Downregulation of miR-19b in pediatric asthma is associated with the activation of nuclear factor-kappa B signaling pathway

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Abstract: miR-19b is associated with asthma while nuclear factor-kappa B (NF-kappa B) signaling pathway plays an important role in asthma progression. Thus, miR-19b may affect asthma via NF-kappa B signaling pathway. All children were assigned into health group and asthma group. B cell line was transferred with miR-19b mimics and inhibitors. MiR-19b level and main molecules of NF-kappa B signaling pathway were measured by Real-time qRT-PCR and Western Blot. NF-kappa B immunoreactivity was measured by using anti-p65 NF-kappa B antibody. qRT-PCR showed that miR-19b level was higher in the health group than the asthma group (P < 0.05; post-test of Kruskal-Wallis and Dunn). Western Blot analyses demonstrated that the levels of phospho-p65 NF-kappa B, granulocyte-macrophage colony-stimulating factor and interleukin-8 were lowest in the health group and highest in the asthma group (P < 0.05). miR-19b overexpression reduced the levels of phospho-p65 NF-kappa B, GM-CSF and IL-8 when compared with the controls. In contrast, miR-19b inhibitor increased the levels of phospho-p65 NF-kappa B, GM-CSF and IL-8, when compared with the controls (P < 0.05). The percentage of B cells with p65 NF-kappa B staining was higher in the cells coinfected with the inhibitors (median, 25-75 percentiles: 49, 46-52) and wild type (median, 25-75 percentiles: 38, 25-36) than in the cells co-infected with the mimics (median, 25-75 percentiles: 8.5, 6-11) (P < 0.05). The results suggest miR-19b prevents the phosphorylation of p-65 NF-kappa B, miR-19b ameliorates pediatric asthma by suppressing NF-kappa B signaling, which activates inflammatory mediators. Serum miR-19b may be a potential drug for the therapy of pediatric asthma.

Keywords: Asthma, inflammation, nuclear factor-kappa B, granulocyte-macrophage colony-stimulating factor, interleukin-8

Introduction

Asthma is global burden of respiratory infections in children but the related molecular mechanisms remain widely unknown. The transcription factor nuclear factor-kappa B (NF-kappa B) regulates the expression of various inflammatory factors and can be activated by inflammatory stimuli [1]. NF-kappa B is a dimer consisting of subunits that include c-Rel, RelA (p65), RelB, p50 and p52 [2]. In most cells, the NF-kappa B prototype is a heterodimer consisting of p65 and p50, which is a subunit with transactivation function [3]. An endogenous inhibitor Ikappa B regulates NF-kappa B activation by complexing with the transcription factor and capturing it in the cytoplasm [4]. Ikappa B molecules form different protein families, including IkappaBα, IkappaBβ, IkappaBε, IkappaBφ, IkappaBδ and p105 [5]. The most characteristic NF-kappa B inhibitor is IkappaBα [6]. This protein strongly binds to the p65 subunit of NF-kappa B by its ankyrin repeat domain associated with the nuclear localization signal and the immunoglobulin-like domain of p65 [7].

Activation of NF-kappa B results in increased expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) [8] and IL-8 [9]. By inhibiting NF-kappa B activity, the expression of these cytokines is usually inhibited by...
MicroRNAs (miRNAs) are small non-coding RNAs that fine-tune post-transcriptional gene expression by mRNA degradation or translation inhibition [15]. miR-19 is a major component of the miR-17~92 cluster, accelerating the production of Th2 cytokines, and amplifying the JAK-STAT signaling pathway to drive asthma pathogenic inflammation [16, 17]. NF-kappa B plays a key role in cytokine signaling [18]. MiR-19b expression reduces the levels of inflammatory cytokines [19], which may be caused by inhibiting phosphorylation of NF-kappa B. Important inflammatory cytokines such as IL-8 and GM-CSF can be activated by NF-kappa B. It appears that miR-19b may inhibit the production of multiple cytokines by down-regulating NF-kappa B, resulting in the reduction of asthma complications. Therefore, the effects of miR-19b on asthma were explored by investigating NF-kappa B signaling pathway.

