Intronic RNA (lncRNA) PCGEM1 strengthens anti-inflammatory and lung protective effects of montelukast sodium in children with cough-variant asthma

Zhenxing Xu1, Lingling Meng2, Yuejuan Xie1, and Wei Guo1

1Department of Pediatrics, The Affiliated Hospital of Yangzhou University, Yangzhou University, Jiangsu, Yangzhou, China
2Pulmonary Function Test Room of Children, The Affiliated Hospital of Yangzhou University, Yangzhou University, Jiangsu, Yangzhou, China

Abstract

Montelukast sodium is an effective and well-tolerated anti-asthmatic drug. Long non-coding RNAs (lncRNAs) are involved in the treatment of asthma. Therefore, this study aimed to investigate the effect of montelukast sodium on children with cough-variant asthma (CVA) and the role of lncRNA prostate cancer gene expression marker 1 (PCGEM1) in drug efficacy. The efficacy of montelukast sodium was evaluated by assessing the release of inflammatory factors and pulmonary function in CVA children after a 3-month treatment. An ovalbumin (OVA)-sensitized mouse model was developed to simulate asthmatic conditions. PCGEM1 expression in clinical peripheral blood samples and lung tissues of asthmatic mice was determined. Asthmatic mice experienced nasal inhalation of PCGEM1 overexpression with simultaneous montelukast sodium to investigate the roles of PCGEM1 in asthma treatment. The NF-κB axis after PCGEM1 overexpression was detected to explore the underlying mechanisms. Consequently, montelukast sodium contributed to reduced levels of pro-inflammatory factors and improved pulmonary function in CVA children. PCGEM1 was poorly expressed in OVA-sensitized asthmatic mice and highly expressed in CVA children with response to the treatment. PCGEM1 overexpression enhanced the anti-inflammatory effects and promoted effects on pulmonary function of montelukast sodium in CVA children and OVA-sensitized asthmatic mice. Furthermore, PCGEM1 inhibited the activation of the NF-κB axis. This study demonstrated the anti-inflammatory and lung-protective effects of montelukast sodium on CVA, which was strengthened by overexpression of PCGEM1. Findings in this study highlighted a potential anti-asthmatic target of montelukast sodium.

Key words: Long non-coding RNA; PCGEM1; Montelukast sodium; Cough-variant asthma; Inflammation; NF-κB signaling pathway

Introduction

Asthma is a disorder with symptoms including wheeze, dyspnea, and cough. However, the presentation of cough-variant asthma (CVA) is atypical, with cough being the only or main symptom (1). CVA is one of the inducers of chronic cough in China (2). Additionally, mucus secretory response is also present in patients with CVA (3). Airway inflammation as well as remodeling characterized with subbasement membrane thickening exhibits associations with CVA and non-asthmatic chronic cough (4). Also, eosinophils and neutrophils are possibly activated and implicated in the pathophysiology of CVA (5). Children with CVA have a high prevalence of inaudible wheezing that may be diminished by inhalation of β2 agonist (6). Montelukast sodium, a potent leukotriene 1 receptor antagonist, has been shown to control chronic non-productive cough effectively in CVA (7). Montelukast sodium has also been applied alone or combined with other anti-asthmatic drugs like budesonide, which is superior to budesonide alone for treating the children with chronic CVA (8). However, little attention has been paid to the mechanisms underlying the therapeutic effect of montelukast sodium on asthma and CVA. From a clinical perspective, long noncoding RNAs (lncRNAs) are possibly of great value as biomarkers for classification and/or efficacy assessment of drugs in respiratory diseases (9).

