



The expression profiling of microRNA in systemic sclerosis-associated pulmonary arterial hypertension

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Background: The role of microRNAs (miRNAs) in the pathogenesis of systemic sclerosis-associated pulmonary arterial hypertension (SSc-PAH) remains to be fully elucidated. This study evaluated the expression profile of miRNAs in the lung tissue of patients with SSc-PAH.

Methods: Lung tissue samples were collected from 3 SSc-PAH patients and 4 healthy controls. A small RNA high throughput sequence approach was used for screening the differentially expressed miRNAs in the lung tissue samples. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to validate 4 highly significant differentially expressed miRNAs. Gene Ontology and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis for mRNAs were performed using the R package clusterProfiler software.

Results: A total of 82 upregulated miRNAs and 35 downregulated miRNAs were detected in the lung tissues of patients with SSc-PAH compared with healthy controls. GO enrichment analysis demonstrated that the upregulated target genes were closely involved in biological processes such as nervous system development, anatomical structure morphogenesis, system development, cellular macromolecule metabolic processes, and cellular processes. The downregulated target genes were involved in the plasma membrane bound cell projection morphogenesis and the regulation of macromolecule metabolic processes. The KEGG enrichment analysis showed that the upregulated genes were associated with important pathways involved in cancer biology, and the target genes of the downregulated miRNAs were involved in axon guidance. High throughput sequencing and qRT-PCR revealed that hsa-miR-205-5p and hsa-miR-539-3p were differentially expressed in SSc-PAH tissue. The target genes of hsa-miR-205-5p and hsa-miR-539-3p, IRF1 and ADCYAP1, respectively, were verified using the high throughput dataset GSE48149.

Conclusions: miRNAs may play an important role in the pathogenesis of SSc-PAH, and hsa-miR-205-5p and hsa-miR-539-3p may be potential therapeutic targets in patients with SSc-PAH.

Keywords: Systemic sclerosis-associated pulmonary arterial hypertension (SSc-PAH); differentially expressed miRNAs; high throughput sequence

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Introduction

Systemic sclerosis (SSc) is an autoimmune connective tissue disease (CTD) characterized by small-vessel vasculopathy, inflammation, and immunodysfunction (1). Pulmonary arterial hypertension (PAH) associated with CTD accounts for about 15–25% of all PAH, and SSc-associated PAH is the most common type of CTD-PAH (2). The prevalence of PAH in SSc patients ranges from 5–12% and is the leading cause of death in these patients, accounting for approximately 15% of SSc-related deaths (3,4). Despite advances in targeted treatment regimens, the survival of SSc-PAH patients remains poor (5), with a mortality rate of about 50% at 3 years (6,7). Recently, it was reported that serum uric acid was a risk factor and associated with the disease severity of the SSc-PAH (8). Furthermore, age, sex, mixed venous oxygen saturation, and WHO functional class were associated with the prognosis of the SSc-PAH (9). In addition to these known parameters, we need to find and understand more underlying risk factors.

SSc-PAH is characterized by abnormal vascular proliferation and remodeling of the small- to medium-sized pulmonary vasculature (3,10). MicroRNAs (miRNAs) are small, non-coding RNA molecules with a size of approximately 22 nucleotides. They are involved in posttranscriptional negative regulation of genes by binding the partially complementary sequences of the target mRNA (11,12). Studies have shown that miRNAs played an important role in the development and regulation of vascular and fibroproliferative alterations associated with SSc pathogenesis (13,14). It has been reported that the dysregulation of miRNAs, including hsa-miR-193b, hsa-miR-21, hsa-miR-31, hsa-miR-155, and hsa-miR-196a, can contribute to the proliferative vasculopathy of SSc (15–17). One previous study showed that the expression levels of plasma miR-20a-5p was lower in SSc-PAH patients, and plasma miR-20a-5p and miR-203a-3p were linked with the occurrence of SSc-PAH in female patients with anti-centromere antibody-positive limited cutaneous SSc (18). Additionally, another study have indicated that miRNAs may play a vital role in the process of vascular inflammation (19), which is believed to be a major cause of poor prognosis in patients with SSc-PAH. However,

the precise role of miRNAs in the development of SSc-PAH has not been fully elucidated and there is a paucity of high throughput studies investigating the expression of miRNAs in the lung tissue of SSc-PAH patients.

This current study used a high throughput approach for screening differentially expressed miRNAs in the lung tissue of SSc-PAH patients and identified their possible roles in the pathogenesis of SSc-PAH. We present the following article in accordance with the MDAR reporting checklist (available at <https://dx.doi.org/10.21037/atm-21-4342>).

Methods

Study population

Lung tissue samples were obtained from SSc-PAH patients (n=3) and healthy donors (n=4) undergoing thoracic surgery for lung transplantation at the Shanghai Pulmonary Hospital and Wuxi People's Hospital, China.

