Original Paper

Mechanism of MicroRNA-146a/*Notch2* Signaling Regulating *IL-6* in Graves Ophthalmopathy

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Key Words

Graves ophthalmopathy • *Notch2* • MicroRNA-146a • *IL-6* • qRT-PCR • Orbital Fibroblast reporter gene

Abstract

Background/Aims: We intended to investigate the significance of microRNA-146a, *Notch2* and *IL-6* on Graves ophthalmopathy (GO) and the relationships among them. **Methods:** About 27 GO patients were incorporated in this study, including 13 patients with inactive GO and 14 patients with active GO. Another 15 patients who had previously received strabismus orthopedics or ophthalmectomy after trauma were selected as the control population. QRT-PCR assay was used to detect microRNA-146a and *Notch2* expression levels in plasma. MTT assay and flow cytometry were respectively used to assess the viability and mitosis of the fibroblasts isolated from connective tissue. Double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was employed to detect serum *IL-6* levels. The dual luciferase reporter gene assay was used to verify the targeting relationship between microRNA-146a and *Notch2*. **Results:** Compared with the control group, the relative expression of miR-146a was significantly increased whereas the relative expression of *Notch2* was significantly decreased (all $P < 0.05$) in GO patients compare with the control. *Notch2* can be directly targeted by microRNA-146a. The over-expression of miR-146a markedly facilitated Orbital Fibroblasts (OFS) viability and mitosis whereas markedly suppressed cell apoptosis (all $P < 0.05$). Continuous microRNA-146a mimics could down-regulate the expression of *Notch2* and up-regulate *IL-6* ($P < 0.05$). The inhibition of microRNA-146 resulted in the elevated expression of *Notch2* and decreased expression of *IL-6* ($P < 0.05$). **Conclusion:** MicroRNA-146a may increase the *IL-6* levels and exacerbate GO by directly targeting *Notch2*.

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Introduction

Graves ophthalmopathy (GO) is a relatively common autoimmune disease that is closely associated with hyperthyroidism due to Graves' disease, the exact etiology and underlying

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mechanism of which still remain to be clarified [1]. GO is characterized by inflammation of the extraocular muscles and orbital connective tissues, which is caused by the infiltration of lymphocytes and macrophages [2, 3]. The infiltrated lymphocytes and macrophages generate cytokines and lead to the tissue inflammation [4]. The inflammation process involves a spectrum of chemokines and cytokines such as *IL-6*. Orbital fibroblasts are particularly susceptible to many pro-inflammatory cytokine activities. The intense immune responses are believed to account for the manifestation of Grave's disease [4]. Therefore, a thorough understanding of the involved cells, the cytokines, and the nature of their interplay is critical to manage GO [5].

IL-6 has been found to be closely correlated with GO pathogenesis, which can stimulate the thyroid stimulating hormone receptor (TSH-R) expression and increase the content in the orbital fat tissue differentiation process of patients [6, 7]. The regulation of *IL-6* may present as a novel management method for GO.

Accumulating evidence shows that microRNAs (miRNAs), a class of small non-coding RNAs, have significant negative modulating effects in pathological and physiological processes of various autoimmune diseases [8]. MiR-146a, a member of the classical miRNAs, has been found to participate in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA), Osteoarthritis (OA), systemic lupus erythematosus (SLE), multiple sclerosis (MS) and GO [3, 9-13]. For instance, MiR-146a was reported to induce TNF α production by targeting TNF- α /TNF receptor-associated factor 6 (TRAF6)/IL-1 receptor-associated kinase 1 (IRAK1) signaling in RA [14]. Again, miR-146a was reported to down-regulate *IL-6* production in orbital fibroblasts [15]. Besides, the overexpression of miR-146a could be induced by IL-10 and TGF- β but reduced by IL-12 and IFN α/β in human carcinoma cells, which then led to the suppression of NK cell-mediated cytotoxicity and IFN γ /TNF α production [16].

