MicroRNA-16 inhibits interleukin-13-induced inflammatory cytokine secretion and mucus production in nasal epithelial cells by suppressing the IκB kinase β/nuclear factor-κB pathway

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Abstract. Chronic inflammation of the nasal mucosal tissue plays important roles in the pathogenesis of allergic rhinitis (AR). Aberrantly expressed microRNAs (miRNAs) have been found to have strong associations with inflammatory reactions in allergic diseases; however, its functional significance and molecular mechanism underlying in AR remains unclear. The aim of the present study was to investigate the biological functions of miRNA and reveal its underlying molecular mechanisms in AR. miRNA microarray was performed to analyze miRNAs expression levels in 3 paired nasal mucosal samples from patients with AR and a control group. Subsequently, human nasal epithelial cells (JME/CF15) were used as an *in vitro* model to further explore the functions of miRNAs. Microarray data revealed that miR-16 was one of the miRNAs being most significantly downregulated. Interleukin (IL)-13 stimulation gradually decreased the levels of miR-16 in JME/CF15 cells. Moreover, upregulation of miR-16 inhibited inflammatory cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF), eotaxin, IL-1β, IL-6 and IL-10 in IL-13-treated JME/CF15 cells. Furthermore, overexpression of miR-16 significantly decreased the mRNA and protein expression levels of mucin 5AC (MUC5AC). IκB kinase β (IKKβ) was identified as a direct target of miR-16 and its expression was negatively regulated by miR-16 at mRNA and protein levels. Notably, forced expression of miR-16 blocked NF-κB signaling by decreasing the expression of nuclear p-p65 and p-IκB-α, as well as increasing the expression of IκB-α in IL-13-treated nasal epithelial cells. Moreover, enhanced IKKβ reactivated the NF-κB pathway that was blocked by miR-16 mimics and then effectively suppressed the miR-16-mediated inhibitory effects on inflammatory response. These findings suggested that miR-16 suppressed the inflammatory response by inhibiting the activation of IKKβ/NF-κB signaling pathways.

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Introduction

Allergic rhinitis (AR) is a common chronic inflammatory disease of the nasal mucosa which is characterized by sneezing, itching, nasal obstruction, and rhinorrhea (1). Symptoms of AR are frequently bothersome, impair the quality of life and increase their socioeconomic burden. Despite numerous experimental therapies that have been made to improve the functional outcome of patients with AR, advances in therapy for this condition is still unsatisfactory (2). Thus, the development of an effective therapy for this disease is essential.

Emerging evidence indicates that allergic diseases including AR are often caused by numerous inflammatory mediators such as histamine, chemokines, and cytokines from immune cells (3,4). Over the past several years, IL-4 and IL-13 produced by T helper type 2 (TH2) cells, have been found to contribute to the pathogenesis of AR (5). For example, IL-13 has been found to promote mucus production and the secretion of inflammatory cytokines in airway epithelial cells (NEC) (6). In addition, IL-1β, IL-6, IL-8 inflammatory molecules produced by mast cell contributed to the influx of immune cells into nasal mucosa tissue of AR, and accelerated inflammatory reactions (7). Recently, Wang *et al* (8), found that platycodin D (PLD) inhibited IL-13 induced inflammatory response in NECs by blocking the activation of NF-κB and MAPK signaling pathways. Therefore, reducing the production of inflammatory molecules may be an effective way to treat AR (9).

MicroRNAs (miRNAs) are a group of small, evolutionarily conserved, noncoding RNAs of 18-25 nucleotides in length that regulate gene expression through mRNA cleavage and/or translation inhibition by binding to the 3'UTR of target genes (10). Recent studies found that a large number of abnormal miRNAs expression was closely associated with multiple allergic inflammatory diseases, such as asthma (11,12), AR (13) and atopic dermatitis (14,15). For example, Case *et al* (16) showed that miR-21 inhibited toll-like receptor 2 (TLR2) agonist-induced

lung inflammation in an animal model of asthma, which indicate the potential therapeutic effects of miR-21 on allergic diseases. Collison *et al* (17), demonstrated that inhibition of miR-145 significantly attenuated allergic inflammation of the nasal mucosa in mite-induced allergic airways disease. However, little attention has been paid on the evaluation of miRNAs function in AR.

In the present study, we found that miR-16 screened by microarray was downregulated in nasal mucosal samples from AR patients and overexpression of miR-16 inhibited inflammatory cytokines and mucus production in IL-13-induced cell model of AR. Furthermore we identified IKKβ, a key catalytic subunit of IKK complex as a direct target of miR-16 and investigated the roles of miR-16 on the activation of NF-κB pathway in IL-13-stimulated nasal epithelial cells.

