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# DIFFERENTIALLY EXPRESSED CIRCULATING LONG-NONCODING RNAS IN PREMATURE INFANTS WITH RESPIRATORY DISTRESS SYNDROME

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## ABSTRACT

**Purpose:** Recent studies have addressed the association between lung development and long-noncoding RNAs (lncRNAs). But few studies have investigated the role of lncRNAs in neonatal respiratory distress syndrome (RDS). Thus, this study aimed to compare the expression profile of circulating lncRNAs between RDS infants and controls.

**Methods:** 10 RDS infants and 5 controls were enrolled. RDS patients were further divided into mild and severe RDS subgroups. Blood samples were collected for the lncRNA expression profile. Subsequently, differentially expressed lncRNAs were screened out. Bioinformatics analysis was applied to establish a co-expression network of differential lncRNAs and mRNAs, and predict the underlying biological functions.

**Results:** A total of 135 differentially expressed lncRNAs were identified, including 108 upregulated and 27 downregulated lncRNAs (fold-change>2 and $P$<0.05) among the three groups (non-RDS, mild RDS and severe RDS groups). Of these lncRNAs, four were selected as showing higher fold changes and validated by qRT-PCR. ENST00000470527.1, ENST00000504497.1, ENST00000417781.5, and ENST00000440408.5 were increased not only in the plasma of total RDS patients but also in the severe RDS subgroup. Gene Ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analyses showed that differentially expressed lncRNAs may play important roles in RDS through regulating PI3K-Akt, RAS, MAPK, and TGF-β signaling pathways.

**Conclusion:** The present results found that ENST00000470527.1, ENST00000504497.1, ENST00000417781.5, and ENST00000440408.5 may be involved in RDS. This could provide new insight into research of the potential pathophysiological mechanisms of preterm RDS.

**Keywords:** LncRNA; Gene sequencing; Respiratory distress syndrome; Preterm infants

## INTRODUCTION

Preterm respiratory distress syndrome (RDS), characterized by immature lung development, has been a severe problem for preterm infants **[1]**. Effective treatments include pulmonary surfactant (PS) replacement and lung-protective ventilatory strategies that could improve oxygenation and minimize ventilator-induced lung injury **[2]**. Recent studies have shown that normal or abnormal lung development is highly regulated by various signal molecules, including fibroblast growth factor (FGF), bone morphogenetic protein-4 (BMP-4), transforming growth factor-beta (TGF-β), etc. **[2-3]**.

Long non-coding RNAs (lncRNAs) could modulate gene expression at the post-transcription level by depredating or translating target mRNAs **[4]**. So far, lncRNAs, characterized by a length longer than 200 nucleotides, have been demonstrated to participate in lung development and related diseases **[5]**. The specific expression patterns of lncRNAs have been explored in fetal lung development. Among distinct embryonic periods of lung development, a total of 687 differentially expressed lncRNAs were identified in our previous study **[6]**. In addition, Herriges et al. further reported that LL18/NANCI (Nkx2.1-associated noncoding intergenic RNA) and LL34 play important roles in lung development by controlling the expression of developmental transcription factors and pathways **[7]**.

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Previous studies have indicated that the components of peripheral cord blood are important clues for the identification of neonatal diseases **[8]**. For example, in our previous study [5 cases in neonatal acute respiratory distress syndrome (NARDS) group and 5 cases in non-NARDS group], circRNA expression profiles, in which 741 circRNAs were downregulated and 588 were upregulated, were screened with circRNA high-throughput sequencing **[9]**. However, the detailed molecular regulatory mechanism still remains unclear. Further exploring the role of RNAs is still important to the field of medical research. To date, few studies have investigated the role of circulating lncRNAs in RDS infants. Therefore, we planned to analyze the expression of plasma lncRNAs by RNA-sequencing and real-time quantitative PCR, then explored the potential function of differentially expressed lncRNAs by Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) in RDS infants. This research could add more useful evidence to the further study of RDS.

## PATIENTS AND METHODS

### Patients

This prospective study enrolled 15 premature infants who were admitted to Jiangyin People's Hospital of Nantong University between April 2019 and October 2019.

*Inclusion and Exclusion Criteria*: Infants were eligible for enrollment in the study if they were (1) with a gestational age less than 36 weeks; (2) admitted within 4 hours after birth; (3) appropriate for gestational age. Patients were excluded for any of the following reasons: (1) severe cyanotic congenital heart diseases; (2) congenital chromosomal diseases or severe congenital malformations; (3) severe asphyxia at delivery (5 min Apgar score <5); (4) early symptoms of sepsis **[10]**.

*Severity grading*: In the present study, the severity of RDS was determined clinically using a combination of PS treatment coupled with a degree of aeration of the lungs on chest X-ray **[11]**. The degree of aeration of the lungs on chest X-ray was graded as follows: (1) slightly reduced radiolucency with still sharp cardiac and diaphragmatic margins; (2) markedly reduced radiolucency with retained cardiac and diaphragmatic margins; (3) severely reduced radiolucency with air bronchogram and blurred cardiac and diaphragmatic margins; and (4) almost completely white lung fields with or without air bronchogram and barely visible cardiac and diaphragmatic margin **[12]**.