Patients were included in the study if they: (I) satisfied the 2017 American College of Rheumatology/European League against Rheumatism (ACR/EULAR) criteria for the classification of SSc (14); and (II) were diagnosed with PAH by right heart catheterization (RHC) measured at rest, with mean pulmonary arterial pressure (mPAP) ≥ 25 mmHg, pulmonary arterial wedge pressure (PAWP) ≤ 15 mmHg, and pulmonary vascular resistance (PVR) ≥ 3 WU based on the 2015 European Society of Cardiology (ESC) and the European Respiratory Society (ERS) guidelines (20). Patients were excluded if they presented with the following: (I) SSc complicated with other syndromes, such as SSc with systemic lupus erythematosus or SSc with Sjogren's syndrome; (II) SSc with post-capillary pulmonary hypertension (PH); (III) chest radiograph with significant parenchymal lung disease; and (IV) echocardiography with evidence of left heart disease.

This study was approved by the institutional review board of the Shanghai Pulmonary Hospital (K20-195Y) and Wuxi People's Hospital (2020-492). All participants provided written informed consent and indicated a willingness to donate their tissue samples for research. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the

Helsinki Declaration (as revised in 2013).

High throughput sequencing

High throughput sequencing was performed by Oebiotech Biotechnology Co. (Shanghai, China). The miRNA raw data was reduced to cleaned sequences by removing low-quality reads and the Q20, Q30, and GC content of the raw data were calculated. Taken together, all the downstream analyses were based on the clean data. Small RNA sequencing procedures and analyses were performed by OEbiotech (Shanghai, China). For preliminary analysis, we determined the length distribution of the clean sequence in the reference genome.

To identify and remove the ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), transfer RNAs (tRNAs), and small nucleolar RNAs (snoRNAs), the clean reads were aligned with the small RNAs from the Rfam database (v2.2.28+) and GenBank database (<http://www.ncbi.nlm.nih.gov/>) using BLAST (v2.2.28+). Known miRNAs were identified by comparing with the miRBase (v21) database (<http://www.mirbase.org/>) and the known miRNA expression patterns in different samples were analyzed.

Identification and functional enrichment analysis of differentially expressed miRNAs

The miRNAs with $P < 0.05$ and $|\log_2 \text{foldchange}| > 1$ were screened as differentially expressed miRNAs. Target genes of differentially expressed miRNAs were predicted using 3 online miRNAs target prediction tools, including Targetscan, miRDB, DIANA-microT-CDS.

To further understand the functional categories and related gene functions, such as metabolic pathways or signal transduction pathways, of the differentially expressed miRNAs, Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the differentially expressed miRNAs was performed using the clusterProfiler in the statistical programming language R (<http://www.R-project.org/>). An adjusted false discovery rate (FDR) $P < 0.05$ was considered statistically significant. The top 10 significantly GO and KEGG pathways were selected.

RNA isolation and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Lung tissue samples were obtained from SSc-PAH lung

transplant patients and healthy donors and stored at -80°C until use. Total RNA was extracted from lung tissue samples using the miRNeasy mini kit (Qiagen, Hilden, Germany). RNA purity was calculated using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) at 260/280 nm (ratio 1.9:2.1).

The highly significant differentially expressed miRNAs were selected based on the stricter criteria of $q\text{-value} < 0.1$ and $|\log_2 \text{foldchange}| > 3$. The expression of the highly significant differentially expressed miRNAs was validated by qRT-PCR. The extracted total RNA ($1\ \mu\text{g}$) was reversely transcribed into cDNA using the ReverTra Ace qPCR RT Kit (TOYOBO FSQ101, Japan) and qRT-PCR was performed with the SYBR Green PCR master mix (TOYOBO, QPK201, Osaka, Japan) according to the manufacturer's instructions. U6 was used for qRT-PCR normalization. The primer pairs used are listed in *Table 1*. All real-time PCR reactions were carried out in duplicates and all data were calculated with the $2^{-\Delta\Delta\text{Ct}}$ equation.

Verification of differentially expressed mRNAs in the SSc-PAH samples based on the GEO database

The mRNAs profile GSE48149 was obtained from the Gene Expression Omnibus (GEO) public database and analyzed. The microarray dataset contained lung tissue samples from 9 healthy controls and 10 SSc-PAH patients. The microarray data from the GSE48149 dataset was downloaded and analyzed using the online web tool GEO2R. The mRNAs in the microarray with a $P < 0.05$ and $|\log_2 \text{foldchange}| > 1$ were considered differentially expressed mRNAs.

Statistic methods

Continuous variables with normal distribution were expressed as mean \pm standard deviation (SD), and non-normal variables were expressed as median (interquartile range). Categorical variables were expressed as numbers (percentage). The identification of differentially expressed miRNAs between SSc-PAH and healthy control tissue samples was performed using the Student's *t*-test and the limma package in the R statistical software. All GO and KEGG analyses, and other computational procedures were carried out using in-house programs written in R and Graph Prism 9.

