MicroRNA-29 mediates anti-inflammatory effects and alleviation of allergic responses and symptoms in mice with allergic rhinitis

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Abstract

Background: To investigate the role of microRNA-29 (miR-29) in mice with allergic rhinitis (AR) and its underlying mechanism.

Methods: AR model was established in BALB/c mice by intraperitoneal sensitization and intranasal challenge with ovalbumin (OVA). miRNA expression was examined in the nasal mucosa tissues of mice and patients with AR, and miRNA-29 was found to be downregulated. To unveil the role of miRNA-29 in AR, it was overexpressed in the nasal mucosa of AR mice by intranasal administration of miRNA-29 agomir. The symptoms of nasal rubbing and sneezing were recorded and evaluated. miR-29 expression, OVA-specific immunoglobulin E (IgE) concentration, pro-inflammatory cytokines levels, eosinophils number, and cleaved caspase-3 and CD276 expression were examined in nasal mucosa tissues and nasal lavage fluid (NALF) by qRT-PCR, ELISA, hematoxylin and eosin staining, western blotting, or immunohistochemistry, respectively. TUNEL assay was used to analyze nasal mucosa cells apoptosis.

Results: Decreased expression of miR-29 was observed in AR, the symptoms of which were alleviated by overexpressing miR-29. In addition, overexpression of miR-29 markedly reduced the concentration of OVA-specific IgE, the levels of IL-4, IL-6, IL-10, and IFN-γ, the pathological alterations and eosinophils infiltration in the nasal mucosa. Furthermore, restoration of miR-29 expression reduced nasal mucosa cell apoptosis. Moreover, overexpression of miR-29 significantly attenuated CD276 mRNA and protein levels in nasal mucosa cells.

Conclusion: MiR-29 mediated anti-inflammatory effects in OVA-induced AR mice by decreasing inflammatory response, probably through targeting CD276. MiRNA-29 may serve as a potential novel therapeutic target for the treatment of AR.

Keywords: miRNA-29, Allergic rhinitis, Nasal mucosa, Cytokines, CD276

Background

Allergic rhinitis (AR) is an inflammatory condition of the nasal mucosa that is mediated by an IgE-associated response to indoor and outdoor environmental allergens [1]. AR is a common disease and a risk factor for the development of other diseases, such as asthma.
serious side effects and do not show sufficient subjective or objective improvements in about 20% of AR patients [5, 6]. Therefore, it is still necessary to identify a more effective treatment strategy for AR patients.

It has been well recognised that inflammatory mechanisms play a key role in the development of AR [7, 8]. The inflammatory response is associated with an increased number of eosinophils and upregulated levels of proinflammatory cytokines, such as interleukin (IL)-4, IL-6, IL-10, and interferon (IFN)-γ [9]. MicroRNAs (miRNAs) are small, single-stranded RNAs that bind to the 3′-untranslated region (UTR) of target messenger RNAs to regulate their expression [10–12]. Accumulating evidence has suggested that miRNAs are involved in the development of tumors, autoimmune diseases, and other systemic diseases [13, 14]. Teng et al. [1] observed that miRNA-143 was significantly downregulated in the nasal mucosal tissues of AR patients and inhibited the production of inflammatory cytokines and mucus by nasal epithelial cells. MiRNA-let-7e has been shown to regulate the progression and development of AR via its anti-inflammatory effects [15]. Together, these reports have demonstrated that miRNAs play a significant role in the regulation of AR.

CD276 (also known as B7-H3) is a member of the B7/CD28 immunoglobulin superfamily that provides crucial co-stimulatory signals that promote T cell functions involved in tumor surveillance, infection response, and autoimmune diseases [16]. CD276 is overexpressed in tumor and tumor-associated cells, making it an interesting therapeutic target [17]. Hong Xu et al. [18] found that miRNA-29a (miR-29a) directly targets the CD276 3′-UTR in HeLa cells, and may have potential applications for immune-based therapy of human solid tumors. In addition, the miR-29c/CD276 axis has been identified to play an important role in childhood asthma via regulation of Th2/Th1 cell differentiation [19]. Another study has reported that miR-29 mediates the innate and adaptive immune responses to bacterial infections by targeting IFN-γ [20]. The plasma levels of miR-29 are reduced in both allergic and asthmatic patients compared to healthy subjects [21]. Together, these studies have suggested that miR-29 may be involved in the allergic and immune processes of AR. However, to our knowledge, there has been no prior study examining the in vivo effect of miR-29 on allergy symptoms and AR. Therefore, this study aimed to investigate the role and significance of miR-29 in mice with OVA-induced AR, and to examine whether miR-29 has any effect on the development of AR.

