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Negative regulation of TIM-3 expression in AML cell line (HL-60) using miR-330-5p

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ABSTRACT

Background: Uncontrolled proliferation and accumulation of leukaemic stem cells (LSCs) in bone marrow leads to acute myeloma leukaemia (AML). T cell immunoglobulin and mucine domain (TIM)-3 is a specific surface marker for LSCs and is highly expressed on LSCs compared with normal bone marrow cells, haematopoietic stem cells. Studies have indicated that microRNAs can affect AML progression through targeting different genes expressions like TIM-3. So, based on bioinformatics assessments, we predicted that miR-330-5p may highly inhibit TIM-3 expression.

The purpose of the present study was to prove the silencing effect of miR-330-5p on TIM-3 gene expression in AML cell line (HL-60) *in vitro*.

Methods: HL-60 cells were cultured in RPMI 1640 supplied with 10% FBS. TIM-3 expression was induced in the cells using phorbol myristate acetate (PMA). The cells were transfected with miR-330-5p and then, the gene and protein expression of TIM-3 were measured using q-RT-PCR and flow-cytometry methods, respectively.

Results: The results of our bioinformatics surveys revealed that miR-330-5p has high predicted ability to silence TIM-3 gene expression. Accordingly, our experiments confirmed that miR-330-5p is able to strongly silence TIM-3 expression (98.15% silencing) in HL-60 cell line ($p = 0.0001$).

Conclusion: According to our results, miR-330-5p has a strong inhibitory effect on TIM-3 expression in AML cell line. Thus, the bioinformatics prediction of Mirwalk and Target Scan softwares for silencing effect of miR-330-5p on TIM-3 is confirmed.

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AML; HL-60; miR-330-5p; molecular medicine; TIM-3

Introduction

Acute myeloma leukaemia (AML) is a heterogenic disease in which the myeloid cell lineage fails to complete its differentiation. This leads to an uncontrolled proliferation of low differentiated cells of this lineage such as myeloid blasts, and accumulation of them in bone marrow. [1] AML is a kind of non-lymphoid leukaemia with a rapid clinical development which mostly leads to death.

TIM-3 is a surface glycoprotein of type I which was firstly discovered as a cell marker for T helper1 (Th1) cells. [2,3] This protein acts as a negative regulator or inhibitor for Th1 cells function.[4] TIM-3 difference with other immune controller molecules is that TIM-3's expression is enhanced on T cells which are differentiated to interferon-γ (IFNγ) producing T helper and T killer 1 cells but not in all activated T cells.[2,3] Accordingly, it is demonstrated that employing anti TIM-3 antibody causes Th1-associated acute autoimmune diseases.[4]

S-type galectin-9 (Gal-9) is known as a ligand for TIM-3 protein. Gal-9 is highly expressed in immune system tissues like bone marrow, lymph nodes, thymus and spleen. According to this liquid molecule, Gal-9 has a high expression in the presence of IFNγ and binds to oligosaccharides on IgV domain of TIM-3 protein. Such a binding in Th1 cells induces apoptosis. Therefore, TIM-3 is considered as a regulator for removing immunological responses generated by Th1 and T cytotoxic 1 (Tc1). [5]

In human cancers, there are some reports of TIM-3 expression on damaged T cells and hence, it could be considered as an index for antitumour responses in various levels.[6,7] Therefore, investigations on TIM-3 in inflammatory diseases and cancers for therapeutic and diagnostic aims are of high importance.

It is reported that TIM-3 is expressed in all AML cytogenetic subgroups. Differential expression of TIM-3 on leukaemic stem cells (LSCs) in AML led to successful separation of haematopoietic stem cells (HSCs) from leukaemic cells in many types of AML. Therefore, TIM-3 is now considered as a potential candidate for AML

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treatment through monoclonal antibodies or other specific inhibitory tools like micro RNAs.[6,7]

MicroRNAs (miRNAs) are noncoding ribonucleic acids which include 18–25 nucleotides in length.[8] They control gene expression after transcription through blockade of protein synthesis by steric hindrance or degeneration of target mRNAs. These molecules control parts of physiologic and pathologic cell processes of which many act as oncogenes or tumour suppressors.[9]