*Grouping*: Of the 15 included infants, 5 were neonates without RDS and 10 were newborns diagnosed with RDS (presenting as cyanosis, groan, intercostal retractions, polypnea, and nasal flaring combined with changed aeration of the lungs on chest X-ray **[11]**). Babies who were worsening when $FiO_2$ >0.30 or positive end-expiratory pressure (PEEP) > 6 cm $H_2O$ were given PS replacement **[11]**. Infants were given PS only once with lung X-ray grade 1or 2 were further defined as mild RDS, while infants who needed PS re-dosing with X-ray grade 3 or 4 were defined as severe RDS. Accordingly, based on the patients' clinical features and chest X-ray results, 10 RDS infants were further divided into mild RDS group (n=5) and severe RDS group (n=5).

*Data collecting*: Data provided by all patients were collected in detail using a standard data collection form, including age, gender, gestational age, weight, 5 min Apgar score, maternal gestational diabetes mellitus, antenatal glucocorticoid use, etc. The collection was completed by two individuals independently and verified by a third person. Patient information has been processed anonymously before statistical analysis.

### Sample preparation and RNA-sequencing

Peripheral blood samples (2ml for each person) were collected from all infants between 1 and 6 hours after birth. Among them, it should be noted that, for RDS patients, samples were drawn before PS replacement. All blood samples were frozen in the −80°C refrigerator following a specific process which includes centrifugation at 3,000 × g for 10 min at 4°C and then separation of clear upper liquid into an RNase-free tube. Total RNA was then extracted from the blood samples using the TRIzol reagent according to the manufacturer's instructions and a previous study **[13]**. After quality control of RNA, the RNA library of each sample was prepared using NEB Next Ultra RNA Library Prep Kit for the Illumina platform (BioLabs Inc., USA). The RNA sequencing analysis was performed by Genminix Informatics Co., Ltd (Shanghai, China) with the GeneChip® Human Transcriptome Array 2.0 (Affymetrix Inc., US) served as a gene expression profiling tool.

### Identification of differentially expressed genes

The expression profile of the lncRNAs were analyzed by Deseq package (Affymetrix Inc., US). Samples were hybridized on the Human Clariom D (Thermo Fisher Scientific) gene chip. Background-adjustment, normalization, and log-transformation of signals intensity were performed with the Signal Space Transformation-Robust Multi-Array Average algorithm (RMA). Raw data were analyzed by the transcriptome analysis console (TAC) 4.0 software (Applied Biosystems, Foster City, CA, USA) awaiting further analysis **[14]**. The differentially expressed lncRNAs and mRNAs were screened according to the criteria of gene differential expression with |log2-fold change| (FC) more than 2 times and adjusted $P$<0.05. The differentially expressed lncRNAs were afterward clustered by a heatmap

package while the hierarchical clustering diagram was drawn to show the results.

**Real-time quantitative PCR validation**

For lncRNA expression analysis, total RNA was transcripted to cDNA using a Reverse Transcription Kit (PrimeScript RT Master Mix, Takara Bio Inc., Otsu, Japan), then real-time quantitative PCR (qRT-PCR) validation was performed using the SYBR method (SYBR® Premix Ex Taq™, Takara Bio Inc., Otsu, Japan) according to the product instructions. An aliquot of 1 μg total RNA was added to each reaction mixture. qRT-PCR was performed on an ABI 7500 thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc. US) with SYBR Green (Roche Diagnostics Co., Ltd. GER). The thermocycling conditions were as follows: 95˚C for 5 min, followed by 40 cycles of 95˚C for 20 sec and 55˚C for 20 sec. At the end of each run, a melting curve analysis was performed at 72˚C to monitor primer dimers and formation of non-specific products. For data analysis, the comparative Ct method ($2^{\Delta\Delta}$Ct) was used. Results were expressed as fold changes of gene expression adjusted to housekeeping gene GAPDH **[15]**. All primers used in the present study were shown in **table 1**.

**LncRNA-mRNA co-expression network**

Multivariate statistical analysis was used to calculate the Pearson correlation coefficient between differentially expressed lncRNAs and mRNAs. The greater the correlation coefficient, the greater possibility that there was a regulatory relationship between certain lncRNAs and mRNAs. The co-expression network was constructed with the Pearson correlation coefficient $r > 0.99$ and $P < 0.05$ as the filtering standard in this study.