Materials and methods

Preparation of nasal mucosal specimens. Nasal mucosal samples were obtained from inferior turbinate sections from 25 patients with perennial AR and 25 patients with nonallergic rhinitis (NAR control group) according to the method of Ruocco *et al* (18). Diagnosed based on the case history, nasal endoscopy, allergen skin-prick tests, and specific IgE assays. No patient had received oral steroids or other medications for 4 weeks prior to study recruitment. The presemt study was approved by the Institutional Review Board at Hangzhou First People's Hospital, Nanjing Medical University. All participants provided written informed consent.

microRNA expression profiling. Total RNA was extracted from nasal mucus from AR patients and NAR controls using the miRNeasy mini kit (Qiagen, West Sussex, UK) according to manufacturer's instructions. Samples were labeled with Hy3 using the miRCURY™ Array Labelling kit (Exiqon, Inc., Woburn, MA, USA) and hybridized to the miRCURY LNA™ Array (v.16.0; Agilent Technologies, Inc., Santa Clara, CA, USA). After washing, the chips were scanned with the Axon GenePix 4000B Microarray Scanner (Molecular Devices, LLC, Sunnyvale, CA, USA). The procedure and images process method as described previously (19). The miRNA expressions of all differentially expressed samples were clearly displayed by a hierarchical clustering heat map.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from nasal mucosal specimens and nasal epithelial cells using the miRNeasy mini kit (Qiagen) and TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. The concentration and purity of RNA were determined spectrophotometrically. Then the RNA was reverse transcribed into cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.). qPCR was performed using the Step One Plus Real-Time PCR System (Thermo Fisher Scientific, Inc.). Primer sequences: miR-16, sense 5'-TAGCAGCACGTAAATATTGGCG-3', antisense 5'-TGCGTGTCGTGGAGTC-3'; U6 sense 5'-TGCGGGTGCTCGCTTCGCAGC-3'; U6 antisense 5'-CCAGTGCAGGGTCCGAGGT-3'; mucin 5AC (MUC5AC) sense 5'-CGACAACTACTTCTGCGGTGC-3', antisense 5'-GCACTCATCCTTCCTGTCGTT-3'; IKKβ, sense 5'-TGTCAGTGGAAGCCCGGATAG-3', and antisense was 3'-AGGTTATGTGCTTCAGCCACCAG-5'; GAPDH, sense 5'-CAAGCTCATTTCCTGGTATGAC-3', antisense, 5'-CAGTGAGGGTCTCTCTCTTCCT-3'. The relative quantification of gene expression was analyzed with $2^{-\Delta\Delta}$CT method, normalized with U6 or GAPDH expression.

Cell cultures and treatment. Human nasal epithelial cell (JME/CF15) and human 293T cell lines were obtained from Academy of Military Medical Science, Beijing, China. JME/CF15 cells were routinely cultured in RPMI 1640 (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 2 mM glutamine, and 1 mM pyruvate and incubated at 37˚C with 5% CO_2 and 95% humidity. 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% FBS and 1% streptomycin-penicillin mix and incubated at 37˚C with 5% CO_2. The JME/CF15 cells were either unstimulated or stimulated with IL-13 (10 ng/ml) for 24 h or 30 min as previously described (8).

Cell transfection. MiR-16 mimics, miR-16 inhibitor and the corresponding negative control (mimics NC and inhibitor NC) were purchased from RiboBio (Guangzhou RiboBio Co., Ltd., Guangzhou, China). For enforced expression of IKKβ in JME/CF15 cells, open reading frame region of human IKKβ gene was amplified and inserted into the pcDNA3.1 eukaryotic expression vector (Invitrogen; Thermo Fisher Scientific, Inc.). The JME/CF15 cells were seeded at 6-well plate and transfection was performed with miR-16 mimics, mimics NC, miR-16 inhibitor and inhibitor NC (100 nmol) or 2 μg/ml IKKβ plasmid using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) on the following day according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA). After treatment, the concentrations of granulocyte-macrophage colony-stimulating factor (GM-CSF), Eotaxin, IL-1β, IL-6 and IL-8 in the cell culture supernatants were measured using ELISA kit in accordance with the manufacturers' instructions. ELISA kits were purchased from R&D Systems, Inc., (Minneapolis, MN, USA). All assays were performed in three independent times.

Luciferase reporter assay. To detect IKKβ as the direct binding target of miR-16, a luciferase reporter assay was performed. 293T cells were cultured in 24-well plate at a density of 1.5×10^6 cells for 24 h, and then miR-16 mimics, miR-16 inhibitor, and IKKβ 3'-UTR wild type (wt) or mutation (mut) of the putative miR-16 target region were co-transfected into this cells using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). Forty-eight hours after transfection, the cells were lysed and their luciferase activity was assayed using dual-luciferase reporter assay system (Promega Corporation, Madison, WI, USA). Luciferase activity was normalized to corresponding Renilla luciferase activity. All experiments were performed in three independent times.

Western blot. Proteins were obtained from cells the using RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Histone protein was extracted from cells using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents from Thermo Fisher Scientific, Inc., according with the manufacturers' instructions. Then the protein concentration was determined using BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Proteins (50 μg) were separated by 8% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Western blotting of MUCA5C, IKKβ, IκB-α, nuclear p-p65, histone 3 and β-actin was performed using a primary rabbit monoclonal anti-MUCA5C antibody, anti-IKKβ (1:500, Abcam, Cambridge, UK), anti-IκB-α (1:1,000), anti-p-IκB-α (1:1,000), anti-nuclear p-p65 (1:1,000), anti-histone 3 (1:1,000; all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-β-actin antibody (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C and followed by incubating with secondary antibody (goat anti-rabbit IgG conjugated with the horseradish peroxidase, 1:4,000 dilution; Cell Signaling Technology, Inc.) for 1 h at room temperature. The protein bands were visualized by ECL detection reagent (GE Healthcare Life Sciences, Piscataway, NJ, USA). Relative band intensities were determined by densitometry using Scion image software (v.4.0).

