Identification of Potential Key Long Non-Coding RNAs and Target Genes Associated with Pneumonia Using Long Non-Coding RNA Sequencing (lncRNA-Seq): A Preliminary Study

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ABFG 2

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Background: This study aimed to identify the potential key long non-coding RNAs (lncRNAs) and target genes associated with pneumonia using lncRNA sequencing (lncRNA-seq).

Material/Methods: A total of 9 peripheral blood samples from patients with mild pneumonia (n=3) and severe pneumonia (n=3), as well as volunteers without pneumonia (n=3), were received for lncRNA-seq. Based on the sequencing data, differentially expressed lncRNAs (DE-lncRNAs) were identified by the limma package. After the functional enrichment analysis, target genes of DE-lncRNAs were predicted, and the regulatory network was constructed.

Results: In total, 99 DE-lncRNAs (14 upregulated and 85 downregulated ones) were identified in the mild pneumonia group and 85 (72 upregulated and 13 downregulated ones) in the severe pneumonia group, compared with the control group. Among these DE-lncRNAs, 9 lncRNAs were upregulated in both the mild and severe pneumonia groups. A set of 868 genes were predicted to be targeted by these 9 DE-lncRNAs. In the network, RP11-248E9.5 and RP11-456D7.1 targeted the majority of genes. RP11-248E9.5 regulated several genes together with CTD-2300H10.2, such as QRFP and EPS8. Both upregulated RP11-456D7.1 and RP11-96C23.9 regulated several genes, such as PDK2. RP11-456D7.1 also positively regulated CCL21.

Conclusions: These novel lncRNAs and their target genes may be closely associated with the progression of pneumonia.

MeSH Keywords: Gene Regulatory Networks • Genes, vif • Pneumonia, Aspiration • RNA, Long Noncoding

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/900783
Background

Pneumonia is defined as inflammation and consolidation of lung tissue due to an infectious agent [1]. It is the leading global cause of death, especially in children and elderly people [2,3]. The typical symptoms of pneumonia are fever, chills, pleuritic chest pain, and cough productive of purulent sputum [4]. A common type of pneumonia, community-acquired pneumonia (CAP), is responsible for high rates of morbidity and mortality worldwide, with an annual incidence of 1.5 to 1.7 per 1000 individuals among adults in Europe [5]. Severe pneumonia is defined as admission to the intensive care unit (ICU), and it results in an extremely high rate of mortality [6]. Therefore, it is very urgent to find more biomarkers associated with pneumonia, thus contributing to the clinical therapy of this disease.

Currently, several molecular mechanisms underlying pneumonia have been found. For instance, the genotype -174 GG of interleukin-6 (IL-6) is associated with lower severity and mortality in patients with pneumococcal CAP [7]. Four risk single-nucleotide polymorphisms (SNPs) located in chromosomes 1 and 17 have been found to be significantly correlated with the susceptibility to development of severe pneumonia in A/H1N1 infection [8]. Previous reports have indicated that severe pneumonia is associated with methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes and the staphylococcal cassette chromosome mec (SCCmec) type IV [9,10]. The activity of metalloproteinase-9 (MMAP-9) in peripheral blood circulation in patients with CAP caused by *Mycoplasma pneumoniæ* is increased in the acute phase of illness compared to the control group [11]. Furthermore, a recent study has reported that high expression of IL-10 and interferon-induced protein (IP)-10 in human immunodeficiency virus (HIV)-infected infants is associated with more severe hypoxic pneumonia [12]. However, currently, no study has reported the association of long non-coding RNAs (lncRNAs) with pneumonia.

LncRNAs have been previously found to be widely transcribed in the genome. Multiple evidence links dysregulations and mutations of lncRNAs to diverse human diseases [13], such as lung diseases (e.g., lung cancer [14,15] and pulmonary fibrosis [16]). Therefore, we suggest a hypothesis that lncRNAs are also correlated with the progression of pneumonia. Thus, in this study, we used a new sequencing technique, lncRNA sequencing (lncRNA-seq), to analyze the lncRNA expression profiling in peripheral blood from patients with mild and severe pneumonia and to identify the potential critical lncRNAs that are associated with the progression of pneumonia. These findings may provide some new information for understanding the molecular functions of lncRNAs in pneumonia and extend the knowledge of the molecular mechanisms underlying pneumonia.

Material and Methods

Clinical samples

This study was approved by the Medical Ethics Committee of the Chinese People’s Liberation Army General Hospital, Beijing, China. A total of 18 patients with pneumonia who received therapy in our hospital from June 2013 to December 2013 were included in this study, including 9 patients with mild pneumonia (MP group) and 9 patients with severe pneumonia (SP group). Another 9 volunteers without pneumonia were enrolled as normal controls (C group) in this study. Here, patients with severe pneumonia must meet at least one of the following criteria: (1) altered mental status; (2) respiratory rate ≥30/min; (3) diastolic blood pressure <60 mm Hg, PaO$_2$/FiO$_2$ <300, and mechanical ventilation; (4) systolic blood pressure <90 mm Hg; (5) septic shock; (6) bilateral or multilobar pneumonia by chest radiograph, or lesion enlargement within 48 h after admission ≥50%; (7) oliguria: urine volume <20 mL/h or <80 mL/4 h, or acute renal failure requiring dialysis treatment [17].

Peripheral blood was sampled from each patient and volunteer. Informed consent was signed before sampling.