**Table 1.** The primers sequence for qPCR

| Gene name | Primer (5′-3′) | |
|---|---|---|
| ENST00000470527.1 | Forward | TGGAATTCGATGGGAACTTT |
| | Reverse | GTCTCGTCCTGGATTGAAGG |
| ENST00000504497.1 | Forward | TCGATTCTCCTGTCAGTGAAC |
| | Reverse | AATGTTTCCAGAGCACCACT |
| ENST00000417781.5 | Forward | GTTGATCGATCCAAGGTCGT |
| | Reverse | GCCTGGAATCCCAGCATTT |
| ENST00000440408.5 | Forward | TGCTTGGACAACAGACATGA |
| | Reverse | GAAGCAATGTAATCCCAGCA |
| GAPDH | Forward | AACTTTGGCATTGTGGAAGG |
| | Reverse | GGATGCAGGGATGATGTTCT |

**GO and KEGG pathway analysis**

GO and KEGG pathway analysis were applied to predict functions of the differentially expressed genes. The GO project offers a controlled vocabulary to label gene and gene product attributes in any organism (geneontology.org). GO results were mainly classified into three subgroups namely biological process, cellular component, and molecular function. GO analysis provides an interpretation of the relevance of genes differentially expressed between the groups. Fisher's exact test and the $\chi 2$ test were performed to calculate the P-value and false discovery rate of each GO term function. KEGG (kegg.jp/kegg/pathway.html) pathway analysis is a functional analysis tool, mapping a set of genes that may be associated with a certain lncRNA to potential pathways. The enrichment probability of a differentially expressed gene set in a term entry was represented by an enrichment score (EC), with a higher EC indicating a higher significance of the entry. The EC was calculated as the negative base 10 log of the P value. The input used in the bioinformatics analysis was the differential mRNA genes co-expressed with lncRNA that were screened in the lncRNA expression profile.

**Statistical analysis**

For clinical results (clinical characteristics), data were analyzed using SPSS 17.0 software. Quantitative data are expressed as mean ± standard deviation (SD). One-way variance analysis was applied to detect differences among the three groups. In terms of qualitative data, the Pearson Chi-square test was performed. Significant differences were considered as $P < 0.05$.

## RESULTS

**Clinical characteristics of the premature infants (G1, G2 and G3)**

This present study was comprised of 15 premature infants in total, 5 cases without RDS for control (named as Group 1, G1), 5 cases with mild RDS (named as Group 2, G2) and 5 with severe RDS (named as Group 3, G3). The recruitment procedures are shown in **Figure 1**, and clinical

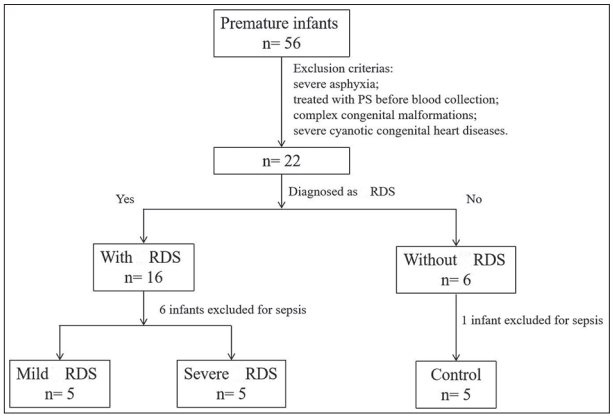


**Figure 1.** The recruitment procedures of the patients.

characteristics of the infants are summarized in **table 2**. There were no significant differences in gestational age, birth weight, 5 min Apgar score, gender, mode of delivery, twin pregnancy, mother of gestational diabetes, and prenatal glucocorticoid ($P$>0.05).

### Expression profile of lncRNAs and mRNAs in three groups (G1, G2, and G3)

Affymetrix Human GeneChip was utilized to determine the expression spectrum of lncRNAs. As a result, the G1 vs. G2 comparison showed a total of 10112 differentially expressed lncRNAs, while the G2 vs. G3 comparison showed a total of 4663 differentially expressed lncRNAs. Of them, 135 lncRNAs were indicated to be differentially expressed among all three groups (G1, G2, and G3) after fold-change filtering (adjusted $P$ value<0.05 and |log2-fold change|>2). The information of the top 10 upregulated and downregulated lncRNAs are listed in **table 3**. A hierarchical clustering map is presented to distinguish lncRNA expression profiles among the three groups. (**Figure 2**)

### Construction of the lncRNA-mRNA co-expression network (G1, G2, and G3)

Furthermore, differentially expressed mRNAs were compared for target prediction. Of them, the comparison between G1 and G2 showed a total of 2520 differentially expressed mRNAs, while the comparison between G2 and G3 showed a total of 530 mRNAs. The comparison of the three groups showed a total of 616 differentially expressed mRNAs. The lncRNA-mRNA co-expression network was constructed and showed a complex interaction between lncRNAs and mRNAs. Our analysis finally identified a total of 278 mRNAs closely

**Table 2.** Clinical characteristics of the three groups

| Group | G1 (N=5) | G2 (N=5) | G3 (N=5) | *P* valve |
|---|---|---|---|---|
| Gestational age (week) | 32.54±2.35 | 32.14±3.47 | 31.11±1.15 | 0.66 |
| Birth weight (g) | 1654.00±540.81 | 1622.00±503.16 | 1473.00±274.94 | 0.81 |
| Apgar score at 5 min | 9.20±0.84 | 8.40±1.14 | 8.40±1.14 | 0.41 |
| Male (%) | 40.00 | 40.00 | 20.00 | 0.78 |
| Cesarean section (%) | 40.00 | 40.00 | 60.00 | 0.80 |
| Twins (%) | 20.00 | 0 | 20.00 | 0.62 |
| Gestational diabetes (%) | 60.00 | 40.00 | 40.00 | 0.80 |
| Without glucocorticoid usage before delivery (%) | 40.00 | 20.00 | 40.00 | 0.78 |

Quantitative data are represented as mean ± SEM. G1 was infants without RDS, G2 was infants with mild RDS and G3 was infants with severe RDS.