Materials and methods

Patients

Sixty-eight patients with asthma were selected according to the American Thoracic Society [20]. The subjects with different-degree asthma: 28 patients with mild intermittent asthma, inhaled short-acting beta2 agonists (100 μg salmeterol daily, Hangzhou Dayangchem Co. Ltd., Hangzhou, China) if needed. Twenty-six patients with moderate asthma, inhaled short-acting beta2 agonists 100 μg salmeterol daily. Fourteen patients with severe uncontrolled asthma, took required high-dose GC (2,500 μg beclomethasone dipropionate (BDP, a kind of GC) daily, Zhejiang Xianju Xianle Pharmaceutical Co., Ltd., Taizhou, China), long-acting beta2 agonists 100 μg salmeterol daily. Fourteen patients with severe uncontrolled asthma, took required high-dose GC (2,500 μg beclomethasone dipropionate (BDP, a kind of GC) daily, Zhejiang Xianju Xianle Pharmaceutical Co., Ltd., Taizhou, China), long-acting beta2 agonists 100 μg salmeterol daily. Results were found between health group and asthma group for age and gender (P > 0.05). The study was approved by the Ethics Committee of Affiliated Hospital of Changchun University of Traditional Chinese Medicine, and written informed consent was obtained from each patient.

Production of cytokines by PBMC

The PBMCs were isolated by Ficoll-Hypaque gradient centrifugation as previously described [21]. Freshly isolated PBMCs were grown in Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing 10% heat inactivated fetal bovine serum (FBS), 100 U penicillin ml⁻¹, 100 μg streptomycin ml⁻¹ and 2 mM glutamine. In the absence or presence of BDP, salmeterol and oral prednisone, the cells were cultured for 3, 12 and 24 h. The supernatants were then harvested to measure the levels of phosphor-p65 NF-kappa B (ab176647), IL-8 (ab46032) and GM-CSF (ab100529) by using the ELISA kits from Abcam (Abcam Inc., Cambridge, MA, USA).

Cell coinfection

B cell lines were purchased from Shanghai cell bank (Shanghai, China). Antagomirs and mimics of miR-19b were synthesized by Sangon biotech co. ltd (Shanghai, China). The cell lines were coinfected with miR-19b antagomirs and mimics via Lipofectamine reagent (GenePharma Co., Shanghai, China). After three-day coinfection, the mutant cells were picked using 100 μg G-418 ml⁻¹. Phospho-p65 NF-kappa B, GM-CSF and IL-8 were measured in the cell lines by using ELISA kits.

Real-time PCR quantification of miR-19b

Total cellular RNA was isolated by using an RNA Isolation Total RNA Isolation Reagent (Shanghai Pufei Biotech Co., Ltd., 3101-100, Shanghai, China). cDNA was synthesized from total RNA by using a reverse transcription kit (TIANGEN, Beijing, China). The levels of miR-19b were measured by using real-time qPCR. MiR-19b, forward primer: 5’-GGGTGTGCAAAT-CCATGCA-3’; reverse primer: 5’-GTGCAGGGTTCCAGGTT-3’. U6, forward primer: 5’-GTGCAGGGTTCCAGGTT-3’. U6 snRNA transcripts were used as internal controls for miRNA expression.

Western blot

The antibodies for Phospho-p65 NF-kappa B (ab86299), GM-CSF (ab14024), IL-8 (ab118-
**Table 1. Clinical characteristics of the subjects in present study**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normal</th>
<th>Mild Controlled</th>
<th>Moderate Uncontrolled</th>
<th>Severe Uncontrolled</th>
</tr>
</thead>
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<tr>
<td>Number</td>
<td>30</td>
<td>28</td>
<td>26</td>
<td>14</td>
</tr>
<tr>
<td>Age, years*</td>
<td>8 (4-14)</td>
<td>9 (5-14)</td>
<td>8 (4-14)</td>
<td>9 (5-14)</td>
</tr>
<tr>
<td>male/female</td>
<td>21/9</td>
<td>20/8</td>
<td>19/7</td>
<td>10/4</td>
</tr>
<tr>
<td>Duration of asthma, years*</td>
<td>NA</td>
<td>5.2 (2.8-10.6)</td>
<td>6.7 (3.4-11.2)</td>
<td>7.5 (4.5-12.3)</td>
</tr>
<tr>
<td>FEV, % predicted*</td>
<td>100 (100-102)</td>
<td>82.3 (81.4-87.5)</td>
<td>56.8 (53.2-62.8)</td>
<td>51.7 (48.5-67.2)</td>
</tr>
<tr>
<td>Oral prednisone dose, mg/day*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>35 (25-60)</td>
</tr>
<tr>
<td>Inhaled GC dose, equivalent BDP mg/day*</td>
<td>0</td>
<td>0</td>
<td>1.100 (1,000-1,400)</td>
<td>2,500</td>
</tr>
</tbody>
</table>