He et al. demonstrated that miR-146a is a negative regulatory factor of LPS-induced *IL-6* production by targeting Notch1 in LPS-induced THP-1 macrophage cells [17]. MiR-146a could also regulate Notch1 to modulate macrophage polarization, which could potentially regulate inflammatory diseases [18]. Notch1/Notch signaling promoted Fc epsilon RI-mediated *IL-4*, *IL-6*, *IL-13* and TNF α production of bone marrow-derived mast cells, which contributed to the differentiation of CD4⁺ T cells into T (H) 2 [19]. Intriguingly, it was found that the overexpression of Notch1 and Notch2 suppressed the production of TLR4-triggered pro-inflammatory cytokines such as *IL-6* and TNF α in macrophages [20]. However, the involvement of Notch2 in the autoimmune thyroiditis has not been clarified.

Notch2 is a putative target of miR-146a, and considering the close relationship between miR-146a or Notch signaling and GO or *IL-6*, we believe that the occurrence and development of GO is likely to be related to their interplay. At present, there is no study reporting the relationship between the miR-146a/Notch2/*IL-6* pathway and the development of GO. However, we believe their interplay could play a significant role during GO pathogenesis. Therefore, we designed this study to clarify the role of microRNA-146a/Notch2/*IL-6* during the development of GO and helped with the understanding of the pathogenesis of GO, which could provide a new intervention strategy for the prevention and treatment of GO.

Material and Methods

Research Subjects

Samples were obtained from the twenty-seven GO patients who accepted surgical treatments in Shanghai General Hospital from August 2014 to October 2014. The baseline characteristics of the objects were shown in Table 1. Patients with strabismus orthopedics or ophthalmectomy due to trauma were selected as the control group with similar age and gender ratios to their counterparts (the patients with GO). All of the patients with other autoimmune diseases were excluded. Ethics Committee of Shanghai General Hospital approved our research, and all the patients signed the consent forms.

Table 1. Demographic and clinical characteristics of patients. FT3: serum free triiodothyronine; FT4: serum free thyroxine; CAS: clinical activity score; ^a applies one-way ANOVA, ^b applies Chi-square test, ^c applies t-test

Characteristics	Normal	Inactive	Active	P value
Number	15	14	13	
Age (years)	48.7±9.2	50.1±9.8	49.5±10.2	0.927 ^a
Gender				0.633 ^b
Male	8	5	6	
Female	7	9	7	
Weight (kg)	64.5±9.2	66.2±9.7	65.3±10.1	0.894 ^a
Systolic blood pressure (mmHg)	124.8±17.7	123.4±17.9	124.5±17.2	0.927 ^a
Diastolic blood pressure (mmHg)	76.3±9.1	78.6±10.2	77.5±10.1	0.851 ^a
Smoking status				0.08 ^b
Yes	3	2	2	
No	12	12	11	
FT3(pg/ml)	/	11	7.5±3.10	0.221 ^c
FT4(ng/dL)	/	46±	4.23±2.93	0.626 ^c
Exophthalmos	/	1.76	19.60±1.94	0.833 ^c
CAS score	/	1.47	4.63±1.32	P<0.001 ^c

Orbital fibroblasts (OFs) separation and cultivation and Fluorescence Activated Cell Sorting (FACS)

The orbital connective tissue of GO patients and control group patients were removed under sterile condition, and thereafter were placed in DMEM/F12 medium immediately. The cell culture was put in 0°C ice water mixture, and transferred to cell culture laboratory. The experiment was then conducted in 5 hours. The samples were placed in Petri dish on the laminar flow bench, and washed three times by PBS (Phosphate Buffered Saline). Then the samples were cut into pieces after the adipose tissue and larger vessels were removed. The pieces were then sucked into 25ml cell culture flasks using a sterile plastic pipette, and they were made evenly distributed on the sidewall. Thereafter the DMEM/F12 medium containing 20% fetal calf serum was added into the flasks. Lastly, the caps were covered, the flasks were flipped and put in a carbon dioxide incubator with 5% CO₂ and 95% humidity at 37°C. After 2-4 hours when the tissue was attached to the sidewall, the flasks were flipped and set flat for static culture. Thenceforth, the culture media were replaced every two to three days. Within 3-5 days, new cells were seen growing from the tissue. The cell morphology, adherence rate and proliferation rate were consistently recorded.

