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Evaluating the Effects of Chemotherapy Drugs and Thiosemicarbazone Complexes on the Alteration of SNHG16 Expression in Acute Lymphoblastic Leukemia

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Abstract

Background: Acute lymphoblastic leukemia (ALL) is a malignant disease that afflicts both children and adults. It starts from the bone marrow (soft and spongy tissue inside the bone) where immature white blood cells or lymphocytes are formed. This study aims to compare chemotherapy drugs, methotrexate (MTX), cyclophosphamide, cytarabine (Ara-C) and mercaptopurine (6mp) with nickel (Ni) and copper (Cu) complexes of thiosemicarbazone (TSCZ) in terms of their effects on the changes of Small Nucleolar RNA Host Gene 16(SNHG16) long non coding RNA (lncRNA) expression in the ALL cell line.

Materials and Methods: this experimental study was conducted on various concentrations of chemotherapy drugs including MTX, CP, Ara-C, 6mp and Ni and Cu complexes of TSCZ. The Jurkat E6.1 cell lines were subjected to passage in different groups and times (24, 48, and 72h) and then treated with the chemotherapy drugs. After RNA extraction and cDNA synthesis, the SNHG16 gene expression was evaluated by Real-Time PCR, and the results were analyzed by the Rest software.

Results: As the results indicated, within 24h, MTX (0.77, 0.72), CP (0.61, 0.7), ara-C (0.78, 0.87), Cu (0.91, 0.95) and Ni (0.94, 0.79) complexes as well as complex 1(0.73) and 2 (0.94) decreased the expression of SNHG16 gene significantly ($P<0.001$). Furthermore within 48h, under the influence of CP (0.86, 0.89) and Ara-C (0.94, 0.97) as well as Cu (0.96) and Ni (0.98) complexes, the gene expression continued to decline($P<0.001$).The greatest effect of chemotherapy drugs belonged to the combination of 1µM of ara-C and 5µM of 6mp.($P<0.001$)**.**

Conclusion: It was found that gene expression analysis is a feasible method to identify the pathways affected by the standard induction chemotherapy in ALL patients. This finding promotes the development of novel targeted drugs and biomarkers to categorize disease aggressiveness and evaluate treatment responses.

Keywords: Acute lymphoblastic leukemia, Chemotherapy, Drugs, Long noncoding RNA

Introduction

Leukemia refers to a group of hematological disorders characterized by abnormal cells with high ability to grow and multiply. Acute lymphoblastic leukemia (ALL) is a cancer originating from a type of white blood cell that is an immature form of lymphocytes in the bone marrow. These cells are not destroyed by apoptosis, grow rapidly in the bone marrow, enter the bloodstream over time, and spread throughout the body. This disease is the most prevalent childhood leukemia but is rarely found in adults. The factors that lead to the development of the disease are unknown (1-4). The Small Nucleolar RNA Host Gene 16(SNHG16) gene is a tumor suppressor gene and cancer-related long non coding RNA (lncRNA). The regulation of this gene is done in various cellular processes, including the inhibition of proliferation, migration and induction of apoptosis. SNHG16 acts as an lncRNA to regulate the corresponding mRNA in cancer, promotes cell proliferation by inhibiting

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the p21 expression, and inhibits apoptosis in some cancer cells. This gene functions by binding to proteins that are active in cell proliferation and migration or miRNAs, and its expression typically increases in various types of cancer, including ALL (5-10). Transcription factors, including c-Myc, STAT3, and AP-2 alpha (TFAP2A), are involved in the positive transcriptional regulation of the SNHG16 gene. In cancer, this gene links to Wnt-controlled transcription factors as an oncogene, and, as a result of knockdown, β-catenin declines in *SNHG16* and c-Myc. The knockdown of c-Myc reduces the expression of *SNHG16*, and its re-regulation increases the expression of the gene. STAT3 reduces the expression of *SNHG16* in cancer, and TFAP2A binds directly to the *SNHG16* promoter region and activates the SNHG16 transcription. The modulatory effects of SNHG16 depend on its position in the cell. This gene is involved in regulating the expression of SCD, p-AKT, MMP9, DKK3, and Wnt/β-catenin. A decrease in the expression of *SNHG16* increases miR-193a-5p levels, and the up regulation of miR-193a-5p increases the survival of leukemic cells. This miRNA is involved in cell proliferation, and its elimination has antitumor effects in leukemia. The gene can also regulate the incidence of leukemia through the (Wilms-1) WT1 tumor (11-15). Depending on the conditions of the patient and the disease, different drugs are used for the treatment of ALL to suppress cancer cells. Methotrexate (MTX) is one of the anticancer drugs used to treat leukemia. It prevents the growth and spread of cancer cells. Cyclophosphamide (CP) is another chemotherapeutic drug as an alkylating agent that prevents cell division by creating a cross-link between the DNA double strands. Mercaptopurine (6MP) enters the S phase of the cell division and is activated in tissues, thereby inhibiting the synthesis of DNA and RNA. Cytarabine (ara-C) plays a role in the cell cycle (S phase) and inhibits DNA synthesis, with the highest positive effect on inhibiting cancer cells. Thiosemicarbazone (TSCZ) complexes, with antiviral, antibacterial and anticancer properties, are used to treat cancer as well (16-26). This study aims to evaluate the changes in the expression of SNHG16 lncRNA in the ALL cell line treated with chemotherapy drugs (MTX, CP, ara-C, and 6mp) as well as Ni and Cu TSCZ complexes at different concentrations and times in comparison to the leukemic cells untreated with drugs.

