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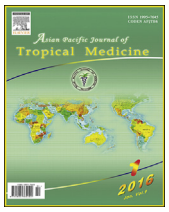

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Anti-inflammatory role of microRNA let-7c in LPS treated alveolar macrophages by targeting STAT3

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ABSTRACT

Objective: To explore the expression of microRNA (miRNA) let-7c and its function in chronic obstructive pulmonary disease (COPD) and alveolar macrophage cells.
Methods: Real time PCR was performed to detect the expression of miRNA let-7c in the lung tissue of COPD patients and COPD model in mice. MiRNA let-7c was overexpressed in alveolar macrophages isolated from mice and its effect was measured by the production of pro-inflammation cytokines and the protein level of signal transducer and activator of transcription 3 (STAT3) as well as phosphorylation level of STAT3 after LPS stimulation. Luciferase assay was used to detect the binding of miRNA let-7c and 3′UTR of STAT3.
Results: MiRNA let-7c expression was significantly lower in patients with COPD compared with control group, and the similar result was found in COPD mice and LPS stimulated alveolar macrophages. Overexpression of miRNA let-7c in alveolar macrophages inhibited LPS-induced increasing of tumor necrosis factor alpha, interleukin-6 and interleukin-1β. Luciferase assay showed STAT3 was a targeting of miRNA let-7c in alveolar macrophages.
Conclusions: MiRNA let-7c low expression in COPD can regulate inflammatory responses by targeting STAT3 in alveolar macrophage, which may provide a new target for COPD treatment strategies.

1. Introduction

Chronic obstructive pulmonary disease (COPD), a high morbidity and mortality pulmonary disease characterized by chronic airway inflammation and emphysematous alveolar destruction, may develop into pulmonary heart disease, respiratory failure and even cancer [1]. Due to the high morbidity and mortality, COPD has become one of the world's three major lethal factors, which brings a heavy burden to the society and a serious threat to the quality of human life [2,3]. Although smoking has been considered an important COPD risk factor, only a small portion of smokers (10%–20%) eventually develop into COPD [4]; its pathogenesis remains to be further studied. The development of COPD must be affected by other factors, such as genetic factors.

Abundant evidences show that the development of COPD is associated with systemic inflammation and chronic inflammatory in the bronchial walls of the small airways. Inflammation is considered to be a markedly increased risk of cardiovascular disease and lung cancer in patients with COPD [5]. Both innate and adaptive immunity is involved in COPD. Plenty of inhibitors of inflammation show potential beneficial effects in COPD. A large number of studies show that participation in the pathogenesis of COPD is associated with a variety of inflammatory cells [6–10], of which the most important are the macrophages, the neutrophils and the lymphocytes. Alveolar macrophages and the release of cytokines play an important role in the pathogenesis of COPD [10]. The present study was designed to investigate the expression of microRNA (miRNA) let-7c in COPD and its role in alveolar macrophage and inflammatory response.

2. Materials and methods

2.1. Animal model

In this study, 40 clean male BALB/C mice were used to build COPD model, 6–8 wk old, weighing 18–20 g. Mice were purchased from Experimental Animal Center of Chongqing Medical University. The animals were randomly divided into control group and COPD model group. Model group were exposed to cigarette smoke, 10 cigarettes per day for 1 h,

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lasting for six months. All treatments and animal care were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Chinese National Committee to the Use of Experimental Animals for Medical Purposes.

2.2. Cell culture

COPD and normal mice were sacrificed by cervical dislocation and bronchial alveolar lavage fluid was collected, followed by centrifugation at 2000 rpm for 10 min. Supernatant was discarded and cells was suspended by RPMI 1640 medium containing 10% fetal calf serum, incubated in 37 °C, 5% CO_2 incubator for 3 h. After that medium was changed to remove the non-adherent cells, and the adherent alveolar macrophages were re-digested and counted; the purified alveolar macrophages were ready to be used.

2.3. Plasmids construction and luciferase assays

Signal transducer and activator of transcription 3 (STAT3) 3′-UTR containing let-7c binding site was cloned into a modified pGL3 vector (Promega, Madison, WI, USA) containing the luciferase gene. Mutations in the 3′-UTR of *STAT3* gene with lect-7c target sites deleted was generated with the QuickChange Site-Directed Mutagenesis kit (Stratagene, CA, USA). About 1×10^5 alveolar macrophages per well were seeded into 24-well plates and co-transfected with 50 ng pGL3 firefly luciferase reporter, 10 ng pRL-TK luciferase reporter and 50 nM let-7c mimics or scramble mimics using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cell lysates were prepared using Passive Lysis Buffer (Promega, Madison, WI, USA) 48 h after transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA).

