Mir-29b mediates the regulation of Nrf2 on airway epithelial remodeling and Th1/Th2 differentiation in COPD rats

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1. Introduction

In patients with chronic obstructive pulmonary disease (COPD), less air flows in and out because of airway restriction (Croft, 2018). Worldwide, COPD is highly prevalent and is a leading cause of disability and mortality (Senior, 2000; Croxton et al., 2003). COPD develops because of multiple reasons, such as thickened and inflamed airways, clogged airways, less elastic airways and air sacs (Yao and Rahman, 2011). When the body encounters external stimulation, CD4+ T helper lymphocytes (Th cells) are activated to develop into a subset of effector cells, including Th1 and Th2 cells (Luckheeram, 2012). Th1 cells mainly mediate cellular immune response and promote the killing effect of cytotoxic T cells; the main function of Th2 cells is to stimulate B cells to produce antibodies, which are related to humoral immunity. The imbalance of Th1/Th2 cell differentiation makes the immune response imbalanced in the body, resulting in persistent inflammatory reactions, airway remodeling, and emphysema (Singh, 2010). Interleukin (IL)-12 and interferon (IFN)-γ form positive feedback, which promotes Th0 to differentiate into Th1; IL-4 promotes Th0 to differentiate into Th2. When COPD occurs, the Th1/Th2 balance is broken, showing Th1 hyperactivity (Biswal, 2012).

Nuclear factor erythroid 2-related factor 2 (Nrf2), as a transcription factor, can also activate the transcription of miR-29b (Kurinna, et al., 2014). Dysregulation of miRNAs is frequently discovered during development of diseases. MiR-29b has been reported to decrease in many diseases.
Detection of plasma samples from COPD patients revealed a decrease in the expression of miR-29b (Soeda et al., 2013). Researches in a variety of malignant tumors also indicated that miR-29b plays a beneficial role against cancers and is associated with survival rate of patients (Yan et al., 2015). MiR-29b is a critical regulator of pulmonary fibrosis in the process of epithelial mesenchymal transition, the secretion of inflammatory factor, collagen deposition, cytoskeleton changes, and gene methylation (Cushing et al., 2011). Therefore, the regulation role of Nrf2 on COPD may be mediated by miR-29b.

Currently the extent and manner of miR-29b regulating COPD occurrence is still not clear, such as the expression level in lung tissues, the regulation by Nrf2 level, the effect on Th1/Th2 differentiation, and the effect on lung epithelial cells. Therefore, we investigated whether miR-29b mediates the regulation of Nrf2 on Th1/Th2 differentiation and airway epithelial remodeling in COPD rats.

2. Materials and methods

2.1. Induction of COPD in rats

Sixteen male SD rats (6–8 weeks, purchased from SLAC, Shang-hai, China) were used. The animal experiment protocols were approved and in compliance with the guidelines for animal research of Cangzhou Central Hospital. The rats were randomly divided into the control group (i.e. normal group) and the model group (i.e. COPD group). To induce COPS, the rats were challenged with intratracheal lipopolysaccharide (LPS, 100 μg dissolved in 0.5 ml of PBS) as described in reference (Zhang et al., 2003). Control animals were challenged with 0.5 ml of PBS. At 3 days after challenge, the rats were sacrificed to collect lung tissues and whole blood samples. Plasma were separated and stored at −80 °C for subsequent assays. For each rat, left lung tissue was fixed and used for histopathological tests; right lung tissue was stored in 4% paraformaldehyde for histopathological tests; right lung tissue was stored in −80 °C and used for RNA and protein extraction.

2.2. Histopathological tests

Thin lung sections underwent hematoxylin and eosin (HE) staining using HE staining kit (Beyotime Biotechnology, Shanghai, China). Lung sections were also subjected to histoimmunochemical analysis to detect expression of Nrf2 and matrix metalloproteinase 2 (MMP2) using SABC-AP kit (for rabbit-labeled IgG, Boster) following the product instruction. Primary rabbit polyclonal antibodies against Nrf2 (1:1000, ab137550) and MMP2 (1:1000, ab37150) were purchased from Abcam (Cambridge, UK). Secondary goat IgG antibody (1:1000) was offered by the kit.

2.3. Cell culture

A549 cells (ATCC, Manassas, USA), derived from human lung cancer, were used as a model of lung epithelial cells. Cells were cultured using DMEM + 10% FBS (all from Thermo Fisher, Carlsbad, USA).

2.4. RT-PCR

To prepare RNA template, lung tissues (grinded in liquid nitrogen) or cells were collected and extracted with total RNA using miRNeasy 96 kit (QIAGEN, Hilden, Germany). Following isolation, RNA integrity was determined with agarose gel electrophoresis. The PrimerScript RT kit was used for cDNA synthesis and SYBR Premix Ex Taq (both by Takara Biomedical Technology, Beijing, China) were used to for mRNA quantification. Amplification reactions were performed on LightCycler480 System (Roche, Mannheim, Germany). U6 or β-actin was selected as normalization control for miR-29b or Nrf2, respectively, and ΔΔCt method was applied for calculating (Livak and Schmittgen, 2001). The sequences of primers validated in this study were summarized in Table 1.