When the cells had covered about 90% of the culture bottle wall within 7-10 days, the media were drained. The culture was then washed twice with PBS and digested by 0.25% trypsin. Then the separated cells were placed into single cell suspension in DMEM/F12 cell culture medium containing 10% fetal calf serum and subcultured.

The cells were examined for fibroblast markers using immunostaining as well as FACS (FACSCalibur, BD Biosciences, NJ, USA). The cells that are morphologically consistent with a fibroblast phenotype, i.e. they demonstrated positive staining of vimentin, collagen and fibroblast antigen but negative staining of desmin and myosin [21-24]. The flow cytometer was also employed to ensure the cell purity (> 90% pure).

Immunofluorescence assay

Well-grown cells were selected. After cells passed on to the third generation, the cells in logarithmic growth phase were selected and seeded in a 6-well plate covered with pre-sterilized coverslips. When cells grew to 70%, all the cells were washed with PBS 3 times. Then air-dried and fixed in situ for 30min with 4% formaldehyde (the volume fraction), all the cells were washed with PBS. Cells were permeabilized with PBS containing 0.2% Triton X-100 for 20 min at room temperature. Mouse anti-human vimentin, desmin, S-100, and keratin cytokeratin monoclonal antibody (1:100 dilution) were added to the well, respectively. Cells were washed 3 times in cold PBS, incubated for 1 h at room temperature with Alexa Fluor F(ab')₂

Table 2. Primer sequence of microRNA-146a and U6. *F: Forward, R: Rever

		Primer sequence
miR-146a	F	5'-CACTCCAGCTGGGTGAGAACCTCAACTGGTGTCTGCTGGA-3'
	R	5'-CTCAACTGGTGTCTGCTGGAGTCGGCAATTCAGTTGAGAACCCATG-3'
Notch2	F	5'-CCAGCATGGCCAGCTCTGG-3'
	R	5'-CATCCAGATCTGTGGCCCTGTT-3'
U6	F	5'-CTCGCTTCGGCAGCACA-3'
	R	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	F	5'-GTGGACCTGACCTGCGTCT-3'
	R	5'-GGAGGAGTGGGTGTCGCTGT-3'

fragment of goat anti-mouse IgG (Invitrogen, CA). After washing, cells were incubated for 1 h in 100 µl of 4',6-diamidino-2-phenyl indole (DAPI) to visualize nuclei, washed again in PBS and mounted on slides. Staining was visualized by fluorescence microscopy in a Carl Zeiss Axioplan 2 immunofluorescence microscope. Images were captured using Axiovision V4 software. The positive staining of Vimentin and negative staining of other proteins were identified as orbital fibroblasts.

Real-time fluorescent quantitative PCR

Total RNA was extracted from sample tissues using the Trizol method according to the manufacturer instructions (Invitrogen, Carlsbad, CA, USA) and the optical density was measured to perform RNA quantitative analysis. PCR was applied for the reverse transcription of RNA templates, Applied Biosystems 7500 Fast Real-Time PCR System (ABI7500 quantitative real-time PCR system, Applied Biosystems, Waltham, MA, USA) was used for Real-time quantitative RT-PCR experiments. The RT-PCR reaction conditions were pre-denaturation at 95°C (10min), denaturation at 95°C (15s), annealing at 60°C (20s, 40 cycles), extension at 72°C (34s). The primer sequences were shown in Table 2. GAPDH and U6 were the reference controls for miRNA and mRNA. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of miRNA or mRNA. The experiment was repeated three times.

Dual luciferase reporter gene

The OFs were seeded in 96-well plates with 10^4 cells per well. Transfection was conducted according to the LipofectamineTM 2000 transfection reagent instructions. 1 µl Lipofectamine 2000, and 1 ng internal reference plasmid pRL-CMV were added to each well. After transfection for 48h, the old medium was sucked away, the cells were washed with PBS and 100 µl PLB was added to each well. The plate was then gently shaken for 10 min at room temperature, and the cell lysates were collected. 100 µl LARII was predisposed into the luminometer tubes to complete the desired number of DLR assays. The luminometer was programmed to perform a 2-second premeasurement delay, followed by a 10-second measurement period for each reporter assay. Up to 20 µl cell lysate was transferred to the luminometer tube containing LARII and mixed by pipetting 2 or 3 times. 100 µl Stop & Glo[®] Reagent was injected into every well. The tube was then put into the luminometer and the program was run by a double fluorescence detection kit (Promega, WI, USA). The data was then exported and saved.

