European Review for Medical and Pharmacological Sciences 2018; 22: 4319-4324

Role of microRNA-218-5p in the pathogenesis of chronic obstructive pulmonary disease

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Abstract. – OBJECTIVE: Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease characterized by inflammatory cell activation and the release of inflammatory mediators. By measuring microRNA expression in the plasma of COPD subjects, we aimed to identify the clinical relevance of plasma miRNA levels in these patients.

PATIENTS AND METHODS: A total of 40 COPD patients and 40 healthy controls were enrolled in the study. The COPD model of C57BL/6 mice was also developed by exposing them to cigarette smoke (CS). The expression of microRNA-218-5p was detected by qRT-PCR in all the subjects and mice. The serum level of IL-18 and TGF-β1 was also detected via ELISA kit. To investigate the effects of miR-218-5p, 10 mg/kg of miR-218-5p inhibitor (miR-218-5p antagonist), a scrambled control or PBS (solvent) was intranasally administered on the first and the fourth exposure day, before the start of CS exposure.

RESULTS: The results showed that miR-218-5p was significantly down-regulated in patients with COPD, compared to normal subjects. There was a negative correlation between the plasma miR-218-5p level and the duration of disease since diagnosis in COPD ex-smokers. CS-induced COPD mice experiments with a miR-218-5p inhibitor demonstrated a protective role of miR-218-5p in cigarette smoke-induced inflammation and COPD.

CONCLUSIONS: These findings supported that miR-218-5p may, therefore, play an important role in the pathogenesis of COPD.

Key Words:
Chronic obstructive pulmonary, microRNA, Plasma, Pathogenesis.

Induction

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease characterized by inflammatory cell activation and the release of inflammatory mediators[1,2]. The main characteristics of COPD are increasing limited obstructive airflow, which is a progressive destructive lung disease. The airflow limitation is due to abnormal inflammatory response relative to poisonous gas or particulate matter exposure[3]. The pathology of COPD is often associated with comorbidities, making COPD a major cause of morbidity and mortality worldwide[4].

MicroRNAs (miRNAs) are a kind of noncoding endogenous single-strand ribonucleic acid (RNA) molecules, on average only 21-25 nucleotides long and are widely found in all eukaryotic cells, including plants, animals and viruses[5]. MiRNAs match with bases in 3'UTR region and silence their target genes post-transcriptionally by inhibiting transcription of target mRNAs or depredating them directly[5]. Therefore, they could regulate the expression of target genes. MiRNAs are involved in many complex life processes, such as growth and development, organogenesis, cell proliferation and apoptosis[6].

Recently, the role of miRNAs in the pathogenesis of respiratory disease has been examined, including COPD, idiopathic pulmonary fibrosis, and lung cancer[7]. In the present study, we investigated the expression levels of miRNA in human plasma and lung tissue as well as in lungs of mice that were exposed to air or cigarette smoke (CS) for 4 or 24 weeks. We also assessed clinical characteristics such as smoking history, duration of disease since diagnosis, and the stages of the COPD patients.

Patients and Methods

Patients and Samples

A total of 80 subjects were invited to participate in this study. Patients were divided into 4 groups: those who had never smoked without COPD (n = 20), current smokers without COPD (n = 20), ex-smokers with COPD (n = 20), and current smokers with COPD (n = 20). Age and gender-matched healthy subjects were also enrolled in this study

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Table I. The characteristics of normal and COPD patients.

	Non-smoker without COPD	Smoker without CODP	COPD ex-smoker	COPD current smoker	*p*-value
No. of subjects	20	20	20	20	
Age (years)	64.5±8.9	67.2±12.5	65.3±8.6	64.8±9.2	0.87
Male (%)	85	90	90	95	0.57
Pack-years of smoking	0	46.8±24.5	68.7±34.5	72.3±54.1	0.67

Data are expressed as the means±SD (standard deviation). *p*-values were determined by the ANOVA test.

(Table I). Patients who had a post-bronchodilator ratio of FEV_1 to forced vital capacity (FVC) of <0.70 were diagnosed as COPD (n = 40). Other exclusion criteria included a diagnosis of asthma, bronchiectasis, lung cancer, or upper or lower respiratory tract infection in the preceding 4 weeks. There were 15 patients in GOLD stage I, 12 in stage II, 10 in stage III, and 3 in stage IV among the 40 COPD patients. This study was approved by the Clinical Research Ethics Committee of The Qingdao University.