Statistical analysis. Statistical analyses were performed with SPSS v.13.0 software (SPSS, Inc., Chicago, IL, USA). All data were recorded as means ± SD. For numerical variables, the results were evaluated by the Student's t-test (comparison between 2 groups) or one way ANOVA to make multiple-group comparisons followed by the post hoc Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-16 was downregulated in nasal mucosal from AR patients. To identify the miRNAs that differed in expression between AR patients and NAR control group. Microarray analysis revealed a significant downregulation of 17 miRNAs and upregulation of 13 miRNAs in AR group compared with NAR control group (Fig. 1A). Among the aberrantly expressed miRNAs, miR-16 was one of the top downregulated miRNAs in nasal mucosal tissues and altered expression of miR-16 has been found to play a critical role in inflammation in several types of diseases (20,21). We also measured its expression in 25 pairs of the nasal mucosal tissues among AR patients and NAR control group by qRT-PCR and observed that miR-16 was significantly decreased in AR group compared with NAR control group (Fig. 1B). Thus, miR-16 was chose as the candidate for further study.

Recently, the IL-13-induced cell model of AR has been found to have similar pathologic changes reminiscent of AR and can be used in preliminary profiling therapies (22-24). Hence, we evaluated the expression of miR-16 in the cell model of IL-13-induced AR. As shown in Fig. 1C, IL-13 decreased the expression of miR-16 in this cell lines in a dose-dependent manner. These results suggest that miR-16 might be associated with the development of AR.

Validation of cell transfection efficiency. qPCR was performed to assess the transfection efficiency of miR-16 mimics or NC and miR-16 inhibitor or inhibitor NC and pcDNA-IKKβ or pcDNA-vector. Compared with the mimics NC group, the expression levels of miR-16 were markedly increased in cells transfected with miR-16 mimics (Fig. 2A). Similarly, compared with the inhibitor NC group, miR-16 expression levels were significantly decreased following transfection with miR-16 inhibitor (Fig. 2B). In addition, compared with empty vector group, the expression levels of IKKβ were obviously increased in cells transfected with pcDNA-IKKβ (Fig. 2C).

Overexpression of miR-16 inhibits the levels of inflammatory cytokines in IL-13-induced JME/CF15 cells. To further determine the protective effect of miR-16 in AR, the expression levels of inflammatory cytokines (TNF-α, IL-6 and IL-1β) were examined using ELISA. The results showed that IL-13 significantly increased the protein levels of GM-CSF, Eotaxin, IL-1β, IL-6 and IL-8 that are common markers for activated NF-κB pathway (7), whereas overexpression of miR-16 inhibited these protein expression levels in IL-13-stimulated JME/CF15 cells (Fig. 2D-H). All these results showed that overexpression of miR-16 could inhibit inflammatory responses by inhibiting these pro-inflammatory cytokines in AR.

Overexpression of miR-16 suppresses IL-13-induced mucus production in JME/CF15 cells. It has been demonstrated that mucus hypersecretion (particularly MUC5AC) is a common feature of allergic airway disorders including AR (25,26). To evaluate the effects of miR-16 on mucus hypersecretion, we measured the mRNA expression of MUC5AC in miR-16 mimics transfected JME/CF15 cells following IL-13 stimulation. Compared with the control group, the mRNA expression levels of MUC5AC were obviously increased by IL-13 stimulation, whereas this promoting effect was suppressed after miR-16 mimics transfection (Fig. 3A). Similarly, the result of Western blot showed that overexpression of miR-16 inhibited IL-13-induced MUC5AC protein expression in JME/CF15 cells (Fig. 3B). All these results showed that overexpression of miR-16 exert its anti-AR activity by inhibiting mucus production in JME/CF15 cells.

IKKβ was a direct target of miR-16. To investigate the molecular mechanism by which miR-16 functions in inflammatory responses in AR, two bioinformatics algorithms (TargetScan and miRanda) were used to search for potential targets of miR-16. According to the results of these analyses, we focused on IKKβ for experimental verification. In addition, IKKβ was reported to be a key catalytic subunit of IKK complex and associated with the activation of NF-κB pathway (27-29). As suggested in Fig. 4A, the complementary sequence of miR-16 was found in the 3'-UTR of IKKβ mRNA. Subsequently, JME/CF15 cells were transfected with miR-16 mimics, miR-16 inhibitor or controls and the mRNA of IKKβ was determined by qRT-PCR. The results showed that overexpression of miR-16 significantly reduced, whereas inhibition of miR-16 promoted the mRNA expression of IKKβ (Fig. 4B). To further test whether that miR-16 could directly target 3'-UTR of IKKβ, a luciferase reporter assay was conducted. The results showed that miR-16 mimics

