



ADRB2 suppresses IL-13-induced allergic rhinitis inflammatory cytokine regulated by miR-15a-5p

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Abstract

Allergic rhinitis (AR) is a common hypersensitive disease that troubles patients a lot. Nasal epithelial cells (NECs), as the outmost protection of inhalation, play an important role in AR allergic response. Adrenoceptor beta 2 (ADRB2) is an important gene in inflammatory response, which has become the hot spot for AR development and treatment in recent years. MiR-15a-5p has been proved to be involved in AR immune response as the upstream regulator of ADRB2. Human primary NECs were isolated and stimulated by IL-13. qRT-PCR assay was used to detect the RNA level of target genes. ELISA and Western blotting were applied to detect target protein levels. Luciferase reporter assay and biotin pull-down assay were performed to test molecules interaction. ADRB2 was highly expressed in nasal mucosa of AR patients and was positively correlated with IL-13 stimulation, and knockdown of ADRB2 inhibited IL-13-induced expression of GM-CSF, eotaxin, and MUC5AC in NECs. ADRB2 was directly targeted by miR-15a-5p, and miR-15a-5p inhibited IL-13-induced expression of GM-CSF, eotaxin, and MUC5AC in NECs. ADRB2 mediated the effect of miR-15a-5p on the regulation of nasal epithelial immune responses. ADRB2 is negatively regulated by miR-15a-5p, which inhibits IL-13-induced nasal epithelial inflammatory responses.

Keywords ADRB2 · miR-15a-5p · IL-13 · NECs · Allergic rhinitis

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Introduction

Allergic rhinitis (AR) is characterized into type I hypersensitivity disease, in which long-term inflammation in nasal mucosa is regulated by non-infectious immunoglobulin E (IgE) [1, 2], further regulated by interleukin-13 (IL-13), a common receptor of STAT6 signaling pathways in many allergic disorders [3–5]. Patients with AR may experience olfactory and/or taste desensitization, sneezing, tears, stuffy nose and irritability [3]. In AR immune response, nasal epithelial cells (NECs) play an important role in the pathogenesis of AR as the first barrier to inhalation defense [4–6].

The role of adrenoceptor beta 2 (ADRB2) in the pathogenesis of inflammation and allergy has been an intensive subject for many years. ADRB2 is widely distributed in the respiratory tract at a very high level [7]. It has been reported to play a range of roles in many respiratory-related physiological processes, including smooth muscle relaxation, inhibition of acetylcholine release from cholinergic nerve terminals, stimulation of serous and mucous cell secretions, increasing ciliary beat frequency, promoting water movement into the airway lumen by stimulation of ion secretion

across the apical membrane of epithelial cells, increasing bronchial blood flow, reducing venular permeability, and inhibiting mediator release of inflammatory cells [8–10].

MicroRNAs (miRNAs), a class of evolutionarily conserved endogenous non-coding RNAs which are 18–25 bp in length, have emerged as key mediators in a variety of biological processes, including cell cycle, proliferation, apoptosis, angiogenesis, tissue invasion, metastasis and inflammation [11–17]. The repressive effect of miRNAs can be powerful in gene expression by binding to the 3'UTR of targets. ADRB2 is an important gene that has been widely studied in inflammation, which plays multiple roles in the immune response to asthma, allergies, and cardiac function [7, 18, 19]. However, its potential function in AR has not been revealed. Here, we demonstrated that ADRB2 could inhibit IL-13-induced nasal epithelial inflammatory responses, which was negatively regulated by miR-15a-5p in NECs. This pathway can provide a reliable strategy for the prevention and treatment of allergic rhinitis.

Materials and methods

Patients and sample collection

Patients with perennial and recrudescence allergic rhinitis (AR) symptoms according to clinical diagnostic criteria deemed to be “long-term” AR patients were recruited into this study. The demographic data of the patients are presented in Table S1. The diagnosis of AR was based on AR history, positive dust mite allergen skin test and the serum-specific IgE levels (UniCAP[®], Phadia, Sweden) were ≥ 0.35 kU/L. Exclusion criteria included the presence of acute or chronic infectious disorders, autoimmune diseases, malignancies or other inflammatory disorders that are contraindications to specific immunotherapy (SIT). Nasal mucosal tissue samples were obtained surgically from the inferior turbinates from patients with perennial AR and nonallergic patients with nasal septum deviation (control group). All samples were immediately preserved in RNAlater Solution (Ambion, USA) after resection and then stored at -20°C until use. No patient had received topical or systemic corticosteroid therapy for 4 weeks prior to study recruitment. All the methods involving humans were carried out in accordance with the approved guidelines. Written informed consent was obtained from all participants. All studies were reviewed and approved by ethics committee of Yuhuangding Hospital (#YHDH1305LC207).

Cell culture

Human primary NECs were isolated and established from the inferior turbinate tissues of 20 patients with AR

following previously reported protocol [20]. The cells were cultured in Bronchial Epithelial Growth Medium (BEGM, Lonza, Walkersville, MD, USA) under submerged conditions at 37°C with 5% CO_2 in humidified air. The NECs were passaged when a 70–80% confluent monolayer appeared, and used for subsequent experiments. HEK291T and HEK293T cells were purchased from Cell Bank of Chinese Academy of Science.