Table 1 Primers for the differentially expressed miRNAs used in stem-loop qRT-PCR

miRNAs	Orientation	Sequences
hsa-miR-205-5p	Forward	CAGTGCGTGTGCGTGGAGT
	Reverse	ACACTCCAGCTGGG TCCTTCATTCCAC
hsa-miR-31-5p	Forward	CAGTGCGTGTGCGTGGAGT
	Reverse	ACACTCCAGCTGGG AGGCAAGATGCTG
hsa-miR-6510-3p	Forward	CAGTGCGTGTGCGTGGAGT
	Reverse	ACACTCCAGCTGGG CACCGACTCTGTCT
hsa-miR-31-3p	Forward	CAGTGCGTGTGCGTGGAGT
	Reverse	ACACTCCAGCTGGG TGCTATGCCAACAT
hsa-miR-376a-3p	Forward	CAGTGCGTGTGCGTGGAGT
	Reverse	ACACTCCAGCTGGG ATCATAGAGGAAA
hsa-miR-514a-3p	Forward	CAGTGCGTGTGCGTGGAGT
	Reverse	ACACTCCAGCTGGG ATTGACACTTCTGT
hsa-miR-539-3p	Forward	CAGTGCGTGTGCGTGGAGT
	Reverse	ACACTCCAGCTGGGATCATAAAGGACA
hsa-miR-154-3p	Forward	CAGTGCGTGTGCGTGGAGT
	Reverse	ACACTCCAGCTGGGAATCATAACGTTG
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT

qRT-PCR, quantitative reverse transcription polymerase chain reaction.

Table 2 The clinical characteristic of 3 patients with systemic sclerosis-associated pulmonary arterial hypertension

Characteristics	Mean [range] or No. (%)
Mean age (years)	54.5 [52–65]
Mean PAP* (mmHg)	61.3 [56–64]
Gender	
Male	3 (100.0)
Co-existing conditions	
SSc-PF	3 (100.0)
Diabetes	1 (33.3)
Treatment	
Systematic glucocorticoid	3 (100.0)
Cyclophosphamide	2 (66.7)

*, pulmonary artery pressure measured by echocardiography. PAP, pulmonary artery pressure; SSc-PF, systemic sclerosis-associated pulmonary fibrosis.

Results

Clinical characteristics of the SSc-PAH patients

A total of 3 SSc-PAH patients were enrolled in the study and all patients were male, with a mean age of 54.5 years. The clinical characteristics of the patients are listed in *Table 2*.

Analysis of the differentially expressed miRNAs

The results of the chip detection revealed 117 differentially expressed miRNAs that satisfied the criteria of $P < 0.05$ and $|\log_2 \text{foldchange}| > 1$. The distribution of miRNA regulation is shown in the volcano plot in *Figure 1*. A total of 82 miRNAs were upregulated and 35 were downregulated. A heatmap was constructed based on the differentially expressed miRNAs between the SSc-PAH patients and the healthy controls by using unsupervised