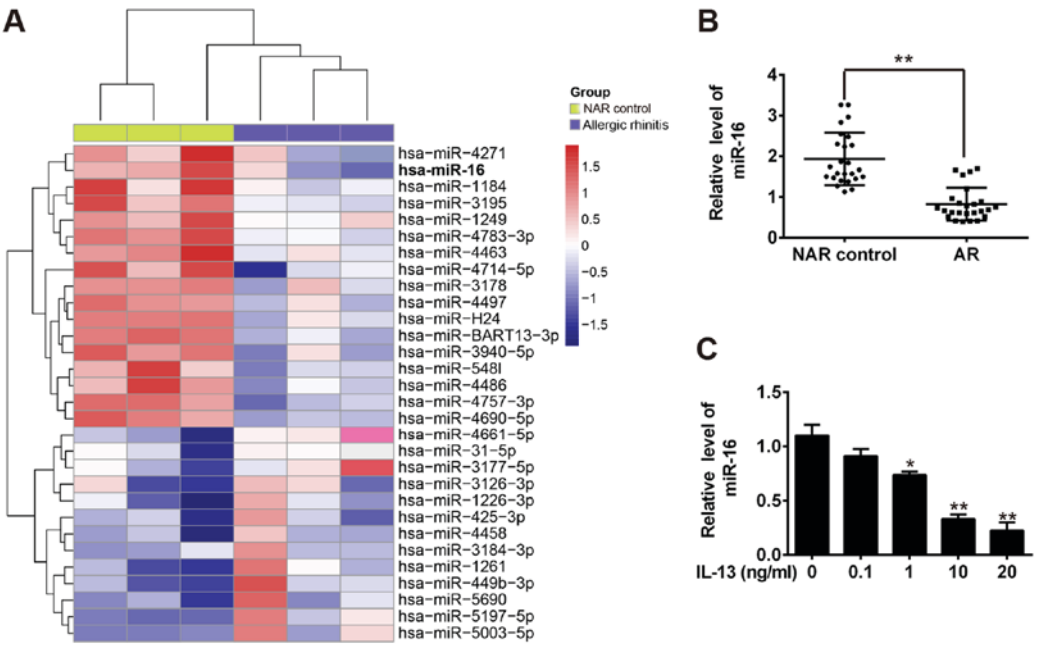

Figure 1. miR-16 was downregulated in nasal mucosal from AR patients. (A) Heatmap of normalized expression levels of miRNAs in 3 paired nasal mucosal tissues among AR patients and NAR controls. (B) RT-qPCR was performed to determine the expression levels of miR-16 in nasal mucosal tissues from 25 pairs of AR patients and NAR controls. **P<0.01 vs. NAR control group, t test. (C) Human JME/CF15 cells were treated with different concentrations of IL-13 (0, 0.1, 1, 10 and 20 ng/ml) for 24 h, then the expression levels of miR-16 was detected by RT-qPCR. Data represent the mean ± SD of three independent experiments. *P<0.05, **P<0.01 compared with IL-13 non-treatment group, one-way ANOVA, Tukey's post hoc test. AR, allergic rhinitis.

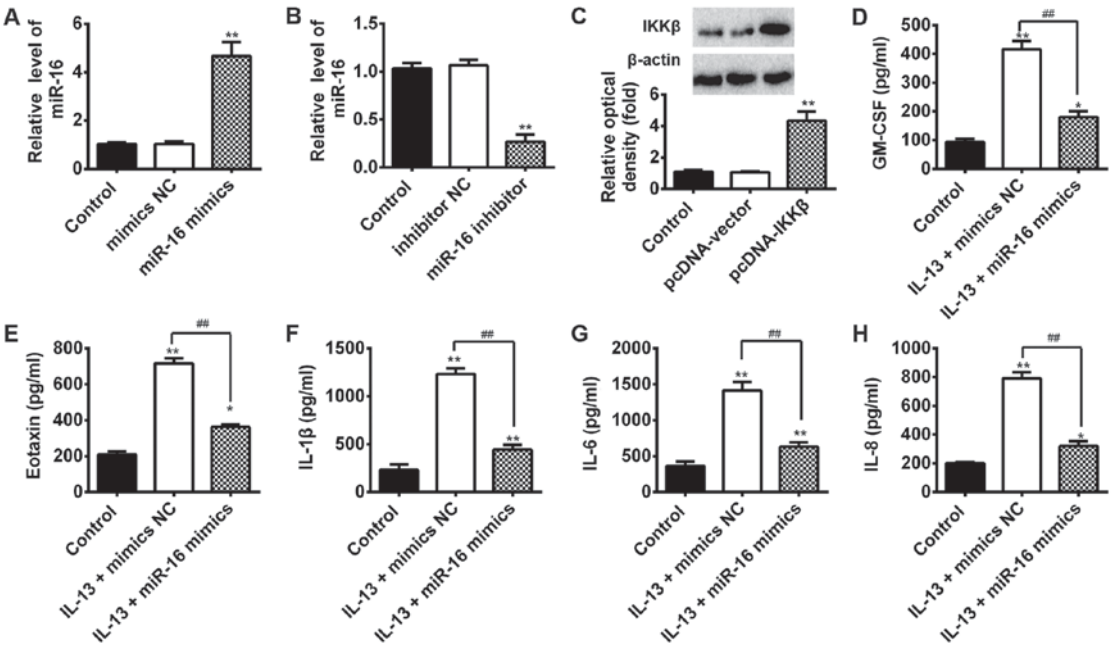

Figure 2. Overexpression of miR-16 inhibits the protein levels of inflammatory cytokines in IL-13-induced JME/CF15 cells. (A and B) RT-qPCR was performed to determine the expression of miR-16 in JME/CF15 cells after miR-16 mimics or miR-16 inhibitor transfection. (C) The protein levels of IKKβ in JME/CF15 cells transfected with IKKβ overexpression vector. D-H. The protein levels of (D) GM-CSF, (E) eotaxin, (F) IL-1β, (G) IL-6 and (H) IL-8 were determined by ELISA after miR-16 mimics transfection prior to treatment with IL-13 (10 ng/ml). Data represent the mean ± SD of three independent experiments. **P<0.01 compared with control group; $^{\#\#}$P<0.01 compared with IL-13 + mimics NC group. one-way ANOVA, Tukey's post hoc test. GM-CSF, granulocyte-macrophage colony-stimulating factor; IKKβ, IκB kinase β; NC, negative control.

