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Original Article

MicroRNA-146a contributes to CD4+ T lymphocyte differentiation in patients with thyroid ophthalmopathy

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Abstract: MicroRNA-146a (miR-146a) is associated with human inflammatory disease, such as thyroid-associated ophthalmopathy (TAO), but its role in human T cells and relevance to TAO remains ambiguous. In this study, T cells of TAO patients showed downregulated expression of miR-146a. We characterized miR-146a in T cells and examined miR-146a as a critical inhibitor of Th1 differentiation processes. MiR-146a inhibited Th1 differentiation processes and cell proliferation of T-lymphocytes. Thus, the results showed that miR-146a was a potent inhibitor of Th1 differentiation and cell proliferation of human T cells and dysregulation of miR-146a contributed to the pathogenesis of TAO.

Keywords: MiR-146a, CD4+ T lymphocyte, differentiation, thyroid ophthalmopathy

Introduction

Thyroid-associated ophthalmopathy (TAO) is an inflammatory autoimmune disorder of the orbit, which occurs in 25% to 50% of patients with Graves' disease (GD) [1]. The manifestations of TAO are periorbital edema, apoptosis, and visual impairment or optic nerve compression, which are caused by inflammation of orbital connective tissue, accumulation of extracellular matrix, overproduction of glycosaminoglycans, and enhanced adipogenesis [2]. Despite substantial recent findings in its cellular and molecular underpinnings, the pathogenesis of TAO remains unclear Accumulating data suggest that the initiation and perpetuation of immune reaction were involved by the CD4+ T cells [3, 4]. On the other hand, microRNAs have emerged as important modulators of immunity and cellular physiology [5, 6].

The role of miRNAs in the regulation of physiological and pathological processes in autoimmune and inflammatory diseases has been extensively examined in recent years [7-10]. MiR-146a has been found to participate in the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus (SLE), Sjogren's syndrome (SS), Graves' orbitopathy, rheumatoid arthritis (RA), and multiple sclerosis [11-14]. MiR-146a is downregulated in SLE and SS. Interestingly, miR-146a is upregulated in different cell types and tissues in RA patients. Chan et al. showed that overexpression of miR-146a contributes to the control of pro-inflammatory cytokine production and confers cross-tolerance in innate immune cells [15]. The deficiency of miR-146a in Treg cells resulted in a breakdown of immunological tolerance manifested in fatal IFNγ-dependent immune-mediated lesions in various organs [16]. However, whether miR-146a plays a role in TAO is ambiguous.

In this study, we investigated the expression of miR-146a in human T cells of TAO patients and elucidated the function of miR-146a alterations. We investigated the potential impact of miR-146a on T cell functions in TAO patients. TAO is characterized by severe inflammation with inappropriate host immune responses. The T cells of TAO patients exhibited significantly downregulated miR-146a expression. The

results also showed that miR-146a is an inhibitor of T-helper 1 (Th1) differentiation and proliferation in human T cells. This process may promote the development of excessive Th1 response.

Materials and methods

Ethics statement

All experimental procedures were approved by the Institutional Review Board of the First Affiliated Hospital of Henan University of Science and Technology (China). Written informed consent was obtained from all participants.

Clinical samples

Untreated TAO patients (n=20) and GD patients (n=20) were included in this study. Healthy volunteers with similar ages, genders, and smoking statuses were also considered as controls. Each patient was diagnosed with TAO or GD from 2013 to 2015 in the First Affiliated Ho-s pital of Henan University of Science and Technology according to the current WHO criteria. All patients were euthyroid for at least 8 weeks, and no patient was treated with radioiodine, immunosuppressive therapy, orbital surgery, or orbital radiotherapy within one year prior to the study or administered systemic steroids in six months prior to the study. Healthy control subjects had no history of autoimmune disorders. Peripheral blood was obtained from patients and healthy volunteers.

Cell isolation and culture

Heparin was added to the peripheral blood samples for lymphocyte isolation. After dilution with equivalent PBS, the blood with heparin was transferred to the centrifuge tubes with lymphocyte separation medium (Tianjin Haoyang Biological Manufacture Co. Ltd, Tianjin, China). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation for further study. The serum mixtures from the TAO patients were mixed, centrifuged, and collected.

