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ITGα4 Inhibition by miR-30d as a Potential Target in Relapsing Form of MS Therapy

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ABSTRACT Multiple sclerosis (MS) is caused by demyelination of neurons. Dysfunction of α4-integrin (*ITGα4*) in lymphocyte surface is associated with neuron demyelination. Herein, inhibitory effect of hsa-miR-30d on *ITGα4* gene expression in HEK293T cells has been evaluated. Bioinformatics approaches were used to identify the miRNAs that can potentially target *ITGα4*. miR-30d was transfected into HEK293T cells using TurboFect reagent. Flow cytometry analysis was performed to evaluate *ITGα4* and miRNAs transfection. *ITGα4* expression level was surveyed in the transfected cells using Q-RT-PCR. MTT assay was carried out in the HEK293T cells. *In silico* analysis predicted that the miR-30 family targets *ITGα4*. Flow cytometry analysis showed that *ITGα4* expression in HEK293T cell surface decreased after miR-30d transfection. The expression of *ITGα4* decreased in transfected cells by miR-30d. Thus, miR-30d can down regulate *ITGα4* in the HEK293T cells. It can be considered as a silencing approach to decrease *ITGα4* expression in MS patients and cancers.

INTRODUCTION

Multiple sclerosis (MS), a progressive autoimmune disease is defined as inflammation in the brain and spinal cord, which damages the myelin sheath and causes demyelination of neurons. In MS patients, neuronal signal transmission is impaired by demyelination, which in turn leads to different physical and cognitive inabilities, such as sensory disorder, motor dysfunction, optic neuritis, and coordination problems (Kawamoto et al. 2012).

Studies indicate that α4-integrin (ITGα4) plays an important role in lymphocyte transmigration into the central nervous system (CNS) (Glatigny et al. 2015). Thus, pathogenesis of this molecule is considerable in MS patients suffering from demyelination of neurons (Schwab et al. 2015). To reduce relapse and progression of this disease, a wide range of treatments have been used (Lorefice et al. 2014). However, effective treatments remain limited (Kawamoto et al. 2012). Natalizumab is a humanized monoclonal antibody (Nicholas et al. 2014) that targets several biological pathways involved in MS and has been approved (Lorefice et al. 2014) for the treatment of the relapsing form of MS (Brandstadter and Katz Sand 2017). Natalizumab blocks α4-integrin receptors in leukocytes and decreases migration of T lymphocytes (Nicholas et al. 2014) across the blood-brain barrier (Lorefice et al. 2014). α4-integrins (α4β1 and α4β7) are implicated in the transmigration of T cells into CNS through the interaction with extracellular matrix ligands (Nicholas et al. 2014). Natalizumab, when used as a first-line treatment, is associated with adverse effects, such as progressive multifocal leukoencephalopathy (PML), mortality, and morbidity risks. Thus, these risks have contributed to the reservation of NTZ for second-line use in therapy (Bargiela et al. 2017).

MicroRNAs (miRNAs) are a class of small non-coding RNAs, which post-transcriptionally controls gene expression (Ranji et al. 2013). MiRNAs bind themselves to target mRNAs, which results in mRNA degradation or translational repression (Ranji et al. 2015). It has been estimated that more than 700 identified miRNAs (Ranji et al. 2014) regulate the expression of ap-

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proximately one-third of the mRNAs found in mammalian cells (Furer et al. 2010; Ranji et al. 2014). MiRNAs bind to 3′-UTR of target mRNAs with perfect or imperfect complementarity and lead to mRNA degradation or translational repression, respectively (Ranji et al. 2014; Rouleau et al. 2017).

Nowadays, many researchers focus on miRNA targets in different pathways in cells (Zhang et al. 2015; Maleki Zadeh et al. 2016; Zhang et al. 2016; Zhang et al. 2017). Hsa-miR-30d belongs to the miR-30 family, which consists of six members (hsa-miR-30a-f). This family has different functions, including inhibition of mitochondrial fission, apoptosis induction (Li et al. 2010), epithelial-to-mesenchymal transition in pancreatic cells, osteogenic differentiation, and endothelial cell behavior during angiogenesis and cardiac function (Moreno-Moya et al. 2014).

MiRNAs can be potential therapeutic targets in the near future. The *in silico* analysis conducted during this research predicted that miR-30d might play a role in the inhibition of α4-integrin. The aim of this study was to evaluate the effect of miR-30d on α4-integrin expression in the HEK-293T cell line.

METHODOLOGY

In Silico Analysis of Potential Targets of miR-30d

Approaches based on bioinformatics were used to determine the potential targets of miR 30d. Their targets were predicted using some algorithms, such as TargetScan (http://www.targetscan.org/), miRWalk (http://www.umm.uni heidelberg.de/apps/zmf/mirwalk), and Diana microT (http://diana.imis.athena-innovation.gr/). These algorithms survey putative miRNA targets based on the presence of complementary binding sites in their 3´untranslated region (3´-UTR) or the minimum binding energies, and determines whether the interaction between the miRNA and its targets are thermodynamically favorable.

