


Negative Regulation of Semaphorin-3A Expression in Peripheral Blood Mononuclear Cells Using MicroRNA-497-5p

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What's Known

- Sema3A and miR-497-5p are important molecules in autoimmune diseases.
- Previous findings have proven that miR-497-5p is upregulated and Sema3A is downregulated in some autoimmune disorders.

What's New

- miR-497-5p has a high suppressive effect on Sema3A expression, and both Sema3A and miR-497-5p can be considered critical targets for further studies on future therapeutic attempts for the treatment of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.

Abstract

Background: Semaphorin-3A (Sema3A), as a secreted semaphorin, is an immune modulator molecule participating in the pathogenesis of autoimmune diseases. MicroRNAs (miRNAs) modulate the target-gene expression at the post-transcriptional level. It has been proposed that miRNAs may be crucial to the modulation of the function of semaphorins. Previous findings have proven that miR-497-5p is upregulated and Sema3A is downregulated in some autoimmune disorders. Thus, we aimed to examine the presence of any correlation between Sema3A and miR-497-5p in peripheral blood mononuclear cells (PBMCs).

Methods: PBMCs were cultured and transfected with miR-497-5p mimic using the X-tremeGENE™ reagent. The expression level of Sema3A was assessed after 48 hours in supernatants and cells via the enzyme-linked immunosorbent assay and quantitative real-time polymerase chain reaction, respectively. Cell viability was evaluated using the methylthiazol tetrazolium assay. All the experiments were done in triplicate, and the statistical analyses were performed with SPSS, version 20. P values equal to or less than 0.05 were considered significant.

Results: We observed downregulation of the Sema3A gene (P=0.0001) and its secretion (P=0.032) in the PBMCs through miR-497-5p transfection. Moreover, transfection with miR-497-5p mimic and downregulation of Sema3A did not affect the viability of the PBMCs (P=0.061).

Conclusion: Based on the obtained results, we suggest that miR-497-5p has a high suppressive effect on Sema3A expression and both Sema3A and miR-497-5p can be considered critical targets for further studies on future therapeutic attempts for the treatment of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.

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Keywords • Autoimmune disease • MicroRNAs • Mononuclear phagocyte system • Semaphorin-3A

Introduction

Semaphorins are a family of evolutionally conserved proteins which are divided into 8 classes: 1–7 and Viral semaphorins.¹⁻³ Class I and II semaphorins exist in invertebrates, class III to VII semaphorins are seen in vertebrates, and class VIII semaphorins are virally encoded.⁴ Up to now, more than 20 types of semaphorins

have been discovered which play crucial roles in many biological functions including epithelial growth and invasion,⁵ cardiogenesis,⁶ vasculogenesis,⁸⁻¹⁰ osteoclastogenesis,¹¹ tumor progression,¹²⁻¹⁴ and immune regulation.^{4, 15} *Sema3A* is expressed by the majority of immune cells including activated dendritic cells, differentiating macrophages, T cells, regulatory T cells, and regulatory B cells. Moreover, other cells such as endothelial cells and neurons in the peripheral nervous system can express *Sema3A*.^{2,4, 16-18} *Sema3A*, which is a secreted protein in vertebrates, has a prominent role in the immune system. A role is emerging for *Sema3A* in the enhanced migration of dendritic cells into lymph nodes. *Sema3A* inhibits actin cytoskeletal reorganization and downregulates the signaling of mitogen-activated protein kinases via R-RAS inactivation in T cells, resulting in the suppression of T-cell proliferation and reduction of pro-inflammatory cytokine production by T cells.^{18, 19} Additionally, *Sema3A* is capable of inhibiting T-cell proliferation through neuropilin-1 binding, which suggests that *Sema3A*/neuropilin-1 interactions participate in terminating the immune response, thus restraining overactivation.^{2, 20} Apoptosis induction in both leukemic cells and monocyte-derived macrophage colony-stimulating factor-differentiated macrophages is another function for *Sema3A*.² Furthermore, *Sema3A* can suppress the overactivity of both T- and B-cell autoimmunity and enhance the regulatory properties of T and B cells; it, therefore, has an important role in downregulating autoimmune diseases.¹⁸