**Table 3.** The differentially expressed lncRNAs (Fold change> 2)

| LncRNA | Gene | Trend | CHR | Strand |
|---|---|---|---|---|
| ENST00000417781.5 | CSE1L-AS1 | Up | chr20 | - |
| ENST00000418924.6 | RIN3 | Up | chr14 | + |
| ENST00000440408.5 | TTTY15 | Up | chrY | + |
| ENST00000467315.5 | PFKL | Up | chr21 | + |
| ENST00000470527.1 | CACHD1 | Up | chr1 | + |
| ENST00000481985.5 | RPL3 | Up | chr22 | - |
| ENST00000488606.5 | MRPS15 | Up | chr1 | - |
| ENST00000497617.1 | TSFM | Up | chr12 | + |
| ENST00000504497.1 | DMXL1 | Up | chr5 | + |
| ENST00000530931.1 | CD82 | Up | chr11 | + |
| ENST00000460278.5 | ANKRD28 | Down | chr3 | - |
| ENST00000544168.5 | AKT1 | Down | chr14 | - |
| ENST00000611549.4 | RAP1GAP | Down | chr1 | - |
| ENST00000491117.5 | GNA12 | Down | chr7 | - |
| ENST00000610076.1 | KCNT2 | Down | chr1 | - |
| ENST00000601034.2 | INTS6-AS1 | Down | chr13 | + |
| ENST00000570265.5 | C15orf41 | Down | chr15 | + |
| ENST00000592944.1 | ITGA2B | Down | chr17 | - |
| ENST00000494731.5 | ZDHHC20 | Down | chr13 | - |
| ENST00000628791.1 | AC093495.1 | Down | chr3 | + |

