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RESEARCH ARTICLE

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The effect of decitabine on the expression and methylation of the *PPP1CA*, *BTG2*, and *PTEN* in association with changes in miR-125b, miR-17, and miR-181b in NALM6 cell line

Asma Vafadar[1] | Pooneh Mokaram[2] | Mehran Erfani[2] | Zahra Yousefi[3] | Ali Farhadi[4] | Tehrani Elham Shirazi[5] | Gholamhossein Tamaddon[6]

[1]Department of Medical Biotechnology, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran

[2]Department of Biochemistry, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

[3]Department of Immunology, Faculty of Medicine, Isfahan University of Medical Science, Isfahan, Iran

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

[5]Department of Pharmacology & Toxicology, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran

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

Correspondence
Gholamhossein Tamaddon, PhD, Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran.
Email: tamaddon.g@gmail.com

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Abstract

Precursor B-cell acute lymphoblastic leukemia (B-ALL) is the most prevalent pediatric cancer. DNA methylation and changes in the microRNAs (miRNAs) expression are known to be important causes of B-ALL. Decitabine as a DNA methyltransferase inhibitor agent is able to induce hypomethylation in several tumor suppressor genes. Much evidence has proven *BTG2, PPP1CA*, and *PTEN* act as tumor suppressor genes in many malignancies. In this case control study, the messenger RNA (mRNA) expression of *PPP1CA, BTG2*, and *PTEN* genes using quantitative real-time polymerase chain reaction (rRT-PCR) in Nalm6 cell line and five patients suffer from ALL with mean age 5.6 years were determined in compare with seven normal healthy donors age and sex matched. qRT-PCR analysis revealed that the expression levels of *PPP1CA, BTG2*, and *PTEN* genes were significantly decreased in Nalm6 ([FC] = 0.46, [FC] = 0.046, [FC] = 0.54) and according to the Methylation-specific PCR (MSP) analysis, these genes were hypermethylated in Nalm6. In next step, the effects of decitabine treatment on the methylation and expression of these genes in association with changes in miR-125b, miR-17, and miR-181b expression levels were evaluated in optimal concentration 2.5 μM of decitabine. Our data showed that decitabine is able to restore the expression levels of aforementioned genes and downregulate expression levels of oncomiRs; including miR-125b, miR-17, and miR-181b in Nalm6 cell line. Therefore, it seems that decitabine can be used as a potential drug for the first line treatment of patients with B-ALL, but further in vivo investigation is necessary.

KEYWORDS

B-ALL, decitabine, DNA methylation, microRNA, NALM6

Abbreviations: ALL, Acute lymphoblastic leukemia; BM, Bone Marrow; CSF, Cerebrospinal Fluid; DAC, Decitabine; miRNA, microRNA; ncRNAs, noncoding RNAs; WHO, World Health Organization.

1 | INTRODUCTION

Precursor B-cell acute lymphoblastic leukemia (B-ALL) is a type of ALL and one of the most prevalent pediatric cancers.[1] This hematological malignancy usually affects children aged 2 to 5 years, but can also be characterized in the adult population.[2,3] Numerous studies have confirmed that in addition to genetic abnormalities, epigenetic modifications are also important in the development of B-ALL.[4] Recent evidence demonstrates that among these epigenetic modifications, aberrant promoter DNA methylation and microRNAs (miRNAs) have been a fundamental phenomenon in the development of B-ALL.[5,6]

DNA methylation is the best-characterized epigenetic aberration, involved in cellular processing.[7] Alternation in DNA methylation patterns appears in two ways; hypermethylation in CpG islands of tumor suppressor genes (TSG), leading to transcriptional gene silencing and global DNA hypomethylation.[8,9]

Recent studies have introduced some genes, involved in this type of leukemia, but in spite of the important role of *PPP1CA*, *BTG2*, and *PTEN* in various malignancies, their role has not yet been studied in B-ALL.

PPP1CA (protein phosphatase 1 catalytic subunit alpha) is referred to the alpha subunit of PP1 protein can act as a tumor suppressor. Protein phosphatase 1 (PP1) is a eukaryotic protein that has an important role in regulating cell cycle and many cellular processes.[10]

B cell translocation gene 2 (*BTG2*) belongs to the family of the BTG/TOB anti-proliferation gene. *BTG2* known as a TSG increased apoptosis through suppression of G1/S and G2/M transition, cell differentiation, repair of DNA damage, and cell proliferation.[11]

Phosphatase and tensin homolog (*PTEN*) is known as a multifunctional TSG in many cancers. Several factors have the important effects in the inactivation of the *PTEN*.[12]

In addition to DNA methylation, changes in the expression levels of miRNAs are also known to be common causes of this leukemia.[6] miRNAs are a class of small, endogenous noncoding RNAs with approximately 22 to 24 nucleotides (nt) that play a significant role as biological regulators in many cellular processes.[13,14] They target the mRNAs of protein-coding genes, attach to a region in the 3′-untranslated regions (3′-UTRs), and suppress the expression of the gene, by either mRNA degradation, inhibiting translation, or both during the posttranscriptional stage.[15] Accordingly, miR-125b, miR-17, and miR-181b, can target *PPP1CA*, *BTG2*, and *PTEN,* respectively, based on miRNAs databases, which they were chosen in this study for further investigation.

Decitabine (DAC), or 5-aza-2′-deoxycytidine is the most powerful hypomethylating agent that inhibits DNA methylation by repressing the activity of DNA methyltransferase. This drug is used for treating myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). Several studies have demonstrated that decitabine can cause re-expression of the TSGs that are aberrantly methylated.[16,17]

The efficacy of DAC in B-ALL has not yet been completely investigated, also few studies have been conducted experiments on the clinical effect of decitabine on patients with relapsed and refractory B-ALL.[18]

With this approach, many evidence have proven that *BTG2*, *PPP1CA*, and *PTEN* act as TSGs in many malignancies, the expression and methylation status of *BTG2, PPP1CA*, and *PTEN* in Nalm-6 cell line have not been studied yet. The aims of this study were to determine the expression and promoter methylation status of *BTG2, PPP1CA*, and *PTEN* in Nalm-6 cell line. We also investigated the effect of treatment with decitabine, on the methylation and expression of these genes, in association with changes in miR-125b, miR-17, and miR-181b expression levels.

