Overexpression of miR-224-5p alleviates allergic rhinitis in mice via the TLR4/MyD88/NF-κB pathway

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Abstract: Inflammatory allergic reaction is the main cause of allergic rhinitis (AR). Previous studies indicated that miR-224-5p was downregulated in the nasal mucosa of patients with AR, while the function of miR-224-5p in AR remains unclear. To explore this issue, AR mouse model was established using ovalbumin (OVA). For treatment group, lentivirus (LV)-miR-224-5p or its control was intranasally administrated to AR mice. miR-224-5p expression was detected by reverse transcription-quantitative PCR, followed by assessing the immunoglobulin E (IgE) level. Pathological alterations in nasal mucosa were detected using Hematoxylin-Eosin staining and Sirius red staining, followed by assessing the levels of inflammatory cells and factors. The NLRP3 inflammasome and TLR4/MyD88/NF-κB pathway were measured by Western blot, and then the relationship between miR-224-5p and toll-like receptor 4 (TLR4) was verified. The results showed that miR-224-5p was significantly decreased in nasal mucosa of AR mice. AR mice exhibited increased sneezing and nasal rubbing events, IgE level in serum, and pathological alterations in nasal mucosa, while overexpression of miR-224-5p markedly attenuated these changes. The levels of inflammatory cells in nasal lavage fluid and pro-inflammatory factors in serum and nasal mucosa were significantly increased in AR mice, which were reduced by miR-224-5p overexpression. Of note, LV-miR-224-5p treatment remarkably suppressed the activations of NLRP3 inflammasome and the TLR4/MyD88/NF-κB pathway in AR mice. Furthermore, miR-224-5p could bind to 3’-untranslated region (3’-UTR) of TLR4 and negatively regulate TLR4 level. Overall, we conclude that miR-224-5p may relieve AR by negatively regulating TLR4/MyD88/NF-κB pathway, indicating that miR-224-5p may be a promising target for AR treatment.

Key words: allergic rhinitis, inflammatory response, miR-224-5p, TLR4/MyD88/NF-κB pathway

Introduction

Allergic rhinitis (AR) is a nasal allergic disease with many common allergens, including dust mites, molds, pollen, pets, and smoke [1]. AR does not directly threaten the patient’s life, but seriously affects the patient’s quality of life, including sleep, cognitive function, and academic performance [2–4]. Conventional treatment methods for AR mainly include avoidance of allergens, medications, and immunotherapy [5, 6]. However, drug side effects and treatment complications are still inevitable. At present, AR has become a global health problem, it is necessary to explore the potential therapeutic targets for the treatment of AR.

MicroRNA (miRNA) is a small non-coding RNA that inhibits the translation of transcripts by binding to the 3’-untranslated region (UTR) [7]. Due to this characteristic, miRNA is involved in the development of multiple...
diseases. It has been reported that miR-224-5p overexpression inhibited the development of cancer, relieved the particulate matter 2.5 (PM2.5)-induced asthma, and improved the cerebral ischemia-reperfusion-induced inflammatory response and apoptosis [8–10]. The findings indicated the diversity of miR-224-5p function. Additionally, early studies have shown that miR-224-5p was downregulated in nasal mucosa of asthmatic patients with AR [11]. Moreover, the decreased miR-224-5p expression was also observed in the nasal mucosa of patients with AR [12]. Those findings suggest that miR-224-5p may play an important role in AR, but the specific mechanism is still unclear.

It is known that miRNAs play a role in many biological processes by targeting their downstream substrates. For example, miR-224-5p could alleviate PM2.5-induced asthma by targeting toll-like receptor 2 [10]. Bioinformatics analysis revealed that there were binding sites between miR-224-5p and the 3’-UTR of toll-like receptor 4 (TLR4). It was previously found that the mRNA and protein levels of TLR4 were significantly increased in nasal mucosa of patients with pollen-induced AR [13]. TLR4 inhibition alleviated allergic responses in mice with AR [14]. Previous results indicate that TLR4 plays a vital role in AR.

Based on the above, the present study mainly investigated the role of miR-224-5p in AR and its underlying mechanism. We hypothesized that miR-224-5p may play a role in AR by targeting TLR4.

