Decreased microRNA-146a in CD4+T cells promote ocular inflammation in thyroid-associated ophthalmopathy by targeting NUMB


1Department of Ophthalmology, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, China
2Department of Endocrinology, The Affiliated National Hospital of Guangxi, Medical University, Guangxi, Zhuang Region, China

Abstract. - OBJECTIVE: The aim of the study was to explore the functional role of miR-146a in CD4+T cells of active thyroid-associated ophthalmopathy (TAO) patients and to identify the possible molecular mechanism for the modulation of miR-146a in TAO.

PATIENTS AND METHODS: The experimental group collected six cases of peripheral venous blood of patients with active TAO. The healthy control group collected six cases of normal peripheral venous blood. All specimens excluded other eye diseases and autoimmune diseases, tumors, asthma, chronic inflammation, Human Immunodeficiency Virus (HIV), recent history of trauma, infection, and showed normal thyroid function. All patients with active TAO and age- and sex-matched healthy control subjects in this study.

RESULTS: miR-146a is downregulated in active TAO CD4+T cells. NUMB was a target of miR-146a.

CONCLUSIONS: We observed that miR-146a expression was downregulated in CD4+T cells during the active stage of patients with TAO. At the same time, it was found that NUMB can be targeted by miR-146a in CD4+T cells in TAO patients. Decreased microRNA-146a in CD4+T cells promotes ocular inflammation in active TAO by targeting NUMB.

Key Words: TAO, MicroRNA-146a, CD4+T cells, NUMB.

Introduction

Thyroid-associated ophthalmopathy (TAO) is the most common orbital disease. Despite substantial new findings in its cellular and molecular underpinnings, the pathogenesis of TAO remains unclear. Many patients with TAO have to endure the condition for long periods, and some severely affected patients are resistant to current treatment regimens. TAO is an inflammatory autoimmune disease of the orbit. Signs and symptoms of TAO are caused by inflammation of orbital connective tissue, increased orbital volume due to overproduction of glycosaminoglycans, and enhanced adipogenesis. CD4+T cells are involved in the immune and proliferative responses, which play an important role in the occurrence and development of TAO.

MicroRNAs (miRNAs) are small single-stranded noncoding RNAs, about 21-25 nucleotides in length. They control gene expression by targeting the 3',5' UTR or the coding sequences of specific mRNAs and triggering either translation repression or RNA degradation. miRNAs have emerged as important modulators of immunity and cellular physiology. miR-146a with multiple target genes is a typical multifunctional miRNA that is in the same cellular pathways according to different conditions, and it plays various life processes, characterized in terms of its regulatory role in immune regulation, cell proliferation, differentiation, apoptosis, and extracellular matrix metabolism. MiR-146a can be induced by PI3K / NF-kappa B pathway; in turn, miR-146a in PI3K /NF-kappa B pathway has multiple target genes, negative to the regulation of the immune response. CD4+T cells are involved in the immune and proliferative responses, which play an important role in the occurrence and development...
of TAO. MiR-146a has some potential targets for the immune and proliferative response of TAO. A previous study showed low expression for miR-146a in TAO patients with orbital tissue and peripheral blood mononuclear cells (PBMC). The aim of this study was to explore the functional role of miR-146a in CD4+ T cells of active TAO patients and to identify the possible molecular mechanism for the modulation of miR-146a in TAO.

**Patients and Methods**

**Patients**

Diagnoses of TAO and disease activity were based on Bartley and Gorman criteria. The experimental group collected six cases of peripheral venous blood of patients with active TAO (n = 6, four female and two male cases; mean ± SD age 30.16 ± 3.76 years). The healthy control group collected six cases of normal peripheral venous blood (n = 6, three female and three male, mean ± SD age 27.66 ± 4.08 years). All specimens excluded other eye diseases and autoimmune diseases, tumors, asthma, chronic inflammation, HIV, recent history of trauma, and infection, and normal thyroid function. All patients with active TAO and age- and sex-matched healthy control subjects were included in this study. The patients with active TAO were collected from the First Affiliated Hospital of Guangxi Medical University’s Outpatient Department and Inpatient Ward. The healthy control subjects were recruited from among the graduate students and medical staff at the Guangxi Medical University. The study protocol was approved by the Ethics Committee of Guangxi Medical University, and written informed consent was obtained from each patient before the study started.

**Cell Isolation and Culture**

A total of 120 ml of peripheral venous blood was obtained from each subject and preserved in heparin. CD4+ T cells were isolated using human CD4+ T cell enrichment cocktail (Stem Cell, Catalog #15062, Vancouver, BC, Canada). Human CD4+ T cells were maintained in immunocult-XF T cell expansion medium without the addition of xeno medium and serum in the medium (Stem Cell, Catalog #10982, Vancouver, BC, Canada).