significantly decreased, whereas miR-16 inhibitor increased the luciferase activity of IKKβ with wt 3'-UTR (Fig. 4C). The luciferase activity of mutant IKKβ was not affected by miR-16. (Fig. 4C). In addition, we also examined whether miR-16 could modulate the protein expression of IKKβ in JME/CF15 cells. The results of Western blot showed that overexpression of miR-16 significantly reduced, whereas inhibition of miR-16 promoted the protein expressions of IKKβ (Fig. 4D). These results indicate that IKKβ is a main functional target of miR-16 in the development of AR.

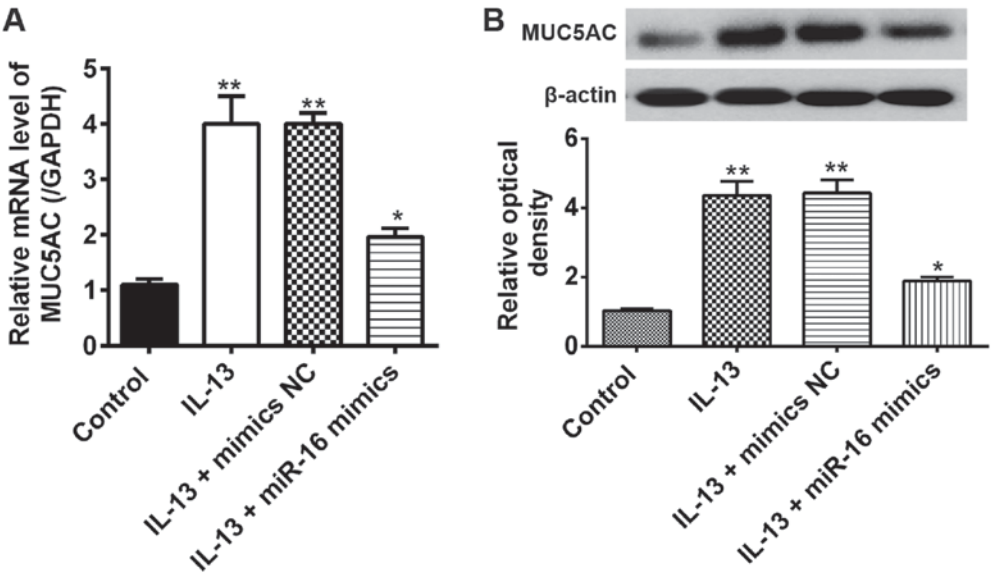

Figure 3. Overexpression of miR-16 suppresses IL-13-induced mucus production in JME/CF15 cells. Human JME/CF15 cells were transfected with miR-16 mimics or mimics negative control prior to treatment with IL-13 (10 ng/ml) for 24 h. (A) RT-qPCR was performed to determine the mRNA levels of MUC5AC in IL-13 stimulated JME/CF15 cells. (B) Western Blot analysis was performed to determine the protein levels of MUC5AC in IL-13 stimulated JME/CF15 cells. Data represent the mean ± SD of three independent experiments. *P<0.05, **P<0.01 compared with control group, one-way ANOVA, Tukey's post hoc test. MUC5AC, mucin 5AC.

