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Epigenetic effects of decitabine on acute lymphoblastic and acute promyelocytic leukemia cells

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Abstract

Background: Decitabine (5-aza-2'-deoxycytidine, DAC) is a deoxycytidine analog currently used as an effective drug against myelodysplastic syndromes and acute myeloid leukemia. Although various studies have pointed out the epigenetic effects of this drug, its epigenetic mechanisms in different leukemic cell lines are not specified. In this lab trial study, possible epigenetic effects of decitabine on leukemia cell lines Hl-60 (acute promyelocytic leukemia) and Nalm-6 (acute pre-B cell lymphoblastic leukemia) vs. normal peripheral blood mononuclear cells (PBMCs) are compared.

Materias and Methods: At the logarithmic phase of growth, the cultured cells Hl-60 and Nalm-6 obtained from Tehran Pasteur Institute, Iran, were treated for 24 hr with 1 µM of decitabine, a dose selected from literature and the MTT viability assay. Normal PBMCs were obtained from a pool of 3 healthy adult volunteer males, and cultured simultaneously in the same manner. The gene expressions of epigenetic enzymes DNA methyltransferases (DNMTs) were assessed with the real-time PCR technique before and after treatment. The GAPDH gene expression served as the calibrator, while normal PBMCs were used for comparison.

Results: The expressions of DNMT1 and DNMT3B in lymphoblasts were significantly ($p=0.0017$ and $p=0.0489$, respectively) decreased after treatment with decitabine, while the expression of DNMT3A was significantly ($p=0.0022$) increased. In leukemic promyelocytes the expressions of DNMT1 and DNMT3B in lymphoblasts were significantly ($p=0.0222$ and $p=0.0452$, respectively) decreased after treatment with decitabine, while the expression of DNMT3A was significantly ($p=0.0013$) increased.

Conclusion: One of the mechanisms by decitabine to inhibit the proliferation of both myeloid and lymphoid acute leukemia cells maybe by altering the gene expression of DNMTs.

Keywords: Decitabine; DNA methyltransferase 1; Epigenesis; Gene expression; Leukemia

Introduction

Acquired hematopoietic disorders are a group of blood diseases known to be induced by epigenetic modifications and can be characterized by changes in DNA methylation (1, 2). Acute myeloblastic leukemia (AML) is a malignancy of the hematopoietic myeloid cells, characterized by the rapid proliferation of genetically abnormal leukocytes that build up in the bone marrow and interfere with the production of normal blood cells. AML is the most common acute leukemia affecting adults, and its incidence increases with age (3).

In addition to genetic variations, AML can also be characterized by epigenetic changes in the neoplastic cells. One of the

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most significant epigenetic alterations in these malignancies occurs in DNA methylation and histone modifications which affect the gene expression throughout the cell cycle (3, 4). The relationship between aberrant DNA methylation and various types of human cancers has been indicated over the past decade and has been a major research field aimed at finding effective drugs for cancer therapy (5, 6).

Aberrant DNA methylation can result in the silencing of tumor suppressor genes which likely contributes to the pathogenesis of AML. Aberrant DNA methylation can be reversed pharmacologically by inhibitors of DNA methyltransferases (DNMTs) (7, 8). Aza-nucleotide analogs such as decitabine (5-aza-2'-deoxycytidine, DAC) have been introduced as DNA anti-methylation agents that act as DNMT inhibitors. Decitabine has been an effective drug and approved for the treatment of patients with myelodysplastic syndromes (MDS) as well as AML (8-15). Decitabine is transported into the cells where it is phosphorylated by deoxycytidine kinase (dCK) followed by conversion to active metabolite decitabine-triphosphate (decitabine-TP) which competes with endogenous dCTPs for incorporation into the DNA (16-18). It seems that a reduction in overall activity of DNMTs may be a strong demethylation activity by decitabine at zones that are normally hypermethylated, such as promoters of tumor suppressors or the genes necessary to control the cellular differentiation and multiplication (17, 19). However, the exact mechanisms of this DNMT inhibitor activity have not yet been clearly defined (9, 13).

