

Overexpression of microRNA-630 in Acute Leukemic T-cell line

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Abstract

Background: MicroRNAs (miRNAs) are noncoding RNAs that control the expression of their target mRNAs. It affects cancer cell proliferation and apoptosis as oncogenes or tumor suppressors. Dysregulation of miRNAs expression leads to the development of various cancers. Therefore, for the first time in this field, this study investigated the effect of overexpression of microRNA-630 on the Jurkat cells.

Materials and methods: In this experimental study, the Jurkat cells were divided into the four groups, i.e. non-transfected control group (A), scramble (B), transfected with 50 nM concentrations of miR-630 (C), and treated with 100 nM miR-630 (D). MiR-630 transfection was performed by lipofectamine 2000. Cancer cell growth in each group was analyzed with MTT assay. Flow cytometry investigated percent of viable, necrotic, and apoptotic cells. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) measured the expression of P53, P21, and BCL2 genes. SPSS (version 21) especially Kruskal-Wallis and Mann-Whitney U tests were utilized for data analysis.

Results: The results of MTT assay showed that the cell growth rates in C (118%) and D (136%) groups were significantly higher than that in the control group ($P=0.037$ vs. 0.034). The percentage of early and late apoptosis in C (3.1% $P=0.01$, 4.2% $P=0.02$) and D (0.5% $P=0.008$, 0.4% $P=0.006$) groups were significantly lower than those in the control group. The expression of p53 and p21 in C (0.7 $P=0.037$, 0.62 $P=0.034$) and D (0.44 $P=0.034$, 0.53 $P=0.038$) groups were significantly decreased compared with the control group. The expression of B-cell lymphoma-2 (Bcl2) in C (1.85) and D (3.26) groups were significantly increased compared with the control group ($P=0.037$ vs. 0.024).

Conclusion: Overexpression of miR-630 led induction of T-ALL cell growth and reduction of their apoptosis. These results emphasized that miR-630 contributed as an oncogenic microRNA in T-ALL cells.

Keywords: Acute Lymphoblastic Leukemia, MiR-630, Overexpression, p53 Genes

Introduction

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer that often occurs at 2 to 5 years of age. Increasing of cancerous B and T lymphoid precursors in the bone marrow is the major component of ALL (1). Almost 35% of patients aged 18 to 60 years old stay alive and their treatment process has been slow (2). Environmental (especially benzene and ionizing radiation), cytogenetic, epigenetic, and infection can be the cause of ALL (3-5). Finding novel therapy is extremely important because drug toxicity, resistance to chemotherapy and radiotherapy or relapse are the weaknesses of current treatments (6- 8).

T cell acute lymphoblastic leukemia (T-ALL) is the malignancy of immature T cell. It is 10%–15% of pediatric cancers and 25% of adult ALL cases. T-ALL in children and juvenile has a good prognosis and 60-75% of them have 5 years of relapse-free survival rate (9). T-ALL can be classified into different molecular genetic subtypes. Patients with T-ALL have a higher risk than B-precursor ALL for relapse (10).

MicroRNAs (miRNAs or miRs) are defined as 21–25 nucleotide RNA molecules that bind to the 3' untranslated region (UTR) and regulate the expression of mRNAs (11, 12). MiRNAs mainly modulate mRNA stability and protein

translation, thereby exerting post-transcription effects (11, 13). MiRs have a specific involvement in cellular proliferation and apoptosis (14-16). Different expressions of special miRs might become a master key for the diagnosis and treatment of malignancy (17).

MiR-630 is a type of micro-RNA that has 22 nucleotides and acts through its various signaling pathways to inhibit or inductance, thereby contributing to the improvement or progression of certain cancers. Depending on the tumor types, miR-630 has reportedly functioned as a tumor-suppressive or an oncogenic miRNA. In some malignancies, overexpression of miR-630 is associated with proliferation, invasion, and metastases (18, 19) but it induced cancer cell apoptosis in another form of malignancy (20-22). Recently, it has been shown that various types of leukemia, such as ALL, have different expressions of microRNA, and this is promising potential biomarker for diagnosis and prognosis (23-28). It has also been shown that the expression of different microRNAs is related to the rate of survival and prognosis in ALL (24). Previous studies have expressed the effects of miR-630 on solid tumors, such as pancreatic tumors(29), prostate(30), and colon cancer(31). However, the function of miRNA 630 is not specified in T ALL disease.