PCR Amplification of 3′ UTR of *ITGα4*

Oligonucleotide primers containing *XhoI* restriction site at 5' flanks of *ITGα4* transcript was designed to amplify the 3′ UTR of *ITGα4*. The sequences of forward and reverse primers were (5′-GTGCTGTGGACCTCAATGC-3′) and (5'-CATTCATTACTGCTCCCGAG-3′), respectively. PCR was performed in a total volume of 50 µl containing 5 µl 10x *Pfu* buffer, 10 µM dNTPs, 10µM of each primer, 100 ng of genomic DNA, and 1.5U of *Pfu* DNA polymerase. Thermocycling profile was adjusted at 95°C for 5 min, followed by 30 cycles of 95°C for 30s, 63°C for 30s, 72°C for 2 min, with a final extension step at 72°C for 5 min. The oligonucleotide primers used for PCR have been described in Table 1. To insert 3'UTR of *ITGα4* to vector, *XhoI* site was added to the 5' site of forward and reverse primers.

Construction of ITGα4.3'UTR Expression Vectors

The PCR product was initially cloned into EX-Z2827-M67 vector (GeneCopoeia, USA) (Fig.1). 3'UTR of ITGα4 was cloned upstream of ORF through the MCZ site to hygromycin coding region. The new construct (EX-Z2827-M67-3'UTR.ITGα4) was transformed into *E. coli* Top10 F´ strain for cloning and amplification. After 12-16 hours of incubation, EX-Z2827-M67-3'UTR.ITGα4 was purified using Solgent™ Plasmid mini prep (Solgent Co. LTD., South Korea). Concentration of the purified DNA was determined with the help of Nanolytik® NanoSpec Cube (Biophotometer). To confirm the insertion of ITGα4-3'UTR, two methods were used - 1) XhoI enzyme digestion and, 2) colony PCR test. For method 1, the PCR program was as follows: 96°C for 3 min, 30 cycles at 96°C for 30 sec, 60°C for 30 sec, 72°C for 90 sec, and a final extension

Table 1: Primers used for PCR

Name	*Sequence*	*PCR product length (bp)*
ITGα4-F	5'- GGACTTCTTTCAAATTGAGAGAATG -3'	869
ITGα4-R	5'- AATCAGCGTGTATCAGGTAAAGTG -3'	
ITGα4-F by enzyme site	5'- TAACTCGAGGGACTTCTTTCAAATTGAGAGAATG-3'	
ITGα4-R by enzyme site	5'- TCTCGAGAATCAGCGTGTATCAGGTAAAGTG-3'	

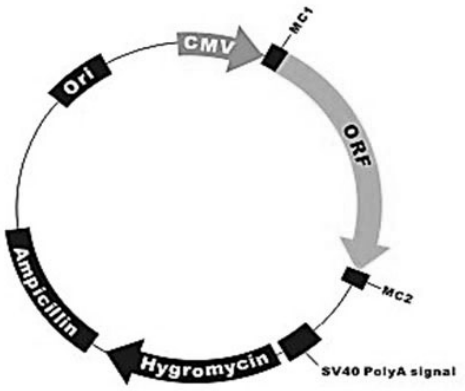

Fig. 1. EX-Z2827-M67 construct - 3′UTR of *ITGα4* was cloned upstream of ORF through the MCZ site to hygromycin coding region

step at 72°C for 5 min. The purified DNA was treated with XhoI enzyme (Fermentas, USA) and run in agarose gel. Then, the plasmid backbone fragment was extracted from the gel using a DNA extraction kit (Bioneer, Korea). The linear plasmid was treated with calf intestinal alkaline phosphatase enzyme (Fermentas, USA). The two fragments were then ligated using T4 DNA ligase (Fermentas, USA). The ligation product (EX-Z2827-M67-3′UTR.ITGα 4) was subsequently transformed into competent *E. coli* TOP10F (Li et al. 2010). The *E. coli* transformants were cultured in LB agar (Merck, Germany) containing 100 µg/ml of ampicillin and incubated overnight at 37°C. Then, the colony PCR test (method 2) was performed by the *ITGα4* 3′UTR forward and hygromycin reverse primers (Table 2), and the master mix. The PCR products were analyzed through sequencing (Bioneer, Korea). The positive clone containing EX-Z2827-M67-3′UTR.ITGα4 plasmid was cultured in LB broth with 100 µg/ml of ampicillin and incubated overnight at 37°C and 250 rpm . Its plasmid was then extracted sequentially with a miniprep extraction kit (SolGent, Korea).