*Results were presented as medians with 25-75 percentiles shown in parentheses. FEV, Forced expiratory ratio; BDP, beclomethasone dipropionate; GC, glucocorticoid; NA, not applicable.

**Immunocytochemistry analysis**

B cells with different treatments were collected by centrifuge and fixed in cold acetone-chloroform (1:1). NF-kappa B immunoreactivity was measured by using an anti-p65 NF-kappa B antibody (Cat no. ab16502, Abcam). Relative protein levels were measured by using labeled streptavidin-biotin kit system (BIOSS, Beijing, China). p65 localization was expressed as the percentage of B cells showing a p65 nuclear staining.

**Statistical analysis**

Kruskal-Wallis and the Dunn’s post tests were used for between-group comparisons. Mann-Whitney test was used for unpaired comparisons. Wilcoxon test was used for paired comparisons. Correlations were analyzed by Spearman rank test. Statistical significance was set at $P < 0.05$.

**Results**

**Baseline characteristics of subjects**

The demographic characteristics of normal subjects, mildly controlled, moderately uncontrolled, severely uncontrolled asthma children were shown in **Table 1**. Severe uncontrolled asthma patients had similar age with other children ($P > 0.05$; $P < 0.05$, Wilcoxon test). The children with severe uncontrolled asthma had a longer duration, and were significantly different from other subjects ($P < 0.05$; Wilcoxon test). Despite chronic GC therapy, severe uncontrolled airway injury in asthma children remains high and higher than moderate asthma children ($P < 0.05$; Wilcoxon test). In addition, all moderate asthma patients responded to oral GC trials because they had...
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Serum levels of miR-19b were reduced with the asthma progression and reached the lowest level in a severe asthma group (Figure 1, \( P < 0.05 \); post-test of Kruskal-Wallis and Dunn). The results suggest that low-level of miR-19b may be associated with asthma development.

The levels of phospho-p65 NF-kappa B, GM-CSF and IL-8 in different groups

Among four different groups, the levels of phospho-p65 NF-kappa B were lowest in a health group. The serum levels of phospho-p65 NF-kappa B were increased with the asthma progression and reached the highest level in a severe asthma group (\( P < 0.05 \), Figure 2A). The results suggest that high-level of phospho-p65 NF-kappa B may be associated with asthma development. Similarly, among four different groups, the levels of GM-CSF and IL-8 were lowest in a health group. The serum levels of GM-CSF and IL-8 were increased with the asthma progression and reached the highest level in a severe asthma group (Figure 2B and 2C, \( P < 0.05 \); post-test of Kruskal-Wallis and Dunn). The results suggest that high-level of cytokines GM-CSF and IL-8 may be associated with asthma development.

The effects of BDP, salmeterol and prednisone on PBMCs

The levels of miR-19b were stable in the presence or absence of BDP, salmeterol and prednisone (Figure 3A). In contrast, BDP, salmeterol and prednisone reduced the levels of phospho-p65 NF-kappa B (Figure 3B), GM-CSF (Figure 3C) and IL-8 (Figure 3D) when compared with control groups (\( P < 0.05 \); Mann-Whitney test) (Figure 3). The results suggest that all the medicine treatment inhibits the release of phospho-p65 NF-kappa B, GM-CSF and IL-8 significantly but not miR-19b.

