



miR-375 prevents nasal mucosa cells from apoptosis and ameliorates allergic rhinitis via inhibiting JAK2/STAT3 pathway



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ARTICLE INFO

Keywords:

MiR-375 nasal mucosa rhinitis JAK2/STAT3 ovalbumin

ABSTRACT

This study aims to explore the roles, and related mechanisms of miR-375 in nasal mucosa cells apoptosis and allergic rhinitis. Here, miR-375 was found to be decreased in the epithelia of nasal mucosa of allergic rhinitis mice and TNF- α -stimulated nasal mucosa cells, while JAK2 expression exhibited an opposite effect. Mechanistically, miR-375/JAK2 regulatory axis was identified in nasal mucosa cells via luciferase reporter, qRT-PCR, western blot and RNA immune co-precipitation (RIP) assays. Pre- or post-injection of miR-375 agomir following ovalbumin (OVA) treatment attenuated OVA-induced allergic rhinitis, characterized as the down-regulation of IL-6 and TNF- α secretion, and upregulation of IL-10 secretion, these effects were attenuated by infection with JAK2 adenovirus through nasal cavity inhalation. Additionally, overexpression of miR-375 reversed TNF- α -mediated nasal mucosa cells apoptosis, which was attenuated by JAK2 overexpression. Therefore, our results indicate that miR-375 prevents nasal mucosa cells from apoptosis and ameliorates allergic rhinitis via inhibiting JAK2/STAT3 pathway.

1. Introduction

Allergic rhinitis is a common disease of otolaryngology, and its incidence is increasing along with global industrialization and 30% of world's population is troubled by it, especially in children and young people [1]. Although there are many drugs including antihistamines, corticosteroids, decongestants, and intranasal anticholinergic drugs that are used for treating allergic rhinitis, they either have high side effects or low therapeutic effects [2]. Thus, it is an urgent task to find novel targets or markers for treating or diagnosing allergic rhinitis.

MiRNAs are a kind of short non-coding RNA that modulates transcripts expression via promoting mRNAs degradation or inhibiting their translation [3]. MiRNAs play important roles in various physiological and pathological processes, like cancer progression [4], inflammation [5] and diabetes [6]. MiR-375 has been found to inhibit *Helicobacter pylori*-induced gastric carcinogenesis by blocking JAK2-STAT3 signaling [7]. And miR-375 could ameliorate morphine analgesic tolerance in mouse dorsal root ganglia [8]. Additionally, miR-375 could negatively regulate porcine preadipocyte differentiation by targeting BMPR2 [9], and miR-375 could ameliorate sepsis by downregulating miR-21 level [10]. Previous study has shown that MiR-375 is down-regulated in epithelial cells after IL-13 stimulation and regulates an IL-

13 induced epithelial transcriptome [11]. Notably, miR-375 could modulate bone marrow-derived progenitor cell-mediated myocardial repair and function after myocardial infarction [12] and post-myocardial infarction inflammatory response and left ventricular dysfunction via PDK-1-AKT signalling axis [13]. However, the roles and related mechanisms of miR-375 in allergic rhinitis are still unclear.

In addition to inflammation, aberrant apoptosis of nasal mucosa cells also contributes to allergic rhinitis [14]. JAK2/STAT3 pathway has also been shown to engage in apoptosis, like Yu J et al showed that advanced glycation end products could induce apoptosis in mouse podocytes through CXCL9-mediated JAK2/STAT3 pathway activation [15]. Moreover, the effects of tanshinone IIA on hypoxia/reoxygenation-induced myocardial microvascular endothelial cell apoptosis in rats are through the JAK2/STAT3 signaling pathway [16]. JAK2 has been proved to be a potential target of miR-375 in various studies [7,8,10]. However, whether the miR-375/JAK2/STAT3 axis exists in nasal mucosa cells apoptosis and is involved in allergic rhinitis are still confused.

Therefore, in this work, we attempted to explore the roles and related mechanisms of miR-375 in nasal mucosa cells apoptosis under OVA-induced allergic rhinitis.

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2. Materials and methods

2.1. Animals and treatments

This study was approved by the Ethics Committee of Shanghai Jiao Tong University. Thirty female BALB/C mice (5 weeks old), purchased from the Model Animal Research Center Of Nanjing University (Nanjing, China) were housed under conventional conditions for 1 week prior to any experiments. Then, the mice were randomly divided into four groups and received corresponding treatments. Normal group, mice were sensitized and challenged by saline alone. Allergic rhinitis group (OVA), mice were sensitized and challenged by ovalbumin (OVA). OVA + miR-375, OVA + JAK2 knockdown adenoviruses (GenePharma, Shanghai, China), mice were sensitized and challenged by ovalbumin (OVA) followed by injection of miR-375 agomir (20 μ M, 5 μ l per mouse, BIOTEND, Shanghai, China) for another 4 days. OVA + miR-375 + JAK2, mice were sensitized and challenged by ovalbumin (OVA) followed by injection of miR-375 agomir plus nasal infection of JAK2 overexpression adenoviruses (GenePharma) for another 4 days.

