Original Article *Iran J Ped Hematol Oncol. 2024, Vol 14, No 2, 78-93*

Changes in the Expression of DNMTs Before and After Treatment with Methotrexate (MTX)/Mercaptopurine (6-MP) in B-Cell ALL Children

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Received: 16 July 2023 **Accepted:** 18 February 2024

Abstract

Background: DNA methylation is catalyzed by DNA methyltransferases (DNMTs) which are encoded by DNMT1, DNMT3A, and DNMT3B. DNMTs play a major role in the abnormal methylation of tumor suppressors and cancer-related genes. Herein, this study explored the expression profile of DNMTs in pediatric patients with B-cell acute lymphoblastic leukemia (ALL), before and after methotrexate (MTX)/mercaptopurine (6-MP) treatment.

Materials and Methods: This before-after prospective study included 30 matched children in sex and age (20 children with B-cell ALL and 10 healthy children used as a control or calibrator group). The expression profile of DNMTs was assessed at two-time points; at the diagnosis time and after MTX/6-MP treatment in the consolidation-maintenance phase of therapy. Notable, all pediatric patients included in this study continued the therapy without adverse events, except two children who were excluded from the study.

Results: The average age of the patient group was 7.1 ± 1.3 years (in the range of 4-9 years), and the average age of the control group was 8.3 ± 1.7 years (6-10 years). The expression profile of DNMTs in B-cell ALL children was obtained completely different from that in the healthy group. After MTX/6-MP treatment of B-cell ALL children, the expression levels of DNMT1 and 3A were increased ($p < 0.01$ & 0.04, respectively), and the expression level of DNMT3B was decreased ($p < 0.01$), significantly.

Conclusions: In ALL, the expression profile of DNMTs would be changed whereby contribute to abnormal growth and maturation capacity of leukemic stem cells and MTX/6-MP treatment could reverse this profile from a cancerous phenotype to the normal one.

Keywords: DNA Methyltransferases (DNMTs), Methotrexate (MTX), Mercaptopurine (6-MP)

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[DOI: 10.18502/ijpho.v14i2.15267]

Introduction

Cancer in children becomes more Acute lymphoblastic leukemia (ALL) is the most common cancer diagnosed in children, with certain subsets experiencing greater than 98% cure rate. Most ALL pediatric patients show B-cell immunophenotype (~ 80%) which, in turn, encompasses a broad range of patients with a high cure rate in chemotherapy (1, 2). Today, the standard protocols for ALL chemotherapy start with an induction regimen (~1 month), followed by a consolidation phase (~1-2 months), and finally end with the methotrexate (MTX)/mercaptopurine (6-MP) maintenance therapy which is given until 2 to 3 years from diagnosis. Consolidation-maintenance therapy with anti-metabolites of folate (methotrexate (MTX)) and

purines (6-mercaptopurine (6-MP)) as pioneering anticancer agents has been significantly approved in the long-term remissions or/and cure of childhood B-cell ALL (2-4).

6-MP as an effective anticancer drug belongs to thiopurine agents with remarkable success in clinical practice, especially in the treatment of ALL in children. Thiopurines and MTX are widely understood to act as DNA anti-methylating agents (4-6). DNA is methylated at CpG dinucleotides whose clusters (CpG islands) are concentrated in the upstream regions of many anti-cancer genes including tumor suppressors wherein CpG sites are mainly un-methylated and their increased methylation is associated with reduced expression of downstream genes. In cancers, hypermethylation of CpG islands in the upstream regions of tumor suppressors and DNA-repair genes is common, as compared with normal tissues. DNA repair genes are frequently repressed in cancers due to hypermethylation of CpG islands within their promoters. In cancers, loss of expression of genes occurs about 10 times more frequently by hypermethylation of promoter CpG islands than by mutations (7, 8). Methylation of CpG islands is catalyzed by DNA methyltransferases (DNMTs) whose changes in expression have been observed in multiple human cancers, in particular in leukemia. The human genome encodes DNMT1, DNMT3A, and DNMT3B whose activity is responsible for the methylation of DNA and dysregulation results in abnormal methylation of CpG islands (8-10).

Accordingly, MTX/6-MP treatment causes a serious decrease in S-adenosylmethionine (SAM) levels which can directly reduce DNMTs activity and would be closely reflected in the demethylation of CpG islands. Herein, SAM is needed by DNMTs over-expressed in tumors to methylate DNA at special promoters wherein the restriction in SAM levels would result in a reduction in the activity of DNMTs. This could be associated with increased expression of genes inhibiting in tumor cells (e.g. Tumor suppressors (TS), DNA repair, DNMT3A,) (5-8, 11). Additionally, the presence of 6-MP at CpG sites (CpTG), simultaneously may enhance hypermethylation of oncogenic sites. The above-mentioned mechanisms may lead to a reversal of abnormal methylation patterns toward normal ones; anti-methylating functions may help to maintain an active chromatin configuration at CpGs-enriched target loci, for example at the promoter of anti-tumor genes or tumor suppressors which are CpGs rich (12-14).

Hence, to gain biochemical evidence for the hypothesis that DNMTs have an essential role in the cancerous state of ALL and response to chemotherapy, we assessed the expression profile of DNMT1, 3A, & 3B in specimens obtained from B-cell ALL children at diagnosis time and after 3 months of treatment course when pediatric patients were on the consolidation-maintenance phase of MTX/6-MP therapy, and then compared to those in healthy matched children. DNMT expression profile was assessed using Quantitative Real-Time PCR (QRT-PCR) method. The results are expected to lead to a better understanding of the molecular basis of ALL and the epigenetic action of effective chemotherapy. Epigenetic drugs have been of great interest, not only for basic research but also for further understanding of disease pathogenesis and the development of new therapies (10, 15). Therefore, the gene expression levels of DNMT1, 3A, & 3B in specimens obtained from B-cell ALL children were assessed and differences between before and after the treatment and also between healthy and sick people were calculated and compared.