2.4. Real time PCR

Total RNA was extracted from tissues and cells by Trizol according to the manufacturer's instructions. Reverse transcription of 1 μg of total RNA was performed using Takara reverse transcription kit. Expression of individual genes was then analyzed by semi-quantitative qRT-PCR using SYBR green technology in ABI Prism Sequencher 7500 (Applied Biosystems, Foster City, CA, USA). Let-7c expression was measured by using the TaqMan MiRNA Assay with specific primers for hsa-let-7c. U6 snRNA was used for normalization of the relative abundance of let-7c. Specific probe was designed and synthesized by Lifescience. Primer pairs for tumor necrosis factor alpha (*TNF-α*) sense: 5′-CAT CTT CTC AAA ATT CGA GTG ACA A-3′, antisense: 5′- CCA GCT GCT CCT CCA CTT G-3′; interleukin (*IL*)-*6* sense: 5′- CAC AGA GGA TAC CAC TCC CAA CA-3′, antisense: 5′- TCC ACG ATT TCC CAG AGA ACA-3′; *IL-1β* sense: 5′- AAG CCT CGT GCT GTC GGA CC-3′, antisense: 5′- TGA GGC CCA AGG CCA CAG GT-3′;*β-actin* was amplified using the following primer: sense: 5′- CGT GAA AAG ACC CAG ATC A-3′, antisense: 5′- CAC AGC CTG GAT GGC TAC GT-3′. The mRNA expression of *TNFα*, *IL-1β* and *IL-6* was normalized versus *β-actin* mRNA. The relative expression was quantified with the 2-ΔΔCt method. Experiments were independently repeated at least three times.

2.5. Western blot analysis

After the cells were harvested, 1 × RIPA buffer containing protease and phosphatase inhibitors was used to lysate cells. Polyacrylamide gel electrophoresis was performed after addition of SDS loading buffer and boiled for 5 min, and then proteins was transferred to a PVDF membrane (Milipore). After blocked by 5% non-fat milk at room temperature, primary antibody was incubated overnight at 4 °C (1: 1000), followed by incubation with horseradish peroxidase-coupled secondary antibody, and target band was detected by chemiluminescence. Protease inhibitors and phosphatase inhibitors were purchased from Roche Company; rabbit antibody against STAT3 p-STAT3 were purchased from Cell Signaling Technology; mouse antibody against β-actin was purchased from Santa Cruz Biotechnology; chemiluminescence were purchased from Thermo Pierce.

2.6. Statistical analysis

All data were shown as the mean ± standard deviation of three or more independent experiments. Results were analyzed using SPSS 18.0 software (SPSS, Chicago, IL, USA) and PRISM 6 (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Expression of miRNA let-7c in COPD

The cells from alveolar macrophages in the BALF of mice with COPD were isolated and total RNA was extracted to detect the expression level of microRNA let-7c. Real-time PCR showed that the level of let-7c in alveolar macrophages of mice with COPD was significantly lower than the normal mice (Table 1).

3.2. MiRNA let-7c expression in LPS treated alveolar macrophages

To further explore the potential role of let-7c in COPD, the cells from alveolar macrophages in the bronchial alveolar lavage fluid of mice with COPD were isolated. In addition, alveolar macrophages form normal mice were treated with 100 ng/mL LPS for 6 h, 12 h and 24 h. we also found that inflammatory cytokines *TNF-α*, *IL-1*β, and *IL-6* expression were significantly up-regulated, while the expression of let-7c was significantly down-regulated (Table 2).

3.3. Inhibition of LPS-induced pro-inflammation cytokines production by let-7c

To further investigate the function of let-7c in alveolar macrophages, let-7c mimics were synthesized and transfected into

Table 1
Expression of microRNA *let-7c* in alveolar macrophages.

Group	*Let-7c* relative expressison
Control	1.083 ± 0.348
COPD	0.356 ± 0.039*

*$P < 0.05$, compared with control.

Table 2
Expression of *let-7c* and cytokines in LPS treated alveolar macrophages.

Group	Control group	6 h	12 h	24 h
Let-7c	1.021 ± 0.242	0.532 ± 0.121*	0.195 ± 0.024*	0.253 ± 0.031*
TNF-α	1.127 ± 0.032	5.351 ± 0.284*	6.723 ± 0.521*	6.272 ± 0.807*
IL-1β	1.097 ± 0.018	6.351 ± 0.412*	9.230 ± 1.031*	12.313 ± 2.24*
IL-6	1.023 ± 0.062	4.826 ± 0.234*	6.237 ± 0.523*	5.826 ± 1.231*

alveolar macrophages. Real-time PCR results showed that expression of let-7c was greatly boosted after transfected with let-7c mimics but not in non-specific small RNA fragments transfected cells. After transfection, alveolar macrophages were treated with LPS for 12 h; it was found that high expression of *TNFα*, *IL-1β* and *IL-6* induced by LPS was significantly suppressed by let-7c. In addition, the phosphorylation level of STAT3, a critical transcription factor in inflammatory signaling pathways that could always be activated, was also inhibited by overexpression of let-7c.

