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Effects of L-asparaginase on The Expression of *EBF1* Gene, miR-181b, and miR-17 and Its Association with Apoptosis in the Nalm-6 Cell Line

Golnaz Minaeeian[1], Reza Ranjbaran[1], Parisa Tandel[1], Farahnaz Zare[1] and Gholamhossein Tamaddon[1,2*]

[1]Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran

[2]Division of Hematology and Blood Bank, Department of Laboratory Sciences, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran

[*]Corresponding e-mail: tamaddon.g@gmail.com

ABSTRACT

Background: *L-asparaginase is one of the most important and common drugs in the treatment of children and adults with acute lymphoblastic leukemia. Transcription factors play a key role in the prognosis, treatment, and recurrence of cancer. The EBF1 transcription factor is essential for B cell commitment and differentiation. Moreover, miRNAs are involved in many cellular processes such as cancer.* ***Aim of the work:*** *This study aimed to evaluate the effect of L-asparaginase on the EBF1 gene, miR-181b, and miR-17 expression and to examine its relationship with apoptosis in the Nalm-6 cell line.* ***Material and Methods:*** *In this experimental study, the Nalm-6 cell line was cultured in optimal condition, and treated with L-asparaginase. An ideal concentration of L-asparaginase for the Nalm-6 cell was determined by using the MTT assay. Before and after treatment with L-asparaginase, Apoptosis was evaluated by using flow cytometry, also the expression of EBF1 gene, miR-181b, and miR-17 was performed by real-time PCR assay.* ***Results:*** *The results showed significant percentages of apoptotic cells at 48 (34.35%) and 72 hours (67.30%) after treatment with L-asparaginase. There was no significant difference in the EBF1 gene expression before and after treatment ($p<0.07$). The expression of miR-181b ($p<0.0001$) and miR-17 ($p<0.0001$) was significantly decreased in the treated Nalm-6 cells as compared to the untreated Nalm-6 cells.* ***Conclusion:*** *After treatment Nalm-6 by L-asparaginase, there was a remarkable increase in apoptosis which was time-dependent. The expression of miR-181b and miR-17 was also notably reduced but there was no significant change in EBF1gene expression.*

Keywords: B-ALL, EBF1, L-asparaginase, microRNA, Nalm-6

INTRODUCTION

Acute Lymphoblastic Leukemia (ALL) is a common hematological malignancy in children, and about 85% of cases are precursor B cell phenotypes [1]. Treatment of leukemia is based on chemotherapy. L-asparaginase is one of the most important and common drugs in the treatment of ALL children and adults [2]. There are two general mechanisms involved in leukemia, that is, oncogenic protein activation and oncogenic hybrid gene generation. In both mechanisms, the protein products are often transcription factors [3]. Therefore, transcription factors play an important role in the progression, relapse, and even treatment of cancer [1].

In more than 80 cases of ALL, mutation of transcription factors, such as *IKZF1* (the most frequent one), *PAX5*, and *EBF1*, was observed, which may involve the development and survival of B cells [4]. Considering the significant role of transcription factors, such as *EBF1*, in the regulation and differentiation of normal B lymphocytes, dysfunction of these factors may result in growth retardation and development of B-ALL [5].

The miRNAs are short, non-encoding sequences with 20-24 nucleotides that play an important role in all processes in mammals and multicellular organisms. Also, miRNAs are involved in several cancer-related processes, including cell proliferation, cell cycle control, apoptosis, differentiation, migration, and metabolism [6,7]. In recent years, miRNAs

have emerged as important biomarkers in cancers, including ALL, and several recent studies have demonstrated their role in leukemia, diseases, and mortality [8]. Six members of the miR-181 family have been identified in the human genome, including miR-181a and miR-181b on chromosome 1. Which are involved in lymphopoiesis [9]. The miR17-92 cluster includes miR17, miR18a, miR19a, miR20a, miR19b-1, and miR92a-1, the expression of which increased in ALL patients. Many studies have evaluated the miRNA expression in children with ALL [10].

The present study aimed to evaluate the effect of L-asparaginase on the *EBF1* gene miR-181b expression, miR-17 expression and to examine its relationship with apoptosis in the Nalm-6 cell line.

