European Review for Medical and Pharmacological Sciences 2019; 23: 5432-5440

Effects of miR-26a/miR-146a/miR-31 on airway inflammation of asthma mice and asthma children

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Abstract. – OBJECTIVE: This study detected the expressions of microRNA-26a (miR-26a), miR-146a and miR-31 in lung tissues and BALF (bronchoalveolar lavage fluid) of asthma mice and children. Besides, cytokine levels of interleukin-5 (IL-5), IL-8, IL-12 and tumor necrosis factor-α (TNF-α) were detected as well. We aim to provide an experimental basis for clinical treatment of asthma.

PATIENTS AND METHODS: Forty female BALB/c mice were randomly assigned into control group and asthma group, respectively. Mice in asthma group (n=20) were immunized by intraperitoneal injection of OVA (ovalbumin) and provoked by atomization inhalation of OVA from the 15th day for 10 days. Mice in control group (n=20) were immunized and provoked with isodose saline during the same period. At the 26th day, mice were sacrificed for collecting lung tissues and BALF. Besides, we enrolled 17 cases of asthma children and 13 cases of children with airway foreign body as controls. BALF of each subject was collected. Total cellular score and differential counting in BALF were recorded. Expression levels of miR-26a, miR-146a, and miR-31 were detected by reverse transcription-polymerase chain reaction (RT-PCR). Levels of IL-5, IL-8, IL-12, and TNF-α were detected by enzyme-linked immunosorbent assay (ELISA).

RESULTS: The total cellular score in BALF of asthma mice and asthma children was higher than that of controls ($p<0.05$). Percentages of eosinophils, neutrophils, and lymphocytes in BALF of asthma mice and asthma children were higher than those of controls, whereas the percentage of macrophages was lower ($p<0.05$). Levels of IL-5, IL-8, IL-12, and TNF-α in lung tissues of asthma mice were markedly elevated compared with those of controls ($p<0.05$). Similarly, levels of IL-5, IL-8, IL-12, and TNF-α were higher in BALF of asthma children than controls ($p<0.05$). RT-PCR data showed higher mRNA levels of miR-26a, miR-146a, and miR-31 in lung tissues of asthma mice than controls ($p<0.05$). The mRNA levels of miR-26a, miR-146a, and miR-31 in BALF of asthma children were highly expressed compared with those of controls as well ($p<0.05$).

CONCLUSIONS: MiR-26a, miR-146a, and miR-31 are involved in asthma progression mainly through regulating inflammatory factors and cells.

Key Words:
MicroRNA, Asthma in children, Inflammatory factors.

Introduction

Asthma is a common chronic inflammatory disease of the airways. Reversible airway obstruction, airway hyper responsiveness, and chronic inflammation are the major features of asthma[1-3]. However, the pathogenesis of asthma is still not fully understood. Glucocorticoids are commonly applied for asthma treatment. Although glucocorticoids could control the symptoms of asthma to a certain extent, their long-term use would lead to adverse events, thus seriously affecting the life quality of asthma patients[4,5]. Therefore, it is of great significance to elucidate the pathogenesis of asthma, so as to develop new therapeutic targets. Currently, it is believed that asthma is caused by a complex interaction between environmental

