

## The silencing effect of miR-30a on *ITGA4* gene expression *in vitro*: an approach for gene therapy

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### Abstract

Integrins are adhesion molecules which play crucial roles in cell-cell and cell-extracellular matrix interactions. Very late antigen-4 or  $\alpha 4\beta 1$  and lymphocyte Peyer's patch adhesion molecule-1 or  $\alpha 4\beta 7$ , are key factors in the invasion of tumor cells and metastasis. Based on the previous reports, integrin  $\alpha 4$  (*ITGA4*) is overexpressed in some immune disorders and cancers. Thus, inhibition of *ITGA4* could be a therapeutic strategy. In the present study, miR-30a was selected in order to suppress *ITGA4* expression. The *ITGA4* 3' UTR was amplified, cloned in the Z2827-M67-(*ITGA4*) plasmid and named as Z2827-M67/3'UTR. HeLa cells were divided into five groups; (1) untreated without any transfection, (2) mock with Z2827-M67/3'UTR transfection and X-tremeGENE reagent, (3) negative control with Z2827-M67/3'UTR transfection alone, (4) test with miR-30a mimic and Z2827-M67/3'UTR transfection and (5) scramble with miR-30a scramble and Z2827-M67/3'UTR transfection. The MTT assay was performed to evaluate cell survival and cytotoxicity in each group. Real-time RT-PCR was applied for the *ITGA4* expression analysis. The findings of this study showed that miR-30a downregulated *ITGA4* expression and had no effect on the cell survival. Due to the silencing effect of miR-30a on the *ITGA4* gene expression, this agent could be considered as a potential tool for cancer and immune disorders therapy.

**Keywords:** Cancer; *ITGA4*; Metastasis; miR-30a; Non-coding RNA

### INTRODUCTION

Integrins are members of type I transmembrane glycoproteins which are heterodimeric molecules with two non-covalently bound subunits including  $\alpha$  and  $\beta$ . To date, 24 different integrins have been identified based on various combinations of 18  $\alpha$  and 8  $\beta$  subunit types (1, 2). These adhesion molecules play crucial roles in cell-cell and cell-extracellular matrix (ECM) interaction, signaling pathways, inflammation,

neovascularization, invasion and metastasis (3-6). Among integrins, very late antigen-4 (VLA-4) or  $\alpha 4\beta 1$  and lymphocyte Peyer's patch adhesion molecule-1 (LPAM-1) or  $\alpha 4\beta 7$  are considered critical factors in the invasion of tumor cells and metastasis (1, 7). Overexpression of integrin  $\alpha 4$  (*ITGA4*) has been reported in several cancers which mediates migration of cancerous cells (8, 9).

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This process is facilitated through binding of VLA-4 and LPAM-1 to vascular cell adhesion protein-1 (VCAM-1) and mucosal vascular address in cell adhesion molecule-1 (MAdCAM-1) on endothelium of cancerous tissue, respectively, or to fibronectin in the extracellular matrix (1, 4, 10, 11). Moreover, VLA-4 shows function in neovascularization and blood vessel formation during malignancy progression (12, 13).

Based on the structural joining role of  $\alpha 4$  in both VLA-4 and LPAM-1, suppression of  $\alpha 4$  expression level and blockade of  $\alpha 4$ -ligand complex can be considered a potential therapeutic approach (14, 15). Several biological agents, including small molecules (15), monoclonal antibodies (14, 16), siRNAs and microRNAs (miRNA) (17) have been developed for the treatment of  $\alpha 4$  integrin-related disorders.

MicroRNA as a class of small non-coding RNA, containing 18-23 nucleotides, binds to the complementary sequence of the 3' untranslated region (3'UTR) of mRNAs and modulates gene expression at the post-transcriptional level (18, 19). Translation inhibition and mRNA degradation are two mechanisms that miRNA employs to regulate gene expression. Complete binding of miRNA seed to 3'UTR leads to target mRNA degradation, and the incomplete attachment results in translation inhibition (18, 20). The essential role of miRNA has been reported in several cell processes including differentiation, proliferation, development, migration, signaling pathways and cell cycle (21). miRNAs can act as oncogenes or tumor suppressors involved in stimulation or prevention of cancer, respectively, and can also trigger tumor cells invasion and metastasis due to induction of migratory process. Therefore, dysregulation of miRNA expression contributes to cancer initiation and progression (18).

