Changes in circulating microRNA-126 levels are associated with immune imbalance in children with acute asthma

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Abstract
Regulation of the immune response in asthma is complex. MicroRNA-126 (miR-126) expression has been implicated in this response, so we sought to determine the clinical significance of miR-126 measured in the peripheral blood. A total of 80 children with acute asthma were selected to participate in the study and were compared to 80 healthy children. The relative circulating miR-126 levels, interleukin (IL)-4 levels, and the CD4+CD25+Treg cell percentage were significantly higher than those in the control group, while the interferon (IFN)-γ levels and the CD4+CD25+Treg cell percentage were significantly lower than those in the control group. Along with the aggravation of the disease, the relative levels of miR-126 and IL-4 and the percentage of Th17 cells increased gradually, while the IFN-γ levels and the CD4+CD25+Treg cell percentage decreased. The relative level of miR-126 in the peripheral blood of children with asthma was positively correlated with IL-4 and the Th17 cell percentage and was negatively correlated with IFN-γ levels, CD4+CD25+Treg cell percentage and lung function indicators. The relative level of miR-126 was correlated with the Th17 cell percentage in the peripheral blood, forced vital capacity (FVC), and forced expiratory flow (FEF)75% of the children with asthma. The relative levels of miR-126 and IL-4 and the Th17 cell percentage were positively correlated with the severity of the asthma, while IFN-γ levels and the CD4+CD25+Treg cell percentage were negatively correlated with the severity of the asthma. The relative level of miR-126 in acute asthma is correlated with signs of immune imbalance and is predictive of the severity of the disease, suggesting that it could be used as a potential serological marker for asthma diagnosis and evaluation.

Keywords
acute attack, asthma, children, cytokine, immune cell, lung function, microRNA-126

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Introduction
CD4+ T cells play a critical role in the pathogenesis of asthma. Th1 cells mainly secrete interleukin (IL)-2 and interferon (IFN)-γ, while Th2 cells secrete IL-4, IL-5, IL-10, and IL-13. When Th1 dominates, the immune response is localized; when Th2 dominates, the immune response diffuses. If the Th1/Th2 systems are imbalanced, a Th2 preponderance response leads to airflow restriction, airway inflammation, and airway hyper-responsiveness.1 The Th1/Th2 paradigm was a cornerstone for our understanding of the pathogenesis of asthma for more than two
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decades because T-helper was known to be limited to both subsets.

Th17 cells are a newly discovered subgroup of effector CD4+ T-cells that secrete IL-17. Th17 cells can enhance inflammatory responses and the body’s defense capability simultaneously and act as pro-inflammatory cells. The upregulated expression of IL-17 can aggravate airway inflammation and airway hyper-responsiveness in a mouse model of asthma.2 CD4+CD25+ Treg cells are key regulating factors in the body’s immune response system, and all CD4+CD25+ Treg cells have a significant immunosuppressive effect.3 If their numbers are reduced or there are changes in function, the absence of their inhibiting effect on Th2 cells produces large amounts of pro-inflammatory cytokines leading to airway inflammation, airway hyper-responsiveness, and ultimately the onset and progression of asthma.4,5

MicroRNAs (miRNAs), a class of small, non-coding RNA molecules able to identify specific target messenger RNA (mRNA) and regulate the expression of target genes at the post-transcriptional level, are of emerging interest as regulators of immune responses. A growing number of studies describe miRNA functions in the development of asthma and related inflammatory processes. Pua and Ansel6 recently summarized the identified miRNAs with altered expression in bulk lesional tissue to include let-7c, miR-21, miR-29, miR-135, miR-142, miR-146, miR-150, miR-155, miR-181, miR-193, miR-223, miR-365, miR-375, miR-452, and miR-615. Similarly, Rebane and Akdis7 reviewed functions of miRNAs in allergy and indicated that miR-126, miR-221, miR-145, miR-106a, let-7a, miR-21, miR-146a, miR-375, miR-133a, miR-1, miR-155, miR-125b, and miR-150 contribute.

