Downregulation of microRNA-30a in bronchoalveolar lavage fluid from idiopathic pulmonary fibrosis patients

BAO LIU[1*], TINGSHU JIANG[2*], XINGANG HU[1], ZHIDA LIU[1], LIMING ZHAO[1], HONGMEI LIU[2], ZHAIHUA LIU[3] and LIJUN MA[1]

[1]Department of Respiratory Medicine, People's Hospital of Zhengzhou University, Zhengzhou, Henan 450000; [2]Respiratory Department, The Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College, Yantai, Shandong 264000; [3]Institute of Basic Theory of Traditional Chinese Medicine, China Academy of Chinese Medical Sciences, Beijing 100700, P.R. China

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Abstract. MicroRNAs (miRs) are short, highly conserved small noncoding RNA molecules with fundamental roles in regulating gene expression. To identify miR biomarkers associated with idiopathic pulmonary fibrosis (IPF), the expression pattern of miRs in exosomes from bronchoalveolar lavage fluid (BALF) of elderly patients with IPF were evaluated. High-throughput quantitative detection of miR expression using a microarray indicated that miR-125b, miR-128, miR-21, miR-100, miR-140-3p and miR-374b were upregulated in patients with IPF, while let-7d, miR-103, miR-26 and miR-30a-5p were downregulated. The expression level of miR-30a-5p was further examined, and its potential target genes were predicted using target gene prediction analysis software. A direct regulatory association was confirmed between miR-30a-5p and TGF-β activated kinase 1/MAP3K7 binding protein 3 (TAB3) via a dual-luciferase reporter assay. Overexpression of miR-30a-5p decreased TAB3, α-smooth muscle actin and fibronectin expression in A549 cells with or without transforming growth factor-β1 treatment. The decreased expression of miR-30a in the BALF of patients with IPF, along with the consequential increase in TAB3 expression, may be a crucial factor in IPF progression.

Correspondence to: Dr Zhaihua Liu, Institute of Basic Theory of Traditional Chinese Medicine, China Academy of Chinese Medical Sciences, 16 Dongzhimenneinanxiaojie Street, Dongcheng, Beijing 100700, P.R. China
E-mail: zhaihua_liu@sina.com

Dr Lijun Ma, Department of Respiratory Medicine, People's Hospital of Zhengzhou University, 7 Weiwu Road, Jinshui, Zhengzhou, Henan 450000, P.R. China
E-mail: malijun1961@sina.com

*Contributed equally

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a type of interstitial lung disease (ILD) (1). This chronic and progressively fatal disease is characterized by the formation of scar tissue deep in the lung, causing the thickening of the pleural lining and leading to the irreversible loss of pulmonary function (2,3). It usually affects adults between 50 and 70 years of age, with a higher incidence in men than in women. The incidence rate of IPF is five times higher than that of cystic fibrosis or Lou Gehrig's disease (also termed amyotrophic lateral sclerosis), with a poor prognosis, a median survival time of 3 years and fatality rate of 2/3 in 5 years (4,5); however this disease remains largely unknown to the general public. Although other ILDs may be attributed to the exposure to asbestos or certain medications, IPF has not been linked to any known pathogenic factors. Potential risk factors for IPF may include smoking, and exposure to wood or metal dust (6,7).

MicroRNAs (miRNAs/miRs) have been proposed to have fundamental roles in the pathogenesis of several pulmonary diseases, including interstitial lung disease, chronic obstructive pulmonary disease and IPF (8). miRs are small noncoding RNAs that subtly regulate gene expression by inhibiting mRNA translation or mediating mRNA degradation. Exosomes are present in the majority of bodily fluids, and their composition differs depending on their cellular origins (8). Studies have demonstrated that miRs encapsulated in exosomes in certain bodily fluids, including saliva (9) and serum (10), can be used as biomarkers for disease screening and prognosis purposes (11).

Potential alterations of exosome miR contents caused by IPF have not yet been investigated. In the present study, the differences between bronchoalveolar lavage fluid (BALF) exosomal miRs from patients with IPF and healthy participants were characterized. Exosomes were collected from patients with IPF or healthy participants by bronchoalveolar lavage prior to exosomal miR extraction. Quantitative analysis of miR expression profiles revealed that miR-125b-5p,

miR-128-3p, miR-21-5p, miR-100-5p, miR-140-3p and miR-374b-5p were upregulated by more than 2-fold in patients with IPF compared with healthy participants; while let-7d-5p, miR-103-3p, miR-27b-3p and miR-30a-5p were downregulated. The expression level of miR-30a-5p was further examined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Target gene prediction analysis of miR-30a-5p indicated that it may directly interact with TGF-β activated kinase 1/MAP3K7 binding protein 3 (TAB3) mRNA. Further examination using a dual-luciferase reporter assay and western blotting analysis confirmed the association between miR-30a-5p and TAB3 mRNA. Functional assays demonstrated that miR-30a-5p overexpression attenuated transforming growth factor-β1 (TGF-β1)-induced upregulation of TAB3, α-SMA and fibronectin expression in 293T cells. The decreased expression of miR-30a-5p in patients with IPF, along with the consequential increase in TAB3 expression may be a key factor in IPF progression.

