MiR-27a-3p downregulation contributes to the development of occlusive bronchiolitis

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Abstract

The only effective clinical treatment for many end-stage lung diseases is lung transplantation. However, chronic rejection of transplanted lung affects the long-term efficacy of lung transplantation to a large extent, thereby limiting the clinical application of lung transplantation. Occlusive bronchiolitis (OB) is a major cause of chronic functional loss of the transplanted lung. However, the OB pathogenesis remains unclear. Therefore, studying the OB pathogenesis and finding effective intervention methods are highly important. This study analyzed changes in the expression profile of microRNAs and transcription factors in mice with OB after orthotopic tracheal transplantation. miR-27a-3p was upregulated in lung tissue 20 days after transplantation. Transcription factor microarray analysis revealed that Smad3 was significantly downregulated. A miRNA–mRNA interaction network was constructed, and specific regulatory effects of miR-27a-3p on Smad3 were found. Smad3 was strongly associated with tumorigenesis and organ fibrosis. Compared with the control group, miR-27a-3p inhibited the epithelial–mesenchymal transformation (EMT) of lung epithelial cells. In addition, miR-27a-3p inhibition promoted the invasion and migration of lung epithelial cells. Dual luciferase reporter gene assay showed that miR-27a-3p can regulate the promoter activity of Smad3. MiR-27a-3p also inhibited the expression of inflammatory cytokines. Western blot results showed that miR-27a-3p can upregulate the E-cadherin expression and downregulate the expression of vimentin, fibronectin, and α -SMA. By studying the OB pathogenesis, we found that inhibition or alteration of the occurrence of EMT may reduce the proportion of chronic rejection of lung transplantation. MiR-27a-3p may also be developed as a new drug for the OB therapy. This finding will provide many possibilities for OB treatment and improve the prognosis of patients with OB.

Keywords Occlusive bronchiolitis · Lung transplantation · miR-27a-3p · Epithelial-mesenchymal transformation

Introduction

Occlusive bronchiolitis (bronchiolitis obliterans, OB) is a type of limited bronchial inflammation caused by chronic airflow syndrome (Dudek et al. 2003). Conventional bronchial diastole therapy is ineffective. Thus, the patient's physical health and quality of life are affected. OB is a common complication

of lung transplantation (Estenne and Hertz 2002). OB is characterized by atypical clinical manifestations, unclear pathogenesis, insensitive diagnostic methods, and uncertain therapeutic effects, resulting in the poor prognosis and high fatality rate of patients (Bergeron and Cheng 2017). To date, OB pathogenesis remains unclear. Early treatment can block the disease progression. However, the curative effect of late treatment is poor because of irreversible fibrosis changes and airway obstruction. Therefore, studying the OB pathogenesis and finding effective interventions are highly important.

miRNAs can help diagnose and treat OB (Mirzaei et al. 2018). Therapeutic strategies based on miRNA molecular level mechanism are significant in preventing and treating complications after organ transplantation and in improving long-term survival rate after transplantation (Shan et al. 2011). miRNA chip technology was applied to detect and analyze the expression characteristics of miRNAs in the graft and control tissues to search for differentially expressed miRNAs in the pathological

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pathogenesis of OB after lung transplantation. Predicting the target genes of miRNAs and exploring the OB pathogenesis after lung transplantation at the molecular level are important to elucidate OB's underlying mechanism.

Studies have shown that epithelial–mesenchymal transformation (EMT) is involved in multiple airway remodeling diseases (Chen et al. 2016; Hackett 2012). Therefore, EMT may play an important role in OB after lung transplantation. EMT refers to the fact that normal epithelial cells lose their original characteristics and instead display some characteristics of mesenchymal cells (Nieto et al. 2016). From the perspective of molecular surface markers, the expression of E-cadherin protein, a marker of epithelial cells, decreases or not during EMT. On the contrary, the expression of mesenchymal markers, such as vimentin and SMA, is upregulated, and their biological functions change (Brabletz et al. 2018).