To date, the function of miR-25, 139, 143 and 34 have been validated in the invasion and metastasis of esophageal squamous cell carcinoma (22), colorectal cancer (CRC) (23, 24), osteosarcoma (25), and liver cancer (26), respectively. Among the miRNAs, miR-30 family regulates multiple targets acting in

tumor invasion and cell migration in different malignancies such as breast cancer (27), hepatocellular carcinoma (HCC) (28, 29) and CRC (30). miR-30a, as a member of this family, has been validated to be remarkably downregulated in invasive CRC (30), breast cancer (31), melanoma (32), neuroblastoma (NB) (33) and HCC (29). Overexpression of miR-30a in these cancers leads to less degrees of invasion and metastasis.

Based on *ITGA4* role in metastatic progression, it could be considered as a potential target. Due to its significantly different expression in tumor vs non-tumor cells (33) and miRNA target prediction *in silico* (34, 35), miR-30a was selected as a therapeutic tool against *ITGA4*. Therefore, this *in vitro* study was launched to investigate the inhibitory effect of miR-30a on *ITGA4* expression level.

## MATERIALS AND METHODS

### PCR amplification

The sequence of *ITGA4* 3'UTR was amplified using *pfu* enzyme (mXwell, Iran) and by forward (5'-TAACGAGGGACTTCTTTCAAATTGAGA GAATG-3') and reverse (5'-TCTCGAGAATCAGCGTGTATCAGGTAAGTG-3') specific primers containing *XhoI* restriction site in 5' flanks (GeneCust, Luxembourg). The PCR program included a primary denaturation at 95 °C for 5 min followed by 30 cycles of 94 °C for 30s, 65 °C for 30 s, 72 °C for 2 min, with a final extension step at 72 °C for 10 min.

### Plasmid preparation

The human *ITGA4* expression vector, Z2827-M67-(*ITGA4*), (GeneCopoeia, USA) was transformed into the host *Escherichia coli* (*E. coli*) strain TOP10F' in Luria-Bertani broth (LB) (Merck, Germany) containing 100 µg/mL ampicillin. The plasmid was extracted using a miniprep extraction kit (SolGent, Korea).

The *ITGA4* 3'UTR fragment and the *ITGA4* expression vector were digested by *XhoI* restriction enzyme (Fermentas, USA) at 37 °C for overnight, and the linear plasmid was treated with calf intestinal alkaline

phosphatase enzyme (Fermentas, USA) and ligated by T4 DNA ligase (Fermentas, USA). The ligation product (Z2827-M67/3'UTR) was transformed into competent *E. coli* TOP10F' using the chemical CaCl<sub>2</sub> method (36). The *E. coli* transformants were cultured on LB agar (Merck, Germany) containing 100 µg/mL ampicillin and incubated at 37 °C for overnight. Colony PCR was performed using *ITGA4* 3'UTR forward primer and hygro reverse primer (5' GCGTCTGCTGCTCCATAC 3') to accomplish selection direction assay and to verify the presence of the Z2827-M67/3'UTR plasmid.

The positive clones were further evaluated by *Xho*I digestion and nucleotide sequence analysis (Bioneer, Korea).

For plasmid extraction, the positive clone containing the Z2827-M67/3'UTR plasmid was cultured in LB broth with 100 µg/mL ampicillin, incubated at 37 °C and 250 rpm for overnight.

### **Cell culture**

The HeLa cell line was purchased from the National Cell Bank of Pasteur Institute, Tehran, Iran. The cells were cultured in Dulbecco's modified Eagle's medium-high glucose (DMEM), supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin, and incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. All cell culture reagents were purchased from Gibco-life technologies, USA.

