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Increased miR-223-3p in Leukocytes Positively Correlated with IL-17A in Plasma of Asthmatic Patients

Wenjuan Xu[1], Yimin Wang[1], Chen Wang[2], Ying Ma[1], Shaojun He[1], Yanmeng Kang[3], and Jiong Yang[1]

[1] Department of Respiratory and Critical Care Medicine, Zhongnan Hospital of Wuhan University, Wuhan, Hubei, China
[2] Clinical Laboratory, Zhongnan Hospital of Wuhan University, Wuhan, Hubei, China
[3] Department of Respiratory Medicine, Shandong Provincial Qianfoshan Hospital, Shandong University, Jinan, Shandong, China

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ABSTRACT

Asthma is a common airway inflammation with an intricate underlying mechanism. The role played by circulating miRNAs in asthma remains unclear. In the present study, we aimed to investigate the role of miR-223-3p in leukocytes of asthma and identify the relationship between miR-223-3p and inflammatory cytokines in asthma.

Using real-time polymerase chain reaction (RT-PCR), we detected miR-223-3p expression in peripheral blood leukocytes from 23 asthmatic patients and 20 healthy controls. The levels of IFN-γ (Th1 cytokine), IL-4 (Th2 cytokine), IL-17A (Th17 cytokine) in plasma were examined using enzyme-linked immunosorbent assay (ELISA). Analysis of variance (ANOVA) and Spearman's test was used for statistical analysis.

The expression of miR-223-3p in peripheral blood leukocytes was upregulated in the asthmatic patients compared with that in the healthy controls. Increased miR-223-3p expression was associated with forced expiratory volume in 1-second percent predicted (FEV1% predicted). A positive correlation was noted between miR-223-3p and IL-17A.

The findings of this study showed that miR-223-3p plays a vital role in the pathogenesis of asthma and can serve as a novel biomarker for asthma.

Keywords: Asthma; Interleukin-17; miR-223-3p

INTRODUCTION

Asthma is a commonly occurring chronic airway inflammatory disease with increasing morbidity,[1] and is characterized by airway inflammation, airway hyperresponsiveness, and reversible airway obstruction.[2] The pathogenesis involves both geneticfactors and environmental triggers.[3] Although numerous rigorous studies have been conducted, the underlying mechanisms leading to asthma remain unclear. Most asthmatic patients can obtain good control with the present treatment options, but asthma still follows a chronic disease course; also, the effectiveness of the present treatments is unsatisfactory for some patients. This necessitates the urgency of

Corresponding Author: Jiong Yang,
Department of Respiratory and Critical Care Medicine, Zhongnan Hospital of Wuhan University, 169 Donghu Road, Wuhan 430071, China. Tel: (+86 27) 67813 523, Fax: (+86 27) 68758 766, E-mail: yangjiongwh@126.com

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research regarding the molecular mechanisms of asthma and finding more effective therapies.[4]

MicroRNAs (miRNA)s comprise a class of highly conserved, small, single-stranded, non-coding RNAs of approximately 17-24 nucleotides (nt) that play a crucial role in the regulation of gene expression by binding the 3'-untranslated regions of target messenger RNAs (mRNAs), which leads to targeting mRNA cleavage or translation repression[5-10]. In this way, miRNAs are involved not only in biological processes, but also in the pathological conditions, such as cancer, rheumatic diseases, and leukemia.[11-14] Several studies have demonstrated that miRNAs may play an essential role in the pathogenesis of asthma.[15-20] The anti-miR-126 treatment has been shown to inhibit the function of Th2 cells and attenuate airway inflammation.[18] In addition, the antagonism of miR-145 has been suggested to suppress airway inflammation.[19] Moreover, the inhibition of let-7 markedly suppressed the production of cytokines[21]. Thus, miRNAs play a vital role in the pathogenesis of asthma.

Several types of cells produce miRNAs, which are secreted into serum/plasma, saliva, bronchoalveolar lavage fluid (BALF), and other body fluids[22-24]. In addition, miRNAs are stably present in circulating blood or other body fluids, which exert biological functions through body fluid circulation, and are used as non-invasive biomarkers for the diagnosis of various diseases, including asthma. Recently, more attention has been paid to multifunctional miR-223 because of the vital roles it plays in the immune system.[25,26] Tania found that miR-223-3p is increased in the sputum of 26 asthmatic patients compared with the levels in 10 healthy subjects and was linked to neutrophilic airway inflammation[27]. However, the relationship between the expression of miR-223-3p in the peripheral blood leukocytes of asthmatic patients and the relationship with inflammatory cytokines remains unclear. We aimed to investigate the role played by miR-223-3p in asthma and its relationship with asthmatic inflammatory factors.

