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Up-Regulation of miR-625-5p Correlates with Suppressed Sox2, Increased Apoptosis, and Cell Cycle Arrest via The PI3K/AKT Signalling Pathway in Acute Myeloid Leukaemia

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ABSTRACT

Background: Up-regulation of the microRNA-625 and abnormal expression of the Sox2 gene have been studied and seen in several tumors. Few reports have also shown the aberrant expression of miR-625 and Sox2 expression in various cancers. Several studies have also confirmed that phosphatidylinositol 3' -kinase /protein kinase B pathways regulate hematological malignancies, including Acute Myeloid Leukemia (AML). Thus, this study aimed to investigate the effects of mir-625 up-regulation on proliferation, apoptosis, and cell cycle by targeting the Sox2 gene via the downstream Akt signaling pathway and cell cycle regulators, such as p21, p27, and cyclin E in the KG-1 cell line.

Materials and Methods: Cells obtained from the KG-1 cell line were cultured and transfected with plasmid DNA (miR-625) and scrambled as the control using the Lonza electroporation system. Flow cytometry was used to evaluate cell cycle, proliferation, and apoptosis. Relative gene expression was validated by qRT-PCR. All data were analyzed using graph pad prism 7.01 and REST 2009.

Results: KG-1 cells transfected with the mir625-GFP construct showed decreased proliferation, increased apoptosis, and induced cell cycle arrest. Low levels of Sox2, p21, cyclin E, and up-regulation of p27 were confirmed and validated by qRT-PCR ($P < 0.05$).

Conclusion: MiR-625 can be a promising approach to aid in the treatment of AML. However, further studies are required in this field.

Keywords: Acute myeloid leukaemia; PI3K/AKT signaling pathway; miR-625; Sox2; Proliferation

INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive hematological cancer due to uncontrolled proliferation and impaired differentiation of myeloid progenitor cells resulting in more immature blast cells in the bone marrow and peripheral blood. AML is a more common type of acute leukemia in adults and comprises less than 20% in children with the lowest survival rate of all leukemia. Leukaemia has been considered the sixth most common cancer in Iran both in males and females. AML is the most common type of leukemia in adults, and leukemia is

the 13th most common disorder in the world[1-3]. The most common type of acute leukemia is AML, and its incidence and prognosis increase with age. Accordingly, explaining the potential mechanism of tumor expansion would help us understand the progression and treatment of AML. An understanding of the roles of specific genes and regulatory molecules, such as miRNAs and transcription factors, is crucial for developing targeted therapies in AML.

MicroRNAs (miRNAs) are small, non-coding RNAs with ~ 22 nucleotides (nt) in length involved in regulating gene expression. About ~30% of human genes have been suggested to be regulated by miRNAs [4]. The function of miRNAs is huge in different biological processes, including cell cycle, differentiation, development, and metabolism. It also plays a vital role in human diseases, including diabetes, immuno- or neurodegenerative disorders, and cancer. The signatures of miRNAs were found in both hematological malignancies and solid tumors associated with cancer prognosis and progression[5]. The roles of miR-625 in cancers have been shown in recent investigations. For instance, decreased expression of microRNA-625 is linked with tumor metastasis and poor prognosis in patients with colorectal cancer[6]. Down-regulated miR-625 represses incursion and metastasis of gastric cancer by targeting ILK[6]. However, its specific role and significance have not been thoroughly explored in AML, making it a potential focal point for understanding disease mechanisms and therapeutic targets.