### **Study plan**

HeLa cells were transfected by Z2827-M67/3'UTR at the primary step and divided into four groups according to second transfection step. The first group, without the second transfection, was considered negative control. The second group was transfected with 12.5 µL of miR-30a mimic with 20 µM concentration (Qiagen, Germany) (test group). The third set was transfected with miR-30a scramble in equal amount with miR-30a mimic (scramble group). The scramble is conjugated with FITC to measure the transfection rate. In the fourth group, cells were transfected by transfection reagent only (mock group). In addition, a set of HeLa cells was utilized as

untreated group for technique calibration without any primary or secondary transfection (untreated group).

### **Plasmid/miRNA transfection**

A day before the primary transfection,  $1.5 \times 10^6$  cells were seeded in the exponential growth phase to 70-80% confluency on flask T25 cm<sup>2</sup> (SPL, Korea). The cells were transfected by the Z2827-M67/3'UTR plasmid, using TurboFect reagent (Fermentas, USA) according to manufacture's instruction. About 24 h after Z2827-M67/3'UTR transfection, the cells were resuspended and  $10^5$  cells were seeded in each well of the 12-well plates. miR-30a mimic and scramble delivery was performed using 5 µL/well X-tremeGENE reagent (Fermentas, USA) in the test and scramble groups, respectively. The final concentration of miR-30a mimic and the scramble in culture media was 50 nM. In the mock group, cells were transfected by the same amount of X-tremeGENE reagent. MTT and Real-time PCR analyses were performed in triplicate.

### **Transfection rate**

For evaluation of second transfection rate, fluorescent intensity of fluorescein isothiocyanate (FITC)-conjugated scrambled transfected cells were determined by FACSCallibur flow cytometer (BD bioscience, USA).

### **The MTT assay**

Cell survival assay was done by 3-[4, 5 dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) reagent (Sigma, USA), to evaluate the cytotoxic effect of miR-30a mimic, scramble and X-tremeGENE reagent. About 48 h after the second transfection, MTT assay was performed with 40 µL MTT (5 mg/mL) per well, and the treated cells were incubated at 37 °C for 4 h in triplicate for each group.

The medium was replaced with 400 µL dimethyl sulfoxide (DMSO) for solubilizing formazan crystals. The absorbance of soluble crystal was determined at 570 nm (A570) using a microplate reader MRX II (Dynex Technologies, USA). Percentage of cell

survival was calculated using the following equation:

% Cell survival =

$$\frac{\text{Absorbance}_{\text{Mean}}(\text{treated cells}) - \text{Absorbance}_{\text{Mean}}(\text{blank})}{\text{Absorbance}_{\text{Mean}}(\text{control}) - \text{Absorbance}_{\text{Mean}}(\text{blank})} \times 100$$

### Relative quantitation of *ITGA4* transcript

Total RNA of  $10^6$  cells in each group was extracted using RNX plus kit (SinaClon, Iran) and stored at  $-80^\circ\text{C}$ . The RNA concentration was assessed by NanoDrop (Goldenlab, Indonesia) at 260 nm, and then, cDNA was synthesized according to the first strand cDNA synthesis kit instructions (Thermo Scientific, USA). *ITGA4* mRNA was quantified by SYBR Green-based real time PCR (Applied Biosystem, USA) using forward (5'-TTCCAGAGCCAAATCCAAGAGTAA-3') and reverse (5'-AAGCCAGCCTTCCACATAACAT-3') primers. *Beta actin* (F: 5'-TTCGAGCAAGAGATGGCCAT-3', R: 5'-CACAGGACTCCATGCCAG-3') was applied as the endogenous control for gene expression analysis.

### Statistical analysis

Data were analyzed using SPSS (version 20) software. The cell survival percentage and relative quantitative are shown as mean  $\pm$  SD, and were compared using independent t-test. Using MS Office Excel 2010 (Microsoft, USA), graphs were drawn.

## RESULTS

### PCR amplification

The designed primers for the *ITGA4* 3'UTR fragment amplified an 888 bp band which was observed in agarose gel electrophoresis (Fig. 1).