The present research aimed to examine the proliferation and viability of the T ALL cell line (Jurkat) after overexpression of miR-630. Besides, the effect of this miRNA on P53 pathway genes like P21, P53, and prosurvival B-cell lymphoma-2 (BCL-2) was investigated by quantitative real-time polymerase chain reaction (qRT-PCR).

Materials and Methods

Cell culture

This study was accomplished at the Bushehr University of Medical Sciences

(research ethics code: IR.BPUMS.REC.1396.66). The Jurkat cell line was obtained from the Iranian Stem Cell Technology Research Center (Tehran, Iran). It cultured in RPMI-1640 (CBSA; cell biotechnology saba arna, Fars, Iran) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, USA) and 1% antibiotic-antimycotic 100x (caisson; caisson laboratories, Inc., Smithfield, USA) at 37°C in a 5% CO₂ humidified incubator. Finally, suspension cultured cells were used for transfection at ~80% confluence(32).

Transfection

The Jurkat cell lines were divided into the following four groups, i.e. non-treated control group (A), scramble (B), treated with 50 nM concentrations of miR-630 (C), and treated with 100 nM miR-630 (D). (Eurofins Genomics, Germany). Cells were cultured in 96-well plate (4×10^4 cells/well) in 85% humidified and 5% CO₂. The first stage was done one day before transfection, where Jurkat cells were seeded in 24-well plates at a concentration of 2×10^5 cells/well in medium with serum, but without antibiotics. In the second stage, miR-630 was diluted in 50 µl OptiMEM (Gibco, USA) in two tubes. After that, Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc. USA) (1.5 µl) was diluted into 50 µl OptiMEM and incubated it for 5 min at room temperature. Next, the diluted miR-630 from stage 2 was mixed with the diluted Lipofectamine 2000 from stage 3. The compound incubated at room temperature for 20 min. Then, miR-630-Lipofectamine 2000 complexes from stage 4 (100 µl) added directly to each well-containing cells (from stage 1), and mixed the compound gently by rocking the plate back and forth. Finally, the compound was incubated at 37°C in a CO₂ incubator. Non-transfected Jurkat cell line was considered as the control group. siRNA conjugated with FITC (Santa Cruz Biotechnology,

Inc. Texas, USA) was utilized as a scramble to closely monitor the transfection states.

Cell growth assessment

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) solution (10 μ l from 5 mg/ml stock) was added to the wells 48 hours after transfection. The cells were incubated for 4 hours at 37°C. Then, the medium was discarded and 150 μ l of DMSO was added to each well, and plates were shaken for 15 min. The absorbance of generated formazan was quantified by spectrophotometry (ELISA reader) at 570 nm. Three independent experiments were performed in triplicate.

Cell apoptosis assay

Jurkat cells were cultured in 48-well plates (8×10^4 cells/well) in antibiotic-free medium and transfected. After 48 hours, cells were collected, washed twice, and stained with FITC-Annexin V and 7-AAD using the FITC-Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend, San Diego, CA; USA) according to the manufacturer's manual (33). First, cells were washed with cold BioLegend's cell staining buffer and then re-suspended in binding buffer. Subsequently, cells were incubated with 5 μ l Annexin V/FITC and 5 μ l 7-AAD for 15 min at room temperature in the dark. Then, 400 μ l of Annexin V binding buffer was added to each tube of the cell. FACSCalibur flow cytometer (BD Bioscience, USA) measured apoptotic cells. Three independent experiments were performed.