SRY (sex-determining region Y)-box 2 (SOX2) has been identified to be involved in embryonic stem cells. It has the ability to maintain its pluripotency whose overexpression has been reported to be able to restore pluripotency in human somatic cells and stem cell-like phenotype in cancer cells[7,8]. It has been reported that the low expression of miR-625 promotes cell proliferation and invasion while its high expression has been shown to down-regulate the SOX2 gene expression[9]. Another similar study has also indicated that the effect of miR-625 roles targeted SOX2 as a tumor suppressor of malignant cell development and progression in malignant melanoma[10]. Newly, several investigations have described the contribution of Sox2 to tumorigenesis, tumor progression, and metastasis of various types of tumors, including lung cancer[11], breast cancer[12], esophageal cancer[13], and ovarian cancer[14]. SOX2 is a key transcription factor involved in maintaining stemness properties in cells, and its aberrant expression is associated with poor prognosis in several cancers. In AML, the interplay between miR-625 and SOX2 may be crucial for understanding the mechanisms underlying disease progression and identifying potential therapeutic targets.

The balance between phosphorylation and dephosphorylation tightly regulates cell signaling. Signaling through tyrosine kinases plays an important role in many biological activities, such as cell growth, cell shape, cell cycle control, transcription, proliferation, survival, apoptosis, embryogenesis, and development. Other studies have described that the activation of PI3K/Akt signaling is most commonly found in numerous cancer cells, including AML. The phosphorylation of both Thr 308 and Ser 473 Akt have accounted for about 50-70% of patients with AML. Approximately 75% of AML patients presented PTEN phosphorylation, which was significantly associated with Akt phosphorylation and shorter overall survival. These signaling pathways not only influence cell survival and proliferation but also play a role in the regulation of transcription factors, such as SOX2[15].

The aberrant expression of the SOX2 gene has been observed in many other tumors, including AML. However, the up-regulation of mir-625 roles has not been fully investigated for its effect on proliferation and apoptosis by the SOX2 gene in the KG-1 cell line[2,16]. In this study, we have found out that transfection with miR-625 up-regulation has lowered the relative expression of SOX2, p21, p27, and cyclin E, which has significantly increased apoptosis and induced cell cycle via the Akt signaling pathway and cell cycle. Thus, it reduced proliferation, induced apoptosis, and arrested cell cycle, suggesting that targeting SOX2 provides an alternative therapeutic target in AML in this instance.

MATERIALS AND METHODS

Cell culture

Cells obtained from the KG-1 cell line (Pasteur Institute, Tehran, Iran) were cultured in the Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS) (Gibco-BRL, Eggenstein, Germany) at 37 °C in a humidified 5% CO2 atmosphere. After several passages, cells were evaluated to examine their viability by the trypan blue dye exclusion test to determine the number of viable cells present in a cell suspension under a microscope to determine cell viability before transfection and other functional assays.

Plasmid DNA extractions

A stock culture was prepared by growing 4-6 ml of plasmid-containing bacterial cells in the LB broth medium with appropriate antibiotic(s) (ampicillin) overnight (12-16 hours) at 37 °C with agitation. Cultured bacteria (*Escherichia coli*) were labeled as miR-625 and scramble for the control. Plasmid DNA was extracted from the cultured bacteria as miR-625 and scrambled for the control using a GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific 168 Third Avenue Waltham, MA USA 02451) as per manufacturer's instruction. Next, 1.5-5 ml of bacterial culture containing the plasmid was pelleted by centrifugation, followed by discarding the supernatant. The procedures were carried out following the order described in the plasmid extraction kit manufacturer's instructions.

Transient transfection

The cells were transfected with oligonucleotides miR-625 and scrambled as the control using a Cell Line NucleofectorTM Kit R (North America, Canada) Lonza electroporation system. DEAE-dextran, a water-soluble and polycationic substance with multiple positive charges, was added to the transfection solution containing the DNA, and its efficiency and cell viability were validated by flow cytometry and qRT-PCR.

Total RNA extraction and quantitative RT-PCR

Total RNA was extracted from the cultivated KG-1 cell line using Trizol Reagent (Thermo Fisher 168 Third Avenue Waltham, MA USA 02451) and reversely transcribed to complementary DNA (cDNA) by a complementary DNA synthesis kit (Thermo Fisher 168 Third Avenue Waltham, MA, USA 02451). The expression levels of targeted genes were assessed by the TaqMan stem-loop RT-PCR method for gene-specific primers. Relative expression levels of Sox2, Akt, p21, p27, and cyclin E genes were examined by SYBR Green real-time PCR (qRT-PCR LightCycler 96, Roche, Vedbaek, Denmark) and normalized to GAPDH as the control based on the manufacturer's manual. PCR primer sequences are given in Table 1.