**Methods**

**Tissue samples**

This study included tissues samples from 9 AR patients and 9 healthy control patients. The diagnosis and treatment of AR were carried out by their physicians. Out of the 9 AR patients, 5 were males and 4 were females with a mean age of 40.2 years (range: 26–57 years). There were 3 cases with positive skin prick tests, 2 cases with family history of asthma and 1 cases with seasonal asthma. There was no history of smoking and no use of hormone drugs in the past 2 weeks. The results of chest X-ray examination were normal. The healthy control group included 7 males and 2 females with a mean age of 38 years (range: 24–58 years). There was no history of rheumatism or AR. There was no upper respiratory tract infection, no use of hormones and anti allergic drugs in the past 1 month, and the results of chest X-ray examination were normal. The epithelial samples were gently scraped from the surface of the inferior nasal turbinate using a plastic curette. All participants have signed an informed consent form and been informed of all the experimental details in advance.

**Animals**

A total of 32 8-week-old pathogen-free male BALB/c mice (18–20 g) were purchased from the Shanghai Xinmao Experimental Animal Center (Shanghai, China) and divided randomly into four groups of 8 mice each: control (Con), OVA-induced AR (OVA), OVA-induced AR with miR-29 agomir treatment (OVA+miR-29), and OVA-induced AR with mismatched agomir (MA) treatment (OVA+miR-MA). All mice were housed in the campus animal facility under standard conditions, including 12 h light/dark cycles, average temperature of 18–22 °C, and mild humidity (50–60%), as well as free access to food and water ad libitum. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. At the end of the experiment, mice were anesthetized with 1.25% pentobarbital (40 mg/kg) and sacrificed by CO2 gas asphyxiation for tissue collection.

**AR model establishment and treatments**

The OVA-induced AR mouse model was generated as previously described [22]. Briefly, mice were injected intraperitoneally with 200 μL saline including 50 μg OVA (Grade V, Sigma, St. Louis, MO, USA) adsorbed to 1 mg aluminum hydroxide (Thermo Scientific, Rockford, MD, USA) on days 1, 8, and 15. From days 22 to 28, the mice received a daily intranasal challenge with 20μL of 1 mg/mL OVA per nostril. Animal experiments were repeated three times.
**Symptom scores**

Thirty minutes after the final OVA challenge, we examined the behavior of the AR mice, including the amount of nasal rubbing and sneezing motions in a given time. A video monitoring device recorded all of the symptoms within a 10 min period. The analysis of behavior was then conducted by professional personnel who were blinded to the groups.

**Intranasal administration of miR-29 agomir**

The miR-29 agomir and the corresponding mismatched agomir (miR-MA) were purchased from Shanghai GeneChem (Shanghai, China). On days 22–28, the miR-29 agomir was diluted to 5 pmol/μL in 20 μL saline and intranasally injected daily into each nostril of the mice in the OVA + miR-29 group 3 h before the OVA challenge. The OVA + miR-MA group was intranasally injected with the same dose of miR-MA. The OVA and Con groups were intranasally treated with saline.

**Quantitative real-time qPCR**

After euthanasia, nasal mucosal tissues were collected and DNA was extracted using an RNeasy Mini Kit (Qiagen, CA, USA) according to the manufacturer's instructions. The expression of genes of interest was analyzed by fluorescence quantitative polymerase chain reaction (qPCR). Gene expression levels were measured using the 2^−ΔΔCt method and normalized to the internal reference gene of β-actin or U6 expression. The primers used for qPCR analyses are listed in Table 1.

**Quantitative measurement of cytokines**

The nasal lavage fluid (NALF) was collected after irrigation and was centrifuged at 8000 × g at 4 °C for 15 min to obtain the supernatant. To evaluate the allergic reaction, cytokines (IL-4, IL-6, IgE, and IFN-γ) were measured using ELISA kits (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. While the concentrations of Eotaxin and RANTES were measured using the DuoSet Mouse ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

**Histological analyses**

Fresh nasal mucosal tissue was isolated, fixed with formalin, and then embedded in paraffin. Tissue sections (5 μm thick) were then stained with hematoxylin and eosin (H&E) according to the manufacturer’s instructions. The eosinophils in nasal lavage fluid and nasal mucosa were studied by HE staining. We observed the morphology of the nasal mucosal epithelium and the number and distribution of eosinophils in the nasal mucosa. Apoptosis was detected by in situ labeling using a TUNEL kit (Beyotime, Jiangsu, China) according to the manufacturer’s instructions. The TUNEL-positive cells were observed under a fluorescence microscope (Leica DM4000, Wetzlar, Germany). The apoptosis rate was quantified by counting TUNEL-positive cells from five random fields of view.