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and genetic factors. Genetic susceptibility is an important pathogenic factor for asthma. A large number of studies[6] have found that expression changes in cytokines, IgE, matrix metalloprotein-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1), and Sonic Hedgehog may lead to infiltration of inflammatory cells, smooth muscle hyperplasia, and excessive mucus secretion. The specific molecular mechanisms of these genes in asthma development are needed to be further elucidated. MicroRNAs are a class of highly conserved, non-coding RNAs with approximately 22 nucleotides in length. They are widely found in plants, animals, and viruses. MicroRNAs could regulate target gene expressions by degrading mRNA or inhibiting translation through binding to the 3'UTR of their corresponding target genes[7,8]. Some microRNAs are differentially expressed in asthma patients, participating in the regulation of inflammatory cell infiltration, the function of bronchial epithelial cells and airway smooth muscle cells[9]. Experimental results also showed divergent expressed microRNAs in an animal asthma model, as well as in sputum or peripheral blood of asthma patients[10-12]. A large number of signaling pathways are affected by microRNAs. For example, interleukin-13 (IL-13), IL-6, signal transducers and activators of tranion 6 (STAT6) and mitogen-activated protein kinase (MAPK) pathways are activated and further regulate immune cell development and inflammatory response during the pathological process of asthma[13-15]. This study established asthma mouse model and enrolled asthma children. The primary purpose of this study was to determine expression levels of inflammatory factors and microRNAs in lung tissues and BALF (bronchoalveolar lavage fluid) of asthma mice and asthma children. Our study aims to provide new ideas for preventing and treating asthma.

Patients and Methods

Experimental Animals

Forty female BALB/c mice with 6 to 8 weeks old were housed in an environment with 22°C of temperature, 50% of humidity and 8 times/h of ventilation. Mice were randomly assigned into control group (n=20) and asthma group (n=20). Mice in asthma group were immunized at the first time by intraperitoneal injection of 1 mL of $Al(OH)_3$ solution containing 10% OVA. They were provoked by atomization inhalation of 1% OVA from the 15th day. OVA provocation was performed every other day for 10 times, with 20 min for each time. Mice in control group were immunized and provoked with isodose saline. The animal experiment was approved by the Animal Ethics Committee of Binzhou Medical University Animal Center.

Patients

Seventeen asthma children diagnosed at the Respiratory Department, People's Hospital of Gaoqing County from January 2018 to March 2018 were collected, including 11 males and 6 females with an average age of 15.87±2.10 years. Disease condition of enrolled asthma children did not alleviate after glucocorticoids treatment. Fiberoptic bronchoscopy was performed to exclude airway foreign bodies and tuberculosis. Besides, 13 cases of children with airway foreign body were collected as controls, including 10 males and 3 females with an average age of 20.67±2.81 years. The airway foreign body was taken out within 24 h. BALF was collected from each subject. This study was approved by the Ethics Committee of the People's Hospital of Gaoqing County. Signed written informed consents were obtained from all participants before the study.

Sample Collection

Mice were anesthetized with intraperitoneal injection of 0.4 mL/100 g urethane (25%). Alveolar lavage of the right bronchus was performed, and 5 mL of 0.9% NaCl was slowly injected for 3 times. Finally, the recycled fluid was collected in a 15 mL centrifuge tube. The left tissues of mice were washed with normal saline and preserved in liquid nitrogen.

Cell Counting in BALF

BALF was collected from asthma mice and children, and it was centrifuged at 1500 r/min for 10 min. The supernatant was resuspended in 0.5 mL of phosphate-buffered saline (PBS) and diluted for 20 times. 10 μL of diluted BALF was utilized for cell counting.

Meanwhile, 30 μL of resuspended BALF without dilution was selected for Giemsa staining (Leagene, Beijing, China). Based on the morphology, the percentages of eosinophils, neutrophils, lymphocytes, and monocytes in 200 white blood cells were calculated.

Enzyme-Linked Immunosorbent Assay (ELISA)

BALF was collected and centrifuged. The supernatant was harvested for detecting levels of IL-5, IL-8, IL-12, and tumor necrosis factor-α (TNF-α) based on the instructions of ELISA determination kit (Beyotime, Shanghai, China).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

