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Original Research Article

Influence of miR155 on allergic conjunctivitis in mice via regulation of NF-κB signal pathway

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Abstract

Purpose: *To investigate the effect of miR-155 on allergic conjunctivitis (AC) in mice, and to elucidate the mechanism of action.*
Methods: *Sixty (60) Balb/c mice were randomly divided into three groups with 20 mice per group. Ovalbumin (OVA) was used to induce experimental model of AC in mice. Mice in the AC+miR-155 siRNA group were given miR-SiRNA once daily for 2 weeks before inducing AC. The expressions of miR-155 in conjunctival tissue of the control and AC groups were assayed with reverse transcription-polymerase chain reaction (RT-PCR). In addition, anti-OVA IgE antibody, eotaxin, IL-13 and IFN-γ levels were determined using ELISA (enzyme-linked immunosorbent assay). The regulatory effect of miR-155 on the NF-κB signal pathway in mice conjunctiva tissue with AC was determined using immunoblotting.*
Results: *Higher miR-155 expression was seen in serum of AC group than in that of control group ($p < 0.05$). Inhibition of miR-155 mitigated AC-induced pathological injury, reduced infiltration of eosinophils, lowered serum levels of anti-AVO IgE antibody eotaxin and Il-13, and increased IFN-γ level ($p < 0.05$). Phosphorylation of P65 of conjunctiva tissue of AC mice was blocked after inhibition of miR-155.*
Conclusion: *The inhibition of miR-155 ameliorates AC in mice most likely via a mechanism related to the inhibition of phosphorylation of P65. This provides a theoretical basis for new drug research and development.*

Keywords: *Allergic conjunctivitis, Mice, miR-155, Phosphorylation of P65, Ovalbumin*

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INTRODUCTION

Allergic conjunctivitis (AC) is a category of conjunctivitis caused by air allergens. The symptoms of AC include itching, overflow of tears, increased secretions, and conjunctival congestion [1]. The disease, which usually affects young people, is related to other allergic diseases such as allergic rhinitis and bronchial asthma [2]. There are three types of AC: acute allergic conjunctivitis (ACC), seasonal allergic conjunctivitis (SAC) and perennial allergic conjunctivitis (PAC) [3]. The mechanism of onset of AC is related to immunological reactions

mediated by Ig-E and Th2 cells [4].

Allergic conjunctivitis (AC) is mediated by IgE which is usually the specific allergen that causes early reaction (pollen, animal dander and house dust mite). This is followed by infiltration of large amounts of eosinocytes (which serve as biomarkers of allergy) in tears and conjunctival tissue, the population of which correlates with the degree of allergy manifested [5]. The pathogenesis of AC is not yet completely elucidated due to its complexity and involvement of multiple genes and abnormally-expressed proteins. Therefore, studies on the pathogenesis of AC are of great clinical importance for its early prevention and effective treatment.

MicroRNAs are a group of small single-stranded, non-coding RNAs of length 20 to 24 nucleotides which are involved in multiple regulatory roles [5]. They regulate multiple gene expressions by combining with specific gene targets. Thus, they perform important roles in physiological processes such as cell proliferation, differentiation and apoptosis [6-8]. Studies have shown that miR-146a effectively reduced AC symptoms in experimental mice by regulating $CD4^+CD25^-$T cells [9]. In addition, inhibition of miR-146a induces AC in mice by improving TSLP [10]. However, there are no extant studies on the effect of miR-155 on AC, and the mechanism involved.

The present study was carried out to investigate the expression levels of miR-155 in conjunctival tissues of healthy mice and AC mice. Moreover, siRNA eye drop was used to knock-down miR-155 expression level in conjunctival tissue, and the effect of miR-155 knock-down on symptoms of AC in mice was studied.