IncRNAs are a group of RNAs with more than 200 nucleotides that can regulate physiological functions and influence disease development in various organ systems, such as cardiovascular, endocrine, digestive, respiratory, etc. (10). Several of them are dysregulated after asthma induction and some are co-expressed with inflammatory cytokines and receptors that are involved in airway allergic
inflammation (11). For instance, IncRNA plasmacytoma variant translocation 1 (PVT1) regulates the proliferation of airway smooth muscle cells and release of IL-6 in the patients suffering from severe asthma (12). A prostate-specific IncRNA prostate cancer gene expression marker 1 (PCGEM1) is a powerful indicator that distinguishes early osteoarthritis from late-stage osteoarthritis (13). IncRNA PCGEM1 is reported to be a mediator of multiple metabolic pathways, such as glucose and glutamine metabolism (14). A preliminary screening of dysregulated IncRNAs presents a low expression of PCGEM1 in the serum samples of patients with asthma (15). In this study, we aimed to investigate the significance of PCGEM1 in the treatment with montelukast sodium for children with CVA, so as to develop novel therapies that can be better targeted toward CVA-specific characteristics.

Material and Methods

Ethics statement

Experiments involving human beings were conducted with approval of the Ethics Committee of the Affiliated Hospital of Yangzhou University and in line with the Declaration of Helsinki. Informed consents were obtained from the relatives of all participants. Animal experiments were performed in compliance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Ethics Committee of the Affiliated Hospital of Yangzhou University.

Study subjects

A total of 60 children diagnosed with CVA from March 2017 to March 2018 were enrolled in this study, including 33 males and 27 females, aged from 2 to 9 years (mean age 5.5 ± 1.7 years). Disease severity was defined by the Global Initiative for Asthma (GINA) 2016 (http://www.ginasthma.com), and all children were defined with mild asthma (GINA 1 or 2). The disease duration ranged from 7 months to 21 months, with a mean duration of 14.4 ± 2.3 months. Additionally, 60 healthy children were enrolled as normal controls, with 31 males and 29 females, aged from 1 to 10 years with a mean age of 5.9 ± 1.4 years. Children with other types of respiratory tract infections and hormonal contraindications were excluded. All the patients received standard treatment for the management of an acute attack of bronchial asthma as per the GINA guidelines. These included parenteral steroids, short-acting beta 2 agonists with inhaled anti-cholinergics by nebulization every 4–6 hourly depending on severity, intravenous theophylline derivatives, oxygen therapy, and other supportive therapy (16). They were administered with one piece of montelukast sodium (BaiSanPing; 10 mg, orally) (China OTSUKA Pharmaceutical Co., Ltd., China; SFDA approval number: H20064370) daily at bedtime. One month indicated one course, and children experienced 3 consecutive courses (3 months).

Measurement of inflammatory cytokines and evaluation of pulmonary function

Pulmonary function was evaluated before and after treatment and three months after treatment, the levels of inflammatory factors in the peripheral blood of children were measured. Fasting venous blood was collected before and after treatment, and interleukin (IL)-4, IL-3, and interferon (IFN)-γ levels were measured by enzyme-linked immunosorbent assay (ELISA) kits (Shanghai ExCell Biological Product Co., Ltd., China). Pulmonary function indicators including peak expiratory flow (PEF), forced vital capacity (FVC), instantaneous maximum expiratory flow after 50% expiration of the FVC (MEP50), and forced expiratory volume in first second (FEV1) were recorded by a pulmonary function detector (MIR Spirolab III srl, Italy) before and after treatment.

Evaluation of therapeutic effects

Clinical efficacy was evaluated based on the Chinese guidelines for the diagnosis and prevention of childhood bronchial asthma (17) 3 months after treatment. Remarkably effective cases: basically no episode of shortness of breath and wheezing, occasional or no cough; valid cases: asthma attack but significantly reduced frequency (<2 times/week); invalid cases: no significant amelioration in the frequency or severity of asthma attacks. In the subsequent experiments, the effective and remarkably effective cases were classified as valid, and the rest were classified as invalid. The expression of IncRNA PCGEM1 in these two groups was determined.

Mouse model of CVA

Thirty male Balb/c mice aged 6–8 weeks were purchased from the Experimental Animal Center of Xi’an Jiaotong University Health Science Center and were acclimated for 3 days before the experiment. Twenty-four mice were randomly selected for asthma model construction and the remaining 6 mice were selected for normal control (MOCK).