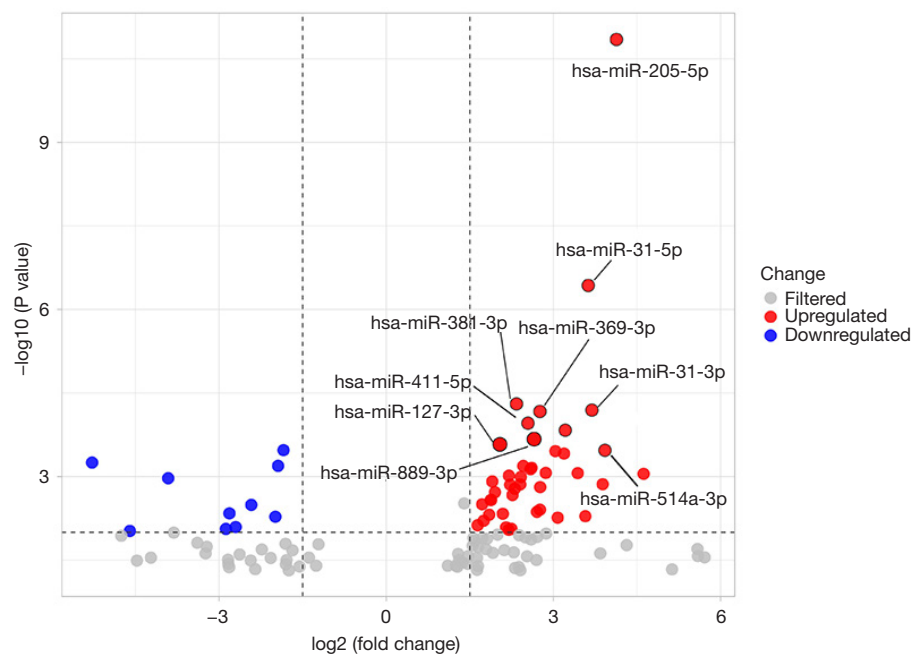


Figure 1 Volcano plot highlighting the significant genes. The y-axis corresponds to negative log 10 (adjusted P), and the x-axis displays the log₂foldchange value. The red dots represent the upregulated differentially expressed genes ($P < 0.05$, log₂foldchange > 1) between normal and SSc-PAH lung tissues. The blue dots represent the downregulated genes ($P < 0.05$, log₂ foldchange < -1). SSc-PAH, systemic sclerosis-associated pulmonary arterial hypertension.

hierarchical clustering analysis (Figure 2). The highly significant differentially expressed miRNAs are listed in Table 3. The q-value of the 4 miRNAs: miR-205-5p, miR-6510-3p, miR-31-5p, and miR-31-3p were less than 0.05.

GO enrichment analysis of target genes of differentially expressed miRNAs

For upregulated miRNAs, GO annotations indicated that predicted target genes were closely related to biological processes, including nervous system development, anatomical structure morphogenesis, system development, cellular macromolecule metabolic processes, and cellular processes (Figure 3A). The results of the KEGG pathway enrichment analysis indicated that the important pathways were involved in cancer biology, which suggested that SSc-PAH was a cancer-like disease (Figure 3B).

For the downregulated miRNAs, GO analysis showed that target genes were closely related to axon guidance, neuron projection guidance, cell morphogenesis involved in neuron differentiation, and anatomical structure morphogenesis (Figure 3C). KEGG analysis suggested that the significant pathways were involved in axon guidance

(Figure 3D).

qRT-PCR analysis for highly significant differentially expressed miRNAs

The qRT-PCR validation showed that the expression of hsa-miR-205-5p and hsa-miR-539-3p were significantly elevated in the tissue samples of SSc-PAH patients compared with healthy donors ($P = 0.00167$ and $P < 0.001$, respectively; Figure 4).

Identification of the candidate genes of hsa-miR-205-5p and hsa-miR-539-3p

The target genes of hsa-miR-205-5p and hsa-539-3p were predicted using 3 online prediction tools, including miRDB, Targetscan, and DIANA-microT-CDs (Figure 5). The differentially expressed mRNAs in the dataset GSE48149 were used to verify the targets genes of hsa-miR-205-5p and hsa-miR-539-3p. A total of 68 differentially downregulated genes were identified in the validation dataset GSE48149. Interferon regulatory factor 1 (IRF1) and adenylate cyclase activating polypeptide 1 (ADCYAP1) were identified as

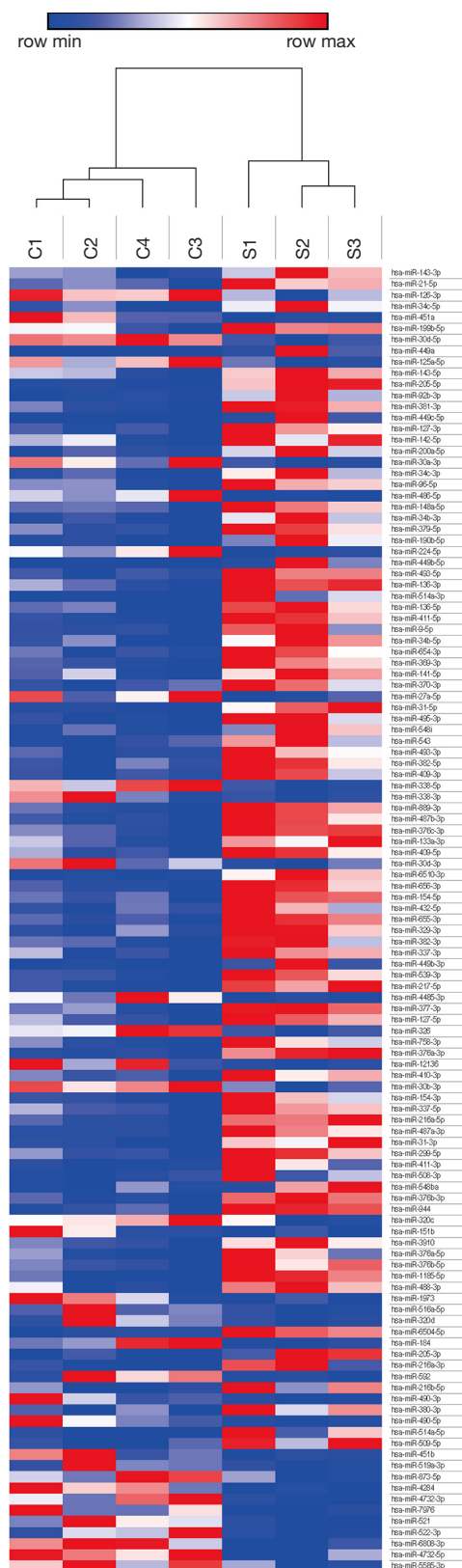


Figure 2 Heatmap of all the differentially expressed miRNAs.

the target genes of hsa-miR-205-5p and hsa-miR-539-3p, respectively (Figure 6).