MicroRNAs (miRNAs), which can be identified in a large scale of samples including tissue biopsies, whole blood, blood cells, serum, plasma, and urine, are small endogenous, non-coding and single-stranded RNAs that have ~21–25 nucleotides length.^{21, 22} miRNAs can modulate the target-gene expression at the post-transcriptional level, either through the inhibition of translation or the cleavage of target mRNAs.^{23, 24} Accordingly, these non-coding RNAs play significant roles in many physiologic processes such as development, differentiation, proliferation, metabolism, apoptosis, angiogenesis, inflammation, and immunity.^{23, 24} Thus, abnormalities in the expression and function of miRNAs can lead to several human diseases including cancer, neurodegeneration, and autoimmunity.^{23, 25} For instance, abnormal miRNA regulation can cause instability in regulatory T cells, loss of the suppressor function in regulatory T cells, and lymphocyte accumulation, leading to the loss of tolerance and development of autoimmunity.²⁶

The fact that both semaphorins and miRNAs play significant roles in similar biological systems suggests that miRNAs could be crucial modulators of the functions of semaphorins. One of these miRNAs is miR-497-5p, which is predicted to be in a correlation with *Sema3A*. The role of miR-497-5p in autoimmune diseases has yet to be explored.²⁷ It has been shown that miR-497-5p upregulates in CD4⁺ T cells and B cells in patients with multiple sclerosis compared with a control group.²⁸

According to our bioinformatics and experimental findings, as well as the importance of *Sema3A* and miR-497-5p in autoimmune disorders such as multiple sclerosis, it could be hypothesized that miR-497-5p upregulation leads to the downregulation of *Sema3A*, which in turn results in the elevation of inflammatory condition in the body. This process may predispose to autoimmunity. Thus, it is possible that upregulation of the *Sema3A* gene expression in the immune system could be an attractive and promising therapeutic approach against these diseases. Consequently, in this work, for the first time, we aimed to determine the interaction between *Sema3A* and miR-497-5p through targeting *Sema3A* by miR-497-5p mimic.

Materials and Methods

Bioinformatics Prediction

Firstly, miRWalk 2.0 (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/>) was applied for the bioinformatics prediction of miRNA-*Sema3A* interaction. In the “predicted targets module”, “Gene-miRNA target” was selected and then the *Sema3A* RefSeq ID (NM_006080.2) was put in the relevant box. The input parameters were adjusted on finding 3'-UTR, and all the databases were chosen. After that, TargetScan 7.0 (<http://www.targetscan.org/>) was used to confirm the bioinformatics prediction performed by miRWalk. The gene symbol of *Sema3A* was put in the relevant box and subsequently the query was submitted.

Cell Culture

The PBMCs were isolated from buffy coats obtained from every single healthy donor using the density-gradient centrifugation on Ficoll (Lymphodex, Germany). The cells were cultured in a 6-well plate at a density of 1×10^6 cells per well in the RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 2% penicillin/streptomycin (Bio-Idea, Iran) and maintained in a humidified 5% CO₂ incubator at 37 °C. In addition, phytohemagglutinin ([PHA]; 7.5 µg/mL) was used to induce T-cell

proliferation. Forty-eight hours after culturing, the cells were subcultured without FBS and penicillin/streptomycin.