RNA extraction

First, plasma was separated from each of the 9 sequencing samples. Total RNA was extracted and purified from the plasma samples using miRNeasy Serum/Plasma Kit (Qiagen, Germany). Subsequently, ribosome RNA (rRNA) was removed from the total RNA using Epicentre Ribo-Zero™ RNA Removal Kit (Epicentre, Madison, Wisconsin, USA), and the remaining RNA was collected and purified. To obtain sufficient quantities of high-quality RNA for sequencing, three RNA samples of equal quantity were randomly pooled into one sample for sequencing. Thus, three samples were generated for each group: WLL1–3 for the SP group, WLL4–6 for the MP group, and WLL7–9 for the C group. The 9 RNA samples were interrupted into short fragments by fragmentation buffer (Agilent Technologies, California, USA). Afterwards, the RNA fragments were reverse transcribed into cDNAs. The concentration of cDNAs in the library was quantified into 1 ng/µL with a Qubit 2.0 fluorometer, and then cDNAs were detected using the Agilent Bioanalyzer 2100 (Agilent Technologies, California, USA). According to the data size and effective cDNA concentration, libraries were pooled. Clusters of the cDNA libraries were generated on an Illumina cBot. Finally, the cDNA libraries were sequenced on an Illumina HiSeq™ 4000 with the model of 2×150 bp. The raw sequencing data have been uploaded to the public database NCBI (the National Center for Biotechnology Information) under the BioProject Accession PRNA324335.
Data filtering

Raw reads were cleaned by removing the empty reads, adapter sequences, reads with Q-value <10 in the both terminals, reads containing fewer than 80% of bases with Q-value >20, reads with length <50 nt, and reads with unknown sequences ‘N’. In addition, the reads of rRNA were removed. The above quality control was conducted using FASTX-Toolkit (available at http://hannonlab.cshl.edu/fastx_toolkit/).

Statistics and alignment of reads

Both Q20 and length of raw and clean reads were summarized to ensure the validity and reliability of the sequencing data. Furthermore, clean reads were aligned to the human genome (hg19) using TopHat 2.1.1 (available at http://ccb.jhu.edu/software/tophat/index.shtml). Sequence mapping was performed by alignment of clean reads to the human genome (hg19) using TopHat 2.1.1 (available at http://ccb.jhu.edu/software/tophat/index.shtml). The clean reads were aligned to the human genome using TopHat 2.1.1 (available at http://ccb.jhu.edu/software/tophat/index.shtml).

Differential expression analysis of lncRNAs

Based on the annotation information of genes and lncRNAs in the GENCODE database (available at http://www.gencodegenes.org/), FPKM (fragments per kilobase of exon per million fragments mapped) of mRNAs and lncRNAs, as well as the read number of lncRNAs mapped, was calculated using the StringTie tool (available at http://ccb.jhu.edu/software/stringtie/).

Differentially expressed lncRNAs (DE-lncRNAs) in the comparison groups of SP versus C, MP versus C, and SP versus MP were identified using the limma package (available at http://www.bioconductor.org/packages/release/bioc/html/limma.html). Only the lncRNAs with the criteria of \(|\log_{2} FC| >1\) and \(p < 0.05\) were identified as DE-lncRNAs.

Prediction of DE-lncRNA target genes

The Pearson correlation coefficient (PCC) was calculated to evaluate the coexpression relationships between DE-lncRNAs and mRNAs. The coexpression pairs with PCC >0.8 and \(p < 0.05\) were selected for the construction of the regulatory network, which was visualized using Cytoscape 3.3.0 (available at http://www.cytoscape.org/).

Functional analysis of DE-lncRNA target genes

GO (Gene Ontology) functional and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analyses were performed for the target genes of DE-lncRNAs using clusterProfiler 3.0.1 in R (available at http://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html). GO enrichment analysis contains three categories, including molecular function (MF), biological process (BP), and cellular component (CC). Only the GO and pathway terms with a \(p < 0.05\) were considered significant.

Results

Data summary of quality control and sequence alignment

In total, 346 G of raw data were generated from the 9 samples. Q20 of reads in both terminals of all samples was at least 99.97% and 96.38%, respectively. The clean rate (clean reads/raw reads) of the both terminals was more than 95% and 75% (Table 1). The results indicated a high quality of the sequencing data.

Furthermore, map rate of reads in most samples was about 70%; read coverage in most samples was more than 80%; and depth of sequencing was more than 3.4, 4–5.5 for most samples (Table 2).

Identification of DE-lncRNAs

Among the 9 samples, there were 34,764 mRNAs and 5496 lncRNAs with FPKM >0 in at least one sample. Based on the criteria of differential expression analysis, 99 DE-lncRNAs (14 upregulated and 85 downregulated ones) were identified in the MP group and 85 (72 upregulated and 13 downregulated ones) in the SP group, compared with the C group. Nine lncRNAs were upregulated in both the MP and SP groups, compared with the C group. Furthermore, there were 159 upregulated and 8 downregulated lncRNAs in the SP group, compared with the MP group. These DE-lncRNAs were able to distinguish the two group samples (Figure 1A–1C).

Target genes of the DE-lncRNAs

To further reveal the potential regulatory relationships between DE-lncRNAs and downstream genes, target genes of the 175 DE-lncRNAs in the MP and SP groups were predicted with the PCC method. In total, 4908 genes were targeted by those DE-lncRNAs. The regulatory network consisted of 175 DE-lncRNAs, 4908 genes, and 17,385 regulatory relationships (Supplementary Figure 1).