Materials and Methods

The current *in vitro* experimental study was conducted at the Research and Science Center of the Islamic Azad University of Zanjan from October to March 2020. Human Jurkat E6.1 ALL cell lines in passage 1 with 80% density (2×10^5 cells cm^{-2}) were purchased from the Pasteur Institute of Iran.

Cell line cultivation and drug treatment

First, the Jurkat E6.1 ALL cell line was cultured in Dulbecco's Modified Eagle's Medium (RPMI 90%) containing 10% FBS and then incubated at 37°C with 5% CO_2 for six days. Three passages were applied to the cells once two days, and the cells were transferred to flasks with a fresh culture medium in each passage. Then, the cells of passage 4 were selected for the next steps. These cells were counted, stained with trypan blue, and found to be 3×10^4 per cm^{-2}. They were divided into control and sample groups. One 6mp tablet (50 g) was dissolved in 10 cc of distilled water. Ni and Cu (0.001 g of each) were dissolved in 1 cc of water. To prepare MTX and CP, one tablet of each was dissolved in 10 cc of DMSO and 10 cc of distilled water, respectively. The ara-C was prepared by the addition of 5 cc of DMSO to a drug vial. Then, the treatment groups with specific concentrations were used for durations of 24, 48, and 72 h. No drug was used for the control cells (Table 1).

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RNA extraction, cDNA synthesis, and Real-Time (RT) PCR

To examine the gene expression after the mentioned durations, RNA was first extracted with a highly pure viral nucleic acid kit (Roche Diagnostics Gmbh, Manheim, Germany, Cat #11858874001) according to the instructions. From the resulting RNA, cDNA was synthesized using a commercial kit (Takara Bio, Otsu, Japan, Cat #RR037Q) according to the instructions. For the synthesis, the required components (i.e., 2 µl of 5X Primer script Buffer 1X, 0.5 µl of Primer script RT enzyme Mix1, 0.5 µl of Oligo dT, 0.5 µl of Random 6mers, 3.5 µl of Total RNA, and 3 µl of RNase-free dH_2O) were mixed and incubated at 37°C for 15 min. The mixture was then subjected to the RT-PCR cycle at 85°C for 8-16 sec. The target and reference genes (SNHG16 and GAPDH) were amplified three times using a Rotor-Gene Q real-time PCR cycler (Qiagen, Hilden, Germany) based on the Syber green instruction (Takara Bio, Otsu, Japan, Cat # RR820Q).

The sequences of primers (5'-3') used to amplify SNHG16 and GAPDH genes were F: CAG TCA GCC TCA GTT TCC AA

R: AGG CAG GGC TGT GCT GAT- 3′ and F: ACC ACA GTC CAT GCC ATC AC / R: TCC ACC ACC CTG TTG CTG TA-, respectively. The reactions were set to a volume of 20 µl. Then, a mixture was prepared using 10 µl of Cybergreen (1X), 1 µl of forward and reverse primers (0.4 µl), 2 µl of cDNA, and 6 µl of deionized water. In the initial denaturation program, DNA fragments were amplified at 95°C for 10 min in 40 cycles (denaturation at 95°C for 5 seconds, annealing 52.2, 30 sec for *SNHG16*, and expansion and elongation at 72°C for 30 seconds: the steps were repeated three times for each gene).