Mammals have five DNMT enzymes (DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L) but only DNMT1, DNMT3A and DNMT3B have a DNA methylation activity. In differentiating cells, the DNA methylation template is determined during primary development by de novo methyltransferases DNMT3A and DNMT3B. However, in proliferating cells, the template of DNA methylation is propagated through excessive loyalty by the preservation of methyltransferase DNMT1 (20-24).

Though the level of DNMT expression and activity in normal cells is relatively stable, wide changes are expected to happen in cancer cells that lead to universal hypomethylation of the genome along with focal hypermethylation at promoter CG islands leading to silencing of the most critical tumor-suppressor genes including p21, RB1 and CDKN2A (25-27). Also, several studies report increases in the expression of de novo DNMTs in various cancers, which can contribute to the development of leukemia by inducing hypermethylation of tumor suppressor genes (28-30). As a result, abnormal expression of de novo DNMTs may serve as a marker for cancer cells and also as a potential target for future cancer therapy.

Although various studies have pointed out the epigenetic effects of this drug, its epigenetic mechanisms in different cell lines are not specified. The aim of this lab trial study was to investigate possible impact of the effective chemotherapy drug decitabine on the expression profile of DNMTs in leukemic cell lines Hl-60 (acute promyelocytic leukemia) and Nalm-6 (acute pre-B cell lymphoblastic leukemia) compared to normal blood cells.

Materials and Methods

Cell cultures

This study was approved by the ethics committee in Shahid Sadoughi University of Medical Sciences, Yazd, Iran (IR.SSU.MEDICINE.REC.1396.55). The human leukemia promyelocyte cell line Hl-60 and lymphoblastic cell line Nalm-6 were obtained from the Tehran Pasteur Institute, Iran. Normal peripheral blood mononuclear cells (PBMCs) were obtained from a pool of 3 healthy adult volunteer

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males who had no disease sign or symptom and didn't have medication use. The cells were cultured in RPMI 1640 medium (GIBCO Invitrogen, US) containing 10% fetal bovine serum (GIBCO Invitrogen, US) and 1% penicillin/streptomycin (Sigma-Aldrich, US) in a humidified condition at 37 °C with 5% CO_2.

Cell treatment with decitabine

The cells were seeded at a density of 10^5 cells/mL in 6-well plates (Jet Biofiltration Co., China). When the cells arrived at logarithmic growth phase, the cultures were treated with 0.1, 1 and 5 µM of decitabine (Sigma-Aldrich, US) for 24, 48 and 72 h, with final selection of the optimal time regarding average proliferations. Finally, the selected dose was 1 µM according to literature and our data obtained from MTT viability assay which showed the highest possible (more than 90%) viability for normal cells. All the experiments were performed in independent triplicates (31, 32).

MTT assay

The cytotoxicity of decitabine was investigated by the MTT assay kit. After incubation of the cells in 96-well plate, they were treated with decitabine with concentrations of 0.5, 1 and 5 µL for 24, 48 and 72 hours. For testing, 10 µL of the MTT (Sigma-Aldrich, US) reagent at 5 mg/mL concentrations were added to each well and the plates were placed in the incubator for 4 hours. In the next step, the culture medium was removed and 100 µL of DMSO 100 added to each well and placed in the incubator to dissolve the formazan purple crystals. The absorbance of wells at 578 nm was read by the enzyme-linked immunosorbent assay (ELISA) reader (BioTek, US), and the growth percentage of the cells calculated. Finally, the obtained mean of three times (24, 48 and 72 h) was used for calculation of LC_{50} and the cells were treated with this dose.