Enrichment analyses of the target genes

To further investigate the potential biological functions of the identified DE-lncRNAs, GO and KEGG pathway enrichment analyses were carried out for the targets of these DE-lncRNAs. For the target genes of DE-lncRNAs in the MP group, the targets of the upregulated lncRNAs were mainly associated with GO functions, such as response to stimulus and regulation of cellular process; meanwhile, the targets of the downregulated lncRNAs...
### Table 1. Summary of the sequencing data after quality control.

| Sample | Raw reads | Raw base | Q20 | Clean reads | Clean base | Clean rate |
|--------|-----------|----------|-----|-------------|------------|------------|
| WLL1   | 52831743  | 7928532000 | 99.99% | 50735720 | 760966306 | 0.960326446 |
| WLL1   | 58572433  | 7928532000 | 97.10% | 44421351 | 666212116 | 0.758400304 |
| WLL2   | 103035876 | 15455381400 | 99.98% | 50735720 | 760966306 | 0.960326446 |
| WLL2   | 103035876 | 15455381400 | 97.50% | 44421351 | 666212116 | 0.758400304 |
| WLL3   | 47936143  | 7190421450 | 99.97% | 45842678 | 687583167 | 0.956328047 |
| WLL3   | 47936143  | 7190421450 | 97.10% | 45842678 | 687583167 | 0.956328047 |
| WLL4   | 55117713  | 8267656950 | 99.98% | 52810671 | 792093105 | 0.958143365 |
| WLL4   | 55117713  | 8267656950 | 97.50% | 52810671 | 792093105 | 0.958143365 |
| WLL5   | 42414859  | 636228850  | 99.97% | 40625314 | 609330357 | 0.957808536 |
| WLL5   | 42414859  | 636228850  | 96.49% | 40625314 | 609330357 | 0.957808536 |
| WLL6   | 58572433  | 8785864950 | 99.99% | 56266066 | 843936516 | 0.960623678 |
| WLL6   | 58572433  | 8785864950 | 97.30% | 56266066 | 843936516 | 0.960623678 |
| WLL7   | 42047930  | 6307189500 | 99.99% | 40469826 | 607008095 | 0.962468925 |
| WLL7   | 42047930  | 6307189500 | 97.23% | 40469826 | 607008095 | 0.962468925 |

WLL1–3 represent the samples in the severe pneumonia; WLL4–6 represent the samples in the mild pneumonia; WLL7–9 represent the control samples. Clean rate = Clean reads/raw reads.

### Table 2. Data summary of the sequence alignment.

| Sample | Mapped-reads | Unique-mapped reads | Left mapped reads | Right mapped reads | Map rate | Unique map rate | Coverage | Depth |
|--------|--------------|---------------------|-------------------|-------------------|----------|----------------|----------|-------|
| WLL1   | 66658130     | 65814534            | 37288693          | 29369437          | 0.700506324 | 0.691641024 | 0.8124469 | 4.656258953 |
| WLL2   | 106477220    | 105906423           | 61493861          | 50675720          | 0.570337791 | 0.567280357 | 0.8897298 | 7.498857573 |
| WLL3   | 60484089     | 59601427            | 34143118          | 26340971          | 0.711187619 | 0.700809051 | 0.7974211 | 4.401589422 |
| WLL4   | 62030490     | 61253756            | 35429803          | 26600867          | 0.678350320 | 0.667059873 | 0.8028072 | 5.141637117 |
| WLL5   | 55225190     | 54350513            | 30940910          | 24284280          | 0.732627356 | 0.721023733 | 0.7059120 | 4.090939095 |
| WLL6   | 62740507     | 66192609            | 37900851          | 29399656          | 0.71700156 | 0.704546451 | 0.8028797 | 5.359257339 |
| WLL7   | 62030490     | 61253756            | 35429803          | 26600867          | 0.678350320 | 0.667059873 | 0.8028072 | 5.141637117 |
| WLL8   | 58572433     | 8785864950          | 97.30%            | 48447586          | 0.82173923 | 0.82173923 | 0.82173923 | 0.82173923 |
| WLL9   | 42047930     | 6307189500          | 99.99%            | 40469826          | 0.96469826 | 0.96469826 | 0.96469826 | 0.96469826 |

WLL1–3 represent the samples in the severe pneumonia; WLL4–6 represent the samples in the mild pneumonia; WLL7–9 represent the control samples.
were significantly correlated with multiple biological functions, such as protein binding and catalytic activity (Table 3).

Additionally, the target genes of both upregulated and downregulated lncRNAs in the SP group were significantly related to GO functions, such as protein binding and catalytic activity. The targets of the downregulated lncRNAs were also implicated in the pathways of ribosome and apoptosis (Table 4).

**Analysis of the common 9 upregulated lncRNAs in the mild and severe pneumonia groups**

The 9 lncRNAs that were upregulated in both the MP and SP groups were analyzed in detail. A total of 868 genes were predicted to be targeted by these 9 DE-lncRNAs (Figure 2). Among them, RP11-248E9.5 targeted the most genes, such as QRFP and EPS8; these two genes were also targeted by CTD-2300H10.2. RP11-248E9.5 also targeted a set of genes encoding zinc finger proteins (ZFPs), such as ZNF717, ZNF460, ZNF687, and ZNF37CP. Furthermore, RP11-456D7.1 regulated a series of genes, such as PDK2, which were also targeted by RP11-96C23.9. RP11-456D7.1 also regulated genes like CCL21.