2 | MATERIALS AND METHODS

2.1 | Cell line and blood samples

The B-cell precursor leukemia cell line (Nalm-6) was kindly provided by Dr. Majid Safa (Iran University of Medical Sciences, Tehran, Iran) which previously was purchased from ATCC. All materials and reagents used for cell culture were purchased from Gibco Life Technologies (Waltham, MA) and Sigma-Aldrich (Munich, Germany). The Cell line was cultured in RIPM-1640, supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin, and 2 mM L-glutamine supplied by Invitrogen at 37°C, under a 5% CO_2 atmosphere. Culture medium was changed, according to standard cell culture techniques to ensure cellular integrity. Trypan blue exclusion methodology was used to assess cell viability.

Five newly-diagnosed patients with ALL were included in this study. The mean age of ALL patients was 5.6 years (range 1.5-13 years). In addition, heparinized peripheral blood (PB) samples were collected from seven normal healthy donors with a median age of 6.5 years (range 4-10 years). All subjects received the consent letter. Diagnosis of patients was based on cytomorphological and immunophenotypic features of bone marrow (BM) leukemic cells. Sampling was conducted at first display before the therapeutic interruption. This study

was approved by the Ethics Committee (1396-01-10-14618), Shiraz University of Medical Sciences.

2.2 | Isolation of PBMCs and B lymphocyte preparation

Peripheral blood mononuclear cells (PBMC) were isolated from all subjects, using Ficoll-Paque density centrifugation (Courtaboeuf, France, Paris). The human B cells were obtained from PBMCs, based on MACS positive selection and anti-CD19 microbead and LS column (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of cells was > 95%, as determined by flow cytometry.

Isolated B cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 15% FBS, 100 U/mL penicillin-streptomycin, 2 mM L-glutamine, nonessential amino acids and 0.5 μM CpG-ODN 2006 (Miltenyi Biotec, Bergisch Gladbach, Germany) for 6 days in 5% CO_2 atmosphere at 37°C.

2.3 | MTT assay

Nalm6 cell line was seeded onto 96-well cell culture plates, 1×10^4 cells/well in 200 μL growth medium, and then treated with different 5-aza-2′-deoxycytidine (DAC) (0,1, 2.5, 5, and 10 μM) for various time-points (72 and 96 hours). Following treatment, cell viability was determined using 20 μL of sterile 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, Germany, Darmstadt) assay, after incubation at 37°C for 4 hours, the MTT solution was removed, and 150 μL dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes. Spectrometric absorbance at 570 nm was measured using a BioTek ELx800 microplate photometer (BioTek ELx800, SN211805; BioTek, Winooski, VT). We determined an optimal concentration of DAC by investigating its IC-50 for Nalm6 cell line by MTT assay. At least three independent experiments were performed in quadruplicate.

2.4 | 5-aza-2′-deoxycytidine (DAC) treatment

To study the effect of epigenetic modulation, Nalm6 cell line was seeded at 3×10^5 cells/100-mm dish for 24 hours before the treatment in RIPM-1640 supplemented with 10% FBS at 37°C and 5% CO_2. Cells were exposed for 96 hours to 2.5 μM DAC (Sigma-Aldrich). The culture media was replaced every 24 hours with fresh media containing DAC. Stock solutions of DAC were prepared fresh every day, sterilized by filtration, and kept at 0°C until needed. Control cells were cultured in the same medium containing DMSO in the same concentration as present in the drug treated cells. For drug treatment, DAC was dissolved in DMSO. After the treatment, cells were washed with PBS, centrifuged at 1000 *g* for 5 minutes and subjected for gDNA and RNA isolation.

2.5 | RNA isolation

Total RNA was extracted using TRIzol (Thermo Fisher Scientific, MA) according to the manufacturer's instructions. The concentrations of extracted RNA were determined using Nano Drop instrument (Hellma, NY).

2.6 | Real-time PCR assays for *PPP1CA*, *BTG2*, and *PTEN*

Complementary DNA (cDNA) was synthesized by reverse transcription using an appropriate amount of total RNA and using a PrimeScriptTM RT reagent Kit (Takara, Tokyo, Japan). Quantitative real-time PCR was performed using a SYBR Premix Ex Taq (Takara, Japan) on the applied biosystems ™ ABI (Applied Biosystems, MA). Relative quantification of gene expression was performed using *GAPDH* gene as the internal control. The qRT-PCR always included a no-template sample as a negative control. The experiments were repeated twice. The comparative $2^{-\Delta\Delta Ct}$ method was used for relative quantification of gene expression on duplicate of each reaction. The primers were used are listed in Table 1.

TABLE 1 Primers used in reverse transcription-quantitative polymerase chain reaction.

Gene	Forward/reverse	Sequence	Product size (bp)
PPP1CA	Forward	5′-GCCAGCATCAACCGCATC-3′	236
	Reverse	5′-CACAGCAGGTCACACAGC-3′	
BTG2	Forward	5′-GAGCCACGGGAAGGGAAC-3′	217
	Reverse	5′-CTTGTGGTTGATGCGAATGC-3′	
PTEN	Forward	5′-CCAGTCAGAGGCGCTATGTG-3′	207
	Reverse	5′-ACTTGTCTTCCCGTCGTGTG-3′	

Abbreviations: Bp, base pair; nt, nucleotide.

2.7 | Real-time PCR assays for mature miRNAs

For the reverse transcription (RT) reaction of the miRNAs, specific RT primers (Exiqon, Vedbaek, Denmark) and cDNA synthesis kit (Exiqon) were used following their protocol. QRT-PCRs of the miR-215b, miR-17, and miR-181b were performed using the Real-time PCR Master Mix (Exiqon), specific primers (Exiqon) and athe pplied biosystems ™ ABI (Applied Biosystems) according to the manufacturer's instructions. 5 seconds ribosomal RNA (rRNA) was used as endogenous control and normalizing the miRNAs expressions. The q-RT-PCR was run under the following conditions: initial denaturation at 95°C for 5 minutes that followed by 40 cycles of 95°C for 5 seconds, 63°C for 20 seconds, and 72°C for 30 seconds. The fold changes were calculated by the relative quantification $2^{-\Delta\Delta Ct}$ method. All qRT-PCR reactions were conducted in triplicate and repeated twice.