Quantitative polymerase chain reaction (qPCR)

Total cellular RNA was extracted using a TRIzol® kit (Invitrogen, Thermo Fisher Scientific, US). The cDNA was obtained from the cDNA Synthesis Kit (Thermo Fisher Scientific, US). For qPCR, cDNA (1 µL) was mixed with EvaGreen® qPCR Mix Plus (Solis BioDyne, Estonia), and the following primers were used: DNMT1 forward: 5'-CCT CAG GGA CCA CAT CTG TA- 3' and reverse: 5'-TCT TCC TGT CAT GGT GGG TA-3'; DNMT3A forward: 5'- GCC CAA GGT CAA GGA GAT TA- 3' and reverse: 5'- CAG ATG TCC TCA ATG TTC CG-3'; DNMT3B forward: 5'- CCT TCT TCA CCA GTG ACA CG-3' and reverse: 5'- CCA TGA CAG GAC TCG AAT GG- 3'; which were designed and synthesized by Pishgam Biotech Co, Iran. Temperature protocol for qPCR was: 95 °C for 10 min, 95 °C for 15 sec, 60 °C for 20 sec, 72 °C for 25 sec, 72 °C for 10 min. Real-time PCR reactions were performed in triplicate. The statistical tests performed were t-test and analysis of variance. The relative gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ formula.

Results

Cell treatments, MTT assay

In the first step, to assess the real effect of decitabine on the gene expression of *DNMTs*, we tried to carefully create a reversible and low-toxicity situation for cells to investigate changes occurred in *DNMTs'* gene expression after treatment with the drug. The Hl-60 and Nalm-6 cells were treated with the selected dose (1 µM)

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of decitabine for 24, 48 and 72 h based on MTT test results. This low concentration of the drug allows the intracellular cycles to be active. A substantial cellular toxicity would be associated with higher concentrations (33, 34) and was undesirable because of secondary effects related to apoptosis that would interfere with the primary gene expression changes induced by the drug. Furthermore, high-dose protocols of DNMT inhibitors are no longer recommended clinically. The low dose of decitabine assessed in this experiment is comparable to the potential concentration of decitabine in the plasma in the previous treatment regimen therapies and may induce hypomethylation of DNA (35).

Confirming the specificity and selectivity of the qRT-PCR method for gene expression

After the decitabine treatment and confirming the cell viability of more than 90%, the expression profile of the epigenetic genes DNMT1, 3A, 3B was investigated. Also, to confirm the real-time PCR assay specificity and selectivity, the qRT-PCR products were loaded onto the gel. Figure 1 shows the bands, confirming the real-time PCR specificity and selectivity.

The expression profiles of *DNMT1*, *3A*, *3B* in Hl-60 and Nalm-6 cells compared to the normal cells

After normalization with GAPDH and in comparison to the expression profile of these genes in normal PBMCs, the fold changes in expression of *DNMT1*, *3A* and *3B* in Hl-60 cells and also in Nalm-6 cells were obtained, which reveal significant ($p<0.05$) alteration toward their level in normal cells after treatment with decitabine (Table I and Figures 2, 3, 4, 5, 6, 7). Since the relative fold changes vary, the figures are not merged. In all cases, the difference in gene expression of *DNMTs* between normal PBMCs and leukemic cells before treatment were significant (P values not shown).

Table I: Fold changes in expression of DNMT1, 3A, 3B in decitabine-treated Hl-60 and Nalm-6 cell lines vs. the healthy control group (as a calibrator), after normalization to GAPDH (P values compare before treatment vs. after treatment).

Gene	Control group	Cell line	Cell line before treatment	Cell line after treatment	P-value
DNMT1, mean ± SD	1.005 ± 0.147	Hl-60	5.238 ± 1.345	1.379 ± 0.222	0.0222
DNMT1, mean ± SD	1.002 ± 0.093	Nalm-6	8.063 ± 0.513	1.837 ± 0.776	0.0017
DNMT3A, mean ± SD	1.02 ± 0.286	Hl-60	0.179 ± 0.059	3.438 ± 0.235	0.0013
DNMT3A, mean ± SD	1.020 ± 0.286	Nalm-6	0.400 ± 0.289	4.25 ± 0.062	0.0022
DNMT3B, mean ± SD	0.966 ± 0.061	Hl-60	5.790 ± 1.569	0.715 ± 0.021	0.0452
DNMT3B, mean ± SD	1.003 ± 0.122	Nalm-6	5.790 ± 1.569	2.65 ± 0.49	0.0489