COPD Mice Model

C57BL/6 mice were randomly divided into CS exposure group and control group. The CS exposure group was exposed to cigarette smoke passively in PAB-S200 Animal Passive Smoking Exposure System (BioLab Technology Co. Ltd., Beijing, China) for 8 weeks. The CS exposure group was exposed 5 days a week to the mainstream cigarette smoke of 5 cigarettes, 4 times a day with a 10-min smoke-free interval between exposures. The control group was exposed to room air simultaneously. At the end, the FVC, $FEV_{0.1}$, and $FEV_{0.1}$/FVC were recorded using the AniRes 2005 Lung Function system (BioLab Technology Co. Ltd., Beijing, China) including CS exposure and control groups. The mice of $FEV_{0.1}$/FVC below 0.7 in the CS exposure were selected as COPD group.

RNA Isolation and miRNA Expression

Total RNA was extracted from plasma using TRIzol reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions). To assess the specific miRNAs in plasma and lung tissue samples, a pre-amplification step was set up according to the manufacturer's recommendations. MiRNAs were reverse transcribed using the Megaplex™ Primer Pools. MiRNA expression was assessed with the Qiagen miScript PCR system. RNA was converted to cDNA using the miScript II RT kit and cDNA was amplified according to the miScript PCR protocol for miR-218-5p.

Plasma Cytokine Measurements

Plasma collected from the 40 COPD patients and 20 healthy controls were used for the measurement of IL-18 and TGF-β1 using a commercially available ELISA kit according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN, USA).

miR-218-5p Mediation in CS-induced Mice

To investigate the effects of miR-218-5p, 10 mg/kg of miR-218-5p inhibitor (miR-218-5p antagonist), a scrambled control or phosphate-buffered saline (PBS) (solvent) was intranasal administration on the first and the fourth exposure day, before the start of CS exposure. The day after the last CS exposure, the mice were sacrificed, and bronchoalveolar lavage fluid was investigated.

Statistical Analysis

Data are expressed as means ± standard deviation (SD). Unless specified, data are representative of the indicated number of subjects or independent experiments. Student's *t-tests* were performed to analysis the differences between groups. Parts of the paired data were analyzed by using paired Student's *t-test*. For comparing means of more than two independent groups one way ANOVA (analysis of variance) and Kruskal-Wallis ANOVA were used. Post-hoc comparisons were performed by using Bonferroni corrected Mann-Whitney U tests. Values of $p < 0.05$ were considered as statistically significant.

Results

Plasma miR-218-5p Levels were Significantly Downregulated in COPD Patients

The expression level of plasma miR-218-5p in COPD patients was significantly lower than in normal controls. Moreover, the expression levels of plasma miR-218-5p were significantly

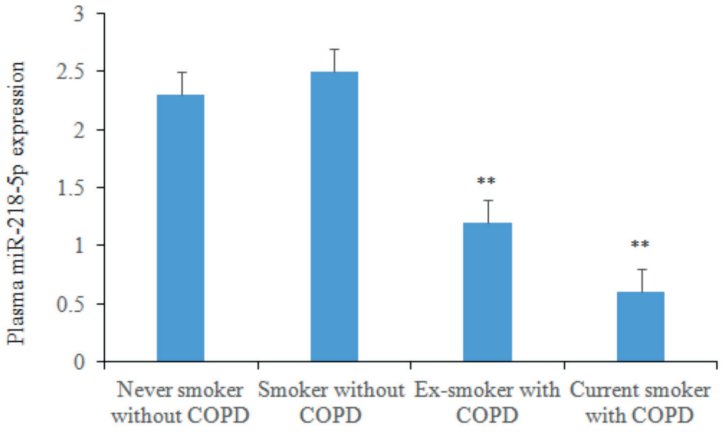

Figure 1. Expression of plasma miR-218-5p levels in patients with COPD and controls. Expression of miR-218-5p was determined by qRT-PCR. The plasma miR-218-5p level in ex-smokers with COPD was significantly downregulated compared with that in current smokers without COPD ($p<0.01$). The plasma miR-218-5p level in current smokers with COPD was significantly lower than that in current smokers without COPD ($p<0.01$). Among the COPD subjects, the level of miR-218-5p in current smokers was significantly lower than that in ex-smokers ($p<0.05$).

decreased in COPD current smoker and ex-smoker compared with smokers without COPD (Figure 1).