2.8 | DNA extraction

Genomic DNA of the Nalm6 cell line was extracted using the DNA extraction kit (Cinagene, Tehran,Iran).

2.9 | Bisulfite treatment and methylation-specific PCR (MSP)

Sodium bisulfite conversion of DNA for methylation analysis was conducted by the EpiTect®Bisulfite Kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. Promoter methylation status of *PPP1CA*, *BTG2*, and *PTEN* genes was determined by the methylation-specific PCR (MSP) technique as described by Herman et al.[19] In brief, after the treatment of 2 μg of DNA with sodium bisulfite, unmethylated cytosine is converted to uracils, but methylated cytosine remains unchanged. MSP employs this alteration to definitely amplify either methylated or unmethylated DNA. Primer sequences, annealing temperature, and PCR products size of each gene were reported in Table 2. "Hot-start" PCR was performed in 40 cycles in 50 μL reaction volume containing 25 pmol of forward and reverse primers, 0.2 mM/L dNTPs, and 80 μg bisulfite-modified DNA in 1 × PCR buffer provided by Taq enzyme supplier. The reaction mixture was denatured at 95°C for 5 minutes, and 30 seconds polymerization at 72°C, followed by a single 10-minute extension at 72°C. Epi-Tect®PCR Control set Kit (Qiagen, Germany) was purchased for use as the positive and negative control in methylation-specific PCR assay. Then, 10 μL of amplified PCR products were mixed with 5 μL of loading dye and electrophoresed on 2.5% agarose gel containing gel red with TBE buffer and visualized under UV illumination. This result was always confirmed by repeat MSP.

2.10 | Flow cytometry

Apoptosis assay was conducted using a PE Annexin V Apoptosis Detection kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Briefly, cells were seeded and treated with the 2.5 μmol/L DAC for 72 hours to 96 hours. Afterward, the cells were washed two times with phosphate-buffered saline (PBS) and 1×10^6 cells were resuspended in 1 mL of 1 × Annexin V binding buffer. Cells undergoing apoptotic cell death were analyzed by counting the cells that stained positive for PE Annexin V and negative for 7-AAD, and late stage of apoptosis as PE Annexin V and 7-AAD positive using

TABLE 2 Methylated and unmethylated primers used for used methylation-specific PCR (MSP) for amplification of *PPP1CA*, *BTG2*, and *PTEN* genes

Gene	Sequence	Annealing T (°C)	Product size (bp)
PPP1CA	MF: 5′-TAGCGAGGTTTCGTGGTC-3′	50	175
	MR: 5′-ACCGAACTTCCCGAATTTCT-3′		
	UF: 5′-GGGTAGTGAGGTTTTGTGGTT-3′	55	175
	UR: 5′-ACCAAACTTCCCAAATTTCTCC-3′		
BTG2	MF: 5′-TTCGAGTTTTAAAAATGGGC-3′	46	156
	MR: 5′- CGCTCGCTATCGTCAATA-3′		
	UF: 5′- TAATTTGAGTTTTAAAAATGGGT-3′	53	156
	UR: 5′- TCACTCACTATCATCAATACT-3′		
	MF: 5′-GGTTTCGGAGGTCGTCGGC-3′	60	155
PTEN	MR: 5′-CAACCGAATAATAACTACTACGACG -3′		
	UF: 5′- TGGGTTTTGGAGGTTGTTGGT-3′	58	173
	UR: 5′- ACTTAACTCTAAACCACAACCA-3′		

Abbreviations: M, methylated; U, UN methylated.

FACS Calibur flow cytometer (BD Biosciences). For analysis of Flow cytometry data FlowJo (TreeStar LLC) was used.

2.11 | Statistics

All experiments were repeated three times and were expressed as mean ± SD. All analyses were performed using the GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA). *P* values were calculated using student *t* test and considered significant if *P* value was < 0.05.

3 | RESULTS

3.1 | mRNA expression pattern of *PPP1CA, BTG2*, and *PTEN* in Nalm6 cell line

For determination of expression levels of *PPP1CA, BTG2* and *PTEN* genes in Nalm6 cell line, we analyzed *PPP1CA, BTG2*, and *PTEN* mRNAs level, comparing Nalm6 cell line with mRNA samples extracted from B lymphocytes obtained from healthy donors. RT-qPCR analysis revealed that *PPP1CA, BTG2*, and *PTEN* mRNA levels were significantly lower in B-ALL cell line when compared with normal lymphocytes. *PPP1CA* (median fold change of expression [FC] = 0.46, $P < 0.0001$), *BTG2* ([FC] = 0.046, $P < 0.0001$), and *PTEN* ([FC] = 0.54, $P = 0.0180$) in samples from patients with ALL compared with healthy controls. In this experiment, we also compared the expression levels of *PPP1CA*, *BTG2*, and *PTEN* genes in normal B cells from healthy donors with B Lymphocytes isolated from patients with B-ALL. Analysis of gene expression in B-ALL showed similar result with Nalm6 cell line. Downregulation of *PPP1CA* (median fold-change of expression [FC] = 0.7, $P = 0.02$), *BTG2* ([FC] = 0.51, $P = 0.001$), *PTEN* ([FC] = 0.56, $P = 0.02$) in normal B cells of healthy donors was observed when compared with B Lymphocytes isolated from patients with B-ALL (Figure 1).

3.2 | Analysis the effect of decitabine on cell viability

According to the results, decitabine could inhibit the growth of Nalm6 cells both in time- and dose-dependent manners. The effect of decitabine (1-10 μM) on cell proliferation after 72 to 96 hours of exposure was assessed by the MTT assay (Figure 2). We determined an optimal concentration of decitabine by investigating its IC-50. On the basis of IC-50 results, Nalm6 cells could hardly grow in the presence of 2.5 μmol decitabine for 96 hours.