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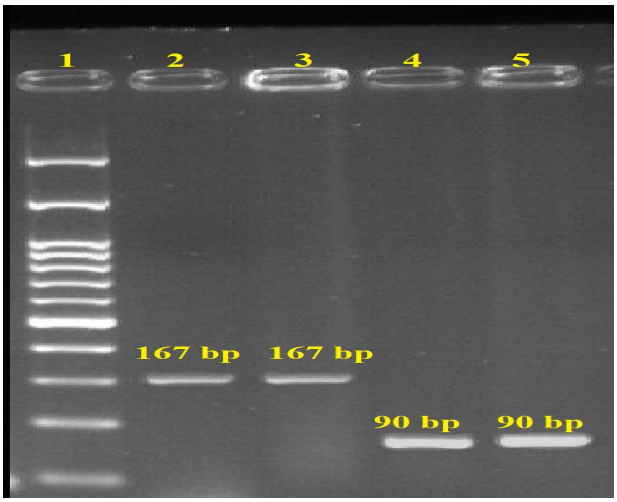

Figure 1. Gel patterns of real-time PCR (qRT-PCR) products of *DNMT1, 3A, 3B* obtained under non-saturating conditions on a 3% agarose gel stained with ethidium bromide. 1 (ladder 50 bp), 2 (*DNMT1* gene in Hl-60 cell line), 3 (*DNMT1* gene in Nalm-6), 4 (*DNMT3A* gene in Hl-60 cell line), 5 (*DNMT3A* gene in Nalm-6), 6 (*DNMT3B* gene in Hl-60 cell line), 7 (ladder 50 bp), 8 (*DNMT3B* gene in Nalm-6).

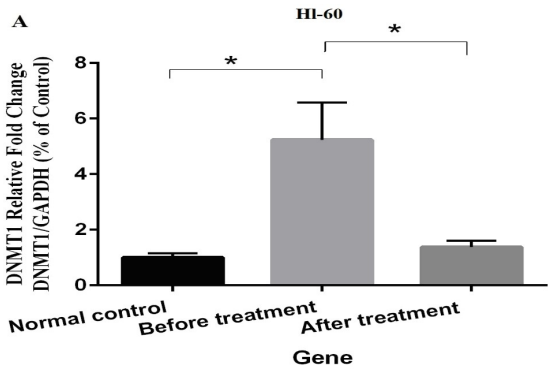

Figure 2. The relative fold change in expression of *DNMT1* in the decitabine-treated Hl-60 cell line compared to the normal PBMCs, after normalization to GAPDH which is expressed constantly in every cell type. Data were considered significantly different with $p<0.05$.

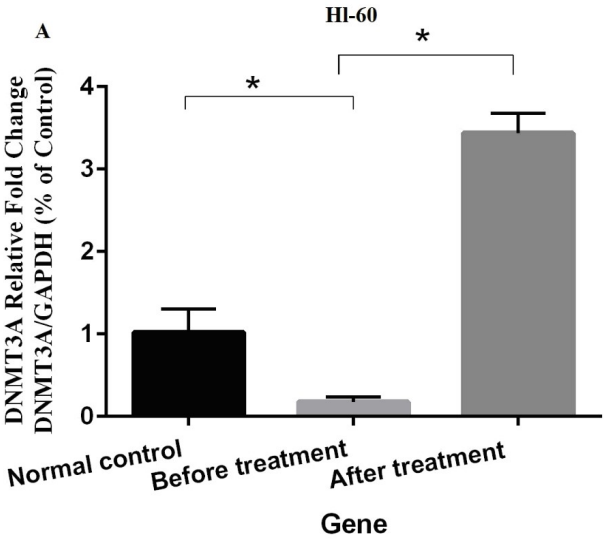

Figure 3. The relative fold change in expression of *DNMT3A* in the decitabine-treated Hl-60 cell line compared to the normal PBMCs, after normalization to GAPDH which is expressed constantly in every cell type. Data were considered significantly different with $p<0.05$.