MATERIAL AND METHODS

Cell Culture and Treatment

This experimental study was approved by the local ethics committee of medical experiments on human subjects of Shiraz University of Medical Sciences (IR.SUMS.REC.1398.1131). The B-cell precursor leukemia cell line (Nalm-6 cell line) was purchased from the American Type Culture Collection (ATCC; Gaithersburg, Maryland, USA). All materials and reagents used for the cell culture were purchased from Gibco™ Life Technologies (Waltham, MA, USA) and Sigma-Aldrich (Munich, Germany). The cell line was cultured in RPMI-1640 medium, supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL of the penicillin-streptomycin solution, and 2 mM L-glutamine (Invitrogen) at 37°C in a 5% CO_2 atmosphere. The culture medium was changed according to the standard cell culture techniques to ensure cellular integrity.

Trypan Blue Exclusion Assay

To assess the inhibitory effect of L-asparaginase on cell growth and viability, the Nalm-6 cells were seeded at 1×10^3 cells/mL in a complete growth medium and treated with different concentrations of L-asparaginase. Next, the cell suspension was centrifuged, and the cell pellet was re-suspended in the complete growth medium. The cell suspension was then mixed with 0.4% trypan blue and loaded onto the chamber of the Neubauer hemocytometer. Viability (%) was measured as follows:

Viability (%): Viable cell count/total cell count × 100

MTT Assay

The Nalm-6 cell line was seeded onto 96-well cell culture plates (13×10^3 cells/well) in 100 µL of complete growth medium. The cells were treated with different concentrations of L-asparaginase (8, 10, 15, 20, 40, and 60) µM for various time intervals (48 hours and 72 hours). Cell viability was determined using 10 µL of a 5-mg/ml 3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyl-tetrazolium bromide (MTT) solution (Melford, Ipswich, UK). After incubation at 37°C for four hours, the MTT solution was removed, and 150 µL of Dimethyl Sulfoxide (DMSO) was added to dissolve formazan crystals, based on the conversion of MTT to MTT formazan by mitochondrial enzymes. Spectrometric absorbance was measured at 545 nm, using a Stat Fax 2100 Microplate Photometer (Stat Fax 2100, SKU: 8036-10-0020). The optimal concentration of L-asparaginase was determined by measuring the half-maximal inhibitory concentration (IC_{50}) for the Nalm-6 cell line using the MTT assay. At least three independent experiments were performed in quadruplicate.

Assessment of Apoptosis by Flow Cytometry

To confirm the MTT assay results, 1×10^{13} cells were seeded on a 24-well plate and treated with different concentrations of L-asparaginase (8 µM and 20 µM; untreated as negative control) for 48 hours and 72 hours. Cell survival was determined using the PE/Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA). The cells were washed with Phosphate-Buffered Saline (PBS) and suspended in a total volume of 100 µL of binding buffer. Next, annexin V (2/5 µl/sample) and PI (2 µl/sample) was added, and cell suspensions were incubated for 20 minutes in darkness. The cells were then topped with 400 µL of binding buffer and analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) for one hour. The FlowJo.7 software (TreeStar LLC, USA) was used for the analysis of data.

RNA Isolation

Total RNA was extracted using TRIzol LS reagent (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. Quantification of RNA was also performed with a NanoDrop instrument (Hellma Analytics, NY, USA).

Real-time PCR Assay for *EBF1* Gene

Complementary DNA (cDNA) was synthesized by reverse transcription using an appropriate amount of total RNA and the Prime Script™ RT reagent Kit (Yektatajhiz Azma, Iran). Quantitative real-time PCR of the *EBF1* gene was performed using specific gene primers and SYBR Green qPCR Master Mix (Yekta Tajhiz Azma, Iran), according to the manufacturer's instructions in a Rotor-Gene Q system (Qiagen, Hilden, USA). The qRT-PCR assay was run under the following conditions: initial denaturation at 95°C for five minutes, followed by 40 cycles of 95°C for five seconds, 59°C for 20 seconds, and 72°C for 30 seconds. Each sample was analyzed in triplicate, and the comparative Ct ($2^{-\Delta\Delta CT}$) method was used to determine the relative expression level of the *EBF1* gene. The primers used are listed in Table 1.