TRIzol kit (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from lung tissues and BALF, which was then reversely transcribed into complementary Deoxyribose Nucleic Acid (cDNA). After cDNA amplification, quantitative Real-time polymerase chain reaction (qRT-PCR) was performed to detect the expressions of related genes. QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was expressed by $2^{-\Delta\Delta Ct}$. Primer sequences used in this study were as follows: miR-26a, F: 5'-GGATCCGCAGAAACTCCAGAGA-3', R: 5'-TTGGAGGAAAGACGATTTCCGT-3'; miR-146a, F: 5'-GGGTGAGAACTGAATTCCA-3', R: 5'-CAGTGCGTGTCGTGGAGT-3'; miR-31, F: 5'-TATTCATAGGCAAGATGCTGGC-3', R: 5'-TATGGTTGTTCTCGTCTCCTTCTC-3'. U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Statistical Analysis

We used Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). The *t*-test was used for comparing differences between the two groups. Comparison between groups was done using One-way ANOVA test, followed by Least Significant Difference Post Hoc Test. $p<0.05$ was considered statistically significant.

Results

Basic Performances of Asthma Mice

During the two-week OVA provocation, asthma mice started to present asthmatic symptoms, including dysphoria, nose cleaning, sneezing, cyanosis of lips, ecphysesis, nodding respiration, and hand-to-face movements. By comparison, mice in control group did not show abnormal performances. At the late stage of provocation, asthma mice showed significant behavioral changes, body weight loss, hair shaft phenomenon, ecphysesis, and nodding respiration. The basic performances of mice in asthma group indicated the successful construction of asthma model in mice.

Total Cellular Score and Differential Counting in BALF

The total cellular score in BALF of asthma mice was higher than that of controls ($p<0.05$). Percentages of eosinophils, neutrophils, and lymphocytes in BALF of asthma mice were higher than those of the controls, whereas the percentage of macrophages was lower ($p<0.05$, Figure 1A-1E). Similar results were obtained in detecting these indicators in BALF of asthma children. The higher total cellular score was detected in BALF of asthma children than that of controls. Besides, higher percentages of eosinophils, neutrophils, and lymphocytes were found in BALF of asthma children as well ($p<0.05$, Figure 2A-2E).

Levels of Inflammatory Factors in Lung Tissues of Asthma Mice and BALF of Asthma Children

Levels of inflammatory factors in lung tissues of asthma mice and BALF of asthma children were detected by ELISA. It is found that levels of IL-5, IL-8, IL-12, and TNF-α in lung tissues of asthma mice were markedly elevated compared with those of controls ($p<0.05$, Figure 3A-3D). Similarly, levels of IL-5, IL-8, IL-12, and TNF-α were higher in BALF of asthma children than controls ($p<0.05$, Figure 4A-4D).

Expressions of miR-26a, miR-146a, and miR-31 in Lung Tissues of Asthma Tissues and BALF of Asthma Children

RT-PCR data showed higher mRNA levels of miR-26a, miR-146a and miR-31 in lung tissues of asthma mice than controls ($p<0.05$, Figure 5A-5C). Similarly, mRNA levels of miR-26a, miR-146a and miR-31 in BALF of asthma children were highly expressed compared with those of controls ($p<0.05$, Figure 5D-5F).

Discussion

Asthma is a chronic airway inflammatory disease with genetic predisposition, involving a variety of immune-related cells, cytokines,