An asthma mouse model was established by ovalbumin (OVA) sensitization (Sigma Chemical, USA). In details, each mouse was intraperitoneally injected with 200 μL phosphate buffer saline (PBS) containing 50 μg OVA and 2 mg aluminum hydroxide gel on the 1st and 14th day, followed by atomization inhalation with OVA for 20 minutes from the 21st to 26th day to establish an asthma model (18).

Among the 24 mice with OVA-induced asthma, 6 mice were randomly selected and intraperitoneally administered montelukast sodium (3 mg/kg; (19)) 30 min before atomization inhalation from the 21st to 26th day, for a total of 7 times. Another 6 OVA-sensitized mice experienced nasal inhalation of PCGEM1 negative control (NC) plasmid
(4 mg/kg) and montelukast sodium treatment from the 21st to 26th day, for a total of 7 times (20,21). Another 6 OVA-sensitized mice experienced nasal inhalation of PCGEM1 mimic plasmid (4 mg/kg; Shanghai GenePharma Co., Ltd, China) and montelukast sodium treatment from the 21st to 26th day.

**Assessment of bronchial hyperresponsiveness**

Total airway resistance in mice was assessed 24 h after the last atomization inhalation by a mouse pulmonary function instrument (RC System, Buxco Electronics Inc., USA). The spontaneous respiration of the mice in the awake state was recorded by plethysmography. The mice were atomized with methacholine at different concentrations (0, 6.25, 12.5, 25, and 100 mg/mL) for 3 min, and connected with a MP150 multichannel physiological recorder (BIOPAC Systems, Inc., Goleta, USA). The data were recorded and averaged. The data are reported as increased airway resistance (Penh).

**Pulmonary function test**

The mice were anesthetized with 5% pentobarbital sodium solution, after which their limbs were exposed and intubated to a ventilator (HX-300, Chengdu Techman Software Co., Ltd., China) with a respiratory frequency of 90 times/min, a tidal volume of 5.6 mL/kg, and respiratory pressure of ~8 cmH₂O (1 cmH₂O=0.098 kPa). The dynamic transpulmonary pressure, flow, and tidal volume were recorded. PEF and the ratio of forced expiratory volume in 0.4 s (FEV0.4) to FVC were calculated when the ventilation was performed with 5 times the tidal volume, and expiration was assisted with ~8 cmH₂O pressure.

**Cell counting in bronchoalveolar lavage fluid (BALF)**

The mice were intubated and fixed after anesthesia. BALF was performed with ice-cold PBS, 0.8 mL/time for 3 times. BALF was collected and centrifuged at 500 g at 4°C for 5 min. The supernatant was collected and stored at low temperature for later use. The total inflammatory cells, lymphocytes, macrophages, neutrophils, and eosinophils were counted by Wright’s staining (Beijing Solarbio Technology Co., Ltd., China) after lysis of red blood cells.

**Hematoxylin-eosin (HE) staining**

The blood of the lung surface was washed with ice-cold PBS buffer after sterilization. The left lung was fixed in 10% neutral formalin for 24 h, routinely embedded in paraffin and sectioned at 4 μm for HE staining, periodic acid-schiff (PAS) staining (Beijing Solarbio Technology Co., Ltd.), and immunofluorescence to observe the pathological changes of lung tissues in mice. The right lung was preserved in an ultra-low temperature refrigerator.

**RT-qPCR**

Total RNA was extracted from the lungs using TRIzol kit (Invitrogen, USA). The concentration and purity of total RNA were determined using a Nanodrop 2000 ultramicro spectrophotometer (Thermofisher Scientific, UK). Then cDNA was synthesized using the reverse transcription kit (GeneCopeia, USA). The expression of each gene in Table 1 was detected using SYBR PCR Master Mix kit (Applied Biosystems, USA) on the PCR system (Applied Biosystems). With β-actin as the internal reference, the relative expression of the gene was expressed by 2^(-ΔΔCt). All primers were synthesized by Shanghai Biotechnology (Shanghai, China).

**ELISA**

The contents of inflammatory cytokines IL-4, IL-13, and IFN-γ were measured according to the instructions of ELISA kits (Shanghai ExCell Biological Product Co., Ltd., China).