Materials and Methods

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Patients Study groups

This before-after prospective study included 20 pediatric patients with B-cell ALL referred to the Pediatric Oncology Branch of the Children's medical center of Tehran between 2015 and 2016, besides 10 healthy matched children as a control group.

According to similar studies that have reported the mean and standard deviation of DNMT3 gene expression in patients (0.69+0.51) and healthy people (0.35+0.47) with 95% confidence and 80% power for a difference of 0.5 units after the intervention, the number of samples 18 cases were estimated (9).

Therefore, gene expression differences in children with newly diagnosed B-cell acute lymphoblastic leukemia before and after treatment, as well as between healthy and diseased group, were calculated and compared.

The control group consisted of 10 healthy children (8 girls and 2 boys, in the age range of 6-10 years) referred to the central laboratory of Yazd city, and according to the diagnosis of the pediatric oncologist were free of any type of malignancy or disease. The case group included eligible pediatric patients with newly diagnosed B-cell ALL (10 girls and 10 boys, in the age range of 4-9 years) who were previously untreated. Children with any previous chemotherapy or those treated with systemic corticosteroids within 1 month before diagnosis were excluded. Patients with lymphoma syndrome, with massive splenomegaly, or any other malignancy were not eligible for this study. Patients were matched at diagnosis and met the above criteria. Participants were enrolled by convenience sampling, and their blood samples were taken two times. In the case of pediatric patients, the first sampling was at the pre-treatment point when they were newly diagnosed with B-cell ALL and the second one was after 3 months of treatment when patients were receiving dexamethasone (Dex)/vincristine (Vin)/ methotrexate (MTX)/ mercaptopurine (6-MP) chemotherapy drugs for the maintenance therapy, according to the standard regimen protocol invested in the center (2-4, 16).

Sampling and Diagnosis

The research was carried out after obtaining permission of Committee of Ethics in Human Research with code: (IR.SSU.MEDICINE.REC.1394.198-201.

All admitted B-cell ALL children met World Health Organization (WHO) criteria and guidelines for B-cell ALL and were diagnosed and introduced by a pediatric leukemia specialist. About 5 mL of peripheral blood was taken from both pediatric groups, after obtaining written informed consent from all participants' parents.

RNA Isolation

White blood cells (WBC) were isolated by sedimentation on Ficoll-Hypaque gradients. Total RNA was participated and isolated with a Ribo EX kit (Geneall, Daejeon, Korea), according to the manufacturer's instructions. The quantity and purity of RNA content were evaluated using Nanodrop-2000 (Thermo Fisher Scientific, Waltham, MA, USA). Absorbance ratios and concentrations were determined as indicators of sample yield. The integrity and quality of total RNA were controlled with the 1% agarose gel electrophoresis. To eliminate DNA contamination, the isolated RNA solution was treated with DNase (DNA-free, Ambion, Austin-USA) (Figure 1). Gene expression of *DNMT1*, *3A*, and *3B* was then assessed by QRT-PCR and analyzed by SPSS15.

Quantitative Real-Time PCR (QRT-PCR)

First-strand cDNA was synthesized from 2 µg RNA in a 20 µL reaction mixture containing random hexamers as primers using a cDNA synthesis kit (MBI Fermentas, Ontario–Canada Revert Aid, First Strand cDNA Synthesis Kit). The relative expression of target genes was

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performed by Step One Plus Real-time PCR (Applied Biosystems) in triplicate; primer specificity was confirmed by melting curves (Figure 2). QRT-PCR was done using HOT FIREPol® EvaGreen qPCR Mix (Solis BioDyne, Tartu, Estonia) according to the SYBR Green method. Briefly, QRT-PCR was carried out in 20 µL PCR buffer (10 mM Tris– HCl (pH 9.5), 50 mM KCL, % 0.1 Triton X), 0.2 mM dNTP mix, 1.5 mM MgCl2, 10 pmol of each primer and 2U of Taq-DNA polymerase. The PCR cycle was 94 ºC for 30s, 60 ºC for 30s and 72 ºC for 60s. The primers and their characteristics are described in Table I. To check the primers and cDNA synthesis, the expression of *GAPDH* (Glyceraldehyde Phosphate Dehydrogenase) and *DNMT*s were analyzed by melting curve, and QRT-PCR products were run on a 1% agarose gel stained with EtBr (Figure 1). Relative fold changes in the expression levels of genes were calculated according to the $2^{-\Delta\Delta Ct}$ formula (below), after normalization with *GAPDH* as a reference gene and then were analyzed by One-way ANOVA.

Statistical Analysis

All the PCR's were carried out in triplicate and the signal intensities were correlated almost linearly with the amount of mRNA dilutions. The expression levels of DNMTs were normalized with glyceraldehyde phosphate dehydrogenase (GAPDH) and then the gene expression differences before and after the treatment and also between healthy and sick people were calculated and compared with a t-Test, using SPSS software version 21 (SPSS 21, IBM Corp., Armonk, NY, USA). Differences were considered significant at *p*-values < 0.05.

Results

Patient Characteristics

The study recruited a total of 30 eligible nearly matched children including 18 girls (10 in the patient group and 8 in the healthy group) and 12 boys (10 in the patient group and 2 in the healthy group). However, two of ALL children were excluded from the study because they were not able to continue the treatment and declined participation in the study. All admitted B-cell ALL children met World Health Organization (WHO) criteria and guidelines for B-cell ALL and were diagnosed and introduced by a pediatric leukemia specialist. The mean age of the patient group was 7.1±1.3 years (a range of 4-9 years), and the mean age of the control group was 8.3±1.7 years (a range of 6-10 years) (Table II). Newly diagnosed children with B-cell ALL were sampled before starting the treatment and after 3 months when they were on consolidation-maintenance therapy and receiving dexamethasone (Dex)/vincristine (Vin)/MTX/6-MP chemotherapy drugs, according to the standard regimen protocol invested in the children's medical center (2-4, 16). Except for two children who were excluded from the study, all pediatric patients who were included in this study showed no adverse effects and were responding to the consolidation-maintenance regimen. Also, there was no significant difference between the patients in showing remission. Herein, the experiment was divided into two parts: first to investigate the expression profile of DNMT1, 3A, and 3B in B-cell ALL children at diagnosis time before receiving any treatment and compare it with that in healthy children; second, to assess possible changes that occurred in the expression profile of DNMT1, DNMT3A, and DNMT3B in pediatric patients, when they were on MTX/6-MP consolidation-maintenance therapy.

