Pulmonary arterial hypertension (PAH) is a chronic progressive cardiovascular disease characterized by vascular remodeling and leading to right-heart failure. The purpose of this research was to further study the pathogenesis of PAH and to detect potential prognostic signatures. Differentially expressed genes (DEGs) selected from GSE38267 were mostly enriched in inflammation-related pathways, suggesting inflammation may be involved in the occurrence and development of PAH. Through the prediction and verification of related miRNAs and long noncoding RNAs using online databases and Gene Expression Omnibus (GEO) datasets, CCR7 and its related molecules, including hsa-let-7e-5p and SNHG12, were identified as possible targets. The expression levels of CCR7, hsa-let-7e-5p and SNHG12 were then verified by quantitative RT-PCR in vivo and in vitro. Further study showed that silencing of SNHG12 decreased the expression of CCR7 under hypoxia treatment in vitro. Dual-luciferase reporter assays were used to verify the relationship between hsa-let-7e-5p and SNHG12. Collectively, our research reveals that a long noncoding RNA–miRNA–mRNA interaction network may be involved in the pathogenesis of PAH and suggests SNHG12, hsa-let-7e-5p and CCR7 as potential biomarkers for PAH.
a type of ncRNA no shorter than 200 nucleotides [8], which is involved in plenty of cellular activities. Correspondingly, Sun et al. [9] pointed out that metformin could prohibit PAH from developing and IncRNA NONRATT015587.2 could possibly be a biomarker for the diagnosis of PAH. Competing endogenous RNA (ceRNA) refers to a new gene-regulatory mechanism that may cause mediation and alternations in key subpathway regions [10]. In previous research, Feng et al. [10] identified several ceRNA-mediated subpathways that were relevant with PAH via a method termed as ce-Subpathway. However, there is still insufficient evidence on the ceRNA regulatory mechanism for PAH, and these inspire our great interest.

In this study, we used bioinformatics methods to analyze and compare the raw sequencing data of the whole blood of patients with PAH and healthy people, hoping to screen out mRNA, miRNA and lncRNA, which may play key roles in PAH, so as to uncover the pathogenesis of PAH from the molecular level and explore potential biomarkers.

Material and methods

The identification of DEGs

The microarray dataset (GSE38267) based on platform GPL13607 was downloaded using "GEOquery" package in the R software. GSE38267 is a microarray analysis performed with homogeneous groups of patients with cystic fibrosis (n = 23), patients with PAH (n = 13) and healthy control subjects (HCs; n = 28). To explore PAH-related molecules, we selected blood samples from patients with PAH and HCs. For genes represented by more than one probe set, we took the average of the expression values of these probe sets. Then the "limma" package was used for standardization. The DEGs were screened out using the "limma" package by the following selection criteria: P < 0.05 and fold change (FC) > 2.

Enrichment analyses

The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of the DEGs, which are commonly used approaches for functional studies, were conducted with the “clusterprofiler” package in R software. The results were visualized by “GOplot” package.

Gene set enrichment analysis (GSEA), a widely used software package, was also performed using the "clusterprofiler" package in R software. We chose "h.all.v7.1.symbols.gmt" and "c2.all.v7.1.symbols.gmt" downloaded from MSigDB (http://software.broadinstitute.org/gsea/msigdb/index.jsp) as the reference gene sets, which were read into R software using the "GSEABase" package. The top three GSEA terms were visualized by “ggplot2” package.

Protein protein interaction network construction and gene clusters identification

A protein protein interaction (PPI) network based on the DEGs was carried out with STRING (https://string-db.org/) and the interaction scores > 0.400. Then the result of STRING analysis was uploaded into CYTOSCAPE v3.7.1, and MCODE plug-in was performed to screen out densely connected clusters in the PPI network. Furthermore, the genes in the top three gene clusters were separately imported into STRING to generate the PPI network and explore related biological processes.