Evaluation of gene existence and statistical analysis

The presence of *SNHG16* gene was confirmed through the electrophoresis of PCR products on a 2% agarose gel. The resulting fragments with the length of 300 pb were sequenced (Gen Fanavaran Co.) and confirmed in a straight direction. After the reaction, the difference between the ct target gene and the reference gene was calculated as Δct for each sample, and then $2^{-\Delta\Delta ct}$ (fold change) was obtained for each sample. The expression of the *SNHG16* gene was evaluated by RT-PCR, the Livak method, and the Rest software (2009) at the significance level of $P < 0.05$. Graphs were drawn using GraphPad Prism v9.0

Ethical consideration

The experiments were conducted based on the ethical code number of IR.IAU.Z.REC.1398.063.

Results

The treatment groups were compared in terms of the reference gene. The correlation coefficients and the standard deviations were also calculated, and the same value was obtained for the $2^{-\Delta\Delta ct}$ and the ratio. These operations indicated that the reference gene was not affected by the drugs.

Changes in the *SNHG16* gene expression in different durations and concentrations of MTX treatment

The statistical analyses revealed a significant increase in the expression of the *SNHG16* gene in the Jurkat E6.1 cell line of the treatment groups with 1 µM and 10 µM of MTX for 48 h (1.008 and 1.002, respectively) and 72 h (1.883 and 1.198, respectively) compared to the cells of the control group ($P < 0.001$) (Figs. 2 and 3). The expression of the *SNHG16* gene significantly increased at 1 µM and 10 µM of MTX within 48 and 72 h in comparison to the control gene (Figs. 2 and 3).

Changes in the *SNHG16* gene expression in different durations and CP concentrations

The statistical analyses of the changes in the *SNHG16* gene expression showed a

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significant enhancement in the cell treatment with 20 μM and 50 μM of CP (1.023 and 1.046, respectively) after 72 h, as compared to the control group (Fig. 3). A significant increase was also observed in the expression of the *SNHG16* gene at 20 μM and 50 μM of CP after 72 h, compared to the control gene (Fig. 3).

Changes in the *SNHG16* gene expression in different durations and ara-C concentrations

During 72 h at the concentrations of 1 μM (1.008) and 5 μM (1.026) of ara-C, the expression of the gene significantly increased in the Jurkat E6.1 cells compared to the cells of the control group (Fig. 3). A significant increase was also detected in the expression of the *SNHG16* gene compared to the control gene at 1 μM and 5 μM of ara-C after 72 h in comparison to the control gene (Fig. 3).

Changes in the *SNHG16* gene expression in different durations and 6mp concentrations

The statistical analyses indicated significant increases in the *SNHG16* gene expression through 6MP treatment at the concentration of 5 μM (1.060) after 24 h, at 5 μM (1.657) and 10 μM (1.367) after 48 h, and at 5 μM (2.089) and 10 μM (1.627) after 72 h in the cells of this group compared to those of the control group ($P < 0.001$) (Figs. 1, 2, and 3). The *SNHG16* gene expression increased significantly in the 6MP treatment at the concentration of 5 μM after 24 h, and 5 μM and 10 μM after 48 and 72 h compared to the control gene (Figs. 1, 2, and 3).

Changes in the *SNHG16* gene expression in different durations of treatment with the Cu and Ni complexes of TSCZ

In the treatment with the Cu and NI complexes of TSCZ, the *SNHG16* gene expression significantly increased at the concentration of 17 μM (1.083) after 48 and at 15 μM (1.035) and 16 μM (1.634) after 72 h ($P < 0.001$). According to the statistical analyses, there were significant increases in the *SNHG16* gene expression during the treatment with the Cu and NI complexes of TSCZ at the concentration of 51 μM (1.087) after 48 h and at 46 μM (1.167) and 48 μM (1.035) after 72 h in cells of this group ($P < 0.001$) in comparison to those of the control group (Figs. 2 and 3).

Changes in the *SNHG16* gene expression in different durations of treatment with the complex

The *SNHG16* gene expression significantly increased in treatments with different drug complexes including Complex 3 (1.001) after 24 h, Complexes 1 (1.039), 2 (1.144) and 3 (1.291) after 48 h, and Complexes 1 (1.493), 2 (1.761) and 3 (1.818) after 72 h ($P < 0.001$) (Figs. 1, 2, and 3).