Transfection

Four rows were designed: 1) positive control (PBMCs treated with PHA), 2) mock-transfection control (PBMCs treated with PHA and the X-tremeGENE™ reagent), 3) scrambled control (PBMCs treated with PHA, the X-tremeGENE™ reagent, and *Label IT*® RNAi Delivery Control), and 4) mimic transfection (PBMCs treated with PHA, the X-tremeGENE™ reagent, and miR-497-5p mimic). Seventy-two hours after culturing, the cells were transfected with a 50-nM final concentration of miR-497-5p mimic (Qiagen, Germany) at 60% confluency using the X-tremeGENE™ reagent (Roche, Germany). The *Label IT*® RNAi Delivery Control-FITC (Mirus, USA) was transfected as a transfection efficiency indicator and scramble siRNA, as it does not react with any known mammalian mRNA. After 4 hours, incubation in 5% CO₂ and at 37 °C, 10% FBS and 1% penicillin²/streptomycin were added to the cells. The plate was incubated at 37 °C for 48 hours. Thereafter, the cells of each well were added to a tube and were centrifuged for 20 minutes at 2000 rpm (448×g) to isolate the supernatants and cells for further experiments. In addition, transfection efficiency was assessed using the FACSCalibur flow cytometer (Becton Dickinson, USA) and CellQuest™ Pro software.

Evaluation of Sema3A Secretion

The supernatants were assessed for the presence of Sema3A with a commercial enzyme-linked immunosorbent assay (ELISA) kit (ElabScience, China) according to the manufacturer's instructions (Sandwich method).

Evaluation of the Sema3A mRNA Level

Quantitative real-time polymerase chain reaction (qRT-PCR) was applied to quantify Sema3A mRNA expression. Total RNA was isolated using the RNXTM-PLUS solution (CinnaGen, Iran) according to the manufacturer's protocol and was reversed-transcribed into cDNA using a first-strand cDNA synthesis kit (Thermo Fisher, USA). Afterward, real-time

PCR was performed using a Maxima SYBR green/ROX qPCR kit (Thermo Fisher, USA). The sequences of the primers used in this study are listed in table 1. β -actin served as an endogenous control for each sample. Quantitative real-time PCRs were done on a StepOnePlus device (Applied Biosystems, USA). qRT-PCR was conducted in 1 cycle at 95 °C for 10 minutes and then in 40 cycles at 95 °C for 15 seconds and 61 °C for 1 minute. The gene expression data were normalized to β -actin. The relative expression was determined using the formula $2^{-\Delta\Delta Ct}$.

Methylthiazol Tetrazolium (MTT) Viability Assay

Assessment of cell viability was performed using the MTT assay. Twenty-four hours after transfection, 90 μ L of a medium containing 10⁵ cells was placed in a 96-well plate, and 10 μ L of a 5-mg/mL MTT solution (Sigma-Aldrich, Germany) in a PBS buffer was added to each well. After 1 hour's incubation at 37°C, the medium was removed and the cells were frozen for 1 hour at -80 °C. Next, the purple formazan product was dissolved in 100 μ L/well dimethyl sulfoxide (Sigma-Aldrich, Germany) at 37 °C for 30 minutes while being shaken and absorbance was photometrically measured at 590 nm.

Statistical Analysis

SPSS, version 20 (SPSS Company, Chicago, USA), was applied for the statistical analyses. The Kolmogorov–Smirnov and Shapiro–Wilk tests were employed to define the normality of data distribution. One-way ANOVA and Kruskal–Wallis tests were utilized for comparisons between the treated groups for the parametric and nonparametric values, respectively. P values equal to or less than 0.05 were considered statistically significant. All the experiments were done in triplicate, and each test was repeated 3 times separately.