Discussion

Though with the advanced therapies, the prognosis of SSc-PAH was still poor. Recently, more and more studies have used the multi-omics analysis to identify the differently miRNAs and mRNAs in the serum and lung tissue of SSc-PAH, however, there was still lacking the specific biomarkers for SSc-PAH, the studies related to the miRNAs in the lung tissue of SSc-PAH was still limited. To our knowledge, this is the first study to use high throughput sequencing to examine the miRNA expression profile in the lung tissue of SSc-PAH patients. The present study identified hsa-miR-205-5p and hsa-miR-539-3p as differentially expressed miRNAs in the lung tissue of SSc-PAH patients, which might help us understand the roles of miRNAs in the pathogenesis of SSc-PAH. Previous studies also indicated that the abnormal hsa-miR-205-5p and hsa-miR-539-3p expressions existed in the various cancers (21,22). These results demonstrated that the two miRNAs may be the potential biomarkers of the early diagnosis, disease evaluation and prognosis judgment in SSc-PAH.

The GO analysis results showed that both the up- and downregulated genes are involved in cellular processes, suggesting that dysfunction in cellular processes may be critical in the development of SSc-PAH. There is evidence that endothelial to mesenchyme transition is a cellular process that contributes to the pathogenesis of PAH (23). Moreover, during PAH development, cells in the pulmonary vascular wall become hyperproliferative which leads to cell proliferation and an increased remodeling of the pulmonary vasculature (24). In addition, the common GO term in both up- and downregulated miRNAs was anatomical structure morphogenesis. KEGG analysis showed that the target genes of upregulated and downregulated miRNAs are both closely associated with the axon guidance pathway. Furthermore, the significant pathways of the target genes of the upregulated miRNAs are linked to cancer biology. The upregulated miRNAs are also linked to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor resistance, Forkhead box O isoforms (FoxO) signaling pathway, and signaling pathways regulating pluripotency of stem cells. FoxOs are reportedly involved in vascular structural maintenance (25-27) and FoxO1 has been shown to be critical to the hyperproliferation of pulmonary artery smooth muscle

Table 3 The highly significant differentially expressed miRNAs

miRNA	log2foldchange	P	Q	Regulation
hsa-miR-205-5p	4.13	0.0000	0.0000	Up
hsa-miR-31-5p	3.62	0.0000	0.0004	Up
hsa-miR-6510-3p	4.13	0.0000	0.0004	Up
hsa-miR-31-3p	3.69	0.0001	0.0259	Up
hsa-miR-376a-3p	3.21	0.0001	0.0413	Up
hsa-miR-514a-3p	3.93	0.0003	0.0636	Up
hsa-miR-539-3p	3.04	0.0003	0.0636	Up
hsa-miR-154-3p	3.19	0.0004	0.0655	Up