Cell cycle assay

The cell cycle assay was performed by fixing the cells in 70% ethanol at 4 °C overnight and treated with 0.2% Triton X-100 and RNase before staining with Propidium Iodide (PI). The cell cycle was analyzed by flow cytometry (Accuri Cytometers Inc.; Ann Arbor, MI, USA) 48 hours after transfection following the manufacturer's instructions.

Apoptosis assay

The cell apoptosis was assayed using an Annexin-V/FITC Kit (BD Biosciences; San Jose, CA, USA). Transfected cells were collected and washed in ice-cold PBS before staining with Annexin V and the PI solution in the dark. The flow cytometer assays analyzed cell apoptosis 48 hours after transfection following the manufacturer's instructions.

Data analysis

The data were tripled, reviewed, and analyzed using the t-test with graph pad prism 7.01 (La Jolla, CA, USA). Results are presented as the mean ± standard error of the mean (SEM) at a statistically significant difference of $P < 0.05$. Data in qRT-PCR were analyzed by Rest 2009.

Table 1: Primers used for real-time PC

Gene	Sequence Forward	Sequence Reverse
Sox2	5'- CATGGACAGTTACGCGCAC-3'	5'-TTCATGTAGGTCTGCGAGCTG-3'
Akt	5'-CCTGGACTACCTGCACTCGG-3'	5'-CTTATTGTGCCCGTCCTTGT-3'
p21	5'-CGATGGAACTTCGACTTTCA-3'	5'-GCACAAGGGTACAAGACAGTG -3'
p27	5'-AGGACACGCATTTGGTGGA-3'	5' GAAGAATCGTCGGTTGCAGGT-3'
Cyclin E	5'-GCCAGCCTTGGGACAATAATG-3'	5'-AGTTTGGGTAAACCCGGTCAT-3'

RESULTS

Overexpression of miR-625 in KG-1 cells

The viability of cell culture was above 97%, and the total concentration of plasmid DNA extraction was well over 4000 ng/uL for both miR-625. Scramble and quality were confirmed by agarose gel electrophoresis. The transfection efficiency of KG1 cells with miR-625 and scramble was visualized using green fluorescent protein (GFP) fluorescence microscopy. GFP-positive cells were observed in miR-625 (37%) and the scramble (34.1%) after transfection. The results showed that transfection of KG1 cells with miR-625 and scramble yielded significant GFP-positive cells well over a mean value of about 35%. The plasmid contains several reporter genes, most notably for GFP and the ampicillin resistance gene. The efficiency calculation for each specific gene and GAPDH for a 5-point standard curve were all above 97%, which was used to gauge a real-time PCR assay's performance. The relative expression of miR-625 was up-regulated significantly compared to scramble. The down-regulated expression levels of Sox2, p21, p27, and cyclin E were confirmed and validated by qRT-PCR as illustrated in Figures 1 and 2.

The effects of miR-625 on the cell cycle and proliferation in the KG-1 cell line

A flow cytometer of cell cycle assay recorded a sub-G1 scramble average mean of 17.8% and miR-625 mean of 30.81% as shown in Figure 3. However, the cell cycle decreases at S and G2/M more predominantly in miR-625 of 8.34% at G2/M than the scramble of 13%. In this study, the flow cytometer histograms showed a significant difference in cell cycle and proliferation of KG1 cells before and after transfection with the up-regulation of miR-625.

These results have revealed that the transfection of KG1 cell lines with miR-625 has decreased cell proliferation and mainly induced G1 cell cycle arrest.