HEK293T Transfection with 3′UTR.ITGα4

The HEK293T cell line was purchased from the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran). The cells (8×10^5 cells) were seeded in DMEM media (Bio India Co., India) supplemented with ten percent fetal bovine serum (FBS) and pen/strep (100 U/mL penicillin and 100 µg/mL streptomycin) (Bio India Co., India) in a T25 flask at 37°C in a humidified atmosphere containing five percent CO_2. To transfect, EX-Z2827-M67-3′UTR.ITGα4 vector was linearized using the EcoRV enzyme. The transfection soluble was comprised from 30 µl (250 µg/ml) of EX-Z2827-M67-3′UTR.ITGα4 vector added to 750 µl of DMEM and 15 µl of TurboFect™ Transfection Reagent (Thermo Sientific). After 20 min, the transfection soluble was added to the HEK293T cells cultured in the T25 flask and incubated at 37°C for 24 hours.

Evaluation of 3′UTR.ITGα4 Transfection to HEK293T Cells

Flow cytometry analysis was used to evaluate the percentage of HEK293T cells transfected by EX-Z2827-M67-3′UTR.ITGα4 vector. To achieve this, the transfected and un-transfected cells were incubated with anti-ITGα4-PE (eBioscience, USA) for 45 min at 4°C. After washing the cell, the samples were read using the FACS Callibur Flow cytometer (BD bioscience, USA).

Ectopic Expression of hsa-miR-30d to Transfected HEK293T Cells

The HEK293T cells (1×10^5 cells/well) were seeded in 24-well plates and incubated at 37°C and five percent CO_2 for 24 hours. Then, the EX-Z2827-M67-3′UTR.ITGα4 plasmid was transfected into the cells using the TurboFect reagent (Fermentas, USA), following the manufacture's instruction. The cells were resuspended approximately 24 hours after transfection, and 10^5 cells/well were seeded in 12 well plates and transfected using the x-tremegene siRNA transfection reagent (sigma Aldrich, Germany). In vial A, 240 µl of scramble miScript miRNA Mimic-FITC (Qiagen, Germany), 75 µl of 10X buffer, and 600 µl of RNase-free water were mixed. Then, 360 µl of optimum medium was added to the vial in a

Table 2: Primers used for colony PCR

Name	*Sequence*	*PCR product length (bp)*
ITGα4-F	5'- TAACTCGAGGGACTTCTTTCAAATTGAGAGAATG-3'	2600
Hygromycin -R	5'- GCGTCTGCTGCTCCATAC -3'	

dark environment and incubated in RT for 5 min. In vial B, 60 µl of x-tremegene siRNA transfection reagent and 540 µl of optimum medium were mixed and incubated in RT for 5 min. Then, vial A and vial B were mixed (transfection mixture-1) and incubated in RT for 15 min. The cultured cells from 11 wells were incubated with 100 µl of transfection mixture-1 for 48 hours. In vial C, 10 µl of Syn-hsa-mir-30d-5p miScript miRNA mimic-FITC (Qiagen) and 490 µl of optimum medium were mixed and incubated in RT for 5 min. In vial D, 50 µl of x-tremegene siRNA transfection reagent and 450 µl of optimum medium were mixed and incubated in RT for 5 min. Then, vial C and vial D were mixed (transfection mixture-2) and incubated in RT for 15 min. The cultured cells from nine wells were incubated with 100 µl of transfection mixture-2 and collected 48 hours later for use in further assays. X-tremegene siRNA transfection reagent was added to eight wells of cultured cells. As negative control, 100 µl of optimum medium was added to nine wells of cultured cells and incubated for 48 hours. All the transfections were carried out in triplicates.

Transfection Analysis by Flow Cytometry

To confirm miRNA transfection, flow cytometry analysis was performed in the transfected cells using FITC labeled scrambled miRNA after 8 hours. Also, flow cytometry analysis was carried out using anti-ITGα4-PE antibody after 48 hours to evaluate the ITGα4 protein level in transfected HEK293T cells by employing miR-30d mimics or scramble miRNA.