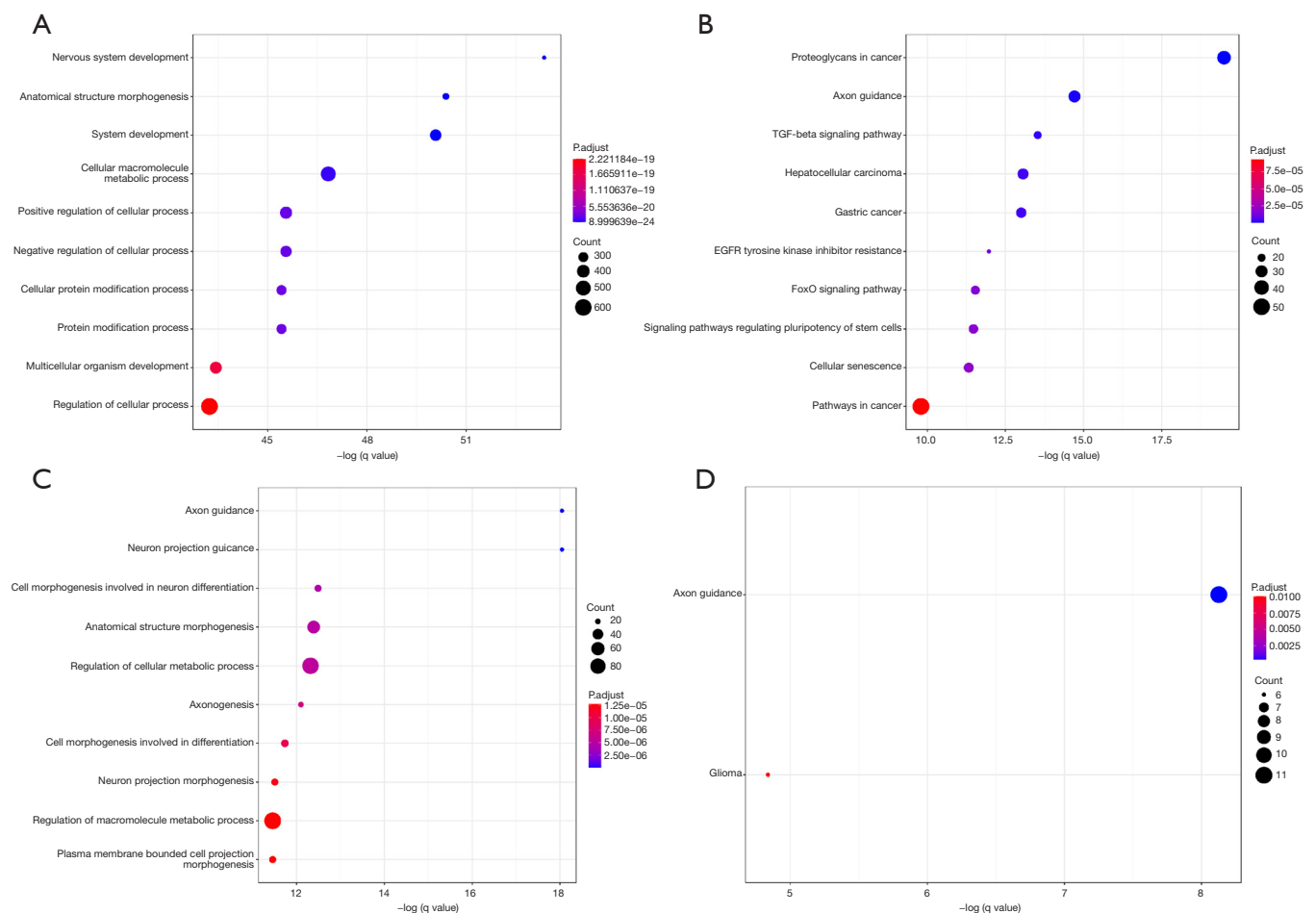


Figure 3 GO and KEGG enrichment analysis of differentially expressed miRNA. (A) The top10 enriched GO terms for the predicted target genes of upregulated differentially expressed miRNAs. FDR corrected $P < 0.05$ was used as a threshold to select significant GO pathways. (B) The top 10 enriched KEGG terms for the predicted target genes of upregulated differentially expressed miRNAs. FDR corrected $P < 0.05$ was used as a threshold to select significant GO pathways. (C) The top 10 enriched GO terms for the predicted target genes of downregulated differentially expressed miRNAs. FDR corrected $P < 0.05$ was used as a threshold to select significant GO pathways. (D) The significant KEGG pathways related to the predicted target genes of downregulated differentially expressed miRNAs. FDR corrected $P < 0.05$ was used as a threshold to select significant GO pathways. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate.

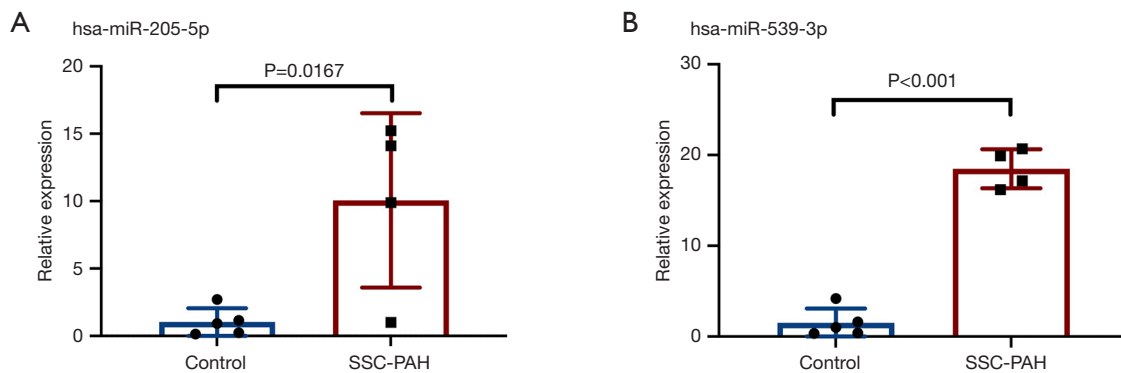


Figure 4 Real-time qRT-PCR verification results of hsa-miR-205-5p and hsa-miR-539-3p in 4 patients with SSC-PAH and 5 healthy controls. (A) The expression of hsa-miR-205-5p verified by real-time qRT-PCR. (B) The expression of hsa-miR-539-3p verified by real-time qRT-PCR. $P < 0.05$ was considered statistically significant. SSC-PAH, systemic sclerosis-associated pulmonary arterial hypertension; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