3. OVA challenge protocol

Mice received an *i.p.* injection of 100 μ l of sensitization fluid containing 0.5 mg/ml OVA and 20 mg/ml $\text{Al}(\text{OH})_3$ (1:1) three times one week. After two weeks, 50 μ l of OVA solution (2 mg/ml) was given through pulverization inhalation for 30 min once a day for one week. Mice in normal group were sensitized and challenged with 0.9% normal saline.

4. TUNEL staining

After different treatment, nasal tissues were rinsed with saline, fixed with 10% formalin, embedded in paraffin and cut at a thickness of 5 μ m. Apoptosis was detected by *in situ* labeling using a TUNEL kit (Sangon Biotech, Shanghai, China) following the manufacturer's instructions. The slides were photographed by light microscopy and TUNEL-positive cells in five random visual fields were counted.

5. ELISA assay

The concentration of IL-6, IL-10 and TNF- α in mice plasma was measured using ELISA kits according to manufacturer's recommendation. ELISA kits were purchased from Abcam (ab209519 for IL-10; ab100712 for IL-6; ab100747 for TNF- α).

6. Cell culture and treatment

Human nasal mucosa cells HNEpC were purchased from American Type Culture Collection (ATCC) and maintained in Minimum Essential Medium (MEM) supplemented with 10% FCS (fetal calf serum) in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. HNEpC cells were transfected with miR-375 mimics followed by treatment with TNF- α (10 ng/ml) or transfected with miR-375 mimics plus infection of JAK2 overexpression adenoviruses followed by TNF- α treatment. After 48 h, cell apoptosis was examined using flow cytometry, and cell apoptosis markers expression (Cleaved caspase 3 and Cleaved PARP) was measured by western blot assay.

7. Cell apoptosis assay

After HNEpC cells being treated differently, cell apoptosis was measured using flow cytometry analysis with AnnexinV-FITC and propidium iodide (PI) (Cat# C1062, Beyotime, China). Cells were harvested, and washed with ice-cold PBS. Then cells were stained with Annexin V-FITC and PI (BD Biosciences, USA) following the

manufacturer's recommendation. Flow cytometry analysis was performed by flow cytometry (BD Biosciences).

8. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from nasal tissues or HNEpC cells using TRIzolTM Reagent (Cat # 15596026, Invitrogen, USA) following the manufacturer's instructions. Then total RNA was reverse transcribed into cDNA using one-step miRNA RT kit (Cat # 1801, Haigene, China) and EasyScript Reverse Transcriptase (TRANSGEN BIOTECH, China) following the standard protocols. Afterwards, the miR-375 and mRNA expression levels were detected according to the protocols of SYBR Premix Ex TaqTM kit (TaKaRa Bio Inc., China) and Hairpin-itTM MicroRNAs Quantitation (GenePharma), and performed on an ABI Prism 7500 Detection System (Applied Biosystems, Inc., USA). Then, the expression of miR-375 and each transcript was calculated using the $2^{-\Delta\Delta\text{ct}}$ method.

9. Western blot

Detailed procedure was described elsewhere [17]. The antibody against JAK2 (ab108596), Cleaved caspase 3 (ab2302), Cleaved PARP (ab32064), Caspase 3 (ab13847), PARP (ab32138), p-STAT3 (ab30647), STAT3 (ab119352) and β -actin (ab8227) were purchased from Abcam. Blots were washed and incubated with a peroxidase-conjugated antibody, and chemiluminescence was detected using an enhanced chemiluminescence kit (Millipore) followed by exposure with Tanon 5200.

10. Immunohistochemical staining analysis

Paraffin sections were prepared. After deparaffinization and rehydrated, the slides were blocked with 5% fetal bovine serum for 30 min at room temperature. Then, sections were incubated with primary antibodies for JAK2 and p-STAT3 overnight at 37 °C, and followed by incubation with biotinylated secondary antibody for 30 min at room temperature, and detected with the ABC immunodetection kits (Vector Laboratories). Sections were counterstained with Harris hematoxylin solution (Sigma), passed through a dehydration process and cover slipped.

11. Luciferase reporter assay

The detailed procedure was referred to the previous study [3]. pMIR-Reporter was used to introduce the fragments of JAK2-3'UTR containing the wild-type (wt) and mutant binding sites (mut) for miR-375, named as Luc-JAK2-wt and Luc-JAK2-mut, respectively. miR-375 antagomir was purchased from BIOTEND. Transfection efficiency was normalized with β -gal expression.