Materials and Methods

Establishment of AR model in mice

The animal experiments were carried out according to the National Institutes of Health Guide for the care and use of laboratory animals and with the approval of the ethic committee of Shandong Provincial ENT Hospital Affiliated to Shandong University. The mice were housed at a temperature of 25 ± 1°C with a 12 h dark/light cycle. Male 8-week-old BALB/c mice were housed at a temperature of 25 ± 1°C with a 12 h dark/light cycle. All mice had free access to food and water. Mice were randomly divided into 4 groups after one week of adaptive feeding, namely: Sham group, AR group, AR+NC group, and AR+miR-224-5p group.

To establish a model of AR, mice were injected intraperitoneally with saline (200 µl) containing 25 µg ovalbumin (OVA) and 2 mg aluminum hydroxide on days 0, 7, and 14 to promote primary sensitization. One week after the primary sensitization, the secondary immunization was built. Mice were intranasally challenged with 3% OVA diluted in 20 µl of saline daily from 21 to 35 days. The mice in Sham group were given the same dose of normal saline. For the treatment group, the lentivirus with miR-224-5p overexpression or the blank lentivirus (1 × 10⁷ IFUs) was intranasally administrated to mice before 3 h of OVA challenge daily on days 28–34. After 35 days of OVA administration, the number of mice rubbing and sneezing within 10 min was recorded. Nasal lavage fluid (NLF) was collected 12 h after OVA treatment under anesthesia. In detail, mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). The skin on the neck of mice was cut, the trachea was separated, and the catheter was inserted into the nasopharynx. Pre-cooled PBS (1ml) was injected into the catheter and flow out through bilateral anterior nostrils. Repeat 3 times to collect NLF. The mice were then euthanized and nasal mucosa and blood samples were collected for subsequent experiments.

Reverse transcription-quantitative PCR

The total RNA extraction kit (Tiangen Biotech, Beijing, China) was used to extract total RNA from nasal mucosa. The concentration of RNA in each sample was determined using the UV spectrophotometer NANO 2000 (Thermo Fisher, Waltham, MA, USA). The RNA was reverse transcribed using RT Primer, dNTP, and RNase inhibitor. Quantitative real-time PCR was then performed with the help of cDNA template, primers, SYBR GREEN, and 2xTaq PCR MasterMix. The expression level of miR-225-5p was normalized by U6. The primers used in present study were purchased from GenScript Biotechnology Co., Ltd. (Nanjing, China).

Measurement of cytokines and immunoglobulin E (IgE)

Serum and tissue homogenate supernatant were obtained from blood and nasal mucosa, respectively. The protein concentration was detected using a microplate reader (Biotek, Winooski, VT, USA) and diluted to 1 mg/ml. Subsequently, the concentrations of IgE in serum and interleukin (IL)-4, IL-5, and IL-13 in serum and nasal mucosa were detected using the corresponding mouse ELISA Kits (Multi Sciences, Shanghai, China).

Histological analysis

The nasal mucosa tissues were embedded with paraffin and sectioned (5 µm). Hematoxylin-Eosin (H&E) staining and Sirius red staining were then performed. The morphology of the nasal mucosa was observed and the eosinophils infiltration in the nasal mucosa was detected.

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Inflammation cells counting
The eosinophils, neutrophils, and lymphocytes in nLF were stained by Wright’s-Giemsa assay for cell counting.

Western blot analysis
Total protein was extracted from nasal mucosa using RIPA lysis buffer and PMSF. Protein concentration was determined using the Bicinchoninic acid (BCA) Protein Assay kit (Solarbio, Beijing, China). Equal amount of protein (10–20 µg) from each sample was isolated using SDS-PAGE (8%, 12%, and 15% gel) and transferred to the polyvinylidene difluoride (PVDF) membranes. After blocking with 5% nonfat milk for 1 h at room temperature, the membranes were incubated with primary antibodies at 4°C overnight, followed by the incubation of horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3,000, Solarbio) for 1 h at 37°C. Subsequently, the membranes were visualized with electrochemiluminescence (Solarbio) for luminescence generation. GAPDH served as inner control for normalization. The primary antibodies used in present study were: the nucleotide binding and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3, 1:1,000, ABclonal, Wuhan, China), apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC, 1:1,000, ABclonal), cleaved caspase-1 (1:500, ABclonal), IL-1β (1:1,000, ABclonal), TLR4 (1:1,000, ABclonal), MyD88 (1:1,000, ABclonal), p65/p-p65 (Ser536) (1:1,000, Affinity, Cincinnati, OH, USA), GAPDH (1:10,000, Proteintech, Wuhan, China), and Histone H3 (1:1,000, GeneTex, San Antonio, TX, USA).