Materials and methods

Patients and clinical specimens. The present study was approved by the institutional review board of People's Hospital of Zhengzhou University (Zhengzhou, China). A total of 30 patients (2016.10-2017.09) diagnosed with IPF were selected as the IPF group based on their positive IPF diagnosis prior to enrollment. A total of 16 healthy participants were selected as a control group in the same facility (People's Hospital of Zhengzhou University) based on their negative diagnosis results in routine blood test, chest X-ray, chest and abdominal ultrasound examination. All patients and healthy participants were diagnosed as free from structural heart disease or brain, liver, kidney and endocrine associated metabolic diseases. Informed consent was obtained from each participant in written form prior to undergoing BALF collection, following the Chinese medical association alveolar lavage operation instructions (12). Clinical data of patients with IPF and healthy participants is summarized in Table I.

Exosome collection and miR extraction. Exosomes in the BALF obtained from each subject were isolated using 100 ml BALF by ExoQuick-TC™ exosome precipitation (System Biosciences, LLC, Palo Alto, CA, USA) following the manufacturer's protocol. The isolated exosomes were resuspended in lysis buffer provided in NucleoSpin miRNA extraction kit (Macherey-Nagel GmbH & Co., KG, Düren, Germany) and stored at -80˚C for no longer than 24 h prior to miR extraction. Total miRs from each subject's exosome sample were extracted using NucleoSpin miRNA extraction kit (Macherey-Nagel GmbH & Co., KG) following the manufacturer's protocol. A 100 μl exosome sample from each group prior to and following precipitation, as well as supernatant solution from precipitation, were reserved for cluster of differentiation 63 (CD63) detection by western blotting.

Exosomal miR profiling and miR-30a-5p expression level. Half of the total miRs extracted from each BALF sample were subject to miR expression profile analysis using miProfile™ human miRNome miRNA qPCR Array kit (Genecopoeia, Inc., Rockville, MD, USA) following the manufacturer's protocol. Each 96-well plate included 12 wells that contained different types of controls for monitoring the performance of the entire experimental process. Particularly, the wells include two negative controls, six different housekeeping controls for normalization (snRNAs), two RT and two PCR controls. The expression level of each detected miR was first normalized to small nuclear (sn)RNA U6 using the $2^{-\Delta\Delta Cq}$ method (13), and the fold-change ($|\log_2^n|>1$) of normalized miR expression is calculated by comparing the expression level of each detected miR in IPF exosome lysates to that in exosome lysates from healthy participants. The other half of extracted miRs from each sample was subjected to evaluation of the miR-30a-5p expression level using a custom-built hsa-miR-30a-5p RT-qPCR assay kit (Genecopoeia, Inc.) following the manufacturer's protocol. miR-30a-5p expression level of each sample was first normalized to snRNA U6 using the $2^{-\Delta\Delta Cq}$ method, and the expressional difference was revealed by calculating the fold-change of the mean value of miR-30a expression level in exosome lysates from healthy participants compared with that of patients with IPF. The receiver operating characteristic curve of miR-30a-5p expression in BALF exosomes of IPF patients was analyzed with reference to the expression level of miR-30a-5p in healthy participants.

Cell culture and preparation. A549 and 293T cells previously purchased from the American Type Culture Collection, (Manassas, VA, USA) and stored in liquid nitrogen were resuscitated and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (HyClone; GE Healthcare, Life Sciences, Logan, UT, USA) and penicillin-streptomycin cocktail at 37˚C in a humidified incubator with 5% CO_2. Cells were transfected with miR-30a-5p mimic (5'-UGUAAACAU CCUCGACUGGAAG-3', 250 μg/ml) or non-targeting miR mimic (mimic control, 5'-UCACAACCUCCUAGAAAGAGU AGA-3') vector (Genecopoeia, Inc.) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 12 h according to the manufacturer's protocol prior to subsequent assays. Recombinant human TGF-β1 cytokine (5 ng/ml for 24 h) for cell treatment was purchased from Invitrogen (Thermo Fisher Scientific, Inc.) and applied according to the manufacturer's protocol.

Dual-luciferase reporter assay. For the firefly luciferase reporter assay, firefly luciferase vector carrying wild type (WT) or mutant (MT) human TAB3 3'untranslated region (UTR) were purchased from Genecopoeia, Inc. Briefly, the firefly luciferase reporter vectors were constructed by inserting the amplified WT or MT TAB3 3'UTR cDNA sequence downstream of a secreted firefly luciferase gene in pEZX-MT01 vector (Genecopoeia, Inc.). 293T cells were transfected with 2 nmol/ml either miR-30a-5p mimic vector, mimic control vector or empty vehicle as blank control, in combination with 2.5 μg/ml of both the reporter vector and *Renilla* luciferase control vector pRL-TK (Genecopoeia, Inc.) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Luciferase reporter assay was performed 24 h following transfection. Firefly and *Renilla* luciferase activities were measured consecutively by GLO-MAX 20/20 fluorescence detector

Table I. Clinical data of patients with IPF and healthy participants.