EXPERIMENTAL

Animal grouping and model establishment

Sixty male Bal/c mice aged 8 – 10 weeks (mean weight = 24.31 ± 0.66 g) were randomly divided into three groups using the random number table: control group, AC group and AC + miR-155 siRNA group (AC + miR-155 inhibition group). Each group had 20 mice. Mice in AC+miR-155 siRNA group were injected intraperitoneally with miR-155 siRNA for two weeks, while mice in the AC group and AC+miR-155 siRNA group were sensitized with intraperitoneal injection of 100 µg ovalbumin (OVA, ALUM; Pierce, Rockford, IL, America) for 15 consecutive days. From day 15 to day 18, ovalbumin (0.1 g/mL in PBS) eye suspension (5 mL) was used to treat the mice.

This research was approved by the Animal Ethical Committee of Fourth Affiliated Hospital of Kunming Medical University (Approval No. KMUAE201893), and was performed according to "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [11].

Scoring of AC clinical symptoms and grading

Clinical symptoms of conjunctivitis in mice in the 3 groups were scored at the end of administration of the eye drops, in line with the scoring scale of AC clinical symptoms (Table 1).

Table 1: Scoring scale and grading format for clinical symptoms of AC

Symptom	None	Mild	Moderate	Severe
Swelling of eye lid	0	1	2	3
Conjunctival redness	0	1	2	3
Lacrimation	0	1	2	3
Bruise	0	1	2	3

Histological examination of conjunctival tissue

Conjunctival tissues from the 3 groups of mice were subjected ti fixation in 10 % formalin overnight, and were subsequently dehydrated and embedded in paraffin blocks. Using a microtome, the blocks were cut into 5-µm slices which were fixed on glass slides. The slides were stained in H & E using standard histological procedures. They were thereafter examined under a light microscope and photographed. The pathological changes and the number of eosinophils were observed and recorded.

Assay of expression of miR-155 in conjunctival tissue using RT-PCR

Total RNA of conjunctival tissue was extracted using Trizol reagent. Trichloromethane extraction was carried out, and the clear aqueous phase in the upper layer was obtained through centrifuging at 12000 g for 15 min at 4 ℃. The RNA was precipitated using isopropanol, and the RNA precipitate was rinsed with 75 % ethanol and dried at room temperature for 10 min. It was then dissolved in 11.2 µL of diethyl pyrocarbonate, and the concentration of the pure RNA was determined in an ultraviolet spectrophotometer. The purity was assessed by determining the ratio of absorbance at 260 nm and 280nm. The RNA was accepted as pure at

A260/A280 values of 1.8 to 2.0. Then, 1 μg the pure RNA was reverse-transcribed into cDNA and kept at -80 °C. The PCR reaction system included 10 × buffer (2.5 μL), cDNA (2 μL), 20 μmol/L forward primer (0.25 μL), 20 μmol/L reverse primer (0.25 μL), 10 mmol/L dNTPs (0.5 μL), 2 × 10^6 U/L Taq polymerase (0.5 μL) and ddH_2O (19 μL). The amplification system of RT-PCR was the same in each group. After 4 times dilution of the reverse transcription product, 1 μL of cDNA was taken for PCR reaction.

Immunofluorescence studies on interleukin-4 and interleukin-5 expressions in vascular tissue

Slices of conjunctiva were oven-dried at 60 °C for 30 min. Then, the tissues were dewaxed three times in xylene for 5 min, and hydrated through a gradient of ethyl alcohol (100, 95 and 70 %). Endogenous peroxidase activity was inhibited with hydrogen peroxide containing 3 % methyl alcohol. The tissues were then blocked with goat blood for 1 h. Anti-Bax antibody was diluted 1:200 with PBS, and incubated with the tissues overnight at 4 °C, followed by washing 4 times with PBS on a shaking table. After adding FITC- double antibody, the tissues were incubated for 1 h at 37 °C, and then stained with DAPI (nuclear stain). After color development, 6 samples were randomly selected from each group, and 5 fields of view were photographed for each sample under fluorescence microscope at x200 magnification.