Prediction of related miRNAs

Three online bioinformatics prediction tools (TargetScan-v7.1, miRDB, miRWalk) were used to predict the target miRNAs of the candidate genes cluster. To ensure the accuracy of the prediction, we took the intersection of three online databases as the final result. Further study was performed by CYTOSCAPE v3.7.1, and miRNAs that regulate more than two genes were selected for the following research.

miRNAs enrichment analysis

miEAA2.0 (https://ecb-compute2.cs.uni-saarland.de/mieaa2) is a website that provides miRNA overrepresentation/un-derrepresentation analysis and GSEA for 10 species by the enrichment methods: overrepresentation analysis or GSEA. The pivotal miRNAs were imported to the miEAA2.0 tool.

Prediction of miRNA–target lncRNA

lncRNAs regulated by the selected miRNAs were predicted in starBase (http://starbase.sysu.edu.cn), which is a significant database for researching ncRNAs. The obtained results were comparable with the differentially expressed lncRNAs analyzed from lncRNA datasets (GSE38267).

Preparation of the animal models

Male wild-type (WT) C57BL/6J mice (20–25 g) were purchased from Vital River Laboratory Animal Technology (Beijing, China). All mice were housed under standard conditions (temperature, 20–24 °C; 12 : 12-hour light : dark cycle) and fed with a standard rodent diet and water in a specific pathogen-free animal facility. All procedures were approved by the Animal Ethics Committee of Wenzhou Medical University.

As for chronic intermittent hypoxia (CIH)-induced pulmonary hypertension models, after adapting for 1 week, all
mice were randomly assigned to two groups, with n = 6 per group: (a) normoxia (Nx) group and (b) CIH. The CIH group was housed in a closed hypoxia chamber (8–11% O₂, 8 h per day, 6 days per week), while the Nx group was exposed to room air. The hypoxia exposure continued for 4 weeks.

As for the Sugen 5416–hypoxia (Su/Hx)-induced pulmonary hypertension mouse model, after adapting for 1 week, all mice were randomly assigned to two groups, with n = 5 per group: (a) Nx group and (b) Su/Hx group. The mice in the Su/Hx group experienced a weekly subcutaneous injection of Sugen 5416 (20 mg/kg, 11% O₂, 8 h per day, 7 days per week), while the Nx group was exposed to room air. The Su/Hx exposure continued for 3 weeks.

**Measurement of hemodynamics and right ventricular hypertrophy**

After CIH-induced pulmonary hypertension model establishment, mice were anesthetized and fixed on an operating table. The right ventricular systolic pressure (RVSP) was measured by pressure transducers (PowerLab 8/35 multi-channel biological signal recording system; AD Instruments, Australia). To further evaluate the right ventricular hypertrophy, the left ventricle plus the interventricular septum (LV+S) and the right ventricle (RV) tissue were dissected and weighed separately.

**Hematoxylin and eosin staining**

The right lung was separated and fixed with 4% paraformaldehyde for 48 h. Then the samples were embedded in paraffin, sectioned routinely and hematoxylin and eosin stained. The pulmonary arteries were captured randomly in paraffin, sectioned and hematoxylin and eosin-stained. The pulmonary arteries were captured randomly and then reverse transcribed into cDNA using the iScript cDNA Synthesis Kit. Total miRNA was extracted from lung tissue of mice and hPASMCs using SanStep Column microRNA Extraction Kit (Sangon Biotech, Shanghai, China) and then reverse transcribed into cDNA by miRNA First Strand cDNA Synthesis (stem-loop method) (Sangon Biotech). Amplification of cDNA was carried out by a quantitative RT-PCR (CFX96 Real-Time System; Bio-Rad, Hercules, CA, USA).

The target gene mRNA and IncRNA expression were standardized with the housekeeping gene GAPDH, while U6 was used as the endogenous control of miRNA. All samples were tested in triplicate. The primer sequences were shown in Table 1.