**Cell culture and miRNA transfections**

The cell line from the nasal mucosa of mice was purchased from American Type Culture Collection (ATCC) and cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% FBS, 100 mg/mL streptomycin, and 100 units/mL penicillin at 37 °C in an atmosphere of 5% CO2 and 95% relative humidity. For transfection of these cells, miR-29 mimics or their corresponding negative controls (Rio Biotechnology, Guangzhou, China) were diluted in OptiMEM I medium (Invitrogen, Carlsbad, CA, USA) and transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) transfection reagent according to the manufacturer's instructions.

**Luciferase experiments**

The luciferase reporter assays were performed to examine the direct binding of miR-29 to the target CD276 mRNA. Wild-type and mutant reporter plasmids of CD276 (Gene ID: 102,657, GenBank) which containing a wild or mutant miR-29 binding sites were synthesized by GenePharma and amplified and cloned into the pMIR-ReportTM vector (Ribobio Co., Guangzhou, China). Nasal mucosal...
cells from mice were co-transfected with the 3′-UTR of CD276 (with either wild-type or mutant miR-29 binding sites) and either miR-29 mimics or miR-29 negative controls using Lipofectamine 2000 (Invitrogen). After incubation for 48 h, the Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) was performed to examine whether miR-29 directly binds CD276. Renilla luciferase activity was used as an internal control for normalization.

**Western blotting**

Total protein was extracted from the nasal mucosal cells of mice using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Shanghai, China). Equal amounts of the protein samples were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was blocked by 5% non-fat milk for 2 h at 25 °C and incubated with the primary antibodies overnight at 4 °C. The next day, the membrane was washed 5 times with 1× PBST and then incubated with the secondary antibody for 1 h at 25 °C. The membrane was developed using enhanced luminol-based chemiluminescence. The results were photographed using a UVP BioSpectrum Imaging System (BioSpectrum, Orangevale, CA, USA).

**Statistics**

All data are presented as mean±SEM and compared by one-way ANOVA with Tukey’s post-hoc test. All statistical analyses were performed using the SPSS 16.0 software and graphical representations were generated using GraphPad Prism 5 (San Diego, CA, USA) software. Results were considered significant when they reached a 95% confidence level (P < 0.05).

**Results**

The levels of miR-29 are suppressed in the nasal mucosa of AR patients and AR mice

We collected the nasal mucosal tissues of AR patients and matched healthy controls, and performed qPCR to detect the amount of miR-29 in each of the samples. MiR-29 levels were much lower in the AR patients than in the healthy controls (Fig. 1a, t = 7.43, p < 0.001). The levels of miR-29 were also significantly lower in AR mouse tissues than in control tissues (Fig. 1b, t = 6.64, p < 0.001). To further investigate the role of miR-29, we administrated mir-29 in mice AR model (Fig. 1c).
The overexpression of miR-29 alleviates the symptoms of AR
We overexpressed miR-29 in the nasal mucosa of AR mice via intranasal administration of miR-29 agomir. As a control, some mice were intranasally treated with mismatched agomir (miR-MA). Compared to the control group, AR symptoms in the OVA-induced group were more severe, causing a significant increase in nasal friction (Fig. 2a, F = 6.732, p < 0.001, one-way ANOVA) and sneezing (Fig. 2b, F = 8.543, p < 0.001, one-way ANOVA). However, the symptoms of AR were alleviated in the miR-29 overexpression group. After the behavioral test, nasal mucosa tissues were collected to perform RT-qPCR test to verify the effect of miR-29 agomir. The miR-29 agomir treatment did increase the miR-29 level in nasal mucosa (Fig. 2c). These results indicated that miR-29 overexpression is able to reduce AR symptoms.

Restoration of miR-29 reduces the levels of cytokines in the NALF of AR mice
To determine the effect of miR-29 on local inflammation, we collected the serum and NALF from the different mouse groups and measured the levels of inflammatory cytokines. The levels of cytokines were substantially increased in the NALF of AR mice compared to that in the NALF of control mice. However, treatment with miR-29 agomir reduced the levels of these cytokines IL-4 (Fig. 3a, F = 8.554, p < 0.001, one-way ANOVA), IL-6 (Fig. 3b, F = 7.356, p < 0.001, one-way ANOVA), and IFN-γ (Fig. 3c, F = 6.976, p < 0.001, one-way ANOVA), whereas that with miR-MA had no significant effect. Next, we examined the levels of eosinophils and the secreted mediator proteins in the NALF of the mice. Eosinophils (Fig. 3d, F = 7.875, p < 0.001, one-way ANOVA), Eotaxin (Fig. 3e, F = 9.452, p < 0.001, one-way ANOVA), and RANTES (Fig. 3f, F = 5.355, p < 0.001, one-way ANOVA) levels were all elevated in the AR mice compared to the control mice, but significantly reduced in mice treated with miR-29 agomir.