MicroRNA-126 (miR-126) is an endothelial cell–specific miRNA that is expressed in the respiratory, hematopoietic, cardiovascular, reproductive, and digestive systems. One of the initial studies in this area demonstrated that inhibition of miR-126 expression resulted in abolished airway hyper-reactivity (AHR), impaired Th2 responses, and reduced allergic inflammation in a house dust mite–induced asthmatic model in mice.8 In a model of chronic asthma sensitized with ovalbumin (OVA), miR-126 was upregulated in the airway wall; in contrast, inhibition of miR-126 by long-term administration of an antagonist suppressed eosinophil recruitment into the airways.9

This study was aimed to understand the mechanism of asthma pathogenesis and progression, and we sought to clarify the role of miR-126 by investigating the effect of miR-126 on the Th1/Th2 and Th17/CD4+CD25+ Treg systems.

Materials and methods

Study participants

This study used a cross-sectional case-control design. Eighty children with acute asthma admitted to Children’s Hospital of Nanjing Medical University (Nanjing, China) between June 2015 and August 2016 were selected to participate in our study. There were 37 males and 43 females aged 2–7 years (average 4.6 ± 1.1 years). In all, 35 cases were considered to be mild asthma, 31 were considered to be moderate, and 14 cases were considered to be severe. All participants had recently been diagnosed or had relapsed at least 3 months after the discontinuation of standardized hormone inhalation. The diagnostic and disease grading criteria used to evaluate patients were described in our previous report.10

The diagnostic criteria for asthma in children were as follows: (1) repeated sharp breaths and age over 3 years. (2) At the onset, both lungs had expiratory wheezing by auscultation, with extended expiratory phase. (3) Treatment with a bronchial dilation agent produced improvement. (4) Other diseases associated with sharp breaths, chest tightness, and cough were excluded, such as chronic obstructive pulmonary disease (COPD). Patients who met all four of the criteria were diagnosed with asthma. Other diseases, including heart, liver, kidney, and other respiratory tract diseases, were ruled out.

An additional 80 healthy children who received a physical examination at the same hospital during the same time period were selected as the control group. The control group consisted of 35 males and 45 females aged 2–8 years (mean ± standard deviation (SD) = 4.8 ± 1.3 years). All healthy control participants underwent clinical examination to exclude respiratory diseases. Children in both groups were negative for immune-related complications, vital organ dysfunction, and infection within 2 months prior to enrollment, and none received adrenocortical hormone or immune-modulator treatment within 3 months prior to enrollment. This study was approved by Ethics Committee of the Children’s Hospital of Nanjing...
Fasting peripheral blood samples were collected from children in both groups. Real-time quantitative fluorescence polymerase chain reaction (q-RT PCR) was used to determine the relative levels of miR-126 in plasma. TRIzol reagent was used to extract total RNA, which was then stored in a refrigerator at −70 °C. The RevertAid™ First Strand complementary DNA (cDNA) synthesis kit (Thermo Scientific, Cat. No. K1621) was used for inverse transcription to synthesize cDNA. Then, q-RT PCR was used to determine the relative level of miR-126 using U6 as an internal reference as in a previous report.11 The primer sequences are provided in Table 1. The reaction system was 20 μL in total: 10 μL of SYBR premix Ex Taq™ (2x), 2 μL PCR Forward Primer (5 μmol/L), 2 μL PCR Reverse Primer (5 μmol/L), 2 μL reverse transcription product, and 4 μL RNA polymerase-free water. The reaction took place over 45 cycles, with degeneration at 95 °C for 30 s, followed by annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The relative expression quantity of target miRNA was based on the difference between the cycle thresholds (Ct value) of target miRNA and Ct values of the reference genes. The 2^−ΔΔCt method was used to determine relative expression levels.

**IL-4 and IFN-γ measurement**

The serum was isolated by centrifugation for 5 min at 3000 r/min. IFN-γ and IL-4 contents in serum samples were detected with the human IFN-γ or IL-4 SunnyELISA Assay Kit (Multi Sciences, Hangzhou, China) following the manufacturer’s instructions.