	IPF (n=30)	Healthy (n=16)	P-value
Age (year)[a]	64.38±9.88	66.90±7.48	0.18
Gender (male/female)	27/3	12/4	0.54
Smoking (current/ever/never)	0/21/9	0/13/3	0.19
Height (cm)[a]	167.30±0.75	153.40±0.23	0.36
Weight (kg)[a]	62.75±6.74	58.77±8.66	0.66
BMI (kg/m^2)[a]	11.95±1.61	19.12±1.79	0.45
FVC (% predicted)[a]	59.80±15.30	N/A	N/A
TLC (% predicted)[a]	62.40±7.90	N/A	N/A
DLCO [Hb] (% predicted)[a]	44.60±21.10	N/A	N/A

[a]Data presented as the mean ± standard deviation. IPF, idiopathic pulmonary fibrosis; healthy, healthy participants; BMI, body mass index; FVC, forced vital capacity; TLC, total lung capacity; DLCO, diffusing capacity of the lung for carbon monoxide; Hb, hemoglobin; N/A, data not available.

device (Promega Corporation) using Secrete Pair™ Dual Luminescence assay kit (Lipofectamine® 2000) following the manufacturers' protocol. The firefly luciferase activity was normalized to *Renilla* luciferase activity.

RT-qPCR and western blotting assay. For evaluating the miR-30a-5p expression level and TAB3, α-SMA and fibronectin mRNA expression level in A549 cells with different treatments, custom-built RT-qPCR kits (Genecopoeia, Inc.) were used following manufacturer's protocol. The sequences of miR-30a-5p primer: 5'-TTCAGTCGGATGTTTGCAGCAAA-3'. Detection was achieved by SYBR green qPCR with the following conditions: 95˚C for 10 min followed by 40 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 20 sec. The miRNA expression levels were normalized to levels of U6 snRNA (5'-AAATTCGTGAAGCGTTCC-3') using the $2^{-\Delta\Delta Cq}$ method (13). For detecting exosomal CD63, the same volume of 30 μl each exosome lysate sample or supernatant sample was used. For detecting cellular TAB3 and β-actin protein expression level in A549 and 293T cells 24 h after transfection, cells cultured in 12-well-plates were lysed with pre-chilled NP-40 lysis buffer (200 μl per well; Thermo Fisher Scientific, Inc.) by pipetting on ice, and a same volume of 30 μl each cell lysate sample was used following centrifugation at 10,000 x g for 3 min at 4˚C to remove cell debris. Protein concentrations were determined using a BCA assay kit (Thermo Fisher Scientific, Inc.). The total protein of each cell group (20 μl) was separated using polyacrylamide gel (15%) electrophoresis under reducing conditions, followed by transferral onto nitrocellulose membranes. Following blocking at 4˚C overnight with non-fat milk and preparation with PBS. The membranes were probed with antibodies, incubated 16 h at 4˚C. And then incubated with horseradish peroxidase conjugated secondary antibodies, incubated 1 h at room temperature, and colorized using the Pierce ECL Plus substrate (Pierce; Thermo Fisher Scientific, Inc.). The image of each sample was captured using chemo-fluorescence-sensitive film and the gray value of each band was analyzed. The quantity of target protein was evaluated by the ratio of its gray value to the internal reference using ImageJ software, version 1.48 (National Institutes of Health, Bethesda, MD, USA). Antibodies against human CD63 (cat. no. ab134045; 1:1,000), TAB3 (cat. no. ab124723; 1:2,000), β-actin (cat. no. ab8227; 1:1,000) and secondary antibody goat anti-rabbit IgG H&L (cat. no. ab6721; 1:5,000) were purchased from Abcam (Cambridge, UK). Antibodies against human CD63, TAB3 and β-actin incubated 16 h at 4˚C. The secondary antibody incubated 1 h at room temperature.

Data visualization and statistical analysis. Data were visualized and statistically analyzed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) and presented as the mean ± standard deviation, unless otherwise indicated. An unpaired parametric t-test was used for analysis. All P-values are two-tailed. $P<0.05$ was considered to indicate a statistically significant difference. Online tools for miRNA analysis are miRDB (http://www.mirdb.org/) online database and the STRING online platform (https://string-db.org/cgi/input.pl, version 10.5). The target genes of these increased miRs were predicted using FunRich software (http://www.funrich.org/; ver. 3).