Significant increase of apoptosis in KG-1 cells

The measurement of cell apoptosis using annexin-V stain before the transfection of KG1 cells showed live cells of 83.3% as reported. miR-625 has a mean of 17% of early apoptosis and increased to an average of 38% of late apoptosis while scramble has 10.5% of early apoptosis and 22.1% of late apoptosis. miR-625 has steadily increased apoptosis from early to late apoptosis by more than 12% compared to scramble (Figure 4).

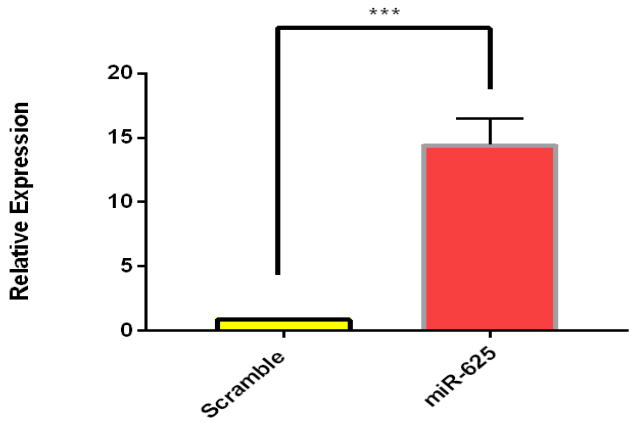

Figure 1. miR-625 is up-regulated. The relative expression level of miR-625 was up-regulated in transfected compared to control scramble was evaluated by qRT-PCR (*P<0.05). Relative expression of miR-625 was up-regulated significantly compared to scramble, which has confirmed the transfection of up-regulation miR-625 into KG1 cell as the main focus of this study.

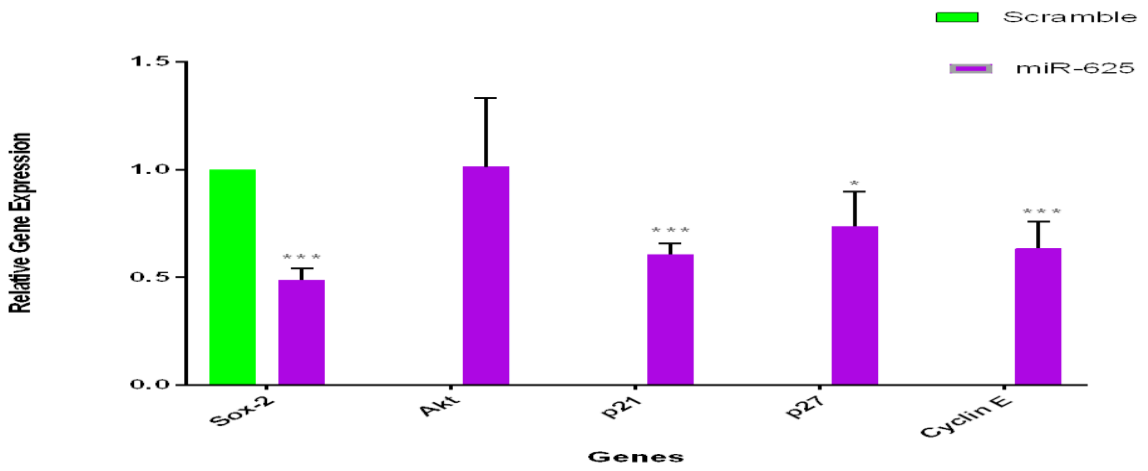

Figure 2. Sox2, p21, p27 and cyclin E are all down-regulated. After transfection of KG1 cells with up-regulated miR-625, the relative expression of Sox2 p21, p27 and cyclin E to control GAPDH were all decreased except for Akt. All resulted were validated by qRT-PCR, which shows (*P<0.05). The up-regulation of miR-625 plays a significant role in suppressing the expression of Sox2, p21, p27 and cyclin E, which all relative genes expressions were validated by qRT-PCR.