Relationship Between miR-218-5p Expression and Duration of Disease

The plasma miR-218-5p expression was inversely correlated with duration of disease since diagnosis in ex-smokers with COPD. However, there was no relationship between the plasma miR-218-5p level and duration of disease since diagnosis in COPD with current smokers (Figure 2). In addition, plasma miR-218-5p expression showed no relationship with age, BMI, FEV_1 (% of predicted), FEV_1/FVC, or duration of smoking cessation, suggesting that no relationship existed between plasma miR-218-5p and the severity of airflow limitation (Table II).

IL-18 and TGF-β1 levels were Increased in COPD Patients

We also compared the plasma IL-18 and TGF-β1 levels in COPD patients and the normal controls. The plasma IL-18 levels in COPD group were significantly higher than the normal control group ($p < 0.01$, Figure 3). However, the plasma TGF-β1 level was not significantly elevated in COPD patients compared with normal controls ($p > 0.05$, Figure 3).

Effect of miR-218-5p Treatment on COPD Mice

To investigate the *in vivo* effect of miR-218-5p in lung tissue during CS exposure, we intranasal administered miR-218-5p inhibitor, scrambled control or PBS (solvent) to CS-exposed mice. The results showed that miR-218-5p inhibitor significantly aggravated the CS-induced increase in inflammatory cells in BAL, such as neutrophils, inflammatory monocytes, dendritic cells and

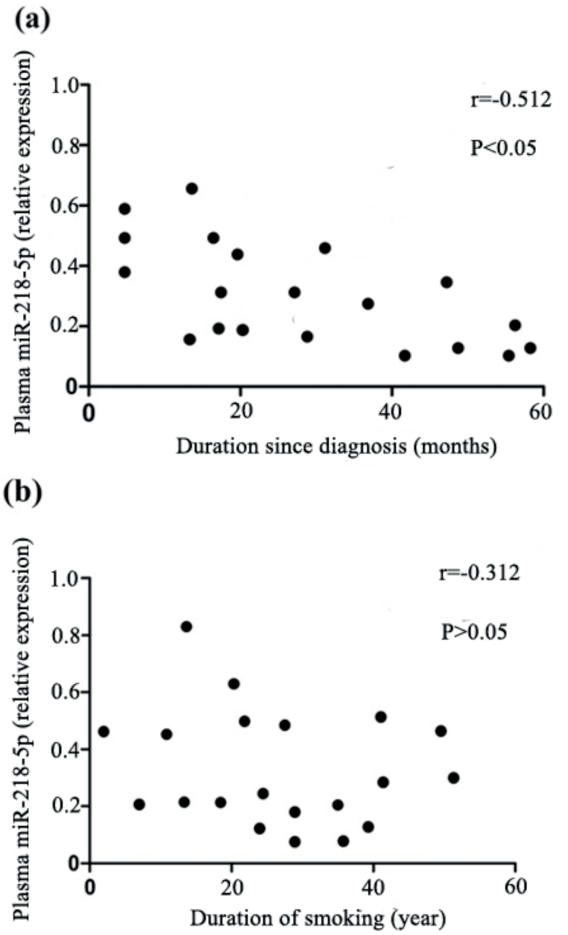

Figure 2. Relationship of plasma miR-218-5p expression in COPD patients and smoking history. ***(a)*** Correlation between the levels of plasma miR-218-5p and duration of disease since diagnosis in ex-smokers with COPD. ***(b)*** Correlation between the levels of plasma miR-218-5p and duration of smoking in current smokers with COPD.