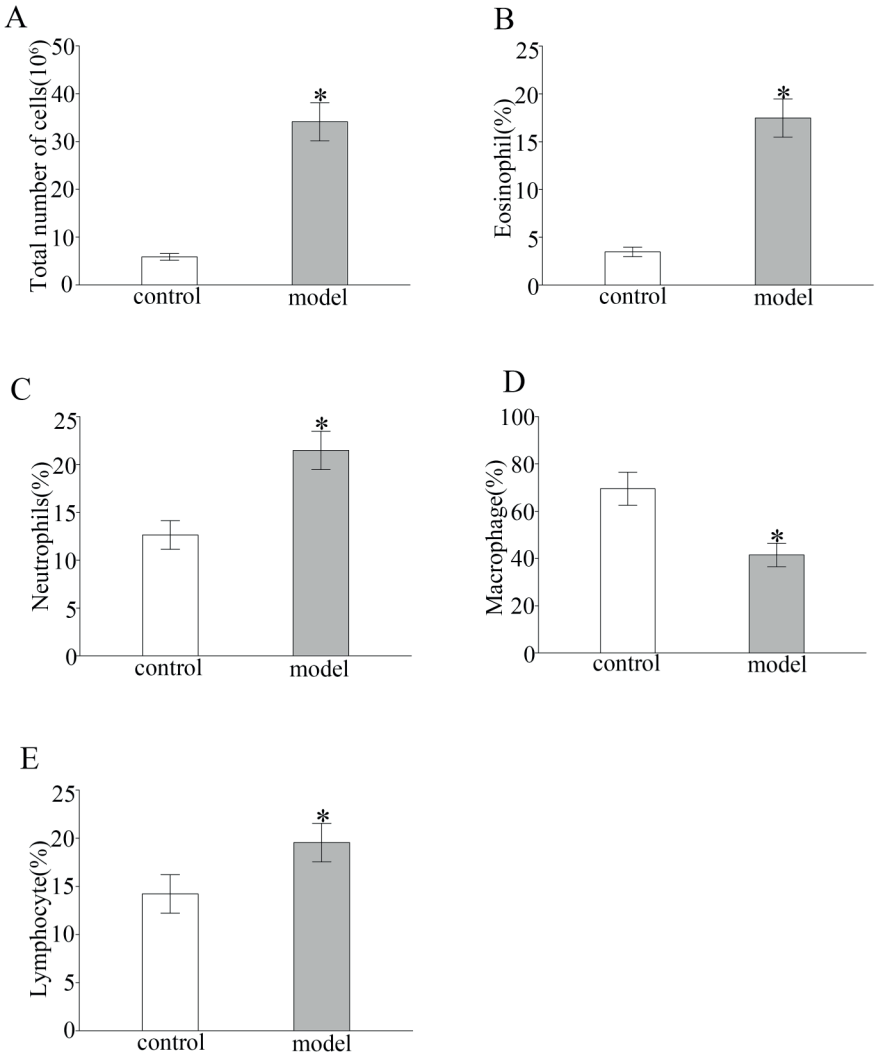

Figure 1. Total cellular score and differential counting in BALF of asthma mice. ***A,*** Comparison in the total cellular score in BALF of asthma mice and control mice. ***B,*** Comparison in the percentage of eosinophils in BALF of asthma mice and control mice. ***C,*** Comparison in the percentage of neutrophils in BALF of asthma mice and control mice. ***D,*** Comparison in the percentage of macrophages in BALF of asthma mice and control mice. ***E,*** Comparison in the percentage of lymphocytes in BALF of asthma mice and control mice. *$p<0.05$, compared with control group.

mediators and signals. Asthma may be caused and provoked by environmental allergens and infections[16-18]. MicroRNAs are post-transcriptional regulators of gene expressions[19]. It is reported that vertebrate microRNAs could regulate up to 200 predicted target genes[20]. In recent years, accumulating studies have shown significant roles of microRNAs in the immune regulation of asthma[21, 22]. He et al[23] showed that there are 66 differentially expressed microRNAs in patients with mild asthma. Yamakuchi et al[24] found 217 abnormally expressed microRNAs in asthma patients undergoing treatment. Through literature review, we speculated that microRNAs are involved in the occurrence and progression of asthma.