**Luciferase reporter assay**

The sequences of SNHG12 contain the let-7e-5p target site, and their corresponding mutations (MUT) were designed, synthesized and then inserted into luciferase reporter vector GP-miRGLO, named SNHG12-WT and SNHG12-MUT, respectively (RIBOBIO, Guangzhou, China). All these plasmids were cotransfected with hsa-let-7e-5p mimics or hsa-let-7e-5p mimics negative control (NC). The relative luciferase activity was examined by Dual Luciferase Assay Kit (Promega, USA) according to the manufacturer’s protocol.

**Statistical analysis**

GraphPad Prism 6.0 (GraphPad Software, CA, USA) was performed for the statistical analysis. All data were analyzed using the Kolmogorov-Smirnov normality test for normality and presented as the mean standard error of mean. Comparisons between two groups were analyzed by Student’s *t*-test, and multiple comparisons were analyzed by one-way ANOVA. A *P* value < 0.05 was considered significant. Notably, bioinformatics analysis was mostly performed through the earlier-mentioned online databases, bioinformatics tools and R language.

**Results**

**Sample information processing and identification of DEGs**

The general workflow is depicted in a flowchart (Fig. 1). First, the raw data of GSE38267 were downloaded from the GEO database. A total of 41 blood samples were chosen from GSE38267 containing 13...
PAH samples and 28 HC samples. The original data were imputed into R software and normalized by the “limma” package (Fig. 2A,B).

According to the data matrix, 165 DEGs were extracted from the samples, among which 129 genes were up-regulated and 36 genes were down-regulated (\(P < 0.05, |FC| > 2\)). Based on the analysis of gene expression, a volcano plot was used to visualize DEGs between two groups (Fig. 2C).

The DEGs in the PAH samples were mostly enriched in immune-related response

To comprehensively explore the potential biological functions and pathways of DEGs, we performed GO and KEGG pathway enrichment analyses, respectively. GO enrichment analysis of DEGs revealed that these genes were mainly involved in myeloid cell differentiation, homeostasis of number of cells and erythrocyte homeostasis (\(P < 0.05\)) (Fig. 3A). As to KEGG, the nuclear factor-κB (NF-κB) signaling pathway and primary immunodeficiency were mostly associated with the DEGs (Fig. 3B). To further validate our results, we performed GSEA. As shown in Fig. 3C, GSEA on the hallmark gene set proclaimed that the genes were mainly enriched in heme metabolism, interferon γ response and TNF-α signaling via NF-κB. As for GSEA using the KEGG gene set, the result showed high enrichment of complement and coagulation cascades, extracellular matrix receptor interaction and olfactory transduction (Fig. 3D). Taken together, the results showed that inflammation might play an important role in the occurrence and development of PAH.

PPI network construction and excavation of gene clusters

To pick out the core genes, we uploaded 167 DEGs to the STRING for PPI network analysis. Then the data were treated with CYTOSCAPE, and gene clusters were identified using MCODE (Fig. 4A and Table 2). It was found that genes in cluster 1 with the highest score were primarily enriched in erythrocyte differentiation, hemoglobin metabolic process and protoporphyrinogen IX metabolic process within the biological process (Fig. 4B). Genes in cluster 2 were mainly involved in positive regulation of T cell activation, positive regulation of immune system process and lymphocyte activation (Fig. 4C). Biological process enrichment analysis of genes in cluster 3 showed that genes were significantly enriched in the translation, peptide metabolic process and ribosome assembly (Fig. 4D). In view of the DEGs enrichment analyses results presented earlier that DEGs in the PAH samples were mostly enriched in immune-related response, genes in cluster 2 (BCL2L1, CCR7, CD5, CD6, CD19, CD40LG, IL7R and LCK) related to immune modulation were selected and then verified with KEGG pathway enrichment analysis in STRING. As indicated in Fig. 4E, the result manifested that genes in cluster 2 mainly participated in primary immunodeficiency, hematopoietic cell lineage and NF-κB signaling.