Results

Characteristics of patients with IPF and healthy participants. The physiological and pathological data of 30 patients with IPF and 16 healthy participants involved in the present study are summarized for comparison in Table I. Patients were diagnosed with IPF by high-resolution computed tomography and other causes of interstitial lung disease in medical history, including family environment, occupational exposure, connective tissue disease and drug-induced pulmonary lesion were excluded (14,15). No statistically significant ($P>0.05$) difference in age, height, weight or body mass index was detected between the two groups.

Alterations in BALF exosomal miRNA expression patterns between IPF pathological and healthy conditions. BALF exosomes from patients with IPF and healthy participants were obtained by precipitation as described. High-throughput quantitative analysis of miR expression using a microarray

was performed to reveal the differentially expressed BALF exosomal miRs between patients with IPF and healthy participants (Fig. 1). A total of 69 miRs exhibited significantly different expression levels in BALF exosomes between the two groups. Of these 69 miRs there was a greater than 2-fold increase in the expression level of miR-125b-5p, miR-128-3p, miR-21-5p, miR-100-5p, miR-140-3p and miR-374b-5p, and a greater than 2-fold decrease in let-7d, miR-103-3p, miR-30a-5p and miR-27b-3p. The majority of miRs with significant expression differences have been previously reported as potential regulators and biomarkers in IPF progression or IPF-associated carcinogenesis (16-23), among which miR-30a-5p demonstrated the most significance and was selected for further investigation in the present study (P<0.001).

Decreases in exosomal miR-30a-5p expression in patients with IPF compared with healthy participants. The miR-30a-5p expression level in BALF exosomes obtained from patients with IPF and healthy participants was validated by RT-qPCR (Fig. 2A). miR-30a-5p expression was significantly decreased under IPF pathological conditions, which is consistent with the result of the miR profiling assay (P<0.001). These data suggested that miR-30a-5p may be a potential regulator involved in IPF pathogenesis, and the downregulation of miR-30a-5p may contribute to IPF progression. The receiver operating characteristic curve analysis based on miR-30a-5p expression level in two groups further demonstrated that decreased miR-30a-5p expression may be a biomarker for IPF (Fig. 2B).

TAB3 as direct regulatory target of miR-30a-5p. To investigate the regulatory role of miR-30a-5p in IPF, potential target mRNAs of miR-30a-5p were predicted using miRDB online database. miRNA-30a-5p was predicted to interact with 842 genes, among which 111 genes were involved in intracellular signal transduction, as revealed by functional enrichment analysis using the STRING online platform. The potential target genes were filtered further based on their association with TGF-β1 signaling pathway, which serves vital role in fibrogenesis and IPF progression via manual literature searching (24,25). Protein interaction data mining using the STRING online platform indicated that TAB3 may directly interact with TGF-β1 receptor and mitogen-activated protein kinase 7 (Fig. 3A), which has been demonstrated to serve an important role in fibrosis process in various tissues (26,27). Sequence analysis of TAB3 mRNA and miR-30a-5p also demonstrated that miR-30a-5p may directly interact with the 3'UTR region in TAB3 mRNA (Fig. 3B). Based on these analysis results, TAB3 was selected for further investigation.

A dual-luciferase reporter assay was performed to confirm this interaction (Fig. 3C). Firefly luciferase activity in 293T cells co-transfected with TAB3 3'UTR-flanked firefly luciferase vector and miR-30a-5p mimic was significantly lower compared the control group co-transfected with TAB3 3'UTR flanked firefly luciferase and non-targeting mimic control (P<0.001), suggesting a direct interaction between miR-30a-5p and the 3'UTR region of TAB3 mRNA. This miR targeting mechanism was confirmed by inducing three consecutive point mutations within the predicted miR-30a-5p binding site on 3'UTR region of TAB3 mRNA (Fig. 3B and D). Firefly luciferase was significantly decreased in 293T cells co-transfected

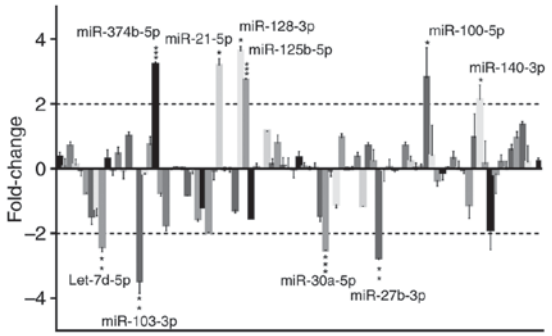

Figure 1. Exosomal miR expression pattern alterations between patients with IPF (n=30) and healthy participants (n=16). The expression level of each miR was presented as fold-change relative to that in healthy participants. *P<0.05; **P<0.01; ***P<0.001, IPF vs. healthy controls. miR, miRNA; IPF, idiopathic pulmonary fibrosis.