In addition, according to the GO and pathway enrichment analyses, the target genes of RP11-248E9.5 (e.g., GPR75 and QRFP) were significantly enriched in the GO functions like G-protein coupled receptor signaling pathway; the target genes of RP11-456D7.1 were mainly enriched in the molecular function. Furthermore, the target genes of CTD-2300H10.2 (e.g., RC3H1, IHH, and IL4) were significantly enriched in the GO functions like negative regulation of alpha-beta T cell differentiation (Table 5).

**Discussion**

In the present study, 99 DE-lncRNAs (14 upregulated and 85 downregulated ones) were identified in the MP group and 85 (72 upregulated and 13 downregulated ones) in the SP group,
compared with the C group. Among these DE-lncRNAs, 9 lncRNAs were upregulated in both the MP and SP groups, compared with the C group. According to the coexpression analysis between DE-lncRNAs and mRNAs, 868 genes were predicted to be targeted by the 9 lncRNAs. RP11-248E9.5 and RP11-456D7.1 targeted the majority of genes.

In the regulatory network, RP11-248E9.5 regulated several genes together with CTD-2300H10.2, such as QRFP and EPS8. QRFP encodes pyroglutamylated RFamide peptide, which is proteolytically processed to generate multiple protein products [18]. In this study, QRFP was predicted to be relevant to the G-protein coupled receptor signaling pathway. A previous study has found that G-protein coupled receptor kinase-5 (GRK5) deficiency improves pulmonary infection and inflammation in *Escherichia coli*-induced pneumonia [19]. Furthermore, G-protein coupled receptors have been suggested to be associated with inflammation [20–22]. Although there is no evidence to show the role of

### Table 3. The enriched Gene Ontology and pathway terms of differentially expressed lncRNAs in the mild pneumonia compared with the controls.

| Category of lncRNAs | Category of functional terms | ID      | Term                        | Gene count | FDR             |
|---------------------|------------------------------|---------|-----------------------------|------------|-----------------|
| Upregulated         | GO-BP                        | GO:0008150 | Biological_process          | 469        | 3.46E-22        |
|                     | GO-BP                        | GO:0009987 | Cellular_process            | 434        | 8.85E-12        |
|                     | GO-BP                        | GO:0050896 | Response to stimulus        | 261        | 0.0004501       |
|                     | GO-BP                        | GO:0050794 | Regulation of cellular process | 303        | 0.003139471    |
|                     | GO-BP                        | GO:0044699 | Single-organism process     | 378        | 0.004578225     |
|                     | GO-CC                        | GO:0005575 | Cellular_component          | 510        | 2.68E-09        |
|                     | GO-MF                        | GO:0003674 | Molecular_function          | 481        | 1.52E-22        |
|                     | GO-MF                        | GO:0005488 | Binding                     | 399        | 0.002334936     |
|                     | GO-MF                        | GO:0060089 | Molecular transducer activity | 74         | 0.016441823     |
|                     | GO-BP                        | GO:0008150 | Biological_process          | 1852       | 2.03E-100       |
|                     | GO-BP                        | GO:0009987 | Cellular_process            | 1685       | 8.04E-41        |
|                     | GO-BP                        | GO:0044699 | Single-organism process     | 1517       | 2.59E-23        |
|                     | GO-BP                        | GO:0044763 | Single-organism cellular process | 1386       | 2.07E-18        |
|                     | GO-CC                        | GO:0008152 | Metabolic process           | 1337       | 4.89E-18        |
|                     | GO-CC                        | GO:0005575 | Cellular_component          | 1965       | 2.83E-43        |
|                     | GO-CC                        | GO:0005623 | Cell                        | 1758       | 1.73E-08        |
|                     | GO-CC                        | GO:0044464 | Cell part                   | 1753       | 2.42E-08        |
|                     | GO-CC                        | GO:0005622 | Intracellular               | 1546       | 9.50E-07        |
|                     | GO-CC                        | GO:0044424 | Intracellular part          | 1504       | 1.05E-05        |
|                     | GO-MF                        | GO:0003674 | Molecular_function          | 1849       | 1.12E-96        |
|                     | GO-MF                        | GO:0005488 | Binding                     | 1554       | 3.18E-22        |
|                     | GO-MF                        | GO:0005515 | Protein binding             | 1174       | 6.27E-10        |
|                     | GO-MF                        | GO:0003824 | Catalytic activity          | 675        | 6.37E-10        |
|                     | GO-MF                        | GO:0016740 | Transferase activity        | 292        | 9.63E-07        |
| Downregulated       | GO-CC                        | GO:0044424 | Intracellular               | 1504       | 1.05E-05        |
|                     | GO-MF                        | GO:0003674 | Molecular_function          | 1849       | 1.12E-96        |
|                     | GO-MF                        | GO:0005488 | Binding                     | 1554       | 3.18E-22        |
|                     | GO-MF                        | GO:0005515 | Protein binding             | 1174       | 6.27E-10        |
|                     | GO-MF                        | GO:0003824 | Catalytic activity          | 675        | 6.37E-10        |
|                     | GO-MF                        | GO:0016740 | Transferase activity        | 292        | 9.63E-07        |
|                     | GO-CC                        | GO:0044424 | Intracellular               | 1504       | 1.05E-05        |
|                     | GO-MF                        | GO:0003674 | Molecular_function          | 1849       | 1.12E-96        |
|                     | GO-MF                        | GO:0005488 | Binding                     | 1554       | 3.18E-22        |
|                     | GO-MF                        | GO:0005515 | Protein binding             | 1174       | 6.27E-10        |
|                     | GO-MF                        | GO:0003824 | Catalytic activity          | 675        | 6.37E-10        |
|                     | GO-MF                        | GO:0016740 | Transferase activity        | 292        | 9.63E-07        |
|                     | KEGG                         | hsa03010 | Ribosyme                   | 32         | 0.009558616     |