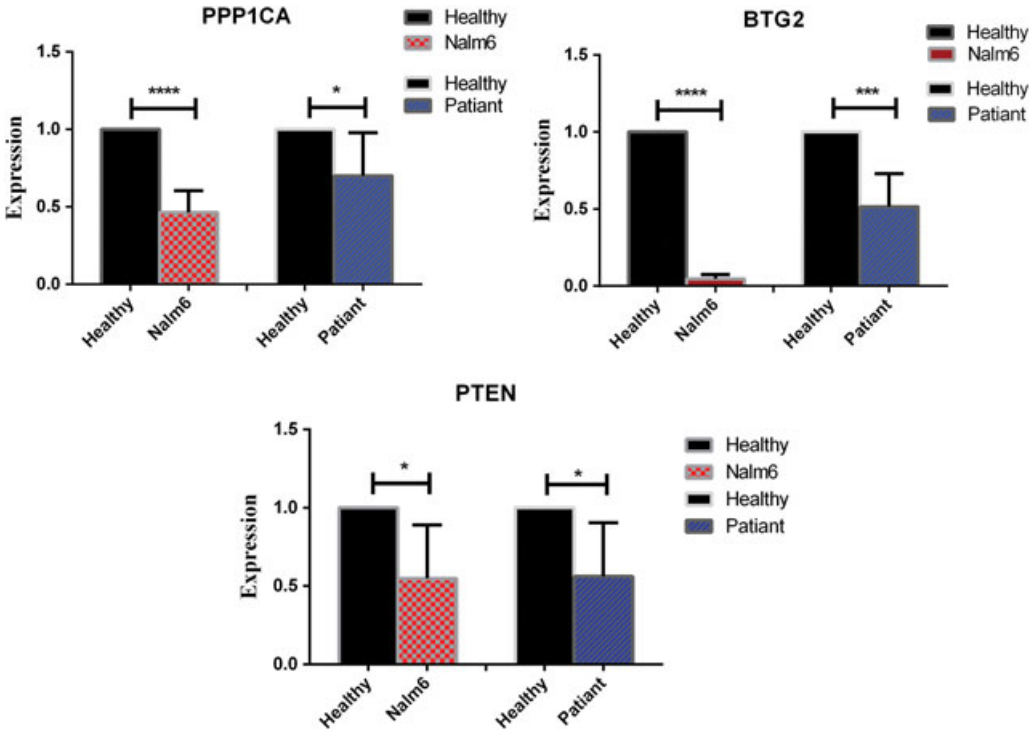

FIGURE 1 Expression of the *PPP1CA, BTG2*, and *PTEN* genes decreased in Nalm6 cell line compared with normal B cells as control. *PPP1CA, BTG2*, and *PTEN* mRNA levels were analyzed in Nalm6 cell line and compared with B lymphocytes as control using RT-qPCR. Result are the average ± SD of three independent experiments in triplicate. (*$P = 0.01$, ***$P = 0.001$, ****$P < 0.0001$). mRNA, messenger RNA; RT-qPCR, quantitative real-time polymerase chain reaction

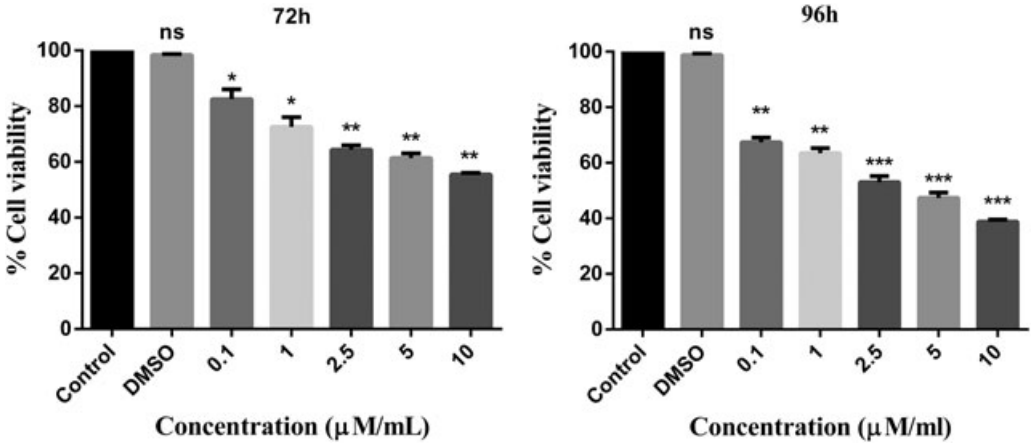

FIGURE 2 Decitabine inhibits cell proliferation in Nalm6 cells in a dose- and time-dependent manner. Nalm6 cells were treated with varying doses of decitabine for 72 and 96 hours and assayed by the MTT assay. Absorbance was read at 570 nM. Data represent one of three independent experiments done in quadruplicate that gave similar results. (**$P < 0.005$, ***$P < 0.0005$, ns: nonsignificant).
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

Therefore, we used 2.5 μmol of decitabine after 96 hours for all remaining experiments.