Table 1. Primers used for quantitative RT-PCR in this study. F, forward; R, reverse.

| Primer sets name | Reverse transcriptase primer (5′-3′) | Real-time quantitative PCR primer (5′-3′) |
|------------------|-----------------------------------|------------------------------------------|
| CCR7 (mouse)    | /                                 | F: GCTGAGATGTCCTCACTGGTCA                |
|                  |                                   | R: AGCCAGATGACGGCATAGGA                  |
| CCR7 (human)    | /                                 | F: CACACAGATCTCAAATGCTCA                 |
|                  |                                   | R: CTTTTTCCTCCACGGAAGCAGA               |
| SNHG12 (mouse)  | /                                 | F: GATTTTTCCCTCGTGTGTTCCA               |
|                  |                                   | R: TGTTCCCTCCCTGACACATC                |
| SNHG12 (human)  | /                                 | F: AAGCCCTCTGCTGCTGCTTC                 |
|                  |                                   | R: GCCACATTTCACCACCATC                  |
| GAPDH            | /                                 | F: GACCGGAGGTTCCTACG                   |
|                  |                                   | R: GCTGCAGATTCCGAGGAAGGTA               |
| mmu-let-7e-5p    | GTCGTATCCAGTGCAGGGTC               | F: GCCGGTTCAGAGGTAGGGTTGTA             |
|                  | CGAGGTATTCGACACTGG                 | R: CAGTGAGAGCTGCCAGTTA                 |
|                  | ATACGACACTAT                       |                                          |
| hsa-let-7e-5p    | GTCGTATCCAGTGCAGGGT               | F: GCCGTTAGGGGAGTCTGTA                 |
|                  | CCGAGGTATTCGACACTGG                | R: CAGTGAGAGCTGCCAGTTA                 |
|                  | ATACGACACTAT                       |                                          |
| U6               | GTCGTATCCAGTGCAGGGT               | F: TCCGGTCTCAGGTCTGCCAGG               |
|                  | GGTATTCGCCACTGGGATACGCAAATGGAAC   | R: CAGTGAGAGCTGCCAGTTA                 |

PAH-related molecules

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pathway. Consequently, a total of eight genes in cluster 2, including *BCL2L1*, *CCR7*, *CD5*, *CD6*, *CD19*, *CD40LG*, *IL7R* and *LCK*, were obtained for further analysis.

**Potential miRNA mining and enrichment analysis**

Based on previous work, eight genes in cluster 2 were respectively uploaded into miRWalk, miRDB and TargetScan, and the intersection of miRNA forecasted by three online tools was chosen as the prediction result (Fig. 5). In addition, the interaction network of a total of 263 miRNAs was reconstructed by CYTOSCAPE v3.7.1, and 23 miRNAs were identified with a high number of gene cross-links (≥2) (Fig. 6A and Table 3). To further evaluate the potential enrichment pathways of candidate miRNAs, we performed miEAA 2.0 with 23 miRNAs. The result showed that miRNAs were mainly enriched in the inflammation-related terms, such as CD40 receptor binding (Fig. 6B).

In addition, for further screening of meaningful miRNA, raw data of GSE44145 and GSE67597 were downloaded from the GEO database, and DEGs were identified using R software (*P* < 0.05, |FC| >1). As shown in Table 4, it was found that *hsa-miR-149-3p* and *has-let-7e-5p* were up-regulated in PAH samples in both the dataset from GSE44145 and the dataset from GSE67597, whereas *hsa-miR-646* was down-regulated in both the dataset from GSE44145 and the dataset from GSE67597. However, expression of other...
miRNAs had no meaningful difference between control samples and PAH samples, or the expression differences of miRNAs in the two datasets were inconsistent. Therefore, hsa-miR-149-3p, has-let-7e-5p and hsa-miR-646 had higher reliability.