RNA extraction

Total RNA was extracted from cells in different groups using Total RNA isolation kit (Santa Cruz Biotechnology, Inc., Texas, USA) and according to the manufacturer's manual. RNA concentration was determined after extraction using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc., USA) and electrophoresis on 2% agarose gel.

cDNA Synthesis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

cDNA of miR and other genes were generated using a high sensitive cDNA Synthesis kit (BONbiotech, Inc. Iran) and cDNA Synthesis H Minus First Strand Revert Aid kit (Thermo Fisher Scientific, Inc. USA), respectively and according to the manufacturer's manual. Specific primers for PCR amplification were synthesized by Bonbiotech Company (Bonbiotech, Inc, Iran) as represented in Table I. The expression level of miR-630 and mRNA in the cells were confirmed on the Step One Plus Real-Time PCR systems (ABI, US). By using a 2X Real MOD Green PCR Master Mix kit (Takara Bio, Inc., Otsu, Japan), expressions of *P53*, *P21*, and *BCL2* mRNA in Jurkat cells were quantified. To put it in a nutshell, in a final volume of 20 μ l, containing 10 pmol of each primer, 10 μ l 2 \times reaction mixture of SYBR Green and 7.4 μ l sterile deionized water, 2 μ l cDNA product was diluted (34). The cycling program was as follows: an initial denaturation step at 95°C for 10 min, followed by 40 cycles including a denaturation step at 95°C for 10 sec, annealing, and extension at 55°C for 40 sec.

Hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and *U6* snRNA were selected as internal housekeeping controls. All PCRs were performed in triplicate. The relative expression levels of miR-630 and *P53*, *P21*, and *Bcl2* mRNA were calculated by the $2^{-\Delta\Delta CT}$ method (35).

Statistical analysis

Results were expressed as the mean \pm standard deviation (SD) and analyzed using SPSS (v. 21.0) (SPSS, Inc. Chicago, IL, USA). Differences between groups were evaluated by Kruskal-Wallis tests, and a comparison of studied groups for the

expression of the desired genes was assessed by Mann-Whitney U tests. $P < 0.05$ was considered to indicate a statistically significant difference (34).

Results

Transfection accuracy

Six hours after transfection, the scramble group (absorbance: 490 nm, emission: 514 nm) was examined by a green filter fluorescent microscope. The transfection accuracy was assured by observing the greenlight fluorescein inside the cells (Figure 1).

The effect of miR-630 on cell growth

The cell growth means of studied groups with triple repetition are presented in Table II and Figure 2. The MTT assay showed that the growth of scramble group was not significantly different from that of the control group ($P = 0.48$). In addition, upregulation of miR-630 significantly stimulated the proliferation of C and D groups compared with the negative control group ($P = 0.037, 0.034$). The growth rate was significantly different between C and D groups ($P = 0.046$).

Reduction of cell apoptosis following overexpression of miR-630

The place of viable, apoptotic, and necrotic cells in flow cytometry quadrant (according to the type of absorbent stains) are given in Table III. The percentage of the viable cells in C and D groups was 90.5% and 97.6%, respectively.

By increase of the miR-630 transfection, the viable cells increased significantly compared with the control group and the scramble. * $p < 0.05$.

Viable cells in C and D groups were significantly different compared with control ($P = 0.034, 0.022$ respectively). As shown in Figure 3, the percentage of live cells increased with increase of miR-630 transfection doses. Early apoptotic cells in C and D groups were significantly different compared with control ($P = 0.01$

and 0.008 respectively). Also, late apoptotic cells in C and D groups were significantly different compared with control ($P = 0.02$ and 0.006 respectively). According to Figure 5, the percentage of necrotic cells was decreased by elevating of miR transfection doses; whereas, C and D groups didn't has significant difference compared with control ($P = 0.38$ and 0.24 respectively) and there wasn't significant difference between C and D groups in necrotic cells ($P = 0.5$). There were significant difference between C and D groups in viable, early apoptotic, and late apoptotic cells ($P = 0.037, 0.03, \text{ and } 0.02$ respectively).

Overexpression of miR-630 in treated groups

Fold changes of the miR-630 in C (50 nM) and D (100 nM) groups were 4.4 and 12.7, respectively. The expression of miR in C and D groups were significantly different compared with control ($P = 0.037, 0.037$). In addition, there was a significant difference between C and D groups ($P = 0.04$). The cycle threshold of target miR was normalized against *U6* as an internal control. The fold change is presented in Figure 6 and Table IV.