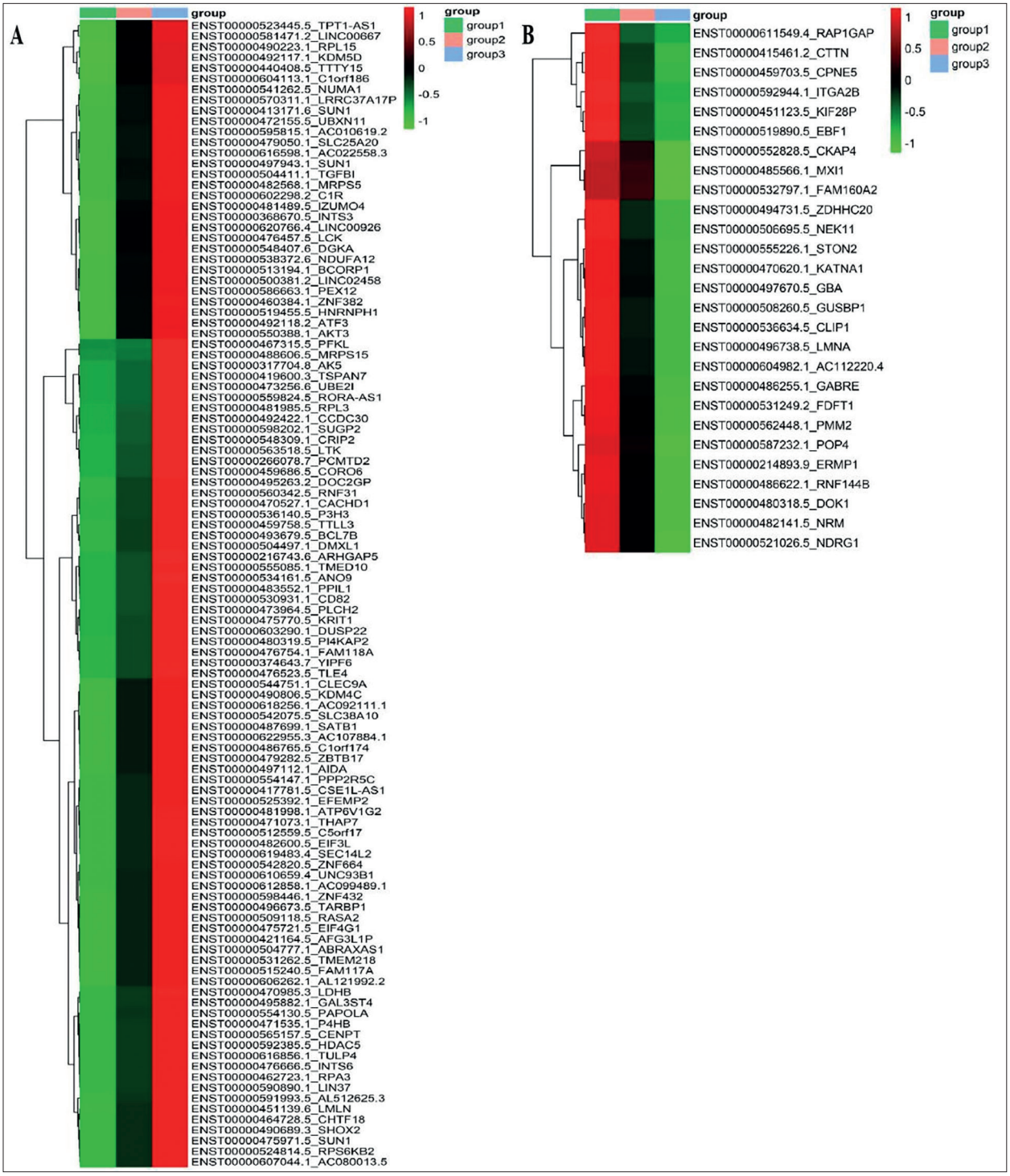


**Figure 2. Hierarchical clustering of lncRNA expression among the three groups.** (A) 108 upregulated lncRNAs, (B) 27 downregulated lncRNAs. Red indicates significantly increased expression. Green indicates significantly reduced expression, and black indicates no difference in expression levels.

**Table 4.** The differentially expressed mRNAs (Fold change> 2)

| mRNA | lncRNA | Trend | CHR | Strand |
|---|---|---|---|---|
| AC027796.3 | ENSG00000262304.2 | Up | chr17 | - |
| AQP7 | ENSG00000165269.12 | Up | chr9 | - |
| ARNT2 | ENSG00000172379.20 | Up | chr15 | + |
| DGCR6 | ENSG00000183628.12 | Up | chr22 | + |
| GRID2IP | ENSG00000215045.8 | Up | chr7 | - |
| MICU3 | ENSG00000155970.11 | Up | chr8 | + |
| MROH7-TTC4 | ENSG00000271723.5 | Up | chr1 | + |
| RHOXF1 | ENSG00000101883.4 | Up | chrX | - |
| ZNF683 | ENSG00000176083.17 | Up | chr1 | - |
| AC046185.1 | ENSG00000125695.12 | Down | chr17 | - |
| AC137834.1 | ENSG00000258830.1 | Down | chr12 | - |
| AL136295.1 | ENSG00000254692.1 | Down | chr14 | - |
| BLOC1S5-TXNDC5 | ENSG00000259040.5 | Down | chr6 | - |
| CTSV | ENSG00000136943.10 | Down | chr9 | - |
| CYP3A5 | ENSG00000106258.13 | Down | chr7 | - |
| GABRE | ENSG00000102287.18 | Down | chrX | - |
| GSTM5 | ENSG00000134201.10 | Down | chr1 | + |
| KCNT2 | ENSG00000162687.16 | Down | chr1 | - |
| MRAP2 | ENSG00000135324.5 | Down | chr6 | + |
| MYZAP | ENSG00000263155.5 | Down | chr15 | + |
| PKDCC | ENSG00000162878.12 | Down | chr2 | + |
| PPP1R14C | ENSG00000198729.4 | Down | chr6 | + |
| SH3D19 | ENSG00000109686.17 | Down | chr4 | - |
| SLC2A14 | ENSG00000173262.11 | Down | chr12 | - |

related to 108 upregulated lncRNAs and 27 downregulated lncRNAs. These mRNAs with FC> 2 are shown in **table 4**.

**GO and KEGG analysis of lncRNAs and mRNAs**

GO and KEGG analysis were further performed to annotate the biological functions of differentially expressed mRNAs. The GO analysis indicated that the mRNAs co-expressed with 108 upregulated lncRNAs were associated with 247 GO terms. The top 25 enriched terms are shown in **Figures 3A** and **3B**.

Additionally, a KEGG pathway analysis was performed to investigate the possible roles of the lncRNA-associated mRNA genes. The most significant pathways enriched in the set of upregulated protein-coding genes included PI3 kinase/Akt (PI3K-Akt), RAS, and mitogen-activated protein kinase (MAPK) signal pathways, while the most significant KEGG pathways of the downregulated protein-coding genes were mainly related to metabolic pathways, etc. The bubble diagrams of the top KEGG pathways of mRNAs co-expressed with upregulated and downregulated lncRNAs are shown in **Figure 3C** and **3D**.

**Differentially expressed lncRNAs verified by qRT-PCR**

Following the screening, four lncRNAs including ENST00000470527.1, ENST00000504497.1, ENST00000417781.5, and ENST00000440408.5 were further confirmed by qRT-PCR. Compared with G2 and G1, the expression levels of these four lncRNAs were increased in G3, which is consistent with the results of RNA sequencing. The relative expression levels are shown in **Figure 4**.