IL-13 stimulation

The prepared NECs were either unstimulated or stimulated with IL-13 (50 ng/mL) for 14 h, 24 h or 48 h in BEGM without hydrocortisone [21]. The medium was replaced every other day. Cell pellets and supernatants were collected for quantitative real-time PCR (qRT-PCR) and Western blot analysis.

qRT-PCR

Total RNA of NECs was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The RNA extracts were reverse transcribed into cDNA and then quantified by SYBR Green assay (Promega GoTaq[®] qPCR Master Mix, Promega, Madison, WI, USA) on an Agilent Mx3000P qPCR system. GAPDH and U6 were used as endogenous references for mRNAs and miRNAs, respectively. All primer sequences are presented in Supplementary Table S2. Each assay was run in triplicate, and relative expression was calculated using the comparative cycle threshold method [22].

Western blot analysis

The NECs were lysed and determined by a BCA Protein Assay kit (Sigma, St. Louis, MO, USA). Protein samples were separated by SDS-PAGE and transferred to PVDF membranes. After blocking in 5% skim milk for 1 h at room temperature, target blots were incubated in primary antibodies targeting beta2AR (1:1000, 182136, Abcam) and Actin (1:1000, ab209857, Abcam) overnight at 4°C . Membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies. Target signals were detected by enhanced chemiluminescence (Millipore, Billerica, MA). All experiments were performed in triplicate.

siRNA transfection

NECs were plated in 6-well plates 1 day before being transfected with siRNA oligonucleotides and plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol [23]. MiR-15a-5p mimics and inhibitors were obtained by chemical synthesis (Liangtai Biotech, Shanghai,

China). The nucleotide sequences were shown in Supplementary Table S2.

Enzyme-linked immunosorbent assay (ELISA)

Granulocyte–macrophage colony-stimulating factor (GM-CSF), eotaxin, and mucin 5AC (MUC5AC) levels in the cell culture supernatants were measured using the GM-CSF Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA), CCL11/Eotaxin Quantikine ELISA Kit (R&D Systems), and Mucin-5 subtype AC ELISA Kit (R&D Systems), according to the manufacturer's protocol, respectively [21].

Luciferase reporter assay

The 3'-UTR binding sites of ADRB2 with wild-type (ADRB2 3'UTR-WT) or mutant (ADRB2 3'UTR-MT) or miR-15a-5p were amplified and cloned into psi-CHECK2 vectors (Supplementary Table 1). NECs and HEK291T cells were plated 24 h prior to transfection at a density of $2 \sim 3 \times 10^4/\text{cm}^2$ and transfected with miR-15a-5p mimics or miR-NC. Transfected cells were lysed after 48 h, and the activities of Luciferase were determined with a Dual-Luciferase® Reporter Assay System (Promega) [24].

Biotin miRNA pull-down assay

NECs were harvested and extracted with 0.5 mL of 25 mM Tris–HCl (pH 7.5), 70 mM KCl, 2.5 mM EDTA, 0.05% NP-40, 80 U/mL RNase Inhibitor (Life Technologies), and $1 \times$ protease inhibitor cocktail (Sigma-Aldrich) on ice for 20 min and centrifuged at $12,000 \times g$ for 15 min at 4 °C [25]. Biotinylated double-stranded RNA (8 nM) of miR-15a-5p was added to the supernatant of NECs and incubated at 4 °C for 30 min and then 30 °C for 1 h. The biotin–miRNA/mRNA complex was eluted with 250 μL of 20 mM Tris–HCl (pH 7.4), 400 mM KCl, 0.5% NP-40, 5 mM biotin, and 80 U/mL RNase inhibitor at 42 °C for 5 min.

Statistical analysis

All data were measured as mean $\pm$ standard deviation and tested by Student's *t* test or one-way ANOVA followed by a Bonferroni post hoc test. Analysis was performed using the SPSS 17.0 and Graph Prism statistical systems, and $P < 0.05$ was considered statistically significant.

Results

ADRB2 was upregulated in nasal mucosal tissues from AR patients and positively correlated to IL-13 stimulation

To investigate the potential role of ADRB2 in AR, we detected the expression level of ADRB2 in nasal mucosa of 20 patients with long-term AR and 20 patients with non-AR by qRT-PCR. We found that the mRNA expression level of ADRB2 in nasal mucosa of patients with AR was significantly higher than that in non-AR patients (Fig. 1a).

Previous studies have shown that IL-13 induction could effectively simulate the inflammatory response of AR. Based on these reports, we used IL-13 to stimulate primary isolated NECs to further determine the effect of ADRB2 in AR. The expression level of ADRB2 were detected by qRT-PCR and Western blot at 12, 24 and 48 h after IL-13 stimulation, respectively, and it was shown that both the mRNA and protein levels of ADRB2 were upregulated with stimulation of IL-13 (Fig. 1b, c) indicating that ADRB2 is a positive regulator in AR and responds to IL-13.