Table 1 Primers used in reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay

Product size (bp)	Sequence	Gene
145	ATGCTGGTCTGGAGTGAGTTG	*EBF1-F*
	CTGTATAAATGAATCTGCCTGGTGTT	*EBF1-R*
284	TATGCCTGCCGTGTGAAC	*β2M-F*
	CTACCTGTGGAGCAACCTG	*β2M-R*

Real-time PCR Assay for miR-181b and miR-17 Expression

The miRNA-specific cDNA was synthesized, using the cDNA Synthesis Kit (Exiqon, Vedbaek, Denmark) with specific primers (Exiqon, Vedbaek, Denmark). First, a poly (A) tail was added to miRNAs with a poly (A) polymerase at 37°C. Next, a Reverse Transcription (RT) enzyme, a reaction buffer, and miR-specific primers for cDNA synthesis were mixed with the RNA poly (A) tail. The mixture was then incubated at 45°C for 60 minutes and inactivated at 85ºC for one minute. The cDNA aliquots were used for quantitative RT-PCR (qRT-PCR), using RT-PCR Master Mix (Exiqon, Vedbaek, Denmark) in a Real time-PCR Thermal Cycler device (Applied Biosystem, USA), according to the manufacturer's instructions.

The qRT-PCR assay was run under the following conditions: initial denaturation at 95°C for five minutes, followed by 40 cycles of 95°C for five seconds, 63°C for 20 seconds, and 72°C for 30 seconds. All qRT-PCR assays were performed in triplicate. Each sample was analyzed in triplicate, and the comparative Ct ($2^{-\Delta\Delta CT}$) method was used to determine the relative expression of miR-181b and miR-17. The U6 as an internal control was used to normalize the cDNA level. The used primers are listed in Table 2.

Table 2 The miR-specific primers for the cDNA synthesis reaction

miRNAs	Sequences
miR-181b	5′ AACAUUCAUUGCUGUCGGUGGGU 3′
miR-17-5P	5′ CAAAGUGCUUACAGUGCAGGUAG 3′

Statistical Analysis

The miRNA and gene expression levels were calculated, using the $2^{-\Delta\Delta CT}$ method. All analyses were performed using SPSS v.26 and Graph Pad Prism 7.05 (Graph Pad Software Inc., San Diego, CA, USA). All experiments were repeated three times and expressed as mean ± SD. The means of the groups were compared with the student's paired t-test and one-way Analysis Of Variance (ANOVA). The correlation between the expression level of genes, miRNAs, and apoptosis was analyzed by Pearson's correlation test. p-value less than 0.05 was considered statistically significant.

RESULTS

Analysis of the Effect of L-asparaginase on Cell Viability

The effects of L-asparaginase on cell proliferation in the concentration range of 8 µM to 20 µM after 48 to 72 hours of

exposure to L-asparaginase were assessed by the MTT assay (Figure 1). The optimal concentration of L-asparaginase was determined by measuring its IC_{50}.

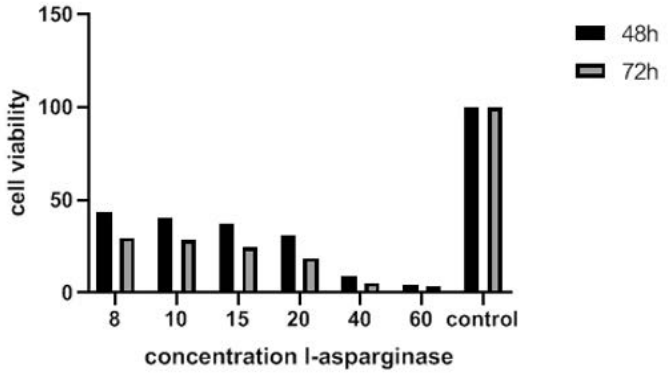

Figure 1 The Nalm-6 cells were treated with varying doses of L-asparaginase for 48 and 72 hours and assayed by the MTT assay; absorbance was read at 545 nm; data have presented the mean of three independent experiments performed in quadruplicate

L-asparaginase Induced Apoptosis in the Nalm-6 Cell Line

To determine early and late apoptotic populations in the Nalm-6 cell line induced by L-asparaginase, flow cytometry was performed using Annexin V-PI apoptosis labeling. The Nalm-6 cells were incubated with 8 µM and 20 µM L-asparaginase for 48 hours and 72 hours. The results showed a significant percentage of early and late apoptotic cells after 48 hours and 72 hours of treatment with L-asparaginase (34.35% and 67.30%, respectively; $p<0.0001$ and $p<0.0001$, respectively) (Figure 2).