**lncRNA prediction and ceRNA network construction**

To explore the corresponding lncRNA of miRNA, we uploaded 23 miRNAs to starBase to predict lncRNAs, whereas only hsa-let-7e-5p, hsa-let-7b-5p and hsa-let-7c-5p can forecast lncRNAs (Table 4). Combined with previous research that hsa-miR-149-3p, has-let-7e-5p and hsa-miR-646 had relatively high reliability, hsa-let-7e-5p was chosen to do further research, and its related 129 lncRNAs were selected with the highest reliability (very high stringency ≥ 5) via starBase. To further identify promising targets of hsa-let-7e-5p, we performed differentially expressed lncRNAs from GSE38267. After cross-linking between 129 lncRNAs predicted by starBase and 291 differentially expressed lncRNAs of GSE38267, SNHG12 was found to be a more creditable biomarker (Fig. 6C).

Because miRNA targeting mRNA often results in mRNA degradation, there is a reverse expression relationship between miRNA and mRNA. In our study, the expression of hsa-let-7e-5p targeting BCL2L1 and CCR7 was up-regulated in PAH samples. Simultaneously, through the analysis of DEGs from GSE38267, BCL2L1 was elevated in blood of patients with PAH, whereas the expression of CCR7 was remarkably reduced in the patients with PAH. Therefore, CCR7 might act as a reliable potential biomarker for PAH following the successful construction of a ceRNA network (Fig. 7).

**Verification of potential biomarkers expression using qRT-PCR**

To verify successful establishment of the CIH-induced pulmonary hypertension model, we measured the
hemodynamics and right ventricular hypertrophy of the Nx group and CIH group. As shown in Fig. 8A,B, RVSP in the CIH group was increased compared with the Nx group. Similarly, the RV/LV + S in the CIH group was notably higher than in the Nx group (Fig. 8C). Moreover, the results of hematoxylin and eosin staining showed that the thickness of the pulmonary artery wall and the degree of muscularization in the CIH group were higher than in the Nx group (Fig. 8D–F). The aforementioned results indicated that the CIH model was successfully established.

To unravel the expression levels of CCR7, hsa-let-7e-5p and SNHG12 in vivo, we performed qRT-PCR analysis. As for CIH models, consistent with our prediction, the results showed that there was obvious decline of CCR7 and SNHG12 in the CIH group (Fig. 8G,H), whereas the expression of let-7e-5p significantly increased under intermittent hypoxia treatment (Fig. 8I). Then, we further assessed the expression of CCR7, let-7e-5p and SNHG12 in the Su/Hx-induced pulmonary hypertension mouse model. As shown, the expression of CCR7 and SNHG12 exhibited a significant decrease in the Su/Hx group concomitant with an increase in let-7e-5p expression (Fig. 8J–L). To verify our results, we carried out in vitro experiments in hPASMCs. As expected, CCR7 and SNHG12 were obviously decreased with hypoxia exposure compared with the Nx group in hPASMCs (Fig. 8M,N).
there was significant up-regulation of let-7e-5p in hPASMCs under hypoxia treatment (Fig. 8O). As such, consistent with previous research, these results offered important insights into the possibility of SNHG12, hsa-let-7e-5p and CCR7 acting as potential biomarkers of PAH.

IncRNA SNHG12 played a ceRNA role in regulating CCR7 expression by binding to hsa-let-7e-5p

To further explore the interaction among SNHG12, hsa-let-7e-5p and CCR7, we performed a series of experiments. As shown in Fig. 8P, SNHG12 expression was knocked down by a specific siRNA. Subsequently, the results showed that the knockdown of SNHG12 reduced the expression of CCR7 after hypoxia treatment (Fig. 8Q). Then, to confirm the bioinformatics prediction analysis, we applied dual-luciferase reporter assay in 293T cells. The SNHG12-WT and mutant version without hsa-let-7e-5p binding sites were subcloned into GP-miRGLO plasmids (Fig. 8R). The results indicated the luciferase activity of the SNHG12-WT group obviously decreased in the hsa-let-7e-5p mimics group, although there was no difference among hsa-let-7e-5p mimics group and NC group in the luciferase activity of the SNHG12-MUT group (Fig. 8S). Rounding up, IncRNA SNHG12 might function as a molecular sponge in modulating the expression of hsa-let-7e-5p to further regulate CCR7.
Fig. 5. Predictive miRNA. Venn diagram of the predicted miRNA for each mRNA (BCL2L1, CCR7, CD6, CD6, CD19, CD40LG, IL7R, LCK) using three online databases (miRWalk, miRDB and TargetScan).