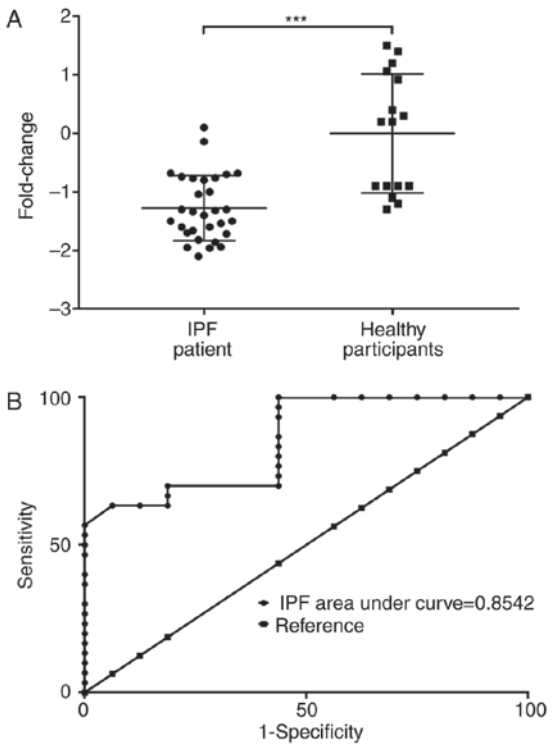

Figure 2. Differential expression of miR-30a-5p in BALF exosomes extracted from IPF patients and healthy participants. (A) miR-30a-5p expression level in BALF exosomes obtained from 30 IPF patients and 16 healthy participants. Data presented as fold-change; (B) Receiver operating characteristics curve analysis of miR-30a-5p expression level in IPF patients' BALF exosomes using that from healthy participants as reference. ***P<0.001. IPF, idiopathic pulmonary fibrosis; miR, microRNA; BALF, bronchoalveolar lavage fluid.

with WT TAB3 3'UTR flanked firefly luciferase vector and miR-30a-5p mimic compared with mimic control (P<0.001). Firefly luciferase was no significant in TAB3 3'UTR MT miR-30a-5p mimic compared with mimic control. This mechanism was further verified by western blotting analysis evaluating the expression level of TAB3 in A549 and 293T cells with or without miR-30a-5p mimic transfection (Fig. 4). miR-30a-5p mimic transfection significantly decreased the TAB3 protein expression level compared with non-targeting mimic control transfection in A549 (P<0.05) and 293T cells