TGF- β plays an important role in EMT. TGF- β , produced primarily by activated inflammatory cells, including macrophages, reaches the site of damage, binds to the TGF2 receptor (TGFBR2), and then forms a complex structure with the TGFBR1 receptor (TGFBR1) (Weiss and Attisano 2013). This complex initiates a signaling cascade; it activates Smad2 and Smad3 complexes, and subsequently, Smad4 (Nakao et al. 1997). The activated complex Smads is transported to the nucleus and induces gene transcription. Therefore, Smad protein plays an important role in EMT and OB.

In the present study, we compared the changes in miRNAs and transcription factors after tracheal transplantation in mice on different days. miR-27a-3p was significantly upregulated after tracheal transplantation. The specific regulatory effects of miR-27a-3p on Smad3 were determined through the construction of a miRNA–mRNA interaction network. In addition, the results of transcriptional factor microarray analysis after transplantation showed that Smad3 was significantly downregulated. The outcomes of cell-level experiments showed that miR-27a-3p can inhibit the EMT of lung epithelial cells, and miR-27a-3p inhibition can promote the invasion and migration of lung epithelial cells. MiR-27a-3p can upregulate the E-cadherin expression and downregulate the expression of vimentin, fibronectin, and α -SMA. Activation of the molecular pathway related to EMT is involved in the occurrence and outcome of OB after tracheal transplantation. Moreover, inhibition or alteration of the EMT occurrence may reduce the proportion of chronic rejection of tracheal transplantation. Therefore, miR-27a-3p is a potential drug target.

Materials and methods

Transcription factor array and microarray analysis

As we described previously (Dong et al. 2015), female C57BL/6 mice weighing 20–24 g were used. Each donor

tracheal graft was orthotopically implanted. Eight pairs of animals in each group were transplanted. After 20 days of allogeneic or syngeneic transplantation, the trachea grafts were obtained. The nuclear proteins were extracted from some of the obtained tissues for further transcription factor array following the manufacturer's instructions, as we described previously. The total RNA was extracted from the obtained tissues for further microarray analysis following the manufacturer's instructions. Differentially expressed genes were screened based on a $|\text{fold change}| \geq 1.5$.

Cell culture

Airway epithelial cells, LLC cells, and NIH-3T3 cells were cultured in 1640 medium supplemented with 10% fetal bovine serum (Sijiqing, Hangzhou, China) and then incubated at 37 °C in a humidified incubator with 5% CO₂.

Cell viability assay

Approximately 2000 cells were seeded in each well of 96-well plate and cultured in a 37 °C, 5% CO₂ incubator. Two groups were designed with three copies of each group. Cell viability was detected at 0, 1, 3, 5, and 7 days. At the test point, each well was digested and counted.

Wound healing assay

LLC cells were seeded in 6-well plates and then incubated for 24 h. Then, a linear wound was created by dragging a 1-mL pipette tip through the monolayer prior to transfection. Cellular debris was removed by gently washing with culture medium followed by transfection. In addition, the cells were allowed to migrate for a further 48 h. Healing was dynamically photographed after the wound was introduced using a microscope (Nikon, Japan). Migration distance was measured from images (three fields) taken at each indicated time point. The gap size was analyzed using Image-Pro Plus 6.0 software. The residual gap between the migrating cells from the opposing wound edge was expressed as a ratio to the initial gap size.

Invasion assay

The capabilities of cell invasion were assessed by Transwell chamber assay. LLC cells were harvested and resuspended in serum-free medium at a concentration of 1×10^5 cells/mL, and 200 μ L was added to the top chamber. The chambers were incubated for 24 h at 37 °C in an incubator with 5% CO₂. The noninvasive cells on the upper surface of the membrane were removed by wiping with cotton-tipped swabs. An 8- μ m pore-size membrane was fixed and stained, and the population of the invaded cells was counted by light microscopy. All experiments were performed in triplicate.