Table I. Drug combinations used in the treatment

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24 Hours	48 Hours	72 Hours
5 & 10 µM of 6mp	5 & 10 µM of 6mp	5 & 10 µM of 6mp
1 & 10 µM of MTX	1 & 10 µM of MTX	1 & 10 µM of MTX
20 & 50 µM of CP	20 & 50 µM of CP	20 & 50 µM of CP
1 & 5 µM of ara-C	1 & 5 µM of ara-C	1 & 5 µM of ara-C
17.5 & 20 µM of Cu drug	15.5 & 17 µM of Cu drug	15 & 16 µM of Cu drug
5, 100, & 104 µM of Ni drug	51 & 61 µM of Ni drug	46 & 48 µM of Ni drug
Complex 1: 1 µM of MTX and 20 µM of CP	Complex 1: 1 µM of MTX and 20 µM of CP	Complex 1: 1 µM of MTX and 20 µM of CP
Complex 2: 20 µM of CP and 1 µM of ara-C	Complex 2: 20 µM of CP and 1 µM of ara-C	Complex 2: 20 µM of CP and 1 µM of ara-C
Complex 3: 1 µM of ara-C and 5 µM of 6mp	Complex 3: 1 µM of ara-C and 5 µM of 6mp	Complex 3: 1 µM of ara-C and 5 µM of 6mp

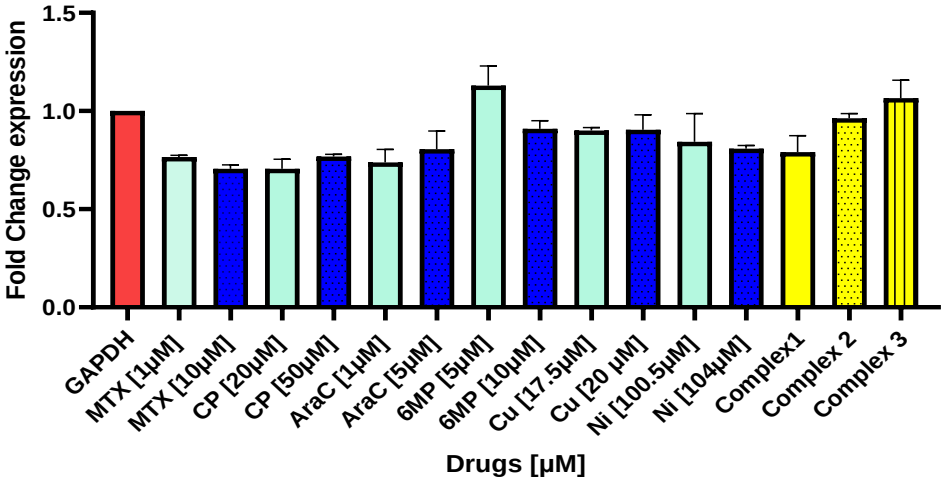

Figure 1. Bar chart of the changes measured at the expression levels of the *SNHG16* gene and the GAPDH reference gene treated with MTX, CP, AraC, 6MP, Cu, Ni, and drug complexes: The values are reported for various concentrations and in terms of 24 h.

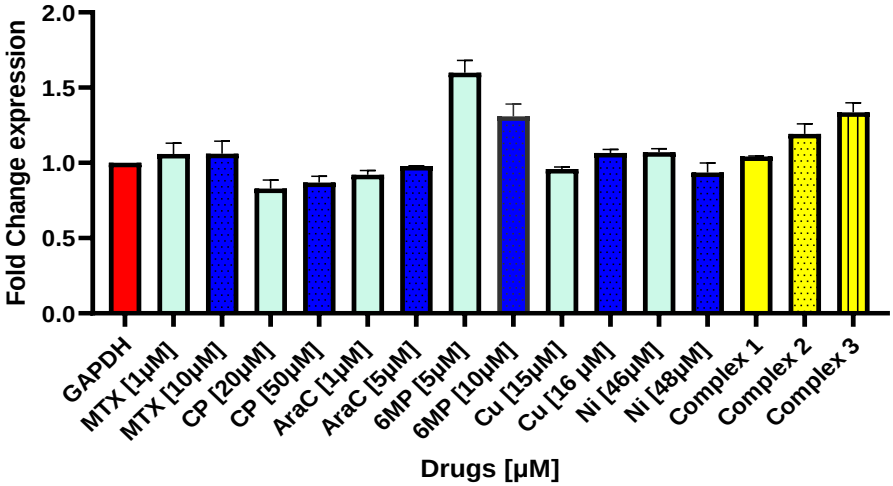

Figure 2. Bar chart of the changes measured at the expression levels of the *SNHG16* gene and the GAPDH reference gene treated with MTX, CP, AraC, 6MP, Cu, Ni, and drug complexes in terms of concentration and time (48 h)