Cell grouping and transfection

OFs were used to conduct the experiment and they were divided into five groups: control group (cells received no treatment), miR-146a mimics group (cells transfected with miR-146a mimics sequence), miR-146a inhibitors group (cells transfected with miR-146a inhibitor sequence), NC (miR-146a negative control duplex) group and p-Notch2 group (cells transfected with pcDNA3.1-Notch2). Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). MiR-146a mimics, miR-146a inhibitors and Negative Control duplex products were purchased from Hai Jima biotechnology company, Shanghai.

Western blot

The cultured cells were washed 3 times using pre-cooled PBS. Subsequently, 100 µl/50ml cells were placed on ice for 30 min. The cells were centrifuged at 12,000 rpm for 10min under 4°C. The suspension was then collected into a 0.5ml centrifuge tube and stored under -20°C. The 2 µg/µl BSA standard protein

solution was serially diluted with PBS to 20 µg/µl, 15 µg/µl, 10 µg/µl, 5 µg/µl, 2.5 µg/µl, and 0 µg/µl. The BCA kit (Pierce, Rockford, IL, USA) were diluted by 50:1. 2 µl lysed protein samples were diluted with 18 µl double distilled water. On a 96-well plate, 200 µl testing solution was added to each of the wells as well as 10 µl reference protein solution and 10 µl solution ready for examination. The plate was then incubated under 37°C for 30 min and cooled down to room temperature. The OD was measured at 490 nm. Protein samples were then stored under 70°C. Electrophoresis (60V) was performed before the separating gel electrophoresis (120V, 1-2h at 4°C). Subsequently, the proteins were transferred from gel to a PVDF membrane (1-2 h), which was then incubated with mouse anti-human primary antibodies of *Notch2* and *IL-6* (Shanghai SUNBIO Ltd., China) overnight at 4°C. The next day, the membrane was washed 3 times with TBST (10min every time). Then the rabbit anti-mouse secondary antibodies were added and samples were incubated for 1h. The membrane was then washed with TBST three times, 10 min every time. In the dark room, the X-ray film was developed and fixed. Finally, ImageJ was used to extract the intensity data.

MTT cell proliferation assay

When the density of transfected cells reached about 80%, cells were washed twice with PBS. Cells were digested with trypsin and suspended. The number of cells was counted using a cell counter. Cells were seeded into a 96-well plate with 3×10^3 - 6×10^3 /200 µl volume per well. MTT (5 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) were placed into each well and the cells were cultured at 37°C in an incubator with 5% CO₂. Subsequently, dimethyl sulfoxide (DMSO, 100 µl) was added to each well and cells were gently shaken for about 10 minutes in order to dissolve the crystals. A plate reader was used to detect the absorbance value of each well at a wavelength of 490 nm at the same time for five days. The experiment was repeated for three times.

Flow cytometric analysis

3×10^5 cells from each group were collected and incubated with 70% cold ethanol overnight at 4°C. Cells were then washed with PBS three times and treated with 100 µg/ml RNase A for 0.5 hour. The cells were then treated with 50 µg/ml propidium iodide (PI) for 1 hour in the dark. The DNA distribution was measured with a FACS can flow cytometer (BD Biosciences, USA). The cells were stained with Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, USA) during flow cytometry and the apoptosis rates of the samples were measured.

ELISA analysis

The expression level of *IL-6* was measured using specific ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction manuals. The optic absorption of the samples was measured using the Multiskan microplate spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0. Measurement data were expressed as mean ± standard deviation. The Chi-square test (χ^2) was conducted to analyze the count data. T-test was used to analyze the measurement data between two groups. One-way ANOVA was used to analyze the measurement data among more than two groups. Spearman rank correlation method was conducted to analyze the correlations between groups. $P < 0.05$ was considered significantly different.