MATERIALS AND METHODS

Subjects

All voluntary patients were recruited for this study from September 10, 2017, to July 10, 2018. Asthmatic patients were enrolled at the Department of Respiratory and Critical Care Medicine, and healthy subjects were recruited from the Physical Examination Center of Zhongnan Hospital of Wuhan University (Wuhan, China). The inclusion criteria for asthmatic patients were as follows: 1) Asthma was diagnosed according to the Global Initiative for Asthma (GINA) guidelines and defined as a ≥12% increase in forced expiratory volume in 1 second (FEV1) and FEV1 change>200 mL after inhalation of salbutamol[28] and 2) without treatment with corticosteroid for the past 3 months. The exclusion criteria of asthmatic patients were as follows: respiratory infection for the past 6 weeks, other respiratory diseases, pregnancy, and severe organ failure. Healthy subjects had no history of respiratory diseases and atopic diseases. Atopy was diagnosed based on positive test results for≥1 specific immunoglobulin (Ig) E (>0.35 kU/L) toward common aeroallergens, had a positive skin prick test response, or both. None of the patients had a history of smoking. This study was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (2016024), and written informed consent was obtained from all participants. Samples of the participants were identified by numbers instead of names. Personally, identifiable information was not disclosed to anyone who was not a member of the research team.

Blood Collection

Five mL whole blood from each participant was collected, placed into an EDTA tube, and separated into plasma and cellular fractions by centrifugation at 3000 rpm for 10 minutes at 4°C within 1h after venous blood was obtained. The cellular fractions were stored in liquid nitrogen for further analysis. The collected plasma was centrifuged again at 16,000×*g* for 10 minutes at 4°C for removing cell debris, and the supernatant was collected into a new tube and stored at −80°C for further analysis.

RNA Extraction

Total RNA was isolated from blood cell samples using the R6814 Blood RNA kit (OMEGA, USA). RNase-free DNase I set (OMEGA, USA) was used in the RNA extraction process. Each RNA was quantified by using a spectrophotometer (NanoDrop 2000, ThermoScientific, Wilmington, DE, USA). cDNA was synthesized using the miRNA Reverse Transcription kit (Thermo Scientific, USA) according to the manufacturer's protocol.

Real-time Polymerase Chain Reaction

RT-PCR was performed using the SYBR Premix ExTaq (Takara, Dalian, China) on a CFX96Touch (BIORAD, USA). All reactions were performed in triplicated. The relative expression levels were calculated using the $2^{-\triangle\triangle CT}$ method. The miRNA expression levels of peripheral blood leukocytes were normalized to U6. The sequences of primers were as follows: U6 forward: 5′-CTCGCTTCGGCAGCACA-3', reverse: 5′-AACGCTTCACGAATTTGCGT-3′'; miR-223-3p forward: 5′-GCGCGTGTCAGTTTGTCAAAT-3′, reverse: 5'-AGTGCAGGGTCCGAGGTATT-3′.

Enzyme-linked Immunosorbent Assay

The expressions of IL-4, IL-17A, and interferon (IFN)-γ in plasma were respectively measured using the enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA, USA). Specific operation steps were performed following the manufacturer's instructions, and the absorbance intensity was read at 450 nm using a microplate reader (Tecan, Clontarf, Australia). The concentrations of cytokines in plasma were calculated by optical density value.

Statistical Analyses

All data are presented as means±SD. Statistical analysis was performed by using SPSS17.0 statistical software (SPSS; IBM, Armonk, NY, USA). A t-test was used for the comparisons of two groups of means, one-way analysis of variance was used for comparisons of multiple sets of means, pairwise comparison was performed used Tukey's post hoc test, and correlation analysis was used Spearman's test when the differences were considered to be statistically significant (p<0.05). All experimental data were repeated at least three times.

RESULTS

Demographic Characteristics

Table 1 shows the detailed clinical demographic characteristics of the 43 subjects. This study included 20 healthy subjects and 23 asthmatic patients. No significant differences were note in age, sex, and body mass index (BMI) between the two groups. The plasma IgE levels, blood eosinophils, and fraction of exhaled nitric oxide (F_{ENO}) values in the asthmatic patients were significantly higher than in healthy subjects (p<0.05). The FEV1% predicted values were significantly lower in asthmatic patients than in the healthy subjects (p<0.05).