3.3 | Epigenetic inactivation of *PPP1CA, BTG2*, and *PTEN* genes in Nalm6 cell line

As shown in Figure 1, *PPP1CA, BTG2*, and *PTEN* genes are weakly expressed in Nalm6 cell line. However, *PPP1CA, BTG2*, and *PTEN* are robustly expressed in B lymphocyte of normal healthy donors. The promoter of these three genes were found to be methylated in Nalm6 cell line by the MSP assay (Figure 3). To evaluate whether aberrant promoter methylation is involved in modulating transcriptional expression of *PPP1CA, BTG2*, and *PTEN* genes, Nalm6 cell line was treated with decitabine. Gene expression analysis showed significantly enhanced expression of *PPP1CA* (median fold-change of expression [FC] = 2, $P < 0.0001$), *BTG2* ([FC] = 6.556, $P < 0.0002$), *PTEN* ([FC] = 3.100, $P < 0.0005$) in treated Nalm6 compared with untreated Nalm6 (Figure 4). This result suggests that

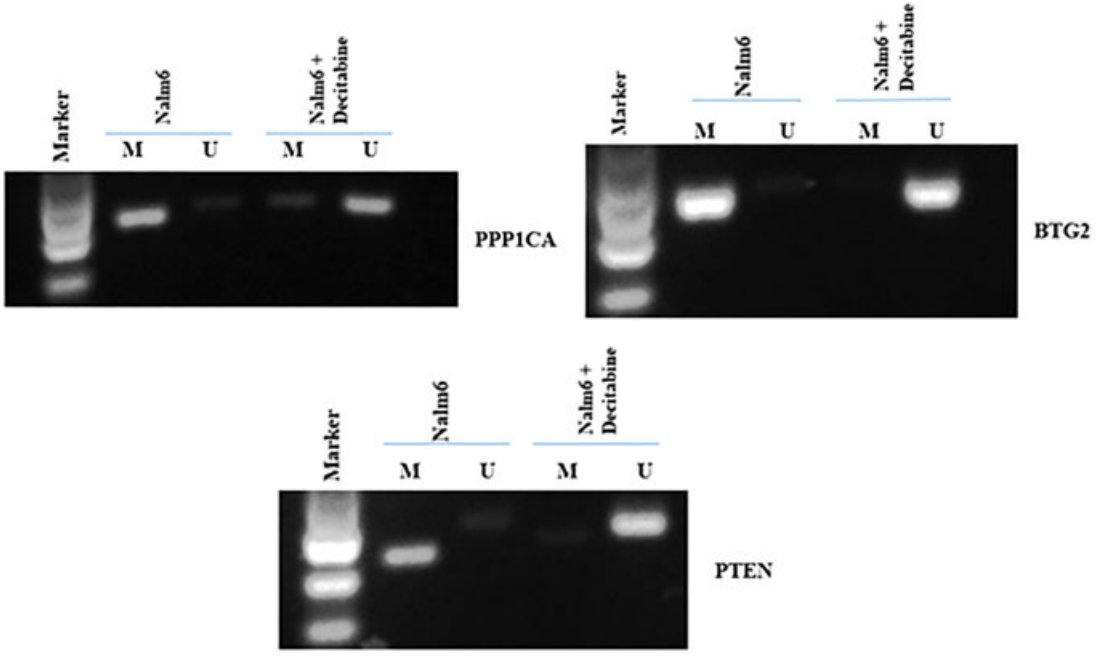

FIGURE 3 DNA methylation status of the *BTG2, PTEN*, and *PPP1CA* promoter in Nalm6 cell line before and after the treatment with decitabine. Cells were treated with or without decitabine (2.5 μM) for 96 hours. Methylation status of the *BTG2, PTEN*, and *PPP1CA* promoter in Nalm6 cell line were examined using MSP-PCR. Bands in Lanes U and M are PCR products amplified with unmethylated and methylated gene-specific primers, respectively. MSP-PCR, methylation-specific polymerase chain reaction

PPP1CA, BTG2, and *PTEN* transcription in this cell line were manipulated by promoter methylation or demethylation (Figure 3). In addition, these results indicate that aberrant methylation of promoter decreased the *PPP1CA, BTG2, and PTEN* expression. The MSP analysis showed that the treatment Nalm6 cell line with decitabine induced demethylation in *PPP1CA, BTG2*, and *PTEN* genes (Figure 3).

3.4 | The effect of decitabine on the miR-125, miR-17, and miR-181b expression

The previous studies, has been suggested that miR-125, miR-17, and miR-181b act as oncomir in Nalm6 cells.[20] In this study, the expression status of these miRNAs in decitabine-treated Nalm6 cell line was followed by quantitative RT-PCR. Following up the real-time PCR results, miR-125 (median fold-change of expression [FC] = 0.460, $P < 0.0001$), miR-17 ([FC] = 0.155, $P < 0.0001$), and miR-181b ([FC] = 0.60, $P = 0.0152$) significantly decreased in treated Nalm6 cells compared with untreated Nalm6 cells (Figure 5).

3.5 | The effect of decitabine induced cells to undergo apoptosis

To determine early and late apoptotic populations in Nalm6 cell line induced by the decitabine treatment, we performed flow cytometry using Annexin V-PE Apoptosis. Nalm6 cells were incubated with 2.5 μM decitabine for 72 and 96-hours. As shown in Figure 6, decitabine showed the significant percentage of early and late-stage apoptotic cells after 72 and 96-hour treatment that was 29.44% and 39.84%, respectively.

4 | DISCUSSION

B-ALL is the most prevalent hematological malignancy in children.[1] Several studies have characterized in addition to genetic abnormalities, epigenetic modifications play an important role in the occurrence of B-ALL.[6] In the present study, we investigated two mechanisms of epigenetic alteration, including DNA methylation and modification in the expression of miRNAs. We also analyzed the effect of decitabine as DNA methylation