Normal T cells were isolated from the PBMCs of healthy volunteers. Isolated primary T cells were cultured in RPMI 1640 medium supplemented with 15% FBS, 20 mM HEPES, 200 Units/mL penicillin, 50 mg/mL streptomycin, 0.05 mM 2-mercaptoethanol, 2 mM glutamine, 10 ng/mL phorbol 12-myristate 13-acetate (all these reagents were from Sigma, St Louis, MO, USA) and 10 ng/mL recombinant human interleukin-2 (Peprotech, Rocky Hill, NJ, USA) [17].

$CD4^+$ T cells were sorted using microbeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The quantity of $CD4^+$ T cells should be at least half million, and the purity should be at least 90%. Jurkat cell line was purchased from ATCC (Manassas, VA, USA) and cultured in RPMI 1640 medium supplemented with 10% FBS. Then, the Jurkat and normal $CD4^+$ T cells were stimulated by serum diluted to 1:2 for 48 h.

Cell transfection

MiR-146a mimics or inhibitor, or their negative control RNAs were synthesized by Qiagen (Hilden, Germany). Transfection was performed with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. For cell differentiation, the primary T cells were transfected with 100 nM miR-146a mimics or inhibitor or their negative control RNAs in six-well plates. To determine cell cycle and apoptosis determination, Jurkat cells were stimulated by the sera from TAO patients diluted to 1:2 for 48 h and then transfected with 100 nM miR-146a mimics or inhibitor or their negative control RNAs in six-well plates.

Cell cycle and apoptosis determination

The Jurkat cells were seeded into six-well plates and stimulated by sera from the TAO patients diluted to 1:2 for 48 h. After transfection with RNA oligonucleotides, the cells were fixed with 70% ice-coldethanol and treated with 1 mg/mL RNaseat 4°C overnight. Subsequently, intracellular DNA was labeled with 10 μL of propidium iodide (PI, 50 μg/mL; Sigma) at 37°C for 30 min and then analyzed using a BD FACSCaliburflow cytometer (BD Technologies, Carlsbad, CA, USA). The distribution of cells in the G0/G1, S, and G2/M phases were calculated using ModFit software (Verity Software House Inc., Topsham, ME, USA). Apoptosis assay was performed using PI staining (BD Bioscience, San Diego, CA, USA) according to the manufacturer's protocol. Cells were harvested, centrifuged, and resuspended in 100 μL of binding buffer. Approximately 5 μL of ready-to-use PI were

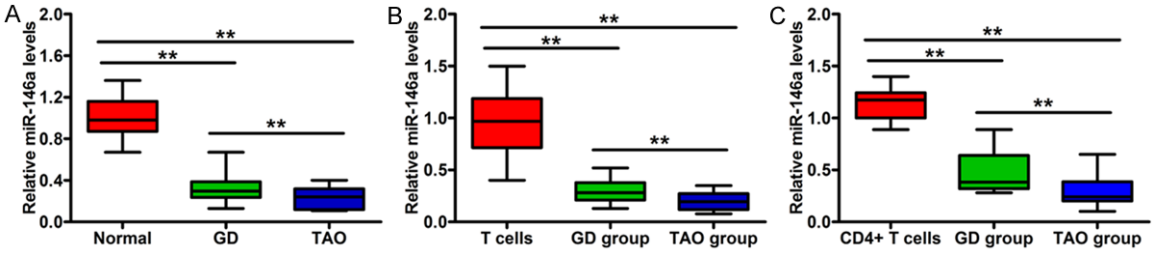

Figure 1. MiR-146a in human T cells of thyroid-associated ophthalmopathy (TAO) patients. A. MiR-146a levels were measured by qPCR with TaqManmiRNA assays relative to U6 in the peripheral blood mononuclear cells of healthy volunteers (n=25), Graves' disease patients without ophthalmopathy (n=20) and TAO patients (n=20); B. Comparisons of the expression levels of miR-146a in T cells; C. Expression levels of miR-146a in $CD4^+$ T cells. **P<0.01, GD group vs. healthy volunteers; TAO vs. GD group; TAO patients vs. healthy volunteers. All data are presented as the mean ± SD of three replicates.