12. RNA immunoprecipitation (RIP) of Ago2

The detailed procedure was referred to the previous study [10]. Briefly, HNEpC cells transfected with different plasmids were lysed with 25 mM Tris-HCl buffer (pH 7.5) and 100 U/ml RNase inhibitor (Sigma), and then incubated with protein-A Sepharose beads pre-coated with 3 μ g anti-Ago2 antibody or control rabbit IgG for 1.5 h at 4 °C. The RNA-protein complexes were pulled-down by protein A/G agarose beads and RNA was extracted with Trizol, followed by detecting miR-375 level with qRT-PCR.

13. Statistical analysis

All data were presented as the mean $\pm$ SD (standard deviation). Datasets with only two groups were analyzed using a student's *t*-test. The differences between the groups were analyzed using One-way

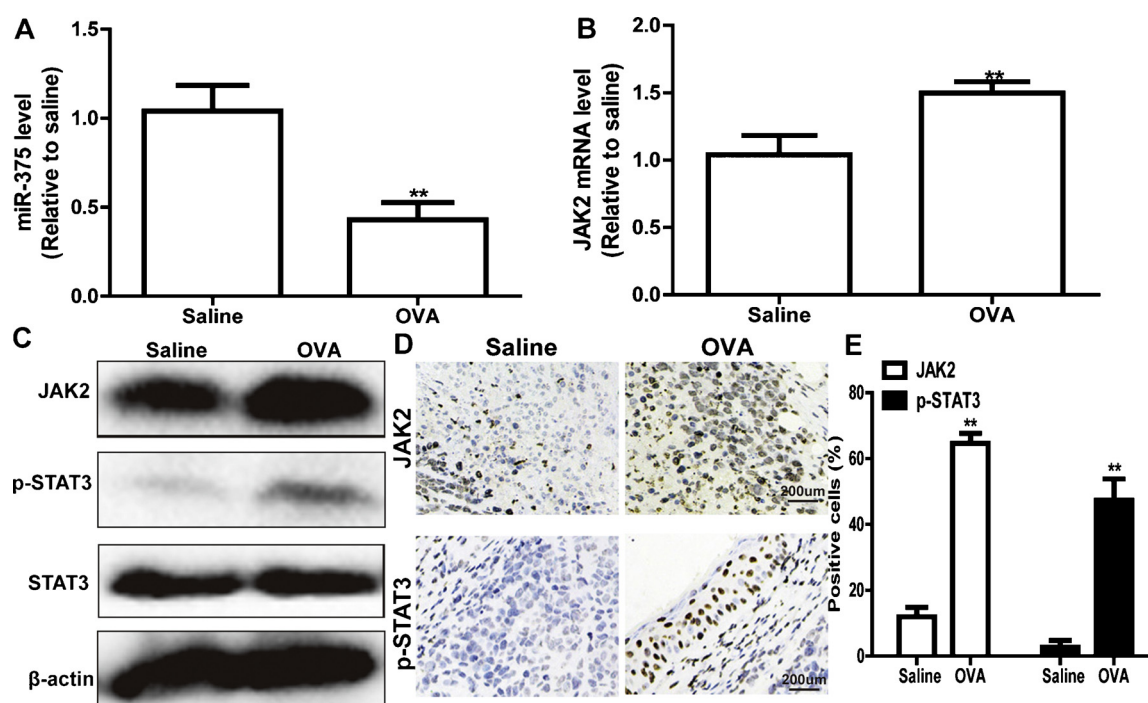


Fig. 1. miR-375 and JAK2 display opposite expression pattern in allergic rhinitis mice. (A and B) miR-375 (A) and JAK2 mRNA (B) level was detected in epithelia of nasal mucosa of mice treated with OVA or not via qRT-PCR. (C) JAK2, p-STAT3 and STAT3 protein level was examined in epithelia of nasal mucosa of mice treated with OVA or not via western blot. (D and E) JAK2 and p-STAT3 expression was measured in epithelia of nasal mucosa of mice treated with OVA or not via immunohistochemistry. Data were presented as mean $\pm$ s.d.; **P < 0.01 vs. Saline.