In-depth bioinformatics analysis of lncRNAs showed all the four lncRNAs were involved in the MAPK signaling pathway by down-regulating gene *GRB2* and *MECOM*. Moreover, ENST00000417781.5 and ENST00000440408.5 may regulate the MAPK signaling pathway by down-regulating gene *IGF2*, while ENST00000440408.5 and ENST00000504497.1 may target the MAPK signaling pathway by up-regulating gene *EFNA1* and *PLA2G4F*, respectively. This study also showed that the above four lncRNAs participate in PI3K-Akt and RAS signaling pathway by down-regulating *GRB2*, while ENST00000417781.5 and ENST00000440408.5

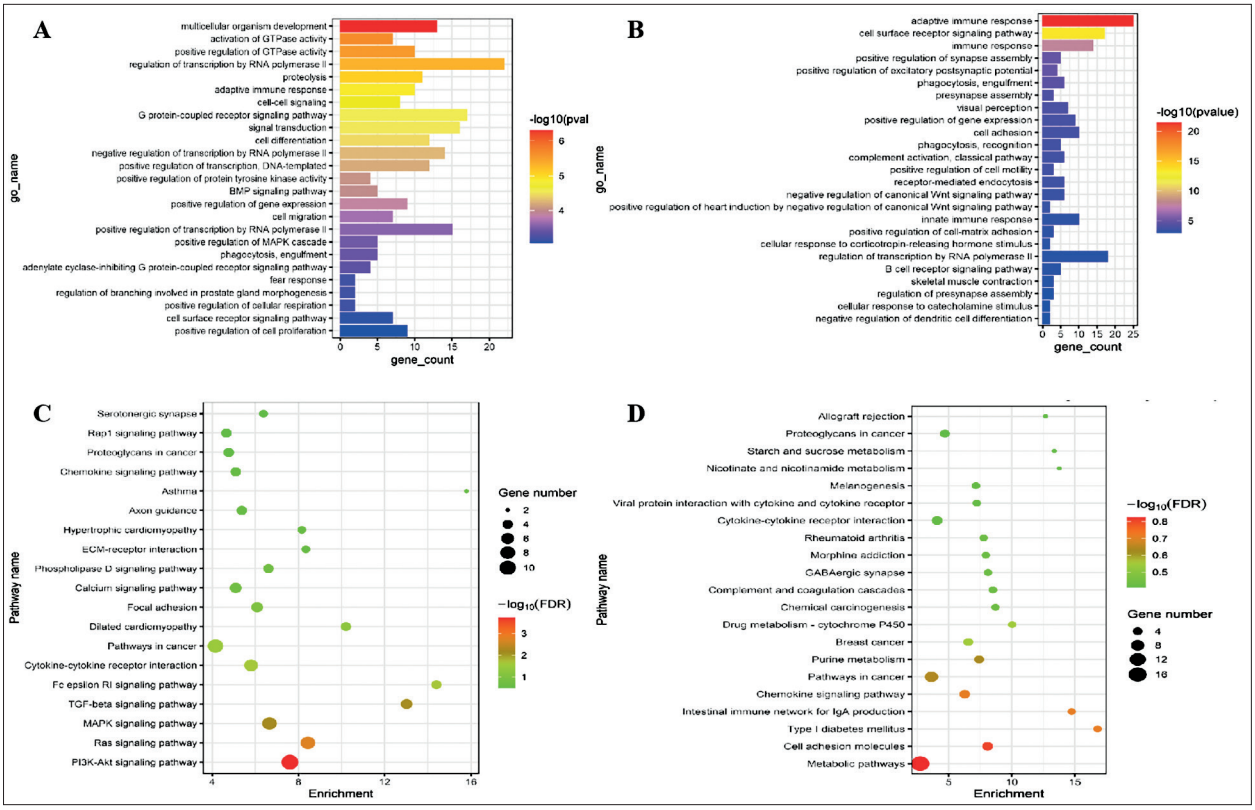


**Figure 3. GO and KEGG analysis of lncRNA function-related mRNAs.** (A) GO terms associated with mRNAs related to upregulated lncRNAs on biological process. (B) GO terms associated with mRNAs related to downregulated lncRNAs on biological process. (C) Bubble Diagram of the KEGG pathways of mRNAs associated with upregulated lncRNAs. (D) Bubble Diagram of the KEGG pathways of mRNAs associated with downregulated lncRNAs.