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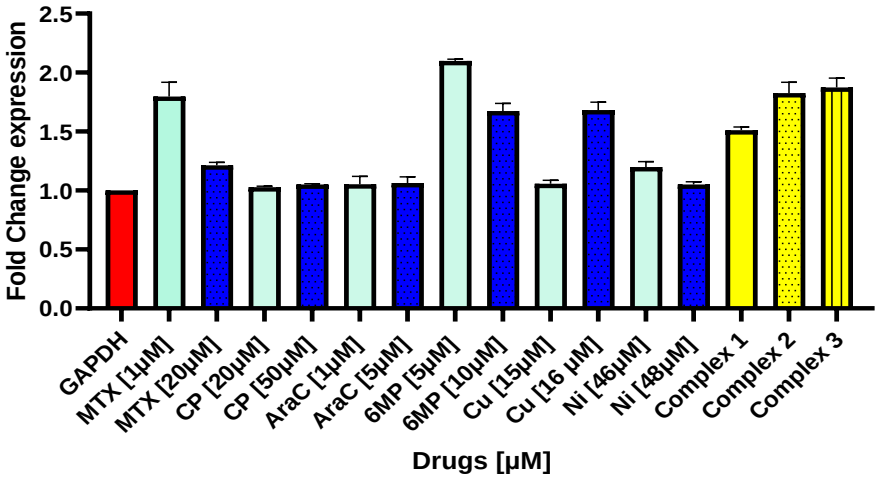

Figure 3. Bar chart of the changes measured at the expression levels of the *SNHG16* gene and the GAPDH reference gene treated with MTX, CP, AraC, 6MP, Cu, Ni, and drug complexes in terms of concentration and time (72 h)

Discussion

In Complex 3 drugs containing 1 μM of ara-C and 5 μM of 6mp, a greater effect was observed on the *SNHG16* gene expression with 5 μM of 6mp than with the other drugs, and the expression of the gene increased in the treatment with Complex 3 and 6mp in all the durations. CP at the concentration of 20 μM and 50 μM and ara-C at the concentration of 1 μM and 5 μM had less effect on the gene expression and caused no significant change in it.

In cancer, the *SNHG16* gene links to Wnt-controlled transcription factors and, as a result of knockdown, β-catenin declines in *SNHG16* and c-Myc. The knockdown of c-Myc reduces the expression of *SNHG16,* and its re-regulation increases its expression. STAT3 reduces the expression of *SNHG16* in cancer, and TFAP2A binds directly to the *SNHG16* promoter region and activates the *SNHG16* transcription. The modulatory effects of *SNHG16* depend on its position in the cell, and the gene is involved in regulating the expression of SCD, p-AKT, MMP9, DKK3, and Wnt/β-catenin (11-13).

Tianxin Yang et al. (2019) investigated the role of *SNHG16* in ALL. Based on their results, the regulation of this gene suppresses cell proliferation and migration in ALL, and it is considered as a tumor suppressor gene in leukemia by an inverse interaction with hsa-miR-124-3p. Yongbo Yu et al. (2019) evaluated the function of the *SNHG16* gene in neuroblastoma to investigate cell proliferation and migration status, colony formation, apoptosis, and cell cycle progression. The silencing of *SNHG16* inhibited the suppressed cell proliferation and migration and arrested the cell cycle in the G0/G1 phase of SH-SY5Y cells. Besides, apoptosis was undetectable in SH-SY5Y cells following the *SNHG16* silencing. *SNHG16* also regulates cell proliferation in NB through transcription and translation pathways. Lu Y.F et al. (2018) studied the *SNHG16* gene in glioma. According to their study, the knockdown of the *SNHG16* gene induces apoptosis in glioma cells and regulates the expression of the target genes miR-4518 and PRMT5. It also affects the expression of proteins from the Bcl-2 family and the activation of the PI3K/Akt signaling pathway. Piao and Zhang (2020) also investigated the *SNHG16* gene in

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leukemia. It was found that this gene is abnormally expressed in acute myeloblastic leukemia cell lines and that the loss of the gene weakens the life of leukemia cells, suppresses proliferation and promotes cell apoptosis (30).