Western blot

Vascular endothelial tissue of mice in various groups were ground in lysis buffer. The total protein content of the supernatant recovered after centrifugation was determined using the bicinchoninic assay method. The protein was subjected to SDS-polyacrylamide gel electrophoresis, and the separated bands were transferred to PVDF membrane and incubated with primary antibodies overnight at 4 °C. Then, they were incubated with goat anti-rabbit antibody in the dark for 1 h. Odyssey membrane sweep instrument was used to scan and quantify the protein bands relative to GAPDH which served as internal control.

Determination of expression level of related proteins in serum

The eye of mouse was removed and 3 mL of whole blood sample was taken. The blood was kept at room temperature for 1 h, and centrifuged at 3500 rpm 15 min at 4 ℃. The serum was used to prepare the standard sample according to the specification of ELISA kit. The standard samples and samples to be tested were added separately to reaction wells. Streptavidin-HRP was added and incubated with proteins, followed by rinsing and color development. After terminating the reaction, wavelength was measured in a UV spectrophotometer.

Statistical analysis

All data were analyzed using SPSS 22.0 software and are presented as mean ± standard deviation (SD). Comparison between two groups was done using *t*-test. and values of $p < 0.05$ were considered statistically significant.

RESULTS

Expression levels of miR-155 in conjunctival tissue in mice

The expression levels of miR-155 in conjunctival tissues of healthy mice and AC mice were determined using RT-PCR. The miR-155 expression level in conjunctival tissue was significantly increased (almost 13-fold) after AC induction in mice, relative to healthy mice ($p < 0.05$).

Relief of clinical symptoms of AC in mice

Eye symptoms of AC in mice in the various groups were observed using the naked eye and given graded scoring (Figure 1). The knock-down of miR-155 in conjunctival tissue of mice inhibited multiple clinical symptoms of AC in the eyes, including tear production, swelling and increased eye secretions. Furthermore, through scoring of eye symptoms, it was found that clinical scores were markedly lower in AC+miR-155 siRNA mice than in AC mice ($p < 0.05$).

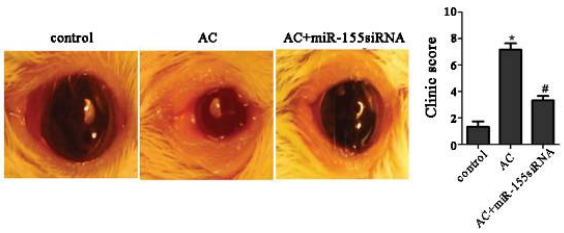

Figure 1: Effect of miR-155 knock-down on clinical symptoms of AC in mice. *$p < 0.05$, relative to control mice, #$p < 0.05$, relative to AC mice

Histological features of mouse conjunctival tissue

Eosinophils are involved in allergy-related inflammation and pathology of conjunctival secretions. Therefore, the pathological changes and infiltration of conjunctival secretions of mice

in the various groups were assessed. As shown in Figure 2, the conjunctiva of AC group had obvious infiltration of eosinophils, while miR-155 knock-down inhibited infiltration of eosinophils in conjunctiva ($p < 0.05$).

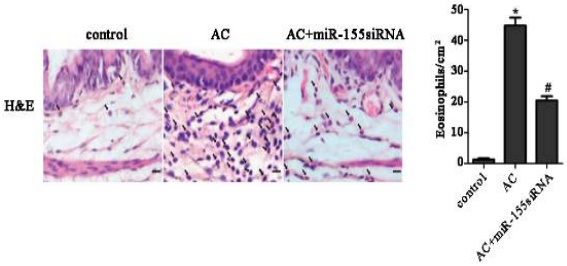

Figure 2: Infiltration of eosinophils in different groups. *p < 0.05, compared to control group, #p < 0.05, compared to AC group

Concentrations of anti-OVA IgE antibody and activated chemotactic factor in serum

The serum expressions of anti-OVA IgE antibody and activated chemotactic factor in mice in AC group were higher than those in mice in the control group (Figure 3). The OVA antibody and eotaxin expression levels were decreased after inhibition of miR-155.