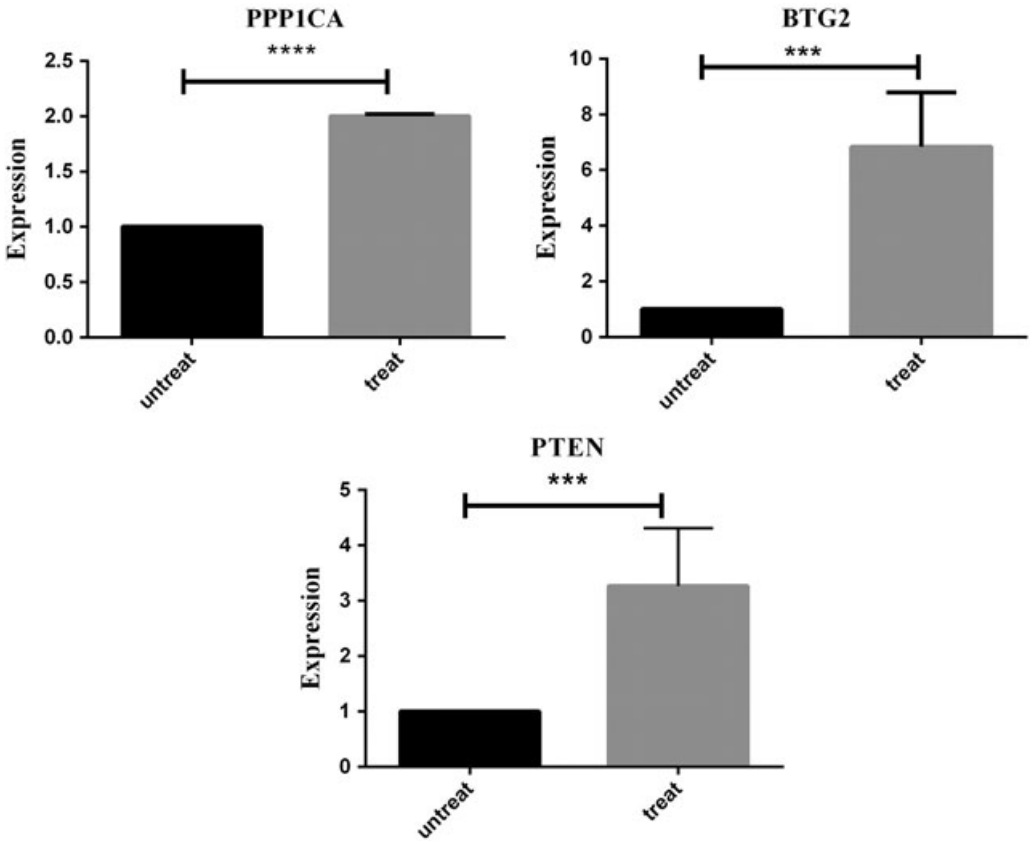

FIGURE 4 The levels of *PPP1CA, BTG2*, and *PTEN* gene expressions in decitabine-treated Nalm6 cell line. *PPP1CA, BTG2*, and *PTEN* transcript levels were measured before and after treatment by quantitative RT-PCR. Expression levels were normalized to GAPDH expression (error bars are standard deviations). RT-PCR, real-time polymerase chain reaction

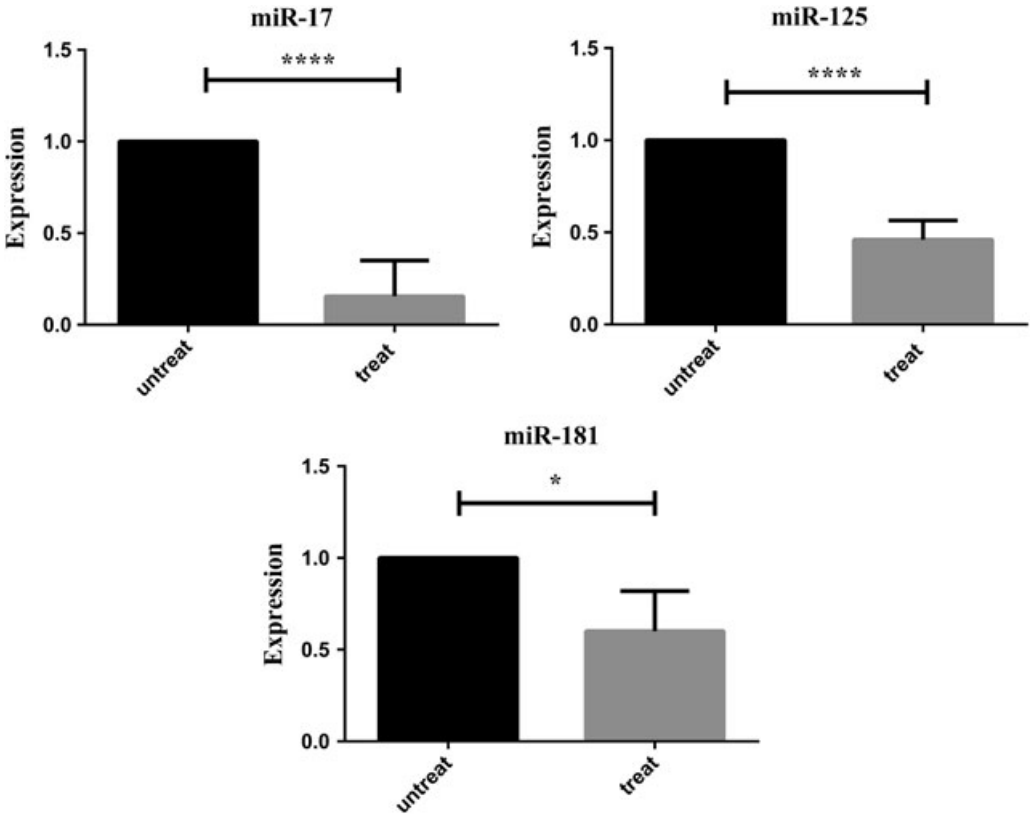

FIGURE 5 The expression levels of miR-125, miR-17, and miR-181b in decitabine-treated Nalm6 cell line. Significant differences of alteration in the expression levels of miR-125, miR-17, and miR-181b were measured between untreated and treated Nalm6 cells by quantitative RT-PCR. 5 seconds used as internal control (error bars are standard deviations). RT-PCR, real-time polymerase chain reaction

inhibitor on the expression of *PPP1CA, BTG2,* and *PTEN* and miR-125b, miR-17, and miR-181b in Nalm6 cell line.

The spectacular results obtained during our study are as follows: (1) the expression levels of *PPP1CA, BTG2*, and *PTEN* genes were significantly decreased in Nalm6 cell line, compared with the control cells; (2) the promoter of *PPP1CA, BTG2*, and *PTEN* genes are hypermethylated in Nalm6 cell line; (3) the treatment of Nalm6 cell line with decitabine induced demethylation in the promoter regions of candidate genes; (4) the decitabine treatment markedly restores expression of *PPP1CA*, *BTG2*, and *PTEN* in Nalm6 cells; (5) in addition, the decitabine treatment downregulated expression levels of oncomirs 125b, 17, and 181b in Nalm6, compared with the control cells.