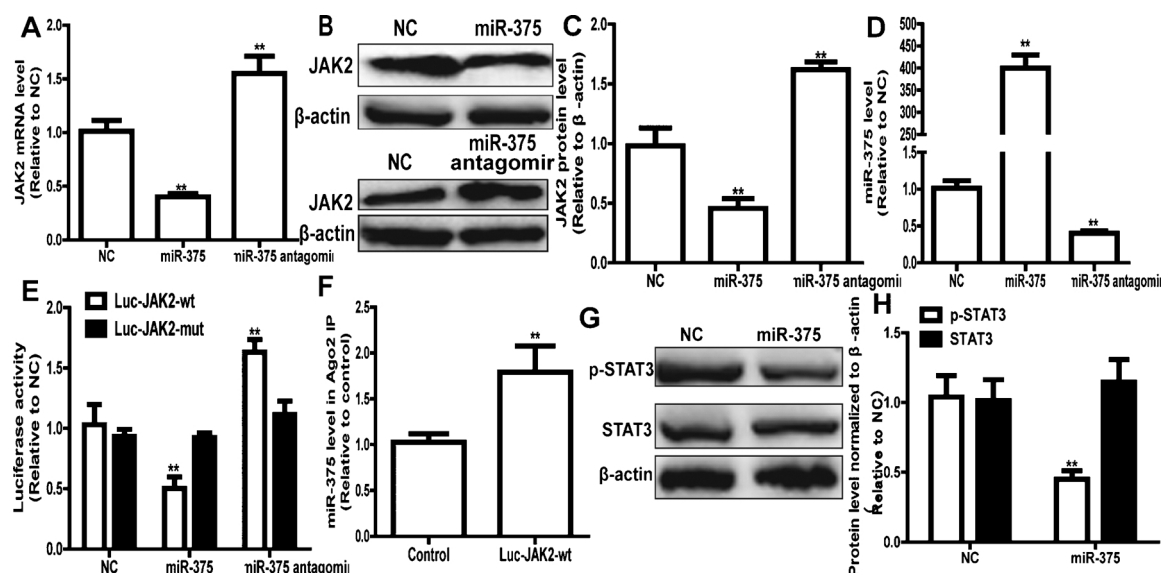


Fig. 2. miR-375 could target JAK2 in nasal mucosa cells. (A–C) JAK2 mRNA (A) and protein (B and C) level was detected in HNEpC cells with miR-375 mimics transfection or antagonist treatment or not. (D) The transfection efficiency of miR-375 mimics and antagonist was confirmed via qRT-PCR assay. (E) HNEpC cells were co-transfected with pMIR-Reporter empty vector (used as a control) or Luc-JAK2-wt or Luc-JAK2-mut, miR-375 mimics or antagonist and β-gal control plasmid for 72 h, then luciferase activity was measured and normalized to β-gal activity. (F) qRT-PCR was used to measure the abundance of miR-375 present in the Ago2-IP materials after the RIP assay in HNEpC cells transfected with Luc-JAK2-wt. Cells without any treatment were used as a control group. (G and H) Protein level of p-STAT3 and STAT3 was examined in cells depicted in (A). Data were presented as mean $\pm$ s.d.; **P < 0.01 vs. NC or Control.

ANOVA with the Tukey–Kramer post-test, and P < 0.05 was considered significant.

14. Results

14.1. miR-375 and JAK2 display opposite expression pattern in allergic rhinitis mice

To explore the roles of miR-375 in allergic rhinitis, we firstly detected miR-375 level in the epithelia of nasal mucosa from mice with OVA induced allergic rhinitis mice. As shown in Fig. 1A, miR-375 level