The higher expression level of DNMT1 in B-cell ALL children

This study was conducted on children with newly-diagnosed B-cell ALL sampled before starting the therapy and then after 3 months when they were on consolidation-

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maintenance therapy and receiving dexamethasone (Dex)/vincristine (Vin)/MTX/6-MP chemotherapy drugs, according to the standard regimen protocol invested in the children's medical center. Blood samples were taken from 20 children newly diagnosed with B-cell ALL before starting the chemotherapy, and also from 10 healthy matched children, as controls. Total mRNAs were isolated from their WBCs and then the expression levels of DNMTs were assessed as described previously. Figure 1 is a representative pattern of QRT-PCR products obtained under non-saturating conditions from samples. For a better understanding of differences in the expression profile of DNMTs in B-cell ALL children, QRT-PCR data were analyzed with appropriate methods whereby the expression of the healthy group was considered as a calibrator and the expression of GAPDH as an internal reference. After normalization with GAPDH, the fold expression level of DNMT1 in the healthy group was 1.03±0.3, which increased to 1.98±0.09 in the patient group (with P~0.02) (Figure 3 & Table III).

The lower expression level of DNMT3A in B-cell ALL children

The human DNMT3A together with DNMT3B have been associated with de novo methylation of CpG dinucleotides whereby they are establishing genomic imprints. Analysis of DNA methylation patterns in normal and tumor cells has revealed that many human tumor cells have hypermethylation and inactivation of tumor suppressor genes such as p16 or p53 **(9, 11).** Hence, this study analyzed the fold expression level of DNMT3A in B-cell ALL children and compared it with that in the normal healthy group. After normalization with GAPDH, the fold expression level of DNMT3A in the healthy control group was 2.72±0.22 which was 1.21±0.6 in B-cell ALL children (with P~0.04) (Figure 3 & Table III)**.**

The higher expression level of DNMT3B in B-cell ALL children

Finally, the fold expression level of DNMT3B in blood cells from B-ALL children was analyzed and compared with the healthy group. The relative expression level of DNMT3B in the healthy control group was 1.26±0.62 which was 3.27±0.3 in B-cell ALL children (with P~0.03) (Figure 3 & Table III). Interestingly, consistent with DNMT1 and unlike DNMT3A, the expression level of DNMT3B was significantly higher in pediatric patients than in healthy children. Besides, consistent with DNMT1 and unlike DNMT3A, the expression level of DNMT3B was significantly higher in B-ALL children than in the healthy group

In the next step, the study aimed to assess changes that appeared in the expression profile of DNMTs, after B-ALL children received MTX/6-MP therapy.

In the next step, the study aimed to evaluate the changes that appeared in the expression level of DNMTs in B-ALL children after receiving MTX/6-MP treatment.

Decreased expression of DNMT1 after MTX/6-MP treatment

Previous studies on the epigenome and drug-targeted mechanisms in tumor cells have emphasized the reversibility of promoter hypermethylation of cancer-related genes. They proposed that hypermethylation of genomic sites can be reversed by treatment with chemotherapy agents and emphasized the role of DNMTs as optimal targets for what is now known as epigenetic therapy (10, 15).

In this part of the study as mentioned earlier, 20 matched B-cell ALL children (10 girls and 10 boys, with an age range of 4-9 years), were included while making sure that the correct types and doses of drugs were given. Except for two children who were excluded from the study, all B-cell ALL children (10 girls and 8 boys) continued participation in this study, showed no adverse effects, responded to

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the chemotherapy, and went on treatment with MTX/6-MP in the consolidation-maintenance phase (according to the protocol regimen in the children's medical center). Thus, it was worthwhile to investigate the expression profile of DNMTs in these patients. In the consolidation-maintenance phase of treatment with MTX/6-MP, the expression of DNMT1 in blood cells from B-cell ALL children showed significantly lowered levels when compared to that obtained at diagnosis time before treatment. The fold change expression of DNMT1 decreased from 1.98±0.09 (before treatment) to 1.58±0.12 (after treatment) (P~0.01) (Figure 4 & Table IV).

Increased expression of DNMT3A after MTX/6-MP treatment

In contrast to DNMT1, DNMT3A was up-regulated in blood cells from B-cell ALL children after MTX/6-MP treatment. Then, the fold change expression of DNMT3A in B-cell ALL children after treatment with MTX/6-MP increased from 1.21±0.6 to 2.3±0.5, with P~0.04, (Figure 4 & Table IV).

Reduced expression of DNMT3B after MTX/6-MP treatment

Similar to DNMT1, the MTX/6-MP treatment lowered significantly the expression of DNMT3B in B-cell ALL children where the fold change expression reached its level in normal cells (Figure 4 & Table IV)**.** Before treatment, the fold change expression of DNMT3B in B-cell ALL children was 3.27±0.62 which was reduced to 1.7±0.08 after treatment with MTX/6-MP (P~0.02) (Figure 4 & Table IV), and thus got close to the fold change expression of normal cells (1.26±0.62)**.**

Table I: Characteristics and sequences of DNMT1, 3A, & 3B and GAPDH primers used in this study.

Gene Name	Forward and Revers primers 5` to 3`	NCBI AC. NO.	cDNA Size
DNMTI	F:CACCAGGCAAACCACCATCAC	NM_001318731.1	168 bp
	R:AGCGGTCTAGCAACTCGTTCTC		
DNMT3A	F: CTCCATCGTCAACCCTGCTC	NM_022552.4	200 bp
	R: TCATCACAGGGTTGGACTCG		
DNMT3B	F: TGCTCTGGAGAAAGCTAGGGT	NM_006892.3	198 bp
	R: TCATCACAGGGTTGGACTCG		
GAPDH	F: GAGCCACATCGCTCAGACAC	NM_001289746.1	150 bp
	R:CATGTAGTTGAGGTCAATGAAGG		

Table II: General characteristics of the healthy group and B-cell ALL children included in this study.