ITGα4-3′UTR Expression Level in HEK293T Cells Transfected by miR-30d

The transcription level of 3′UTR of ITGα4 was analyzed as a putative target of miR-30d using Q-RT-PCR. RNA extraction from the HEK293T transfected cells was performed by miR-30d or scramble miRNA using the RNX-Plus kit (SinaClon, Iran). cDNA was synthesized with a first strand cDNA synthesis kit (Thermo scientific, USA) and oligodT as primer, and following the manufacturer's instructions. Q-RT-PCR analysis of ITGα4-3'UTR was performed using the Power SYBR Green PCR Master Mix kit (Applied Biosystem, USA), and monitored using the Applied Biosystems StepOneTM instrument. The PCR performance was programed with: 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min. The gene expression level was normalized by the β-actin gene, used as an endogenous control. The oligonucleotide primers used for Q-RT-PCR have been described in Table 3. All the reactions were run in triplicate and the fold changes of genes were calculated by the equation $2^{-\Delta\Delta CT.}$

MTT Assay

MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide) assay was performed to determine the viability of HEK293T cells transfected by miR-30d as compared to: 1) cells transfected by scramble miRNA, 2) cells treated with x-tremegene siRNA transfection reagent, and 3) untreated cells (negative control). MTT dye (0.5 mg/mL; Sigma Aldrich) was added to the wells 48 hours after transfection, and incubated at 37°C. The formazan crystals were dissolved by adding dimethyl sulfoxide (DMSO; 100 µL/well). After 1 hour, the optical density was measured at 570 nm using an microplate reader MRX II (Dynex Technologies, Chantilly, VA, USA).

Statistical Analysis

The results have been presented as mean ±SD. Student's t-test was used to compare data between two transfected cell groups. Individual experiments were performed in triplicate. All statistical calculations were performed using the SPSS software (version 20.0).

RESULTS

In Silico Analysis

TargetScan, DIANA-mT, and miRanda algorithms were used for the *in silico* investiga-

Table 3: Primers used for Q-RT-PCR

Name	*Sequence*	*PCR product length (bp)*
ITGα4-F	5'- TTCCAGAGCCAAATCCAAGAGTAA -3'	184
ITGα4-R	5'- AAGCCAGCCTTCCACATAACAT -3'	
β-Actin-F	TTCGAGCAAGAGATGGCCA-3'	151
β-Actin-R	5'-CACAGGACTCCATGCCCAG-3'	

tions (Table 4). Analysis of the collected data showed that several miRNAs target ITGα4, such as the miR-30 family, miR-19-a, miR-19b, miR-20a, miR-20b, miR-106a, miR-106b, and miR-515. In this study, miR-30d was selected to transfect in experiments and further analyses.

Table 4: *In silico* analysis of miRNAs which targeted *ITGα4*

DIANA MT	*Target scan*	*miRanda*
hsa-miR-30a	hsa-miR-30a	hsa-miR-30a
hsa-miR-30b	hsa-miR-30b	hsa-miR-30b
hsa-miR-30c	hsa-miR-30c	hsa-miR-30c
hsa-miR-30d	hsa-miR-30d	hsa-miR-30d
hsa-miR-30e	hsa-miR-30e	hsa-miR-17
hsa-miR-27a	hsa-miR-19a	hsa-miR-106a
hsa-miR-320a	hsa-miR-19b	hsa-miR-106b
hsa-miR-320b	hsa-miR-27a	hsa-miR-20a
hsa-miR-511	hsa-miR-27b	hsa-miR-20b
hsa-miR-515	hsa-miR-520a	hsa-miR-320a
hsa-miR-519e	hsa-miR-520b	hsa-miR-320b
hsa-miR-578	hsa-miR-520c	hsa-miR-320c
hsa-miR-641	hsa-miR-520d	hsa-miR-320d
		hsa-miR-519d

PCR Amplification of 3′UTR of *ITGα4*

To insert 3′UTR of *ITGα4* into vector, *ITGα4*.3′UTR fragment was amplified using the PCR method, and the PCR product was confirmed through agarose gel electrophoresis (Fig. 2).

Cloning Results

To confirm the insertion of 3′UTR.ITGα4 into the EX-Z2827-M67 vector, *E.coli* Top10 F′ strain colony PCR was carried out. Its analysis demonstrated that some cells had 2600 bp band (transfected cells with EX-Z2827-M67-3′UTR.ITGα4 construct), while some did not have any band in agarose gel (untransfected cells) (Fig. 3a). Also, *Xho*I digestion illustrated two different bands (869 bp and 9500 bp), which represent *ITGα4* 3′UTR and plasmid backbone, respectively (Fig. 3b). The positive clones were further verified by DNA sequencing (data not shown).

Flow Cytometry Analysis

ITGα4 expression in HEK293T cells surface was evaluated by flow cytometry analysis. Anti-ITGα4-PE was detected in 42.93 percent of 3′UTR.ITGα4 transfected cells (Fig. 4). Also, 57.61 percent of HEK293T cells were transfected by