Results

Patients characteristics

This study included 42 objects: 15 healthy individuals and 27 GO patients (14 patients with inactive GO and 13 patients with active GO). As shown in Table 1, the age, gender ratios, weight, blood pressure and smoking status of the patients in the three groups did not demonstrate a significant difference ($P > 0.05$). Among the three indexes measured of GO patients, that is, serum free T3, T4 and exophthalmos showed no substantial difference between the inactive and active GO groups ($P > 0.05$). The difference was significant in CAS score between active and inactive GO groups ($P < 0.001$).

Fig. 1. The expressions of microRNA-146a and *Notch2* in patients' tissues. (A) Difference in the expressions of miR-146a among normal tissues, active GO tissues and inactive GO tissues. (B) Difference in the expressions of *Notch2* among normal tissues, active GO tissues and inactive GO tissues. Normal represented the normal control group, Inactive represented inactive GO group and Active represented the active group. Data were presented as mean $\pm$ SD. **, $P < 0.01$ compared with normal group; ##, $P < 0.01$ compared with inactive GO group. GO: Graves ophthalmopathy.

Fig. 2. Immunofluorescence images. (A) The expressions of Vimentin, Desmin, S-100 and Cytokeratin in the control group. (B) The expressions of Vimentin, Desmin, S-100 and Cytokeratin in the GO group. Alexa Fluor 594 goat anti-mouse secondary antibody and DAPI staining for nucleus (1000 $\times$ magnification). GO: Graves ophthalmopathy.

miR-146a and *Notch2* expression levels of miR-146a and *Notch2* in GO tissues

To assess whether the expressions of miR-146a or *Notch2* was increased in the tissues, qRT-PCR was performed in 27 GO tissues samples and 15 normal tissues samples. The expression of miR-146a in GO tissues was significantly up-regulated in comparison with that in normal tissues, and the expression of miR-146a in the active GO group significantly exceeded that in the inactive group (all $P < 0.05$) (Fig. 1A). The expression level of *Notch2* in normal tissues was significantly lower than that in normal tissues, and that in the active group was significantly lower than that in inactive group (all $P < 0.05$) (Fig. 1B).

Immunofluorescence assay results

To distinguish the orbital fibroblasts, immunofluorescence assay was used in the study. As shown in Fig. 2, Vimentin expression of OFs was positive in the GO group and control group and the cytoplasm was stained in red, indicating a mesenchymal origin of the cells. Desmin expression of OFs was negative in the GO group and control group, and the nuclei were stained in blue, indicating that the origins of the cells such as the smooth muscle,

Fig. 3. The effect of miR-146a and *Notch2* on OFs viability was evaluated using MTT assay. Control represented control group (OFs received no treatment), miR-146a mimics represented miR-146a mimics group (OFs transfected with miR-146a mimics sequence), miR-146a inhibitors represented miR-146a inhibitor group (OFs transfected with miR-146a inhibitor sequence), NC represented negative control group (OFs transfected with miR-146a negative control duplex) group and p-Notch2 represented p-Notch2 group (OFs transfected with pcDNA3.1-Notch2). Data were presented as mean $\pm$ SD. **, $P < 0.01$ compared with control group; ##, $P < 0.01$ compared with the NC group; &&, $P < 0.01$ compared with the miR-146a mimics group. OFs: Orbital fibroblasts.

striated muscle and cardiac muscle could be excluded. The negative expressions of S-100 and Cytokeratin also indicated that the origins of the tumor were not skin melanin and epithelium, and mesothelium are excluded respectively.

The effects of miR-146a and Notch2 on cell proliferation

To determine the effects of miR-146a and Notch2 on proliferation *in vitro*, MTT assay was analyzed for their viability. As suggested by MTT assay result (Fig. 3), there was no significant difference in cell viability between the NC and control groups. The miR-146a mimics significantly strengthened the cell viability, whereas the miR-146a inhibitors and exogenous *Notch2* had the reverse effect, compared with the control group or the NC group (all $P < 0.05$). The miR-146a inhibitor and exogenous *Notch2* had identical inhibitory effects on OF viability ($P > 0.05$). Comparing with the miR-146a mimics group, the cells in the miR-146a inhibitor and p-*Notch2* groups were less viable.