LncRNA – long non-coding RNA; GO – Gene Ontology; MF – molecular function; CC – cellular component; BP – biological process; FDR – false discovery rate.
QRFP in pneumonia, we speculate that QRFP may participate in the progression of pneumonia via the G-protein coupled receptor signaling pathway. **EPS8** encodes epidermal growth factor receptor (EGFR) pathway substrate 8 and functions as part of the EGFR pathway [23]. In mycoplasmal pneumonia, the EGFR pathway takes part in the IL-8 production by bronchial epithelial cells stimulated with Mp-Ag [24]. Therefore, **EPS8** may be involved in the progression of pneumonia via the EGFR pathway.

### Table 4. The enriched Gene Ontology and pathway terms of differentially expressed lncRNAs in the severe pneumonia compared with the controls

| Category of lncRNAs | Category of functional terms | ID       | Term                   | Gene count | FDR      |
|---------------------|-----------------------------|----------|------------------------|------------|----------|
| GO-BP               | GO: 0008150                 | Biological_process | 1688       | 8.86E-91 |
| GO-BP               | GO: 0009987                 | Cellular process   | 1543       | 4.84E-40 |
| GO-BP               | GO: 0044699                 | Single-organism process | 1376       | 3.97E-19 |
| GO-BP               | GO: 008152                  | Metabolic process  | 1218       | 5.83E-16 |
| GO-BP               | GO: 0071704                 | Organic substance metabolic process | 1101       | 2.21E-14 |
| GO-GO: 0005575      | Cellular component          | 1818       | 1.08E-39 |
| GO-GO: 0005623      | Cell                        | 1621       | 6.64E-09 |
| GO-GO: 0044464      | Cell part                   | 1628       | 6.82E-09 |
| GO-GO: 0044424      | Intracellular part          | 1401       | 1.78E-06 |
| GO-GO: 0005622      | Intracellular               | 1432       | 1.78E-06 |
| GO-GO: 0003674      | Molecular_function          | 1698       | 3.69E-88 |
| GO-GO: 0005488      | Binding                     | 1421       | 1.71E-18 |
| GO-GO: 0003824      | Catalytic activity          | 621        | 5.29E-09 |
| GO-GO: 0005515      | Protein binding             | 1062       | 9.78E-07 |
| GO-MF               | GO: 0016740                 | Transferase activity | 258        | 0.000259127 |
| GO-BP               | GO: 0008150                 | Biological_process | 588        | 1.18E-28 |
| GO-BP               | GO: 0009987                 | Cellular process   | 538        | 2.41E-12 |
| GO-BP               | GO: 0044699                 | Single-organism process | 495       | 7.99E-10 |
| GO-BP               | GO: 0044763                 | Single-organism cellular process | 456       | 2.16E-08 |
| GO-BP               | GO: 0044237                 | Cellular metabolic process | 393       | 5.52E-08 |
| GO-GO: 0005575      | Cellular_component          | 622        | 9.48E-12 |
| GO-GO: 0005622      | Intracellular               | 1201       | 1.20E-09 |
| GO-GO: 0005624      | Intracellular part          | 512        | 2.29E-08 |
| GO-GO: 0005623      | Cell                        | 578        | 2.29E-08 |
| GO-GO: 0005737      | Cytoplasm                   | 431        | 2.29E-08 |
| GO-GO: 0003674      | Molecular_function          | 587        | 4.87E-28 |
| GO-GO: 0005488      | Binding                     | 501        | 3.95E-08 |
| GO-GO: 0003735      | Structural constituent of ribosome | 22        | 1.40E-06 |
| GO-GO: 0005515      | Protein binding             | 391        | 1.01E-05 |
| GO-GO: 0003824      | Catalytic activity          | 232        | 1.05E-05 |
| KEGG                | hsa03010                    | Ribosome    | 21         |          |
| KEGG                | hsa04210                    | Apoptosis   | 16         | 0.031885823 |

LncRNA – long non-coding RNA; GO – Gene Ontology; MF – molecular function; CC – cellular component; BP – biological process; FDR – false discovery rate.
Figure 2. The regulatory network of the 9 long non-coding RNAs (lncRNAs) that are differentially expressed in both mild and severe pneumonia. Dark red nodes represent the lncRNAs, and purple nodes represent the target genes. Lines represent the regulatory relationships between lncRNAs and target genes.
### Table 5. The enriched Gene Ontology and pathway terms of lncRNAs that are differentially expressed in the both mild and severe pneumonia.