Reporter gene assay

The recombinant luciferase reporter plasmids contained the potential miRNA binding site sequences of the Smad3 3'-untranslated region (UTR). MiRNA mimics or inhibitor was co-transfected into cells. After 48 h of transfection, gaussia luciferase and secreted alkaline phosphatase were measured using the dual-luciferase reporter gene assay kit (GeneCopoeia, China). Renilla luciferase acted as an internal control to normalize the transfection efficiency.

Enzyme-linked immunosorbent assay

The interleukin (IL)-1 β levels in the cell culture medium with different treatments were quantified using sand with ELISA kit, which comprised 96-well microplates coated with an antibody against IL-1 β (R&D Systems, USA). The kits were used in accordance with the manufacturer's instructions. The staining intensity was determined spectrophotometrically at 450 nm by using a Multiskan spectrophotometer (Thermo, USA).

Western blot analysis

The treated cells were lysed, and total cellular protein concentrations were measured by BCA Protein Assay Kit (Thermo, USA). Equal amounts of protein were resolved by electrophoresis through 10% Tris-glycine sodium dodecyl sulfate polyacrylamide gel and then electrotransferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 1% (wt/vol) bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h and then incubated overnight at 4 °C with anti-Smad3 antibody (1:1000), anti-p-Smad3 antibody (1:1000), anti- α -SMA antibody (1:1000), anti-fibronectin antibody (1:1000), anti-E-cadherin antibody (1:1000), anti-vimentin antibody (1:1000), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:1000). All the primary antibodies were bought from Affinity Bioreagents, USA. After washing thrice with TBST, the membrane was incubated for 1 h with 1:10,000 dilutions of a horseradish peroxidase-conjugated IgG antibody (Santa Cruz Biotechnology, USA) in TBST containing 1% BSA. After three washes with TBST, the bands were detected using a Molecular Imager ChemiDoc XRS system (Bio-Rad, USA). As needed, the membrane was stripped in a stripping buffer (CW BIO, China) and then reblotted with an antibody specific to GAPDH as the loading control. The band intensities were semiquantified by densitometry by using Quantity-One software.

Statistical data analysis

Statistical analyses were performed using Graph Pad Prism software. Gap closure was analyzed by one-way ANOVA followed by pairwise comparison with the control groups by paired *t* test. Statistical significance was considered at $P < 0.05$.

Results

Selection of key candidate miRNA and its target gene during OB

The miRNA expression profile data were used to investigate the candidate miRNA. Twenty days after tracheal transplantation, the miRNA expression profile differed from the OB group. Then, the upregulated microRNA of mmu-miR-27a-3p was selected for further analysis (Fig. 1a). Gene expression profiling analysis was also carried out, and a distinct pattern of mRNA expression profile was determined after tracheal transplantation (Fig. 1b). Using the TargetScan software program (Lewis et al. 2003), we found 47 candidate target genes of mmu-miR-27a-3p (Fig. 1c). To further investigate the regulation mechanism of mmu-miR-27a-3p in OB formation, we screened the downregulated transcription factors after tracheal transplantation (Fig. 1d, yellow circle). By Venn analysis, we identified Smad3 in both target genes of mmu-miR-27a-3p and downregulated transcription factors (Fig. 1b and d).

MiR-27a-3p affects OB formation, OB-induced fibrosis, and OB-induced carcinogenesis

The relationship between Smad3 and diseases was analyzed to further confirm the function of miR-27a-3p/Smad3 on OB formation (Fig. 2). Smad3 upregulation correlated with inflammation, fibrosis, neoplasms, and other diseases. Among these, neoplasms and fibrosis ranked in front due to the number of related literatures. miR-27a-3p/Smad3 mediates the OB occurrence and causes further malignant progression.