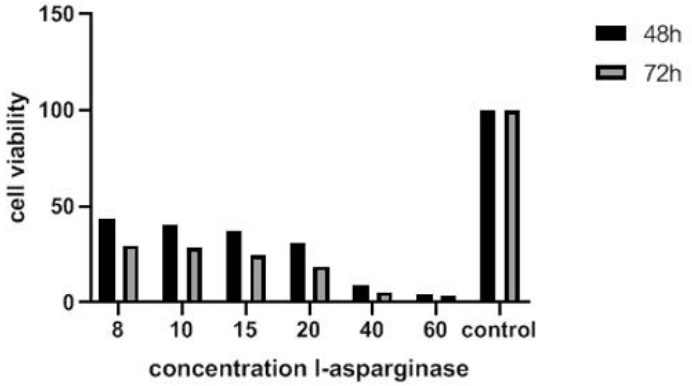

Figure 2 A): The effects of L-asparaginase on the apoptosis of Nalm-6 cells. The cells were incubated with 8 and 20 µM L-asparaginase for 48 hours. They were incubated with 8 and 20 µM L-asparaginase for 72 hours; B): They were incubated with 8 and 20 µM L-asparaginase; C): The apoptosis in the graph is shown as a percentage. Each experiment was performed in triplicate ($p<0.0001$)

EBF1 Gene mRNA Expression in the Nalm-6 Cell Line

The *EBF1* gene expression level in the Nalm-6 cell line was determined and compared with its expression level after treatment with L-asparaginase. Based on the RT-PCR assay, no significant difference was found in the *EBF1* mRNA expression in the B-ALL cell line compared to the post-treatment stage ($p<0.07$) (Figure 3).

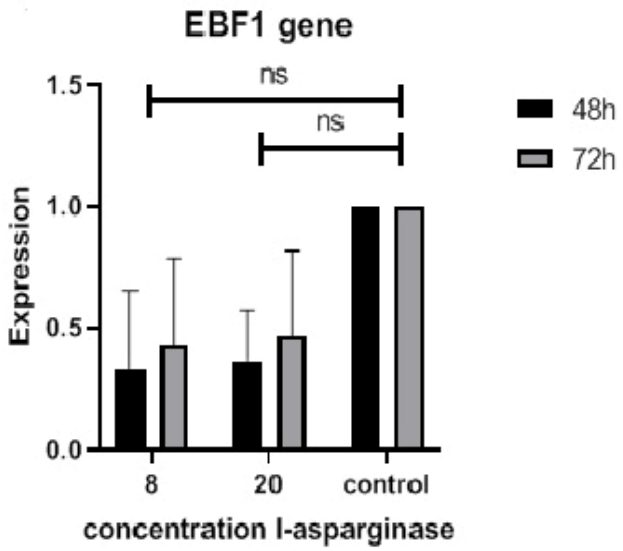

Figure 3 The level of EBF1 in the L-asparaginase-treated Nalm-6 cell line; the expression level was measured before and after treatment by RT-PCR (p<0.007)

Effect of L-asparaginase on the miR-181b and miR-17 Expression

In this study, the expression levels of miR-181b and miR-17 were evaluated in the Nalm-6 cell line before and after treatment with L-asparaginase. The RT-PCR assay showed that the expression of miR-181b (p>0.0001) and miR-17 (p>0.0001) significantly decreased in the treated Nalm-6 cells as compared to the untreated Nalm-6 cells (Figure 4A, and Figure 4B).