Expression Levels of miRNA-223-3p in Peripheral Blood Leukocytes Were Upregulated in Asthmatic Patients

The expression levels of miRNA-223-3p in peripheral blood leukocytes of the asthmatic patients and healthy subjects were measured using real-time-PCR. The results suggested that the expression levels of miRNA-223-3p of blood leukocytes were significantly higher in asthmatic patients than in healthy controls (Figure 1).

MiRNA-223-3p Expression Levels in Peripheral Blood Leukocytes Correlated with Pulmonary Function in Asthmatic Patients

Next, we identified factors that influenced miR-223-3p expression using multiple linear regression analysis. The results showed that miR-223-3p expression was significantly associated with FEV1% predicted, even after controlling for age, BMI, plasma IgE, F_{ENO}, and blood eosinophils (Table 2). Spearman's test was used to analyze the correlations between F_{ENO}, blood eosinophils, FEV1% predicted, and miR-223-3p expression. The results revealed that miR-223-3p was negatively correlated with FEV1% predicted and was not associated with F_{ENO}, and blood eosinophils (Figure 2).

MiRNA-223-3p Expression Levels Correlated with Th1, Th2, and Th17 Cytokine Production in Asthmatic Patients

Th1, Th2, and Th17 cells primarily secreted cytokines IFN-γ, IL-4, and IL-17A, respectively. The expression levels of IFN-γ, IL-4, and IL-17A in plasma from healthy controls and asthmatic patients were detected using ELISA kits following the manufacturers' protocols. Compared with healthy controls, the asthmatic patients showed significantly upregulated IL-4 and IL-17A in plasma expressions, whereas no significant difference was noted in the levels of IFN-γ in plasma between the two groups. (Figure 3A).

For further evaluation of the possible interaction between Th1, Th2, and Th17 cytokines and miR-223-3p expression levels in asthmatic patients, Spearman's test was used for analyzing the correlations between them. The results revealed that miR-223-3p was positively correlated with IL-17A in plasma and was not associated with IL-4 and IFN-γ (Figures 3 B-D).

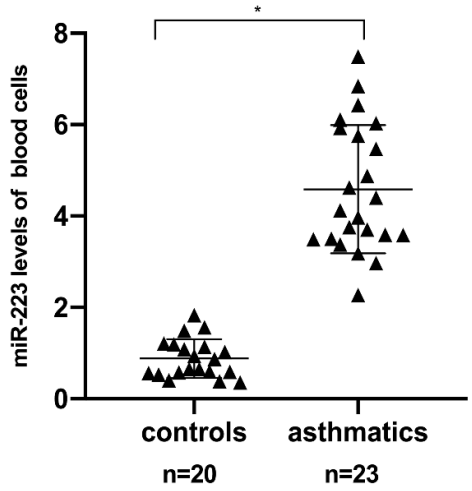

Figure 1. Expression levels of miRNA-223-3p in peripheral blood leukocytes from asthmatic patients and healthy subjects. Data are expressed as $2^{-\Delta\Delta CT}$.*p<0.05.