Mouse airway epithelial cells, NIH-3T3, and LLC cells were then used to explain the regulation role of miR-27a-3p in OB formation, OB-induced fibrosis, and OB-induced carcinogenesis, separately. Transfection of miR-27a-3p inhibitors (anti-miR) significantly increased the proliferation of airway epithelial cells (Fig. 3a). Meanwhile, miR-27a-3p mimics significantly inhibited the proliferations of NIH-3T3 and LLC (Fig. 3b and c). miR-27a-3p upregulation by transfection of miRNA mimics significantly inhibited the migration of LLC cells (Fig. 3d). In addition, the number of LLC cells passing through the transwell chamber was significantly inhibited by miR-27a-3p (Fig. 3e).

a

Day 1 Day 20

mmu-miR-27a-3p

b

Day 1 Day 20

Smad3

c

mmu-miR-27a-3p-Target genes regulatory network

d

Target genes of mmu-miR-27a-3p

Down regulated transcripts

Smad3

46 (16, 20)

1 (0, 4)

237 (83, 56)

The relationship between Smad3 and diseases

Disease	Number of related literature
Neoplasms	165
Neoplasm Metastasis	45
Fibrosis	45
Breast Neoplasms	35
Growth Disorders	33
Focal Nodular Hyperplasia	20
Osteoarthritis	18
Inflammation	18
Kidney Diseases	15
Prostatic Neoplasms	15
Colorectal Neoplasms	15
Carcinoma, Hepatocellular	12
Aneurysm	12
Lung Neoplasms	12
Carcinogenesis	12
Carcinoma	10
Adenocarcinoma	10
Genetic Diseases, Inborn	8
Diabetes Mellitus	8
Disease	8

Fig. 3 MiR-27a-3p affects cell proliferation, migration, and invasion during OB formation and progression. **a** Blocking miR-27a-3p significantly promoted the proliferation of mouse airway epithelial cells. **b** Upregulated miR-27a-3p significantly inhibited the proliferation of mouse embryo fibroblast cells of NIH-3T3. **c** Upregulated miR-27a-3p significantly inhibited the proliferation of mouse lung cancer cells of LLC. **d** Upregulated miR-27a-3p significantly inhibited the migration of LLC cells. **e** Upregulated miR-27a-3p significantly inhibited the invasion of LLC cells. (mean $\pm$ SD; $n = 3$ in triplicate; ** $P < 0.01$)

MiR-27a-3p inhibits OB progression through the EMT pathway

Addition of the miR-27a-3p inhibitor significantly upregulated the transcriptional Smad3 expression (Fig. 4a). We speculated that miR-27a-3p/Smad3 is related to the EMT during OB progression. To test this hypothesis, we conducted several experiments. Western blot analysis showed that miR-27a-3p inhibitor significantly promoted the Smad3 expression and its phosphorylation, promoted the expressions of mesenchymal markers of α -SMA, fibronectin, and vimentin, and inhibited the epithelial marker of E-cadherin in mouse airway epithelial cells (Fig. 4b). Addition of the miR-27a-3p inhibitor significantly promoted the secreted content of inflammation factor IL-1 β in mouse airway epithelial cells (Fig. 4c). In the mouse fibroblast cells of NIH-3T3 and mouse cancer cells of LLC, transfection of miR-27a-3p mimic significantly inhibited the transcriptional activity of Smad3 (Fig. 4d) and inhibited the IL-1 β secretion (Fig. 4g). miR-27a-3p significantly inhibited the expression of Smad3, p-Smad3, α -SMA, fibronectin, and

vimentin and significantly promoted the expression of the epithelial marker of E-cadherin in NIH-3T3 cells (Fig. 4e). Similarly, miR-27a-3p transfection significantly inhibited the expression of Smad3 and p-Smad3 and inhibited the EMT progress in LLC cells (Fig. 4f).