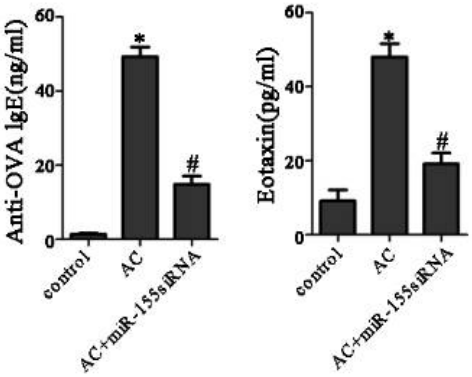

Figure 3: Amounts of serum anti-OVA IgE antibody and activated chemotactic factor in mice. *P < 0.05, relative to control mice, #p < 0.05, relative to AC mice

Expression levels of IL-4 and Il-5 in mouse conjunctival tissue

The knock-down of miR-155 significantly reversed the over-expressions of IL-4 and IL-5 protein of conjunctival tissue in AC mice ($p < 0.05$) (Figure 4).

Knock-down of miR-155 inhibited immune reaction mediated by Th2 cells

As shown in Figure 5, miR-155 knock-down decreased serum IL-13 level in AC mice, and upregulated serum IFN-γ ($p < 0.05$). Thus, inhibition of miR-155 slowed down immune reaction mediated by Th2 cells.

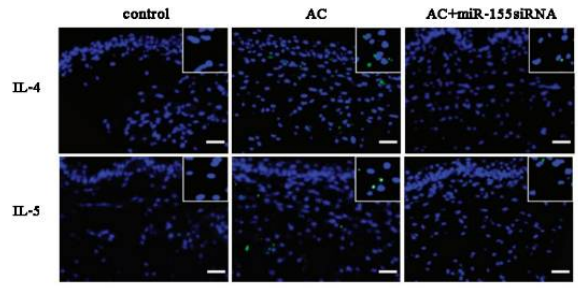

Figure 4: Immunofluorescent staining of IL-4 and IL-5 of conjunctival tissue of mice in the various groups

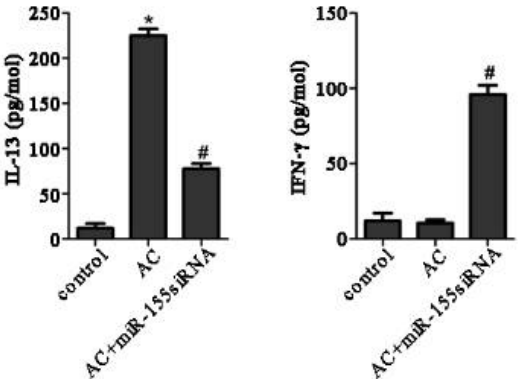

Figure 5: Effect of miR-155 knock-down on Th2 cell-mediated immune reaction. *$p5$ < 0.05, relative to control mice; #p < 0.05, relative to AC mice

Influence of MIR-155 knock-down on NF-κB signal pathway of mouse conjunctival tissue

The results in Figure 6 show that knock-down of miR-155 led to significant decrease in the phosphorylation level of P65 of conjunctival tissue in AC mice ($p < 0.05$).

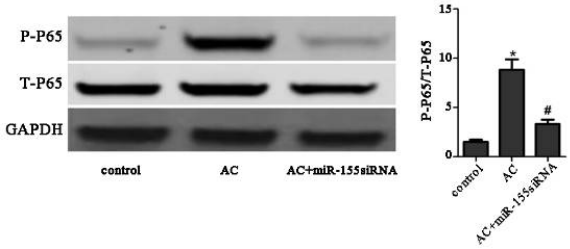

Figure 6: Influence of miR-155 knock-down on NF-κB signal pathway of mice conjunctival tissue. *p < 0.05, relative to control mice; #p < 0.05, relative to AC mice

DISCUSSION

Long-term allergic conjunctivitis (AC), regarded as the most serious allergic eye disease, lowers the standard of life of patients and greatly increases their financial burden [12]. The current use of mast cell stabilizers and corticosteroids for

the treatment of AC has achieved relatively satisfactory curative effects, although long-term use of these drugs produce some adverse side effects [13]. Therefore, there is need for further research to identify treatment targets and diagnostic markers of AC so as to improve clinical effects of treatments.