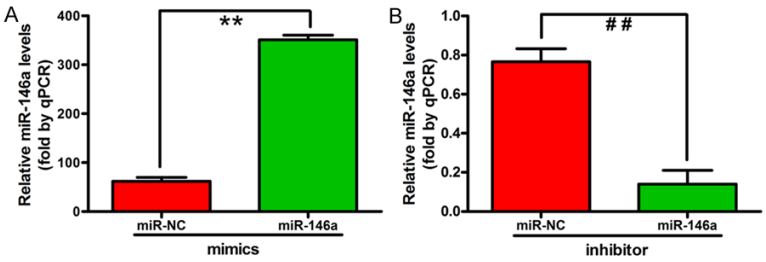

Figure 2. MiR-146a expression in T lymphocytes transfected with miR-146a mimics or inhibitor. A. Expression level of miR-146a in cells transfected with mimics and negative control; B. MiR-146a expression in cells transfected with inhibitor and negative control. Expression of miR-146a was determined using qRT-PCR and was normalized to that of an endogenous control (U6 RNA). All data are presented as the mean ± SD of three replicates. **P<0.01, miR-146a mimics vs. miR-NC, ##P<0.01, miR-146a inhibitor vs. miR-NC.

added into the mixture. Then, the cells were incubated in the dark for 30 min. PI fluorescence was assessed by BD FACSCalibur flow cytometer (BD Technologies) and analyzed by CellQuest software (BD Bioscience). Each sample was prepared in triplicate.

RNA isolation and synthesis of cDNA

Blood samples from the participants were collected in the morning after the patients had fasted for 12 h. The samples were immediately stored on ice at 4°C. The plasma was then separated by centrifugation at 1,500 rpm for 10 min. The serum was separated from the cells by centrifugation at 3,000 rpm for 10 min. Plasma and serum samples were stored at -80°C until used to measure the miR-146a levels. Total RNA was isolated from PBMCs, primary human T cells, and $CD4^+$ T cells using mirVanamiRNA Isolation Kit followed by DNase digestion with the Turbo DNA-free Kit (Ambion). To quantify miR-146a expression, complementary DNA (cDNA) was synthesized with miScript Reverse Transcription Kit (Qiagen). Expression of mature miRNA-146a was measured using TaqMan®MicroRNA Assay (Applied Biosystems, Life Technologies, Darmstadt, Germany) and then amplified using SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) on an Applied Biosystems 7500 system (Applied Biosystems, Warrington, UK). U6 was used as an internal control. Primers of miR-146a used were as follows: forward, CAC TCC AGC TGG GTG AGA ACC TCA ACTGGT GTC GTG GA; and reverse, CTC AAC TGG TGTCGT GGA GTC GGC AAT TCA GTT GAG AACCCA TG; for U6: forward, CTC GCT TCGGCA GCA CA; and reverse, AAC GCT TCA CGA ATTTGC GT.

Cytokine assays

Serum levels of interleukin-2 (IL-2), IL-4, IL-10, TGF-β, and interferon-γ (IFN-γ) were determined by human cytokine ELISA kits (Shanghai Joyee-Biotechnics Co.,Ltd., Shanghai, China) according to manufacturer's instructions. The cytokine concentration in each supernatant was extrapolated from a standard curve.

Statistical analyses

Statistical analysis was performed using Prism 5.0 Software for Macintosh (GraphPad Software, San Diego, California, USA). Normal distribution was controlled by Kolmogorov-Smirnov.