Fig. 6. miRNAs prediction, miRNAs enrichment analysis and lncRNAs prediction. (A) Interaction network between eight genes in cluster 2 and its targeted miRNAs. Genes were colored in red; miRNAs targeting only one gene were colored in blue; 23 miRNAs targeting more than two genes were colored in green. (B) Biological process analysis of 23 miRNAs. The closer the point was to the left, the smaller the $P$ value obtained. (C) The intersection of the 291 differentially expressed lncRNAs selected from GSE38267 and 129 lncRNAs predicted by starBase.
Discussion

PAH is a lethal pulmonary vascular disease with complex pathogenic mechanisms caused by multiple risk factors. In this study, a total of 41 blood samples from GSE38267 were selected to screen out potential biomarkers in PAH. After a series of bioinformatics analysis, SNHG12 served as the ceRNA of CCR7 via sponging hsa-let-7e-5p. Meanwhile, in vivo and in vitro experiments validated the possibility of CCR7, hsa-let-7e-5p and SNHG12 as candidate biomarkers for PAH.

Different tissues have different functions and naturally express different RNAs. Therefore, there is a certain difference of RNA expression in blood and tissues. Meanwhile, a firm association and similar expression patterns are recognized between blood and tissues. Gao et al. [11] found that the expression of cysteine-rich 61 (Cyr61) in the plasma of patients with PAH was highly increased, and similar results were found in lung tissues and PASMCs of the Monocrotaline-induced PAH rat model. Sun et al. [12] found that the endothelial SCUBE1 expression was decreased by known triggers of PAH, and concentrations of SCUBE1 were also decreased in plasma obtained from PAH rodent models and patients with PAH. Besides, the expression trends of CCR7 and

Table 3. miRNAs and their direct target genes.

| miRNA       | Genes targeted by miRNA | Gene count |
|-------------|-------------------------|------------|
| hsa-miR-6753-5p | CD5, IL7R               | 2          |
| hsa-miR-6825-5p | CD5, BCL2L1            | 2          |
| hsa-miR-3664-3p | CD5, LCK               | 2          |
| hsa-miR-3944-5p | CD5, LCK               | 2          |
| hsa-miR-6766-5p | BCL2L1, LCK            | 3          |
| hsa-miR-6756-5p | BCL2L1, LCK, CD6       | 3          |
| hsa-miR-6731-5p | LCK, CCR7              | 2          |
| hsa-miR-8095  | LCK, CCR7              | 2          |
| hsa-miR-4652-5p | CD19, CD6              | 2          |
| hsa-miR-149-3p | CD6, BCL2L1            | 2          |
| hsa-miR-6751-5p | CD6, BCL2L1            | 2          |
| hsa-miR-6829-5p | CD6, BCL2L1            | 2          |
| hsa-miR-6773-5p | CD6, BCL2L1            | 2          |
| hsa-miR-4534  | CD40LG, CD6            | 2          |
| hsa-miR-6785-5p | CD6, CCR7, BCL2L1     | 3          |
| hsa-miR-4728-5p | BCL2L1, CCR7           | 2          |
| hsa-let-7e-5p   | BCL2L1, CCR7           | 2          |
| hsa-let-7b-5p   | BCL2L1, CCR7           | 2          |
| hsa-let-7b-5p   | CCR7, IL7R             | 2          |
| hsa-miR-6646   | CCR7, IL7R             | 2          |
| hsa-miR-1233-5p | CCR7, IL7R             | 2          |

Table 4. Verification of candidate miRNAs through GSE44145 and GSE67597.