Restoration of miR-29 reduces eosinophil infiltration and apoptosis in the nasal mucosa of AR mice
We investigated the histopathological changes in AR mice following overexpression of miR-29. H&E staining demonstrated that there was a significant influx of eosinophils into the nasal mucosa of the AR group compared to the control group. Restoration of miR-29 levels significantly decreased eosinophil infiltration (Fig. 4a, c, F = 8.896, p < 0.001, one-way ANOVA). In addition, TUNEL staining was used to evaluate the level of apoptosis in the nasal mucosa of the mice. OVA administration significantly increased TUNEL-positive cells, indicating increased levels of apoptosis. However, restoration of miR-29 levels efficiently suppressed apoptosis (Fig. 4b, d, F = 7.979, p < 0.001, one-way ANOVA).

Restoration of miR-29 reduces inflammation-related gene expression in the nasal mucosa of AR mice
To investigate the association between miR-29 and inflammation, the amounts of IL-6 and cleaved caspase-3 were examined by immunostaining in each group. The increased amounts of IL-6 (Fig. 5a, b, F = 13.334, p < 0.001, one-way ANOVA) and Cleaved caspase-3 (Fig. 5c, d, F = 11.164, p < 0.001, one-way ANOVA) following OVA challenge were attenuated by miR-29 overexpression. The mRNA expression of relevant inflammatory factors, including IL-6 (Fig. 5e, F = 10.331, p < 0.001, one-way ANOVA), Cleaved caspase-3 (Fig. 5f, F = 14.434, p < 0.001, one-way ANOVA), IL-4 (Fig. 5g, F = 15.423, p < 0.001, one-way ANOVA), IL-10 (Fig. 5h, F = 14.453, p < 0.001, one-way ANOVA), and

Fig. 2 MiRNA-29 inhibited the OVA-induced nasal allergy symptoms. a, b The amount of nasal rubbing (a) and sneezing (b) in a 10 min period following ovalbumin (OVA) challenge. The data are presented as mean ± SEM, n = 8 for each group. ***, P < 0.001 vs. Con; #, P < 0.01 vs. OVA by ANOVA
IFN-γ (Fig. 5i, \( F = 7.534, p < 0.001 \), one-way ANOVA), was assessed and was found to be increased upon administration of OVA and decreased upon treatment with miR-29 agomir. Meanwhile, there was no significant difference between the OVA + miR-MA group and the OVA group.

CD276 is the target gene of miR-29

To investigate the possible target genes of miR-29 in regulating inflammation, we used the miRNA target gene prediction website TargetScan (www.targetscan.org). We identified highly conserved miR-29b binding sites in the 3′-UTR of CD276, and partial sequence alignments of miR-29 and CD276 (Fig. 6a, \( t = 7.24, p < 0.001 \)). Accordingly, a previous study found that miR-29a directly targets the 3′-UTR of CD276 in HeLa cells [18]. To confirm the in silico results, miR-29 mimics or corresponding negative controls were co-transfected with either pCD276-WT or pCD276-MUT (CD276 with a mutated binding site) in murine nasal mucosa cells. A luciferase reporter assay showed that transfection with the miR-29 mimics significantly inhibited the luciferase activity associated with the wild-type, but not the mutated, pCD276 (Fig. 6b, \( t = 7.12, p < 0.001 \)). These results demonstrated that miR-29 binds to and suppresses CD276 expression in a direct and specific manner. The expression of CD276 was also examined by immunohistochemistry of the nasal mucosa. The results of the staining showed that CD276 levels increased significantly in OVA-treated mice compared to those in control mice. Restoration of miR-29 remarkably decreased the expression of CD276 (Fig. 6c, \( F = 7.75, p < 0.001 \)). Immunohistochemistry experiments (Fig. 6d) revealed a consistent pattern with immunoblotting results that upregulation of miR-29 caused reduced CD276 protein expression in the nasal mucosa (Fig. 6e, \( F = 6.643, p < 0.001 \), one-way ANOVA). These data indicate that miR-29 negatively regulates CD276 expression.