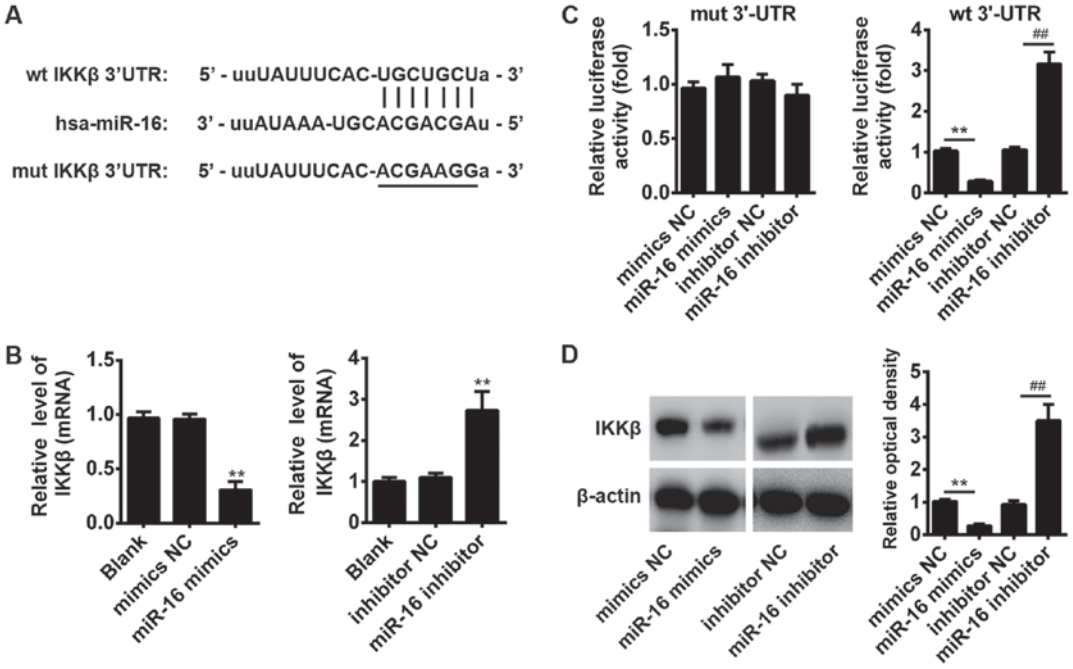

Figure 4. IKKβ was a direct target of miR-16. (A) The predicted miR-16 binding sites on IKKβ. (B) miR-16 mimics, miR-16 inhibitor and controls were transfected into JME/CF15 cells, then the mRNA levels of IKKβ were detected by RT-qPCR. **P<0.01 vs. mimics NC, inhibitor NC or Blank group, one-way ANOVA, Tukey's post hoc test. (C) Luciferase activity in 293T cells co-transfected with miR-16 mimics, miR-16 inhibitor and luciferase reporters containing IKKβ wild type or mutant type (MUT) 3'-UTR. Histogram indicates the values of luciferase measured 48 h after transfection. **P<0.01 vs. mimics NC group; ##P<0.01 vs. inhibitor NC group, one-way ANOVA, Tukey's post hoc test. (D) miR-16 mimics, miR-16 inhibitor and controls were transfected into JME/CF15 cells, then the protein levels of IKKβ were detected by western blot assays. Data represent the mean ± SD of three independent experiments. **P<0.01 vs. mimics NC group; ##P<0.01 vs. inhibitor NC group, one-way ANOVA, Tukey's post hoc test. IKKβ, IκB kinase β; NC, negative control.

Effects of miR-16 on NF-κB signaling pathway in IL-13-induced JME/CF15 cells. Recent studies have been shown that miR-16 negatively modulated the activation of the NF-κB pathway in glioblastoma multiforme (GBM) (30) and in the diabetic retinopathy (31). It is well-known that NF-κB signaling pathway plays important roles in allergic diseases including AR (32-35). Thus, we sought to determine whether NF-κB pathway involves in the protective effect of miR-16 in AR. As shown in Fig. 5A and B, IL-13 treatment significantly increased the protein expression level of IKKβ, p-p65 and p-IκB-α, decreased the expression levels of IκB-α, while overexpression of miR-16 reduced the expression of

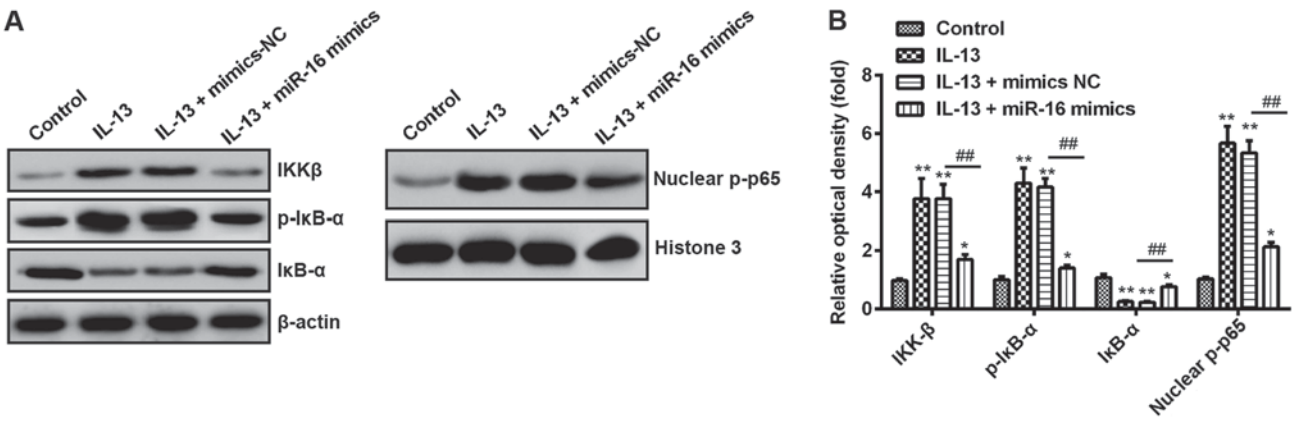

Figure 5. Overexpression of miR-16 blocked the activation of NF-κB signaling pathway in IL-13-induced JME/CF15 cells. Human JME/CF15 cell lines were transfected with miR-16 mimics or mimics NC prior to treatment with IL-13 (10 ng/ml) for 24 h. (A) Protein expressions of IKKβ, p-IκB-α, IκB-α and nuclear p-p65 in IL-13 stimulated JME/CF15 cells were determined by western blot analysis. (B) Bands were semi-quantitatively analyzed by using Image J software, normalized to β-actin or Histone 3 density. Data represent the mean ± SD of three independent experiments. *P<0.05, **P<0.01 vs. control group; $^{\#\#}$P<0.01 vs. IL-13 + mimics NC group, one-way ANOVA, Tukey's post hoc test. IKKβ, IκB kinase β; NC, negative control.