The expression of miR-630 in C (50 nM) and D (100 nM) groups increased significantly compared to the control. This finding confirmed accuracy of miR transfection. * $p < 0.05$.

Inducing *Bcl2* gene expression following overexpression of miR-630

Fold changes in the expression of *p21*, *p53*, and *Bcl2* are shown in Table IV and Figure 7. The cycle threshold of target genes was normalized against *HGPRT* as a reference gene. Results demonstrated that *Bcl2* gene expression increased following elevation of miR-630 (fold changes were 1.86 and 3.26 in C and D groups respectively). *Bcl2* expression was increased significantly in C and D groups compared with control ($P = 0.037$ vs.

0.024). There was a significant difference between C and D groups ($P=0.04$).

Reduction of *p53* gene expression in transfected cells

As shown in Table IV and Figure 7, the gene expression of *p53* were 0.7 and 0.44 in C and D groups respectively. The expression were downregulated significantly in C and D groups compared with control ($P= 0.037, 0.034$ respectively). In addition, the gene expression in C group was significantly higher than that in D group ($P=0.04$).

The Effect of miR-630 upregulation on the expression of *p21*

According to Figure 7, fold changes were 0.62 and 0.53 in C and D groups respectively, so the relative gene expression of *p21* was significantly downregulated in C and D groups compared with control ($P= 0.034$ vs. 0.038). There was no significant difference between C and D groups ($P=0.06$). The expression of Bcl2 was increased significantly in C (50 nM) and D (100 nM) groups compared with control. The expression of p53 and p21 were downregulated significantly in C and D groups compared with control ($p<0.05$).

Table I: Sequence of Real-Time PCR primers

Gene	Direction	Primer Sequence
MicroRNA-630	F	5'-TAAAGGAGGAAGATAAAGG-3'
	R	5'-GTAGCAGTGATAGGCATT-3'
U6 small nuclear RNA	F	5'-CTCGCTTCGGCAGCACATATAC-3'
	R	5'-ACGCTTCACGAATTTGCGTGTC-3'
P53	F	5'-TAACAGTTCCTGCATGGGCGGC-3'
	R	5'-AGGACAGGCACAAACACGCACC-3'
P21	F	5'-GAGGCCGGGATGAGTTGGGAGGAG-3'
	R	5'-CAGCCGGCGTTTGGAGTGGTAGAA-3'
Bcl ₂	F	5'-GGTGGGGTTCATGTGTGTGG-3'
	R	5'-CGGTTTCAGGTAAGTCAAGTCAATCC-3'
HGPRT	F	5'-GGACAGGACTGAACGTCTTG-3'
	R	5'-ATAGCCCCCTTGAGCACAC-3'

HGPRT, Hypoxanthine-guanine phosphoribosyl transferase; Bcl₂, B-cell lymphoma 2 F, forward; R, reverse

Table II: The growth rate of Jurkat cells

Non treated	Treated	
Scramble (B)	50 nM (C)	100 nM (D)
97 ±4.5	118 ±3.0	136 ±2.8

Table III: Place of viable, apoptotic, and necrotic cells in the flow cytometry quadrant according to the type of absorbent stains

stain	Annexin-V	7-AAD
Cell Condition		
Viable (Q1)	Neg	Neg
Early Apoptotic (Q2)	Pos	Neg
Late Apoptotic (Q3)	Pos	Pos
Necrotic (Q4)	Neg	Pos
Pos; Positive, Neg; Negative		

Table IV: The Expression of miR-630, pro-apoptotic genes, and anti-apoptotic genes in cell groups

Gene	miR-630	P53	P21	Bcl2
Group				
Scramble (B)	1.09±0.100	1.20± 0.050	1.06±0.090	1.02±0.060
50 nM (C)	4.40±0.900	0.70±0.015	0.62±0.010	1.85±0.055
100 nM (D)	12.70±1.100	0.44±0.011	0.53±0.011	3.26±0.200