In this study, for the first time and to the best of our knowledge, the expression levels of *PPP1CA*, *BTG2,* and *PTEN* in Nalm6 cell line have been compared with normal B cells, from healthy donors as a control cells. QRT-PCR analysis performed on RNA extracted from Nalm6 cell line showed that *PPP1CA*, *BTG2*, and *PTEN* mRNAs are significantly downregulated, in comparison with the control cells. In addition, to confirm the results, five patient samples were used. Analysis results revealed that the expression of the indicated genes in B-ALL patient's samples was similar to Nalm6 cell line and differ from control cells (Figure 1).

PP1 has four isoforms, including Alpha, Beta, Gama1, and Gama 2. *PPP1CA* is an alpha subunit of PP1.[21] Several previous studies suggesting that *PPP1CA* is a TSG, depending on the situation.[11] Castro et al designed short hairpin RNA (shRNA) against human *PPP1CA* for determination of the role of *PPP1CA* in HCT116 cell line. Based on their result, downregulation of *PPP1CA* increases the growth of tumor cells. They also explained that the re-expression of *PPP1CA* induces apoptosis in cancer cells and confirmed that *PPP1CA* could behave as a putative tumor suppressor.[22] Our data and Castro reported data confirmed each other and show that a reduction in the expression of this gene before the treatment, and an increase after the treatment, not only suggest that this gene is a *TSG*, but also can contribute to the occurrence and development of B-ALL.

Tijchon et al. in their research showed that *BTG2* is a transcriptional cofactor with an important role in regulating B-cell differentiation and they also determined that the *BTG2* act as a TSG in B-cell neoplasms.[23] *BTG2* is the first known gene in BTG/TOB family. In many cancers, *BTG2* is known as a TSG and several studies have been demonstrated that the expression of this gene

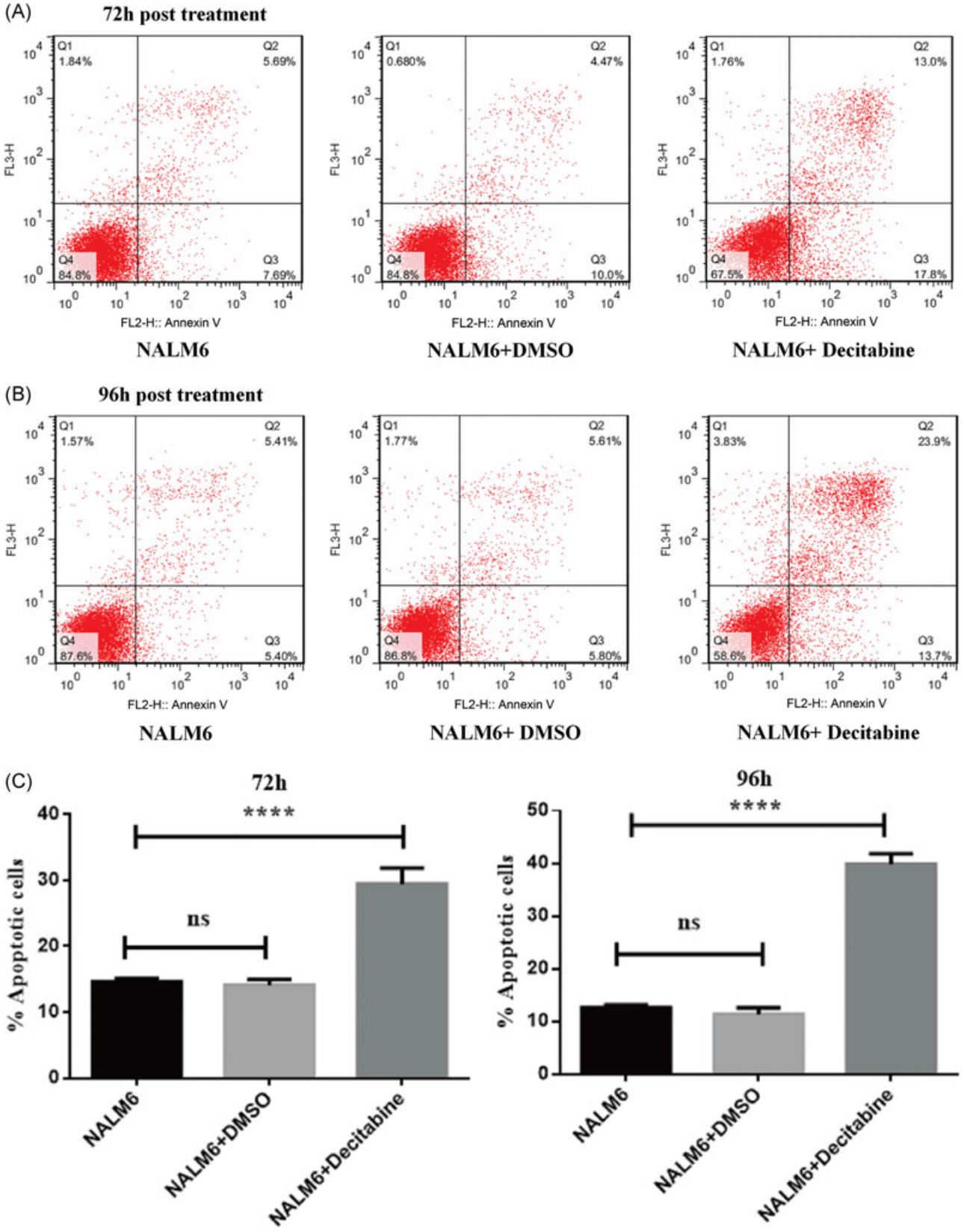

FIGURE 6 The effects of decitabine on apoptosis of Nalm6 cells. Cells were incubated with 2.5 μM decitabine (A) for 72 hours and (B) 96 hours. C, The apoptosis in the graph is shown as the percentage of Annexin V positive cells among all cells counting by flow cytometry. The error bars in the diagram represent the standard deviations of three separate tests. Each experiment was performed in triplicate

can be affected by various factors, including miRNAs, or DNA methylation.[11] It is expected that *BTG2* similar to *PPP1CA* will have the same profile change and our results further confirmed that.