Discussion

As a form of chronic allograft rejection, OB is a serious threat to long-term survival after lung transplantation and affects up to 50–60% of patients who survive 5 years after surgery (Husain and Garrity 2016). Sustained inflammatory reactions can often lead to fibrosis or even carcinogenesis of organs (Liu et al. 2018; Rieder et al. 2010). Activated fibroblasts and myofibroblasts play an important role in fibrosis or carcinogenesis by causing excessive deposition of extracellular matrix proteins, such as fibronectin, collagen, and laminin (Phan 2002; Wang et al. 2017; Willis et al. 2006). Transformation of epithelial cells into fibroblasts through EMT progress is one of

Fig. 4 MiR-27a-3p inhibits OB progression through the EMT pathway. **a** Smad3 transcription was significantly promoted by adding the miR-27a-3p inhibitor in mouse airway epithelial cells. **b** The influence of miR-27a-3p on protein expression levels of p-Smad3, Smad3, and EMT markers in mouse airway epithelial cells was examined using Western blot assay. **c** Adding the miR-27a-3p inhibitor significantly promoted the IL-1β content in the medium of mouse airway epithelial cells. **d** Transfection of miR-27a-3p mimic significantly inhibited Smad3 transcription in NIH-

3T3 and LLC cells. **e** Influence of miR-27a-3p on the protein expression levels of p-Smad3, Smad3, and EMT markers in NIH-3T3 cells was examined using Western blot assay. **f** Transfection of miR-27a-3p mimic significantly inhibited the IL-1β content in the medium of NIH-3T3 and LLC cells. **g** The influence of miR-27a-3p on protein expression levels of p-Smad3, Smad3, and EMT markers in LLC cells was examined using Western blot assay (mean ± SD; $n = 3$ in triplicate; $^{**}P < 0.01$)

the original major explanations of fibroblasts (Chapman 2011). EMT was considered a potential factor of fibrotic diseases in various organs in the past few years (Nieto et al. 2016). In vitro studies reported that EMT can trigger human bronchial epithelial cells, primitive alveolar epithelial cells, and pulmonary-derived epithelial carcinoma. Whether the OB occurrence and further pathological changes in the organ are related to EMT needs further explanation.

MiRNAs are small noncoding RNAs that recognize and bind to partially complementary sites in the 3'-UTR of the target genes, thereby inducing cleavage of the message or inhibiting translation. MiRNAs are involved in multiple diseases by regulating gene expression (Taft et al. 2010). In the present study, miR-27a-3p was screened to be a differentially

expressed miRNA after tracheal transplantation. We speculated that miR-27a-3p affects OB progression. Trachea and lung transplants tend to produce the same inflammatory response in the lung. miR-27a-3p could inhibit the proliferation, invasion, and migration of lung cancer cells. By Venn analysis, Smad3 was screened in transcription factors and target genes of miR-27a-3p between the OB group and the transplantation treatment group. Smad3, which is related to inflammation, fibrosis, and carcinogenesis, was identified in the pathway for transducing TGF-β signals from the cell membrane to the nucleus (Gauldie et al. 2007; Skeen et al. 2012; Yoshida et al. 2014). Considering that fibrosis and carcinogenesis can often be transformed from inflammation, we hypothesized that the decreasing miR-27a-3p expression

Fig. 5 Proposed regulatory mechanism of miR-27a-3p during OB progression

increases the Smad3 level, consequently leading to OB. In addition, OB might convert to pulmonary fibrosis or cancer mediated by miR-27a-3p (Fig. 5).