3.4. Suppression of phosphorylation of STAT3 by let-7

To understand the mechanism of down-regulation of p-STAT3 in alveolar macrophages by let-7c, the target gene of let-7c was predicted (http://www.microrna.org). Interestingly, we found that *STAT3* was predicted to be a potential target of let-7c. Therefore, we conducted luciferase reporter assays with *STAT3* 3′-UTR in let-7c or scramble mimic transfected alveolar macrophages. A significant decrease of luciferase activity upon let-7c transfection was observed, suggesting that let-7c suppressed STAT3 directly in alveolar macrophages. In addition, we also found protein and mRNA expression levels of STAT3 were significantly decreased in let-7c transfected cells.

4. Discussion

MiRNAs, a class of short single-stranded RNA molecules (19- to 25-nucleotide) that negatively regulate gene expression at the post-transcriptional level, play an important regulatory role in many biological processes, including inflammation, cellular proliferation, differentiation, and apoptosis. MiRNAs have been implicated in the pathogenesis of asthma, lung fibrosis and lung cancer by targeting transcription factors [11–13]. Here, we reported miRNA let-7c was significantly lower in patients with COPD compared with healthy subjects, especially in alveolar macrophages. Restored expression of miRNA let-7c in alveolar macrophages could partially reversed LPS induced inflammatory reaction by inhibiting the phosphorylation of STAT3.

Let-7, the first known human miRNA, was originally discovered by Reinhart in 2000 [14]. Let-7 comprise one of the largest family of miRNAs in human, including let-7a-1, let-7a-2, let-7a-3, let-7b, let-7c, let-7d, let-7e, let-7f-1, let-7f-2, let-7g, let-7i, miR-98, and miR-202 [15]. Let-7 miRNAs have been reported to be critical for promoting differentiation and inhibiting cellular proliferation and its down-regulation has been found in many cancers, including breast cancers, prostate cancer and lung cancer [16–18]. In the present study, we found let-7c was obviously lower in patients with COPD in comparison with healthy subjects, and the similar results was observed in mice model, which was consistent with the results reported by Van Pottelberge [19].

It is well known that COPD is characterized by chronic airway inflammation and emphysematous alveolar destruction. The inflammation is heterogeneous and involved with macrophages, neutrophils and T cells [5]. Macrophages are believed to play a key role in the pathogenesis of COPD and found markedly increase in numbers with increasingly strong inflammatory response, in both the airways and lung parenchyma [20,21]. Interestingly, we found let-7c was greatly decreased in alveolar macrophages in mice with COPD compared with control group. Down-regulation of let-7c was also found in isolated alveolar macrophages with LPS stimulation. To assess the effect of let-7c, alveolar macrophages were isolated and transfected with let-7c mimic or scramble shRNA. We observed that overexpression of let-7c suppressed increasing of important pro-inflammation cytokines produced by alveolar macrophages after exposed to LPS, including TNFα, IL-1β and IL-6. These results suggested that let-7c might play an anti-inflammation role in alveolar macrophages.

STAT3 is an important mediator of the inflammatory response. Once activated, STAT3 migrates to the nucleus, activates transcription of downstream genes in a sequence-specific manner and plays a role in cell proliferation, inhibition of apoptosis and inflammatory response. The phosphorylation of STAT3 was enhanced in alveolar macrophages post LPS stimulation inhibited by the expression of let-7c. We found the putative binding site of let-7c in STAT3 3′-UTR by the biological prediction program. Luciferase reporter assays showed that overexpression of let-7c caused a huge reduction of luciferase activity by the luciferase expression constructs carrying the target STAT3 3′-UTR fragment but no difference was observed on STAT3 3′-UTR mutated fragment. Also, the protein and mRNA expression of STAT3 were significantly decreased after transfected with let-7c mimic demonstrating that let-7c can directly target *STAT3* mRNA in alveolar macrophages.

In conclusion, let-7c is significantly lower in patients with COPD and might function as a negative regulator of the inflammatory response in alveolar macrophages by inhibiting the expression and phosphorylation of STAT3. This finding not only helps understand the role and mechanism of miRNA in inflammation, but also provides foundation for the development of targeted inhibitors of inflammation in COPD.

Conflict of interest statement

We declare that we have no conflict of interest.

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