### Plasmid preparation

Colony PCR results showed a 2600 bp band in some transformants which confirmed the construction of the Z2827-M67/3'UTR plasmid in the correct direction (Fig. 2A).

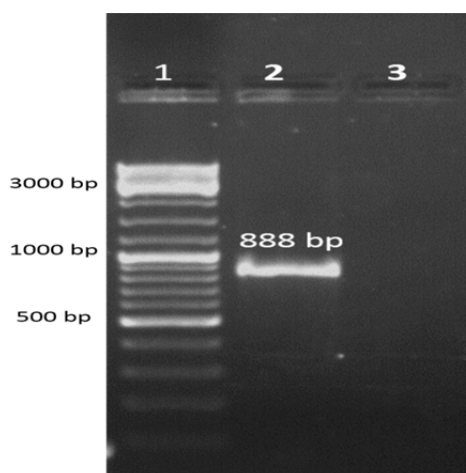
The result of *XhoI* digestion illustrated two different bands, about 880 bp and 9500 bp, which are representative of *ITGA4* 3'UTR and plasmid backbone, respectively (Fig. 2B). The positive clones were further verified by DNA sequencing (data not shown).

### Transfection rate

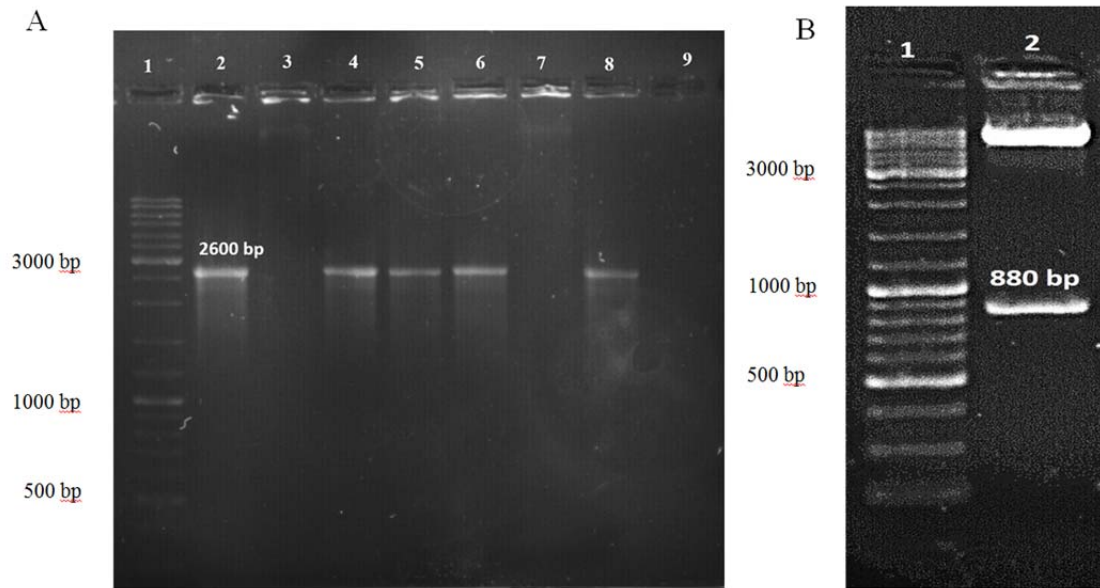
Flow cytometry plots illustrated that 80% of cells were transfected successfully (Fig. 3).