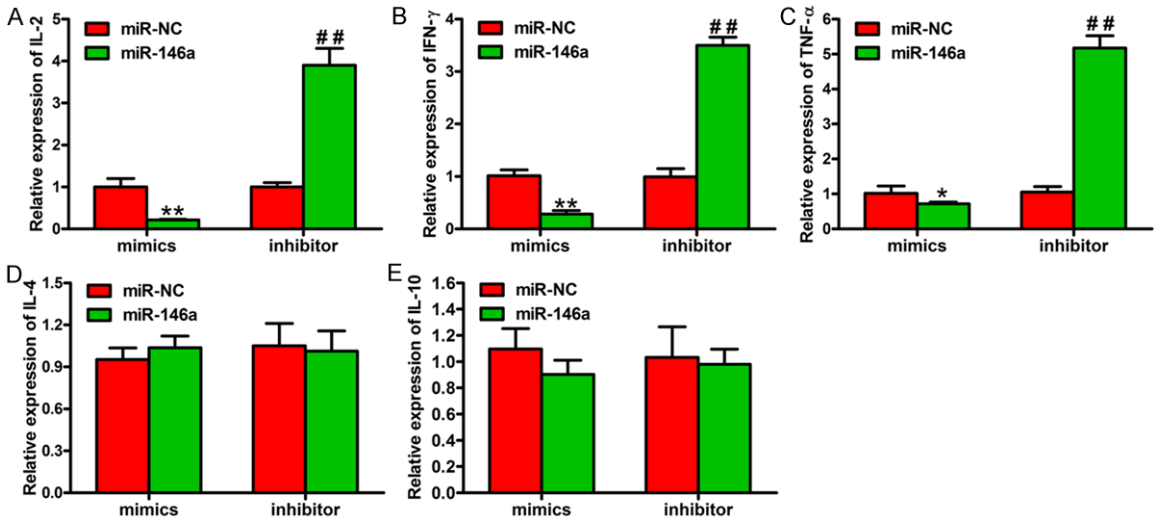

Figure 3. Cytokine protein levels in cells transfected with miR-146a mimics or inhibitor or negative controls. T lymphocytes were transfected with miR-146a mimics or inhibitor or negative controls for 48 h. Protein levels of cytokine IL-2 (A), IFN-γ (B), TNF-α (C), IL-4 (D) and IL-10 (E) were determined by ELISA. All data are presented as the mean ± SD of three replicates. *P<0.05, **P<0.01, miR-146a mimics vs. miR-NC. $^{\#\#}$P<0.01, miR-146a inhibitor vs. miR-NC.

Differences between study cohorts were determined using Student t test. Spearman's rank correlation coefficient was performed to test statistical dependency between two variables. P<0.05 was considered statistically significant.

Results

MiR-146a was downregulated in PBMCs and T cells of TAO patients

To investigate the relative expression of miR-146a in TAO patients, 20 TAO, 20 GD patients without ophthalmopathy, and 25 healthy controls matched with age and gender were enrolled in this study. qRT-PCR was used to detect the relative expression of miR-146a in the PBMCs and T cells. Relative expression of miR-146a in the PBMCs of TAO and GD patients without ophthalmopathy were significantly lower than that of healthy controls. Compared with GD patients, the miR-146a expression in the two other groups was significantly downregulated (**Figure 1A**). Similarly, the relative expression of miR-146a in the T cells of TAO patients was lower than that of GD patients without ophthalmopathy and healthy controls (**Figure 1B**). Therefore, we selected $CD4^+$ lymphocytes for further analyses. MiR-146a level was lowest in TAO patients among all $CD4^+$ T cells isolated from all samples (**Figure 1C**). Thus, we hypothesized that miR-146a may be involved in the $CD4^+$ differentiation process.

MiR-146a was involved in Th1 differentiation of human T cells

To determine the specific functions of miR-146a in $CD4^+$ T cells, the T lymphocytes obtained from healthy controls were transfected with miR-146a mimics or inhibitor or their negative control RNAs. After 48 h post-transfection with RNA oligonucleotides, qRT-PCR was conducted to determine miR-146a levels. **Figure 2A** shows that the expression of miR-146a was obviously higher in the T cells transfected with miR-146a than with miR-NC. Cells transfected with miR-146a inhibitor resulted in reduced production of miR-146a (**Figure 2B**).

Further assays were performed to detect effector cytokine production in response to anti-CD3/CD28 stimulation by ELISA. **Figure 3A** shows that the transfection of miR-146a mimics or inhibitor resulted in reduced or increased production of pro-inflammatory Th1 cytokines IL-2. The levels of two pro-inflammatory Th1 cytokines, namely, IFN-γ and tumor necrosis factor (TNF)-α, were sharply reduced in T cells transfected with miR-146a mimics but significantly enhanced in that transfected with miR-146a inhibitor (**Figure 3B** and **3C**). By contrast, the expression of the Th2 specific cytokines