Discussion

AR is triggered after allergen-specific IgE and Th2 cells recognize inhaled allergens in the environment and elicit an inflammatory process that involves many different inflammatory cells and molecules, including eosinophils, cytokines, and other regulatory molecules [23]. MiR-29 consists of three different isoforms: miR-29a, miR-29b, and miR-29c. These mature isoforms...
of miR-29 are silenced or downregulated in different kinds of cancers [8-10]. It has also been reported that suppression of miR-29 may lead to several inflammatory diseases [24, 25]. In addition, miR-29 has been identified as a potential biomarker and therapeutic target for allergies and asthma [26, 27].

The present study demonstrated that miR-29 was downregulated in human patients and mice with AR when compared with control groups. To avoid the possible systemic complications that could be caused by tail vein injection, we intranasally administered miR-29 agomir in AR mice and investigated its direct effects on the nasal mucosa. A previous study had indicated that mice with OVA-induced AR display nasal allergy symptoms similar to humans [28]. Our research showed that the frequencies of nasal rubbing and sneezing were much lower in AR mice overexpressing miR-29 than in the normal AR model mice, indicating that intranasal administration of miR-29 agomir ameliorates AR symptoms.

MiR-29 overexpression also inhibited the expression levels of OVA-specific IgE and proinflammatory cytokines (IL-4, IL-6, IL-10, and IFN-γ) in the NALF of AR mice. Furthermore, we observed that miR-29 markedly inhibited the pathological changes of OVA-induced AR and reduced the number of infiltrating eosinophils. Eosinophil infiltration in the tissues is the main characteristic of allergic inflammation in humans [29]. IL-4 and IL-10 are Th2 cytokines produced by mast cells, T cells, and macrophages. They play an important role in regulating IgE isotype switching in B cells and the differentiation of T cells into Th2 cells [30]. IFN-γ, a Th1-related cytokine, has been found to regulate IgE-mediated allergies and asthma [31, 32].

The above results indicate that the decrease in Th1
and Th2 cytokines may underlie the anti-inflammatory effect of miR-29 in an AR model.

In addition to inflammation, aberrant apoptosis of nasal mucosa cells can contribute to AR symptoms [33]. Through the use of TUNEL assays, immunohistochemistry, and qPCR of the apoptosis-related gene caspase-3, we determined that miR-29 was able to prevent apoptosis in cells of the nasal mucosa. These results suggest that miR-29 alleviates OVA-induced AR in mice, at least partially, through an anti-apoptotic mechanism.

CD276 (also known as B7-H3) belongs to a family of immune modulators that are expressed in various immune cells, such as dendritic cells (DCs), monocytes, and activated T cells [34]. An anti-CD276 monoclonal antibody has previously been used as an effective treatment to alleviate asthmatic syndromes [35]. Therefore, inhibition of CD276 signals may provide a
novel therapeutic approach for the treatment of allergic asthma. In this study, the luciferase reporter gene assay in murine nasal mucosa cells showed that CD276 is a target gene of miR-29, which is consistent with a previous study by Hong Xu et al. [18]. Thus, the mechanism by which miR-29 overexpression can inhibit the expression of inflammatory cytokines and prevent the development of AR-related histological changes likely involves the ability of miR-29 to reduce expression of CD276. Although there have been no previous studies linking miR-29 to AR, miR-29c was previously reported to be involved in allergic asthma inflammatory diseases [19]. The current study, together with the miR-29c study by Zhang et al. (38), suggests that the miR-29/CD276 regulatory axis may be a general mechanism underlying inflammatory diseases, including allergic airway inflammation diseases.
The role of this regulatory axis in a potentially wide array of inflammatory diseases warrants further investigation.

In conclusion, the results of our study demonstrate that miRNA-29 alleviates the symptoms and allergic responses of OVA-induced AR, potentially by regulating the expression of CD276 and other inflammation-related cytokines.

Abbreviations
mir-29: Microrna-29; AR: Allergic rhinitis; OVA: Ovalbumin; ige: Immunoglobulin E; NALF: Nasal lavage fluid; IFN: Interferon; UTR: Untranslated region; MA: Mismatched agomir; ATCC: American Type Culture Collection; dcs: Dendritic cells.

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All experimental protocols were approved by the Ethics Committee of the Beijing Shijitan Hospital of Capital Medical University (sjtkyll-lx-2020(9)).

Authors' contributions
Y.J-S. conceptualized and designed the research. W.J and L.A-Z. performed experiments, acquired and analyzed the data, and wrote the paper. P.H. prepared the data figures. All authors have read and approved the final manuscript.

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Availability of data and materials
The datasets used or/and analyzed during the current study are available in the corresponding author on reasonable request.

Ethics approval and consent to participate
All experimental protocols were approved by the Ethics Committee of the Beijing Shijitan Hospital of Capital Medical University (sjtkyll-lx-2020(9)). All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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