### MTT assay

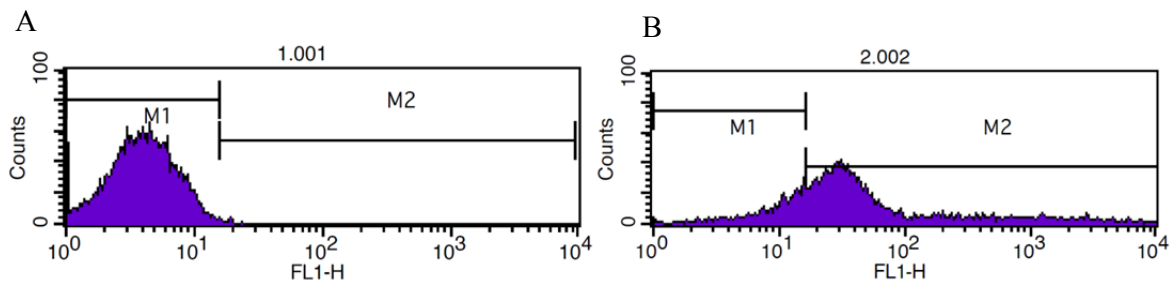
MiR-30a showed no inhibitory effect on cell proliferation when compared to scramble and negative control ( $p = 0.064$ ) groups. Cytotoxicity assay by MTT also illustrated no significant difference between the mock and negative control groups ( $p = 0.057$ ). The XtremeGene reagent did not show any toxicity effect on the cells.



**Fig. 1.** Amplification of the *ITGA4* 3'UTR. Lane 1, DNA ladder mix; lane 2, the 888 bp amplicon representing *ITGA4* 3'UTR; lane 3m negative control.



**Fig. 2.** Verification of the presence of Z2827-M67/3'UTR plasmid. (A) Colony PCR. Lane 1, DNA ladder mix; lanes 2, 4, 5, 6 and 8, a 2600 bp band is related to the presence of the 3'UTR fragment in the correct orientation; lane 9, negative control. (B) Digestion of the plasmid with *Xho*I. Lane 1, DNA ladder mix; Lane 2, the digestion products including the 880 bp and 9500 bp bands are related to the *ITGA4* 3'UTR fragment and backbone plasmid, respectively.



**Fig. 3.** Secondary transfection rate. Flow cytometry plot of cells transfected by FITC-conjugated scramble. (A) The untreated HeLa cells were considered as negative control. (B) Flow cytometry plot showed about 80% of cells were tr

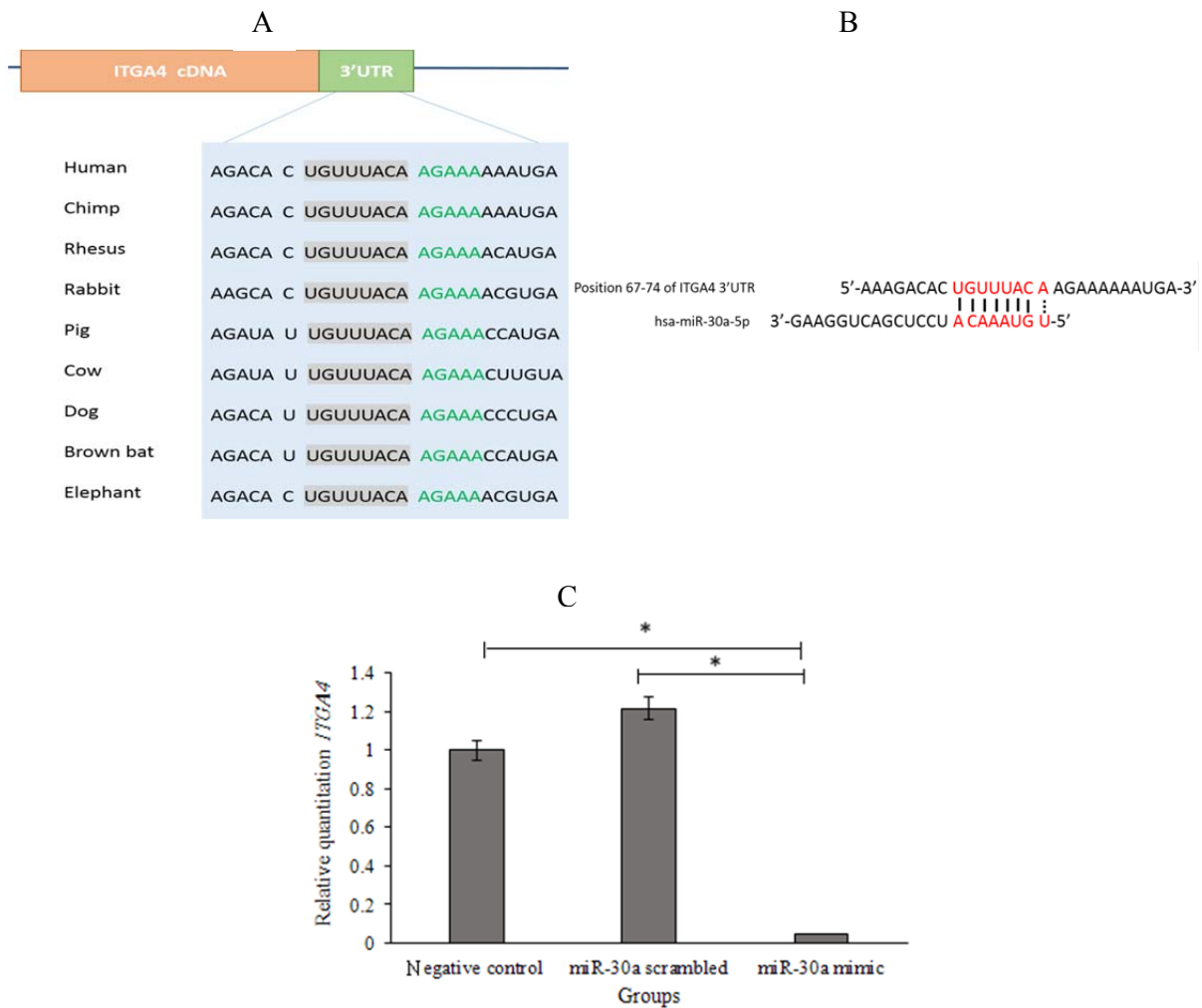
### Relative quantitation of *ITGA4* transcript