**Western blot assay**

The lung tissues frozen at ultra-low temperature were thawed on ice and lysed by radio-immunoprecipitation assay (RIPA) lysis buffer. After homogenization, the supernatant was collected by centrifugation (14,000 g, 15 min, 4°C) to obtain total protein. After the concentration of the protein was measured with the bicinchoninic acid (BCA) protein assay kit (TransGen Biotech, China), 40 μg of total protein per well was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred onto the nitrocellulose membrane. After being blocked with 5% skim milk powder, the membrane was incubated with antibody to NF-κB (1:1000, ab220803, Abcam, USA) and p-NF-κB

**Table 1. Primer sequences for RT-qPCR.**

| Gene Primer sequence | Primer sequence |
|----------------------|-----------------|
| IncRNA PGEM1 F: ACCTTTTTGGCCCTATGCGCT R: ACCTGTAGTCGGCATGCACT | GTCACCTGCTTTCTTCTCGG R: CTCTCTGTGGTGTTCTTCGGT |
| IL-4 F: TCTTGGCTGCTTGGTCCTGTC R: TCTCTGTGGGTGTTTCGTTG | TCGCTGTGGTGTTCTTCGGT |
| IL-13 F: TCTTGGCTGCTTGGTCCTGTC R: TCTCTGTGGTGTTCTTCGGT | TCGCTGTGGTGTTCTTCGGT |
| IFN-γ F: TCAAGTGGCATAGATGTGGAA R: TGGCTGTGCAGGATTTTTCG | TGAAGGGAAGGGGCGCG |
| NF-κB F: GGCCGGAGACCATCTACT R: GCTCATCACCTCGCAGGTTTCGT | CTACAGACACAGCCGAGACCATCTACT |
| β-actin F: GTCATCTCACCCACCTACT R: GCTCATCACCTCGCAGGTTTCGT | GCTATCACCTCCCCTGTGTG |

IncRNA: long non-coding RNA; PGEM1: prostate cancer gene expression marker 1; IL-4: interleukin-4; IL-13: interleukin-13; IFN-γ: interferon γ; NF-κB: nuclear factor κB; F: forward; R: reverse.
(1:1000, ab194908, Abcam) at 4°C overnight, followed by TBST (tris-buffered saline and Tween 20) washing. Next, the membrane was incubated with secondary antibody at room temperature for 45 min, followed by TBST washing. The membrane was stained with electrochemiluminescence (ECL) liquid and photographed. Grayscale analysis was performed to analyze the absorbance value.

**Immunofluorescence assay**

After dewaxing and dehydration with gradient alcohol, antigen repair of lung tissues was carried out. The tissue sections were washed with 0.01M PBS 3 times (5 min/time) and blocked with 2% bovine serum albumin at room temperature for 20 min, and incubated with the primary antibody NF-κB (ab7204) at 4°C overnight. After thorough PBS washing, the sections were incubated with secondary antibody horseradish peroxidase (ab205718) for 40 min. The sections were washed thoroughly with PBS and sealed with glycerin. The fluorescence was observed under fluorescence microscope (BX51; Olympus, Japan).

**Statistical analysis**

Data were analyzed by SPSS 21.0 statistical software (IBM, USA). The data normality was tested by Kolmogorov-Smirnov test. The data are reported as means ± SD. Comparisons between two groups were analyzed by t-test, and among multi-groups by one-way analysis of variance (ANOVA) or two-way ANOVA. The post hoc test was performed by Sidak’s multiple comparisons test or Tukey’s multiple comparisons test. The receiver-operating characteristics (ROC) curve was drawn to evaluate the diagnostic value of PCGEM1 expression for the efficacy of montelukast sodium. A two-tailed P value less than 0.05 indicated statistically significant difference.