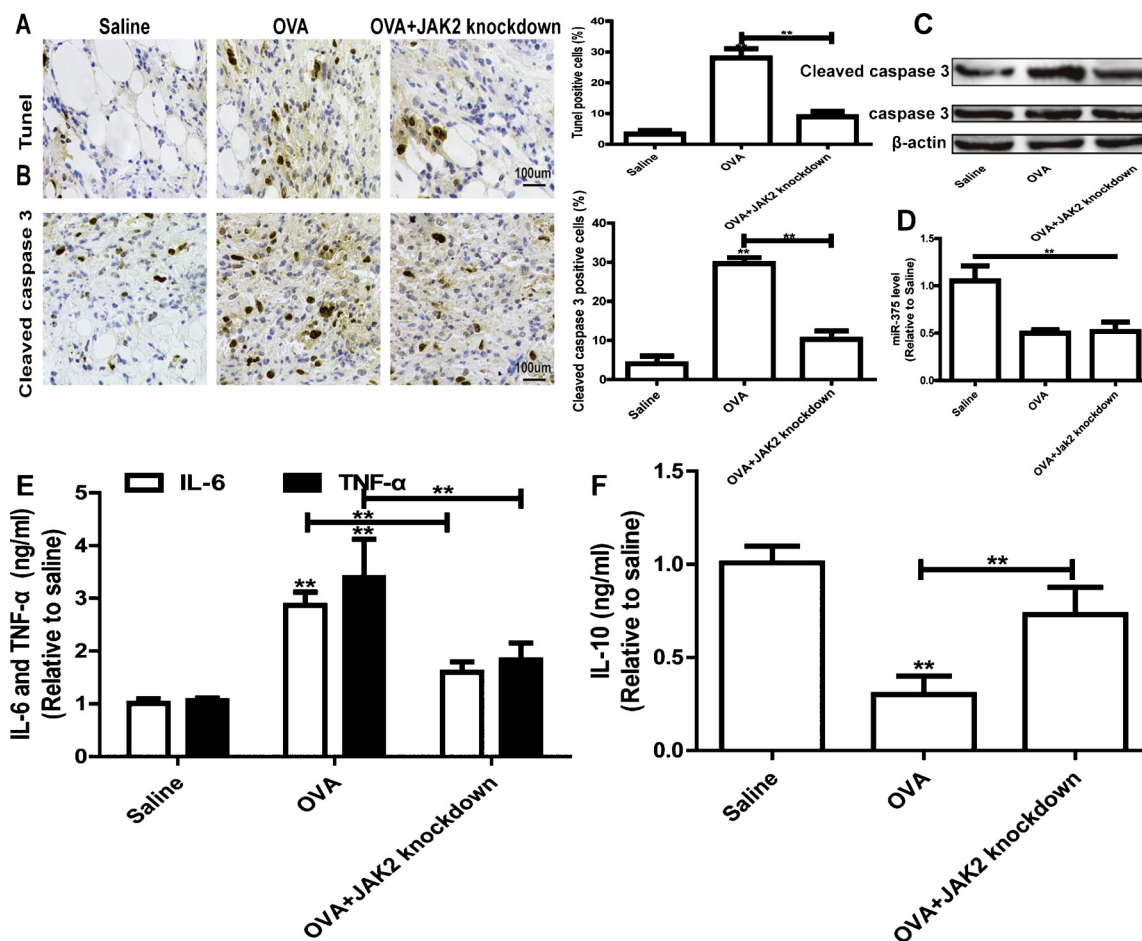


Fig. 3. Increased epithelia apoptosis and inflammation of nasal mucosa in allergic rhinitis mice was suppressed by JAK2 knockdown. (A) TUNEL and Cleaved caspase 3 staining of epithelia of nasal mucosa in mice with OVA treatment plus JAK2 knockdown adenoviruses infection or not. (B) Cleaved caspase 3 staining of epithelia of nasal mucosa in mice depicted in (A) via immunohistochemistry. (C) Protein level of cleaved caspase 3 and total caspase 3 was detected in epithelia of nasal mucosa in mice depicted in (A) via western blot assay. (D) miR-375 level was examined in epithelia of nasal mucosa of mice depicted in (A). (E and F) proinflammatory cytokines (IL-6 and TNF- α) and anti-inflammatory cytokine IL-10 secretion level was examined in mice plasma depicted in (A) via ELISA. Data were presented as mean $\pm$ s.d.; **P < 0.01 vs. NC or Saline.

was remarkably downregulated in the epithelia of nasal mucosa from mice with OVA induced allergic rhinitis compared to saline-treated controls. Since JAK2/STAT3 pathway has been shown to be involved in apoptosis and sepsis, which was linked with allergies [18], JAK2 and downstream effector p-STAT3 expression was further examined. In contrast, JAK2 and p-STAT3 expression was significantly increased in the epithelia of nasal mucosa from mice with OVA induced allergic rhinitis compared to saline-treated controls, evidenced by qRT-PCR (Fig. 1B), western blot (Fig. 1C) and immunohistochemical (Fig. 1D and E) analysis. These results indicated that miR-375 and JAK2 display opposite expression pattern in allergic rhinitis mice.

14.2. miR-375 could target JAK2 in nasal mucosa cells

As miR-375/JAK2 axis has been denoted in gastric cancer, mouse dorsal root ganglia and sepsis, we speculated that this axis also existed in nasal mucosa cells. qRT-PCR and western blot analysis showed that overexpression of miR-375 or knockdown of miR-375 in nasal mucosa cells significantly decreased or increased JAK2 mRNA and protein expression, respectively (Fig. 2A–C). The transfection efficiency of miR-375 mimics and antagomir was confirmed by qRT-PCR assay (Fig. 2D). Further luciferase reporter assay indicated that miR-375 mimics and antagomir decreased or increased the activity of Luc-JAK2-wt, respectively, but had no effect on Luc-JAK2-mut activity (Fig. 2E). To confirm the direct interaction between miR-375 and JAK2 at endogenous levels,

we performed RIP analysis to pull down endogenous miRNAs associated with Ago2 in Luc-JAK2-wt overexpressed HNEpC cells. The precipitated miRNAs were subjected to qRT-PCR analysis and results showed that miR-375 was enriched in RNAs retrieved from Luc-JAK2-wt-overexpressed cells (Fig. 2F), supporting that JAK2 was the bona fide target of miR-375 in nasal mucosa cells. Additionally, the downstream effector of JAK2, p-STAT3 expression was decreased in miR-375-overexpressed HNEpC cells (Fig. 2G and H). Our results suggested that miR-375 could target JAK2 and inactivate the downstream signaling in nasal mucosa cells.