The 3'UTR of *ITGA4* mRNA contains a binding site for the seed region of miR-30a in different species (Fig. 4 A and B) and the binding site is conserved in mammals and this implies the importance of the binding site sequence in the regulation of *ITGA4* expression. Quantitation of *ITGA4* expression, 48 h after miR-30a mimic transfection, showed *ITGA4* mRNA level reduction in HeLa cells compared to the negative control group ( $P < 0.001$ ). miR-30a could regulate *ITGA4* expression directly in this experiment. There was no significant difference in *ITGA4* mRNA between the scramble and negative control groups (Fig. 4C) using the  $2^{-\Delta\Delta CT}$  quantitation method.

### In silico study

To explore miRNAs regulating *ITGA4* expression, TargetScan, as a miRNA target prediction tools was used. Analyses revealed that *ITGA4* is a potential target of miR-30 family especially miR-30a.

The TargetScan algorithm is based on the conserved region of miRNA binding site on the 3'UTR of genes in different species. The results were checked in the miRWalk database. miRWalk and a collection of other tools including TargetScan, miRanda, PICTAR5, miRWalk and DIANAmt emphasized that the *ITGA4* 3'UTR has the binding site for miR-30a.



**Fig. 4.** miR-30a as an *ITGA4*-targeting miRNA. (A) Sequence of the 3'UTR of *ITGA4* mRNA showing the predicted seed region for the binding of miR-30a in different vertebrate species. (B) The predicted position of the *ITGA4* 3'UTR that is targeted by miR-30a is 67-74 nucleotides. (C) Relative quantification of the *ITGA4* transcript. The expression of *ITGA4* was compared between HeLa cells treated with miR-30a mimic and Z2827-M67/3'UTR (test group) and HeLa cells treated with Z2827-M67/3'UTR and miR-30a scramble in comparison to HeLa cells treated with Z2827-M67/3'UTR (negative control) by quantification comparative Ct Real time PCR. All transcript levels were normalized against human beta actin transcript levels. The test group significantly decreased *ITGA4* transcript vs. negative control and scramble groups (\* $P < 0.001$ ). There is no significant difference between negative control and scramble groups. All results are means  $\pm$  SD from three independent experiments. Statistical significance was calculated using the independent student's *t*-test.

