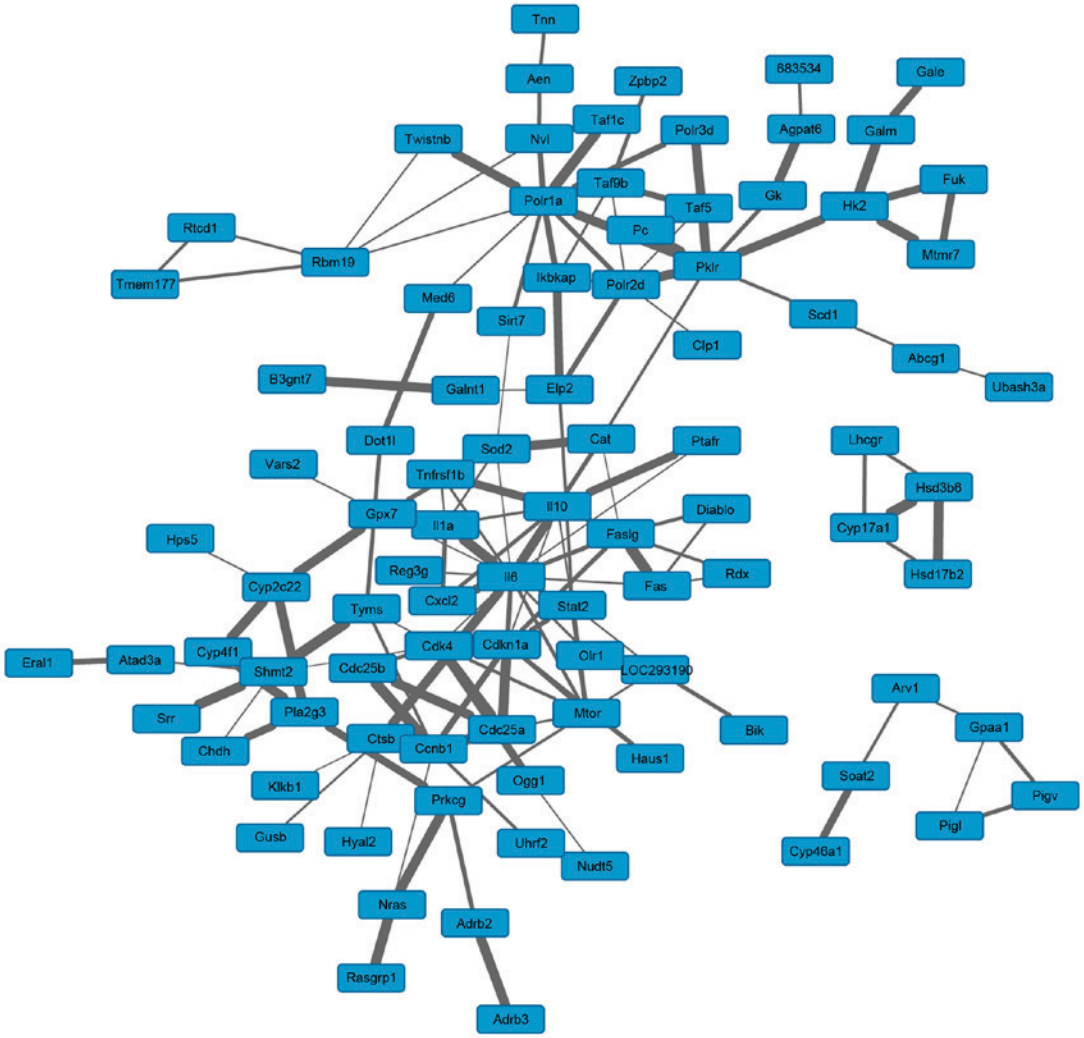

Figure 5. Protein-protein-interaction network of co-regulated target genes of four rno-let-7 microRNAs. Blue rectangles represent the target proteins and the edges represent the interaction between two proteins. The thickness of the edge represents the number of biological process terms of co-regulated target genes.

The highly conserved let-7 miRNA family members are downregulated in multiple types of cancer and the low expression of let-7 is associated with poor prognosis (31,32). By directly targeting oncogenes and inhibiting their expression levels, let-7 can act as a tumor suppressor (33). In addition, let-7 miRNA may directly limit cancer growth in the lung and the loss of its function enhances lung tumor formation (34,35). In humans, let-7 miRNAs consist of 12 family members (let-7a-1, -2, -3; let-7b; let-7c; let-7d; let-7e; let-7f-1, -2; let-7g; let-7i; miR-98), which are located at 8 different chromosomal loci and the coordinate downregulation of multiple let-7 miRNAs has been identified in various tumors (32,36). In the present study, the 4 rno-let-7 family members (rno-let-7a, rno-let-7b, rno-let-7c and rno-let-7f) were all downregulated in ARDS rat lung tissues and had synergistic functions, suggesting that they may synergistically suppress tumor growth associated with ARDS.

Reduced expression levels of let-7 were detected in lung cancer and were determined to be involved in the pathogenesis of this fatal disease (37). Through directly binding to the 3'-UTR of their target genes, the let-7 family members are involved in the regulation of inflammatory pathways and processes. A previous study indicated that let-7 participated in airway inflammation via the direct regulation of IL-13, a cytokine that was associated with the mediation of allergic inflammation (38,39). Another cytokine, IL-6 was also reported to be targeted by let-7 and an inverse association between lower expression levels of let-7a and higher levels of IL-6 was established in various cancer types compared with normal breast, prostate, hepatocellular and lung tissues (40). A feedback loop involving nuclear factor-kB, Lin28 homolog A, let-7 and IL-6 is a critical part of the transient inflammatory signal pathway (33). IL-6 is a cytokine protein that contributes to inflammation and it has been demonstrated that elevated IL-6 expression is closely associated with increased mortality in critically ill patients with ARDS (41). IL-6 has been identified as a potential predictor for the development of ARDS in adults with severe blunt trauma (42). In addition, the high expression levels of cytokines, such as IL-6 and IL-8, were determined to be reliable markers for predicting the development and the degree of severity of the systemic inflammatory response, which may result in ARDS (43). The findings of the present study indicated that IL-6, which was significantly enriched in the inflammatory response BP, is a vital target gene of let-7 miRNAs. Therefore, let-7 miRNAs may be involved in the inflammatory process via the direct regulation of IL-6 during the progression of ARDS.

In conclusion, the downregulated rno-let-7 miRNAs, including rno-let-7a, rno-let-7b, rno-let-7c and rno-let-7f, may have important roles in the inflammatory process during the pathogenesis and progression of ARDS through the synergistic regulation of their target genes, such as IL-6. These findings provided a novel insight into the roles of miRNAs in the mediation of ARDS development. However, further experimental experiments are required to verify the association between IL-6 and rno-let-7 miRNAs, as the current findings were based only on microarray data.

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