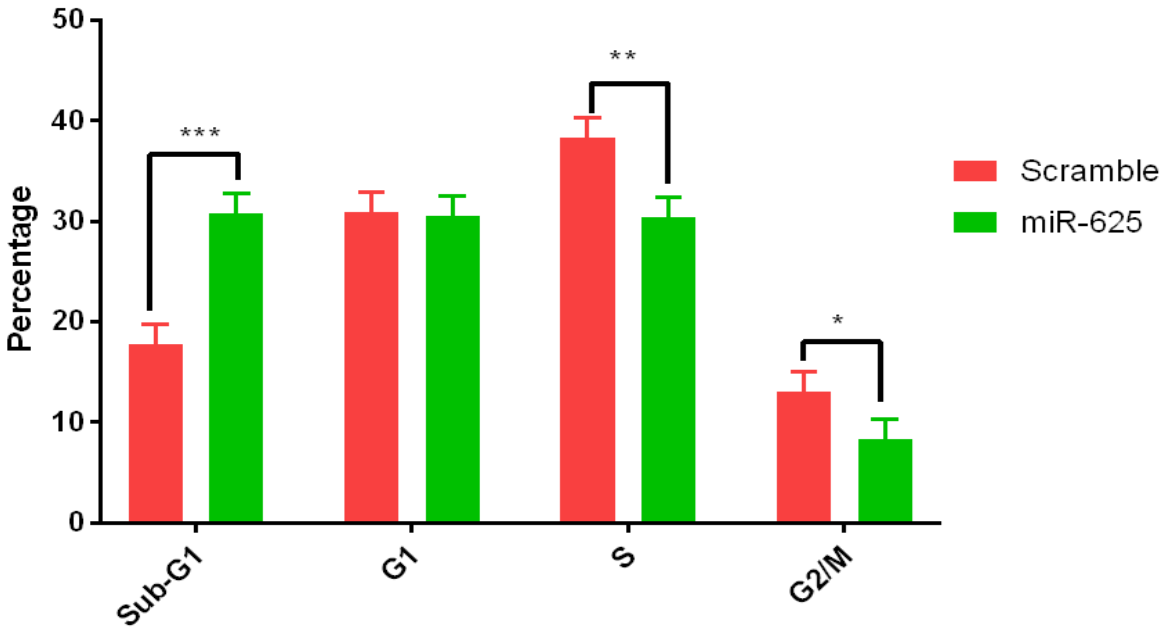

Figure 3. Decrease in the proliferation of DNA content and induce cycle arrest transfection. At sub-G1, scramble average mean was 17.8% and miR-625 mean 30.81%. However, cell cycle decreases at S and G2/M for miR-625 of about 8.34% at G2/M compared to the scramble of 13%. DNA content changes during cell cycle progression at the G_1 phase, where both miR-625 and scramble remains almost the same at G1 at around 30% but dramatically changes at G2/M.