**Cell Transfection**

The CD4+ T cells were seeded in six-well plates and cultured for 24 h before the transfection. We transfected with miR-146a mimic and inhibitor (RibBio, Guangzhou, Guangdong, China) using INTERFERin (Polyplus-transfection, Strasbourg, France) according to the manufacturer’s protocol. Cell samples were collected at 48 h after transfection for further analysis. Total RNA was extracted at 48 h after transfection. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was used to verify miR-146a expression.

**Quantitative RT-PCR**

CD4+ T cells were collected and lysed in RNAiso for small RAN (TaKaRa, Dalian, China) for miRAN extraction using the One-Step primeScript miRNA cDNA Synthesis Kit (TaKaRa, Dalian, China). qPCR was conducted for reverse transcription using the ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) to detect the expression of 146a (Fw: 5'-TTA TTA AGT ATC CAG TGC AGG GTC CGA GG-3' and Rv: 5'-TTG CGG GAC ATC TAA TAC TGC CTG GTC GTA ATG-3') (TaKaRa, Dalian, China), standardized by U6 reference (Fw: 5'-GGA TCG ATA CAG AGA AGA TTA GC-3' and Rv 5'-TGG AAC GCT TCA CGA ATT TGC G-3') (TaKaRa, Dalian, China). Detection was conducted in triplicate for each sample and data were calculated with the 2^\Delta ΔCt method.

**MiRNA Target Prediction**

Bioinformatics methods were applied for the prediction of targeted genes of miR-146a. The bioinformatics algorithm from TargetscanHuman (http://www.targetscan.org) was used.

**Plasmid Construction and Double Luciferase Reporter Assay**

To verify whether NUMB directly binds to miR-146a, a dual Luciferase assay was performed. The 3’UTR of NUMB was amplified by polymerase chain reaction (PCR) genomic DNA. The production was inserted into the downstream of NUMB 3’UTR reporter plasmids (Prl-numb) (Promega, Madison, WI, USA). The whole plasmid was verified by sequencing. The mutation in the miR-146a binding site of the NUMB 3’UTR was constructed by the GenePharma Company (GenePharma, Shanghai, China). The Luciferase reporter-containing mutant was constructed. For Luciferase assays, 293 T cells were transfected with Luciferase reporter plasmid along with miR-146a mimics or negative control by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 48 h after transfection, these cells were analyzed by
miR-146a in CD4+T cells and thyroid ophthalmopathy

Western Blot
All reagents were prepared according to manufacturer’s recommendations for Wes (ProteinSimple, San Jose, CA, USA). Total protein of cells was extracted using radioimmunoprecipitation assay (RIPA) protein extract (Beyotime Biotechnology, Shanghai, China). The extracted protein was quantified using bicinchoninic acid (BCA) protein quantitative detection kit (Thermo-Fisher Scientific, Waltham, MA, USA). Samples were diluted to adjust protein concentration. The final samples of 5 μl each were boiled for 5 min, placed on ice for 5 min, briefly centrifuged, and then applied to proper wells. The Wes is capable of performing loading, separation, washing, blocking, and immune-detection. After plate loading, the separation of proteins by electrophoresis and immune-detection took place in the capillary system and were fully automated. Sample Western analysis was carried out at room temperature and instrument default settings. NUMB (1:50 dilution) primary antibodies (ab177465, Abcam, Cambridge, MA, USA) were diluted with antibody diluent (ProteinSimple, San Jose, CA, USA). The digital images were analyzed with Compass software (ProteinSimple, San Jose, CA, USA) on Wes. Band density differences were expressed as percent of values. Data were collected from three independent experiments.

Statistical Analysis
Data are expressed as the mean ± standard (SD). The Mann-Whitney U-test was used to compare the values of miR-146a between the two groups. The comparison among other groups was estimated by the Student’s t-test. All results were analyzed using SPSS17.0 (SPSS Inc., Chicago, IL, USA) and p<0.05 was considered significant.

Results

**miR-146a is Downregulated in TAO CD4+T cells**
In this study, the expression level of miR-146a was detected by qPCR, and we observed that miR-146a was significantly downregulated in active TAO CD4+T cells compared with the control (Figure 1).

**NUMB was a Target of miR-146a**
By using the database of TargetscanHuman (http://www.targetscan.org) to predict the target gene of miR-146a, we selected the potential target genes of NUMB as miR146a. To further confirm the direct target gene of miR146a for NUMB, we constructed a reporter gene expression vector of the NUMB gene. The 3’-UTR fragment of the NUMB gene was amplified by the PCR method, and then, the fragment was inserted into the expression vector to carry out sequencing to confirm that the vector was constructed correctly. A dual luciferase reporter gene assay system was used to demonstrate that NUMB is a direct target of miR-146a. The results confirmed that NUMB was a validated target of miR-146a (Figure 2).