The present study investigated the role of the *SNHG16* gene in ALL. This gene reduces the proliferation and differentiation of cancer cells as its expression increases in the disease. It leads the cells to apoptosis and can be used as a therapeutic target for the treatment of leukemia. The gene also has a negative effect on tumor growth and promotes the DNA repair

The *SNHG16* gene treatment with 1 μM of MTX after 72 h led to the highest increase in the expression, and the passage of time had a positive effect on the gene expression. However, an increase in the drug concentration did not positively affect the gene expression. In the treatment with CP drug, the passage of time and an increase in the concentration positively affected the gene expression. The highest increase in the expression was observed at the concentration of 50 μM after 72 h. In the treatment with 5 μM of ara-C, the highest gene expression was observed after 72 h, and the increase of the concentration and the passage of time positively influenced the expression of the gene. In the treatment with 6MP, the passage of time had a positive effect on the gene expression, but an increase in the concentration had no positive influence on it. The highest gene expression level was observed at the concentration of 5 μM after 72 h.

In the treatment with Cu, the increase of concentration had a positive effect on the expression of the gene; the concentrations of 17 μM and 16 μM after 48 and 72 h resulted in the highest increase of the expression, respectively. An increase in the Ni drug, however, led to no positive outcome in the gene expression, and the highest gene expression was observed at the concentrations of 51 μM and 46 μM after 48 and 72 h, respectively.In the treatment with Complexes 1, 2 and 3, the highest increase in the expression was observed after 72 h, and the passage of time positively influenced the expression of the gene.

The results of measuring the changes in the expression of *SNHG16* and the GAPDH reference gene treated with MTX showed a time-dependent pattern. Indeed, the expression increased significantly with the increase of time. The most increase occurred after 72 h. The statistical analyses of the changes in the concentration showed that the expression of the gene was not concentration-dependent; the gene expression decreased as the concentration rose. The highest increase in the expression (1.883) occurred at the concentration of 1 μM after 72 h. The treatment with CP also revealed that the expression of the gene was time-dependent; the highest increase in the expression was noticed after 72 h. The expression of the gene was concentration-dependent too. It increased with the rise of the drug concentration, and the highest increase (1.046) occurred at 50 μM of CP after 72 h. The *SNHG16* gene expression in ara-C treatment was time-dependent, and the highest increase in it was observed after 72 h. According to the statistical analyses of the changes in the concentration, the expression of the gene was concentration-dependent; that is, the increase of the concentration positively affected the gene expression, with the highest expression level (1.026) at 5 μM of ara-C after 72 h. In the treatment with 6MP, the *SNHG16* expression was time-dependent. It significantly rose with the increase of the time, and the highest expression occurred after 72 h. The gene expression in the treatment with this drug was not concentration-dependent, and the increased concentration had no positive influence on it. The highest expression of the gene (2.089) was observed at 5 μM of 6MP after 72 h.

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In the treatment with the Cu drug, the gene expression was concentration-dependent, and the highest increase in the expression occurred at 17 μM and 16 μM of the drug after 48 h (1.083) and 72 h (1.634). In the treatment with the Ni drug, the gene expression was not concentration-dependent; it decreased significantly with the rise of the concentration. The highest increases in the expression were detected at 51 μM and 46 μM of the drug after 48 h (1.087) and 72 h (1.167), respectively.

Through the treatments with the studied drug complexes 1, 2 and 3, a time-dependent pattern emerged for the gene expression. The highest expression levels (1.493, 1.761 and 1.818, respectively) were observed after 72 h. As it was found, MTX at the concentration of 1 μM had a great effect on the *SNHG16* gene of cancer cells after 72 h. Also, CP at the concentration of 50 μM after 72 h, ara-C at 5 μM after 72 h, 6MP at 5 μM after 72 h, Cu at 16 μM after 72 h, Ni at 46 μM after 72 h, and Complexes 1, 2, and 3 after 72 h had the greatest effects on the *SNHG16* gene in cancer cells.

Conclusion

This finding promotes the development of novel targeted drugs and biomarkers to categorize disease aggressiveness and evaluate treatment responses. The drug complexes could inhibit cell proliferation, which denotes their important role in the treatment of ALL.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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