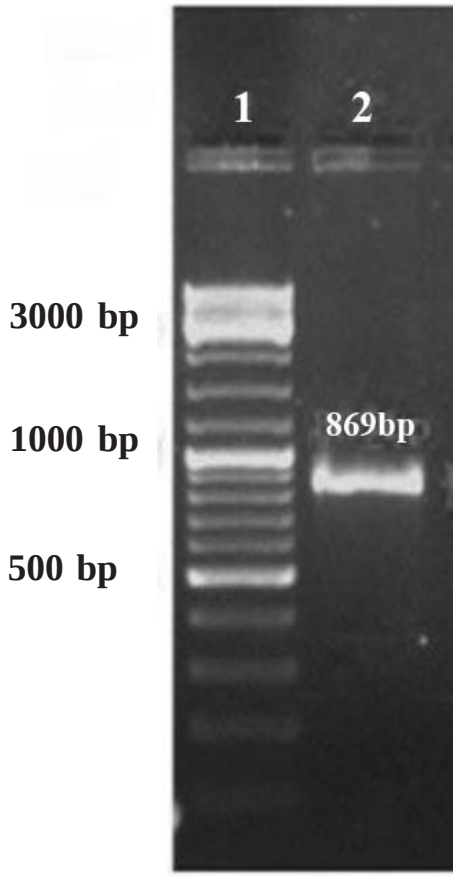

Fig. 2. PCR product of *ITGα4*-3′UTR in agarose gel (1%)-1) DNA ladder, 2) *ITGα4*-3′UTR with 869bp

FITC-conjugated scramble miRNA (Fig. 5), which confirmed the miRNA transfection. Flow cytometry analysis showed that ITGα4 expression in HEK293T cells 48 hours after transfection with miR-30d, scramble, x-tremegene siRNA transfection reagent, and control was 5.73 percent, 18.77 percent, 21.47 percent and 23.93 percent, respectively (Fig. 6a-Fig. 6d).

Cell Viability Assay

The viability of cells, before and after treatment with miR-30d mimic and scramble, was evaluated by applying the MTT assay. miR-30d did not showed any cytotoxicity effect as compared to the scramble and x-tremegene siRNA (P=0.11) groups (Table 5). There was no significant difference between the mimic and negative control groups in terms of cytotoxicity (P=0.49).

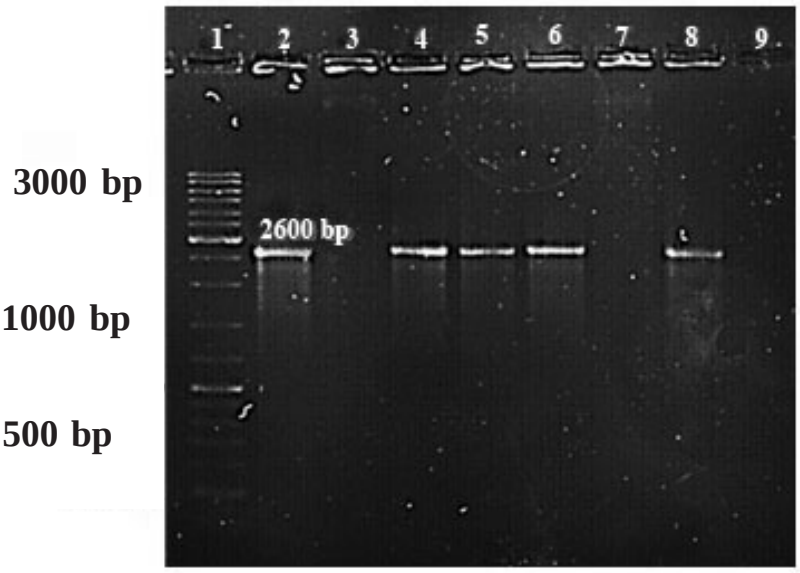

Fig. 3a. Colony PCR analysis demonstrated 2600 bp band composed of a fragment between 3′UTR.ITGα4 to hygromycin. 1) DNA ladder; 2, 4, 5, 6, and 8) transformants consisting of EX-Z2827-M67-3′UTR.ITGα4 construct; 3, 7, and 9) un-transfected cells with the construct

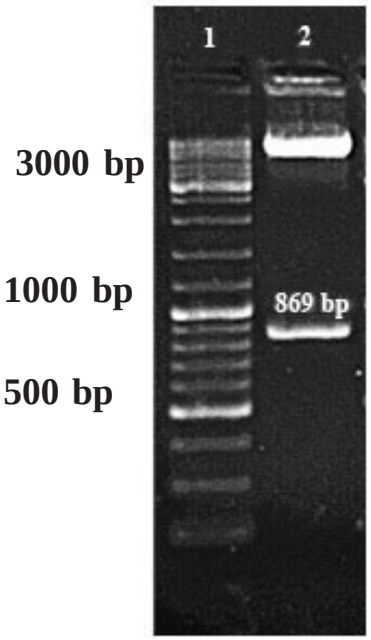

Fig. 3b. Z2827-M67-3′UTR.ITGα4 construct digested by *Xho*I. Enzyme digestion demonstrated two bands, 869 and 9500 bp, which represent *ITGα4* 3′UTR and plasmid backbone, respectively

Table 5: The viability of cells after miRNA transfection - before and after treatment with miR-30d mimic and scramble was evaluated with the help of MTT assay.