2.5. Western blot

Protein was extracted from cells and prepared with RIPA buffer supplemented with a protease inhibitor cocktail (Beyotime), then separated by SDS-PAGE (10% resolving gel) to PVDF membranes. The membranes were subjected to non-fat milk blocking, incubation with primary antibodies, and incubation with secondary antibody. The primary antibodies were used against rabbit polyclonal antibody to Nrf2 (1:2000, ab137550), rabbit polyclonal antibody to MMP2 (1:2000, ab37150), and rabbit polyclonal antibody to β-actin (1:2000, ab8227). All the antibodies were purchased from Abcam (Cambridge, UK).

2.6. Detection of cytokines

IL-2 and IFN-γ were detected as Th1 cytokines; IL-4 and IL-10 were detected as Th2 cytokines. The secretion of these cytokines was quantified in peripheral blood using appropriate ELISA kits for rat (EK0399, EK0406, EK0418, and EK0374). Concentrations of those cytokines were determined by absorbance at 450 nm.

2.7. Flow cytometry

T cells in peripheral blood were stained with FITC-CD3 fluorescence antibody (Biolegend, San Diego, USA) and gated on CD3+ population by flow cytometry (Beckman-Coulter Inc., Brea, USA). The proportion of Th1 cells (INF-γ+) and Th2 cells (IL-4+) in T cells was determined by staining with PE-INF-γ antibody (Biolegend), PE-IL-4 antibody (Biolegend), and PE-Cy5 anti-rat CD4 (BD Biosciences, Franklin Lakes, USA).

2.8. Transfection

To block Nrf2 expression, the siRNA constructs: 5'-AAUGAGUU CACUGUCAACUGGUUCCG-3' and 5'-CCACAGUUGACAGUG AACUCAU-3'. To modulate miR-29b expression, A549 cells were transfected with miR-29b mimic (5'-UAGACCAUUGAAUACAGU GUU-3' and 5'-CACUAGUUUUAUUGGUCGUA-3') or miR-29b inhibitor (5'-AACACUGAUUUUCUUUGGCGUUA-3'). Mocks sequences were: 5'-UUCUCGAACUGUGUCAGCUTT-3' and 5'-ACGU...
GACAGUUCGGAGAATT-3'. All the interference oligonucleotides were synthesized by Gene Pharma (Shanghai, China). The Nrf2 plasmids were transfected by using Lipofectamine 2000 reagent (Thermo Fisher). The blocking effect was tested by RT-PCR.

2.9. Cell proliferation

A549 cells were collected and reseeded in 96-well plates (1000 cells/well). The growth curve was measured by CCK8 method. CCK-8 reagent (Beyotime) was added with reference volume of the instruction, and absorbance at 450 nm was measured to calculate cell density.

2.10. Cell migration

A549 cells were plated in 6-well plates to form confluent monolayer. A linear scratch wound was made on each monolayer by a pipette tip. Floating cells were removed by washes with culture medium. The width of injury line was recorded 24 h after wound generation.

2.11. Statistical analysis

All experimental data were documented, classified, and analyzed by SPSS software version 16.0 and Excel 2007. Independent

![Fig. 1. Expression of Nrf2, miR-29b, and MMP2 were altered in COPD rats. (A), HE staining of lung tissue sections (magnification × 400); (B), relative expression of Nrf2 and miR-29b detected by RT-PCR; (C), expression of Nrf2 and MMP2 in lung tissues detected by Western blot; (D), expression of Nrf2 and MMP2 in lung tissues detected by immunohistochemistry (magnification × 200). *P<0.05 compared with normal control.](image-url)
sample were compared by t-test (two groups) or one-way ANOVA (multiple groups) with LSD correction. $P$ values less than 0.05 was defines as the level of significance.

3. Results

3.1. LPS-induced COPD leads to decreased level of miR-29b in lung tissues of rats

Firstly, we tested whether miR-29b expression was altered in COPD rats. As shown by HE staining (Fig. 1A), inflammation occurred around the airway, perivascular, and intertissue gaps in the lungs of COPD rats. Expression of Nrf2 significantly decreased in the lung tissues of COPD rats (Fig. 1B–D; $P < 0.05$). The expression trend of miR-29b in lung tissues and peripheral blood was consistent with that of Nrf2 (Fig. 1B; $P < 0.05$). MMP2 could be directly regulated by miR-29b and we also tested its expression (Wang et al., 2015). Immunohistochemical assay and Western blot showed that both MMP2 were up-regulated in lung tissues of COPD rats (Fig. 1C and D; $P < 0.05$). Furthermore, the analysis above showed that miR-29b/MMP2 was under regulation of Nrf2 expression.

3.2. Th1/Th2 balance was broken in peripheral blood of COPD rats

Next, we investigated whether Th1/Th2 balance was influenced in COPD rats. As shown by ELISA tests, the expression of the Th1 and Th2 significantly have a significantly higher level in peripheral blood samples of COPD rats (Fig. 2A), compared with normal rats. Ratio of Th1 cells and ratio of Th2 cells were elevated in peripheral blood (Fig. 2B and C). In addition, the ratio of Th1/Th2 was also increased two folds in COPD rats (Fig. 2D). The results indicated that inflammation reactions were triggered during COPD and Th1/Th2 balance was broken.