Our study found differentially expressed microRNAs in lung tissues of asthma mice and BALF of asthma children, indicating the potential roles of microRNAs in the pathological progression of asthma. Based on previous studies, we speculated that these microRNAs may be closely related to the ratio and function of inflammatory cells[25-27], as well as functions of

airway epithelial cells and fibroblasts[28, 29]. It is reported that overexpression of miR-26a leads to hypertrophy of airway smooth muscle cells and increased airway remodeling by inhibition of GSK-3β[30]. MiR-146 was the first discovered regulator of mammalian infections, exerting a crucial role in the activation of T lymphocytes[31]. Navarro *et al*[32] pointed out highly expressed miR-146a in CD4+ T lymphocytes of asthma mice is remarkably downregulated after dexamethasone treatment. In the present study, miR-146a expression in the lung tissues of asthma mice was higher than that of controls, suggesting that miR-146a may regulate asthma progression. Rutledge et al[33] have shown that miR-31 is expressed in airway epithelial

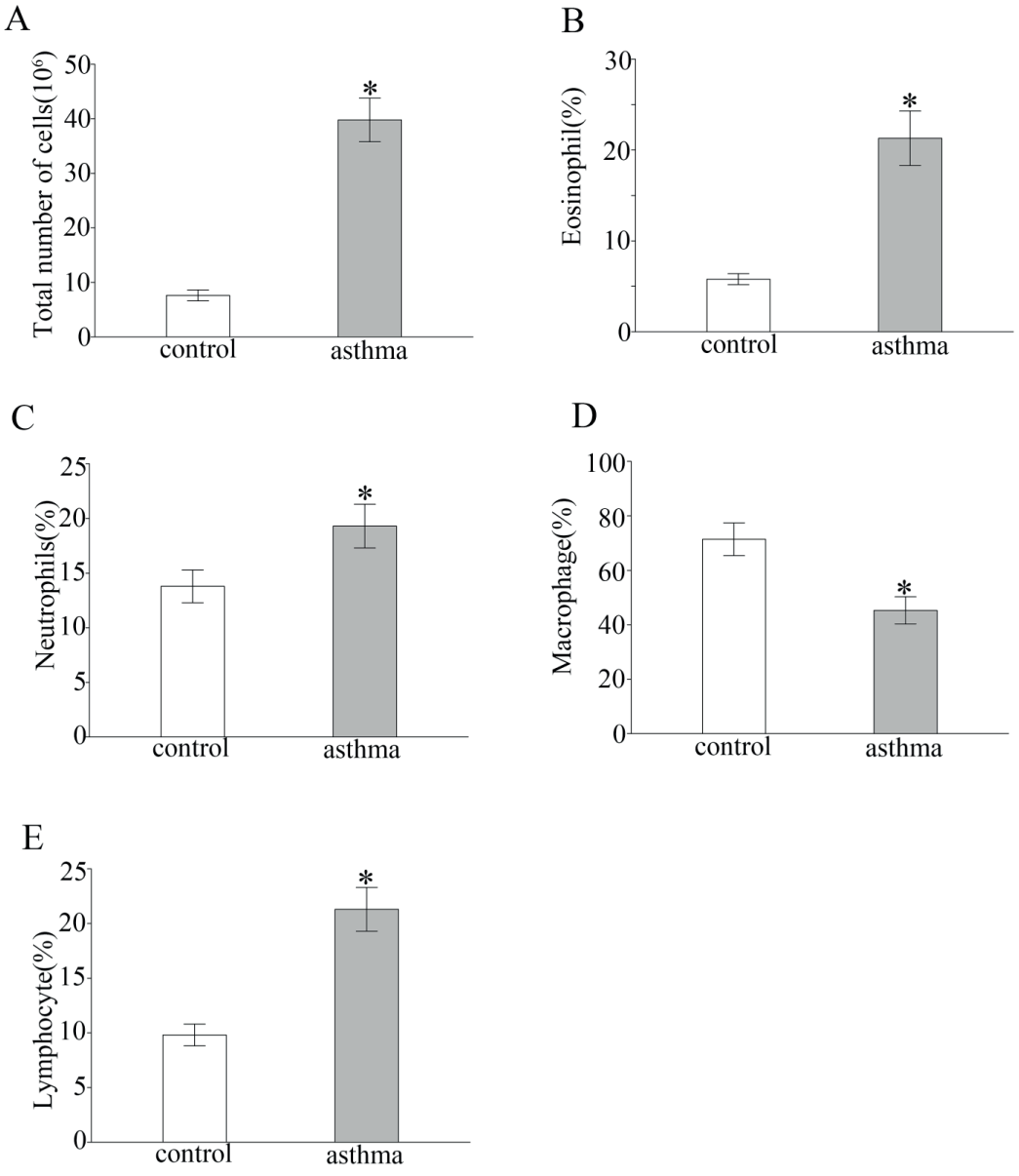

Figure 2. Total cellular score and differential counting in BALF of asthma children. ***A,*** Comparison in the total cellular score in BALF of asthma children and controls. ***B,*** Comparison in the percentage of eosinophils in BALF of asthma children and controls. ***C,*** Comparison in the percentage of neutrophils in BALF of asthma children and controls. ***D,*** Comparison in the percentage of macrophages in BALF of asthma children and controls. ***E,*** Comparison in the percentage of lymphocytes in BALF of asthma children and controls. *$p<0.05$, compared with control group.

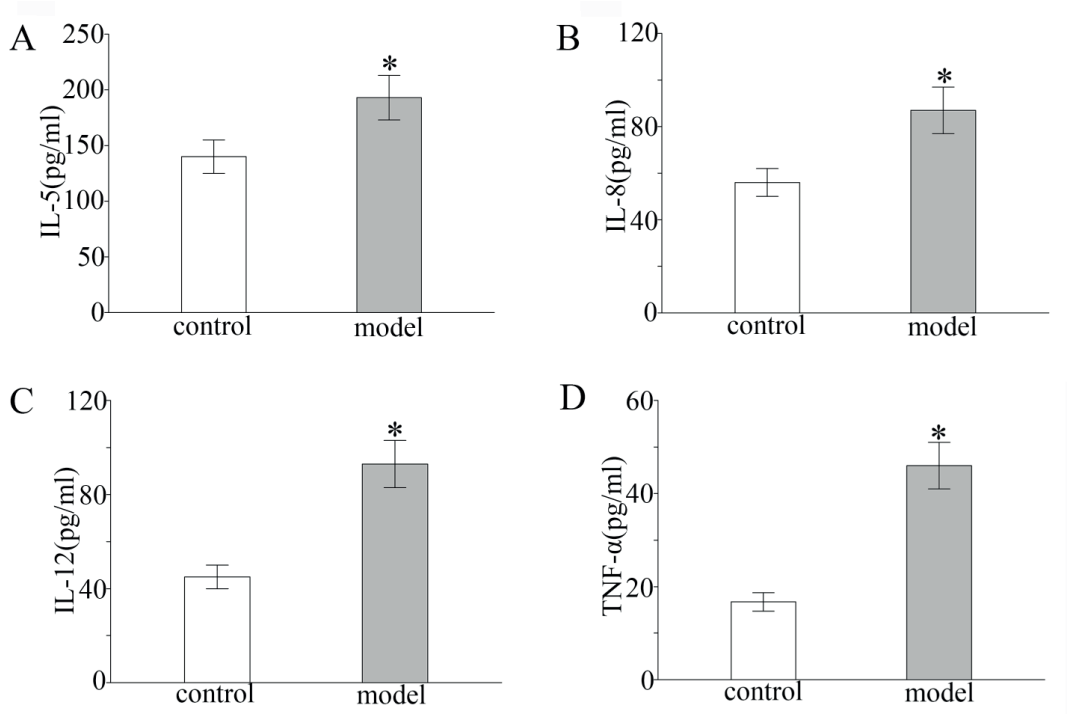

Figure 3. Expression levels of inflammatory factors in lung tissues of asthma mice and BALF of asthma children. ***A,*** IL-5 level in lung tissues of asthma mice and control mice. ***B,*** IL-8 level in lung tissues of asthma mice and control mice. ***C,*** IL-12 level in lung tissues of asthma mice and control mice. ***D,*** TNF-α level in lung tissues of asthma mice and control mice. **p*<0.05, compared with control group.