Experimental groups	*Viability*
Control	100±1.23
Scrambled	83±4.53
Xtremgene	131±3.71
miR-30d	101±4.15

Data represent mean ± SD

Quantitative Analysis of 3′UTR of *ITGα4* after Transfection

In silico analysis showed that the 3′UTR of *ITGα4* mRNA contained a binding site for the seed region of miR-30d (Fig. 7). Thus, the expression level of 3′UTR of *ITGα4* was quantitatively assessed in four cell groups. miR-30d transfected cells had a significant decrease in *ITGα4*-3′UTR expression as compared to the scramble, x-tremegene siRNA, and control groups after 48 hours (Table 6).

DISCUSSION

Integrins are a family of membrane glycoproteins that act as surface receptors in various

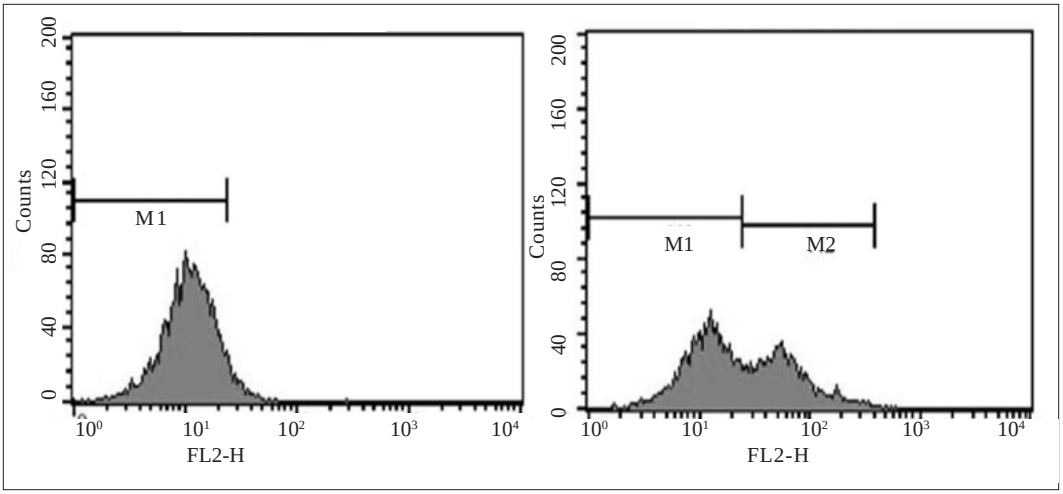

Fig. 4. ITGα4 expression in HEK293T cells. Flow cytometry analysis showed expression level of ITGα4 by anti-ITGα4-PE

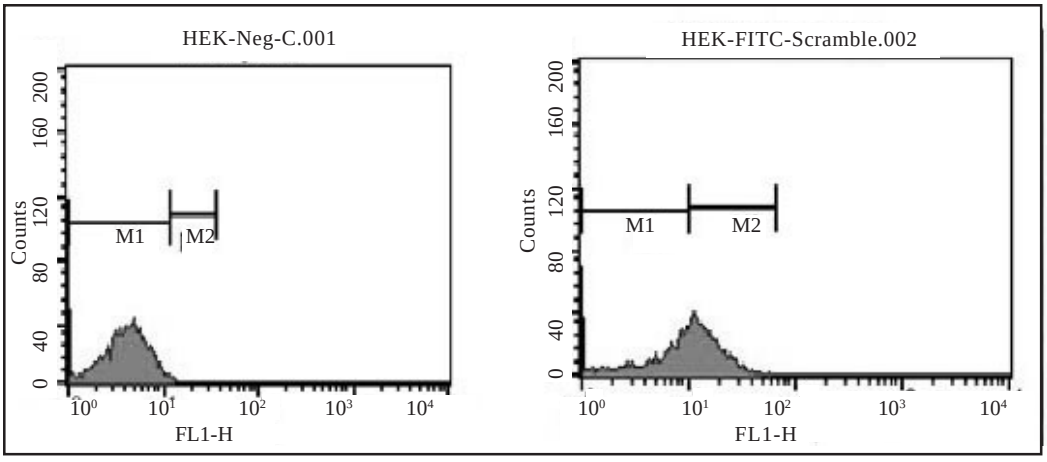

Fig. 5. Evaluation of miRNA transfection by flow cytometry - FITC- conjugated scramble miRNA was used to evaluate transfection percentage in the HEK293T cells

Table 6: Relative expression 3′UTR of *ITGα4* in four cell groups (transfected by miR-30d, scramble miRNA, x-tremegene reagent and un-transfected cells). Relative expression of 3′UTR of *ITGα4* was normalized to β-actin as an endogenous control.