**Lung function assessment**

A pediatric pneumatometer was used to assess the lung function of children in the case group. The pneumatometer measured forced expiratory volume in 1 s (FEV₁), forced vital capacity (FVC), maximal expiratory flow (PEF), forced expiratory flow (FEF)₂₅%, FEF₅₀%, and FEF₇₅%.

**Statistical analysis**

SPSS 23.0 statistical software was used for data analysis. The measurement data were expressed as (mean ± mean ± SD). Two groups were compared by an independent-samples t test. Multiple groups were compared by a one-way analysis of variance. Pairwise comparison was determined by the least significant difference (LSD). Correlation analysis was assessed based on data type, using linear correlation analysis or Spearman rank correlation analysis. Multiple-factor analysis was determined using multiple-linear regression. Comparison of diagnostic values of the different indexes was analyzed by a receiver operating characteristic curve (ROC curve), with area under the curve (AUC) as the evaluation index. A P < 0.05 was considered to be statistically significant.
Results

Relative levels of miR-126 and IL-4 and Th17 cell counts are increased in asthma patient peripheral blood, while IFN-γ and CD4+CD25+Treg cells are decreased

To determine whether circulating levels of miR-126 correlated with asthma, serum levels were determined in children with and without asthma. The relative level of miR-126 in the peripheral blood of children with asthma was significantly higher than in control participants ($P<0.05$; Table 1). IL-4 levels and the proportion of Th17 cells in plasma were also significantly higher in the case group compared to the control group (Table 2). In contrast, IFN-γ and the proportion of CD4+CD25+Treg cells in blood plasma were significantly lower in children with asthma ($P<0.05$; Table 2).

Relative levels of miR-126 and IL-4 and Th17 cell counts in peripheral blood increase with illness severity, while IFN-γ, CD4+CD25+Treg cells, and lung function decrease

The relative levels of miR-126 and IL-4 and the proportion of Th17 cells significantly increased in accordance with the severity of the participants’ asthma. IFN-γ levels, the proportion of CD4+CD25+Treg cells, and lung function indexes gradually decreased in conjunction with illness severity (Table 3).

Relative level of miR-126 is positively correlated with IL-4 and Th17 levels in the peripheral blood of children with asthma and negatively correlated with IFN-γ levels, the proportion of CD4+CD25+Treg cells, and lung function indexes

Linear correlation analysis revealed that the relative level of miR-126 in the peripheral blood of children with asthma was positively correlated with plasma IL-4 levels and the proportion of Th17 cells ($P<0.05$). Relative level of miR-126 was negatively correlated with IFN-γ levels, the proportion of CD4+CD25+Treg cells, and lung function indexes ($P<0.05$) as shown in Table 4. Multiple linear regression analysis revealed that the relative level of miR-126 in asthma patients’ peripheral blood was correlated with the proportion of Th17 cells, FVC, and FEF$_{75\%}$ ($P<0.05$), as shown in Table 5.

The degree of asthma severity is positively correlated with the relative levels of miR-126 and IL-4 and the proportion of Th17 cells and negatively correlated with IFN-γ levels and CD4+CD25+Treg cells

To determine whether miR-126 and immune markers are associated with disease severity, measurements were compared against asthma severity scores. Spearman rank correlation analysis revealed that the degree of asthma severity was positively correlated with the relative levels of miR-126 and IL-4 and the proportion of Th17 cells in the peripheral blood of children with asthma ($P<0.05$). In contrast, the degree of severity was negatively correlated with IFN-γ levels and the proportion of CD4+CD25+Treg cells ($P<0.05$) in peripheral blood, as shown in Table 6.