Results

miR-497-5p Was Predicted as a Sema3A-Silencer miRNA

In the miRWalk output, miR-497-5p was predicted to suppress Sema3A expression by 7/12 of the selected algorithms (miRWalk, miRanda, miRDB, PICTAR2, PITA, RNA hybrid,

Table 1: Specific primers for qPCR

Gene	Sequence of Primers, 5' to 3'	Product Length (bp)
Sema3A	Forward: TGTTGGGACCGTTCTTAAAGTAGT	142
	Reverse: TAGTTGTTGCTGCTTAGTGGAAG	
β -actin	Forward: TGAAGATCAAGATCATTGCTCCTC	170
	Reverse: CAACTAAGTCATAGTCCGCCTAGA	

and TargetScan). These programs predicted 2 positions within the Sema3A-3' UTR sequence for hsa-miR-497-5p (figure 1A) and showed that the predicted complementary sequence in humans and other mammals is conserved (figure 1B). The "validated targets module" showed nothing for miR-497-Sema3A interaction in that it represented that no experimental study had been done to validate this bioinformatics prediction. In the TargetScan output, the context score percentile of miR-497-5p for silencing Sema3A expression was predicted as 87% with an 8-mer seed region.

MiR-497-5p Reduced Sema3A Secretion in the PBMCs

The effects of miR-497-5p on Sema3A in vitro were studied by transfecting miR-497-5p

mimic into the PBMCs to overexpress the miR of interest. There was delay in Sema3A secretion, and the best time for the optimum secretion of Sema3A was obtained with an ELISA kit. Overexpression of miR-497-5p significantly decreased the secretion of Sema3A. As is seen in figure 2, the Sema3A level was lower in the transfected cells than in the control group (0.17±0.01 vs. 0.8±0.01 ng/mL; P=0.032).

Efficiency of Transfection

To confirm our findings, we first determined the efficiency of transfection with the *Label IT*[®] RNAi Delivery Control kit (Mirus, USA), which is labeled with fluorescein. After the transfection of the cells with the *Label IT*[®] RNAi Delivery Control kit, transfection efficiency was identified quantitatively by the flow-cytometry method and

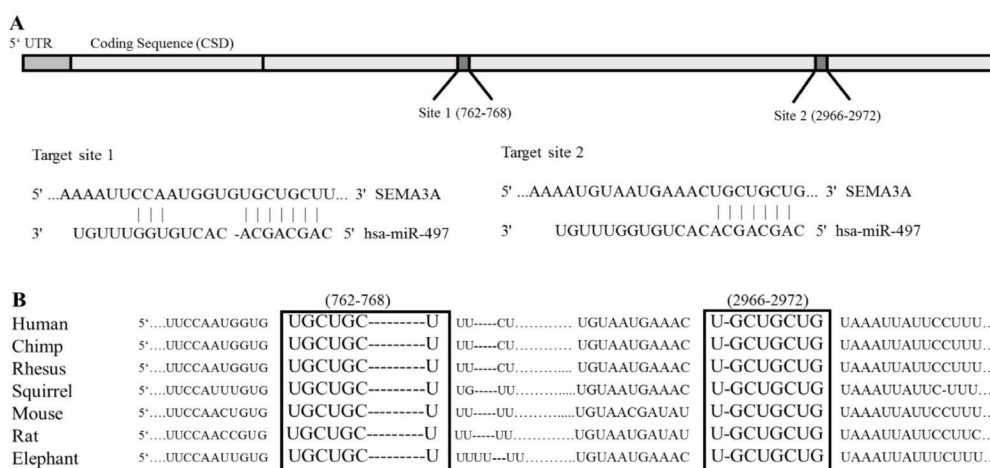


Figure 1: This figure shows the predicted hsa-miR-497-5p complementary sequence in Sema3A mRNA 3'-UTR and its conservation status. Part A shows the positions of the predicted complementary sequence located in Sema3A mRNA 3'-UTR and also the pairing status of hsa-miR-497-5p and the predicted target sequence. Part B shows the conservation status of the predicted complementary sequence in humans and other mammals.