## DISCUSSION

*ITGA4* has been known as a signaling molecule involved in movement of tumor cells whose overexpression in metastatic cancerous cells has been confirmed by several methods (4, 8, 9, 11).

In various studies, upregulation of *ITGA4* has been reported in different malignancies e.g. breast cancer (37), melanoma (9) and neuroblastoma (8), and immune disorders like multiple sclerosis (MS) and Crohn's disease (38). Downregulation of *ITGA4* and its

ligands, or inhibition of *ITGA4*-ligand complex formation, are regarded as potential therapeutic approaches (7). Biological tools such as antibodies, aptamers (39), small molecules and miRNAs could be used to achieve this purpose.

As regard to the overexpression of integrin  $\alpha 4\beta 1$  on  $CD4^+$  T cells in multiple sclerosis patients, some studies have shown that natalizumab, a monoclonal antibody (mAb) against integrin  $\alpha 4$ , reduces or stops plaque formation in the central nervous system (CNS) (14). Also, similar effect of this mAb has been

reported in multiple myeloma in order to reduce the symptoms (40).

R411 (41) and CDP323 (42) are small molecules against integrin  $\alpha 4\beta 1$ , whose mechanism of action are exactly the same as natalizumab in multiple sclerosis. Also, AJM300 as a new small molecule has been tested in Crohn's disease which was able to block binding of integrin  $\alpha 4\beta 7$  to mucosal vascular address in cell adhesion molecule-1 in intestine epithelial cells (43).

Another considerable inhibitory tool against the formation of  $\alpha 4$ -ligand complex is miRNA. Based on miRNA expression profile, the miR-30 family has been downregulated in different types of metastatic cancers (27-31). Among this family, downregulating effect of miR-30c on *ITGA4* expression has been validated (17). Other studies have illustrated that miR-30a enhances invasion and migration through direct binding and downregulating Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta (*PIK3CD*) (a member of Rho GTPase signaling) in colorectal cancer (CRC) (30). Also, reduced expression of miR-30a has been reported in metastatic-related miRNA in hepatocellular carcinoma (HCC) (29). Complementary investigations have shown that miR-30a knockdown triggers tumor invasion and metastasis (27-30, 44).

In this study, the suppressive effect of hsa-miR-30a on *ITGA4* expression was assessed. We observed that miR-30a decreased *ITGA4* at mRNA level. It seems that miR-30a causes degradation of *ITGA4* mRNA by complete attachment to 3'UTR region. Binding of the seed of miRNA to their targets via 8mer and 7mer-m8 leads to mRNA degradation (18, 34). Hence, depletion of *ITGA4* expression by miR-30a mimic may be done via 8mer or 7mer-m8 attachments.

So far, the effect of miR-30 family on *ITGA4* expression has been only assessed by Zhang, et al. They found that miR-30c had inhibitory effect on this gene which is in line with our findings (17). They reported these results based on microarray analysis, while in the present study, direct suppression effect of miR-30a on *ITGA4* expression was assessed using the quantitative Real-time PCR.

Although the luciferase assay has been routinely performed for miRNA target validation (34, 35, 45), cDNA of the target gene was utilized in our study. Moreover, in our study, 3'UTR complete sequence of the target gene was inserted just adjacent to the gene of interest in the construct. Such a specific construct leads to an mRNA secondary structure which mimics natural *ITGA4* transcript.

## CONCLUSION

Due to *ITGA4* roles in the invasion and cancerous cell migration, downregulation of the *ITGA4* gene by miRNAs could be a potential therapeutic approach. Based on this *in silico* study, we found that miR-30a could be considered a silencing tool to reduce *ITGA4* expression. This investigation also revealed the suppressive effect of miR-30a on *ITGA4* expression.

Confirmation of the present findings by migration assay and animal models makes miR-30a applicable for cancer gene therapy in the future.

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