CD4+CD25+Treg cells and miR-126 levels have diagnostic potential

ROC curve analysis showed that the AUC for the proportion of CD4+CD25+Treg cells was the highest (AUC=0.976, $P<0.05$), indicating that it has the potential to help diagnose asthma. The AUC for miR-126 was 0.762 ($P<0.05$), as shown in Table 7. For severe asthma specifically, miR-126 had the most potential for diagnostics, with an AUC of 0.909, $P<0.05$, as shown in Table 8.
Table 3. Peripheral blood profiles of patients with varying degrees of asthma.

| Group  | Number miR-126 | IL-4 (ng/L) | IFN-γ (ng/L) | Th17 | CD4⁺CD25⁺Treg | FEV₁ (%) | FVC (%) | PEF (%) | PEF25% (%) | PEF50% (%) | PEF75% (%) |
|--------|----------------|-------------|--------------|------|--------------|---------|--------|--------|-----------|-----------|-----------|
| Mild   | 35             | 4.67 ± 3.15 | 74.09 ± 47.55 | 5.63 ± 80 | 103.89 ± 10.13 | 108.35 ± 10.89 | 92.47 ± 8.87 | 83.84 ± 11.86 | 71.78 ± 10.19 | 62.80 ± 23.95 | 52.06 ± 22.95 |
| Moderate| 31            | 9.03 ± 3.88 | 133.48 ± 48.30 | 59.81 ± 11.9 | 108.35 ± 10.89 | 92.47 ± 8.87 | 83.84 ± 11.86 | 71.78 ± 10.19 | 62.80 ± 23.95 | 52.06 ± 22.95 |
| Severe | 14             | 13.30 ± 2.62 | 178.30 ± 31.97 | 48.33 ± 8.08 | 108.35 ± 10.89 | 92.47 ± 8.87 | 83.84 ± 11.86 | 71.78 ± 10.19 | 62.80 ± 23.95 | 52.06 ± 22.95 |

Table 4. Linear correlation analysis.

| Variables | r  | P  |
|-----------|----|----|
| IL-4      | 0.905 | <0.001 |
| IFN-γ     | -0.806 | <0.001 |
| Th17      | 0.853 | <0.001 |
| CD4⁺CD25⁺Treg | -0.670 | <0.001 |
| FEV₁      | -0.720 | <0.001 |

Table 5. Multiple linear regression analysis.

| Variables | Std. error | Standardized coefficients (beta) | t  | P  |
|-----------|------------|-----------------------------------|----|----|
| (Constant) | 4.200 | 3.367 | 0.001 |
| IL-4      | 0.008 | 0.179 | 1.721 | 0.090 |
| IFN-γ     | 0.026 | -0.032 | -0.416 | 0.679 |
| Th17      | 0.352 | 0.258 | 3.127 | 0.003 |
| CD4⁺CD25⁺Treg | 0.304 | 0.066 | 1.154 | 0.252 |
| FEV₁      | 0.021 | 0.000 | -0.003 | 0.998 |
| PEF       | 0.051 | 0.258 | 1.601 | 0.114 |
| PEF25%    | 0.018 | -0.144 | -1.875 | 0.065 |
| PEF50%    | 0.015 | -0.103 | -1.435 | 0.156 |
| PEF75%    | 0.018 | -0.242 | -2.502 | 0.015 |

Table 6. Correlation analysis.

| miR-126 | IL-4 | IFN-γ | Th17 | CD4⁺CD25⁺Treg |
|---------|------|-------|------|---------------|
| r       | 0.688 | 0.671 | -0.569 | 0.636 | -0.495 |
| P       | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

Table 7. Diagnostic potential in determining asthma.

| Variables | AUC | Std. error | P  |
|-----------|-----|------------|----|
| miR-126   | 0.762 | 0.039 | <0.001 |
| IL-4      | 0.678 | 0.042 | <0.001 |
| IFN-γ     | 0.834 | 0.030 | <0.001 |
| Th17      | 0.822 | 0.035 | <0.001 |
| CD4⁺CD25⁺Treg | 0.976 | 0.011 | <0.001 |

Discussion

We found that children with acute asthma presented with upregulated IL-4 and an elevated proportion of Th17 cells, contrasted by downregulated IFN-γ levels and a lower proportion of CD4⁺CD25⁺Treg cells. This pathologic state
reflects the exacerbation of systemic inflammation and imbalance of the immune system. Meanwhile, the relative level of miR-126 in peripheral blood increased and was significantly correlated with the degree of illness severity, suggesting that miR-126 may play a role in promoting immune imbalance in asthma.