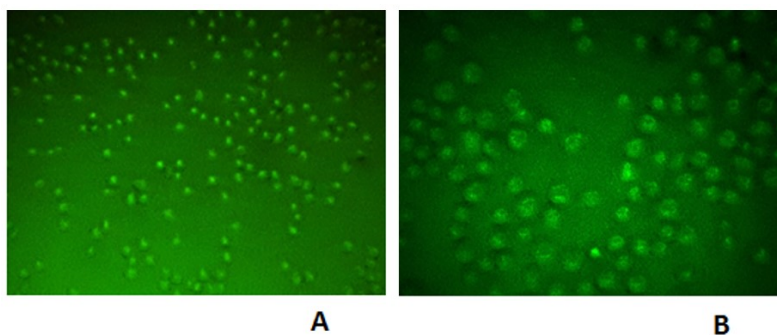


Figure 1. Greenlight of FITC attached to siRNA in the scramble sample

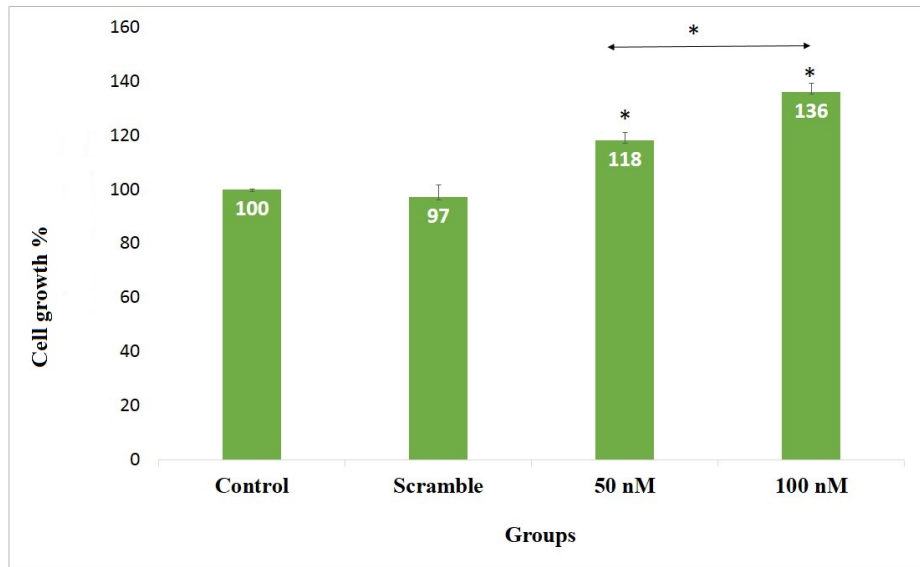


Figure 2. T-ALL cell growth in four groups

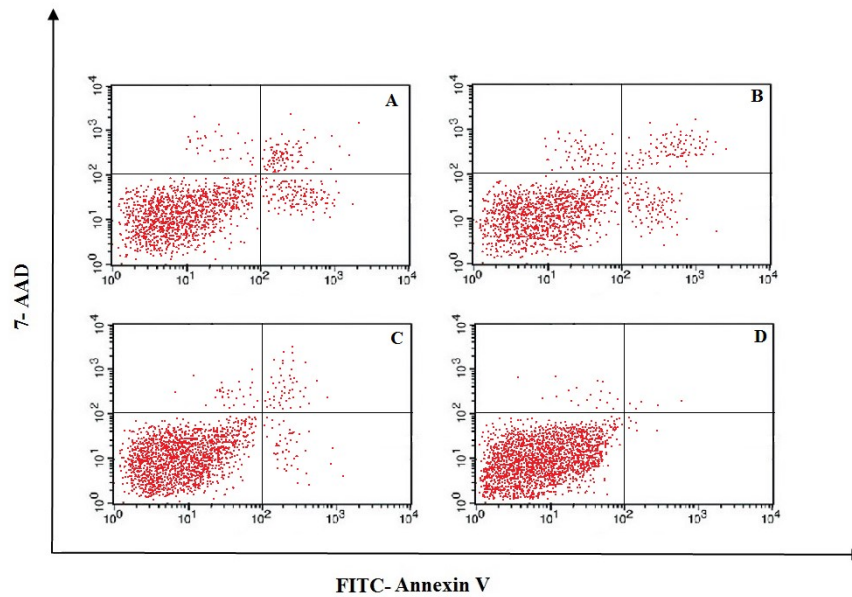


Figure 3. The effect of miR-630 on the apoptosis of Jurkat cells Flow cytometry analysis of