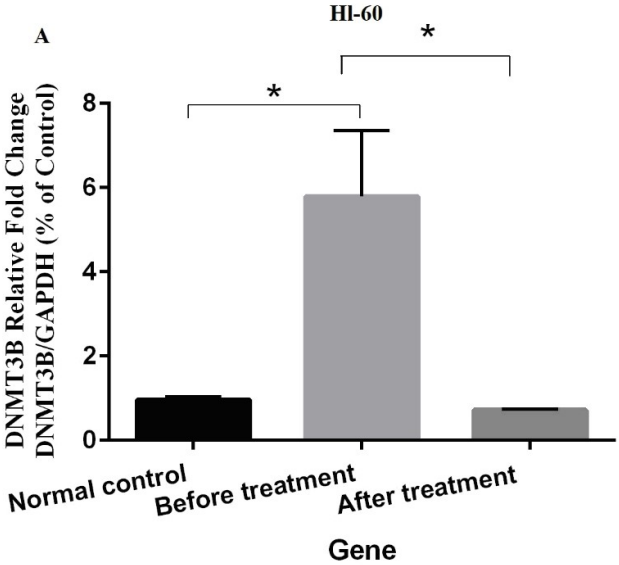

Figure 4. The relative fold change in expression of *DNMT3B* in the decitabine-treated Hl-60 cell line compared to the normal PBMCs, after normalization to GAPDH which is expressed constantly in every cell type. Data were considered significantly different with $p<0.05$.

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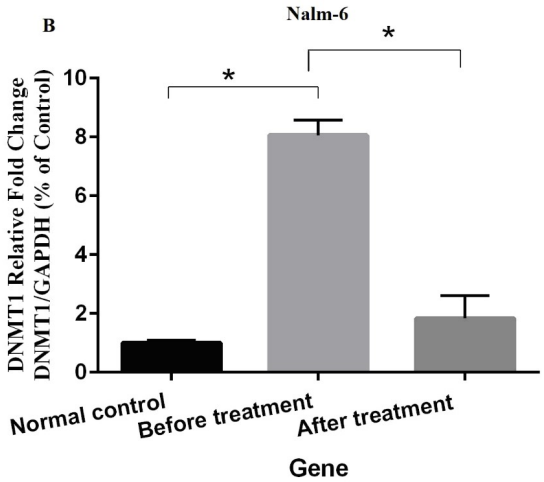

Figure 5. The relative fold change in expression of *DNMT1* in the decitabine-treated Nalm-6 cell line compared to the normal PBMCs, after normalization to GAPDH which is expressed constantly in every cell type. Data were considered significantly different with $p<0.05$.

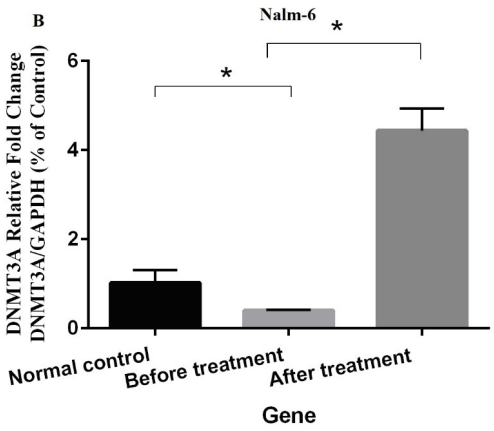

Figure 6. The relative fold change in expression of *DNMT*3A in the decitabine-treated Nalm-6 cell line compared to the normal PBMCs, after normalization to GAPDH which is expressed constantly in every cell type. Data were considered significantly different with $p<0.05$.