3.3. MiR-29b mediates the effect of Nrf2 on remodeling of airway epithelial cells

To further confirm that miR-29b expression was under regulation of Nrf2 level, A549 cells were used as airway epithelial cell model and transfected with Nrf2 siRNA. By blocking Nrf2 expression, miR-29b expression was inhibited accordingly (Fig. 3A). Meanwhile, MMP2 expression was increased (Fig. 3A and B). Because MMP2 is a promoter during remodeling of epithelial cells, we also tested the change of cellular proliferation and migration. MTT assay showed that cellular proliferation was promoted by...
Fig. 3. MiR-29b mediated the effect of Nrf2 on remodeling of airway epithelial cells. (A), relative expression of Nrf2 mRNA, miR-29b, and MMP2 mRNA in different transfectants of A549 cells; (B), protein levels of Nrf2 and MMP2 in different transfectants of A549 cells; (C), cell viabilities of different transfectants detected by MTT method; (D), migration abilities of different transfectants detected by wound healing assay. *$P < 0.05$ compared with normal control; **$P < 0.01$ compared with normal control.
blocking Nrf2 expression (Fig. 3C). Cell migration ability was enhanced when suppressing Nrf2 and miR-29b expression (Fig. 3D). The results suggested that decreased expression of Nrf2 promoted MMP2 expression and epithelial remodeling.

So far the results suggested that the change of MMP2 expression might be due to miR-29b regulation. To verify this, we monitored miR-29b expression in A549 cells by transfection. MiR-29b mimic inhibited MMP2 expression and restricted cellular proliferation and migration; miR-29b inhibitor yielded similar effect as Nrf2 siRNA on MMP2 expression and cell remodeling (Fig. 3). Alteration of miR-29b level did not change the expression of Nrf2 (Fig. 3A). The results suggested that miR-29b was a down-stream factor of Nrf2 regulation and mediated the effect of Nrf2 on MMP2 expression and epithelial cell remodeling.

4. Discussion

COPD is characterized by pulmonary emphysema and inflammation. In this study, exposure to LPS had a negative effect on the production of Nrf2 and miR-29b in the lung tissues of rat models. Sequentially, Th1/Th2 differentiation lost balance and led to substantial accumulation of inflammatory cytokines in peripheral blood of the rats. Blocking Nrf2 expression in A549 cells promoted cellular proliferation and migration, which could also be observed by inhibiting miR-29b expression. Blocking miR-29b expression did not affect Nrf2 expression. We thus conclude that miR-29b mediated the influence of Nrf2 on Th cell differentiation and airway remodeling during COPD.

Nrf2 is an emerging regulator of oxidative stress. Nrf2 mediated antioxidative activity is an important cell defense mechanism. Nrf2 regulation pathway widely participates in the development of COPD (Ma, 2013). Many studies have also suggested that improving Nrf2 activity has beneficial effects against oxidative lung injury (Reddy et al., 2009; Guan et al., 2013; Sun et al., 2018; Cho et al., 2002). Current researches have shown that Nrf2 can sense oxidants, dissociate from Keap1-Nrf2 complex, translocate into nucleus, recognize the AREs (antioxidant response elements) of target genes, and initiate transcription of detoxification enzymes such as thioredoxin (Kensler et al., 2007). The Nrf2-miR-29b regulation pathway participated in the regulation of COPD (Bergeron and Boulet, 2006). Structural changes in airway diseases: characteristics, mechanisms, consequences, and pharmacologic modulation. Chest 129, 1068–1087.

The finding of the present study also showed that miR-29b signaling improves cellular clearance by alveolar macrophages in patients with COPD and in a mouse model. Sci. Transl. Med. 3, 745–752.

In this study, we found increased MMP2 expression in lung tissues of COPD rats, which might be under regulation of miR-29b. Previous studies indicated that cigarette smoking could stimulate MMP2 activity in human lung fibroblasts and in mice bronchoalveolar lavage fluid (Ning, 2007; Seagrave et al., 2004). These studies together suggested that dysregulation of miR-29b and MMP2 was involved in taking part in the regulation of COPD.

5. Conclusion

MiR-29b was a mediator of Nrf2 regulation and dysregulation of Nrf2/miR-29b influenced Th1/Th2 differentiation and remodeling of airway epithelial cell. Our findings provide new possibilities of targeting Nrf2 as a treatment of COPD.

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Pasini et al., 2016; Ban et al., 2018. The scholars speculated that the alleviated Nrf2 expression in blood might be due to the leakage from the nucleus (Ban et al., 2018). These findings lead to another question why miR-29b expression was also down-regulated instead of being activated by Nrf2 in blood, which was observed in our study and Soeda’s (Soeda et al., 2013). The contradiction may suggest that miR-29b is under a complex variety of regulation network rather than a single regulator.

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