Knockdown of ADRB2 inhibited IL-13-induced GM-CSF, eotaxin, and MUC5AC expression in human NECs

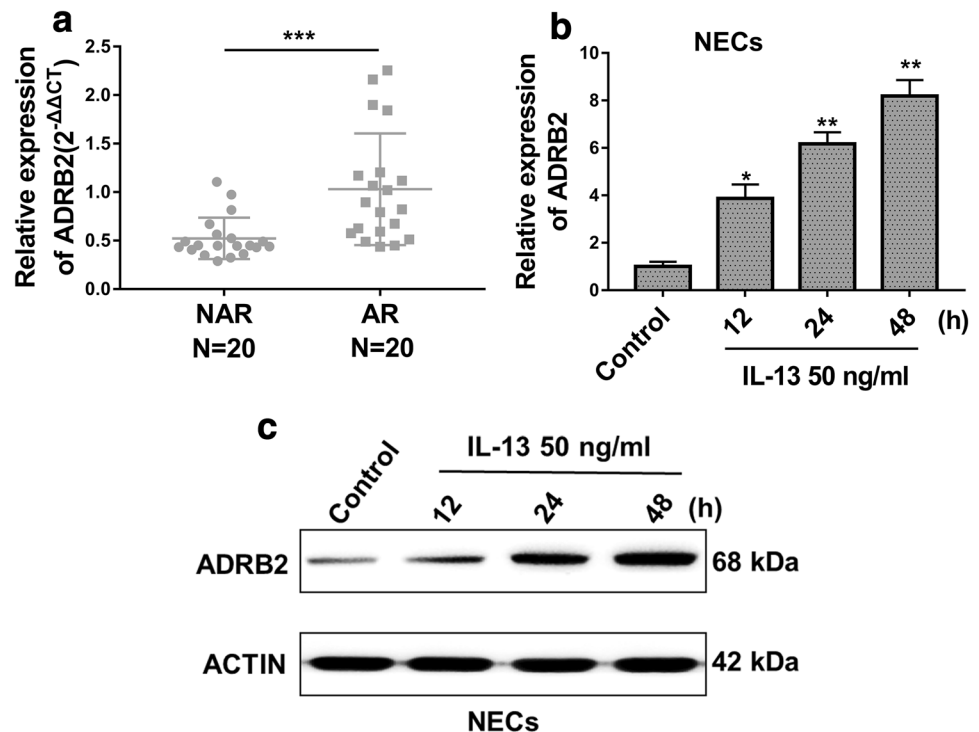
To verify the function of ADRB2 in nasal epithelial cells, we knocked down ADRB2 expression in NECs by siRNA (Fig. 2a, b), and then detected the mRNA and protein levels of inflammatory factors GM-CSF, eotaxin, and mucin MUC5AC by qRT-PCR and ELISA after IL-13 stimulation. Before siRNA transfection of ADRB2, mRNA (Fig. 2c, e) and protein (Fig. 2f–h) levels of inflammatory factors given above significantly increased compared to the normal control, but it reduced after ADRB2 was knocked down. Thus, it was inferred that ADRB2 was an upstream gene of NECs that responded to inflammation.

ADRB2 was directly targeted by miR-15a-5p

To figure out what role that ADRB2 plays in NECs, we focused on its functional mechanism. First, we predicted the potential miRNA associated with ADRB2 by targetscan (http://www.targetscan.org/vert_71/). Among these candidates, miR-15a-5p has been reported in a series of diseases including lung cancer, chronic myeloid leukemia, and hepatocellular carcinoma [26–28]. We detected the binding sites between ADRB2 and miR-15a-5p and established a mutant at the locus to construct the fluorescent reporter vector. We observed that miR-15a-5p inhibited the fluorescence of the

Fig. 1 ADRB2 was upregulated in nasal mucosal tissues from AR patients and positively correlated to IL-13 stimulation.

a ADRB2 expression levels in mucosal tissues from 20 patients with perennial allergic rhinitis (AR) and 20 patients with nonallergic rhinitis (NAR) were measured by qRT-PCR. **b, c** The RNA and protein level of ADRB2 in human nasal epithelial cells treated with 50 ng/mL IL-13 for 12, 24 or 48 h were determined by qRT-PCR and Western blot. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$



wild-type ADRB2 3'UTR but had no effect on the mutant vector (Fig. 3a). By biotin pull-down assay, we confirmed that biotin-labeled miR-15a-5p could efficiently enrich the mRNA of ADRB2 (Fig. 3b). Next, we carried out luciferase assay on NECs and HEK293T cells to compare miR-15a-5p level between ADRB2 3'UTR-WT and ADRB2 3'UTR-MT (Fig. 3c, d). We found that miR-15a-5p overexpression inhibited ADRB2 expression, while miR-15a-5p knockdown promoted the expression of ADRB2 characterized by both qRT-PCR (Fig. 3e) and Western blot (Fig. 3f). Therefore, this suggested that miR-15a-5p is an inhibitor of ADRB2 by directly binding to its 3' UTR.

miR-15a-5p suppressed IL-13-induced GM-CSF, eotaxin, and MUC5AC expression

To explore the regulatory relationship between miR-15a-5p and ADRB2, we also compared the expression of miR-15a-5p in nasal mucosa tissues between long-term AR and non-AR patients, and its expression changed with IL-13 stimulation time. In contrast to ADRB2, miR-15a-5p expression in AR was significantly lower than that in non-AR patients (Fig. 4a). At the same time, expression of miR-15a-5p was downregulated as the IL-13 stimulation time increased (Fig. 4b). Then, we overexpressed miR-15a-5p using miRNA mimics and detected its effect on the inflammatory factors in both mRNA (Fig. 4c–e) and protein (Fig. 4f–h) levels. Consistently, expression of GM-CSF, eotaxin and MUC5AC was significantly downregulated before IL-13 stimulation and

downregulated after. In summary, miR-15a-5p is an inhibitory factor in allergic rhinitis.