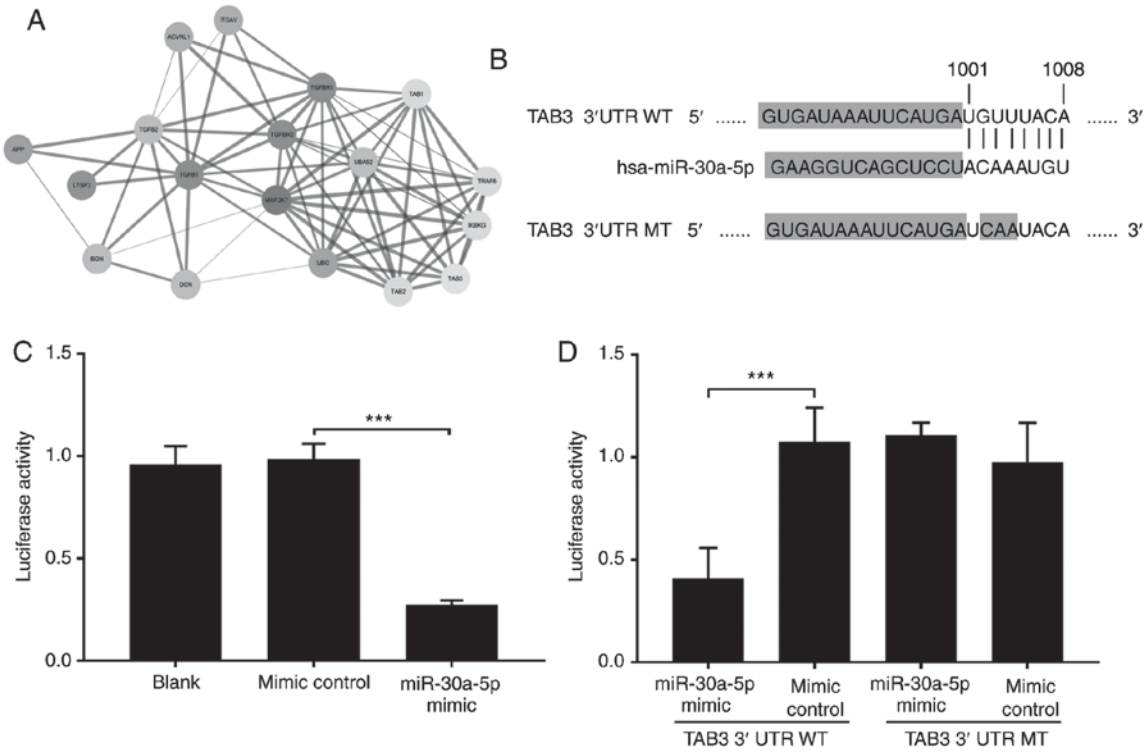

Figure 3. TAB3 3'UTR as a target of miR-30a-5p. (A) Interaction network of TAB3 and TGF-β receptor associated proteins predicted by STRING online platform. Each node represents a protein with its gene ID labeled at node center. Node color represents the connection degree of each protein within the network with red representing high connection degree. The width of the line connecting two nodes represents the combined score of the predicted association between two proteins. (B) Schematics of WT TAB3 3'UTR interacting with miR-30a-5p and MT TAB3 3'UTR with three continuous point mutations. The nucleotide sequences of TAB3 mRNA and miR-30a-5p were acquired from the National Center for Biotechnology Information gene database and miRDB online database, respectively. Dual-luciferase reporter assay in 293T cells showing that (C) miR-30a-5p mimic transfection decreased the expression of firefly luciferase with (D) WT TAB3 3'UTR but not that with MT TAB3 3'UTR. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Each column represents at least five replicate experiments. ***P<0.001. TAB3, transforming growth factor-β activated kinase 1/MAP3K7 binding protein 3; 3'UTR, 3'untranslated region; WT, wild type; hsa-miR-30a-5p, human microRNA-30a-5p; MT, mutant.

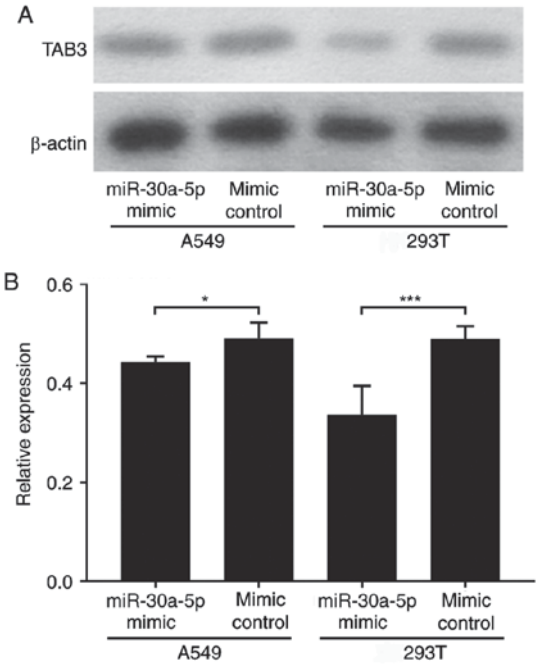

Figure 4. Western blotting analysis of TAB3 expression level in A549 and 293T cells transfected with miR-30a-5p mimic or vehicle. (A) Representative result of western blotting assay. (B) Densitometry analysis of western blotting assay. Each column represents five replicate experiments. *P<0.05. ***P<0.001. TAB3, TGF-β activated kinase 1/MAP3K7 binding protein 3; miR-30a-5p, microRNA-30a-5p.

(P<0.001). Together, these data suggested that TAB3 mRNA is a direct target of miR-30a-5p.

miR-30a-5p overexpression attenuates TGF-β1 stimulated α-SMA and fibronectin expression. Considering the potential association between miR-30a-5p, TAB3 and TGF-β signaling, a preliminary study was performed on the impact of miR-30a-5p overexpression on the mRNA expression level of TAB3, α-SMA and fibronectin in A549 cells, the last two of which have been suggested biomarkers of TGF-β1 induced fibrogenesis in lung epithelial cells (Fig. 5) (28-30). The results of the present study demonstrated that 5 ng/ml TGF-β1 treatment for 24 h increased TAB3, α-SMA and fibronectin expression, which was significantly decreased by artificial miR-30a-5p overexpression (P<0.05; Fig. 5A-C). Notably, it was demonstrated that TGF-β1 treatment could also significantly decrease the miR-30a-5p expression level in A549 cells with the mimic control but not in the cells transfected with the miR-30a-5p mimic, which implied that TGF-β1 signaling may inhibit miR-30a-5p expression in an indirect fashion (Fig. 5D).

Discussion

miRs are small non-coding RNAs that normally serve negative regulatory roles in different cellular events by inducing