14.3. Increased epithelia apoptosis and inflammation of nasal mucosa in allergic rhinitis mice was suppressed by JAK2 knockdown

We sought to investigate whether the allergic rhinitis was indeed led by JAK2 overexpression. To address this, apoptosis in nasal mucosa was evaluated by TUNEL staining and determination of cleaved caspase-3 through immunohistochemical and western blot assay, and results showed that JAK2 knockdown by adenoviruses infection significantly reduced the increased TUNEL- and Cleaved caspase 3-positive cells, and the expression of Cleaved caspase 3 in nasal mucosa from allergic rhinitis mice (Fig. 3A–C). Notably, JAK2 knockdown did not affect the reduction of miR-375 level induced by OVA treatment (Fig. 3D). Additionally, JAK2 knockdown could efficiently decrease the upregulated proinflammatory cytokines (IL-6 and TNF- α), whereas increase the

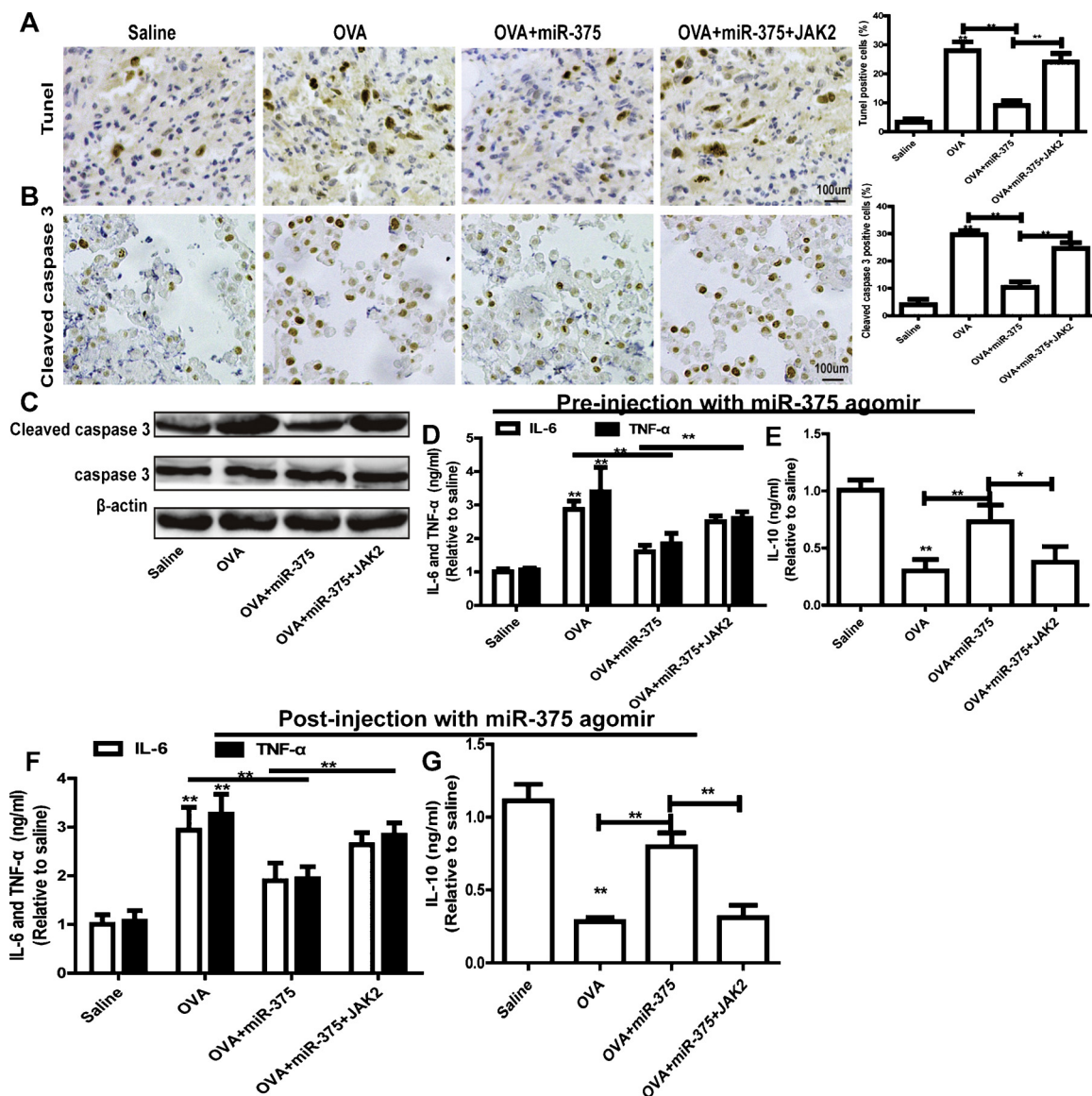


Fig. 4. Increased epithelia apoptosis and inflammation of nasal mucosa in allergic rhinitis mice was suppressed by miR-375 agomir in a JAK2-dependent manner. (A) TUNEL staining of epithelia of nasal mucosa in mice with OVA treatment plus miR-375 agomir pre-injection and JAK2 overexpression adenoviruses infection or not. (B) Cleaved caspase 3 staining of epithelia of nasal mucosa in mice depicted in (A) via immunohistochemistry. (C) Protein level of cleaved caspase 3 and total caspase 3 was detected in epithelia of nasal mucosa in mice depicted in (A) via western blot assay. (D and E) proinflammatory cytokines (IL-6 and TNF-α) and anti-inflammatory cytokine IL-10 secretion level was examined in mice plasma depicted in (A) via ELISA. (F and G) proinflammatory cytokines (IL-6 and TNF-α) and anti-inflammatory cytokine IL-10 secretion level was examined in mice plasma with OVA treatment plus miR-375 agomir post-injection and JAK2 overexpression adenoviruses infection or not via ELISA. Data were presented as mean $\pm$ s.d.; *P < 0.05, **P < 0.01 vs. NC or Saline.