Immunofluorescence staining
The sections were blocked with goat serum for 15 min and then incubated with the primary antibody (p65, 1:200, Affinity) at 4°C overnight. After washed three times with PBS (5 min/time), the sections were incubated with Cy3-labeled goat anti-rabbit secondary antibody (1:200, Beyotime, Shanghai, China) at room temperature for 1 h. After washed three times with PBS (5 min/time), the nucleus was counterstained by DAPI (Aladdin, Shanghai, China). Finally, the stained sections were observed and photographed under a fluorescence microscope (Olympus, Tokyo, Japan, magnification, ×400).

Luciferase Reporter Assays
The human embryonic kidney cell line 293T was used to detect the relationship between miR-224-5p and TLR4. Cells were seeded into 12-well plates and the cell density was about 70%. Wild type (wt) and mutant (mut) type of TLR4 3’-UTR were cloned into pmirGLO plasmids (GenScript, Nanjing, China). 293T cells were co-transfected with miR-224-5p mimic or its negative control (NC). Luciferase detection kit (Keygen Biotech, Nanjing, China) was used to detect luciferase activity. The luciferase activity was detected in a microplate reader.

Statistical Analysis
Statistical analysis was performed using one-way ANOVA with Tukey’s multiple comparisons test. All data were presented as mean ± SD. P<0.05 was considered statistically significant and analyzed by Graphpad Prism.

Results
Overexpression of miR-224-5p alleviates OVA-induced AR in mice
To investigate the role of miR-224-5p in AR, we first detected the expression level of miR-224-5p in the control, model, and treatment groups. As presented in Fig.1a, the expression level of miR-224-5p in aR group was significantly decreased compared with control group, whereas the treatment of LV-miR-224-5p markedly increased miR-224-5p expression compared with aR+ nC group. The sneezing and nasal rubbing in 10 min were measured on day 35 of aR modeling. The results showed that AR mice suffered from severe sneezing and nasal rubbing compared with the control, whereas miR-224-5p upregulation alleviated these symptoms (Figs. 1b and c). The levels of OVA-specific IgE in serum in AR mice were dramatically increased, while miR-224-5p upregulation corrected the abnormal increases (Fig. 1d). Histologic changes of nasal mucosa in AR mice were detected by Hematoxylin-Eosin staining. It was found that AR mice showed hyperemia, edema, necrosis, and eosinophil infiltration in the nasal mucosa epithelium, whilst LV-miR-224-5p treatment alleviated these pathological changes, including the number of eosinophil (Fig. 1e). Overall, these data suggest that miR-224-5p plays an important role in the progression of AR, and overexpression of miR-224-5p may alleviate OVA-induced AR.