ADRB2 mediated the effects of miR-15a-5p on the inflammatory response of NECs

After determining the function of ADRB2 and miR-15a-5p in AR inflammation response, we tried to figure out how they work. We performed a co-transfection assay in nasal epithelial cells: qRT-PCR (Fig. 5a) and Western blot analysis (Fig. 5b) revealed that ADRB2 level was downregulated by miR-15a-5p overexpression and returned to higher levels by addition of ADRB2. At the same time, qRT-PCR and ELISA were used to detect the changes in the related factors of these co-transformed nasal epithelial cells after IL-13 stimulation. It was found that mRNA and protein level of GM-CSF, eotaxin, and MUC5AC decreased after being transfected by miR-15a-5p in nasal epithelial cells based on IL-13 stimulation but reached a higher level after co-transfection with the ADRB2 expression plasmid (Fig. 5c–h). In conclusion, ADRB2 regulating the inflammatory response of nasal epithelial cells after IL-13 stimulation can be mediated by miR-15a-5p.

Discussion

AR is a symptomatic disorder of the nose induced after allergen exposure by IgE-mediated inflammation of the membranes lining the nose [3]. It causes major

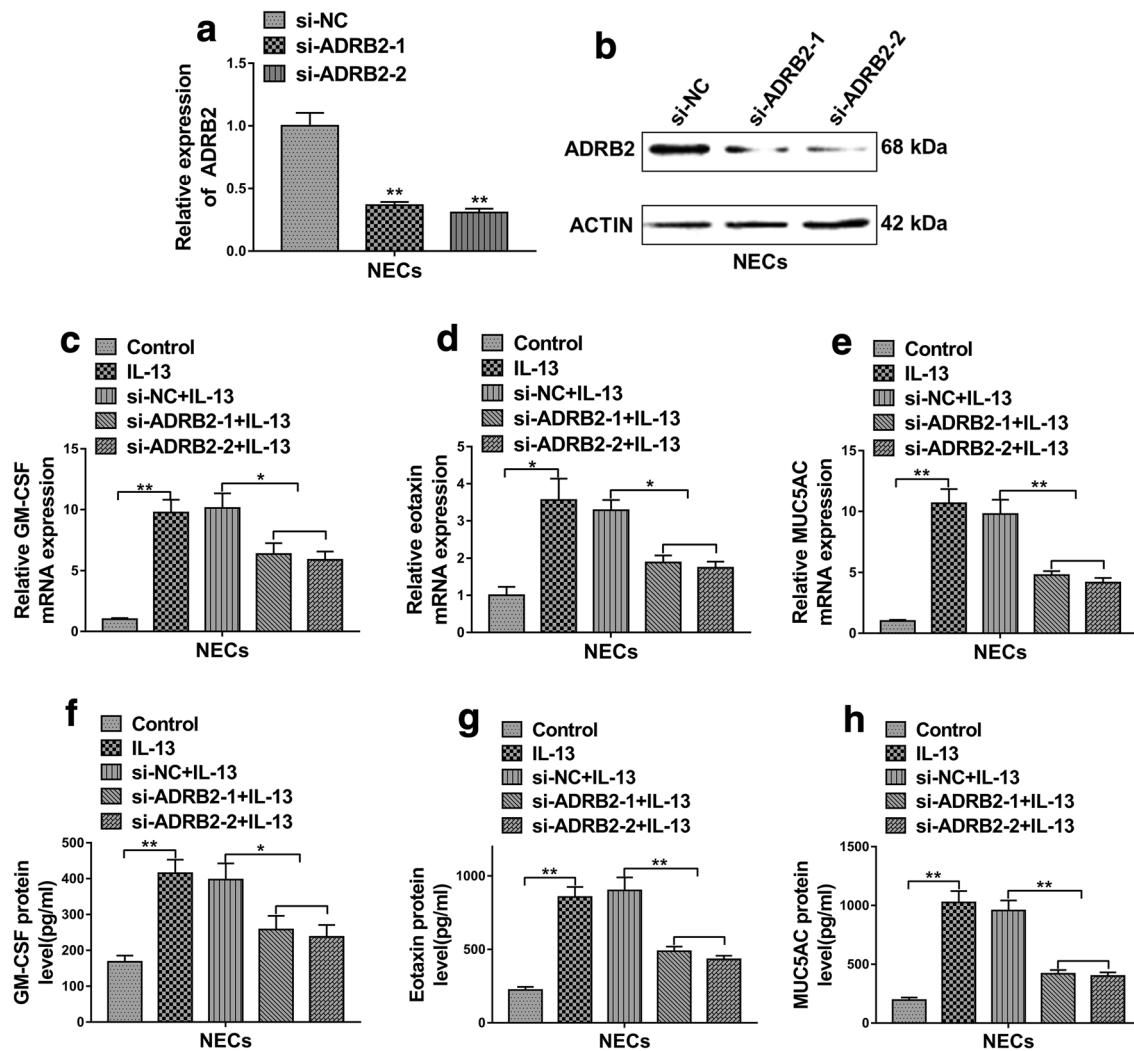


Fig. 2 Knockdown of ADRB2 inhibited IL-13-induced GM-CSF, eotaxin and MUC5AC expression in human nasal epithelial cells. **a, b** The RNA and protein level of ADRB2 in human nasal epithelial cells transfected with ADRB2 si-RNAs (si-ADRB2-1 and si-ADRB2-2) or negative control siRNA (si-NC) were determined by qRT-PCR and Western blot. **c–e** The mRNA expression levels of GM-CSF, eotaxin and MUC5AC in human nasal epithelial cells transfected with ADRB2 si-RNAs (si-ADRB2-1 and si-ADRB2-2) or negative