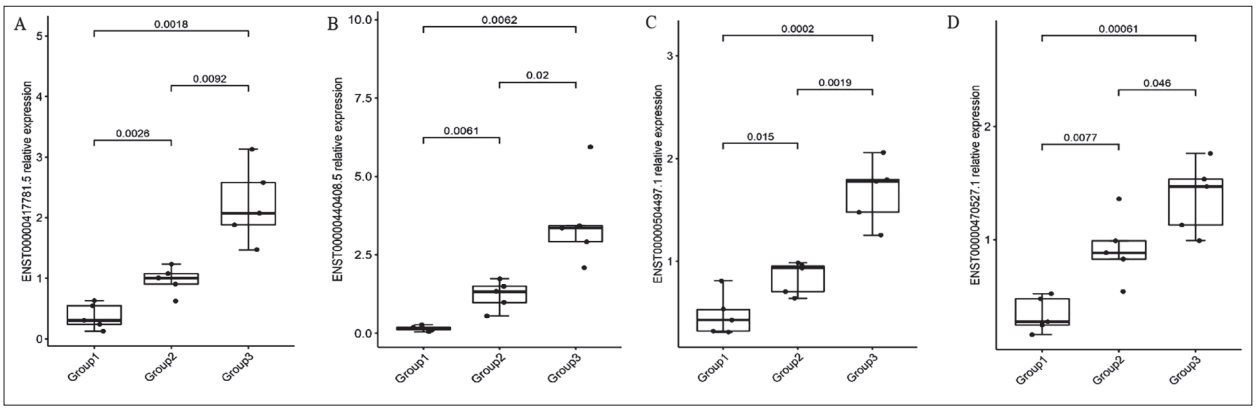


**Figure 4. qRT-PCR of the differentially expressed lncRNAs.** The expression level of differentially expressed four lncRNAs were further determined using qRT-PCR. Group1 was infants without RDS, Group2 was infants with mild RDS, and Group3 was infants with severe RDS. (A) relative expression of ENST00000417781.5, (B) relative expression of ENST00000440408.5, (C) relative expression of ENST00000504497.1, (D) relative expression of ENST00000470527.1

could regulate PI3K-Akt pathway by down-regulating *IGF2* and up-regulating *ITGB8* and *TCL1B*.

In addition, three of lncRNAs including ENST-00000417781.5, ENST00000470527.1, and ENST-00000504497.1 could target the RAS signaling pathway by up-regulating *RASAL1*, while ENST000004177, as well as ENST00000440408.5 could regulate RAS pathway by down-regulating *IGF2*. ENST00000440408.5 and ENST00000504497.1 may be involved in the RAS signaling pathway by up-regulating *EFNA1* and *PLA2G4F*, respectively. LncRNAs including ENST00000417781.5, ENST00000470527.1, and ENST00000504497.1 could

participate in the TGF-β signaling pathway by promoting gene expression of *AMH* and inhibiting *TGIF2*. In addition, ENST00000470527.1 and ENST00000504497.1 could be involved in the TGF-β pathway by up-regulating *GDF7* and down-regulating *GDF6*, while ENST00000440408.5 may down-regulate *FST* to be involved in TGF-β pathway. The pathway regulatory network of four validated lncRNAs are shown in **Figure 5**.

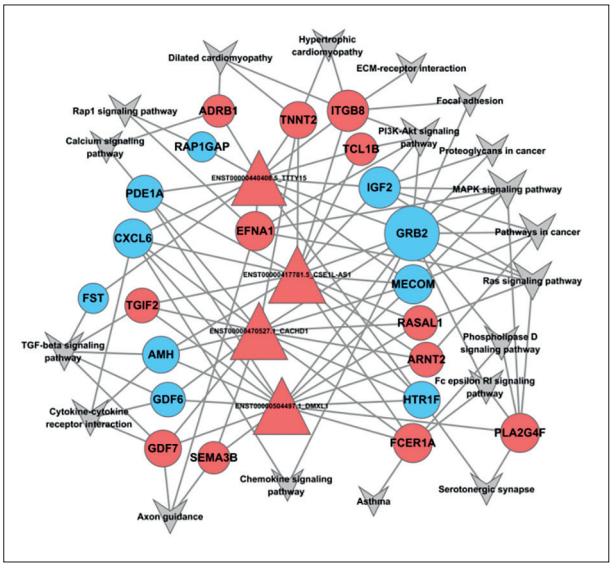


**Figure 5. Pathway regulatory network of four validated lncRNAs.** Red triangles indicate the four selected lncRNAs. Blue circles indicate mRNAs down-regulated by lncRNAs, while red circles indicate mRNAs up-regulated by lncRNAs, respectively. Arrows indicat1e signaling pathways associated with protein-coding genes co-expressed with the four selected lncRNAs.

## DISCUSSION

RDS is one of the most common respiratory disorders in preterm infants, which can induce acute respiratory failure **[16]**. Currently, it has been proven that RDS is a complex disease characterized by immature lung development. The embryonic phase of human lung development begins approximately at the gestational age of 3-4 weeks and originates from the endoderm. Immature fetal embryonic lung development has been recognized in the pseudo-glandular period (7-16 weeks of gestation), canalicular period (16-25 weeks of gestation), and terminal saccular period (25 weeks of gestation to full term) **[17]**. Yet the specific molecular regulatory mechanism of RDS has not yet been fully understood.

LncRNAs are related to many biologic processes, such as cell differentiation and proliferation **[18]**. Previous studies have indicated that lncRNAs are involved in lung development by regulating tracheal branches and differentiation of lung epithelial progenitor cells **[19-20]**. Few studies, however, have investigated the role of lncRNAs in RDS patients. Our study found that lncRNA and mRNA profiles exhibited differential expressions in the plasma of RDS patients. Our results further showed that the expression patterns of mRNAs and lncRNAs were consistent (Figures 2 and 4). Further function analysis of target lncRNAs and mRNAs described that PI3K-Akt, RAS, MAPK, and metabolic pathways might be downstream of the significant lncRNAs, and were potentially involved in the development of RDS.