Interaction between micro RNAs and their target genes reflects their role in growth, apoptosis, differentiation and cell proliferation. Such interaction also magnifies their direct function in developing cancers. In addition, many of them are expressed unnaturally in cancer cells. For instance, in breast, lung, colorectal and colon cancer, different micro RNAs show a higher or lower expression than usual.[10–12] Of course, recognising target genes by micro RNAs is of high importance. Their specificity is laid in 'seed' region which includes nucleotides 2–9 from 5` end of micro RNA.[12,13]

The HL-60 cell line, derived from a patient with acute promyelocytic leukaemia, was first classified as FAB-M3 and then as FAB-M2, with an immunophenotype corresponding to immature myeloid cells.[14] FAB-M2 is acute myeloblastic leukaemia with maturation. Thus, it seems that HL-60 could be a suitable cell line for study on AML.

Based on our bioinformatics predictions and differential expression of TIM-3 on LSCs in AML, we hypothesised that miR-330-5p is able to inhibit TIM-3 expression on TIM-3 expressing AML cells. Suppression of TIM-3 could reduce malignant cells proliferation and help to antitumour immunity. This study might help in developing a new micro-RNA-based treatment for future.

Materials and methods

Bioinformatics prediction

First, Mirwalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) was used for bioinformatics prediction of miRNA-TIM-3 interaction. In the 'predicted targets module', we selected 'Gene-miRNA target' tab, and then put the TIM3 RefSeq ID (NM_032782.4) in the relevant box. Input parameters were adjusted on finding 3'-UTR and all databases were selected. At the next step, Target Scan 7.0 (http://www.targetscan.org/) was applied to confirm bioinformatics prediction of miRNA-TIM-3 interaction predicted by Mirwalk. We put the formal gene symbol of TIM-3 (HAVCR2) in the relevant box and then, submitted the query.

Cell culture

In this study, HL-60 cell line (Pasture Inst., Iran) as an AML cell line was cultured in RPMI-1640 supplied with 10% foetal buvin serum (FBS) and 1% Pen-Strep (PSF1) (Farabi Inst., Iran).

Transfection with miRNA-330-5p mimic

About 24 h before transfection, in each well of 24-well plate, 1–2 × 10^5 HL-60 cells were cultured in 450 µl serum free medium. The cells were treated with 50 ng RMA (Sigma Aldrich, Germany) in order to induce TIM-3 expression. Then, the cells were transfected with hsa-miRNA-330-5p mimic (Qiagene, Germany) with final concentration of 50 nM, using X-tremegene transfection reagent (Roche, Germany). LableIT siRNA delivery control labelled with FITC (Mirus, USA) was applied as both transfection indicator and scramble siRNA as it does not react with any known mammalian mRNA. Cells treated with only X-tremegene transfection reagent and cells without treatment were used as mock and negative control, respectively. After 4 h, 10% FBS and 1% penicillin/streptomycin was added to each well. Cells were incubated overnight. Then, the cell culture medium (except for transfected polyplexes) was refreshed.

Cell viability test

Metabolic activities of HL-60 cells were assessed through methylthiazole terazolium (MTT) assay. MTT assay was done in brief as follows: 10 µl of a 5 mg/ml MTT solution in PBS buffer (Sigma-Aldrich, Germany) was added to each well of 96-well plate (Orang, Belgium) containing 90 µl medium. After 1 h of incubation at 37 °C and 5% CO2, the medium was emptied and wells were covered. Then, the cells were frozen for 1 h at –80 °C (New Brunswick scientific, USA). Next, the purple formazan product was dissolved in 100 µl/well dimethyl sulfoxide (DMSO) (Sigma–Aldrich, Germany) for 30 min while shaking. Optical density was quantified by a microplate reader (M680 Bio rad, USA) at 590 nm (reference wavelength 630 nm) and viability of the cells was reported as a percentage compared with untransfected (negative) control cells.

RNA extraction, cDNA synthesis and quantitative RT-PCR)

Total RNA was extracted from HL-60 cell line through RNX Plus kit (Cinnagen, Iran). Then, cDNA was synthesised using Revert Identify Transcription Kit (Fermentase, Germany) as instructed by the manufacturer. Afterwards, the resulting transcripts quantified with real time quantitative PCR on a Step One Plus™ real time DNA amplification system (Applied Biosystem, USA) along with maxims SYBER Green qPCR master mix kit (Fermentase, Germany) and specific primers for each sample. The expression level of TIM-3 was optimised vs. beta-actin (ACTB) expression as house-keeping gene. The PCR holding stage was performed once for 10 min in 95 °C. The denaturation, annealing and extension cycles temperatures were 95 °C for 15 s, 60 °C for 1 min and 72 °C for 15 s, respectively, for 40 times. Specific primers for TIM-3 and ACTB were

Table 1. The sequence of β-Actin and TIM3 specific primers.