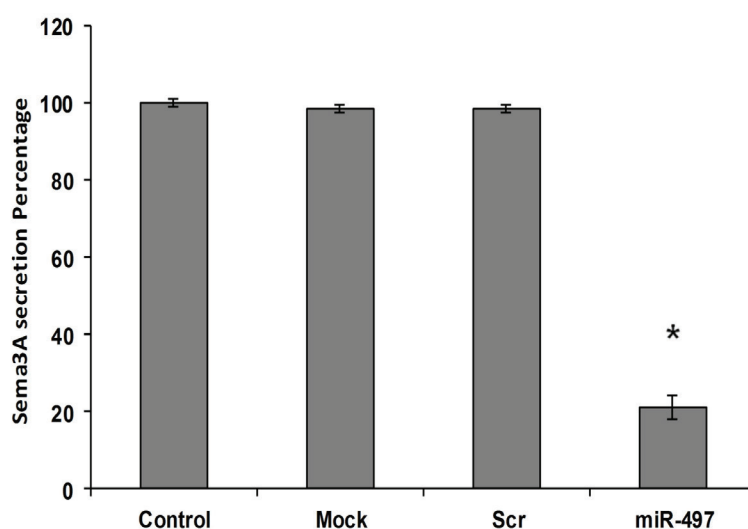


Figure 2: Sema3A level was significantly decreased in the transfected cells compared with the control groups (*P<0.05). Data are presented as the mean±SD of 3 identical repeats of each experiment. Control, PBMCs treated with PHA; Mock, PBMCs treated with PHA and the X-tremeGENE™ reagent; Scr, PBMCs treated with PHA, the X-tremeGENE™ reagent, and Label IT[®] RNAi Delivery Control; miR-497-5p, PBMCs treated with PHA, the X-tremeGENE™ reagent, and miR-497-5p mimic

it was 84% (figure 3). Furthermore, transfection with miR-497-5p mimic and downregulation of Sema3A did not affect the viability of the PBMCs, and no statistically significant difference was observed between the transfected cells and the control group ($P=0.061$) (figure 4).

mir-497-5p Downregulated Sema3A Expression in the PBMCs

To determine whether miR-497-5p had modulated Sema3A gene expression, we evaluated the expression pattern of Sema3A by qRT-PCR before and after transfection with miR-497-5p mimic. The expression data of the target gene were normalized with a corresponding mean value of β -actin as an endogenous gene. We found that the Sema3A expression level was significantly decreased in the transfected cells compared with the control group ($P=0.0001$). As is indicated in figure 5, there was no significant difference between the negative, mock, and

scrambled controls for Sema3A expression, which confirmed that the silencing was specific.

Discussion

In the present study, we applied miR-497-5p mimic and found that Sema3A expression was highly suppressed in the cultured PBMCs. We observed downregulation of the Sema3A gene ($P=0.0001$) and its secretion ($P=0.021$) in the PBMCs through miR-497-5p transfection. Moreover, transfection with miR-497-5p mimic and downregulation of Sema3A did not affect the viability of the PBMCs ($P=0.156$). Hence, the predicted inhibitory interaction of miR-497-5p on Sema3A was experimentally confirmed in this study. Our literature review yielded no study on the inhibitory effect of miR-497-5p on Sema3A in autoimmune diseases, precluding comparison of our results. However, in a similar study, Rezaeepoor et al.²⁹ demonstrated that