| miRNA       | mRNA       | Log(FC) [GSE44145] | Log(FC) [GSE67597] | IncRNA prediction in starBase |
|-------------|------------|---------------------|---------------------|------------------------------|
| hsa-miR-6753-5p | CD5, IL7R  | no significance     | no significance     | No                           |
| hsa-miR-6825-5p | CD5, BCL2L1 | no significance     | no significance     | No                           |
| hsa-miR-3664-3p | CD5, LCK   | 0.3432              | −5.3290E−15         | No                           |
| hsa-miR-3944-5p | CD5, LCK   | 0.3432              | −0.8840             | No                           |
| hsa-miR-6766-5p | BCL2L1, LCK | no significance     | no significance     | No                           |
| hsa-miR-6756-5p | BCL2L1, LCK, CD6 | no significance | no significance     | No                           |
| hsa-miR-6731-5p | LCK, CCR7  | no significance     | no significance     | No                           |
| hsa-miR-8095  | LCK, CCR7  | no significance     | no significance     | No                           |
| hsa-miR-4652-5p | CD19, CD6  | no significance     | −0.6861             | No                           |
| hsa-miR-149-3p | CD6, BCL2L1 | 0.1904              | 0.1252              | No                           |
| hsa-miR-6751-5p | CD6, BCL2L1 | no significance     | no significance     | No                           |
| hsa-miR-6829-5p | CD6, BCL2L1 | no significance     | no significance     | No                           |
| hsa-miR-6773-5p | CD6, BCL2L1 | no significance     | no significance     | No                           |
| hsa-miR-4534  | CD40LG, CD6 | −0.2778             | 0.4174              | No                           |
| hsa-miR-6785-5p | CD6, CCR7, BCL2L1 | no significance | no significance     | No                           |
| hsa-miR-4728-5p | BCL2L1, CCR7 | no significance     | −0.0219             | No                           |
| hsa-let-7e-5p   | BCL2L1, CCR7 | no significance     | 1.8762              | No                           |
| hsa-let-7b-5p   | BCL2L1, CCR7 | no significance     | 0.0953              | Yes                          |
| hsa-let-7b-5p   | BCL2L1, CCR7 | no significance     | −0.0893             | Yes                          |
| hsa-miR-6646   | CCR7, IL7R  | 4.5295              | −0.7849             | No                           |
| hsa-miR-1233-5p | CCR7, IL7R  | no significance     | no significance     | No                           |
SNHG12 were decreased in blood of patients with PAH, lung tissues of PH mouse models and PASMCs under hypoxia exposure, whereas the expression of let-7e-5p was increased. Hence, in combination with previous studies, some molecules in blood and tissues under pathological conditions may have the similar expression trend, which suggests that these molecules may serve as potential markers for diseases.

As is well known, the main pathogenesis of pulmonary hypertension includes pulmonary vasoconstriction and pulmonary vascular structural reconstruction. In recent years, it is believed that the main pathological changes of pulmonary vascular structural reconstruction include pulmonary vascular endothelial cell (EC) injury, excessive proliferation of smooth muscle cells and fibroblasts, reduction of mitochondrial pathway apoptosis and extracellular matrix proliferation. The inflammatory response inducing the proliferation of the PASMCs caused by the injury of pulmonary vascular ECs may be one of the key factors in pulmonary vascular structural reconstruction. Our data also suggested that the occurrence and development of PAH were correlated with the dysregulation of the immune response pathway. It is well known that NF-κB is an important transcriptional regulator with various biological activities, which plays a crucial role in pulmonary vascular structural reconstruction. Our data also suggested that the occurrence and development of PAH were correlated with the dysregulation of the immune response pathway. It is well known that NF-κB is an important transcriptional regulator with various biological activities, which plays a crucial role in immune response, inflammation, cell differentiation and cell proliferation. It has been reported that inflammation of vascular smooth muscle cells played a key role in various vascular disorders, including PAH, restenosis and atherosclerosis [13]. In terms of PAH research, a recent study noted that the up-regulation of miR-335-3p mediated by NF-κB contributes to the inhibition of apelin receptor and development of PAH [14]. Moreover, Li et al. [15] found that NF-κB-mediated miR-130a modulation might play a critical role in excessive lung vascular remodeling, which is a major cause of the increased pulmonary vascular resistance. In summary, the inflammation, especially in the NF-κB pathway, might contribute to the pathogenesis and development of PAH.