Specific primers	Direction	Sequence
Sequence of β-Actin	Forward	5-TTC GAG CAA GAG ATG GCC A-3
	Reverse	5-CAC AGG ACT CCA TGC CCA G-3
Sequence of TIM3	Forward	5′-CCA TCA GAA TAG GCA TCT ACA TC-3′
	Reverse	5′-CCA TCA GAA TAG GCA TCT ACA TC-3′

designed with Allele ID 7.0 and were synthesised by Tag Copenhagen Inc., Denmark (Table 1). Data were analysed using relative quantification ($2^{\Delta\Delta Ct}$) method.

Flow cytometry analysis

The flow cytometric analyses were performed using FACS Callibuor instrument (BD Bioscience, San Jose, USA). The expression of TIM-3 protein on HL-60 cells was measured with antihuman TIM-3-PE (eBiosciences, USA). The results were analysed using Cell Quest Pro software (BD Bioscience, San Jose, USA).

Statistical analysis

All statistical analyses were carried out using SPSS 16.0 software (SPSS Inc, Chicago, USA). One-way ANOVA was used for comparing the groups. All experiments were performed in triplicate. Results are expressed as mean percentage ± relative standard deviation, and p values <0.05 are considered as significant.

Results

The miR-125a-3p was predicted as a TIM-3 silencer miRNA

In the Mirwalk output, miR-330-5p was predicted to suppress TIM-3 expression by 9/12 of chosen algorithms (miRWalk, Microt4, miRanda, miRDB, miRMap, PITA, RNA22, RNAhybrid and Targetscan). 'Validated targets module' showed nothing for miR-330-5p-TIM-3 interaction which represented that no experimental study had been done to validate this bioinformatics prediction. In the Targer Scan output, context score percentile of miR-330-5p for silencing TIM-3 expression was predicted as 98% with an eight mer seed region.

In the current study, we added 50 ng PMA to the cultured HL-60 cells. After 72 h, the expression of TIM-3 protein on Hl-60 cell line was 60% while it was 0.08% without treatment (Figure 1).

Using 50 nM final concentration of LableIT siRNA Delivery Control-FITC for 24 h, we observed that 90% of the cells had been successfully transfected (Figure 2).

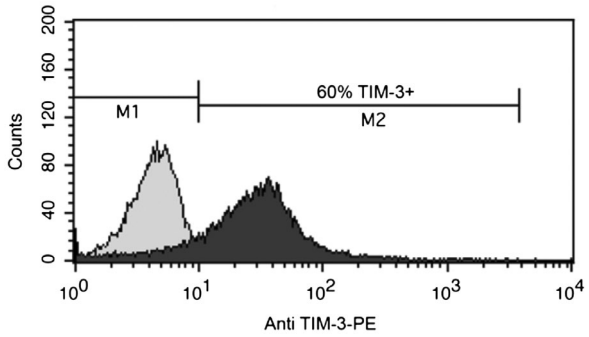

Figure 1. Flow cytometry analysis of TIM-3 expression on HL-60 cell line. Histogram analysis of TIM-3 expression on PMA treated HL-60 cells in compare with un-treated cells after 72 h. The histogram shows that 60% of the cells are expressing TIM-3 on their surface.

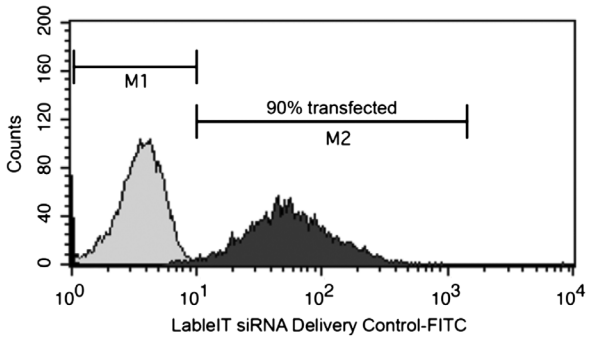

Figure 2. Flow cytometry analysis of HL-60 cells after transfection with FITC labelled siRNA. The histogram shows that 90% of the cells are successfully transfected.