decreased expression of anti-inflammatory cytokine IL-10 in allergic rhinitis mice plasma (Fig. 3E and F). The data suggested that impaired JAK2 expression may suppress the nasal mucosa inflammation in allergic rhinitis mice

14.4. Increased epithelia apoptosis and inflammation of nasal mucosa in allergic rhinitis mice was suppressed by miR-375 agomir in a JAK2-dependent manner

We further explored whether miR-375 could ameliorate nasal mucosa apoptosis and inflammation in allergic rhinitis mice in a JAK2-dependent manner. As shown in Fig. 4A–C, pre-injection with miR-375 agomir significantly decreased apoptosis in nasal mucosa evaluated by TUNEL staining and determination of cleaved caspase-3 through western blot assay, this effect was attenuated by JAK2 overexpression adenoviruses infection. Moreover, pre-injection with miR-375 agomir

decreased the upregulated proinflammatory cytokines (IL-6 and TNF-α), while increased the decreased expression of anti-inflammatory cytokine IL-10 in allergic rhinitis mice plasma, these effects also were attenuated or even reversed by JAK2 overexpression adenoviruses infection (Fig. 4D and E). Importantly, post-injection with miR-375 agomir exerted similar effects (Fig. 4F and G). These data suggested impaired miR-375 may suppress the nasal mucosa inflammation in allergic rhinitis mice partly dependent on JAK2 expression.

15. miR-375 affects the survival and cytokine expression of human nasal mucosa cells HNEpC in a JAK2-dependent manner

To explore the effects of allergic rhinitis on nasal mucosa cells and the role of miR-375/JAK2 axis played in this process, human nasal mucosa cell line HNEpC was stimulated with TNF-α, accompanied by miR-375 mimics transfection with JAK2 overexpression or not.