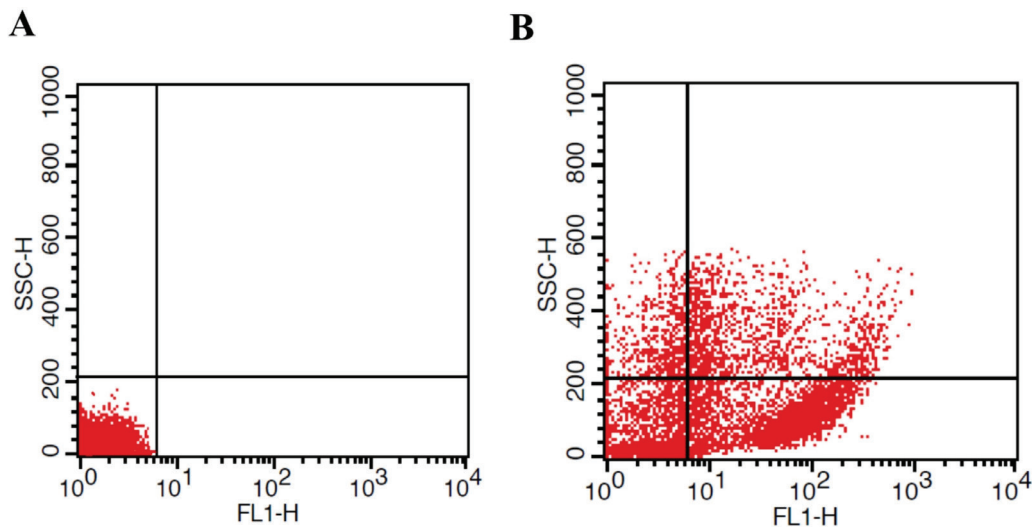


Figure 3: A group of cells were transfected with the *LabelIT*® RNAi Delivery Control kit and were analyzed using a flow-cytometry instrument. The transfection efficiency was 84% (B) in comparison with the negative control (A).

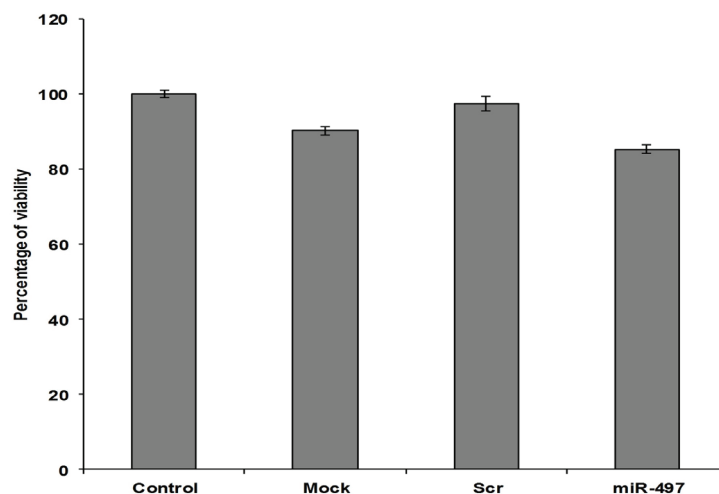


Figure 4: There was no statistically significant difference in the viability of the PBMCs by the MTT assay between the transfected cells and the control groups. Data are presented as the mean \pm SD of 3 identical repeats of each experiment.

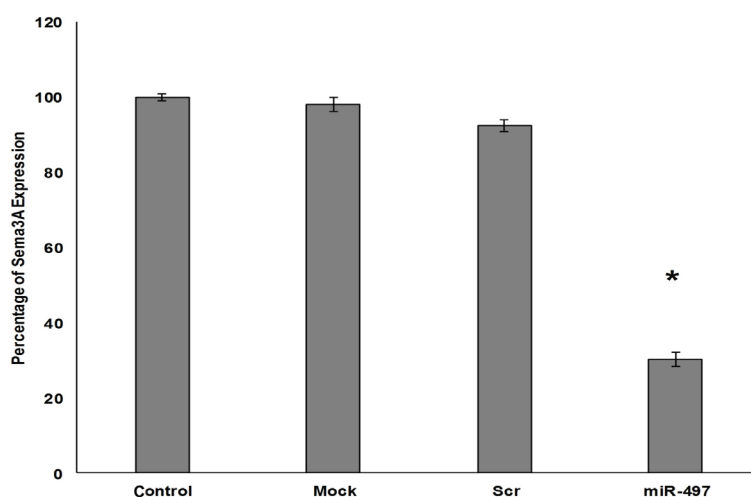


Figure 5: The mRNA levels were normalized to the expression of β -actin as an endogenous control. There was a significant difference between the miR-497-5p transfected cells and the other groups by the quantitative real-time PCR analysis of the Sema3A expression level in the culture of the PBMCs (* $P < 0.05$ vs. control). Data are presented as the mean \pm SD of 3 identical repeats of each experiment.

an increase in miR-145-5p downregulated Sema3A expression at both protein and mRNA levels in PBMCs.