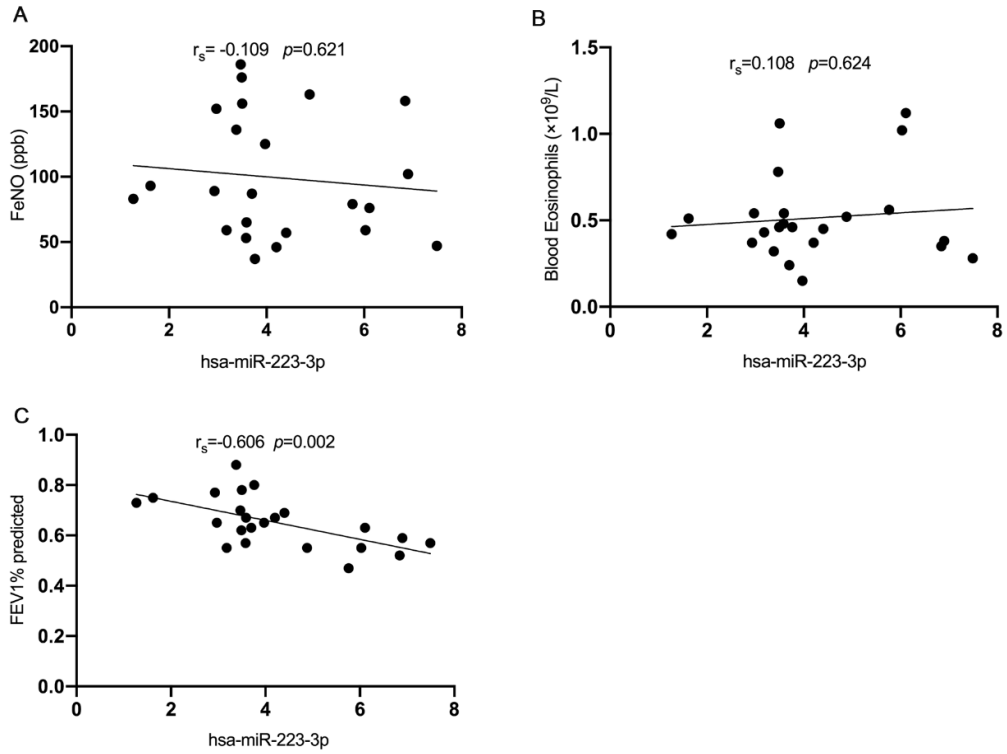

Figure 2. **A-C: Spearman correlation between FeNO, Blood Eosinophils, FEV1% levels and hsa-miR-223-3p expression in peripheral blood leukocytes.**

Table 1. Demographic characteristics

Variables	Healthy subjects	Asthma patients	*p* value
Age (years)	40.90±9.93	43.83±10.73	0.036
Gender (male/female)	9/11	10/13	0.582
BMI(kg/m^2)	22.68±0.58	23.35±0.78	0.505
Blood eosinophils (×109/L)	0.18±0.13	0.51±0.25	0.000**
Plasma IgE (ng/mL)	215.85±17.55	1532.70±367.72	0.000**
FEV1% predicted	104.81±8.95	66.78±14.69	0.000**
FeNO (ppb)	16.50±5.04	99.74±46.48	0.000**

BMI: body mass index; Plasma IgE: plasma immunoglobulin E; FEV1% predicted: forced expiratory volume in 1-second percent predicted; FeNO: fractional exhaled nitric oxide. Data are presented as the mean ± SD. **p<0.01

Table 2. Factors determined by multiple linear regression analysis that influenced miR-223-3p expression

miRNA	adjusted R^2	*p* value model	predictor variable	B	*p* value
Multiple linear regression					
miR-223-3p	0.201	0.011	Age	0.124	0.546
			BMI	-0.150	0.488
			IgE	-0.010	0.968
			FeNO	-0.121	0.577
			Blood eosinophils	0.127	0.544
			FEV1% predicted	-0.564	0.024
Multiple linear regression (stepwise method)					
miR-223-3p	0.337	0.002	FEV1% predicted	-0.606	0.002

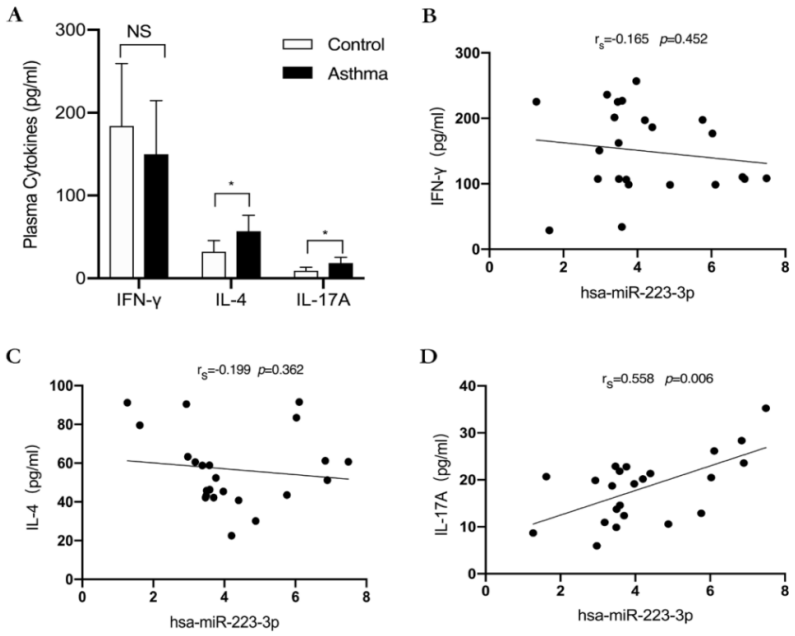

Figure 3. **A: Expression levels of IFN-γ, IL-4, and IL-17A in plasma from healthy controls and asthmatic patients. B-D: Spearman correlation between plasma IFN-γ, IL-4, and IL-17A expression levels and expression of hsa-miR-223-3p in peripheral blood leukocytes.**