Characteristics	*Control group*	*B-cell ALL group**	
Age (years), mean ± SD	8.3 ± 1.7	Before treatment	After treatment
		7.1 ± 1.3	7.5 ± 1.4
Females	8 (80%)	10 (50%)	10 (50%)
Males	2 (20%)	10 (50%)	8 (50%)

* B-cell acute lymphoblastic leukemia (B-cell ALL).

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Table III. The fold change expression of *DNMT1/GAPDH*, *DNMT3A/GAPDH* and *DNMT3B/GAPDH*, in B-cell ALL children and in healthy normal group (as a calibrator), are presented here, after normalization to *GAPDH*.

Gene Name	*Control group*	*Patient group*	*P-values*
***DNMT1*, mean ± SD**	1.03±0.16	1.98±0.09	0.02
***DNMT3A*, mean ± SD**	2.72±0.22	1.21±0.6	0.04
***DNMT3B*, mean ± SD**	1.26±0.62	3.27±0.3	0.03

SD; Standard deviation, GAPDH; glyceraldehyde phosphate dehydrogenase, DNMT; DNA methyltransferase). The fold change expression of each gene in the patient group and the healthy group was analyzed and differences between the groups were compared with t-Test, $P < 0.05$ was considered significant

Table IV: Comparison of the fold change expressions of DNMTs in B-cell ALL children before and after the treatment. DNMT1/GAPDH, DNMT3A/GAPDH and DNMT3B/GAPDH, in B-cell ALL children before and after treatment with Methotrexate (MTX)/Mercaptopurine (6-MP), and in healthy normal group (as a calibrator), are presented here, after normalization to GAPDH.

Gene Name	*Control healthy group*	*B-ALL group, Before treatment*	*B-ALL group, After treatment*	*P-values*
***DNMT1*, mean ± SD**	1.03±0.16	1.98±0.09	1.58±0.12	0.01
***DNMT3A*, mean ± SD**	2.72±0.22	1.21±0.6	2.3±0.5	0.04
***DNMT3B*, mean ± SD**	1.26±0.62	3.27±0.36	1.7±0.08	0.02

SD; Standard deviation, GAPDH; glyceraldehyde phosphate dehydrogenase, DNMT; DNA methyltransferase). The fold change expression of each gene in the patient group, before and after the treatment was analyzed and compared, by using paired t-Test, $P < 0.05$ was considered significant.

Table V: Postulated pathways and related genes are disrupted by promoter hypermethylation and are associated with gene-silencing in cancers.

Pathway	Genes
Cell-Cycle Control	*Rb, p16, p15, p14, p73*
DNA Repair	*MLH1, O6-MGMT, GST-Pi, BRCA1**
Growth Factor Response Inhibition	*ER, RAR-beta, SOCS-1*
Inducing Apoptosis	*DAP kinase, caspase 8, TMS-1*

* *Genes abbreviations*: DNA mismatch repair protein Mlh1 or MutL protein homolog 1 (*MLH1*), O6-alkylguanine DNA alkyltransferase (*O6-MGMT*), Glutathione S-transferases pi (*GST-Pi*) Breast cancer type 1 susceptibility protein (*BRCA1*), Suppressor of cytokine signaling 1 (*SOCS1*), and the inhibitor of STAT-induced and Janus kinase–signal (JAK–STAT) pathway (9, 11, 23-25).

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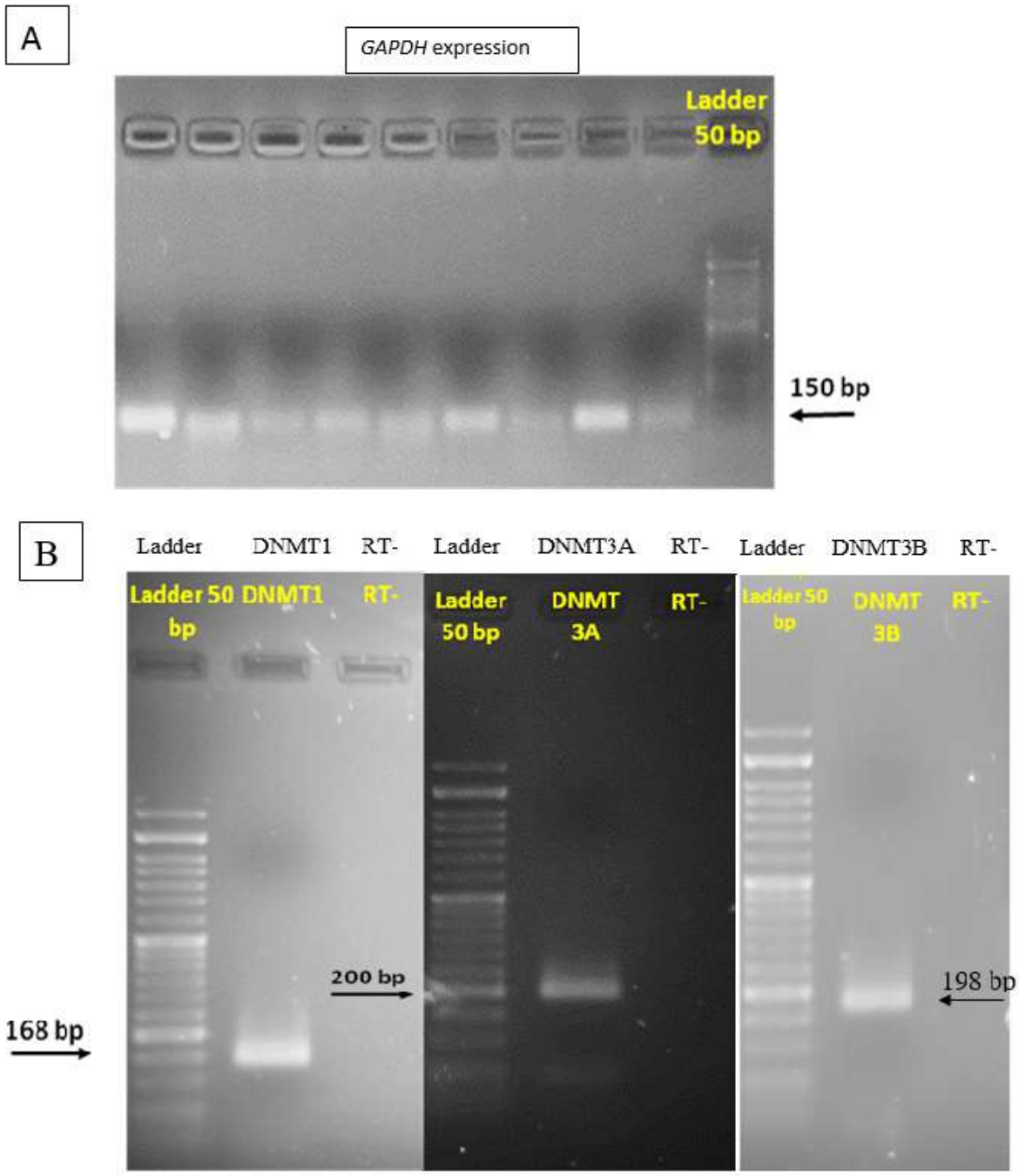