Western blot

Figure 4A showed the relative levels of miR-19b in different groups (CG, control group; SG, miR-19b was silenced by antagonirs. OG, miR-19b was overexpressed). The level was highest in OG and Lowest in SG. To measure the effects of miR-19b on the changes of phospho-p65 NF-kappa B, and phospho-p38 MAPK, Western Blot analysis was performed. Western Blot analysis shows that miR-19b overexpression reduces the levels of phospho-p65 NF-kappa B, GM-CSF and IL-8.
Figure 3. The effects of BDP, salmeterol and prednisone on PBMCs. A. The effects of BDP, salmeterol and prednisone on relative level of miR-19b. B. The effects of BDP, salmeterol and prednisone on the concentration of phospho-p65 NF-κB. C. The effects of BDP, salmeterol and prednisone on the concentration of GM-CSF. D. The effects of BDP, salmeterol and prednisone on the concentration of IL-8. *P < 0.05 vs. a control group without BDP, salmeterol and prednisone treatment.

Subcellular distribution of phospho-p65 NF-κB subunit

To determine the effects of miR-19b on subcellular distribution of p65 NF-κB subunit, we performed immunocytochemistry analysis in B cell line infected with miR-19b mimics or inhibitors. Immunostaining showed that the percentage of B cells with a p65 NF-κB staining was higher in the cells coinfected with the inhibitors (median, 25-75 percentiles: 49, 46-52) and wild type (median, 25-75 percentiles: 38, 25-36) than in the cells coinfected with the mimics (median, 25-75 percentiles: 8.5, 6-11) (P < 0.05; Spearman rank test). The results suggest miR-19b prevents the phosphorylation of p-65 NF-κB.

Discussion

Despite the use of GC for asthma therapy, subjects with severe uncontrolled asthma exhibit persistent airway inflammation characterized by activated neutrophils, T lymphocytes and eosinophils [23]. This inflammation may continue to be increased by the cytokines such as GM-CSF and IL-8, not only in airway cells but also in PBMCs isolated from subjects with severe uncontrolled asthma. We have shown that PBMCs from the subjects with severe uncontrolled asthma can release these inflam-
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Figure 4. The effects of miR-19b on the expression of NF-kappa B signaling molecules. A. The levels of miR-19b in different groups. B. Western Blot analysis of the effects of miR-19b on the expression of NF-kappa B signaling molecules. C. The relative protein level of phospho-p65 NF-kappa B in different groups. D. The relative protein level of GM-CSF in different groups. E. The relative protein level of IL-8 in different groups. CG, control group. SG, miR-19b was silenced by antagomirs. OG, miR-19b was overexpressed. *P < 0.05 vs. a CG group.

Figure 5. The subcellular distribution of phosphor-p65 nuclear factor kappa B (NF-kappa B) subunit by immunocytochemistry. A. p65 is located in the cytosol of B cells of wild type; B. p65 is located in the cytosol and nuclei of B cells infected with miR-19b inhibitors; C. p65 is located in the cytosol of B cells infected with miR-19b mimics.

Inflammatory cytokines due to abnormal expression of NF-kappa B, suggesting that other mechanisms may be associated inflammation responses.

Although the biological and clinical relevance of these results requires further support from greater studies, these data extend previous observations that p65 expression increases in bronchial epithelial cells in patients with severe GC-dependent asthma [24]. Importantly, p65 overexpression increases NF-kappa B transcriptional activity, as shown in the transfection study. The continued activation of the NF-kappa B system appears to be associated with poorly controlled disease of moderate and severe uncontrolled asthma. There may be excessive activity of NF-kappa B receptor in severely uncontrolled asthma patients.

PBMCs have high-level miR-19b in a health group, suggesting that miR-19b may control asthma by reducing inflammatory microenvironment via the inhibition of NF-kappa B. In addition, sustained NF-kappa B activation in severe asthma patients was also demonstrat-
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ed by increased p65 DNA binding and nuclear p65 localization. Although it is believed that the sustained activation of the NF-kappa B system in patients with severe asthma is undesirable. The correlation between low-level serum miR-19b and high-level NF-kappa B level also supports this problem.

The present results show that serum miR-19b can prevent pediatric asthma by inhibiting NF-kappa B signaling pathway, which is associated with asthma development. Serum miR-19b may be a potential drug for the therapy of pediatric asthma. To confirm that, further work is needed to be done in the future.

Disclosure of conflict of interest

None.

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