A. Control group, B. Scramble, C. transfected by 50 nM of miR-630 and D. transfected by 100 nM of miR-630

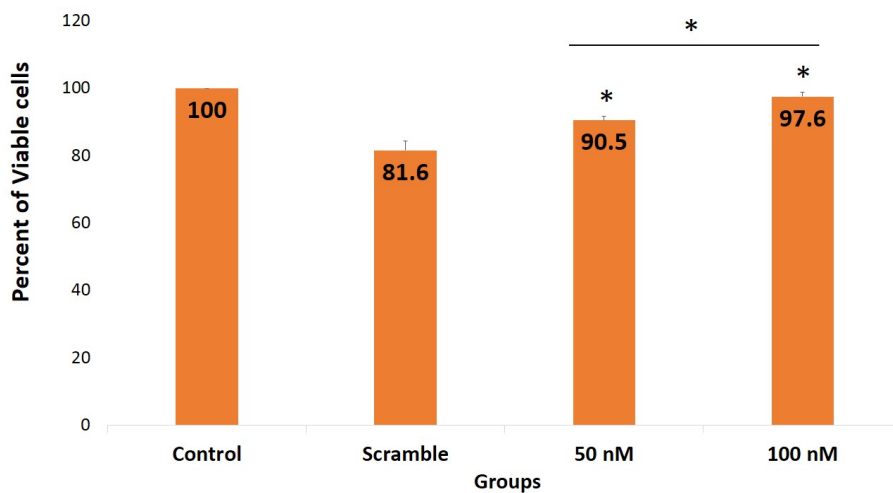


Figure 4. Viable cell in treated and non-treated groups (%)

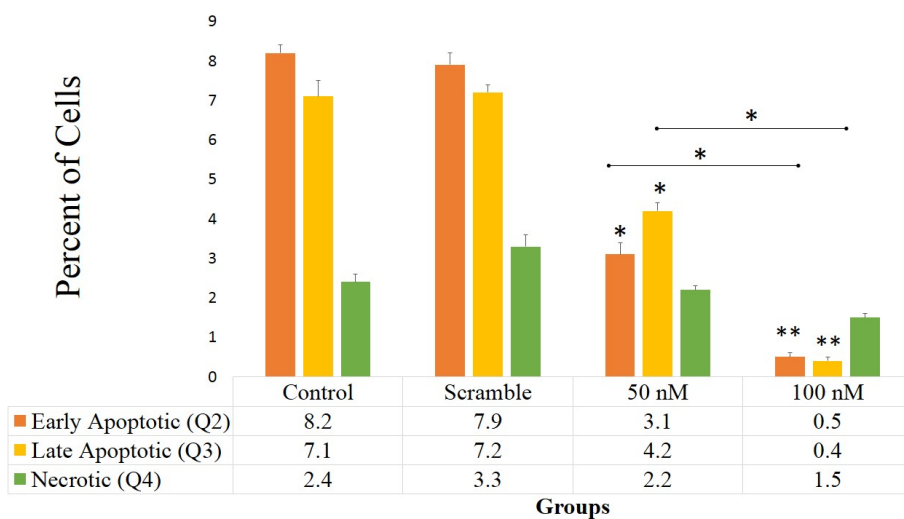


Figure 5. Percentage of early and late apoptotic and necrotic cells in Q2, Q3, Q4