miR-330-5p suppressed TIM3 protein expression on HL-60 cell line

About 24 h after treatment of HL-60 cells with PMA, the cells were transfected with has-miR-330-5p mimic for 24 h. Then, 48 h post transfection (72 h after induction with PMA), the cells were analysed using anti TIM-3-PE antibody for TIM-3 expression. In comparison with untransfected control, flow cytometry analysis showed that only 1.85 ± 0.09 of transfected cells expressed TIM-3 protein which reflects more than 98% ($p = 0.0001$) gene silencing (Figure 3). As it is indicated in Figure 3, there is no significant difference among negative, mock and scrambled controls for TIM-3 expression which confirms that the silencing is specific.

In the present study, transcript level of TIM-3 was also measured in transfected and untransfected HL-60 cells with qRT-PCR. As is shown in Figure 4, TIM3 mRNA level was only 3.5 ± 28% in comparison with negative control ($p = 0.001$), which indicates 96.5% silencing of TIM-3 gene expression. Again no significant difference for TIM-3 transcript level was observed among negative, mock and scrambled controls (Figure 4).

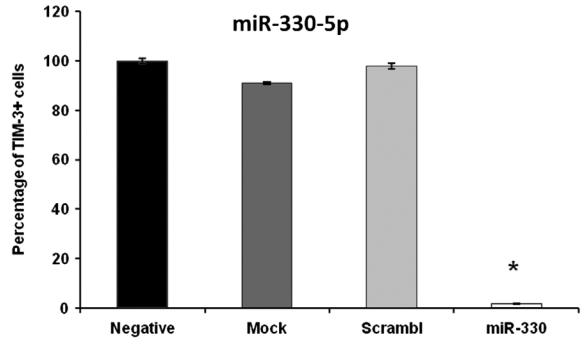

Figure 3. Flow cytometry analysis of TIM3 expression on HL-60 cells after transfection with miR330-5p mimic. TIM-3 expression on transfected cells was greatly inhibited in compare with negative control ($p < 0.05$). There was no meaningful difference among negative, mock and scrambled controls for TIM-3 expression. Data are shown as mean percentage ± relative SD of three identical repeats of each experiment. Asterisk (*) indicates statistical significance.

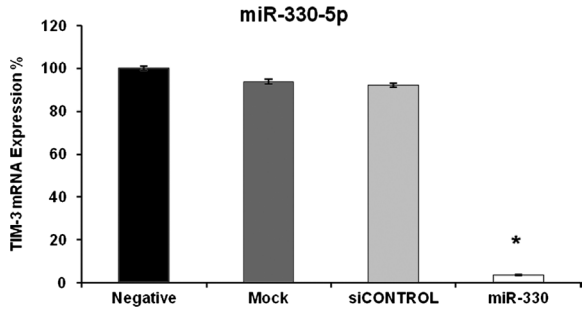

Figure 4. Measurement of TIM-3 mRNA level in HL-60 cells using qRT-PCR after transfection with miR330-5p mimic. TIM-3 mRNA was significantly reduced in transfected cells in compare with negative control ($p < 0.05$). There was no meaningful difference among negative, mock and scrambled controls for TIM-3 mRNA level. Data are shown as mean percentage ± relative SD of three identical repeats of each experiment. Asterisk (*) indicates statistical significance.

Monitoring HL-60 cell line viability after transfection with miR-330-5p mimic

As is indicated in Figure 5, the viability of transfected cells was 85.7 ± 0.18% and MTT assay showed no significant difference between the viability of transfected cells with negative, mock and scrambled controls ($p > 0.05$). This confirmed that no toxic effect of transfection reagent and/or RNA structure has affected TIM-3 gene silencing.

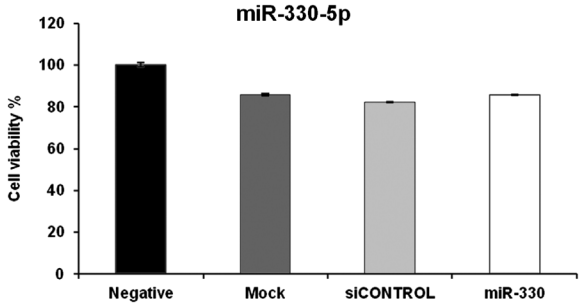

Figure 5. Cell viability assessment with MTT assay. As depicted in the graph, the cell viability in transfected HL-60 cells was 85.7% which is not significantly different from negative control. There was no meaningful difference among negative, mock and scrambled controls for cell viability. Data are shown as mean percentage ± relative SD of three identical repeats of each experiment. Asterisk (*) indicates statistical significance.