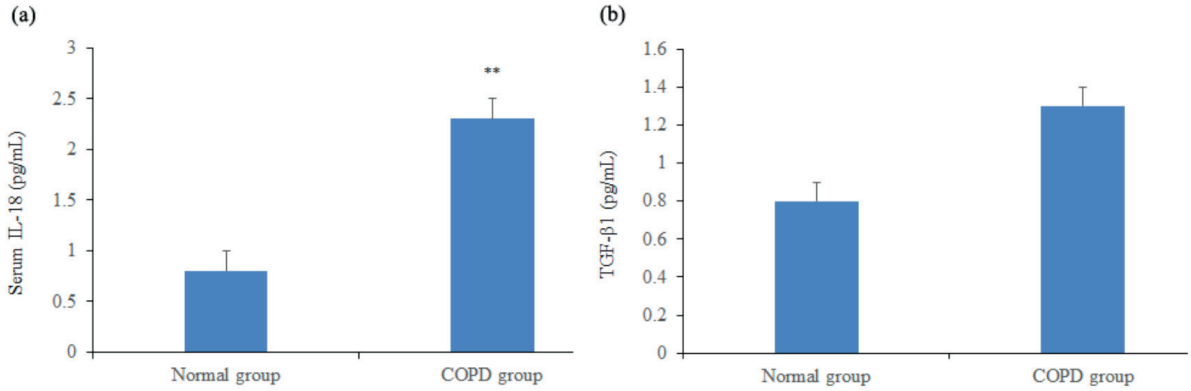

Figure 3. Plasma IL-18 and TGF-β1 levels in COPD patients and control groups by EISLA method. Statistical analysis was used to assess the difference in IL-18 levels, where $^{**}p<0.01$.

CD4+ and CD8+ T cells (Figure 4). Moreover, the level of IL-18 was significantly higher in BAL fluid of CS-exposed mice treated with the miR-218-5p inhibitor (data were not provided).

Discussion

Chronic obstructive pulmonary disease (COPD) is nowadays the fourth leading cause of death; its prevalence is rapidly increasing[8,9]. The pulmonary manifestation of COPD, such as a decrease in FEV_1 and reduction the percentage of FEV_1/FVC, could be due to at least two different pathological manifestations. COPD is characterized by chronic inflammation; thus, the inhaled toxin could induce pulmonary inflammatory reactions[10]. For example, exposure of the lung to cigarette smoke causes an influx of inflammatory cells[11], and increased numbers of neutrophils and macrophages in bronchoalveolar lavage fluid[12].

In the previous study[13], cigarette smoke (CS) has been proofed as the main causative factor of COPD. More reports widely used the well-established experimental model of CS-induced COPD in mice[14], which the CS could contribute to irreversible pathological changes in lung tissue, such as pulmonary emphysema and airway remodeling[15]. However, further studies are needed to fully understand CS-induced COPD animal model.

Aberrant expression of miRNAs has been associated with several pulmonary disorders, suggesting their involvement in the pathogenesis of these diseases[16]. In this study, we explored the expression and functional characteristics of miR-218-5p in plasma in the context of COPD. The results showed that the levels of miR-218-5p in COPD patients were lower than that in smokers without COPD, which indicated that the plasma miR-218-5p level was related to duration since diagnosis of COPD.

The previous study indicated that cigarette smoking activates the inflammatory cells in the airways and airway epithelial cells to release inflammatory peptides, such as interleukin (IL)-1β, IL-8, interferon (IFN)-α, tumor necrosis factor

Table II. Pearson correlation of miR-218-5p and clinical characteristics in patients with COPD.

	COPD			
	Ex-smoker		Current smoker	
	Pearson r	*p*-value	Pearson r	*p*-value
Age (years)	-0.453	0.0734	-0.372	0.345
BMI	0.245	0.678	-0.0782	0.732
FEV_1/FVC (%)	0.372	0.334	0.0482	0.812
FEV1 (% of predicted)	0.129	0.678	-0.123	0.765
Duration of smoking cessation (months)	-0.232	0.384	-	-

Data are expressed as the means±SD (standard deviation). *p*-values were determined by the ANOVA test.