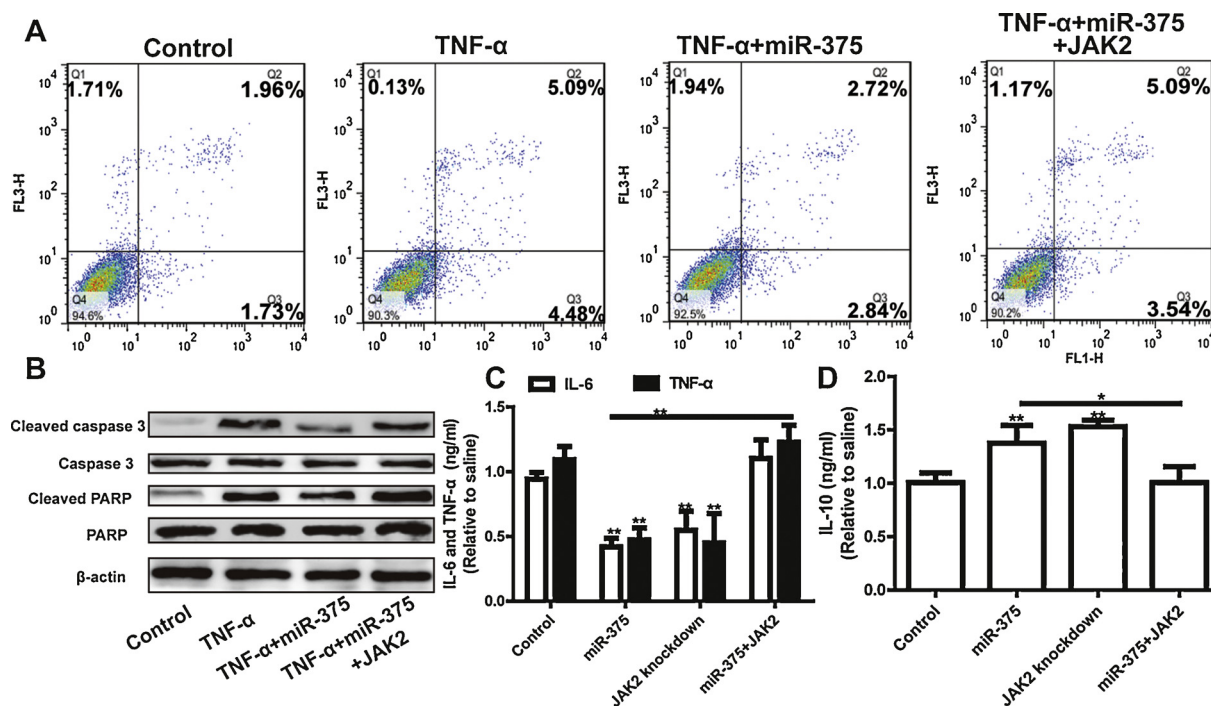


Fig. 5. miR-375 affects the survival and cytokine expression of human nasal mucosa cells HNEpC in a JAK2-dependent manner. (A) HNEpC cells apoptosis was determined with TNF- α plus miR-375 mimics transfection and JAK2 overexpression adenoviruses infection or not using flow cytometry assay. (B) The apoptotic executors (Cleaved caspase 3 and cleaved PARP) expression was measured in cells depicted in (A) via western blot assay. (C and D) proinflammatory cytokines (IL-6 and TNF- α) and anti-inflammatory cytokine IL-10 secretion level was examined in cells depicted in (A) via ELISA. Data were presented as mean $\pm$ s.d.; ** $P < 0.01$ vs. control.

Expression of Cleaved caspase 3 and Cleaved PARP was determined using flow cytometry and western blotting. The results showed that under simulation of TNF- α , miR-375 overexpression significantly inhibited nasal mucosa cells apoptosis, while JAK2 overexpression attenuated these effects (Fig. 5A and B). In addition, miR-375 overexpression or JAK2 knockdown could efficiently decrease the proinflammatory cytokines (IL-6 and TNF- α), whereas increase the expression of anti-inflammatory cytokine IL-10 in HNEpC cells, these effects were rescued by JAK2 overexpression (Fig. 5C and D). These results indicated that miR-375/JAK2 affects the survival and cytokine expression of human nasal mucosa cells HNEpC.

16. Discussion

In the present study, we found that miR-375 and JAK2 expression was significantly decreased or increased in epithelia of nasal mucosa of mice with ovalbumin (OVA) induced allergic rhinitis, respectively. Mechanistically, JAK2 was identified as the direct target of miR-375 in nasal mucosa cells. Overexpression of miR-375 or knockdown of JAK2 could efficiently suppress the increased epithelia apoptosis of nasal mucosa and inflammation in a JAK2-dependent manner. Consistent in vitro results in human nasal mucosa cells were obtained. Our results indicated that miR-375 could prevent nasal mucosa cells from apoptosis and ameliorate allergic rhinitis via inhibiting JAK2/STAT3 pathway.

miR-375 roles have been elucidated in many pathological processes, especially in cancer progression [19,20]. However, investigations about its role in allergic rhinitis are rare. Our findings share broad similarities with an earlier study in which miR-375 is downregulated in epithelial cells after IL-13 stimulation and regulates an IL-13-induced epithelial transcriptome [11]. However, the detailed mechanism is still unclear. Previous study has shown that baicalin could inhibit ovalbumin-induced allergic rhinitis guinea pigs and lipopolysaccharide-stimulated human mast cells via blocking JAK2-STAT5 and NF- κ B signaling pathways [21]. In this work, we identified JAK2 as a direct target of

miR-375 via qRT-PCR, western blot, luciferase reporter and RIP analysis, and found that miR-375 could block JAK/STAT3 pathway. But whether STAT5 or STAT families were blocked needs to be further explored. Importantly, the identified miR-375/JAK2 axis is consistent with various previous reports [7,10,22]. We strongly believe that miR-375/JAK2 axis is a common phenomenon in various pathological processes.

To the best of our knowledge, this is the first study concerning the influence of miR-375 on airway epithelial cells (ECs) apoptosis. In this work, we found that decreased miR-375 expression in human nasal mucosa cell line HNEpC significantly increased its susceptibility to TNF- α induced apoptosis, which is consistent with the in vivo results. Notably, as overexpression of JAK2 just could attenuate miR-375-induced protective effects in allergic rhinitis mice and human nasal mucosa cells, and based on the fact that miR-375 could block or activate other pathways [23,24]. Thus, there might be other pathways involved in miR-375-mediated protective effects in allergic rhinitis, which needs to be further investigated. In conclusion, since miR-375 expression is significantly reduced in epithelia of nasal mucosa of allergic rhinitis mice, which was tightly related to epithelial cell apoptosis and inflammation, miR-375 agomir may be a potential method for allergic rhinitis treatment.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the Shanghai Key Laboratory of Translational Medicine on Ear and Nose diseases (14DZ2260300).

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