| LncRNA | Category | ID     | Term                                      | FDR       | Gene count | Target genes                                                                 |
|--------|----------|--------|-------------------------------------------|-----------|------------|-------------------------------------------------------------------------------|
| AJ006995.3 | BP    | GO: 1901137 | Carbohydrate derivative biosynthetic process | 0.004964  | 5          | NM6E, SEC23A, ADSL, POFUT1, ST6GALNAC5                                         |
|         |         | GO: 0006486 | Protein glycosylation                      | 0.016382  | 3          | SEC23A, POFUT1, ST6GALNAC5                                                   |
|         |         | GO: 0043413 | Macromolecule glycosylation                | 0.016382  | 3          | SEC23A, POFUT1, ST6GALNAC5                                                   |
|         |         | GO: 0070085 | Glycosylation                              | 0.016382  | 3          | SEC23A, POFUT1, ST6GALNAC5                                                   |
|         |         | GO: 1901135 | Carbohydrate metabolic process             | 0.019142  | 6          | NM6E, SEC23A, ADSL, POFUT1, ARHGEF28, ST6GALNAC5                             |
| CTD-2210P24.6 | MF          | GO: 1901363 | Heterocyclic compound binding              | 0.017896  | 16         | FOXP4, POU2F3, MAGI3, DDX25, HOXA13, NLRP9, MTA3, KIAA1586, BMPR1B, SRPK1, UBE2G2, UB1P, METTL16, CGGBP1, USP6, POLR1C |
|         |         | GO: 0097159 | Organic cyclic compound binding            | 0.017896  | 16         | FOXP4, POU2F3, MAGI3, DDX25, HOXA13, NLRP9, MTA3, KIAA1586, BMPR1B, SRPK1, UBE2G2, UB1P, METTL16, CGGBP1, USP6, POLR1C |
|         |         | GO: 0005488 | Binding                                   | 0.017896  | 24         | FOXP4, ADRA1B, NLGN4Y, POU2F3, MAGI3, DDX25, HOXA13, NLRP9, KRT8, RUFY2, MTA3, KIAA1586, BMPR1B, SRPK1, UBE2G2, UB1P, METTL16, FRAS1, CGGBP1, LGR5, EED, USP6, INT54, POLR1C |
|         |         | GO: 0003700 | Sequence-specific DNA binding transcription factor activity | 0.019057  | 6          | FOXP4, POU2F3, HOXA13, MTA3, UB1P, CGGBP1                                     |
|         |         | GO: 001071 | Nucleic acid binding transcription factor activity | 0.019057  | 6          | FOXP4, POU2F3, HOXA13, MTA3, UB1P, CGGBP1                                     |
| CTD-2300H10.2 | BP          | GO: 0046639 | Negative regulation of alpha-beta T cell differentiation | 7.61E-05  | 3          | RC3H1, IHH, IL4                                                               |
|         |         | GO: 0046636 | Negative regulation of alpha-beta T cell activation | 0.000187  | 3          | RC3H1, IHH, IL4                                                               |
|         |         | GO: 0045581 | Negative regulation of T cell differentiation | 0.000269  | 3          | RC3H1, IHH, IL4                                                               |
|         |         | GO: 0045620 | Negative regulation of lymphocyte differentiation | 0.000617  | 3          | RC3H1, IHH, IL4                                                               |
|         |         | GO: 0046637 | Regulation of alpha-beta T cell differentiation | 0.00103   | 3          | RC3H1, IHH, IL4                                                               |
|         |         | GO: 0032587 | Ruffle membrane                           | 0.015463  | 3          | EPS8, PLA2G4F, PDE9A                                                         |
|         |         | GO: 0031256 | Leading edge membrane                     | 0.033811  | 3          | EPS8, PLA2G4F, PDE9A                                                         |
|         |         | GO: 0001726 | Ruffle                                    | 0.039361  | 3          | EPS8, PLA2G4F, PDE9A                                                         |
|         |         | GO: 0052689 | Carboxylic ester hydrolase activity        | 0.000439  | 4          | ACOT2, ESD, ACOT9, PLA2G4F                                                   |
|         |         | GO: 0016790 | Thiolester hydrolase activity             | 0.001389  | 3          | ACOT2, ESD, ACOT9                                                           |
|         |         | GO: 0016788 | Hydrolase activity, acting on ester bonds  | 0.027305  | 5          | ACOT2, ESD, ACOT9, PLA2G4F                                                   |