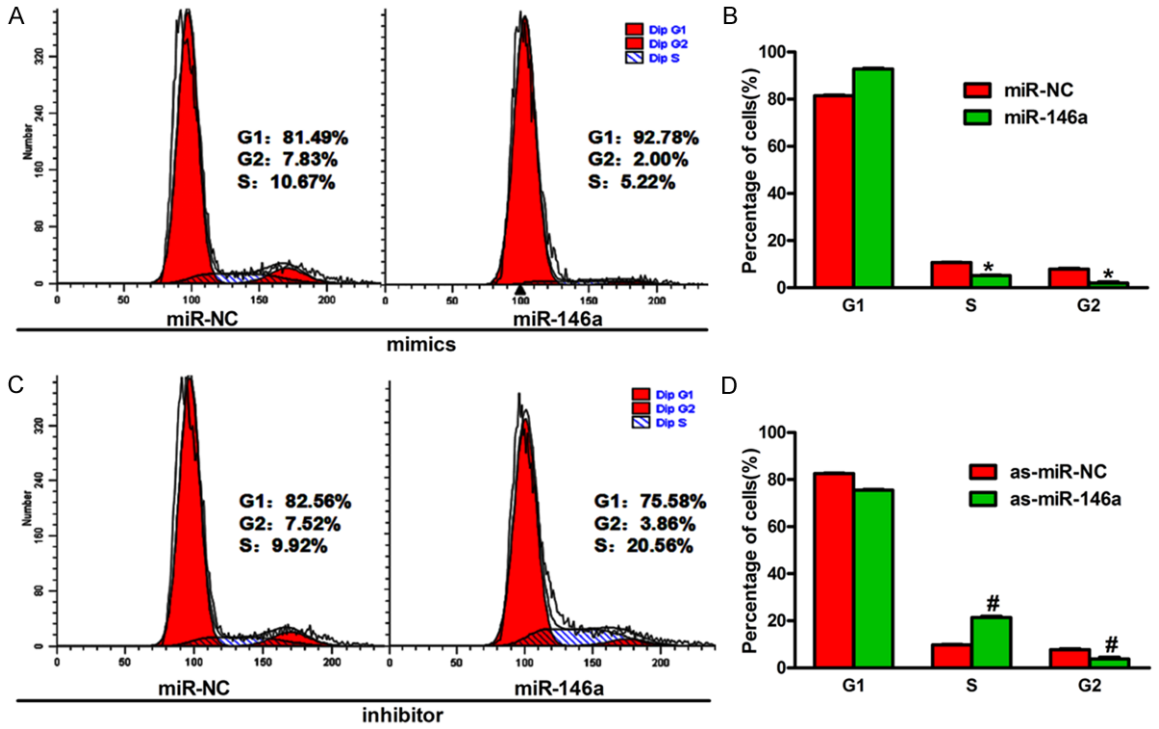

Figure 4. Effect of miR-146a in the proliferation of Jurkat T cells. Flow cytometer detected cell cycle of Jurkat T cells stimulated by the sera of TAO patients and diluted to 1:2 for 48 h. (A) Images of cell cycle progression after 48 h of transfection with miR-146a mimics and miR-NC; (B) Calculated rates of G1, S, and G2 phases in (A); (C) The representative images of cell cycle after 48 h of transfection with miR-146a inhibitor and negative control RNA; (D) Calculated rates of G1, S, and G2 phases in (C). All data are presented as the mean ± SD of three replicates. *P<0.05, miR-146a vs. miR-NC. #P<0.05, miR-146a inhibitor vs. miR-NC.

IL-4 and IL-10 remained unaffected (**Figure 3D** and **3E**), indicating that miR-146a exerts major effects in T effector cells.

MiR-146a efficiently inhibited the proliferation of Jurkat T cells by inducing G1 arrest

To investigate the potential role of miR-146a in T cell functions, we stimulated Jurkat T cells in the sera from the TAO patients diluted to 1:2 for 48 h and then transfected the cells with miR-146a mimics or inhibitor or their negative control RNAs. After 24 h of treatment, the cell cycle of the Jurkat T cells was analyzed using PI staining and flow cytometry (**Figure 4A**). The majority of the cells transfected with miR-146a were found to be in the G1 phase with a relatively low percentage of cells in the S and G2 phases (**Figure 4B**). These data indicated a G1 arrest in suppressed-T cells. As expected, JurkatT cells transfected with miR-146a inhibitor showed a marked decrease in the percentage of G1-phase cells, which was accompanied by a significant increase in the percentages of the S-phase and G2-phase cells (**Figure 4C** and **4D**). These results showed that, consistent with published results, T cells were efficiently suppressed by miR-146a in TAO-serum culturing conditions.

Introduction of miR-146a led to apoptosis in T Cells

To determine whether miR-146a had a potential role in T cells apoptosis, we further analyzed the expression during the incorporation of PI. **Figure 5A** and **5B** show that cells transfected with miR-146a mimics exhibited high levels of apoptosis to the negative control. Meanwhile, miR-146a inhibitor treatment after 48 h did not induce further apoptosis but showed a significant decrease in apoptosis (**Figure 5C** and **5D**). Thus, these data demonstrate that miR-146a promotes the apoptosis of T cells.