control siRNA (si-NC) and stimulated by IL-13 were measured by qRT-PCR. **f–h** The protein levels of GM-CSF, eotaxin and MUC5AC in human nasal epithelial cells transfected with ADRB2 si-RNAs (si-ADRB2-1 and si-ADRB2-2) or negative control siRNA (si-NC) and stimulated by IL-13 were measured by ELISA. The data represent the mean $\pm$ SD from three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

inconvenience in every aspect of the patients' life. Risk factors of AR have been clearly identified and the diagnosis is usually easy, however, the treatment and prevention approaches are still lacking. Therefore, figuring out the pathogenesis and finding some potential targets are very urgent for AR treatment. On the other hand, AR is an outstanding model for studying allergic inflammation, mostly when accompanied by seasonal rhinitis, its triggering factors can be clearly identified. Nasal mucosa is easily accessible for provocation with allergen and recording of responses, both clinical and immunological [1].

In our research, we first established an in vitro AR model by stimulating NECs with IL-13, and determined the effect of ADRB2 on NECs in the AR model after analyzing the expression of ADRB2. NECs are the outmost best safeguards against exoteric inflammatory stimuli and antigens, and also release a series of cytokines. In other studies, they have long been known to be a rich source of proinflammatory cytokines, such as TNF- α , GM-CSF, IL-6, and IL-8, which might play a central role in airway host defense and allergic asthma and rhinitis [29]. So, it is necessary to start with NECs to study AR. Meanwhile, it