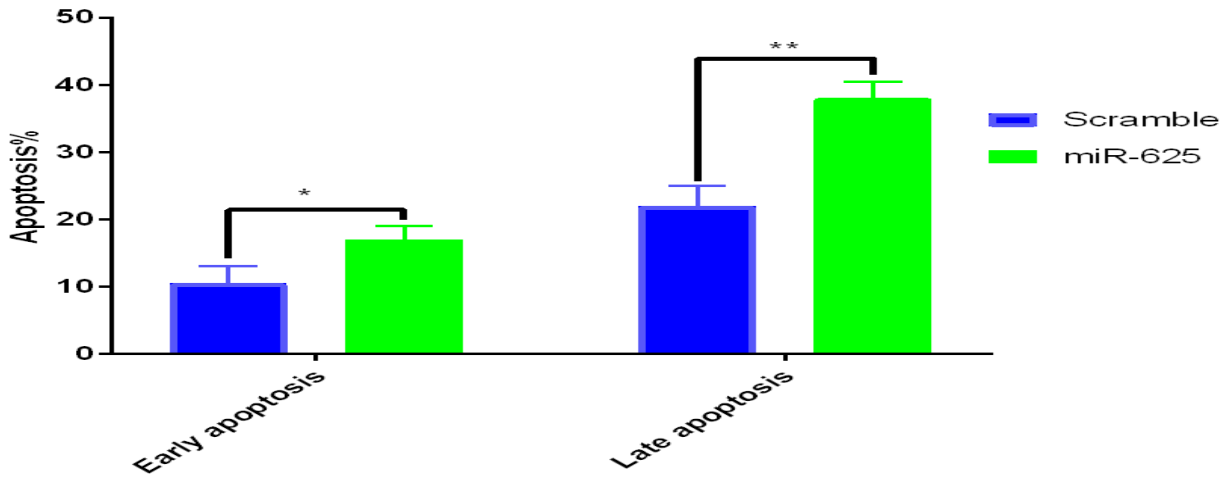

Figure 4. Significant increase of early and late apoptosis phases. miR-625 has a mean of 17% of early apoptosis and increased to 38% of late apoptosis while scramble has 10.5% of early apoptosis 22.1% of late apoptosis. miR-625 has steadily increased apoptosis from early to late apoptosis compared to the scramble.

DISCUSSION

MicroRNAs are involved in regulating gene expression, and bout ~30% of human genes have been suggested to be regulated by miRNAs[4]. The function of miRNAs is huge as they are involved in different biological processes, including cell cycle, differentiation, development, and metabolism. They also play a vital role in human diseases, including diabetes, immuno- or neurodegenerative disorders, and cancer. MicroRNA signatures have been identified in both hematological malignancies and solid tumors, where they are closely linked to cancer prognosis and progression[5]. For instance, miR-625-5p plays a crucial role in cell fate as small, potent molecules. Previous studies have indicated that the expression of miR-625 is reduced in AML cell lines[17]. This reduction has been linked to various cancer types, including AML, where miR-625 acts as a tumor suppressor by targeting key oncogenic pathways. miR-625 has different targets, including NTRK3, ILK, FHIT, etc. ILK is one of the direct targets of miR-625-5p (mirtarbase.mbc.nctu.edu.tw), confirmed with validation methods such as Reporter assay, qPCR, and NGS. Li C et al. identified that ILK is a direct target gene for miR-625 [18]. Through ILK downstream targets AKT, GSK3, NF-κB, Caspase 3, c-fos, β-catenin, and Cyclin D1 and ILK are associated with two key processes in tumorigenesis: cell proliferation and apoptosis. ILK is aberrantly activated in malignancies such as AML [19]. In this study, we confirmed that the up-regulation of miR-625 resulted in the decreased expression of Sox2 and other cell cycle regulatory proteins, such as p21, p27, and cyclin E, through the PI3K/Akt signaling pathway. This finding is consistent with the role of miR-625 as a regulator of cell proliferation and apoptosis in AML cells.

The therapeutic approach based on miRNA has been widely investigated in several cancer cells in various oncogenic pathways, including PI3K/Akt, through different targets such as ILK and Sox2[20,21]. The level of Sox2 expression was higher in AML cells as reviewed by other studies, but it significantly decreased after transfection with the up-regulation of miR-625[22]. Sox2 expression has been shown to increase in KG1 cells and has been identified in many tumor cells. Thus, it provides attractive therapeutic targets for the best alternative treatment for cancer patients[23]. Previous research has identified Sox2 involvement in the Akt signaling pathway, affecting crucial functions in cell survival and growth via direct or indirect regulation of apoptotic factors and cell cycle regulators, such as caspase-9, Bad, Fas, p21, and p27[24]. Few reports have also shown the aberrant expression of microRNA (miR) 625 and Sox2