DISCUSSION

Numerous studies have demonstrated that miRNAs play an important role in regulating the development or response of the immune system, which includes regulating the maturation, proliferation, differentiation, and activation of immune cells, which contribute to the pathogenesis of asthma.[21-29-31] The findings of the present study revealed that the miR-223-3p expression levels in peripheral blood leukocytes were significantly higher in asthmatic patients than in healthy controls. Linear regression analysis revealed that the expression of miR-223-3p was negatively associated with FEV1% predicted. We also demonstrated that the concentrations of Th2- and Th17-related cytokines increased significantly in the plasma of asthmatic patients compared with those in healthy controls. Particularly, miR-223-3p expression in peripheral blood leukocytes of asthmatic patients was not correlated with plasma IgE, F_{ENO} and blood eosinophils, but positively correlated with the IL-17A concentration in the plasma of asthmatic patients, which indicated that miR-223-3p may participate in the pathogenesis of asthma by regulating the IL-17A production.

Previous studies have shown that the expressions of miR-223-3p in bronchial brushings and biopsy specimens of mildly asthmatic patients did not a significant difference compared with those in healthy controls.[32,33] However, another microarray analysis has shown that the expression of miR-223 was significantly upregulated in the sputum of asthmatic patients[27]. Moreover, this present study confirmed using RT-PCR that miR-223-3p expression was significantly increased in the peripheral blood leukocytes of asthmatic patients. Ezzie et al. suggested in their study that miR-223-3p expression was upregulated in smokers with chronic pulmonary disease relative to the expression without airflow obstruction[34]. Similarly, our study also confirmed that upregulated miR-223-3p expression was negatively correlated with FEV1% predicted in asthmatic patients, which showed that the expression of miR-223-3p was associated with the levels of asthmatic severity.

It has previously been clearly shown that an imbalance of Th1/Th2 cell and abnormal Th17 cell immunity plays a vital role in the pathogenesis of asthma.[35] Th2 cells can exacerbate airway inflammation of asthma by secreting Th2 cytokines, such as IL-4, IL-5, and IL-13. Similarly, Th17 cells can lead to severe airway inflammation by the secretion of IL-17A.[36] Consistent with a previous study,[37] the present study demonstrated that the expression levels of IL-4 and IL-17A increased in the plasma of asthmatic patients compared with those in healthy controls, but IFN-γ expression did not significantly differ between the two groups, which suggested that an imbalance of Th1/Th2 cell and abnormal Th17 cell immunity is involved in the pathogenesis of asthma. For evaluating the possible clinical significance of miR-223-3p in asthma, Spearman's test was used for analyzing the correlations between Th1, Th2, and Th17 cytokines and miR-223-3p levels in asthmatic patients. Consequently, our research revealed that there were not correlations between miR-223-3p and IL-4 and IFN-γ. However, we also noted that there was a positive correlation between miR-223-3p and IL-17A levels, which suggested that the upregulation of miR-223-3p levels may play a key role in severe airway inflammation of asthma. In addition, the specific mechanism of miR-223-3p in asthma requires further investigation.

There were several limitations to this study. First, the participants in this study had mild-to-moderate asthma; thus, the expression levels of miR-223-3p in severe asthmatic patients need to be further investigated. Second, the lack of animal studies to clarify the possible molecular mechanism of miR-223-3p regulation of airway inflammation in asthma.

In conclusion, this study showed that the level of miR-223-3p in peripheral blood leukocytes was significantly increased in asthmatic patients. In addition, upregulated miR-223-3p expression was significantly associated with FEV1% predicted and was positively correlated with the IL-17A concentration in plasma. These results suggested that miR-223-3p may play an important role in the pathogenesis of asthma and could serve as a novel biomarker for asthma in the future.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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