Experimental groups	*ITGα4*
Control	1± 0.041
Scrambled	0.94± 0.048
Xtremgene	0.9± 0.044
miR-30d	0.86± 0.042

Data represent mean ± SD

cell types and regulate cell cell and cell extracellular matrix interactions, inflammation, tumor vascularization, and metastasis (Huttenlocher and Horwitz 2011). The key role of α4β1-integrin, specifically in the endothelial transmigration for lymphocytes into the CNS, has provided an important insight into the pathogenesis of human demyelinating disease. Thus, α4β1-integrin can be considered as a target for MS therapy. Natalizumab (NTZ) is a humanized monoclonal antibody against α4-integrin used for the treatment of MS patients (Ferret-Sena et al. 2016). It

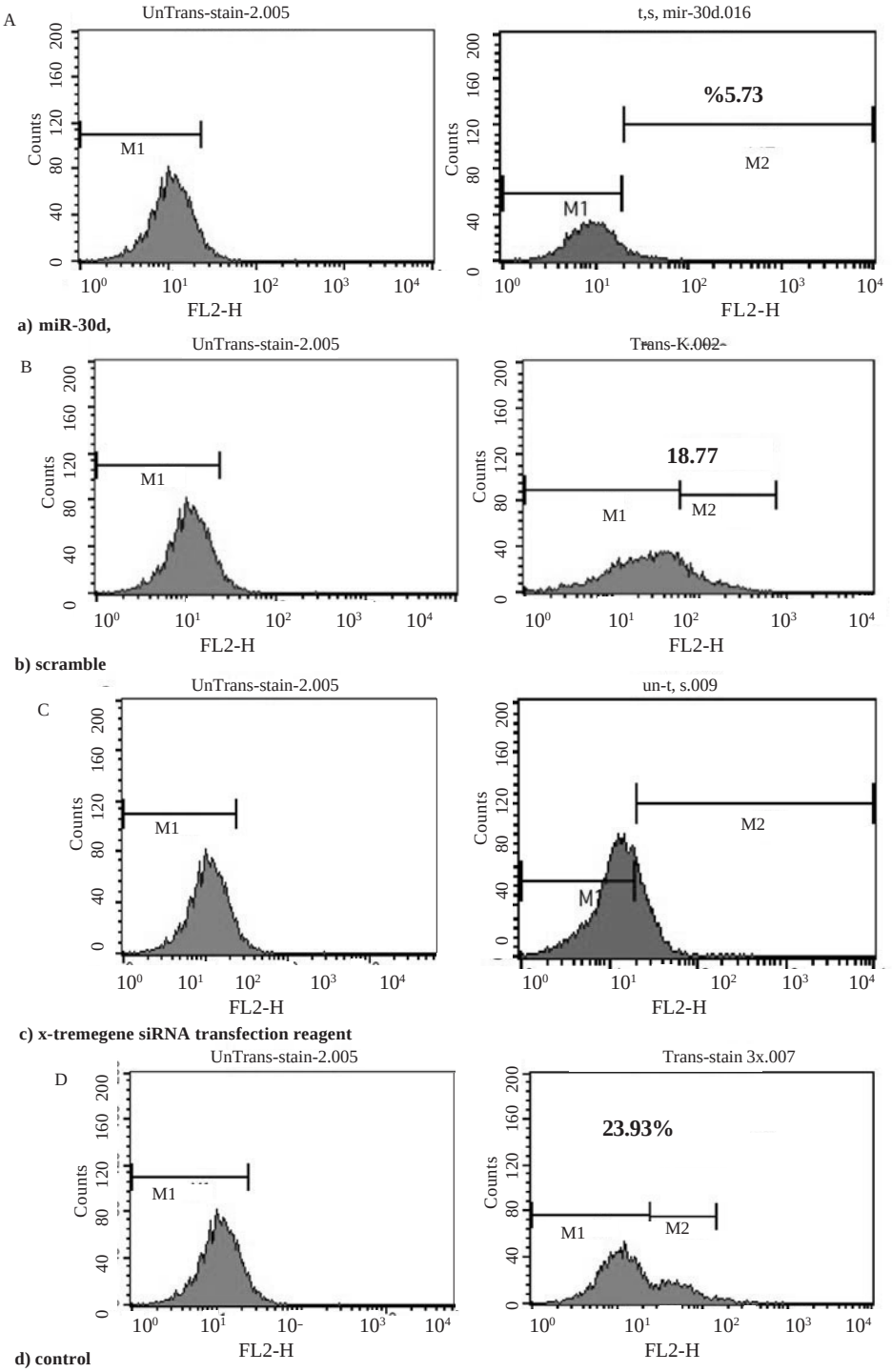

Fig. 6. *ITGα4* expression in HEK293T cells transfected by miR-30d after 48h with a) miR-30d, b) scramble, c) x-tremegene siRNA transfection reagent, and d) control