The role of Sema3A has been identified in some autoimmune disorders such as rheumatoid arthritis, psoriasis, systemic sclerosis, and systemic lupus erythematosus.^{17, 30-33} Two previous studies demonstrated that not only was Sema3A downregulated in T cells derived from patients with rheumatoid arthritis but also a decrease in Sema3A expression in rheumatoid arthritis synovial tissues might contribute to the pathogenesis of rheumatoid arthritis.^{31,33} The results of another study showed that Sema3A expression was low in psoriatic skin samples compared with healthy skin samples.³⁰ Elsewhere, the expression of Sema3A on regulatory T cells was also lower in patients with systemic sclerosis than in healthy controls.³² In a study on patients with systemic lupus erythematosus, it was found that Sema3A expression as well as the expression of its receptor neuropilin-1 was significantly reduced in B reg cells from patients with systemic lupus erythematosus compared with normal individuals.¹⁷ In our previous study, we showed that Sema3A is downregulated in the PBMCs of patients with multiple sclerosis.³⁴ Moreover, Gutiérrez-Franco et al.³⁵ studied experimental autoimmune encephalomyelitis (EAE) mice and showed a decreased Sema3A expression in their immune system. The authors proved that the Sema3A gene expression during EAE development had no differences, although the levels of its protein expression after EAE induction were downregulated. Consequently, this discrepancy in the mRNA and protein levels can be explained by the

regulation of Sema3A expression at a post-transcriptional level. In accordance with these reports and the mentioned roles of Sema3A in the immune system, it seems that alteration in Sema3A expression may play a key role in the pathogenesis of autoimmune diseases. Thus, we decided to investigate one of the possible reasons for Sema3A downregulation.

On the other hand, it has been indicated that miR-497-5p is upregulated in some autoimmune disorders such as multiple sclerosis^{28, 36} and regarding the bioinformatics predictions, we anticipated that miR-497-5p might have a powerful inhibitory effect on Sema3A expression. Additionally, according to these databases, the silencing effect of miR-497-5p on the Sema3A protein has not been validated in vitro, so far.

The recent years have witnessed an increasing interest in discovering miRNA-based biomarkers for autoimmune diseases and several miRNAs have been found to be dysregulated in blood cells, brain lesions, and biological fluids from patients with autoimmunity.²³ In light of the previous studies which have proven that miR-497-5p is upregulated²⁸ and Sema3A is downregulated in some autoimmune disorders³⁴ and also bioinformatics predictions (TargetScan and miRWalk), we found that miR-497-5p has a possible and powerful interaction with Sema3A in PBMCs.

Targeting of specific miRNAs could be another novel and interesting approach for the development of a therapeutic strategy for the regulation of pathogenic gene expression. Recent findings suggest that the use and delivery of modified oligonucleotides mimicking or inhibiting specific miRNAs may eventually be

a treat for autoimmune disorders.²³ Therefore, inhibition of miR-497-5p expression or effect might be helpful as another therapeutic approach.

Although we found a significant inhibitory effect of miR-497-5p on *Sema3A* expression in normal PBMCs, we suggest that similar experiments be conducted on PBMCs from autoimmune patients to validate this impact in disease conditions.

Conclusion

In the current study, we evaluated the effects of miR-497-5p on *Sema3A* expression in PBMCs. Based on the obtained results, we suggest that miR-497-5p has a high suppressive effect on *Sema3A* expression. Since reduced *Sema3A* expression along with increased miR-497-5p level has been observed in autoimmune disorders, both *Sema3A* and miR-497-5p could be considered critical targets for further studies on future therapeutic attempts for the treatment of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.

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Conflict of Interest: None declared.

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