Overexpression of miR-224-5p reduces OVA-induced inflammatory response in mice
We next investigated the effects of miR-224-5p on OVA-induced inflammatory response. The results of Sirius red staining further confirmed the changes of eosinophils in AR mice and miR-224-5p overexpressed AR mice (Fig. 2a). Wright’s-Giemsa staining showed that
Overexpression of miR-224-5p alleviates OVA-induced AR in mice. (a) The expression level of miR-224-5p in mouse nasal mucosa. (b and c) Sneezing and nasal rubbing events in 10 min were recorded. (d) The concentrations of IgE in serum were measured using ELISA assay. (e) The pathological alterations in nasal mucosa were detected using Hematoxylin-Eosin staining. The number of eosinophils was analyzed statistically. Scale bar=100 µm. Data represent means ± SD (n=6). ***P<0.001 compared with sham group. ##P<0.01 and ###P<0.001 compared with AR+NC group. Sham group (normal mice), AR group (OVA-induced AR mice), AR+NC group (AR mice treated with empty lentivirus), AR+miR-224-5p group (AR mice treated with lentiviral-mmu-miR-224-5p). IgE, immunoglobulin E; ELISA, enzyme-linked immunosorbent assay; OVA, ovalbumin; NC, negative control; AR, allergic rhinitis.
Fig. 2. Overexpression of miR-224-5p reduces OVA-induced inflammatory response in mice. (a) The changes of eosinophils in nasal mucosa were assessed using Sirius red staining, and the number of eosinophils was counted. Scale bar=100 μm. (b) The number of eosinophils, neutrophils and lymphocytes in NLF were counted using Wright’s-Giemsa assay. Scale bar=100 μm. Blue arrow represents eosinophils; Red arrow represents neutrophils; Green represents lymphocytes. (c and d) The concentrations of IL-4, IL-5, and IL-13 in serum and nasal mucosa were detected using eLISA assay. Data represent means ± SD (n=6). ***P<0.001 compared with sham group. #P<0.05, ##P<0.01 and ###P<0.001 compared with aR+ nC group. Sham group (normal mice), aR group (OV a-induced aR mice), aR+nC group (aR mice treated with empty lentivirus), aR+miR-224-5p group (aR mice treated with lentiviral-mmu-miR-224-5p). NLF, nasal lavage fluid; IL-4, interleukin-4; IL-5, interleukin-5, IL-13, interleukin-13; eLISA, enzyme-linked immunosorbent assay; OVA, ovalbumin; nC, negative control; AR, allergic rhinitis.
Overexpression of miR-224-5p alleviates allergic rhinitis

The number of eosinophils, lymphocytes, and neutrophils in NLF was significantly increased in AR mice compared with normal mice, whereas miR-224-5p overexpression decreased their levels (Fig. 2b). Meanwhile, the levels of IL-4, IL-5, and IL-13 (pro-inflammatory cytokines) in serum and nasal mucosa were significantly increased in AR group compared with sham group, whereas their levels were decreased by miR-224-5p overexpression (Figs. 2c and d). On the contrary, AR mice exhibited decreased IFN-γ (anti-inflammatory factor) level in nasal mucosa, and miR-224-5p overexpression just slightly increased IFN-γ level (SF. 1). Collectively, these data imply that miR-224-5p can alleviate the inflammatory response of AR mice.

Overexpression of miR-224-5p inhibits the activation of NLRP3 inflammasome in nasal mucosa of AR mice

The effects of miR-224-5p on NLRP3 inflammasome in AR mice were also investigated. As shown in Fig. 3, the protein expression levels of NLRP3, ASC, cleaved caspase 1, and IL-1β in nasal mucosa were dramatically increased in AR mice, which were markedly reduced by the administration of LV-miR-224-5p. Therefore, the results indicate that upregulation of miR-224-5p may inhibit the activation of NLRP3 inflammasome in AR mice.

Overexpression of miR-224-5p blocks the TLR4/MyD88/NF-κB pathway in AR mice

We further explored the specific mechanism by which miR-224-5p regulates the inflammatory response in AR mice. The protein expression levels of TLR4, MyD88, and p-p65 in AR mice were markedly increased compared with normal mice, whereas their levels were significantly decreased by miR-224-5p overexpression (Fig. 4a). AR mice showed increased nuclear translocation of p65 compared with normal mice, which was attenuated by miR-224-5p overexpression (Fig. 4b). Meanwhile, the protein level of p65 in cytoplasm was significantly decreased and p65 protein in nucleus was increased in nasal mucosa of AR mice (Fig. 4c). miR-224-5p overexpression reversed the decrease of p65 protein level in cytoplasm and the increase of p65 level in nucleus (Fig. 4c). These data suggest that miR-224-5p inhibits the activation of the TLR4/MyD88/NF-κB pathway in AR mice.

miR-224-5p regulates the TLR4/MyD88/NF-κB pathway by targeting TLR4 in AR mice

The relationship between miR-224-5p and TLR4 was detected by luciferase reporter assays. The results showed that the transfection of miR-224-5p mimic observably decreased the luciferase activity in cells transfected with wild-type TLR4 3’-UTR reporter, no significant change was observed in other groups (Fig. 5). Therefore, the results indicate that miR-224-5p can target TLR4.