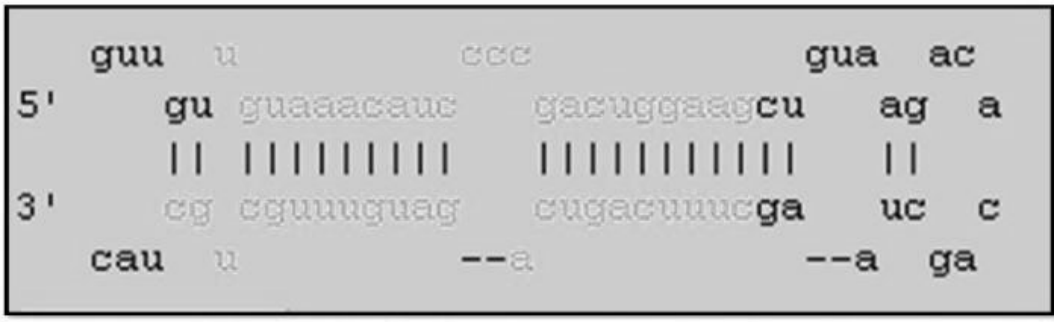

Fig. 7. Diagram of miR-30d binding site in ITGα4-3´UTR

is, however, associated with adverse effects (Bargiela et al. 2017). Researchers are trying to design new drugs for the inhibition of α4-integrin due to the importance of α4β1-integrin blocking in the treatment of relapsing forms of MS.

miRNAs are an important group of negative regulators in different cells that can change the expression of target genes promptly and play an important role in cell fate. Hence, they control some aspects of immune responses and their expression changes during T cell function (Ranji et al. 2014). Decrease of miR30 family members (miR-30a/b/c/d/e) has been observed in several human cancers (Braun and Huttelmaier 2011; Cheng et al. 2012). miR-30d expression reduced notably in some cancers, such as thyroid carcinoma (Visone et al. 2016), prostate cancer et al. 2015), and renal cell carcinoma (Yu et al. 2014). In a study, miR-30c levels were found to be lower in MS patients as compared to healthy controls (Jernas et al. 2013). The *in silico* analysis of this study predicted *ITGα4* as a putative target of the miR-30 family. Thus, the miR-30 family members were transfected (separately in several projects) to the HEK293T cells to evaluate its effect on *ITGα4* expression (data not shown). The expression of *ITGα4* is associated with high cell movement, and increased invasion and migration rates of the cells (Young et al. 2015). Focal inflammatory lesions are a cause of relapsing forms of MS. These lesions consist of activated macrophages, and $CD4^+$T and $CD8^+$ T cells in CNS (Bornsen et al. 2012). The adhesion between endothelial (VCAM-1) and T cells (VLA-4) has been known to be one of the earliest events in MS development, which leads to T-cell transmigration into the CNS across the blood-brain-barrier (Damotte et al. 2014). VLA-4 (very late antigen-4) molecule is composed of ITGβ1 and ITGα4 (integrin subunits) expressed on the surface of most immune cells (Bornsen et al. 2012). In this study, it has been demonstrated that ectopic expression of miR-30d is associated with the decrease of *ITGα4* in mRNA level for HEK293T cells. It seems that miR-30d can act as a negative regulator of *ITGα4* in $CD4^+$T cells.

Despite the risks, such as PML, mortality, and morbidity, Natalizumab therapy is necessary in relapsing forms of MS (Bargiela et al. 2017) applied new therapeutic strategies in second line treatment of these patients. MiRNAs are recently being used as important regulators of genes involved in immune diseases (Ranji et al. 2015) and can be suitable targets of therapy. For example, anti-miR-326 and anti-miR-155 injections reduced the severity of experimental autoimmune encephalomyelitis (EAE). Also, a milder form of disease developed in EAE mice, which were genetically modified through the overexpression of miR-23b (Jr Ode et al. 2012). MiRNA therapy can overcome potential adverse effects of routine therapeutic agents and could be a promising therapeutic candidate for different disorders, such as MS. The results of this study suggests that miR-30d can target *ITGα4* gene in the $CD4^+$T cells of MS patients and inhibit T-cell transmigration into CNS, thus decreasing the formation of lesions in the future.

CONCLUSION

In this study, the expression of *ITGα4* gene as a potential target of miR-30d in HEK293T cells was evaluated. The results revealed that ectopic overexpression of miR-30d decreased the expression of *ITGα4*. It seems that miR-30d can be

a negative regulator of *ITGα4* in CD4⁺T cells and a potential target for MS therapy.

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