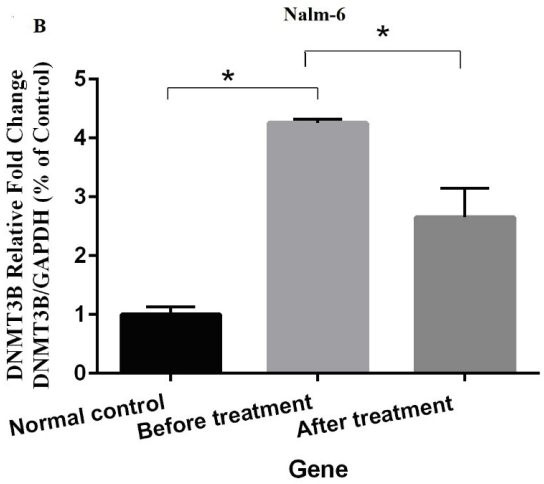

Figure 7. The relative fold change in expression of *DNMT*3B in the decitabine-treated Nalm-6 cell line compared to the normal PBMCs, after normalization to GAPDH which is expressed constantly in every cell type. Data were considered significantly different with $p<0.05$.

Discussion

Epigenetic changes in leukemia, in contrary to genetic lesions, are pharmacologically reversible and therefore a good target for novel therapeutic approaches. According to previous studies, DNMT3A and 3B seem to be essential for neoplastic transformation and tumor progression (28). DNMT3A is known as one of the most frequently mutated genes in hematological cancers. Additionally, it has been observed that eliminating DNMT3B limits tumor growth in vivo. In hematological malignancies, excessive expression of DNMT1 has been shown in 12 leukemia samples including AML, ALL and MDS. It is noteworthy that among the 3 DNMT genes (DNMT1, 3A, 3B), DNMT3B shows the greatest role in AML. As a result, abnormal expression of de novo DNMTs may serve as a marker for cancer cells, as well as a potential aim for future cancer therapy (30).

In the present study, the expression profile of DNMTs in both types of leukemic cells was completely different from those in normal cells, which was consistent with the previous findings (30, 32). Consistent

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with the results of this study, other experiments have also reported that the expression of DNMT1 and DNMT3B are increased in solid tumors such as ovarian tumors (31, 32). In the current study, under the effect of chemotherapy drug decitabine on the cell lines, the expression levels of DNMT1 and DNMT3B were significantly reduced and approached the normal one, a phenomenon which has also been observed with azacitidine (30). Previous studies have also shown that the activity of DNMT3B should be decreased for the inhibition of proliferation of some human cancer cells (36, 37). In the present study, the expression of DNMT3A in leukemic cell lines was lower than the normal cells, which increased after treatment with decitabine, as in some past works (38, 39). The effect of decitabine on a lymphoid cell line (Nalm-6) suggests that further research on its potential applications in lymphoid malignancies would be justified.

One mechanism attributed to decitabine is that it can covalently bind to DNMT, leading to a decrease in the DNA methylation of specific genes involved in the cell cycle (30, 39). Thus, it can be assumed that decitabine causes not only direct DNA demethylation, but also indirect changes in the expression of DNMTs. Although several experiments have been done regarding the effect of drug on gene expression, including in breast and colon cancer, the impact of decitabine on DNMTs in these two leukemia cell types was cleared by the current study.

Side effects of decitabine are many, including pyrexia, anemia, thrombocytopenia, febrile neutropenia, nausea, and diarrhea (40). It is not yet obvious which of them could be attributed to direct or indirect DNA demethylation in specific genes. Attempts have been made in recent years toward development of new delivery systems for enhanced efficacy and ease of use of decitabine (41).

Conclusion

According to qRT-PCR analysis, the expression profiles of DNMT1, 3A, and 3B in leukemic cell lines Hl-60 (i.e., myeloid) and Nalm-6 (i.e., lymphoid) were completely different from those in normal PBMCs. The decitabine treatment changed the cancerous profile of DNMTs in Hl-60 and Nalm-6 cells toward the normal one in PBMCs.

Acknowledgments

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Conflicting of Interest

There is no conflict of interest between the authors

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