**Expression Changes of miR-146a and NUMB protein in CD4+T cell After Transfection**
To assess whether miR-146a directly regulates NUMB, we transfected primary CD4+T cells from healthy subjects and patients with a mimic of miR-146a, an inhibitor of miR-146a, blank control, or negative control. qRT-PCR results showed that the miR-146a mimic group had a higher expression level of miR-146a than the negative control (NC) and the blank groups (p<0.05). The miR-146a inhibitor group had a lower expression level of miR-146a compared to the NC and the blank groups (p < 0.05). There was no diffe-

---

**Figure 1.** miR-146a is expressed at lower levels in CD4+T cells from patients with active TAO compared to normal control subjects. The miR-146a levels in CD4+T cells from TAO and matching adjacent normal control subjects were tested by qRT-PCR. The qRT-PCR experiments were performed three times. Data are mean ± SD *p<0.05.
expression level of miR-146a between the NC group and blank group (p>0.05) (Figures 3A-B). Moreover, decreased levels of the NUMB protein were found in cells transfected with a miR-146a mimic relative to the NC and the blank groups (p<0.05), whereas increased levels of the NUMB protein were observed in cells transfected with a miR-146a inhibitor relative to the NC and the blank groups (p<0.05). There was no difference shown in levels of NUMB protein between the NC group and blank group (p>0.05) (Figure 3C-D). At the same time, we investigated the mRNA expression level of NUMB, and reverse transcriptase-polymerase chain reaction (RT-PCR) results indicated that neither miR-146a mimic nor miR-146a inhibitor influenced the expression of NUMB (p>0.05) (Figures 3E-F).

Expression of NUMB

Then, we detected the NUMB protein levels in the CD4+T cells and found the mean expression of NUMB in active TAO patients was higher than that in healthy subjects (p<0.05) (Figure 4).

Discussion

In this work, we observed that miR-146a expression was downregulated in CD4+T cells in the active stage of TAO patients. At the same time, we found that NUMB can be targeted by miR-146a in CD4+T cells in TAO patients. NUMB is a cell-fate determinant, is a tyrosine-binding domain-containing protein that is linked to the cell’s membrane. It is one of the most important cell-fate determinants in living organisms. NUMB also is widely distributed in mature tissues, widely involved in the balance of differentiation/proliferation, regulation of apoptosis, cell migration, tissue regeneration, and other important physiological processes. Uemura et al. have discovered and identified a new gene in the study of the embryonic tissue of the fruit fly. NUMB is considered essential for the formation of the sensory organ of Drosophila to determine the cell differentiation fate. Subsequent researches have found that the loss of NUMB function will lead to the loss of nerve cells in the sensory organ development. In contrast, overexpression of NUMB leads to abnormal differentiation, which makes the original supporting cells into neural cells, thus determining the role of NUMB as an endogenous cell fate determinant in the asymmetric cell division. Previous studies have demonstrated that miR-146a is implicated in cell apoptosis. It was reported that upregulation of miR-146a induces apoptosis of human chondrocytes and miR-146a regulated the maturation and differentiation of vascular smooth muscle cells. Also miR-146a induces apoptosis in several types of cancer, including non-small cell lung cancer, gastric cancer, and breast cancer. Moreover, miR-146a has been found to participate in the pathogenesis of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis. A recent work has shown that aberrant miRNA expression is associated with the pathogenesis of many diseases.
miR-146a in CD4+T cells and thyroid ophthalmopathy

Figure 3. The expression levels of miR-146a were analyzed after transfection with miR-146a mimic or inhibitor, negative control, and blank. NUMB expression in CD4+T cells compared with the blank control group and negative control group. (A) After transfection miR-146a mimics or inhibitor, negative control, blank, the miR-146a levels in CD4+T cells from patients with active TAO were examined. (B) After transfection miR-146a mimics or inhibitor, negative control, blank, the miR-146a levels in CD4+T cells from healthy control subjects were examined. (C) MiR-146a mimics or inhibitor was transfected into CD4+T cells. At 48 h later, the NUMB protein level in CD4+T cells from patients with active TAO tested by Western blot. (D) MiR-146a mimics or inhibitor was transfected into CD4+T cells. At 48 h later, the NUMB protein level in CD4+T cells from healthy control subjects tested by Western blot. (E) The relative gene expression levels of NUMB from patients with active TAO. (F) The relative gene expression levels of NUMB from healthy control subjects. *p<0.05.
such as tumors. miR-146a with multiple target genes is a typical multifunctional mRNA that is in the same cellular pathways according to different conditions, and it plays various life processes, characterized in terms of their regulatory role in immune regulation, cell proliferation, differentiation, apoptosis, and extracellular matrix metabolism. Further studies were warranted to explore how miR-146a targets certain genes in CD4+T cells with inactive TAO.

**Conclusions**

We not only observed the downregulation of miR-146a expression in CD4+T cells of active TAO patients, but also verified that miR-146a directly regulates the protein expression level of NUMB. We hope that this investigation provides assistance to explore further the pathogenesis of miR-146a in active TAO and in developing new treatment methods.

**Acknowledgments**

This work is supported by grants from the National Natural Science Foundation of China (No.81360152).
miR-146a in CD4+T cells and thyroid ophthalmopathy