The effects of miR-146a and Notch2 on cell apoptosis and cell cycle

To assess the contribution of miR-146a and Notch2 in tumor development, we examined the cell apoptosis and cell cycle in different groups. The results of flow cytometry showed that there was no significant difference in cell cycle and apoptosis between the control and the NC groups (all $P > 0.05$). When compared with the control group, the proportion of cells in G0/G1 phase increased, whereas that in S and G2/M increased in the miR-146a mimics group. The cells in miR-146a inhibitor and p-*Notch2* showed similar cell cycle progression ($P > 0.05$), i.e., the percentage of cells in G0/G1 phase increased significantly and that in S and G2/M decreased compared with the control group ($P < 0.05$, Fig. 4A). Comparing with miR-146a mimics group, cell cycle arrest in the miR-146a inhibitor and p-*Notch2* groups were significantly enhanced.

Consistently, no significant difference was observed between the control and NC groups. Compared with the control group, the cells in the miR-146a mimics group demonstrated significantly lower apoptosis rate (both $P < 0.05$). On the contrary, the apoptosis rate of OFs was significantly higher in the miR-146a inhibitor group and p-*Notch2* group compared with the control group or the NC group (both $P < 0.05$). Meanwhile, there is no difference between the miR-146a inhibitor and the p-*Notch2* groups ($P > 0.05$). Comparing with miR-146a mimics group, cell apoptosis rates in the miR-146a inhibitor and p-*Notch2* groups were substantially higher.

Dual luciferase reporter gene assay results

Dual luciferase reporter gene assay was utilized to verify the target relationship between miR-146a and Notch2. The complementary sequences of *Notch2* 3'UTR and miR-146a as

Fig. 4. The effects of miR-146a and *Notch2* on cell apoptosis and cell cycle. (A) The cell cycle of OFs in different groups. Data were presented as mean ± SD. *, $P < 0.05$, **, $P < 0.01$ compared with control group; #, $P < 0.05$, ##, $P < 0.01$ compared with the NC group; &#, $P < 0.01$ compared with the miR-146a mimics group. (B) The apoptosis of OFs in different groups. Data were presented as mean ± SD. **, $P < 0.01$ compared with control group; #, $P < 0.05$, ##, $P < 0.01$ compared with the NC group; &#, $P < 0.01$ compared with the miR-146a mimics group. Control represented control group (OFs received no treatment), miR-146a mimics represented miR-146a mimics group (OFs transfected with miR-146a mimics sequence), miR-146a inhibitors represented miR-146a inhibitor group (OFs transfected with miR-146a inhibitor sequence), NC represented negative control group (OFs transfected with miR-146a negative control duplex) group and p-Notch2 represented p-Notch2 group (OFs transfected with pcDNA3.1-Notch2). OFs: Orbital fibroblasts.

well as the mutated *Notch2* 3'UTR were shown in Fig. 5A. Only the co-transfection of pGL3-*Notch2*-3'UTR-wt and miR-146a mimics weakened the fluorescence (Fig. 5B). The cells co-transfected with pGL3-*Notch2*-3'UTR-mut and miR-146a mimics did not show significant alteration in fluorescence compared with the NC group ($P > 0.05$).

Fig. 5. Notch2 was a target of miR-146a. (A) Sequence scheme of miR-146a, Notch2 3'-UTR-wt and Notch2 3'-UTR-mut. (B) Fluorescence measurement of cells with different transfections. NC represented negative control group (OFs transfected with miR-146a negative control duplex) group and miR-146a mimics represented miR-146a mimics group (OFs transfected with miR-146a mimics sequence). Data were presented as mean $\pm$ SD. **, $P < 0.01$ compared with the NC group. OFs: Orbital fibroblasts.