Fig. 3. Overexpression of miR-224-5p inhibits the activation of NLRP3 inflammasome in nasal mucosa of AR mice. The protein expression of NLRP3, ASC, cleaved caspase 1 (p20), and IL-1β in nasal mucosa were measured by Western blot. ***P<0.001 compared with sham group. ###P<0.001 compared with AR+NC group. Sham group (normal mice), AR group (OVa-induced AR mice), AR+NC group (AR mice treated with empty lentivirus), AR+miR-224-5p group (AR mice treated with lentiviral-mmu-miR-224-5p). NLRP3, the nucleotide binding and oligomerization domain-like receptor family pyrin domain-containing 3; ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; IL-1β, interleukin-1β; OVA, ovalbumin; NC, negative control; AR, allergic rhinitis.
Fig. 4. Overexpression of miR-224-5p blocks the TLR4/MyD88/NF-κB pathway in AR mice. (a) The protein expression levels of TLR4, MyD88, and p-p65 (Ser536) in nasal mucosa. (b) Immunofluorescence staining of p65 in nasal mucosa. Scale bar=50 µm. (c) The protein levels of p65 in cytoplasm and nucleus were measured by Western blot. Data represent means ± SD (n=6). ***P<0.001 compared with sham group. ###P<0.001 compared with AR+nC group. Sham group (normal mice), AR group (OVa-induced AR mice), AR+NC group (AR mice treated with empty lentivirus), AR+miR-224-5p group (AR mice treated with lentiviral-mmumirR-224-5p). TLR4, toll-like Receptor 4; OVA, ovalbumin; NC, negative control; AR, allergic rhinitis.
Discussion

The present study demonstrates for the first time that overexpression of miR-224-5p can alleviate AR. The results indicated that miR-224-5p was significantly decreased in AR mice and miR-224-5p overexpression alleviated the pathology, inflammatory cell and cytokines disorders, and the activation of NLRP3 inflammasome in AR mice. Furthermore, overexpression of miR-224-5p inhibited the activation of the TLR4/MyD88/NF-κB pathway in the nasal mucosa of AR mice and this inhibitory effect might be achieved by targeting TLR4. Collectively, the present study demonstrates that miR-224-5p may relieve AR by negatively regulating TLR4/MyD88/NF-κB pathway, indicating that miR-224-5p may be a promising target for AR treatment.

AR is a chronic inflammatory disease involving the activation of various immune cells and the release of cytokines [15]. When the upper respiratory tract is exposed to allergens, antigen-presenting cells present the allergens to Th2 lymphocytes, and then induce B cells to differentiate into plasma cells, and further produce IgE antibodies [16–18]. Antigen-specific IgE binds to the high-affinity IgE receptor FcER1 on mast cells and basophils, resulting in the secretion of a variety of pro-inflammatory cytokines and chemokines to trigger inflammatory allergic reactions [5]. Previous studies have shown that miRNAs played a vital role in the progression of AR [19, 20]. Early studies found that miR-224-5p was decreased in nasal biopsies of patients with asthma [11]. Microarray analysis showed that miR-224-5p was significantly decreased in the nasal mucosa of AR patients [12]. These findings indicate that miR-224-5p may play an important role in allergic diseases, including AR. The present study showed that miR-224-5p was markedly decreased in AR mice and the overexpression of miR-224-5p alleviated nasal symptoms (rubbing and sneezing) and histological alteration in AR mice. The results indicated that miR-224-5p can alleviate AR and may become a potential target for AR treatment.