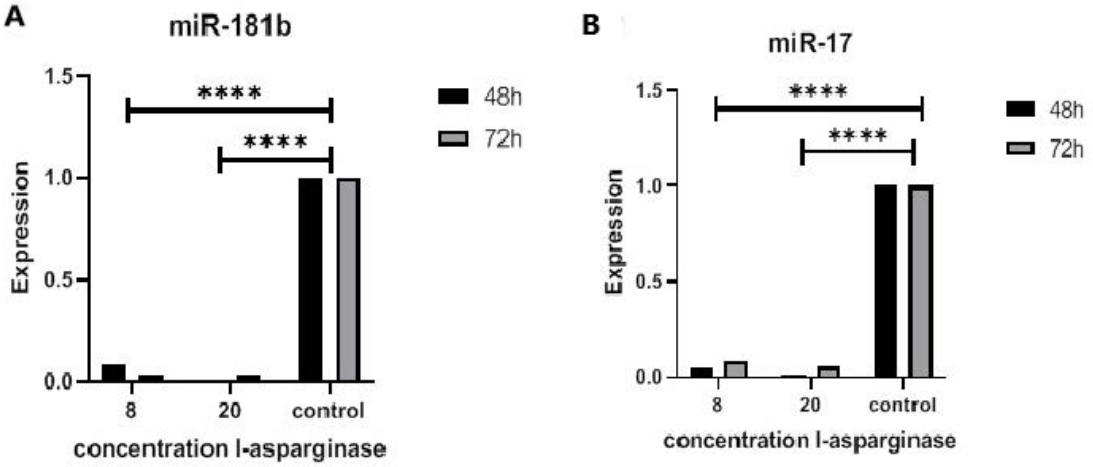

Figure 4 The level of EBF1, miR-181b, miR-17 in the L-asparaginase treated Nalm-6 cell line; the expression level was measured before and after treatment by RT-PCR; A): (p<0.0001); B) (p<0.0001) were respectively

Relationship between *EBF1* Gene and Apoptosis

The relationship between the expression of the *EBF1* gene in the Nalm-6 cell line and apoptosis was evaluated in this study. The results of Pearson's correlation test showed that the expression level of the *EBF1* gene had a significant inverse correlation with apoptosis (p=0.02; r= -0.535) (Figure 5).

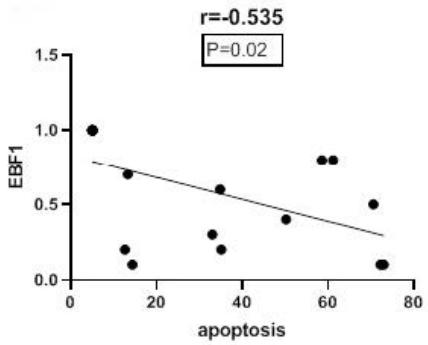

Figure 5 Correlation expression of *EBF1* gene, and apoptosis; these were inversely significant for *EBF1* gene (p=0.02) (r= -0.535)

Relationship between miR-181b and miR-17 Expression and Apoptosis

The relationship between the expression of miR-181b and miR-17 and apoptosis was evaluated in the Nalm-6 cell line. The results of Pearson's test showed that the expression of miR-181b (p=0.03; r= -0.496) and miR-17 (p=0.001; r= -0.691) had a significant inverse correlation with apoptosis (Figure 6A, and Figure 6B).

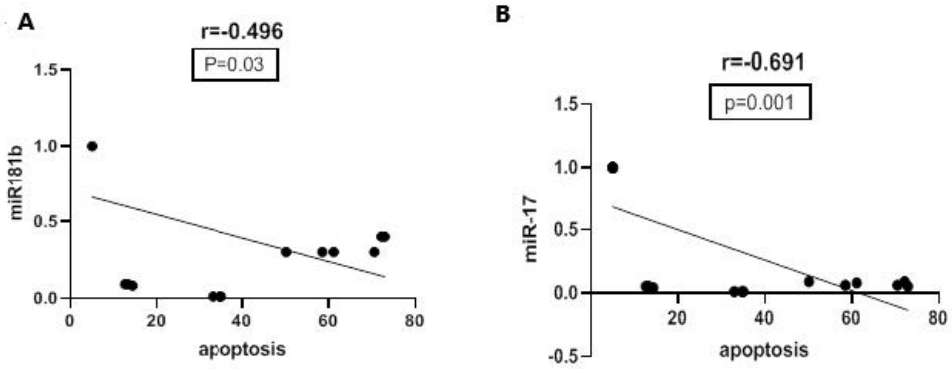

Figure 6 Correlation expressions of miR-181b, miR-17 and apoptosis. These were inversely significant for; A): miR-181b (p=0.03) (r= -0.496); B): miR-17 (p=0.001) (r= -0.691)