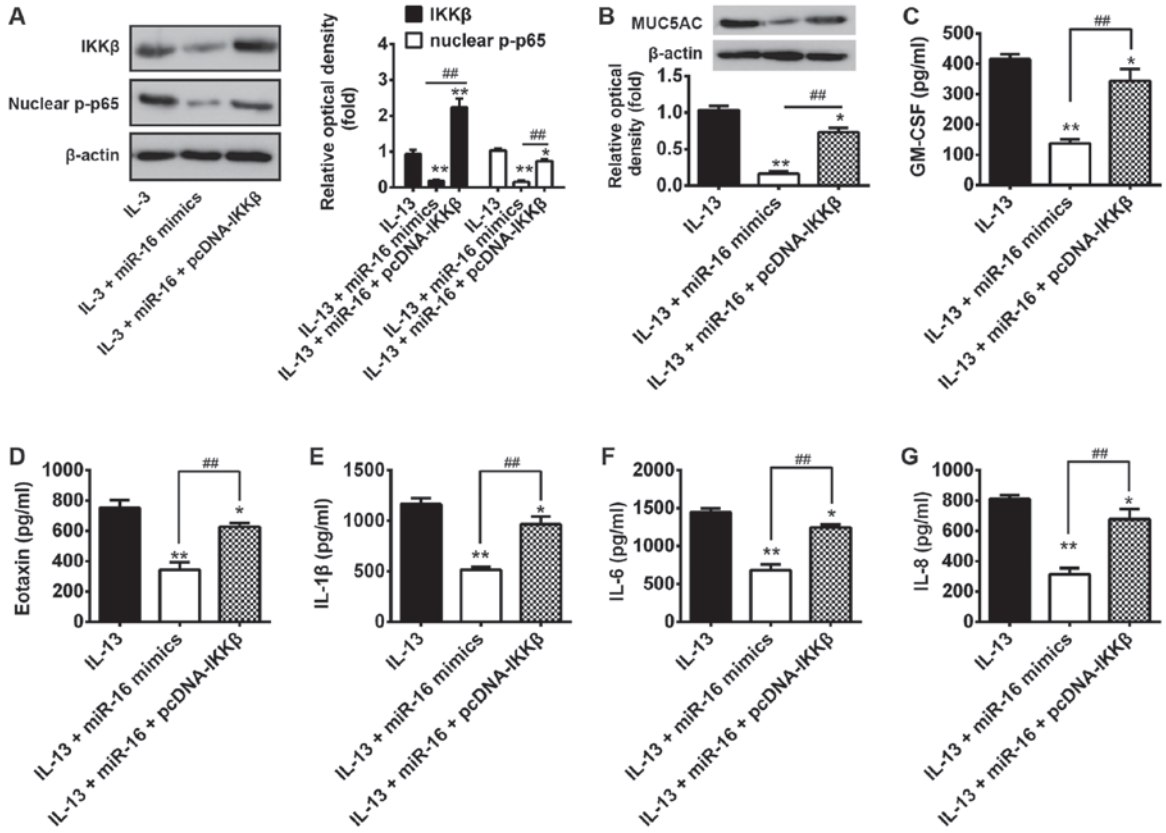

Figure 6. miR-16 inhibits IL-13-induced mucus production and inflammation through IKKβ. Cells were co-transfected with miR-16 mimics and pcDNA-IKKβ for 24 h, then treated with 10 ng/ml IL-13 for another 24 h. (A and B) Protein levels of IKKβ, nuclear p-p65 and MUCA5C were measured by western Blot. (C-G) The levels of cytokines including GM-CSF, eotaxin, IL-1β, IL-6 and IL-8 were determined by ELISA. *P<0.05, **P<0.01 vs. IL-13 alone group; $^{\#\#}$P<0.01 vs. IL-13 + miR-16 mimics group, one-way ANOVA, Tukey's post hoc test. IKKβ, IκB kinase β; GM-CSF, granulocyte-macrophage colony-stimulating factor.

IKKβ, nuclear p-p65, p-IκB-α, and promoted IκB-α expression in IL-13-treated JME/CF15 cells. These results indicate that miR-16 inhibits inflammatory response via blocking the activation of NF-κB signaling pathway.

miR-16 inhibits IL-13-induced inflammation through IKKβ. IKKβ has been shown to play an essential role in the activation of NF-κB signaling pathway (27-29). Thus, we then explored whether IKKβ could rescue the inhibition of NF-κB and pro-inflammatory cytokines mediated by miR-16. We found that overexpression of IKKβ along with miR-16 followed by IL-13 stimulation significantly rescued the inhibition of nuclear p-p65 and MUC5AC expression levels (Fig. 6A and B). Moreover, the levels of the pro-inflammatory cytokines including GM-CSF, Eotaxin, IL-1β, IL-6 and IL-8 were also rescued (Fig. 6C-G). Taken together, these data suggest that IL-13 inhibits miR-16

expression which is involved in the negative regulation of IL-13-stimulated NF-κB activation and pro-inflammatory cytokines production through targeting IKKβ.