We examined the EMT downstream of Smad3, and miR-27a-3p regulated the occurrence and further deterioration of OB through Smad3-mediated EMT. The miR-27a-3p/Smad3 pathway plays an important role in the OB pathogenesis and could be used as a potential target for clinical treatment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Bergeron A, Cheng GS (2017) Bronchiolitis obliterans syndrome and other late pulmonary complications after allogeneic hematopoietic stem cell transplantation. *Clin Chest Med* 38:607–621
- Brabletz T, Kalluri R, Nieto MA, Weinberg RA (2018) EMT in cancer. *Nat Rev Cancer* 18:128–134
- Chapman HA (2011) Epithelial-mesenchymal interactions in pulmonary fibrosis. *Annu Rev Physiol* 73:413–435
- Chen YC, Statt S, Wu R, Chang HT, Liao JW, Wang CN, Shyu WC, Lee CC (2016) High mobility group box 1-induced epithelial mesenchymal transition in human airway epithelial cells. *Sci Rep* 6:18815
- Dong M, Wang X, Zhao HL, Chen XL, Yuan JH, Guo JY, Li KQ, Li G (2015) Integrated analysis of transcription factor, microRNA and lncRNA in an animal model of obliterative bronchiolitis. *Int J Clin Exp Pathol* 8:7050–7058
- Dudek AZ, Mahaseth H, DeFor TE, Weisdorf DJ (2003) Bronchiolitis obliterans in chronic graft-versus-host disease: analysis of risk factors and treatment outcomes. *Biol Blood Marrow Transpl: J Am Soc Blood Marrow Transpl* 9:657–666
- Estenne M, Hertz MI (2002) Bronchiolitis obliterans after human lung transplantation. *Am J Respir Crit Care Med* 166:440–444
- Gauldie J, Bonniaud P, Sime P, Ask K, Kolb M (2007) TGF-beta, Smad3 and the process of progressive fibrosis. *Biochem Soc Trans* 35:661–664

- Hackett TL (2012) Epithelial-mesenchymal transition in the pathophysiology of airway remodelling in asthma. *Curr Opin Allergy Clin Immunol* 12:53–59
- Husain AN, Garrity ER (2016) Lung transplantation: the state of the airways. *Arch Pathol Lab Med* 140:241–244
- Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. *Cell* 115:787–798
- Liu Y, Zhao J, Zhao Y, Zong S, Tian Y, Chen S, Li M, Liu H, Zhang Q, Jing X et al (2018) Therapeutic effects of lentinan on inflammatory bowel disease and colitis-associated cancer. *J Cell Mol Med*
- Mirzaei H, Momeni F, Saadatpour L, Sahebkar A, Goodarzi M, Masoudifar A, Kouhpayeh S, Salehi H, Mirzaei HR, Jaafari MR (2018) MicroRNA: relevance to stroke diagnosis, prognosis, and therapy. *J Cell Physiol* 233:856–865
- Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K, ten Dijke P (1997) TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. *EMBO J* 16:5353–5362
- Nieto MA, Huang RY, Jackson RA, Thiery JP (2016) EMT: 2016. *Cell* 166:21–45
- Phan SH (2002) The myofibroblast in pulmonary fibrosis. *Chest* 122: 286S–289S
- Rieder F, Biancani P, Hammett K, Yeran L, Falk GW (2010) Inflammatory mediators in gastroesophageal reflux disease: impact on esophageal motility, fibrosis, and carcinogenesis. *Am J Physiol Gastrointest Liver Physiol* 298:G571–G581
- Shan J, Feng L, Luo L, Wu W, Li C, Li S, Li Y (2011) MicroRNAs: potential biomarker in organ transplantation. *Transpl Immunol* 24: 210–215
- Skeen VR, Paterson I, Paraskeva C, Williams AC (2012) TGF-beta1 signalling, connecting aberrant inflammation and colorectal tumorigenesis. *Curr Pharm Des* 18:3874–3888
- Taft RJ, Pang KC, Mercer TR, Dinger M, Mattick JS (2010) Non-coding RNAs: regulators of disease. *J Pathol* 220:126–139
- Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, Gong Z, Zhang S, Zhou J, Cao K, Li X, Xiong W, Li G, Zeng Z, Guo C (2017) Role of tumor microenvironment in tumorigenesis. *J Cancer* 8:761–773
- Weiss A, Attisano L (2013) The TGFbeta superfamily signaling pathway. *Wiley Interdiscip Rev Dev Biol* 2:47–63
- Willis BC, duBois RM, Borok Z (2006) Epithelial origin of myofibroblasts during fibrosis in the lung. *Proc Am Thorac Soc* 3: 377–382
- Yoshida K, Murata M, Yamaguchi T, Matsuzaki K (2014) TGF-beta/Smad signaling during hepatic fibro-carcinogenesis (review). *Int J Oncol* 45:1363–1371

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