**Results**

Montelukast sodium reduced inflammation and improved pulmonary function in CVA children

IncRNAs are reported to be involved in the regulation of inflammatory mediators or the expression of cytokines (22). IncRNA PCGEM1 is lowly expressed in the serum of...
Lung tissue of asthmatic mice was slightly thickened, and inflammation in the asthmatic mice was significantly increased (both \( P < 0.05 \)). More noticeable reduction in airway hyperresponsiveness and increase in FEV\(_{0.4}/\)FVC level were found in the asthmatic mice treated with montelukast sodium and PCGEM1 mimic (Figure 4A and B).

HE and PAS staining assays showed disordered lung tissue, thickened alveolar wall, increased inflammatory cell infiltration around the bronchia, increased mucus secretion, and goblet cell hyperplasia in the asthmatic mice. After treatment with montelukast sodium, the lung tissue structure of the asthmatic mice was slightly improved, and mucus secretion and the number of inflammatory cells around goblet cells and bronchi were reduced. After montelukast sodium combined with

**PCGEM1 overexpression enhanced the promotive effects of montelukast sodium on pulmonary function in OVA-sensitized asthmatic mice**

With the increase of the concentration of methacholine, the airway hyperresponsiveness of asthmatic mice increased and the lung function index \( FEV_{0.4}/FVC \) decreased (both \( P < 0.05 \)), but reduced airway hyperresponsiveness and increased \( FEV_{0.4}/FVC \) level were detected in the asthmatic mice treated with montelukast sodium (both \( P < 0.05 \)). More noticeable reduction in airway hyperresponsiveness and increase in \( FEV_{0.4}/FVC \) level were found in the asthmatic mice treated with montelukast sodium and PCGEM1 mimic (Figure 4A and B).

PCGEM1 overexpression contributed to suppression of inflammation in OVA-sensitized asthmatic mice following montelukast sodium treatment.

**Figure 2.** Prostate cancer gene expression marker 1 (PCGEM1) was expressed at a low level in ovalbumin (OVA)-sensitized asthmatic mice and normal control (MOCK). A, Infiltration of inflammatory cells in the lung tissues of asthmatic and control mice detected by HE staining (n=3, scale bar: 50 \( \mu m \)); B, RT-qPCR determination of lncRNA PCGEM1 expression in lung tissues of untreated asthmatic mice (n=3) and after treatment with montelukast sodium (MONT) and PCGEM1. Data are reported as means ± SD. *\( P < 0.05 \) vs the MOCK group; ^\( P < 0.05 \) vs the OVA group (one-way ANOVA, followed by Tukey’s multiple comparisons post hoc test). NC: negative control.
PCGEM1 overexpression, the lung tissue structure of asthmatic mice was significantly improved, and the number of inflammatory cells around the bronchi was significantly reduced (Figure 4C and D). As a result, PCGEM1 overexpression contributed to improvement in the pulmonary function in OVA-sensitized asthmatic mice following montelukast sodium treatment.

PCGEM1 overexpression plus montelukast sodium blocked the NF-κB signaling pathway in the OVA-sensitized asthmatic mice

Nuclear factor-κB (NF-κB) signaling plays an important role in the inflammatory response, and is activated in chronic obstructive pulmonary disease tissues (23). We speculated that PCGEM1 may mediate the expression of NF-κB. It was found that NF-κB mRNA expression, NF-κB protein expression, p-NF-κB expression, and NF-κB fluorescence (Figure 5A–C) were increased in the lung tissues of asthmatic mice, but was reduced in lung tissues of asthmatic mice treated with montelukast sodium and PCGEM1 mimic (P < 0.05). This suggested that IncRNA PCGEM1 may enhance the therapeutic effect of montelukast sodium on asthma by blocking the NF-κB signaling pathway.

**Discussion**

Airway inflammation attributed to eosinophil and T-lymphocyte infiltration is involved in asthma (24). The exploration of underlying mechanisms associated with asthma phenotypes could possibly lead to better molecule-targeted therapies and improved disease prognosis (25). Our study principally evaluated the role of PCGEM1 in the treatment of montelukast sodium for CVA and a ROC showed a good diagnostic value of PCGEM1. Animal experiments were conducted to further investigate the effects of PCGEM1 combined with montelukast sodium on the inflammation and lung function of an OVA-induced asthmatic murine model. The data showed that PCGEM1 strengthened the anti-inflammatory and lung protective effects of montelukast sodium in OVA-sensitized asthmatic mice.