Graphical depiction of the proposed model

Thus, we propose that miR-146a was downregulated in the $CD4^+$ T cells of patients with TAO.

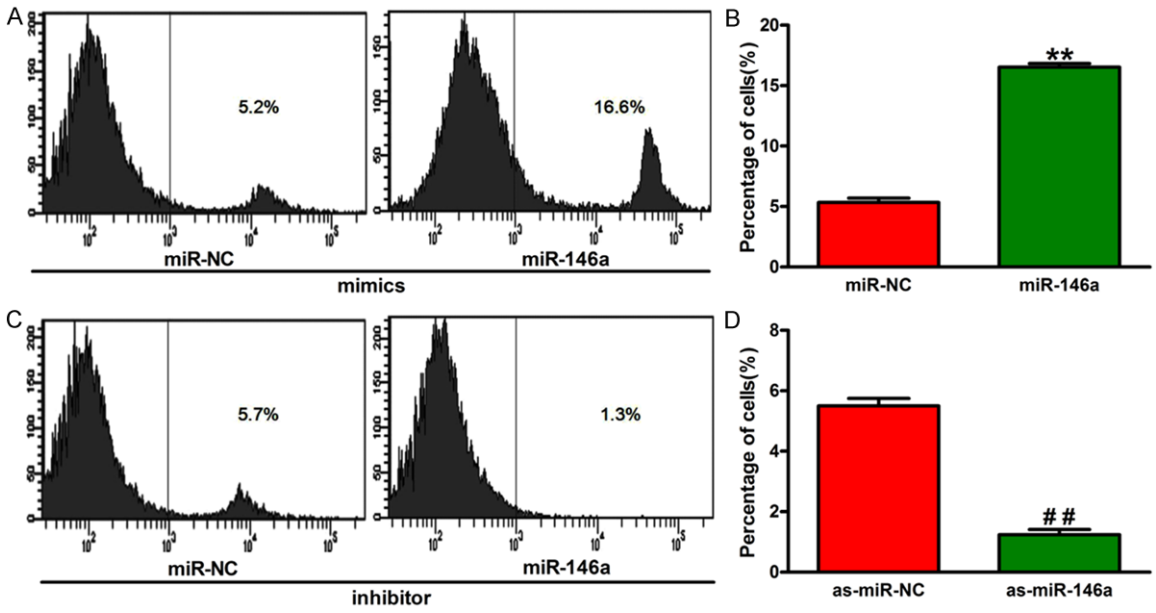

Figure 5. Effect of miR-146a in the apoptosis of Jurkat T cells. Flow cytometer detected cell cycle of Jurkat T cells stimulated by sera of TAO patients diluted to 1:2 for 48 h and transfected with miR-146a mimics (A and B) or inhibitor (C and D) for another 48 h. (A and C) Apoptosis of cells analyzed by flow cytometry; (B and D) Apoptosis rate in (A and C) was calculated. All data are presented as the mean ± SD of three replicates. **P<0.01, miR-146a vs. miR-NC. $^{\#\#}$P<0.01, as-miR-146a inhibitor vs. as-miR-NC.

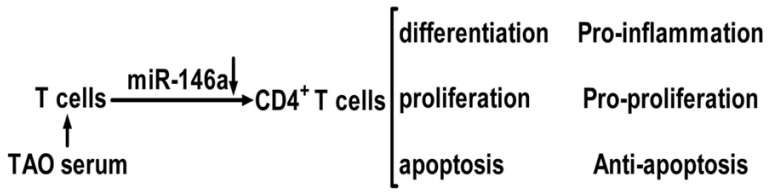

Figure 6. Proposed function of the miR-146a in $CD4^+$ T cells. The miR-146a was downregulated in the T cells of patients with TAO inhibiting $CD4^+$ T cell differentiation and proliferation, thus promoting autoimmune inflammation and proliferation response.

This characteristic promoted autoimmune inflammation and proliferation response (**Figure 6**).