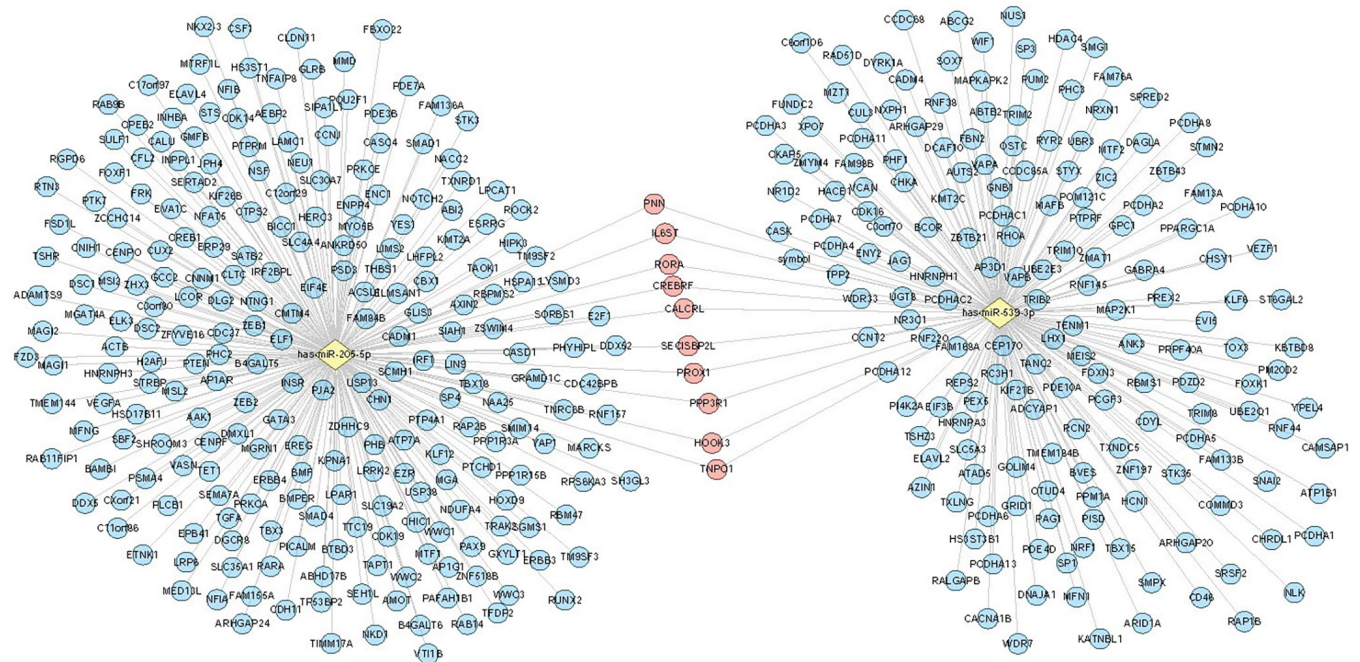


Figure 5 The predicted target genes of hsa-miR-205-5p and hsa-miR-539-3p.

cells (PASMCs) in pulmonary hypertension (27).

Prior studies by Gulei *et al.* demonstrated that upregulation of hsa-miR-205-5p inhibited epithelial to mesenchymal transition in colon cancer cells (28), suggesting that hsa-miR-205-5p plays a vital role in the development of cancer. In addition, hsa-miR-205-5p may play an important role in the development of SSC-PAH.

Studies have suggested that the dysregulation of miRNAs plays important roles in the development of pulmonary hypertension (29). In fact, hsa-miR-205-5p has been shown to be closely associated with the proliferation of PASMCs in PAH. Furthermore, hsa-miR-205-5p can inhibit smooth muscle cell proliferation by activating the ERK1/2 signaling pathway, and thus, may be a potential therapeutic target