IgE has been reported to mediate the immune response of a variety of allergic diseases, including AR [21]. Here, we found that miR-224-5p overexpression reduced the level of IgE, indicating that miR-224-5p overexpression may reduce the immune response of AR mice. Moreover, overexpression of miR-224-5p reduced the number of inflammatory cells and the activation of NLRP3 inflammasome in AR mice. NLRP3 inflammasome has been reported to be related to a variety of diseases, including inflammatory diseases and autoimmune diseases [22]. The activation of NLRP3 inflammasome promoted the development of AR, and knockout of NLRP3 inhibited the inflammatory response in AR mice [23]. The present study indicated that miR-224-5p overexpression inhibited the inflammatory response in AR mice. Additionally, we found that miR-224-5p overexpression alleviated Th2 inflammation, as evidenced by decreased IL-4, IL-5, and IL-13 in LV-miR-224-5p treated AR mice. Meanwhile, we demonstrate that miR-224-5p may not have a significant effect on Th1 inflammation, because no significant change was observed in IFN-γ level after LV-miR-224-5p treatment. The induction of AR is mainly caused by IgE mediated immune response, inflammatory cell and cytokines disorders [24, 25]. Therefore, the results demonstrated that overexpression of miR-224-5p may alleviate AR in mice by suppressing the immune response and Th2 inflammatory response.

We further explored the potential mechanism of miR-224-5p relieving AR in mice. It has been reported that TLRs can mediate the activation of the innate immune system [26]. TLR4 is an important innate immune receptor, which plays a vital role in recognizing foreign antigens, including allergens, and subsequently inducing immune responses [27]. The results of previous studies have shown that TLR4 has an important regulatory role in AR. Specifically, the mRNA and protein levels of TLR4 in the nasal mucosa of patients with pollen-induced allergic rhinitis were significantly increased [13]. In addition, short ragweed pollen could induce AR and increased Th2 cytokine production in a TLR4-dependent manner [28]. The signal transduction pathway mediated...
by TLRs can be composed of MyD88-dependent pathways. The TLR4/MyD88/NF-κB pathway is the positive regulatory pathway of inflammatory response [29]. It has been reported that the blockade of TLR4/MyD88/NF-κB signaling pathway inhibited the inflammatory response induced by lipopolysaccharide [30]. Moreover, TLR4/MyD88/NF-κB pathway mediated the activation of NLRP3 inflammasome [29, 31]. It has been previously reported that the TLR4/MyD88/NF-κB signaling pathway was activated in OVA-induced asthmatic mice [32]. Here, we found that the upregulation of miR-224-5p inhibited the activation of the TLR4/MyD88/NF-κB signaling pathway in AR mice, indicating that miR-224-5p may alleviate AR in mice through blocking the TLR4/MyD88/NF-κB signaling pathway. Moreover, this blockade by miR-224-5p may be achieved by targeting the 3′-UTR of TLR4. Of note, the present study showed that AR mice exhibited increased neutrophils, lymphocytes, and eosinophils in NLF, which was decreased by LV-miR-224-5p treatment, indicating that inflammatory cell infiltration was occurred in nasal mucosa of AR mice, and miR-224-5p overexpression alleviated this phenomenon. It is well known that NF-κB, as a transcription factor, is able to regulate the gene expression of proinflammatory mediators, including chemokines and cytokines, and those factors contribute to the recruitment of inflammatory cells in tissues, such as neutrophil [33, 34]. Thus, the inhibitory effect of miR-224-5p on inflammatory cell infiltration, including neutrophil, may be achieved by inhibiting the release of inflammatory cytokines through blocking the NF-κB pathway. miRNA is considered to perform numerous functions by targeting downstream genes. Here, we found that miR-224-5p could target TLR4 and negatively regulated its expression, indicating that miR-224-5p may regulate the TLR4/MyD88/NF-κB signaling pathway in AR mice by targeting TLR4.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Data Availability Statement

The data will be made available from the corresponding author on reasonable request.

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Author Contribution

Jianhua Wu, Lizhen Wu, Li Zhang, and Kaiyue Sun conceived and designed the experiments. Huanhuan Xu, Min Wang, and Lin Wang performed the experiments and analyzed the data. Jie Chen contributed reagents/materials/analysis tools. Jianhua Wu and Kaiyue Sun wrote the paper.
OVEREXPRESSION OF MIR-224-5P ALLEVIATES ALLERGIC RHINITIS

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