Figure 1. Representative gel patterns of QRT-PCR products of *GAPDH, DNMT1, 3A, & 3B* obtained under non-saturating conditions, run on a 1% agarose gel and stained with EtBr. A: QRT-PCR product of *GAPDH* gene (left to right: patients *GAPDH*, 150 bp, and DNA marker (ladder 50 bp)); B (left to right): The first well is DNA marker (ladder 50 bp). RT-PCR product of the *DNMT1* gene (patients, 168 bp, and negative control (RT-)), RT-PCR product of the *DNMT3A* gene (patients, 200 bp, negative control (RT-)), RT-PCR product of the *DNMT3B* gene (patients, 198 bp, and negative control (RT-)).

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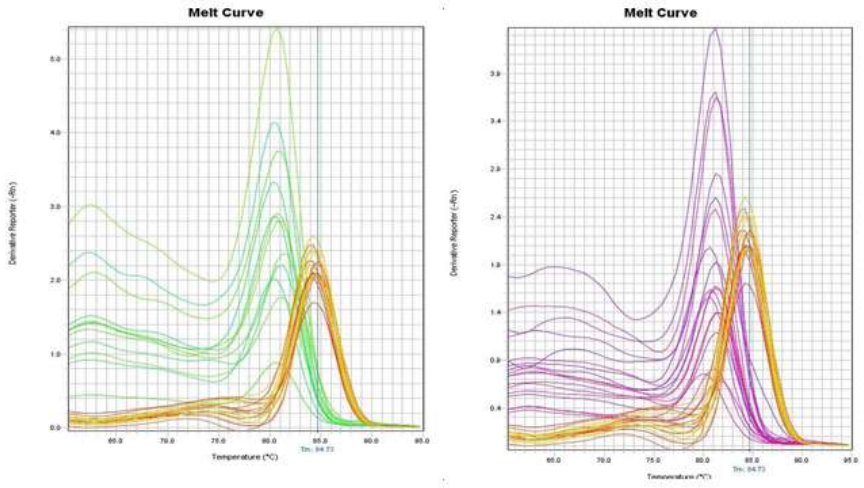

Figure 2. Melting curves of PCR products after cDNA synthesis of GAPDH and DNMT1, 3A and 3B transcripts.

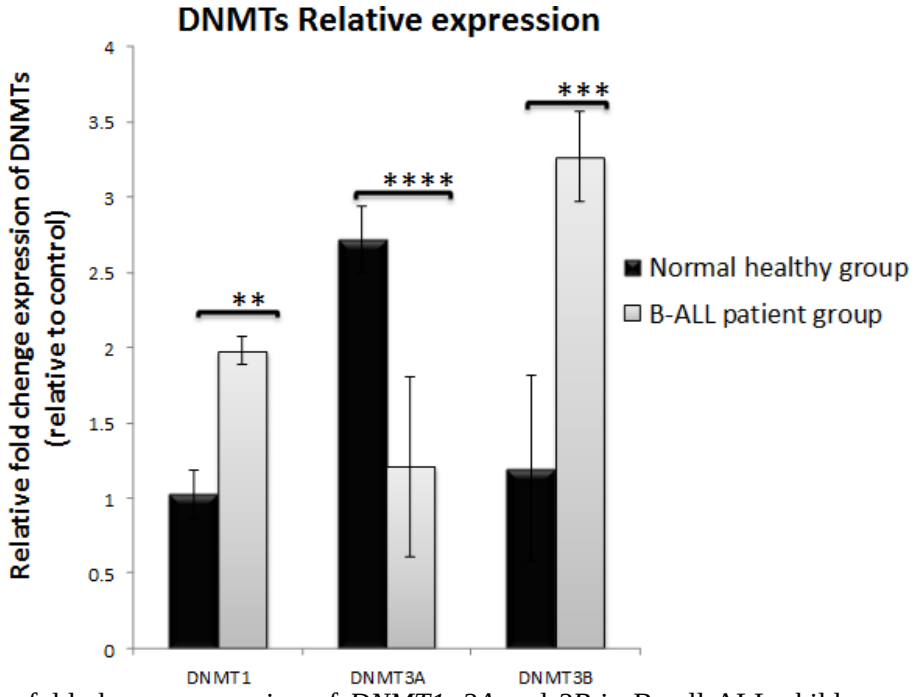

Figure 3. The fold change expression of *DNMT1*, *3A* and *3B* in B-cell ALL children and in normal healthy group (as a control), after normalization to *GAPDH* which was considered as a reference gene and is expressed constantly in every cell type. The fold change expression of each gene in the patient`s group was compared with the same gene in the healthy group, by using t-Test. *; $P < 0.01$ was considered significant. **; $P < 0.02$ was considered significant. ***; $P < 0.03$ was considered significant. ****; $P < 0.04$ was considered significant. GAPDH; glyceraldehyde phosphate dehydrogenase, DNMT; DNA methyltransferase.