Fig. 6. Downregulation of effects of miR-146a on Notch2 and IL-6 expressions in OFs. (A) The expression level of Notch2 protein in OFs. (B) The column diagram of the expression level of Notch2 protein in OFs. Data were presented as mean $\pm$ SD. **, $P < 0.01$ compared with control group; #, $P < 0.01$ compared with the NC group; &, $P < 0.05$ compared with the miR-146a mimics group. (C) The expression level of IL-6 protein in OFs. (D) The column diagram of the expression level of IL-6 protein in OFs. Data were presented as mean $\pm$ SD. **, $P < 0.01$ compared with control group; #, $P < 0.01$ compared with the NC group; &, $P < 0.05$, &&, $P < 0.01$ compared with the miR-146a mimics group. Control represented control group (OFs received no treatment), miR-146a mimics represented miR-146a mimics group (OFs transfected with miR-146a mimics sequence), miR-146a inhibitors represented miR-146a inhibitor group (OFs transfected with miR-146a inhibitor sequence), NC represented negative control group (OFs transfected with miR-146a negative control duplex) group and p-Notch2 represented p-Notch2 group (OFs transfected with pcDNA3.1-Notch2). OFs: Orbital fibroblasts.

The effects of different groups on Notch2 and IL-6 expressions in OFs

To assess the effects of different groups on Notch2 and IL-6 expressions in OFs, we examined the protein expressions of Notch2 and IL-6 by western blot and ELISA. Western

blot and ELISA results showed that 24h after the transfection of miRNA-146a mimics in OFs, *Notch2* was down-regulated whereas IL-6 was up-regulated compared with the control and NC groups ($P < 0.01$, Fig. 6). *Notch2* expressions in miR-146a inhibitor and p-*Notch2* groups were significantly higher than that in the control and NC groups ($P < 0.01$) (Fig. 6A and 6B). IL-6 expressions in the miR-146a inhibitor and p-*Notch2* groups were significantly lower than that in the control and NC groups ($P < 0.05$, Fig. 6C and 6D). Comparing with miR-146a mimics group, the expression of *Notch2* significantly increased whereas the expression of IL-6 dramatically decreased ($P < 0.05$) in the p-*Notch2* and the miR-146a inhibitor group.

Discussion

GO, especially active GO, has long been a major challenge for the endocrine industry. Active GO has more propensity to Th1 cells immune response, while inactive GO mainly has more propensity to Th2 cells immune response [4]. We investigated the mechanism of microRNA-146a/Notch2 signaling pathway regulating IL-6 in GO.

We compared the expression of miR-146a and *Notch2* in active and inactive GO and normal orbital connective tissue. The expression of miR-146a was notably increased in GO tissues compared with that in normal tissues, and miR-146a expression in the active GO tissues significantly exceeded the inactive tissues, while expression level of *Notch2* showed the opposite trend. In addition, we indicated that miR-146a markedly promoted the GO Orbital Fibroblasts' (OFs') viability and mitosis but markedly suppressed cell apoptosis, while *Notch2* showed the opposite effects.

The effect of microRNAs in autoimmune diseases has been attracting increasing interest of numerous researchers. Because GO is an inflammatory autoimmune disease, miR-146a seems to be up-regulated by inflammatory stress as in GO. Similarly, there have several researchers reported that miR-146a expression was increased in diseased tissue of autoimmune diseases. For example, Wang et al. reported that miR-146a expression was increased in rheumatoid arthritis (RA) synovial tissue [25]. Yamasaki et al. reported that miR-146a expression was increased in cartilage of low-grade osteoarthritis [26]. Sonkoly et al. reported that miR-146a expression in psoriatic lesioned skin was notably higher than that in healthy skin [27]. Gauley et al. demonstrated that the expressions of miR-146a in peripheral blood mononuclear cells of RA patients were notably overexpressed compared with healthy controls [28]. Liang et al. indicated that circulating miR-146a levels were markedly decreased in systemic lupus erythematosus compared with healthy controls [29]. Bernecker et al. indicated that miR-146a was significantly decreased in the thyroid tissue of Graves disease (GD) [30]. Intriguingly, Wei et al. indicated that the miR-146a expression in plasma of GD patients was not notably different from that of the control group, and the miR-146a expression in GO patients was not only significantly reduced but also negatively correlated with the clinical activity of GO [3]. The distinction in the expression of miR-146a in autoimmune diseases may be accounted for the complex mechanisms of the different autoimmune diseases.