Relationship between miR-181b and miR-17 Expression and *EBF1* gene

The results of Pearson's test showed that the expression levels of miR-181b (p<0.0001; r=0.797) and miR-17 (p<0.0001; r=0.801) had significant positive correlations with *EBF1* gene expression (Figure 7A, and Figure 7B).

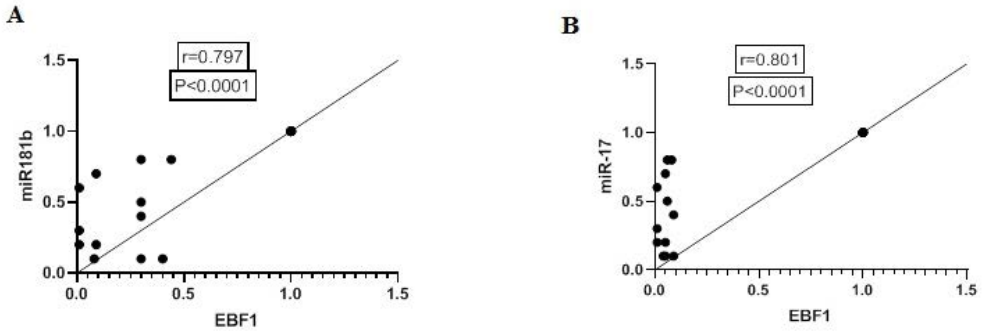

Figure 7 A): The correlation between the expression of miR-181b and *EBF1* gene was positive and significant (p<0.0001; r=0.797); B): The correlation between the expression of miR-17 and *EBF1* gene was positive and significant (p<0.0001; r=0.801)

DISCUSSION

In the present study, the expression of the *EBF1* gene, miR-181b, and miR-17 before and after treatment with L-asparaginase was examined in the Nalm-6 cell line. According to the results, L-asparaginase could inhibit the growth of Nalm-6 cells both in time- and dose-dependent manners.

ALL is a common hematological malignancy in children. In about 85% of ALL cases, B cell precursors are involved [1]. In B-ALL, the B-cell-specific transcription network is disrupted. The EBF1 transcription factor is essential for B cell development, and its absence leads to growth retardation in the Common Lymphoid Progenitor (CLP) stage [11]. This factor is also involved in the early maturation of B cells, as its inactivation greatly reduces the immune response and leads to the destruction of follicular B cells and the germinal centre [12]. Moreover, Wang, et al. showed that the *EBF1* gene is a transcription factor, which plays an important role in regulating B-cell differentiation and is also active as a tumour suppressor gene in CLL [12]. Harris LM, et al. also reported that the *EBF1* gene plays an important role in regulating B-cell differentiation and is also active as a tumour suppressor gene in ALL [13].

In line with the results of the above studies, in the present study, following the exposure of NALM-6 cells to L-asparaginase at concentrations of 8 μM and 20 μM for 48 and 72 hours and inhibition of cell growth, it was observed that the effect of this drug is time-and dose-dependent. But unlike previous results, based on the present results, no significant difference was found in the EBF1 gene expression in the B-ALL cell line before and after the treatment (Figure 3, and Figure 4). It seems that the *EBF1* gene in this cell is not related to the L-asparaginase treatment.