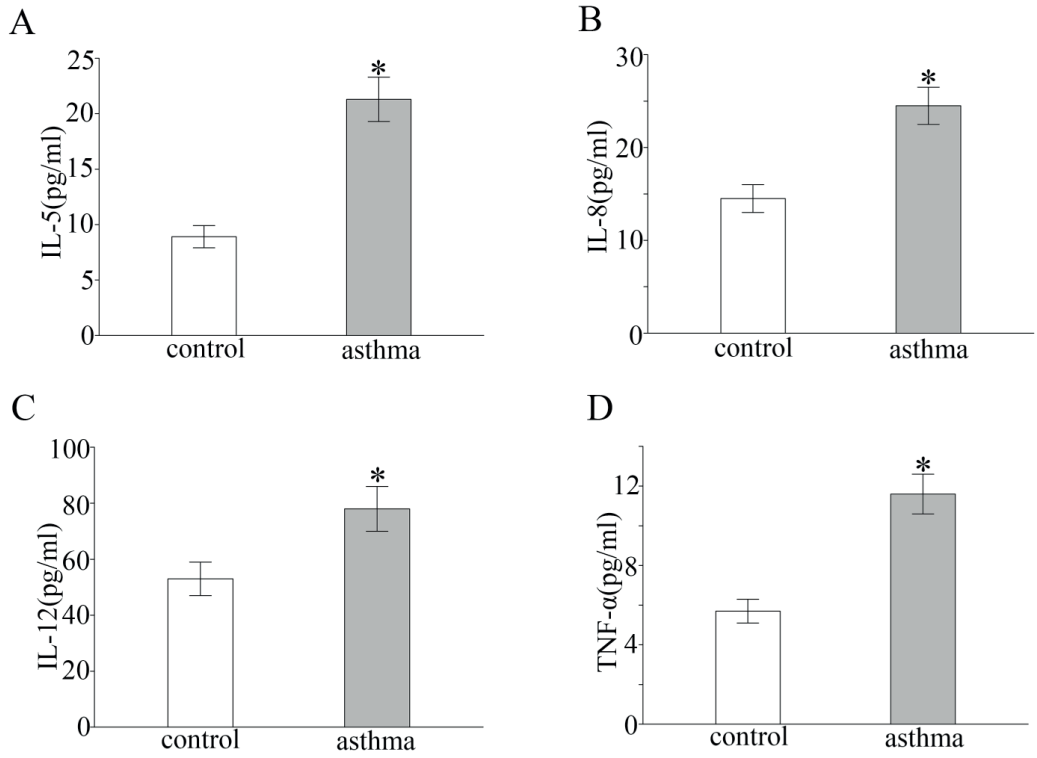

Figure 4. Expression levels of inflammatory factors in BALF of asthma children. ***A,*** IL-5 level in BALF of asthma children and controls. ***B,*** IL-8 level in BALF of asthma children and controls. ***C,*** IL-12 level in BALF of asthma children and controls. ***D,*** TNF-α level in BALF of asthma children and controls. **p*<0.05, compared with control group.