Our data showed that montelukast sodium induced an anti-inflammatory response by increasing the level of IFN-γ together with reducing the levels of IL-3 and IL-4 and the numbers of total inflammatory cells, lymphocytes, macrophages, neutrophils, and eosinophils in the peripheral blood of children with CVA. Consistently, montelukast sodium suppressed eosinophilic inflammation in airways in mild asthmatic children (27). Montelukast sodium statistically elevates the serum level of anti-inflammatory protein IL-10 and reduces peripheral blood eosinophils following a 4-week treatment, providing clinical benefits for children with chronic asthma (28). As demonstrated by RT-qPCR determination, PCGEM1 was lowly expressed in CVA patients and its expression was elevated after montelukast sodium treatment and in the valid cases of CVA. IncRNAs are able to regulate inflammation by mediating recognized inflammatory mediators including...
TNF-α, IL-1, IL-6, and IL-18, as well as cell adhesion molecules such as ICAM-1 and VCAM-1 (29). IL-3-producing basophils that could release IL-4, IL-6, and IL-13 triggered exacerbation of airway hyperresponsiveness in a murine inflammatory model (30). IFN-γ causes reductions in type 2 innate lymphoid cell (ILC2) expansion and IL-13 expression thereby blocking the progression of asthma-like phenotype (31). This study confirmed the enhanced anti-inflammatory effects of montelukast sodium mediated by upregulation of PCGEM1, evidenced by inhibited inflammation in the BALF and reduced IL-4 and IL-13 levels and increased IFN-γ level in the lung tissues.

In addition, a 3-month montelukast sodium treatment improved pulmonary function by elevating pulmonary function indices PEF, FVC, FEV₁, and MEP50. In the murine model of asthma, montelukast sodium reduced airway hyperresponsiveness and increased FEV₀.₄/FVC identically. Pulmonary function indices, FEV₁, and PEF are the main markers of the severity of asthma and response to the treatment. Montelukast sodium elevated FEV₁ after 2 h (32), and simple montelukast sodium for children with CVA contributed to elevations in PEF and FEV₁ after treatment and at follow-up (33), with which our results are in line. Airway hyperresponsiveness and inflammation are causes of airway remodeling in asthma, which could be reversed by montelukast sodium (34). The in vivo experiments demonstrated that upregulation of PCGEM1 strengthened the protective effects of montelukast sodium on pulmonary function by reducing airway hyperresponsiveness and increasing FEV₀.₄/FVC.

Finally, upregulation of PCGEM1 combined with montelukast sodium treatment blocked the activation of NF-κB mRNA and protein after asthma induction. Inhibited NF-κB activation that mediates the production of IL-6, TNF-α, and MCP-1 is also required for the anti-inflammatory effect of montelukast sodium (35). Chrysophanol, another anti-asthmatic drug, ameliorates airway inflammation and remodeling by inhibiting the NF-κB signaling pathway (36). Montelukast sodium reduces Aβ1-42-induced toxicity to primary neurons via targeting CysLT1R-mediated dysfunction.
NF-κB signaling (37). lncRNAs interplay with mRNA and each other to control the mRNA stability and/or translation involved with lung inflammation (38). In a similar way, lncRNA HOTAIR alleviates inflammation in rheumatoid arthritis by targeting the NF-κB signaling pathway (39). Based on the above findings, it is reasonable to conclude that lncRNA PCGEM1 potentially enhances the therapeutic effect of montelukast sodium on asthma by blocking the NF-κB signaling pathway.

Taken together, our findings demonstrated that overexpression of PCGEM1 potentiated the anti-inflammatory effect of montelukast sodium on asthma by blocking the NF-κB signaling pathway. However, the blockade of NF-κB activation in this process remains to be confirmed with further pathway inhibitor experiments. No negative control group of montelukast sodium was included in our experimental study, which may affect our results to some degree. In the near future, further research will be carried out to discuss the molecular mechanism in CVA.

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