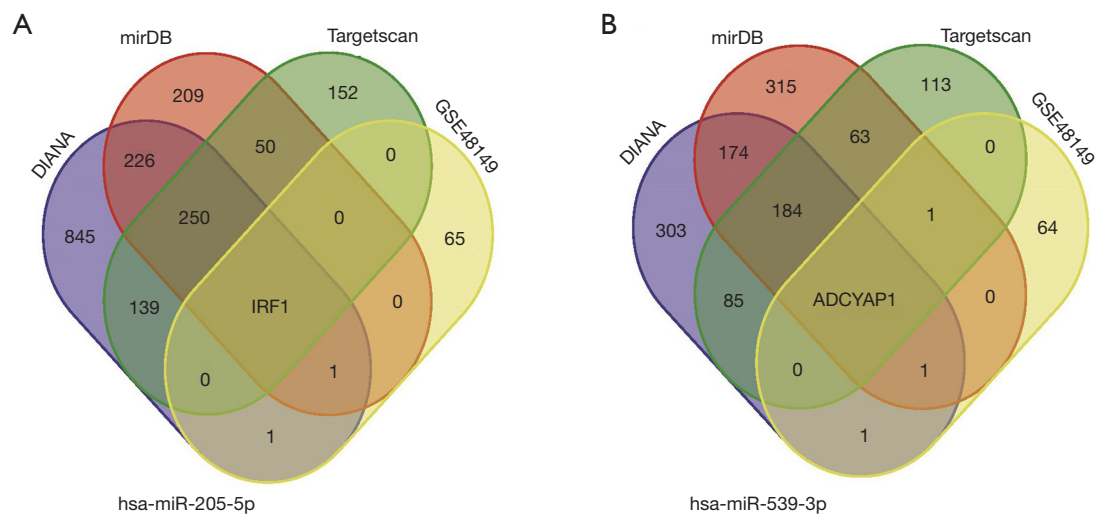


Figure 6 The venn diagram analysis of target genes of hsa-miR-205-5p and hsa-miR-539-3p and differentially expressed mRNAs in the dataset GSE48149. (A) The venn diagram showed the overlap of the target genes of hsa-miR-205-5p miRNAs predicted by three online tools and the differentially expressed mRNAs in the dataset GSE48149 was IRF1. (B) The venn diagram showed the overlap of the target genes of hsa-miR-539-3p predicted by three online tools and in the dataset GSE48149 was ADCYAP1.

for PAH treatment (30). Tao *et al.* suggested that hsa-miR-205-5p was downregulated in mice PSMCs under hypoxia conditions, and inhibited the cell proliferation through regulating ERK1/2 pathway (31). The similar mechanism was also observed in cancer, which might lead to PSMC cancer-like transformation (32). However, to date, there have been few studies examining the expression and role of hsa-miR-205-5p and hsa-miR-539-3p in SSc-PAH.

GO analysis results indicated that the target genes of hsa-miR-205-5p were involved in all GO categories, especially in biological processes, including tube morphogenesis, negative regulation of development processes, and regulation of anatomical structure morphogenesis. This suggested that cellular processes and organ system development play vital roles in the development of SSc-PAH. Reports have suggested that endothelial to mesenchyme transition contributes significantly to the pathogenesis of PAH (20). Moreover, during PAH development, cells in the pulmonary vascular wall become hyperproliferative which leads to cell proliferation and an increase in remodeling of the pulmonary vasculature (24).

This study identified IRF1 and ADCYAP1 as the target genes of the hsa-miR-205-5p and hsa-miR-539-3p, respectively. IRF1 has been reported to be associated with cell growth control and induction of apoptosis (32). Lee *et al.* showed that IRF1 inhibited the proliferation and migration of endothelial cells (33). Besides, IRF1 regulated

the immune cell development (34,35). Both ADCYAP1 and IRF1 have been shown to exert antiproliferative effects in cells of various diseases (36,37). ADCYAP1 was a multifunctional neuropeptide with anti-inflammatory and cardioprotective functions (38). ADCYAP1 could indirectly vasodilate vessel through stimulating NO releasing (39). Therefore, hsa-miR-205-5p and hsa-miR-539-3p may target the two target genes to participate in the process of disease development by involving in the regulation of perivascular inflammatory cell function. Future studies are warranted to further elucidate the roles of IRF1 and ADCYAP1 in SSc-PAH.

Study limitations

There were several limitations to this study. First, there were few tissue samples examined this study, and this may lead to inadequate statistical power. Second, additional subgroup analysis of the gene expression of SSc patients and SSc-PAH patients may lead to further understanding of the development of SSc-PAH. Third, further experiments are required to verify the target genes of the differentially expressed miRNAs.

Conclusions

PAH is an incurable and common complication of

SSc, that contributes to early mortality in SSc patients. Unfortunately, the genetic etiology of SSc-PAH has not been fully elucidated. Therefore, understanding the molecular mechanisms underlying the early development of SSc-PAH, in particular, the role of miRNAs, is crucial. This study demonstrated that the miRNAs hsa-miR-205-5p and hsa-miR-539-3p may play important roles in the pathogenesis of SSc-PAH.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://dx.doi.org/10.21037/atm-21-4342>

Data Sharing Statement: Available at <https://dx.doi.org/10.21037/atm-21-4342>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/atm-21-4342>). The authors have no conflicts of interest to declare.

Ethical Statement: All authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration (as revised in 2013). Shanghai Pulmonary Hospital, Wuxi People's Hospital reviewed and approved the protocol form prior to initiation of the study (approval number: K20-195Y and 2020-492). All participants provided written informed consent and indicated a willingness to donate their tissue samples for research.

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