expression in various cancers including colorectal cancer, breast cancer, hepatocellular carcinoma (HCC), esophageal squamous cell carcinoma (ESCC), gastric cancer, malignant pleural mesothelioma, acute lymphoblastic leukemia, and multiple myeloma. MiR-625 down-regulation and Sox2 up-regulation were also confirmed in esophageal cancer samples[9,10]. It has been reported that the low expression of miR-625 promotes cell proliferation and invasion while high expression of miR-625 has been shown to down-regulate the Sox2 gene expression. Another study has also indicated that in malignant melanoma, the effect of miR-625 roles targeted Sox2 as a tumor suppressor of malignant cell development and progression[10]. It has been obvious that Sox2 has a major role in embryonic and many other cellular development processes. An abnormal high expression of Sox2 has been shown to be involved in increased proliferation, impaired differentiation, and inhibited apoptosis in AML cells[25].

Our results indicate that transfection with miR-625 has led to decreased proliferation, increased apoptosis, and induced cell cycle arrest in KG1 cells. This suggests that Sox2 has fundamental effects on proliferation, apoptosis, and the cell cycle in AML cells. These findings support the potential of targeting Sox2 through miR-625 up-regulation as a therapeutic approach in AML treatment. Sox2 was over-expressed and, ultimately, increased proliferation and inhibited apoptosis in the continuous progression of AML cells as reported in other cancers from the previous studies [26]. However, the DNA content during the cell cycle progression has been shown to decrease after transfection with miR-625 up-regulation by knowing that the Sox2 expression has reduced proliferation, induced apoptosis, and the cell cycle arrest as observed in flow cytometry and confirmed by qRT-PCR.

Several studies have described that the activation of PI3K/Akt signaling is most commonly found in AML cells. The phosphorylation of both Thr 308 and Ser 473 Akt accounts for about 50-70% of patients with AML. Approximately 75% of AML patients had PTEN phosphorylation, which was significantly associated with Akt phosphorylation and shorter overall survival. AML cases found with PI3K/Akt/mTOR up-regulated had shorter survival rates for both disease-free and overall survival rates[25,15]. Thus, the up-regulation of miR-625 and low relative expression levels of Sox2, p21, p27, and cyclin E were all confirmed by quantitative RT-PCR. In this study, the up-regulation of miR-625 significantly decreased proliferation, increased apoptosis, and induced cell cycle arrest. Therefore, targeting Sox2 by up-regulating miR-625 ultimately suppresses AML cell proliferation and induces apoptosis and cell cycle arrest as outlined in Figure 5.

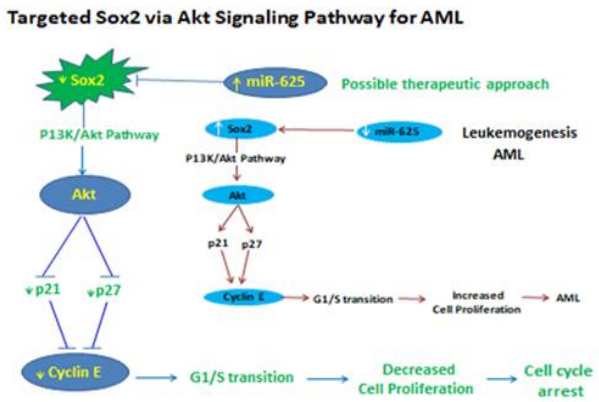

Figure 5. Illustration of possible therapeutic approach targeting Sox2, Akt, p21, p27 and cyclin E to decrease AML cell proliferation, increase apoptosis and induce cell cycle arrest

CONCLUSION

In this study, the up-regulation of miR-625 played an important role in decreasing proliferation, increasing apoptosis, and inducing cell cycle arrest. Low levels of Sox2, p21, and cyclin E and the up-regulation of p27 were confirmed and validated by qRT-PCR. Therefore, targeting Sox2 by up-regulating miR-625 has significantly suppressed AML cell proliferation and induced apoptosis and cell cycle arrest.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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Ethics approval and consent to participate

The present study was approved by The Ethics Committee of the Tehran University of Medical Sciences.

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