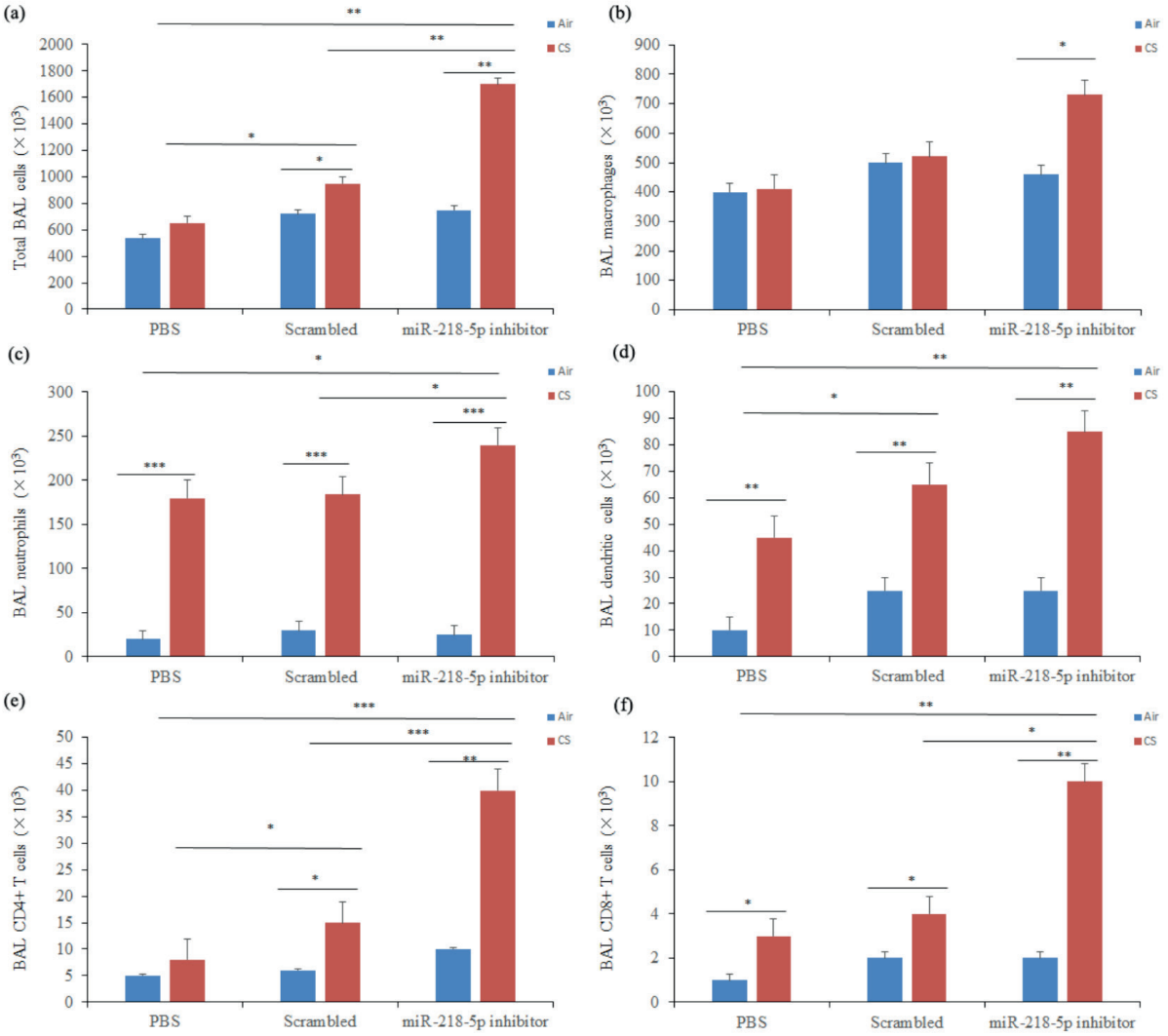

Figure 4. *In vivo* administration of miR-218-5p inhibitor to CS-induced mice. Effect of a miR-218-5p inhibitor, a scrambled control or PBS (solvent) on total bronchoalveolar lavage (BAL) cells and cell differentiation in BAL of male wild type mice exposed to air or CS for 5 days. (a) Total BAL cells, (b) alveolar macrophages, (c) neutrophils, (d) dendritic cells, (e) CD4+ T cells, (f) CD8+ T cells, enumerated by flow cytometry. Results are expressed as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(TNF)-α and IL-18[17-19]. IL-18 has recently been implicated in the pathogenesis of COPD induced by cigarette smoking, via IL-18Rα dependent signaling[20]. We found that IL-18 plasma level has a significant improvement in COPD patients compared to the control group. In contrast, the plasma miR-218-5p level progressively decreased, even after the COPD patients stopped smoking.

Moreover, after inhibiting miR-218-5p in CS-induced mice, we demonstrated an aggravation of the CS-induced inflammation, leading to significantly higher numbers of neutrophils, dendritic cells and T cells, all important cell types in the pathogenesis of COPD. These results indicated that reduced expression of miR-218-5p likely contributes to the CS-induced inflammation.

Conclusions

We showed that miR-218-5p expression was significantly decreased in COPD patients, suggesting that miR-218-5p inhibition may be an initial event in the inflammatory process of COPD and might become an indicator of COPD. There, regulation of miR-218-5p in the early stages of the

disease may be a useful therapeutic method to treat COPD. Although the biological implications of molecules regulated by miR-218-5p need to be clarified in future research, the measurement of the plasma miR-218-5p level could provide important information concerning COPD patients in clinical practice.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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