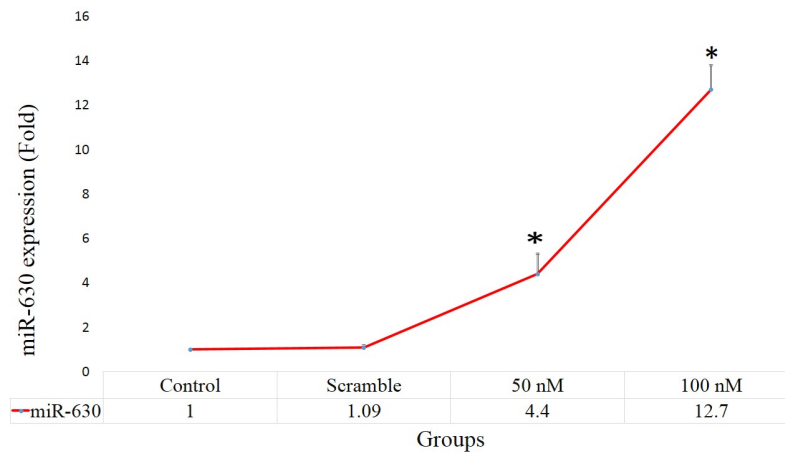


Figure 6. Relative expression of miR-630 in treated and non-treated cell groups.

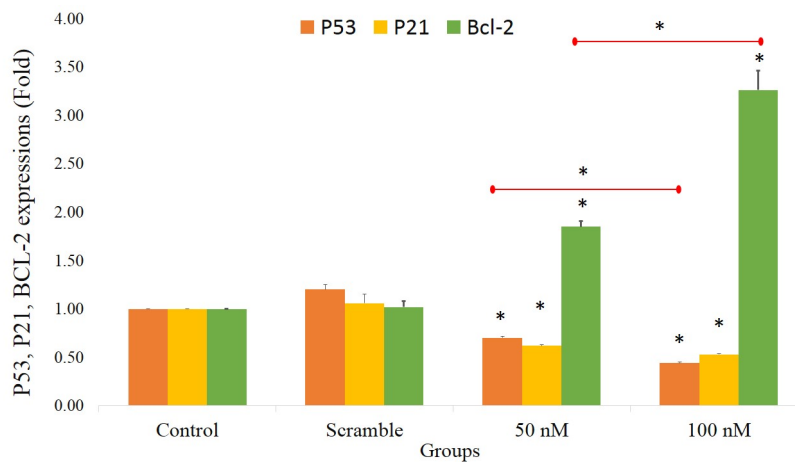


Figure 7. The relative expression of pro-apoptotic and anti-apoptotic genes in treated and non-treated groups

Discussion

Immunophenotypic characterization of According to recent studies, cancer cell growth or apoptosis is modulated following the dysregulation of miRNAs (36). Aberrant expression of miRNAs was specified but an interesting question is, through which molecular pathway does the miR intervene? (37). Depending on the tumor types, miR-630 has reportedly functioned as a tumor-suppressive or an

oncogenic miRNA. In some malignancies, overexpression of miR-630 was associated with proliferation and invasion (18, 19), but it induced cancer cell apoptosis in another form of malignancy (20-22). In this study, to further investigate the effects of miR 630 on T ALL, Jurkat cells were transfected with miR 630, which induced overexpression of miR 630. In the present study, MTT assessed that the cancer cell growth in miR treated groups was

significantly higher than that in the control group. Increase of the miR transfected dosage from 50 to 100 nM significantly induced proliferation. Hence, it proved that overexpression of miR-630 implicated as an oncogene in boosting cell growth and proliferation. Flow cytometry demonstrated that the viable cells rose to 97.6% in the 100 nM group. The number of cells into the primary and secondary apoptosis phase had a significant decrease in both doses compared to control and compared with each other, but necrosis changes were not significant in different groups. In general, overexpression of miR-630 could increase the number of viable cells and led to a decrease in apoptosis.