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| LncRNA     | Category | ID       | Term                                                   | FDR        | Gene count | Target genes                                                                 |
|------------|----------|----------|--------------------------------------------------------|------------|------------|-------------------------------------------------------------------------------|
| RP11-96C23.9 | MF       | GO: 0016773 | Phosphotransferase activity, alcohol group as acceptor | 0.020183   | 3          | SRPK3, NMRK2, PDK2, CNTLN, RELN, PIP5K1B                                    |
|            |          | GO: 0016301 | Kinase activity                                        | 0.020183   | 6          | SRPK3, NMRK2, PDK2, CNTLN, RELN, PIP5K1B                                    |
|            |          | GO: 0016772 | Transferase activity, transferring phosphorus-containing groups | 0.028775   | 6          | SRPK3, NMRK2, PDK2, CNTLN, RELN, PIP5K1B                                    |
|            |          | GO: 0016740 | Transferase activity                                   | 0.028775   | 9          | MBOAT2, PPL2, SRPK3, NMRK2, PDK2, CNTLN, RELN, VCPKMT, PIP5K1B              |
| RP11-248E9.5 | BP       | GO: 0008150 | Biological process                                     | 0.002259   | 101        | ZNF717, ARL4C, ZNF460, GPR75, UGT2A1, CHEK1, ER12, CPE, DHRS13, ZNF418, TCC9B, IL23R, ADRA2B, SNRPNP48, ADSL, DPH2, TIGIT, CERS3, EPS8, FANCF, OR4D11, LHFPL5, MVD16, CDY1B, PRPF40B, LRIG1, STEAP2, MSTM, OR2V1, ANG, CLEC9A, TMPRESS12, KCNRG, GPR22, FRYL, SCA1, GPX5, VN1R2, OR2AG2, TRIM1L, IFNA14, ACTBL2, QRP, OR6B2, OR4K17, OR13C6P, OR51A4, VN1R17P, CEP83, TCEB3B, SCARA3, LARP7, ASB1, PLCB4, SPIN2A, PCDH18, PPP2R3A, CHST12, TNFRSF19, FAM46A, KIF16B, EXOC1, PCDH97, PRDM11, TAS2R38, RIMKLB, ZNF687, BAK1, HRH4, BCL2, RYR2, BLOC155, ARHGEF2B, SH3GL3, SMYD3, RFX7, SNAPC3, SOX3, TFAM, THY1, KRTAP4-8, ZNF140, CEP97, OR4A16, OR12D3, FCRL4, C19orf12, HPS3, DUSP11, SORBS2, PPBP2, RCTA, NRP2, TGF2LX, ANGPT1, HTR3B, MMP20, RIN1, PCDH9, TECPR2, JOSD1 |
|            |          | GO: 0007186 | G-protein coupled receptor signaling pathway           | 0.002613   | 20         | GPR75, CPE, ADRA2B, OR4D11, OR2V1, GPR22, VN1R2, OR2AG2, QRF, OR6B2, OR4K17, OR13C6P, OR51A4, VN1R17P, TAS2R38, HRH4, RYR2, OR4A16, OR12D3, HTR3B |
|            |          | GO: 0009593 | Detection of chemical stimulus                          | 0.017347   | 11         | UGT2A1, OR4D11, OR2V1, OR2AG2, OR6B2, OR4K17, OR51A4, TAS2R38, RYR2, OR4A16, OR12D3 |
|            |          | GO: 0007606 | Sensory perception of chemical stimulus                | 0.017347   | 11         | UGT2A1, OR4D11, OR2V1, OR2AG2, OR6B2, OR4K17, OR13C6P, OR51A4, TAS2R38, OR4A16, OR12D3 |
|            |          | GO: 0007608 | Sensory perception of smell                            | 0.021759   | 10         | UGT2A1, OR4D11, OR2V1, OR2AG2, OR6B2, OR4K17, OR13C6P, OR51A4, OR4A16, OR12D3 |
| LncRNA          | Category | ID        | Term                        | FDR         | Gene count | Target genes                                                                 |
|-----------------|----------|-----------|-----------------------------|-------------|------------|-------------------------------------------------------------------------------|
| RP11-248E9.5    | MF       | GO: 0003674 | molecular_function          | 2.96E-05    | 99         | ZNF717, ARL4C, ZNF460, GPR75, UGT2A1, CHEK1, CHGB, ER12, C1orf131, SLX41P, CPE, DHRS13, ZNF418, TTC9B, IL23R, ADRA2B, SNRPN48, ADSL, TIGIT, CERS3, EPS8, FANC, OR4D11, MYO16, CDY1B, STEAP2, MSTN, OR2V1, ANG, CLEC9A, TMPRRSS12, KCNRRG, GPR22, SCAI, GPX5, VN1R2, OR2AG2, TRIML1, IFNA14, ACTBL2, QRF, OR6B2, OR4K17, OR13C6P, OR51A4, VN1R17P, CEP83, TCEB3B, SCARA3, LARP7, ASB1, PLCB4, SPIN2A, PCDH18, PPP2R3A, CHST12, TNFRSF19, FAM46A, KIF16B, EKOC1, PCDHA7, PRDM11, TAS2R38, RIMKLB, ZNF687, BAK1, HRH4, BCL2, RYR2, BLOC15S, ANKEF1, ARHGEF28, CEP170P1, SH3GL3, SMDY3, RFX7, SNAPC3, SOX3, TFAM, THY1, ZNF140, GPR97, OR4A16, OR12D3, FCLR4, EFCAB7, DUSP11, SORBS2, PPP1BP2, RTCA, NR0P2, TGF2LX, ANGPTL1, HTR3B, MMP20, RIN1, PCDHA9, TECPR2, IOSD1 |
| GO: 0004930     |          |           | G-protein coupled receptor activity | 0.000121    | 17         | GPR75, ADRA2B, OR4D11, OR2V1, GPR22, VN1R2, OR2AG2, OR6B2, OR4K17, OR13C6P, OR51A4, VN1R17P, TAS2R38, HRH4, OR4A16, OR12D3, HTR3B |
| GO: 0004888     |          |           | transmembrane signaling receptor activity | 0.000206    | 20         | GPR75, IL23R, ADRA2B, OR4D11, OR2V1, GPR22, VN1R2, OR2AG2, OR6B2, OR4K17, OR13C6P, OR51A4, VN1R17P, TNFRSF19, TAS2R38, HRH4, OR4A16, OR12D3, NRP2, HTR3B |
| GO: 0038023     |          |           | signaling receptor activity    | 0.000484    | 20         | GPR75, IL23R, ADRA2B, OR4D11, OR2V1, GPR22, VN1R2, OR2AG2, OR6B2, OR4K17, OR13C6P, OR51A4, VN1R17P, TNFRSF19, TAS2R38, HRH4, OR4A16, OR12D3, NRP2, HTR3B |
| GO: 0004872     |          |           | receptor activity              | 0.001039    | 21         | GPR75, IL23R, ADRA2B, OR4D11, OR2V1, GPR22, VN1R2, OR2AG2, OR6B2, OR4K17, OR13C6P, OR51A4, VN1R17P, SCARA3, TNFRSF19, TAS2R38, HRH4, OR4A16, OR12D3, NRP2, HTR3B |
| KEGG hsa04740   |          |           | Olfactory transduction         | 0.00179994  | 7          | OR12D3, OR2AG2, OR4A16, OR4D11, OR4K17, OR51A4, OR6B2 |