Most recently, miR-146a has been found to negatively regulate cytokines [31]. According to literatures, miR-146a had the following targets, including IGF1R, COX-2, TRAF6, FAS, IL-1, IL-6, IL-17, IL-18, IL-21, IL-23, IL-27, IL-31, IL-32, IL-33, IL-34, IL-35, IL-36, IL-37, IL-38, IL-39, IL-40, IL-41, IL-42, IL-43, IL-44, IL-45, IL-46, IL-47, IL-48, IL-49, IL-50, IL-51, IL-52, IL-53, IL-54, IL-55, IL-56, IL-57, IL-58, IL-59, IL-60, IL-61, IL-62, IL-63, IL-64, IL-65, IL-66, IL-67, IL-68, IL-69, IL-70, IL-71, IL-72, IL-73, IL-74, IL-75, IL-76, IL-77, IL-78, IL-79, IL-80, IL-81, IL-82, IL-83, IL-84, IL-85, IL-86, IL-87, IL-88, IL-89, IL-90, IL-91, IL-92, IL-93, IL-94, IL-95, IL-96, IL-97, IL-98, IL-99, IL-100, IL-101, IL-102, IL-103, IL-104, IL-105, IL-106, IL-107, IL-108, IL-109, IL-110, IL-111, IL-112, IL-113, IL-114, IL-115, IL-116, IL-117, IL-118, IL-119, IL-120, IL-121, IL-122, IL-123, IL-124, IL-125, IL-126, IL-127, IL-128, IL-129, IL-130, IL-131, IL-132, IL-133, IL-134, IL-135, IL-136, IL-137, IL-138, IL-139, IL-140, IL-141, IL-142, IL-143, IL-144, IL-145, 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level of miR-146 induced by the rs2910164 polymorphism [36]. Cao et al. indicated that miR-146a was significantly up-regulated in atherosclerotic plaque, which contributed to the acceleration of VSMC growth and cell cycle progression by directly inhibiting *Notch2* and *Jag1* [37]. However, there had no research report on the role of *Notch2* in GO. Herein, we discovered that *Notch2* could be directly regulated by miR-146a in GO Orbital fibroblasts (OFs), which could then result in the strong viability, proliferation, differentiation, and mitosis of GO OFs.

Moreover, we indicated that the IL-6 level in miRNA-146a mimics notably higher than that in control and NC groups, while *Notch2* showed the opposite trend. Nevertheless, IL-6 level in the miRNA-146a inhibitor group significantly lower than that in control and NC groups, while *Notch2* again showed the opposite trend. These results indicated that microRNA-146a could improve the IL-6 level by inhibiting *Notch2* and thereby worsening GO, conversely, the down-regulation of IL-6 by inhibiting microRNA-146a and *Notch2* can improve the progression of GO, which are consistent with a prior study showing that PGE2-dependent IL-6 production up-regulation might promote the progression of GO and thus prove an important therapeutic target in the treatment of GO [41].

IL-6 has been involved in the pathogenesis of several autoimmune diseases previously [42]. For instance, Deon et al. indicated that IL-6 signaling cross-talks with that of IL-1 in synovial fibroblasts from RA patients and play a role in regulating cytokine balance in RA [43]. Molnar et al. indicated that up-regulated IL-6 levels have been observed in GO and hyperthyroidism [44]. Raychaudhuri et al. found that elevated IL-6 expression in orbital fibroblasts could influence inflammatory responses relevant to autoimmune disease affecting the tissues surrounding the eye [41]. Taken together, we believe that the up-regulation of IL-6 resulted from either the excessive miR-146a or the inhibition of *Notch2* could exacerbate GO.

In conclusion, we identified that microRNA-146a could improve the IL-6 level by inhibiting *Notch2* and thus promote the progression of GO. Conversely, the down-regulation of microRNA-146a could serve as a therapeutic target in GO by inhibiting the expression of target gene *Notch2*. However, microRNA-146a-based therapeutic methods are still in the infancy. Further studies should be carried out to investigate the influence of microRNA-146a on other target genes or cellular processes in GO, and to explore how miRNA-146a interacts with IL-6 in the progression of GO.

Disclosure of Interest

The authors declare no conflicts of interest.

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