Discussion

AML is a disease in which the myeloid cell lineage differentiation is defected. This defect leads to an uncontrolled proliferation of low-differentiated cells such as myeloid blasts, and accumulation of them in bone marrow. With regard to recent studies, the increase in TIM-3 gene expression on LSCs and also the therapeutic functions of miRNA, we decided to investigate the influence of miR330-5p on expression of TIM-3 in AML [1].

Using Mirwalk and Target Scan, we found that miR330-5p with a probability of 98% can silence TIM-3. It does it through an 8 mer seat region. Also, information obtained from these databases has shown that the silencing effect of miR330-5p on TIM-3 protein has not been confirmed *in vitro*, so far.

The results of the present study revealed that miR-330-5p has a regulatory effect on TIM-3 coding mRNA and causes a low expression of this protein on HL-60 linage. Based on our qPCR results, miR-330-5p degenerates TIM-3 mRNA and hence, reduces its translation. As the silencing effect was also significant in mRNA level and was nearly equal to its protein level, it seems that miR-330-5p acts as a siRNA and causes TIM-3 mRNA degeneration rather than inhibition of its mRNA translation.

Fraga et al. [15] have made a study on biological function of miR-330-5p on DU145 and PC3 in relation to human prostate cancer. They found that transfection

Table 2. Summary.

What is known about this subject	What this paper adds
• Acute Myeloid Leukemia is a heterogenic disease with high mortality rate (1% of cancer deaths in the world) • Because of many problems that are with conventional therapies of AML, new therapeutic approaches try to target LSCs • TIM-3 is a surface glycoprotein of type I which is expressed on AML LSCs rather than on normal HSCs	• Using MIRWALK and TARGET SCAN, we found that mir330-5p with a probability of 98% can silence TIM-3 • The results of the present study revealed that mir330-5p has a strong regulatory effect on TIM-3 expression on HL-60 cell line

of miR-330-5p to cancer cells has an antimetastatic role and causes low expression of SP-1. This could be almost similar to our experience with miR-330-5p's influence on TIM-3 expression in an AML cell lineage.

Qu et al. [16], in a study, have investigated miR-330-5p function in U251 and U87 human glioblastoma linage. The results obtained from flow cytometry demonstrated that SH3GL2, a tumour suppressor gene with high expression in central nervous system, has low expression in human gliobastoma. At the same time, miR-330-5p is expressed highly in glioblastoma cells and tissues compared to normal brain tissue.Also, SH3GL2 is a special target for miR-330-5p as a tumour suppressor miRNA.

Yuefeng et al. [17] have extensively investigated the effect of miR-330-5p on SW1116 and SW480 cells which are human colorectal cancer cell lines. The results of their study revealed that miR-330-5p caused a reduced expression of Cdc42. Again, this confirms that miR-330-5p may act as a tumour suppressor.

Studies have shown that there are some cellular factors in cancers, like TIM-3 which inhibit the immune system. In cancers, TIM-3 is expressed on Th1 cells and inhibits their antitumour role [18]. TIM-3's high expression in LSCs in AML makes stronger the hypothesis that TIM-3 high expression has a role in leukaemic cells development, proliferation and survival. Previous studies put emphasis on the inhibitory role of miR-330-5p on different cancers which are in line with our findings.

We conclude that miR-330-5p has a powerful suppressive effect on TIM-3 gene expression. As TIM-3 expression is increased in nearly all types of AML LSCs and because of its suppressive effect on antitumour T cell response, miR-330-5p could be considered as a potential and important element for future studies on other aspects of AML including leukaemic cells apoptosis, proliferation and differentiation. Such researches may shed light on the therapeutic approaches for AML and other cancers. This work represents an advancement in biomedical science because we showed miR-330-5p with its strong silencing activity on TIM-3 which could be considered as a potential therapeutic target for AML (Table 2).

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Disclosure statement

The authors report no conflicts of interest.

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