In previous studies, the increase or decrease of miR-630 in some cancer cell lines led to an increase in growth or an increase in cell apoptosis. For example, Sakurai et al in 2014 showed that upregulation of exogenous miR-630 in prostate cancer cells (PC3) stopped the growth of these cells(30). In a study by Zhou et al., the proliferation and invasion of cancer cells were reduced following overexpression of miR-630 in the gonadotropic cell line due to inhibition of Sox4(38). Corcoran et al, in 2014 proved that upregulation of miR-630 in the HER2-resistant breast cancer cell line decreased invasion and migration, but inhibition of this miR to an increase in invasion (39). In another study on breast cancer cell line, Zhou et al., in 2016 found that when the miR-630 was increased, the invasion and metastasis of cancer cells reduced significantly. They also observed decrease of metastases and proliferation following increase of this miR in the animal phase and cell line (40). Jin et al., in 2016 found that overexpression of miR-630 in esophageal squamous cell carcinoma cell lines suppressed proliferation and metastasis. On the contrary, knockdown of miR elevated cell growth and invasion(41). Studies on other cell lines showed that this miR could also act as an oncogene. For example, Zhou et

al., in 2014 and 2016 revealed that expression of miR-630 significantly was upregulated in clear cell renal cell carcinoma (ccRCC). This overexpression was associated with metastasis to lymph nodes. On another hand, decrease in miR levels caused proliferation and migration reduction and increased apoptosis(42, 43). Galluzzi et al., in 2010 yielded that overexpression of miR-630 reduced lung cancer cells apoptosis treated by cisplatin (20). Zou et al., in 2015 investigated the ovarian malignant tumor cell line, and demonstrated that down-regulation of this miR increased apoptosis and reduce the proliferation and invasion of cancer cells (19).

The expression of p53, p21 genes induce cell growth arrest and apoptosis but Bcl2 gene inhibit apoptosis (44). As a transcription factor, P53 regulates multiple cell functions in humans (45). It responds to different stressors to transactivate many downstream genes, such as p21 and PUMA. The p53/p21 complex is created through the interaction between P53 and P21. Then, it binds to prosurvival BCL-2 family proteins (apoptosis regulator) to liberate pro-apoptotic groups, such as Bax and Bak, which in turn promotes apoptosis or cycle arrest (46-48). In many cancers, the functions of p53 pathway components, such as p21 and p53, have been disrupted. Many studies showed that miRNAs might target components of this pathway and potentially inhibit their expression, thereby increasing malignancies (45, 46). Moreover, Bcl2 acts as an apoptosis regulator and has been shown to play a critical role in the pathogenesis of different malignancies (34). These proteins are important because studies suggested that miRs could exert their oncogenic or suppressive effects by inhibition or induction of these genes (29, 49-51). For example, Galluzzi et al., in 2010 studies lung cancer cell line (A549), and concluded that overexpression of miR-630 by reduction of PARP3 / DDIT4 / EP300 was associated with modulation of

apoptosis (20). In a study by Zhang et al., in 2015 on 6 types of colorectal cancer cell lines, it was shown that the up-regulation of miR-630 had effects on p53 and Bcl2 levels (52).

In the present study, the results of real-time PCR indicated that fold changes of miR-630 was elevated following increase of transfection dosage, but expressions of p53 and p21 were decreased significantly. In contrast, the expression of Bcl2 was increased significantly. It was displayed that Bcl2 had a key role in the development of cancers (53). Thus, it can be concluded that miR-630 is able to affect the signaling pathway of pro-apoptotic and anti-apoptotic proteins and hence reduce apoptosis in the Jurkat cell line.

Conclusion

Overexpression of miR-630 in Jurkat cells could promote cell viability via increasing Bcl2 expression. Furthermore, it could reduce cell apoptosis by lowering the expression of p53. The cell growth and proliferation were significantly increased in treated groups compared with untreated (control) and scramble groups. The expression of p21 and p53 genes decreased and the expression of Bcl2 gene increased significantly following overexpression of miR. The study demonstrated that miR-630 acted as an oncogene in the Jurkat cells, which elevated the viability and proliferation and reduced the apoptosis of cancer cells. Further research is needed to define the exact interaction mechanism of this miR on the Bcl2 pathway.

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Conflict of interest

The authors declare no conflict of interest.

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