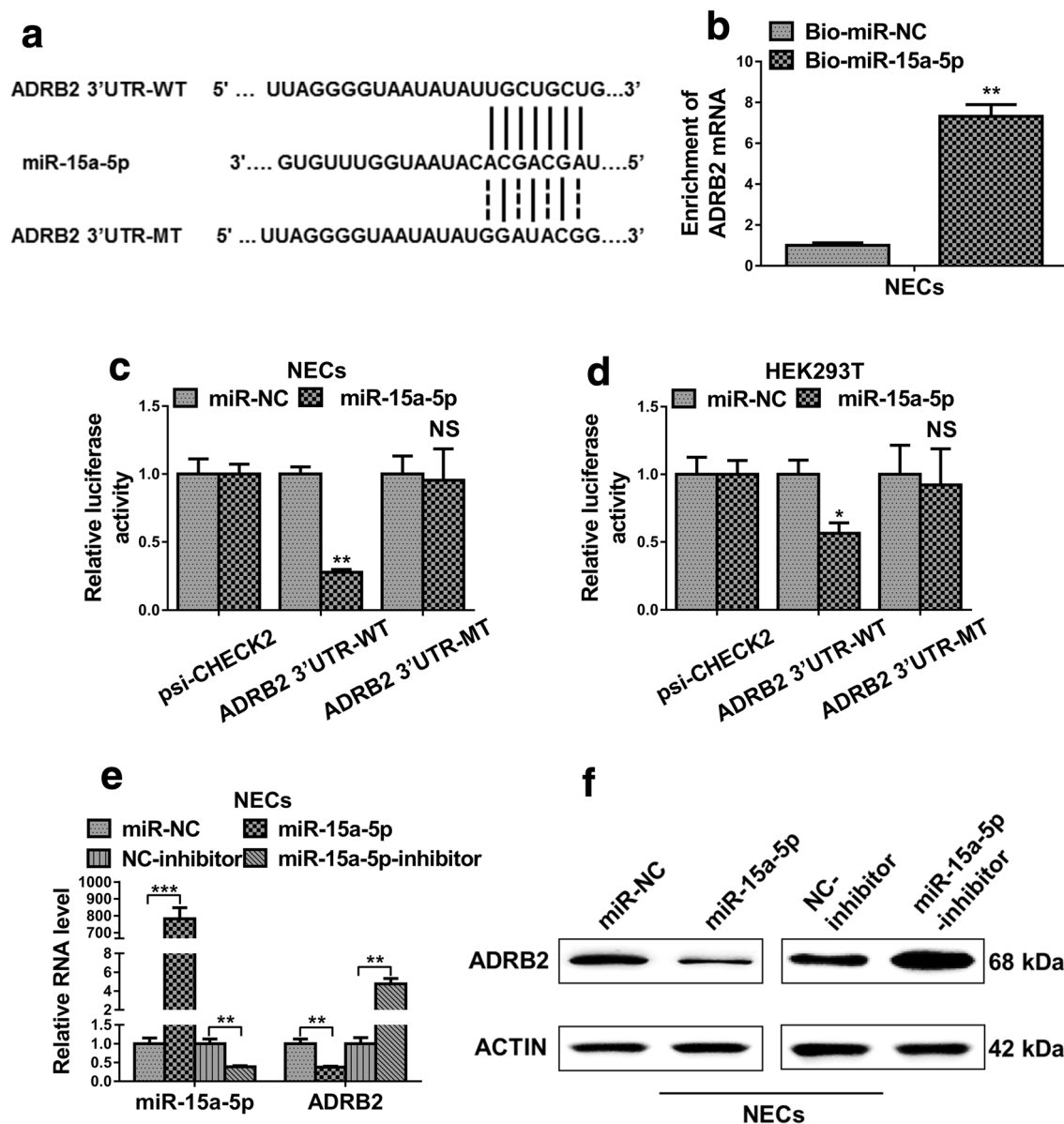


Fig. 3 ADRB2 was directly targeted by miR-15a-5p in human nasal epithelial cells. **a** The prediction for miR-15a-5p binding sites on the 3'UTR of ADRB2 and schematic of luciferase reporter vector constructs wild-type (ADRB2 3'UTR-WT) ADRB2 3'UTR and the miR-15a-5p-binding site mutated (ADRB2 3'UTR-MT) one. **b** Detection of ADRB2 mRNAs in biotinylated miRNA/target mRNA complex by real-time RT-PCR. The relative level of ADRB2 mRNA in the complex pulled down using biotinylated miR-15a-5p (Bio-miR-15a-5p) was compared to that of the complex pulled down using the biotinylated control random RNA (Bio-miR-NC). **c, d** Relative luciferase activities of empty vector (psi-CHECK2), plasmids carrying wild-

type (ADRB2 3'UTR-WT) or mutant (ADRB2 3'UTR-MT) ADRB2 3'UTR in NECs and HEK293T cells co-transfected with miR-15a-5p mimics or miR-NC. **e** The relative RNA levels of miR-15a-5p and ADRB2 in NECs transfected with miR-15a-5p mimics and miR-15a-5p inhibitors or their respective negative controls were detected by qRT-PCR. **f** The protein level of ADRB2 in NECs transfected with miR-15a-5p mimics and miR-15a-5p inhibitors or their respective negative controls were detected by Western blot. The data represent the mean $\pm$ SD from three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS not significant

has been proven that IL-13 is a typical Th2 cytokine and the central mediator in many allergic diseases via activation of its receptor and the associated signal transducer and activator of transcription 6 (STAT6) [20], and many studies have demonstrated that IL-13 could model pathologic

changes of airway allergic diseases, such as eosinophil recruitment, mucus cell metaplasia, subepithelial fibrosis and smooth muscle hypertrophy [30]. On this basis, IL-13 stimulation is the foundation of our research.