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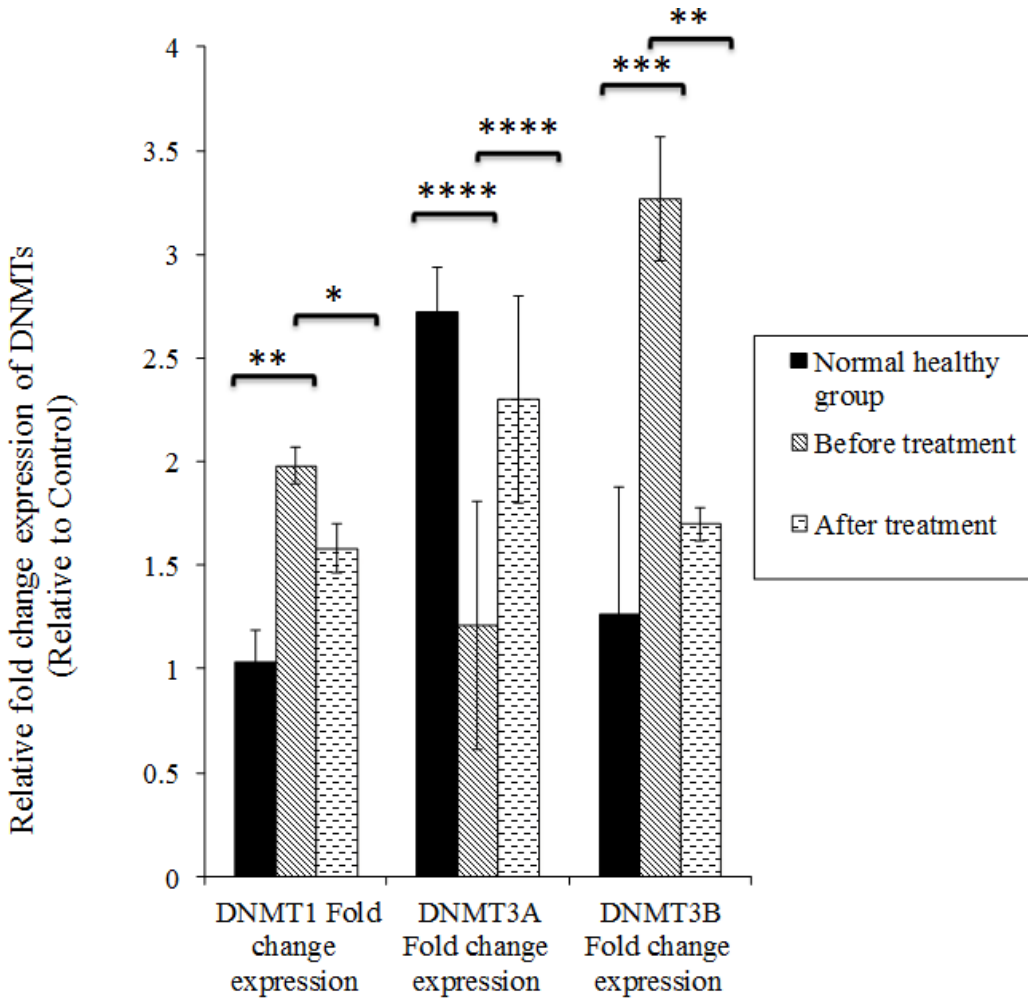

Figure 4. Comparison of the fold change expression of DNMTs, in B-cell ALL children before and after treatment with Methotrexate (MTX)/Mercaptopurine (6-MP) and in healthy normal group (as a calibrator), after normalization to *GAPDH* which was considered as a reference gene and is expressed constantly in every cell type. The fold change expression of each gene in the patient`s group was compared with the same gene in the healthy group, by using t-Test. Also, the fold change expression of each gene in the patient group, before and after the treatment was analyzed and compared, by using t-Test. *; $P < 0.01$ was considered significant. **; $P < 0.02$ was considered significant. ***; $P < 0.03$ was considered significant. ****; $P < 0.04$ was considered significant. GAPDH; glyceraldehyde phosphate dehydrogenase, DNMT; DNA methyltransferase.

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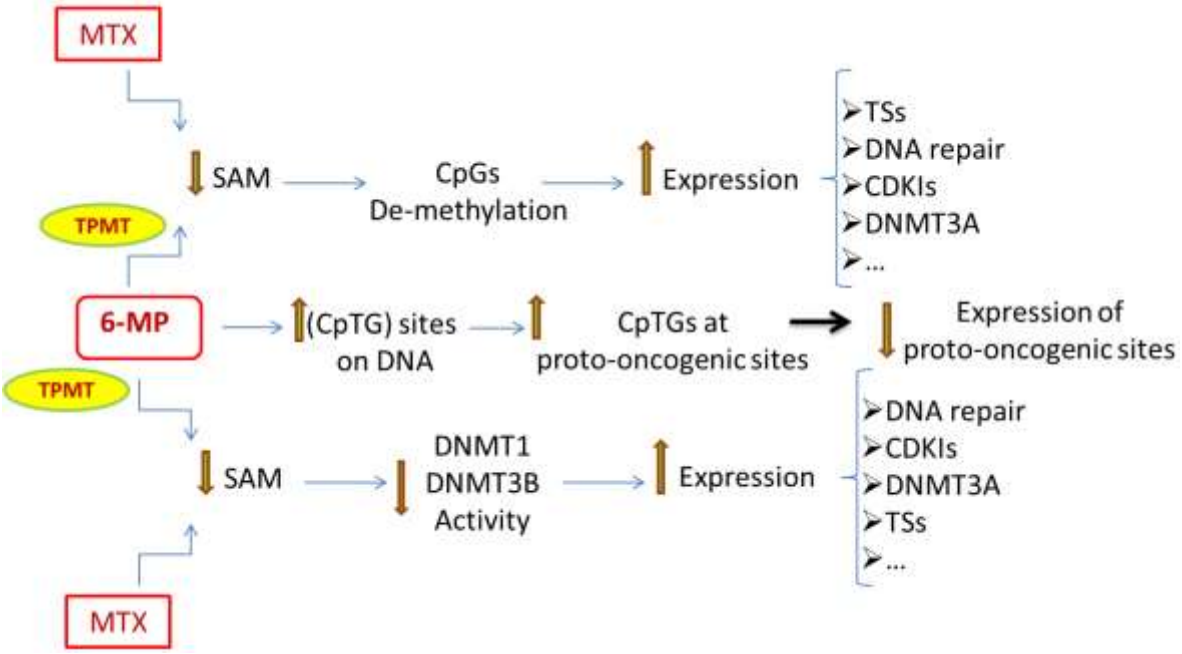