The flow cytometry results showed that L-asparaginase has a significant effect on apoptosis. The present findings showed 13.39% and 34.25% early and late apoptosis after 48 hours at concentrations of 8 μM and 20 μM, respectively ($p<0.0001$). Also, early and late apoptosis percentages of 60.14% and 67.30% were reported after 72 hours at concentrations of 8 μM and 20 μM, respectively ($p<0.0001$) (Figure 2). Ping Song, et al. showed that L-asparaginase could induce growth inhibition and trigger apoptosis in the CML cell lines, KU812 and K562 [14]. Also, Iiboshi, et al. demonstrated that L-asparaginase inactivated cell proliferation. In addition to Jurkat cells, the effects of L-asparaginase on the P70 activity in other human cell lines, including acute B-cell lymphocytic leukemia precursor cells (HAL-01, HYON, NALM-6, ABON, and REH) and the ALL cell line, were examined. L-asparaginase was found to inhibit the P70 activity in these cells at doses similar to Jurkat cells [15]. Like these studies, our findings also show L-asparaginase mainly induces cell death in cancer cells.

The miRNAs play an important role in regulating gene expression, and their lack of regulation is associated with the pathogenesis of solid cancers and hematological malignancies [16]. In the present study, we found that the expression of miR-181b and miR-17 significantly decreased in the Nalm-6 cells after treatment with L-asparaginase, compared to the untreated Nalm-6 cell line (Figure 3, and Figure 4). Wang Yao, et al. reported that by targeting SMAD-7, miR-181b-induced granulosa cell apoptosis acts as an inhibitory and targets the TGFβ signalling pathway. Also, they showed that SMAD-7 is directly inhibited by the corresponding miRNAs [17]. Also, Zhu et al. revealed that the expression of miR-181b was downregulated in SGC7901/VCR and A549/CDDP cell lines as compared to the parental human gastric cancer cell line (SGC7901) and lung cancer cell line (A549) [18]. Also, a study by ZhengY, et al. examined the expression level of miR-181b in breast cancer cells. Their results showed that miR-181b acts as an oncogene possibly by increasing the level of anti-apoptotic Bcl-2 protein in the studied cells. In a study by Zanette, et al. the expression of miR-128, miR-204, miR-218, miR-331, and miR181b-1 was the highest in ALL patients [16,19]. It is essential according to the above studies the target proteins of miR-181b should be examined in the intracellular signalling pathways in NALM-6 to provide the basis for target therapy with the help of this miR-181b. From the results of the present study, it can be concluded that miR-181b can play a biomarker role in the follow-up of treatment. However, it is necessary to examine the samples of treated patients.

Similar to the present study, Scherr M, et al. found that miR-17 was upregulated in the Nalm-6 cell line [20]. Moreover, the results of a study by Yan Hj, et al., which examined the expression level of miR-17 in pancreatic cells, showed that miR-17, through its oncogenicity and decreased level of pro-apoptotic protein BCL11B/Bim, led to the cessation of apoptosis, while no miR-17 increased apoptosis by increasing the proapoptotic proteins, caspase 3 and Bim. In the absence of miR-17, there is a possibility of decreased Bcl-2 expression, increased Bim expression as a pro-apoptotic protein, and, therefore, increased apoptosis [21]. According to the results of the relationship between the expression of miR-17 and apoptosis in the present study, it can be inferred that miR-17 inhibits apoptosis in untreated cells

by targeting apoptotic pathway proteins. With effective treatment, decreased expression of miR-17 was observed, resulting in increased apoptosis. Since the miR17-92 cluster has a common promoter, the seemed to be upregulated in ALL. With proper treatment, this cluster is expected to decrease.

CONCLUSION

Treatment of L-asparaginase in NALM-6 cells increased the rate of apoptosis, but it was time-dependent. Unlike *EBF* a significant decrease in miR-181b and miR-17 expression was found after treatment with L-asparaginase. These two miRNAs can be used as a biomarker to follow the response to the treatment of B-ALL patients. Biologic therapy can also be achieved in subsequent studies by targeting the two miRNAs.

DECLARATIONS

Author Contribution

Gholamhossein Tamaddon contributed to the conception and design of the study. Golnaz Minaeeian and Parisa Tandel performed all procedures and drafted the manuscript. Farahnaz Zare supervised the procedures and performed the statistical analysis. Reza Ranjbaran was involved in interpreting results. All authors read and approved the final manuscript.

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Availability of Data and Materials

Data will be made available on request.

Ethical Approval

This study was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1398.679).

Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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