In mucus, epithelial cells have sensitization effects or immune regulation on dendritic cells, thereby further regulating activity of T cells [14]. The activation of NF-κB in epithelial cells is vital for this process, because abnormal activation of this signal conduction pathway leads to mucous inflammation mediated by T cells [15]. On the other hand, long-term exposure of skin or mucous membrane to toxic environment promotes specific immune response of antigen in local tissue, thereby inducing excessive activation of NF-κB signal pathway in epithelial cells.

Thus, the inhibition of this pathway reduces the severity of allergic diseases [16,17]. Anti-dintrobenzene IgE stimulates mast cells of rats to release TNF-α and IL-4. The TNF-α induces the activation of NF-κB in fibroblasts and epithelial cells in rat and human conjunctiva, leading to production and release of the cytokines IL-8 and RANTES.

However, Il-4 activation induced by STAT6 does not induce the release of these cytokines. Following treatment with TNF-αand IL-4, only fibroblasts produce eosinophil recruitment cytokine eotaxin-1. Allergy stimulation in cultured cells *in vivo* induces degradation of IκB-α in conjunctiva, and infiltration of eosinophils and other inflammatory cells. Only fibroblast cells can release eotaxin after exposure to TNF-α and IL-4. Similar experimental results are usually obtained *in vitro*. Studies have shown that allergic stimulation in *in vivo* model also induced the release of IκB-αin conjunctiva, and infiltration of eosinophil and other inflammatory cells [18]. Therefore, NF-κB may be an important treatment target for allergic conjunctivitis.

It has been demonstrated that MiR-155, an important miRNA factor, is vital for immune regulation. For instance, HBeAg induces miR-155 expression in macrophages and accelerates hepatic injury through enhancement of the production of inflammatory cytokines [19]. Moreover, TRIF controls the production of oxidized low-density lipoprotein (OX- LDL), thereby further inducing inflammatory reaction mediated by macrophages through ERK signal pathway regulated by BIC/miR-155 [20].

In mice with abdominal aortic aneurysm, the inhibition of miR-155 relieved inflammation mediated by macrophages [21]. In addition, it has been reported that silencing of miR-155 relieved inflammatory reaction of psoriasis through inhibiting inflammatory globule NLRP3 activation [22]. In the present study, it was found that miR-155 expression level in conjunctiva of mice with AC was increased, which is consistent with proinflammatory function of miR-155 reported in previous studies.

Furthermore, the pathological injury in conjunctiva tissue of mice was relieved; eosinophil population was decreased in conjunctiva tissue, and conjunctival tissue Il-4 and IL-5 were inhibited through miR-155 silencing. The immune reactions of marker molecules IL13 and IFN-γ mediated by serum Th2 cells were present in mice. This indicates that silencing of miR-155 inhibited immune reaction mediated by Th2 *in vivo* in mice. Through immunoblotting, it was found that silencing of miR-144 inhibited phosphorylation of P65 in conjunctiva.

Study limitations

This study has certain limitations: (1) no direct target for miR-155 was found; (2) there were no verifications of the findings through *in vitro* experiments.

CONCLUSION

The results of this study show that miR-155 inhibition relieves the clinical symptoms of AC in mice through a mechanism related to inhibition of phosphorylation of P65. This finding provides theoretical basis for new drug research and development.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zhulin Hu designed the study and interpreted the results. Mengtian Bai, Yan Li, Yun Li and Zhulin Hu collected data and drafted the manuscript. Mengtian Bai performed the experiments.

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