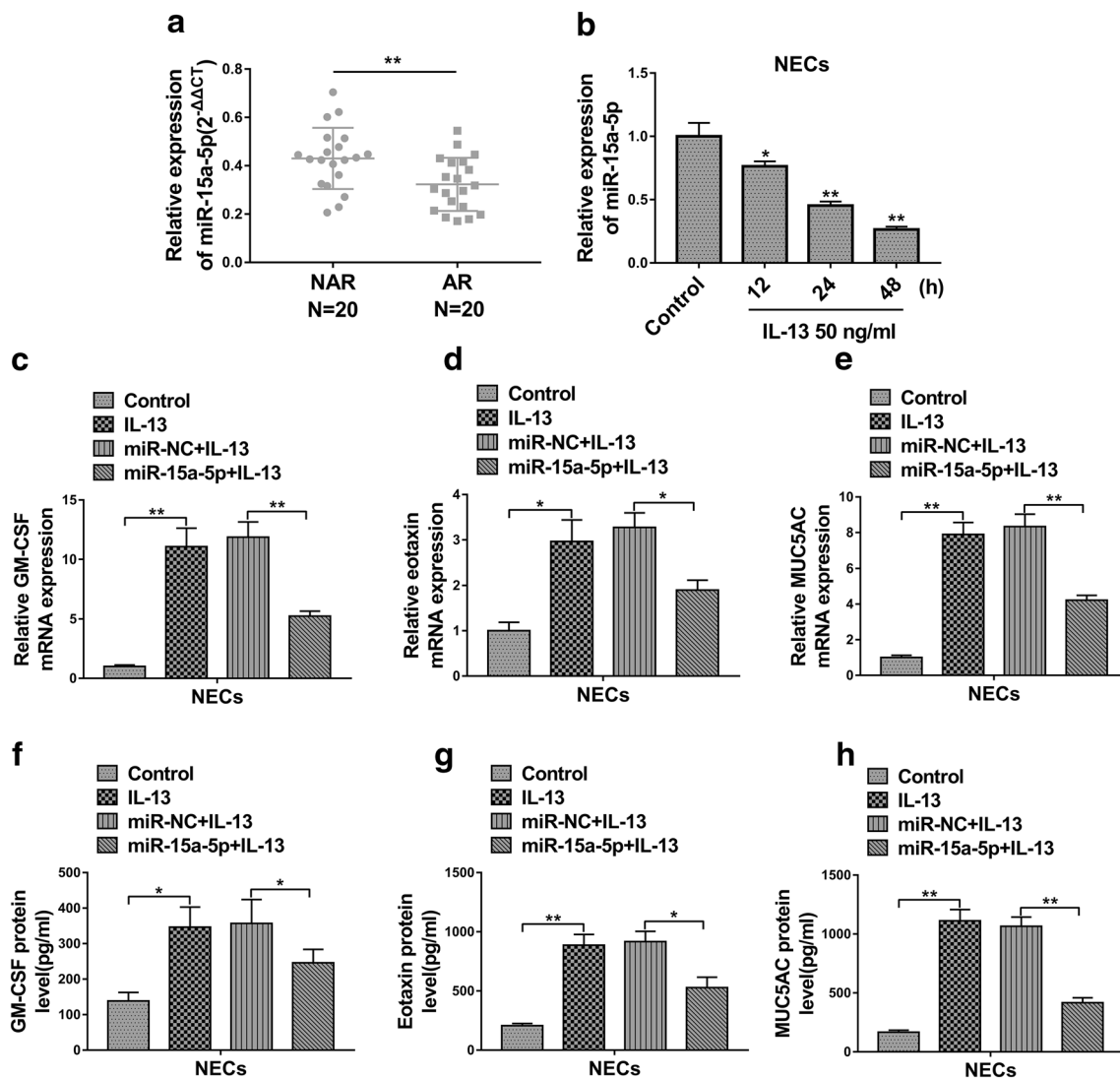


Fig. 4 MiR-15a-5p suppressed IL-13-induced GM-CSF, eotaxin and MUC5AC expression. **a** MiR-15a-5p expression levels in mucosal tissues from 20 patients with perennial allergic rhinitis (AR) and 20 patients with nonallergic rhinitis (NAR) were measured by qRT-PCR. **b** The RNA level of miR-15a-5p in human nasal epithelial cells treated with 50 ng/mL IL-13 for 12, 24 or 48 h were determined by qRT-PCR. **c–e** The mRNA expression levels of GM-CSF, eotaxin

and MUC5AC in human nasal epithelial cells transfected with miR-15a-5p mimics (miR-15a-5p) or mimics negative control (miR-NC) and stimulated by IL-13 were measured by qRT-PCR. **f–h** The protein levels of GM-CSF, eotaxin and MUC5AC in human nasal epithelial cells transfected with miR-15a-5p mimics (miR-15a-5p) or mimics negative control (miR-NC) and stimulated by IL-13 were measured by ELISA. * $P < 0.05$; ** $P < 0.01$

After determining the function of ADRB2, the upstream regulatory factor, miR-15a-5p, and its negative regulation on ADRB2 was found by interaction analysis. MiRNAs are produced by a wide variety of cells in different organs and secreted into the blood and other bodily fluids, where they can exert biological effects [15]. Immune cell response has been linked to increased intracellular cAMP levels, which can communicate with ADRB2 receptors. Activation or interruption of ADRB2 can affect intracellular levels of cAMP, potentially creating a potent inhibitor of immunity response [18]. Over the past 2 decades, many miRNAs have been revealed in various human diseases. Loss and

enhancement of miRNA have been shown to contribute to cancer development through specific mechanisms. In recent years, miRNAs are emerging as targets that play important roles in allergic response. But their function is poorly understood in AR. Most studies have reported that miR-15a-5p is associated with cancer. Its abnormal expression often affects a series of cell physiological process, including proliferation, cell cycle, apoptosis, and even neurotrophics [28]. Here, we first determined the role of ADRB2 in allergic rhinitis and its interaction with miR-15a-5p. Both the targets can be referenced in AR clinical diagnose.