Interestingly, we found that the expression level of lncRNA ENST00000470527.1, ENST00000504497.1, ENST00000417781.5, and ENST00000440408.5 was increased in the plasma of RDS patients, compared with non-RDS controls. Additionally, the level of those four lncRNAs was significantly higher in the severe patients, compared with the mild RDS group. The above results suggest that these four lncRNAs were possibly related to the severity of RDS.

A few studies have investigated lncRNA ENST00000440408.5, also known as Testis-specific transcript Y-linked 15 (TTTY15). A study reported by Zhang et al. demonstrated that TTTY15 knockdown can protect cardiomyocytes against hypoxia-induced apoptosis and mitochondrial energy metabolism dysfunction in vitro through the let-7i-5p/TLR3/NF-κB pathway **[21]**. The let-7 family has been demonstrated to be important in lung development and regulate RAS gene expression **[22]**. Fabro et al. further reported that circulating miRNA-let-7i-5p significantly changed in patients with acute pulmonary embolism and idiopathic pulmonary arterial hypertension compared with healthy controls **[23]**. Let-7i-5p were just regulators of pulmonary arterial adventitial fibroblasts, pulmonary artery endothelial cells, and pulmonary artery smooth muscle cells. Thus, we thought that lncRNA ENST00000440408.5 may be involved in lung development by interacting with miRNA let-7 directly or indirectly.

To our knowledge, the other three lncRNAs (ENST00000470527.1, ENST00000504497.1, and ENST00000417781.5) were reported for the first time. Bioinformatics analysis showed that they may be associated with PI3K-Akt, RAS, MAPK, and TGF-β signaling pathways, which could regulate lung development and PS secretion. Furthermore, the process of transdifferentiation from alveolar epithelial type II to type I cells is also controlled by TGF-β and BMP signaling pathways **[24]**. In our previous study, the results indicated that SMAD4 negatively regulates the expression of surfactant proteins (SPs), and that miR-431 negatively regulates the expression of SPs by inhibiting the BMP4/activin/ TGF-β signaling pathway by targeting *SMAD4* **[25]**. In addition, the PI3K-Akt signaling

pathway synergistically regulates epithelial-mesenchymal transition **[26]**, which is also essential for lung development **[27]**. Zhao M et al. reported that naringenin pre-treatment ameliorated LPS-induced acute lung injury through its anti-oxidative and anti-inflammatory activity and by inhibition of the PI3K/AKT pathway in mice **[28]**. As far as Ras/MAPK signaling pathway is concerned, it affects the FGF signaling cascade, while the FGF signaling pathway is crucial for the dynamic and reciprocal communication between epithelium and mesenchyme during lung development **[29]**.

There were several limitations in our study. Firstly, the sample size is relatively small, a larger sample study could validate the results further. Secondly, the specific functions of four differentially expressed lncRNAs should be deeply explored in future studies to clarify the pathogenesis of RDS.

## CONCLUSION

135 lncRNAs were differentially expressed among non-RDS group, mild RDS group and severe RDS group. LncRNA-mRNA co-expression networks further identified a total of 278 mRNAs that were closely related to the above differentially expressed lncRNAs. Among them, the differential expression of ENST00000470527.1, ENST00000504497.1, ENST00000417781.5, and ENST00000440408.5 were confirmed by qRT-PCR. The above results could provide a new sight for researching the potential pathophysiological mechanisms of RDS.

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**Authors' contributions**

YY and XYZ designed the study. ZDB, WZ, and JW performed the experiments. ZDB, WZ, and JXS analyzed the data. ZDB and YY drafted the manuscript. JW revised the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

All the procedures were followed in accordance with the Declaration of Helsinki. This study was approved by the Ethics Committee of Jiangyin People's Hospital of Nantong University [approval No. [(2019)011], and all the guardians of the patients have provided written informed consent.

**Conflict of interest**

The authors declare no competing interests.

**Acknowledgment**

We are grateful for the parents' understanding and support.

**Availability of data and materials**

The dataset used during this study is available from the corresponding author upon reasonable request.

## ABBREVIATIONS

Respiratory distress syndrome (RDS)
Pulmonary surfactant (PS)
Fibroblast growth factor (FGF)
Bone morphogenetic protein-4 (BMP-4)
Transforming growth factor-beta (TGF-β)
Long non-coding RNAs (lncRNAs)
Nkx2.1-associated noncoding intergenic RNA (NANCI)
Gene ontology (GO)
Kyoto Encyclopedia of Genes and Genomes (KEGG)
Positive end-expiratory pressure (PEEP)
Fold change (FC)
Real-time quantitative PCR (qRT-PCR)
Enrichment score (EC)
Standard deviation (SD)
PI3 kinase/Akt (PI3K-Akt)
Mitogen-activated protein kinase (MAPK)
Testis-specific transcript Y-linked 15 (TTTY15)

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