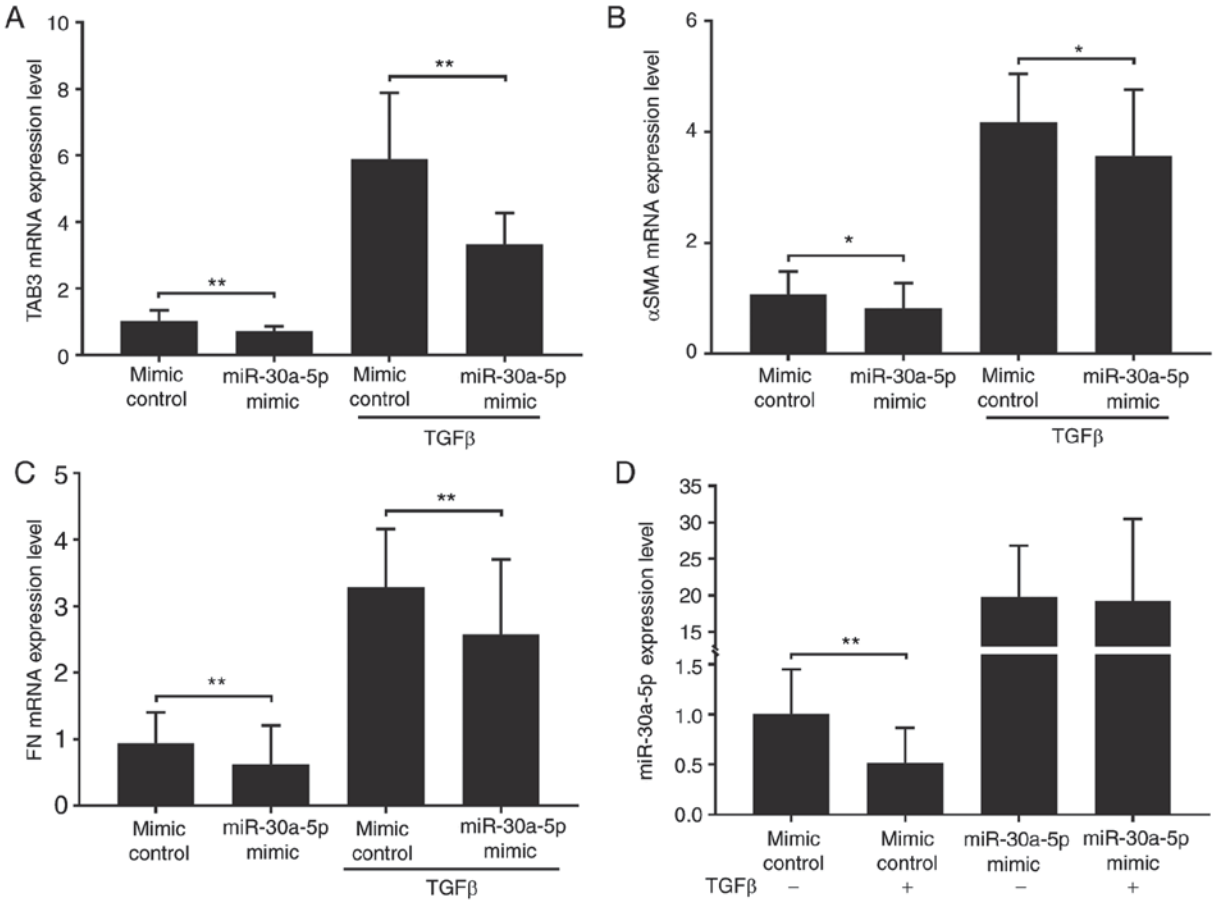

Figure 5. Influences of miR-30a-5p overexpression in A549 cells with or without TGF-β1 treatment (5 ng/ml for 24 h). miR-30a-5p mimic transfection decreased (A) TAB3, (B) α-SMA and (C) FN expression in A549 cells with or without TGF-β1 treatment. Each column represents five replicate experiments. (D) TGF-β1 treatment decreased miR-30a-5p expression in un-transfected A549 cells but not in that transfected with miR-30a-5p mimic. *P<0.05, **P<0.01. miR-30a-5p, microRNA-30a-5p; TGF-β, transforming growth factor-β; TAB3, transforming growth factor-β activated kinase 1/MAP3K7 binding protein 3; α-SMA, smooth muscle actin-α; FN, fibronectin.

post-transcriptional silencing of their target genes (31). The lungs of patients with are characterized by phenotypic alterations of fibroblasts and epithelial cells, due to alterations in gene expression.

The aim of the present study was to detect differentially expressed exosomal miRs in BALF from patients with IPF and healthy participants that may be involved in IPF pathogenesis or progression. miRs were extracted from exosomes in BALF from 30 patients with IPF and 16 healthy participants, and miR profiling was subsequently performed. Microarray and next-generation sequencing (NGS) are the two major high-throughput approaches for genome and transcriptome analysis. The former was chosen to analyze miR expression in the present study, as previous experiences demonstrated that NGS results could not be well replicated by qPCR, and it was hypothesized that this was due to the inevitable sample loss and subsequent proportional alteration in composition during sequencing library construction, which may distort the results. The microarray chip method, however, frequently yields a more accurate outcome because of its simpler process compared with NGS. The major advantage of NGS lies in identifying novel sequences or mutations; however, this was not within the scope of the present study.