Discussion

In the present study, miR-16 was found to be downregulated in the nasal mucosal tissues from AR patients and IL-13-induced cell model of AR. Moreover, we demonstrated that miR-16 exerted its anti-inflammation activity through blocking IL-13-stimulated NF-κB activation in JME/CF15 cells. Our findings suggest that miR-16 may be a potential target for the prevention and treatment of AR.

Previous studies have indicated that miRNAs have key roles in allergic diseases. For example, miR-146a was increased in keratinocytes and chronic lesional skin of patients with atopic dermatitis (AD) and functioned as an important regulator in chronic inflammation (36). Malmhäll *et al* (37), found the involvement of miR-155 in the regulation of allergic airway inflammation by modulating TH2 responses. However, to date, little attention has been paid to the role of miRNAs in the development of AR. A recent report from Yu *et al* (13), analyzed the differentially expressed miRNAs in AR using miRNA microarrays, and listed a catalogue of miRNAs potentially involved in the development of AR. Another study from Teng *et al* (38), found that miR-143 inhibited IL-13-induced inflammatory response in NECs from AR patients by targeting IL13Rα1. Thus, miR-143 may represent a promising therapeutic target for allergic inflammation. These data promote us to determine whether other miRNAs involved in the inflammation response in AR. In this study, we identified a large number of miRNAs that were significantly deregulated in nasal mucosal tissues from AR patients using miRNA microarray. Among them, miR-16 was one of the most downregulated miRNAs and also reported to play different roles in inflammatory diseases (20,21). Thus, we chose miR-16 for further study. Subsequently, we found miR-16 was significantly decreased in AR patients and IL-13 treated JME/CF15 cells. These results indicate that miR-16 may be related to the pathogenesis of AR.

The pathophysiological mechanisms of AR are complicated. During this period, allergic inflammation plays an important role in the process of AR, which can be orchestrated by a variety of cell mediators, such as IL-4 and IL-13 (39,40). Among the key drivers, IL-13 pretreatment could cause severe sepsis and inflammation (41). Proinflammatory cytokines, such as GM-CSF, eotaxin and IL-1β were obviously increased in the NECs following IL-13 stimulation (38). In addition, IL-13-induced cell model of AR has been widely used to simulate the major events of AR. In this model, IL-13 treatment can lead to mucus hypersecretion and inflammatory factors release in airway epithelial cells (22,23). In our experiment, we used this model to explore the roles of miR-16 in inflammatory responses in AR. Consistent with above studies, we found that the levels of MUC5AC and the pro-inflammatory cytokines including GM-CSF, Eotaxin, IL-1β, IL-6 and IL-8 were increased in IL-13-treated JME/CF15 cells, whereas overexpression of miR-16 reversed the promoting effects of IL-13, suggesting the potential protective effect of miR-16 in AR. Although the evidence highlighted the important roles of miR-16 in AR, the underlying mechanism through which miR-16 inhibits inflammatory responses in AR remains unclear.

Recently, many new insights into the core signaling pathways in allergic inflammatory diseases have been made, especially NF-κB signaling pathways (8,34,42). These pathways are often constitutively activated in subsets of human nasal epithelial cells in AR. NF-κB is a pleiotropic transcription factor that plays an important role in the inflammatory process. For example, by regulation NF-κB signaling pathway, PLD attenuated airway inflammation in a mouse model of allergic asthma (36). In allergic asthma, arsenictrioxide, a potent inhibitor of NF-κB, abrogated allergen induced inflammatory response (43). Therefore, targeting NF-κB signaling pathway could have benefit in the inhibition of inflammatory reaction in allergic diseases. Shin *et al* (44), found that miR-16 was directly regulated by NF-κB in gastric cancer. Thus, we hypothesis that miR-16 may exert a significant therapeutic effect on AR through inhibiting the activation of NF-κB pathway. In a previous study, it was found that IKK, by phosphorylating the IκB, played an important role in the activation of NF-κB signaling pathway (29). In our study, we found miR-16 could directly target the 3'-UTR of IKKβ and negatively regulated the expression of IKKβ. Furthermore, we found that overexpression of miR-16 could reverse IL-13-induced the protein expression of NF-κB p65, IKKβ and IκB-α, indicate that miR-16 is involved in the negative regulation of IL-13-stimulated NF-κB activation. More importantly, we found that IKKβ could rescue the inhibition of NF-κB and pro-inflammatory cytokines mediated by miR-16. Based on the above results, we felt safe to conclude that miR-16 inhibited IL-13-induced inflammatory response in AR by inhibiting the activation of IKKβ/NF-κB signaling pathway.

In summary, our results showed that miR-16 was downregulated in AR and IL-13 treated JME/CF15 cells, and overexpression of miR-16 inhibited inflammatory response by blocking the activation of IKKβ/NF-κB signaling pathways. On this basis, it is proposed that the miR-16/IKKβ/NF-κB axis might represent a beneficial way of regulating nasal inflammation and preventing the development of AR.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YQG performed the experiments, contributed to data analysis and wrote the paper. ZZY analyzed the data. YQG conceptualized the study design, contributed to data analysis and experimental materials. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All individuals provided informed consent for the use of human specimens for clinical research. The present study was approved by the Ethics Committee of the Hangzhou First People's Hospital, Nanjing Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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