Figure 5. A proposed mechanism by 6-MP/MTX to reactivate silenced genes/inactivate proto-oncogenic sites is illustrated here. MTX as an analog of folate and 6-MP as an analog of purines cause reducing levels of S-adenosylmethionine (SAM) in the cells. SAM is essentially needed to maintain hypermethylation of specific CpGs by tumor-over-expressed DNMTs, as well as, thiopurine methyltransferase (TPMT) to metabolize 6-MP. TPMT metabolizes 6-MP and like as DNMTs, transfers methyl groups from SAM and generates S-adenosylhomocysteine (SAH). MTX/6-MP treatment causes a serious decrease in SAM levels by which can directly reduce DNMTs activity and would be closely reflected in the demethylation of CpG islands. Herein, SAM is needed by DNMTs over-expressed in tumors to methylate DNA at special promoters wherein the restriction in SAM levels would result in a reduction in the activity of DNMTs and could be associated with increased expression of genes inhibiting in tumor cells (e.g. TS, DNA repair, DNMT3A,...). Additionally, presence of 6-MP at CpG sites (CpTG), simultaneously may enhance inactivation of proto-oncogenic sites. The extent of MTX/6-MP effects is through DNA incorporation of 6-MP and also through reducing concentrations of SAM which is influenced by MTX concentration. CpG, Cytosine-guanine dinucleotide sequence; DNMTs, DNA methyltransferases; SAM, S-adenosylmethionine; 6-MP, 6-Mercaptopurine; MTX, Methotrexate; TPMT, Thiopurine methyltransferase; TG, thioguanine nucleotide; TS, Tumor suppressors.

Discussion

Epigenetic enzymes DNMTs play a major role in promoter methylation and silencing of tumor suppressor genes in human cancer cells. Even more, aberrant methylations by these genes facilitate gene mutation, since the deamination of 5-methylcytosine leads to the formation of thymine which is not repaired by glycosylases (9, 17). Tumor suppressors and DNA repair genes have frequent expression in proliferating cells wherein they are required for genome stability; impairment in their role can lead to the pathogenesis of leukemic stem cells (7-9). Data from ALL patients indicate that there is a significant reduction in the expression of genes involved in DNA repair and that these are responsible for genome stability including tumor suppressor and cell cycle-related genes. These changes are clear in the lymphocytes of ALL patients and are associated with an increased level of mutations in DNA and induction of transformation during the cell cycle (3, 8, 15).

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As a remarkable instance, CpG methylation of p15 and p16 promoters has frequently occurred in acute leukemia. Accordingly, about 93% of AML patients, or 61–94% of all leukemia subtypes display methylation of tumor suppressor promoters (18-20). These highly expressed genes have CpG-rich promoters (high-CpG-density class (HCP)) which are usually un-methylated in normal cells, since these genes are needed to control the cell cycle and growth (8, 20). Studies have identified methylated CpG sites in the promoter of these genes which are frequently inactivated in cancers and also silenced in acute leukemia (7, 9, 11). Methylation of CpG islands is catalyzed by DNMT1, 3A, and 3B whose changes in expression are observed in multiple human cancers, in particular in leukemia (9, 10, 21). DNMT1 is a key methyltransferase in the maintenance of genome methylation, whereas DNMT3A, together with DNMT3B, has an essential role in de novo methylation (7, 11). Additionally, studies have indicated that the promoter regions of DNMTs are also enriched in CpG dinucleotides, the best substrates for DNA methyltransferase activity (13, 14). However, changes in DNMTs expression can be observed in a cancer state or even after the chemotherapy phase wherein it could be accounted as an effect associated with an anti-cancer mechanism of drugs (5, 6, 12).

Accordingly, CpG methylation is reversible by treatment with anti-methylating agents which are regarded as optimal targets in epigenetic therapy. It is apparent from experimental settings that the reactivation of the types of genes epigenetically silenced in cancer would have a profound anti-tumor effect (22-25). De-methylation and reactivation of silenced tumor suppressor genes (e.g. CDKN2A) is possible through chemotherapy by anti-methylating agents (15, 26). DNA anti-methylating agents exhibit remarkable success in clinical practice especially effective in the treatment of ALL. Whether the underlying mechanism leads to global or selective de-methylation is not clearly defined yet (3, 5, 6). In this regard, as anti-methylating agents that negatively affect tumor growth, MTX/6-MP therapy is hypothesized to act through a mechanism directly affecting DNMTs activity followed indirectly by changes in the expression of tumor-related genes (3, 5, 6, 12). Note that, MTX/6-MP treatment seriously reduces the level of S-adenosylmethionine (SAM) in the cells, which is essentially needed by DNMTs to catalyze the addition of methyl groups to CpGs sites and is thereby accounted as a mechanism by these drugs (5, 6, 12). Additionally, the level of SAM is also consumed by another enzyme named thiopurine methyltransferase (TPMT) which metabolizes 6-MP and like DNMTs needs SAM to transfer methyl groups and generate Me-6Mp and S-adenosylhomocysteine (SAH) (3, 5, 6). Accordingly, 6-MP can simultaneously be incorporated into CpG dinucleotides and create CpTG sites; this subsequently causes inhibition of DNMT activity and hypermethylation of CpG islands present at the regions of oncogenic properties, identified frequently hypermethylated, and inactivated in cancers and also silenced in acute leukemia (Table V, Figure 5) (6, 8, 12).