Significant differences in miR expression profile were observed between the two groups. Among the 68 differentially expressed miRs, let-7d-5p, miR-103-3p, miR-27b-3p and miR-30a-5p were downregulated >2-fold in patients with IPF compared with the control, which is consistent with previous studies that these miRs may serve an important role in regulating IPF pathogenesis and IPF-associated carcinogenesis (20,32). For instance, according to a previous study (33), let-7d may target insulin-like growth factor 1 (IGF-1) and insulin-like growth factor receptor 2. The increased expression of IGF-1/2 in interstitial macrophages, cells and epithelial cells may stimulate collagen production (34,35). Downregulation of let-7d results in overexpression of IGF-1 and, therefore, accelerates fibrosis. Although certain miRs are downregulated in IPF, specific others are increased compared with the control, including miR-125b-5p, miR-128-3p, miR-21-5p, miR-100-5p, miR-140-3p and miR-374b-5p (36). The target genes of these increased miRs were also predicted using FunRich software (ver. 3) with an enrichment analysis of these predicted target genes, demonstrating that these genes are involved in the epidermal growth factor receptor, liver kinase B1 (LKB1), vascular endothelial growth factor, sphingosine 1-phosphate (S1P) and TNF-related apoptosis-inducing ligand (TRAIL) signaling network (data not shown), among which the LKB1,

S1P and TRAIL signaling network have been reported to serve negative roles in regulating fibrosis pathogenic processes in different tissues (37-39). These reports supported the veracity of the miRNA array assay results in the present study. The expression profiles of miRs in the lung of patients with pulmonary diseases have frequently been reported to be different to that of healthy lungs. In the present study, the differences in miR expression profiles between patients with IPF and healthy participants have the potential to serve as non-invasive or mini-invasive biomarkers for the screening and prognosis of IPF, discriminating early stage IPF patients from control subjects with high sensitivity and specificity. Using miRs from BALF exosome as biomarkers is more practical and reliable compared with using invasive lung tissue biopsies for IPF diagnosis.

Based on the analysis of the miR expression profile in the present study, miR-30a-5p was chosen as a research focus. The miR-30a-5p family has been reported to suppress cell migration and invasion, and have an anti-fibrotic role in liver fibrosis (40). The miR-30a-5p expression level in the 46 BALF samples from patients with IPF and healthy participants was measured by RT-qPCR. The results of the present study indicated that miR-30a-5p was downregulated by 71.8% in patients with IPF compared with the control. Furthermore, the microarray analysis indicated that the average expression level of miR-30a-5p in patients with IPF was >2-fold lower than that in healthy participants.

TAB3, which was selected for further investigation has been reported to be involved in multiple signaling events in IPF pathogenesis or progression, including chronic inflammatory responses and tissue remodeling, altered fibrosis and tissue repair, and immune responses. It was further hypothesized that miR-30a-5p inhibits TAB3 expression by binding to the 3'UTR region of TAB3 mRNA, and a dual-luciferase reporter assay was performed to confirm this association (41). Following co-transfection of 293T cells with the miR-30a-5p mimic and vectors encoding WT TAB3 3'UTR flanked by the firefly luciferase sequence, the luciferase activity was observed to be decrease by up to 46.3% compared with the negative controls; however, the miR-30a-5p mimic did not influence the firefly luciferase activity when MT TAB3 3'UTR use used. The direct interaction between the miR-30a-5p gene and TAB3 3'UTR indicates that miR-30a-5p overexpression may decrease TAB3 protein expression. As expected, when A549 and 293T cells were transfected with miR-30a-5p, a decrease in TAB3 protein expression level was observed compared with the control. These data suggested that TAB3 upregulation due to decreased miR-30a-5p expression may be an important factor in IPF progression.

As targeting the TGF-β1 signaling pathway is being considered as a strategy in IPF management (42,43), the impact of miR-30a-5p overexpression on the expression level of two TGF-β1 signaling-inducible fibrogenesis biomarkers, α-SMA and fibronectin (44,45) was determined in response to treatment of A549 cells with the potent fibrogenic cytokine, TGF-β1 (46,47). The results of the present study demonstrate that enforced miR-30a-5p expression inhibited TGF-β1-induced α-SMA and fibronectin upregulation, demonstrating a potential inhibitory role in IPF progression. Notably, the results of the present study also demonstrated that TGF-β1 treatment decreased the miR-30a-5p expression level in A549 cells, presumably in an indirect manner, which may be involved in the TGF-β1-stimulated fibrogenesis mechanism.

In conclusion the results of the present study suggested that decreased miR-30a-5p in BALF exosome may be a potential biomarker for IPF diagnosis, and artificially increasing miR-30a-5p level may be a potential strategy for IPF management.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

TJ and XH completed the experiments including the collection of exosomes and extraction of miRNAs. ZDL and LZ performed the experiments including cell culture, RT-qPCR and western blotting. HL and ZHL participated in the Dual-luciferase reporter assay. BL and LM participated in statistical analysis and bioinformatics analysis. BL wrote and revised the manuscript. All authors read and approved the final the manuscript.

Ethics approval and consent to participate

The present study was approved by the institutional review board of the People's Hospital of Zhengzhou University (Zhengzhou, China). Informed consent was obtained from each participant in written form prior to undergoing bronchoalveolar lavage fluid collection following the Chinese medical association alveolar lavage operation instruction.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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