Moreover, to validate our results, we constructed PPI network analysis and further excavation of gene clusters to screen out eight genes (CD40LG, BCL2L1, CD6, CD5, CD19, IL7R, CCR7 and LCK) involved in immune system-related function, and CCR7 was selected as a biomarker candidate. It was reported that CD40LG, which is also known as CD40LG, played an important role in the pathogenesis of PAH through crosstalk between platelets and ECs [16]. The Bcl-2 family includes antiapoptotic proteins, such as Bcl-XL (BCL2L1), and proapoptotic proteins. Chowdhury et al. [17] demonstrated that the deficiency of bone morphogenetic protein receptor type II caused cell-specific effects, including increasing the expression of Bcl-XL transcripts in PASMCs while inhibiting it in ECs in PAH. There has been little direct evidence that CD5, CD6, CD19 and IL7R were involved in the development of PAH, but CD5, CD6, CD19 and IL7R were T cell and B cell surface markers, and the perivascular infiltration of T and B cells correlated with the severity of PAH through aggravating EC

**Fig. 7.** Construction of the ceRNA network and verification of SNHG12, hsa-let-7e-5p and CCR7. (A) The lncRNA–miRNA–mRNA ceRNA network; 8 mRNAs in cluster 2 were labeled green; 23 miRNAs were labeled pink; lncRNAs related to hsa-let-7e-5p were labeled blue.
Together with our results, it strongly suggests potential roles of these molecules in PAH. LCK, a member of the Src protein kinase family, might play the potential role in PAH induced by protein kinase inhibitors [19]. Also, Larsen et al. [20] found that the CCR7 deletion in mice deteriorated pulmonary hypertension, including increased RVSP and reduced pulmonary artery acceleration time.

In our research, miRNA hsa-let-7e-5p was considered to be a potential biomarker for the diagnosis and treatment of pulmonary hypertension through further verification. hsa-let-7e-5p was found to inhibit the progression of head and neck squamous cell carcinoma by significantly decreasing CCR7 protein expression [21]. Also, the expression of hsa-let-7e-5p, targeting FASLG, regulated the function of endothelial progenitor cells in deep vein thrombosis [22]. In addition, accumulating researchers have uncovered the potential value of lncRNAs as biomarkers for disease diagnosis. Based on our current study, lncRNA SNHG12 was selected as a molecular sponge of hsa-let-7e-5p and...
thus regulates its function. The knockdown of SNHG12, highly expressed in the vascular endothelium, could accelerate atherosclerosis [23]. Moreover, Sun et al. [24] revealed that SNHG12 promoted the proliferation and migration of vascular smooth muscle cells by modulating miR-199a-5p/HIF-1α contributing to atherosclerosis. In our study, along with silencing experiments, our dual-luciferase reporter gene assays indicated that SNHG12 could directly target hsa-let-7e-5p. Taken together, CCR7, hsa-let-7e-5p and SNHG12 may be novel biomarkers in PAH.

Conclusion

Through the research on bioinformatics and experiments in vivo and in vitro, we detected the pathways and potential molecules related to PAH. Based on the DEGs enrichment analyses, construction of ceRNA and verification of candidate biomarkers, we identified that signal transduction in the immune system played an important role in the occurrence and development of PAH, and CCR7, hsa-let-7e-5p and SNHG12 were considered to serve as potential biomarkers in PAH.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

MC and XL conceived the idea and wrote the first draft of the manuscript. HD executed the bioinformatics analysis. MC, XL and YW performed the experiments in vitro and in vivo. MC, XL and XH wrote parts of the manuscript and prepared the tables and figures. XH revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Data accessibility

The data that support the findings of this study are available in GE0 dataset at https://www.ncbi.nlm.nih.gov/geo/. These data were derived from the following resources available in the public domain: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38267; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44145; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67597.

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