However, despite their long history of use, the exact epigenetic mechanism of the MTX/6-MP regimen remains to be clearly defined which certainly improves our understanding of the pharmacology property and gives rise to better chemotherapy use (5, 6, 12).

Thus, at the first stage of this experiment, the expression profile of DNMTs in blood cells from B-cell ALL children was determined and compared to those in healthy children who were used as a control or calibrator group. As expected,

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the expression profile of DNMTs in B-cell ALL children was obtained completely different from that in healthy children. In B-cell ALL children, the expression levels of DNMT1 and DNMT3B appeared up-regulated and had significantly higher levels compared to the healthy group. Previous studies have also reported that up-regulation of these DNMTs is associated with tumor progression (20-22). However, the opposite phenotype was observed for DNMT3A which is expected to participate in differentiating events; also, its down-regulation or loss of function has been observed in tumors of lung, breast, prostate, and colorectal carcinomas (11, 20, 21). The second stage of the experiment was to investigate the effect of MTX/6-MP regimen therapy on the expression profile of DNMTs. Then, the expression of DNMT1, 3A, and 3B was assessed at the consolidation-maintenance phase of therapy with MTX/6-MP. The expressions of DNMT1 and 3B appeared coordinately down-regulated, whereas the opposite result was observed for DNMT3A expression. It appeared that the profile was changing from a cancerous phenotype toward the normal one and their expressions were reaching those levels in normal cells. It is notable that all pediatric patients included in this study continued the consolidation-maintenance therapy while showing no adverse effects, except two children who were excluded from the study (Figures 3 & 4). Hence, it is concluded that the MTX/6-MP regimen therapy may act through a mechanism that affects the expression and activity of DNMTs (9, 13, 14, 20). It is important to note that DNMT1 knockdown is understood to cause the induction of genes involved in cell cycle arrest and stress response, whose suppression may have important therapeutic implications. It has been also observed that DNMT3B depletion in human cancer cells induces apoptosis, but not in normal cells. DNMT3B activity seems to be associated with tumor suppressor genes and their promoter silencing (Table V) (22-24). Moreover, according to data, DNMT3B and DNMT1 may act highly cooperative which is needed by tumor cells to methylate anti-cancer and cell-cycle-related promoters (20-23). DNMT3B and DNMT1 have been detected highly enriched at hypermethylated loci of these genes with high and intermediate CpG-density promoters (HCPs & ICPs) (8, 9, 20). Regarding data, one of the optimal targets in tumor cells to reactivate silenced genes and lowering DNA methylation in the promoters seems to be disruption of DNMT1 and DNMTB, whereas DNMT3A has appeared with differentiating properties. DNMT3A has been found in pre-centromeric and telomeric regions and has been supposed to be associated with the methylation of these regions; however, it is essentially needed to contract tumor progression (5, 6, 8). The changes identified in the expression profile of DNMTs from ALL pediatric patients in this study may be used as an indirect mechanism by MTX/6-MP drugs used in cancer treatments.

Conclusion

MTX/6-MP regimen in the consolidation-maintenance phase is an effective anticancer therapy with remarkable success in B-cell ALL remission; however, the accurate mechanism of MTX/6-MP remains to be made clear. Regarding previous studies, the extent of MTX/6-MP effects could be through DNA methylation events and tumor-expressed activity of DNMTs, whereby demethylation of CpG islands is selectively induced at promoters of specific genes (2, 4, 25).

It was observed in the present study that the expression profile of DNMTs in B-cell ALL children was completely different from that in the healthy group and that their expressions changed from the cancerous phenotype toward the normal one, after the MTX/6-MP regimen therapy.

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Somehow, the results of this study are expected to lead to a better understanding of the molecular basis of ALL and the epigenetic action of effective chemotherapy. Epigenetic effects of MTX/6-MP drugs have been of great interest, not only for basic research but also for new therapies. However, the results of this work are not the only mechanism by MTX/6-MP; the confirmation of them requires more in vivo studies.

Ethical Consideration

The study protocol was approved by the Institutional Review Board and also by the Ethics Committee at Shahid Sadoughi University of Medical Sciences. The trial registration in the Institutional Review Board and the Medical Ethics Committee was done on 2015-11-25, with registration NO. IR.SSU.MEDICINE.REC.1394.298 (The vice-chancellor of research, at the School of Medicine, Shahid Sadoughi University of Medical Sciences: E-mail: medschool@ssu.ac.ir).

Author's contiributions

Seyed Hossein Hekmati Moghaddam, Azam Sadat Hashemi, & Nasrin Ghasemi: supervising and advising the research and editing the manuscript text;

Mahmoud Imanei: collecting data, doing experiments, and analyzing data;

Fatemeh Pourrajab: supervising the research and writing the main manuscript text.

Mahmood Vakili, and Kazem Barzegar: writing the main manuscript text.

Conflicts of interests

The authors declare no competing interests in this work.

Acknowledgements

The authors thank Ms. Tahereh Ahmadi Afzadi, Zahra Kinzad and Kolan Rahmani for their kind help and all parents of patients who were referred to the Pediatric Oncology Branch of Children's Medical Center of Tehran and participated at the study. Also, we give thanks to the professionals from the Department of Pediatrics Oncology and Hematology center, Shahid Sadoughi Hospital, Shahid Sadoughi University of Medical Sciences, Yazd, Iran, in particular Dr. Ali Dehghani Firoozabadi for their kind supports.

Funding

The authors have no funding source to declare.

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