PTEN is very commonly inactivated in a wide range of human neoplasms. Several factors have an important effect in inactivation of the *PTEN* gene, such as genetic aberrations, promoter hypermethylation, histone modification, and changes of miRNAs expression levels.[12]

Many studies have shown that *PTEN* is downregulated in several hematological malignancies, and has also been considered the role of *PTEN* as a TSG.[24-26] Our data revealed that expression levels of *PPP1CA*, *BTG2*, and *PTEN* downregulated in Nalm6, compared with the B lymphocytes of healthy controls. The most decrease was related to *BTG2* gene expression (median fold change = 0.51) (Figure 1).

As the aberrant promoter methylation is correlated with gene silencing, in the next step, promoter methylation status of *PPP1CA*, *BTG2,* and *PTEN* genes in Nalm6 cell line were analyzed using MSP. Methylation verification expressed that *PPP1CA, BTG2*, and *PTEN* genes are hypermethylated in Nalm6 cell line. Our results confirmed the association between the reduction of these candidate genes and improper methylation status in gene promoters (Figure 3).

Since epigenetic aberrations are reversible, today the epigenetic therapy is known as one of the attractive therapeutic approaches, as this method creates changes in the level of expression of genes, involved in cancer (tumor suppressors and oncogenes) to fight against cancer cells.[27] The most common antitumor drugs that cause epigenetic alteration in tumor cells are the use of DNA methylation inhibitor. Several studies demonstrated decitabine has the ability to re-express TSGs which silenced by hypermethylation. Decitabine can act on the gene expression, by two mechanisms first: direct approach, in this way, decitabine incorporated into DNA structure and affect genes promoter methylation and expression status,[28] second: indirect approach, in which decitabine altered gene expression through demethylation of upstream genes, also decitabine can reactivate gene silencing via an effect on histone modification.[29] In this study, also the effect of the DNA methylation inhibitor, decitabine on the methylation and expression of *PPP1CA*, *BTG2*, and *PTEN* genes, in association with changes in miR-125b, miR-17, and miR-181b levels, was investigated. Some previous studies reported these miRNAs play important roles in this type of leukemia.[28,30,31]

The effect of decitabine on cell proliferation was carried out using the MTT assay. The IC-50 of decitabine was 2.5 μM in Nalm6 cells, upon exposure for 96 hours. Although the minimal effect was seen after decitabine exposure at 48 hours, a significant dose and time-dependent growth inhibition was observed in 96 hours (Figure 2).

We also investigated the influence of decitabine (2.5 μM) on apoptosis of Nalm6 cells after 72 and 96 hours. The flow cytometry analysis showed, decitabine has a significant impact on inducing apoptosis. Our results described 29.44% and 39.84%, early and late apoptosis after 72 hours and 96 hours, respectively. According to several studies, decitabine is known as a potent agent in inducing cells that undergo apoptosis in many cancers, including different types of leukemia's (Figure 6).[32]

Quantitative RT-PCR was performed for evaluating the expression level of *PPP1CA, BTG2*, and *PTEN* in Nalm6 cell line, before and after the influence of decitabine. Our results showed that the expression of *PPP1CA*, *BTG2*, and *PTEN* genes are significantly increased in Nalm6 cells, after the treatment with decitabine (Figure 6). Although, decitabine is not in the treatment line of ALL, but according to new findings, it can be used as a novel approach to the B-ALL treatment with less side effects and high effectiveness. This study performed on cell line in vitro, to confirm the reduction of side effects of decitabine, it is necessary to study animal and human models.

Recently, it has been reported that in addition to this fact which miRNAs can be methylated in their promoter region, they can modulate epigenetic mechanisms, by affecting DNA methyltransferases and EZH2 (histone-lyzine N methyltransphrase), responsible for DNA methylation and histone alterations, respectively.[6,33] Puissegur et al. stated that miR-125b behave as an oncomir in Nalm6 cell line.[20] In contrast, Wu et al. explained miR-125b acts as an oncomir in gastric cancer tissue and also showed increases in the expression levels of miR-125b resulted in downregulation of the *PPP1CA* expression, but no studies on leukemia have been done so far, and our study seems to be the first to investigate this.[34] Therefore, it can be justified that the decrease in the *PPP1CA* expression in Nalm6 cell line can occur for two reasons, hypermethylation in its promoter region and the upregulation of miR-125b.

Scherr et al. have described miR-17 is upregulated in Nalm6 cell line.[35] In addition, Hu et al have reported that miR-17 significantly was upregulated in the endometrial tissues of patients and have the direct impact on down-regulation of the *PTEN* expression.[36] Furthermore, it is approved that miR-181b functions as an oncomir in childhood acute lymphoblastic leukemia.[37] Our results showed that decitabine reduces the expression of the miR-125b, miR-17, and miR-181b (Figure 5). As noted earlier,

these miRNAs might play a role in the pathogenesis of the B-ALL.

In the present study, miRTarBase, Target Scan, and Miranda databases were used to identify miRNAs and their targeted genes. According to this, miR-125b and miR-181b can also affect the *BTG2* and *PTEN* expression, respectively.

5 | CONCLUSION

From the results of this study, it might be concluded that reduction of the expression levels of *PPP1CA, BTG2*, and *PTEN* genes in Nalm6 cell line are related to hypermethylation of the promoter regions of these genes. Also, the expression levels of these genes and miR-125b, miR-17 and miR-181b oncomirs after exposure to decitabine were examined and surprisingly, the expression of all candidate genes was increased but the expression of oncomirs decreased. These results indicate that decitabine as a demethylation drug, has a potential role, and it is suggested that this drug can be used as a modulator for the first line treatment of patients with B-ALL, but more in vivo investigations are necessary.

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CONFLICT OF INTERESTS

Author declare that they have no conflict of interests.

ORCID

Gholamhossein Tamaddon http://orcid.org/0000-0001-8158-6004

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