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In addition to QRFp and EPS8, RP11-248E9.5 also targeted a series of ZFP coding genes, such as ZNF717, ZNF460, ZNF687, and ZNF37CP. ZNF37CP was also targeted by CTD-2300H10.2. Multiple studies have reported the associations of ZFPs with immunity [25–27], which is involved in pneumonia. In addition, in the network, CTD-2300H10.2 also targeted IL4, which is highly expressed in idiopathic interstitial pneumonias [28]. Currently, the associations of RP11-248E9.5 and CTD-2300H10.2 with pneumonia have not been previously reported, indicating they may be new potential molecules in pneumonia.

Furthermore, in the regulatory network, both upregulated RP11-456D7.1 and RP11-96C23.9 regulated several genes, such as PDK2, which encodes a member of the pyruvate dehydrogenase kinase family and is able to downregulate the activity of the mitochondrial pyruvate dehydrogenase complex. Inhibition of a homologue of PDK2, PDK4, can prevent multiorgan failure in severe influenza accompanied with pneumonia [29]. Moreover, pyruvate dehydrogenase E1 \( \beta \) subunit can act as fibronectin-binding protein in Mycoplasma pneumoniae, helping \( M. \) pneumoniae to locate in the host cells [30]. These evidences indicate that PDK2 may be related to the occurrence and development of pneumonia. In this study, RP11-456D7.1 also positively regulated CCL21, a high-affinity functional ligand for chemokine receptor 7 (CCR7) that is expressed on T and B lymphocytes and plays a key role in the inflammatory response [31,32]. CCL21 was detected at a significantly higher concentration in the bronchoalveolar lavage fluid of patients with eosinophilic pneumonia than in that of controls [33,34], which is similar to the results of this study. Taken together, although the roles of RP11-456D7.1 and RP11-96C23.9 have not been previously proved in pneumonia, we speculate that they may participate in the progression of pneumonia, likely via regulating their downstream genes PDK2 or CCL21.

In addition, according to the results of the enrichment analysis, functions of DE-lncRNAs in the SP group were similar to those in the MP group. However, 167 lncRNAs were identified to be differentially expressed between the SP and MP groups, indicating that lncRNA expression profiling between mild and severe pneumonia is different. In our future study, we will continue to focus on these DE-lncRNAs.

Despite the aforementioned results, this study has several limitations. In this study, the number of samples analyzed was small. Furthermore, the predicted results need to be validated by experimental data.

**Conclusions**

Based on the lncRNA-seq and bioinformatics analysis method, compared with the control, a set of DE-lncRNAs in patients with mild and severe pneumonia was identified. Nine lncRNAs were differentially expressed in both mild and severe pneumonia, such as RP11-248E9.5, CTD-2300H10.2, RP11-456D7.1, and RP11-96C23.9. All of them were predicted to target a set of downstream genes. At present, these lncRNAs have not been demonstrated to be associated with pneumonia by other studies; thus, they are novel lncRNAs that might be related to pneumonia. These results provided new information for further experimental studies.

**Potential conflicts of interest**

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to subject of this article.

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**Table:**

| LncRNA      | Category | ID        | Term           | FDR     | Gene count | Target genes |
|-------------|----------|-----------|----------------|---------|------------|--------------|
| RP11-456D7.1| MF       | GO: 0003674| molecular_function| 0.007716221 | 61         | TANK, USP17L12, DSCR4, SCLM2, SCGN, KHDRBS3, ZBTB6, OSBP57, C1QTNF3, RBP7, LCN4, GID3, OR2734, OR274, COX5B, CCEB1, UPP2, EPHA2, TXLNA, FOXL1, IQSEC2, TNFRSF13B, KIF4A, ATXN10, TRAF3IP1, GMFB, ANXA4, ZACN, LDHC, ATP2B3, OCR1, TAS2R8, PDK2, ACP5, PPP1R3D, PRIM2, PRKAA1, POLR3B, WWC3, PCDHGA7, TMX4, AAR52, NTN4, OPN1LW, RGS13, CCL21, PGA3, ZCCHC18, NMNAT1, SMARCD1, TACR1, CA6, ORS1B2, COLQ, PPFIA4, ENOU, BUB3, ZMYM3, GPR52, FEZ2, IKBKE |

LncRNA – long non-coding RNA; GO – Gene Ontology; MF – molecular function; CC – cellular component; BP – biological process; FDR – false discovery rate.
## Supplementary Figure 1

The regulatory network of the differentially expressed long non-coding RNAs (DE-lncRNAs) and target genes. Light green nodes represent the downregulated lncRNAs in mild pneumonia; light red nodes represent the upregulated lncRNAs in mild pneumonia; green nodes represent the downregulated lncRNAs in severe pneumonia; red nodes represent the upregulated lncRNAs in severe pneumonia; dark red nodes represent the upregulated lncRNAs in both mild and severe pneumonia; purple nodes represent the target genes. Lines represent the regulatory relationships between lncRNAs and target genes.

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