Discussion

T cell functions must be tightly controlled after contact with pathogens to prevent detrimental hyperinflammatory responses [18]. MiR-146a has recently gained attention as a significant potent marker on the adaptive immune system in mice [16, 19, 20]. Its role within the signaling networks is to control human T cell responses, but the effects on inflammatory pathways remain unclear. Thus, we propose a new insight in the function of miR-146a. As an inhibitor of Th1 differentiation in human T cells, miR-146a is considered to be involved in cell differentiation processes. Overexpression of miR-146a contributes to controlling pro-inflammatory Th1 cytokine production, inhibiting cell cycle, and promoting apoptosis of T cells.

Following antigenic stimulation, immature $CD4^+$ T helper (Th) cells proliferate and differentiate to different memory and effector subsets, with responses tailored to specific pathogens [17]. The Th cells have been inferred to be limited to two major subsets, namely, Th1 and Th2 cells [21]. Th1 cells produce IFN-γ, IL-2, while TNF-α and Th2 cells produce IL-4 and IL-10 [22]. This concept is based on their production of specific cytokines (IFN-γ and IL-4). The Th1/Th2 behavior may play a critical role in the development of autoimmune disorders [23]. Maintaining the balance between Th1 and Th2 responses to Th cells is essential to prevent pathological conditions [24, 25]. We examined the expression of Th1 and Th2 cytokines in human T cells. The results showed that miR-146a reduced the pro-

duction of Th1 cytokines in human T cells but did not influence the cytokines produced by Th2. The findings in the T cells of TAO patients are consistent with that in the Th1 cells, which is the key in the response to activate macrophages and are involved in delayed-type hypersensitivity reactions, while Th2 cells are responsible for strong antibody responses [22].

Previous findings showed that miR-146a acted as an inhibitor of Th1 cell differentiation in human T cells by targeting the protein kinase C epsilon (PRKCε). This molecule is a part of a functional complex consisting of STAT4 and PRKCε, which controls Th1 cell differentiation in human $CD4^+$ T lymphocytes [26]. Upregulated expression of miR-146a in human lung alveolar epithelial cells could inhibit the production of inflammatory chemokines [27], suggesting that miR-146a may also play a crucial role in the migration of $CD4^+$ T cells. Interestingly, we identified lower expression of miR-146a in T cells, especially in $CD4^+$ T cells of TAO patients than that in negative controls. In addition, miR-146a level in T cells was increased using miRNA mimic, which significantly inhibited the production of inflammatory cytokines of Th1. In addition, miR-146a is a regulator of IL-2 expression and activation-induced cell death in T lymphocytes [28], implying that miR-146a may participate in the cell changes triggered by T cell receptor engagement. In this study, we found that miR-146a suppressed the proliferation of T cells by inducing G1 arrest, whereas T cells transfected with miR-146a inhibitor enhanced the cell proliferation. This phenomenon suggests that miR-146a in T cells enhance the suppression of T cell proliferation. Contrary to the findings of Zhou et al. [29], we previously showed that miR-146a also promoted the apoptosis of T cells treated by the sera of TAO patients. Therefore, our results suggested that miR-146a could be a major molecular regulator in the development of autoimmune disorders.

Our study has limitations in determining the role of miR-146a in different grades of TAO patients and the molecular mechanisms of miR-146a in TAO progression. In this study, we have elucidated only the regulatory effects of miR-146a on T cell differentiation and proliferation in TAO patients. In our future studies, we shall clarify in detail the molecular mechanisms of miR-146a. Möhnle et al. [26] revealed that miR-146a controls Th1-differentiation of human $CD4^+$ lymphocytes by targeting PRKCε and suggested the possible role of miR-146a in the regulation of human T cell functions during sepsis. However, the influence of miR-146a on cytokine production may partly be inflammatory pathways via a concerted action on multiple transcripts rather than on one single transcript. We expect that our ongoing studies into the molecular mechanisms of miR-146a both in the TAO occurrence and progression will lead to greater insights into its functions. MiR-146a may contribute in determining the expression of inflammatory mediators in vivo, given the role of miRNAs as precise regulators of gene expression. Thus, further studies, such as characterization of T cell subset and analysis of surface markers, are needed to exactly determine the significance of the molecular interactions within the regulatory networks of TAO.

Thus, the results presented some new insights into the possible role of miR-146a in the regulation of human T cell functions during TAO disease. MiR-146a was downregulated in the T cells of TAO patients and is considered as an inhibitor of Th1 differentiation and proliferation in human T cells.

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Disclosure of conflict of interest

None.

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