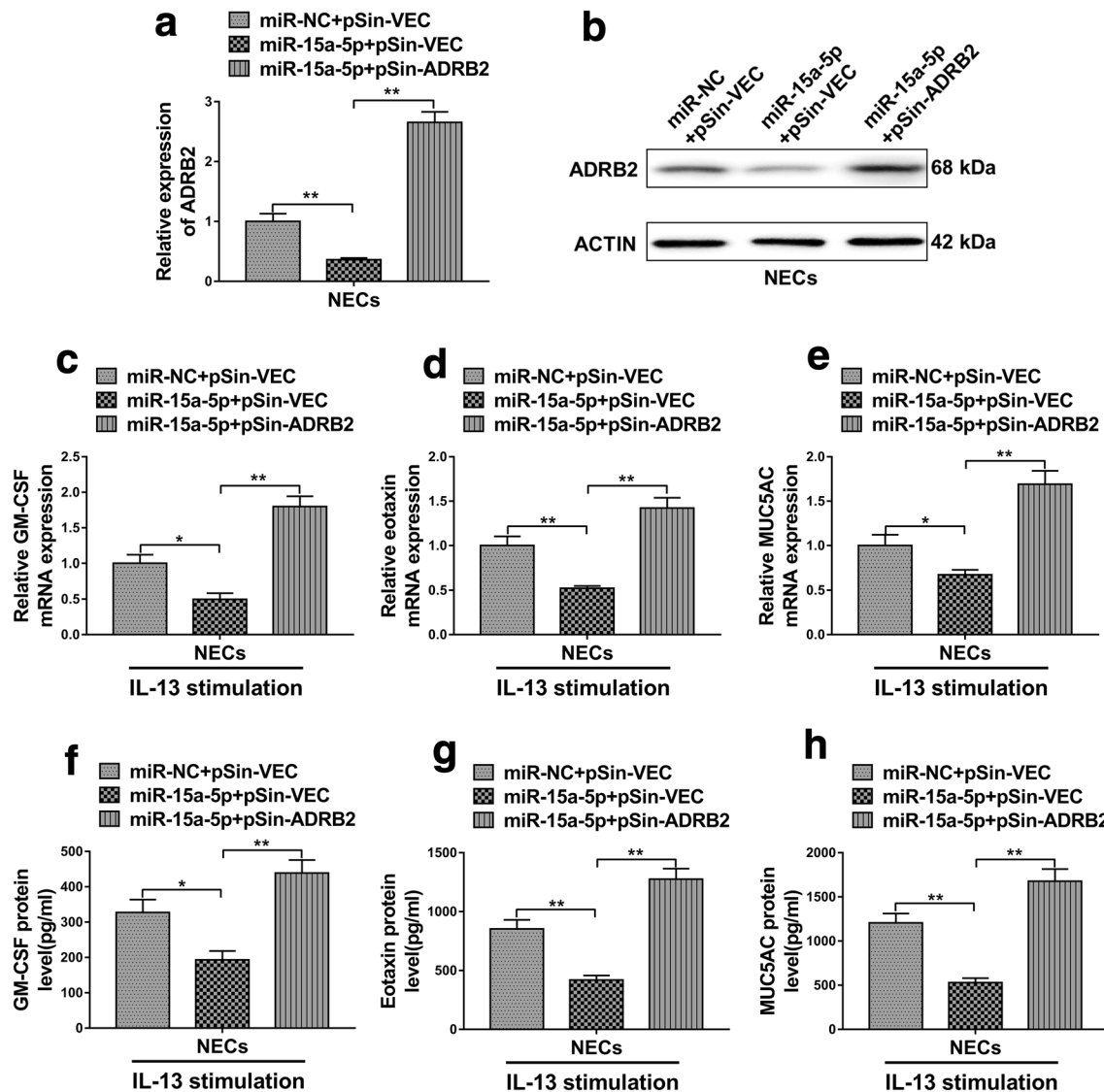


Fig. 5 ADRB2 mediated the effects of miR-15a-5p on the inflammatory response of human nasal epithelial cells. **a, b** The expression levels of ADRB2 were measured by qRT-PCR and Western blot in NECs co-transfected with mimics of negative control and empty vector (miR-NC+pSin-VEC), miR-15a-5p mimics and empty vector (miR-15a-5p+pSin-VEC) or miR-15a-5p mimics and ADRB2 over-expression plasmid (miR-15a-5p+pSin-ADRB2). **c–e** The mRNA

expression levels of GM-CSF, eotaxin and MUC5AC in these co-transfected human nasal epithelial cells and stimulated by IL-13 were measured by qRT-PCR. **f–h** The protein levels of GM-CSF, eotaxin and MUC5AC in these co-transfected human nasal epithelial cells and stimulated by IL-13 were measured by ELISA. The data represent the mean $\pm$ SD from three independent experiments. * $P < 0.05$; ** $P < 0.01$

So far, we have figured out the miR-15a-5p-ADRB2-IL13 pathway in AR. Under the circumstance of IL-13 stimulation, the expression level of ADRB2 significantly increased, indicating the inhibition of NECs in AR. Further, miR-15a-5p showed the opposite performance in this process, so we confirmed through a series of experiments that the inhibitory effect of ADRB2 on IL-13 stimulation is regulated by miR-15a-5p.

Previous study always focused on the exploitation of AR drugs, including oral H1 antihistamines and intranasal corticosteroids used either as immunotherapy, or in combination,

depending on the predominant symptoms and the patient's response to therapy [31]. But little was known about its pathological mechanisms and potential gene therapy targets. However, based on our previous research, ADRB2 seems to be very promising for allergic diseases such as AR. The role of ADRB2 in the pathogenesis of inflammation and allergy, and especially, in the treatment of asthma has been a subject of intense speculation and investigation for many years [7]. ADRB2 is a transmembrane prototypical G protein-coupled receptor (GPCR), which is an important target for endogenous circulating catecholamines and exogenous

adrenergic agonists. It has been reported as a candidate gene for asthma, however, its function in AR was first discussed in this research.

Currently, our research is only concerned with the pathology of AR, especially in the case of NECs. Moreover, the model of IL-13-stimulated NECs can only be used for the study of molecular mechanisms in vitro, but cannot completely imitate the actual conditions of patients. In future studies, on the one hand, we want to find a model that is closer to the situation in vivo. On the other hand, we may focus on developing drugs targeting ADRB2 and miR-15a-5p, or blocking the ADRB2-miR-15a-5p-IL-13 pathway. Compared with traditional immunosuppressive drugs, this drug may be better able to fundamentally treat AR allergic reactions.

In summary, ADRB2 inhibits the inflammatory response of NECs in AR stimulated by IL-13 stimulation, while miR-15a-5p has a negative regulatory effect on ADRB2. We identified the miR-15a-5p-ADRB2-IL-13 pathway and provide potential targets and novel thoughts for the clinical treatment of allergic rhinitis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals The procedures were approved by the human ethics committee of Yuhuangding Hospital.

Informed consent Informed consent was derived from all participants.

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