Several studies\textsuperscript{6,12} support the idea that gene expression can significantly influence autocrine and paracrine effects on multiple inflammatory mediators and that transcriptional regulation of gene expression plays an important role in the manifestation of asthma. During the pathogenesis of asthma, miRNAs are regulators of the allergic response, regulating the survival, proliferation, differentiation, and functional development of Th2 cells. Their ability to control the intensity of the immune response makes miRNAs of interest as non-invasive biomarkers of asthma. Another study\textsuperscript{8} confirmed that the overexpression of miR-126 in bronchial epithelial cells can promote increased levels of the Th2 cytokine IL-13, suggesting that miR-126 should be studied as a factor related to excessive activation of Th2 cells in asthmatic children. We found that the relative level of miR-126 was independently associated with the proportion of Th17 cells, indicating that the mechanism of miR-126 overexpression in promoting asthma may also be related to the proportion of Th17 cells.

The results of our study showed that the relative level of miR-126 in peripheral blood of asthma children was associated with the degree of severity of the illness, which was highly valuable in the diagnosis of asthma, especially severe asthma. Therefore, we propose that miR-126 can be used as a potential serological marker to predict the severity of asthma in children. Indeed, numerous other miRNAs have been reported to be upregulated in asthma patients and related to the severity of the disease.\textsuperscript{13–20} These miRNAs are thought to act by regulating the expression of inflammatory factors such as transforming growth factor beta (TGF-\(\beta\)), IL-6, IL-8, and IFN-\(\gamma\). A few studies have pointed to a role for miR-126, as it is abnormally expressed in allergic respiratory system disease.\textsuperscript{21} Another study reported upregulation of miR-126 in the bronchial epithelial cells of asthma patients as well as an association with IL-13.\textsuperscript{22} Alterations in miRNA expression associated with asthma have been shown to improve following treatment, suggesting that changes in miRNA are essential in the continuation of asthma symptoms.\textsuperscript{9}

This study has some limitations. First, we used a cross-sectional study design. Further studies that assess miRNA expression longitudinally are needed to validate our conclusions. While we found an association of miR-126 levels and disease severity, whether miR-126 levels are correlated with long-term clinical outcomes remains unknown. In addition, miRNA expression is altered in many disease states, and thus, it represents a sensitive, rather than specific, marker for disease. Finally, studies of other cohorts and larger populations are needed to validate our findings.

In summary, we found that miR-126 expression was upregulated in the peripheral blood of children with acute asthma. Its relative expression was associated with immune imbalance, lung function, and the degree of illness severity, leading us to propose that miR-126 could be used as a potential serological marker in the auxiliary diagnosis and assessment of asthma. Further studies are needed to validate our conclusions and explore the mechanism through which miR-126 and other miRNAs impact asthma pathogenesis and progression. Inhibiting miR-126 expression in conjunction with other similarly functioning miRNAs may hinder the infiltration of eosinophilic granulocytes in the airway, which could lead to the development of possible treatment options.

### Table 8. Diagnostic potential in determining severe asthma.

| Variables          | AUC  | Std. error | \(P\)  | 95% confidence interval | Lower bound | Upper bound |
|--------------------|------|------------|--------|-------------------------|-------------|-------------|
| miR-126            | 0.909| 0.040      | <0.001 | 0.831                   | 0.987       |             |
| IL-4               | 0.882| 0.046      | <0.001 | 0.792                   | 0.972       |             |
| IFN-\(\gamma\)     | 0.864| 0.048      | <0.001 | 0.771                   | 0.958       |             |
| Th17               | 0.841| 0.055      | <0.001 | 0.734                   | 0.948       |             |
| CD4\(^+\)CD25\(^+\)Treg | 0.779| 0.073      | 0.001  | 0.637                   | 0.921       |             |

AUC: area under the curve.
Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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