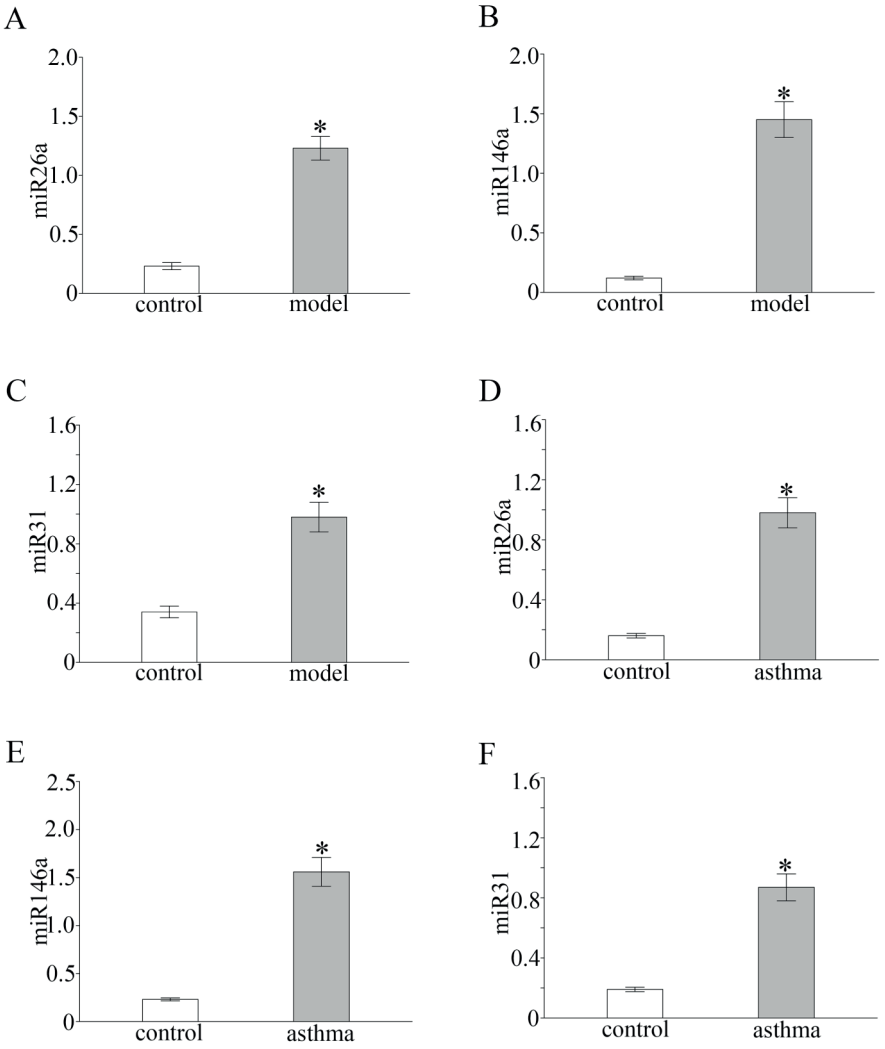

Figure 5. Expression levels of miR-26a, miR-146a and miR-31 in lung tissues of asthma mice and BALF of asthma children. ***A,*** MiR-26a expression in lung tissues of asthma mice and control mice. ***B,*** MiR-146a expression in lung tissues of asthma mice and control mice. ***C,*** MiR-31 expression in lung tissues of asthma mice and control mice. ***D,*** MiR-26a expression in BALF of asthma children and controls. ***E,*** MiR-146a expression in BALF of asthma children and controls. ***F,*** MiR-31 expression in BALF of asthma children and controls. *$p<0.05$, compared with control group.

cells, and exerts its biological function in regulating neutrophilic inflammation, predicting that miR-31 is a potential regulator of airway inflammation. Our study showed that miR-26a, miR-146a and miR-31 were all highly expressed in lung tissues of asthma mice and BALF of asthma children, indicating their crucial roles in asthmatic airway inflammation.

To sum up, we observed that miR-26a, miR-146a, and miR-31 are involved in asthma progression mainly through regulating inflammatory factors and cells. Inhibitors or mimics based on these certain microRNAs may be potential therapeutic targets for clinical treatment of asthma.

Conclusions

We showed that miR-26a, miR-146a, and miR-31 are involved in asthma progression mainly through regulating inflammatory factors and cells.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Funding Acknowledgements

This work was supported by Natural Science Foundation of Shandong Province (ZR2016HB29) and Scientific Research Fund of Binzhou Medical University (BY2012KYQD02).

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