

Suppressive Effect of Genistein on Childhood Cell Lineage Acute Lymphoblastic Leukemia by Inducing Dicer and AGO2

Fatemeh Piroozian

Hormozgan University of Medical Sciences

Hoda Bagheri Varkiyani

Hormozgan University of Medical Sciences

Afshin Samiei

Hormozgan University of Medical Sciences

Amirhosein Bradaran Najar

Isfahan University of Technology

Masoumeh Afsa

Hormozgan University of Medical Sciences

Kianoosh MalekZadeh (✉ keyanoosh@gmail.com)

Hormozgan University of Medical Sciences <https://orcid.org/0000-0001-7011-5637>

Research

Keywords: Genistein, Dicer, AGO2, Acute Lymphoblastic Leukemia, MicroRNA Biogenesis

Posted Date: May 11th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-27251/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background Childhood Acute Lymphoblastic Leukemia (ALL) is one of the most prevalent malignancies. Dysregulation of microRNAs in some cancers suggests their role in pathogenesis of the disease. Dicer and AGO2, two factors participate in biogenesis of miRNAs can exert a crucial function in development of the cancers. On the other hand, it has been proved that, genistein has anticancer effects against some cancer cells.

Methods In the present study, it was attempted to assess alteration in the mRNA expression of the Dicer and AGO2 genes, and then evaluating inhibitory effect of genistein on Dicer and AGO2 genes. Up-regulation of Dicer and down-regulation of AGO2 were observed in 40 patients with childhood ALL compared to 35 healthy controls.

Results Alteration in the expression of these genes directed to a correlation with progression of the disease. Genistein had anti-proliferative effect against ALL cancer cells through increasing mortality rate via induction of apoptosis and decreasing growth rate of malignant cells. The genistein significantly increased the mRNA level of Dicer particularly in two cells (Molt-17 and Nalm-6). Up-regulation of AGO2 that occurred as a result of genistein administration was significant in the 4 cell lines compared to non-treated cells.

Conclusions The concordance in alteration of AGO2 and Dicer mRNA expressions in B-ALL cell lines caused by genistein administration suggests existence of another mechanism of this compound as chemotherapeutic agent against ALL cell line.

Background

Leukemia is the most prevalent cancer in childhood around the world (Iacobucci & Mullighan, 2017). Among different types of leukemia, the Acute Lymphoblastic Leukemia (ALL) accounts for 25% of all Pediatric cancers worldwide (Simioni, Martelli, Zauli, Melloni, & Neri, 2019). Generally, ALL is divided into two types, B-cell ALL and T-cell ALL (Iacobucci & Mullighan, 2017). Mammalian immune system is mainly consisted of three kinds of lymphoid cells including Natural killer cells, T-cells, and B-cells. These cellular populations originate from progenitor cells. In ALL, many of the stem cells are haphazardly turned into lymphoblasts, which are proliferative and immortalized cells. Lymphoblasts are not capable of fighting against infections. This may lead to infection, anemia, or self-hemorrhage (Carroll, Loh, Biondi, & Willman, 2011). Overall, development of ALL is associated with both genetic and environmental factors (Wiemels, 2012).

MicroRNAs belong to a group of small non-coding RNAs, role of which has been recognized in primary cellular activities including proliferation, differentiation, apoptosis, and response to stress, which any disturbance in their natural way can form a tumor and cause a cancer (Gebert & MacRae, 2019). For instance, it has been shown that, dysregulation of miRNA-100 and miRNA-99a expressions is related with survival of the patients with ALL (Li, et al., 2013). As synthesis pathway of miRNAs can influence and

change their expression, it is essential to pay special attention to the genes involved in this pathway including Drosha, DGCR8, Exportin V, Dicer, AGO2, and TRBP. MicroRNA biogenesis involves two main processing phases, which its first phase occurs in the nucleus and the subsequent one occurs in the cytoplasm. Dicer and AGO2 enzymes in the cell cytoplasm generally influence maturation trend of microRNAs (Drobna, Szarzyńska-Zawadzka, & Dawidowska, 2018; Kwon, et al., 2019).

Both Dicer and AGO2 have functions in cytoplasm of cells for microRNAs maturity. AGO2 enzyme as a member of the Argonaut family (Ago) is one of the components of RNA-Induced Silencing Complexes (RISCs). MicroRNAs need to connect to the RISCs to apply their function. Therefore, it is so clear that any imbalance in the expression of AGO2 can have pathological effect on different types of cancer (Pokornowska, et al., 2019). Dicer as an RNase-III family enzymes cleaves double-stranded RNAs. The mature and functional microRNAs which is acquired by action of Drosha and DGCR8 on long pri-microRNA and consequently is digested by Dicer. Several studies have shown that, Dicer is also involved in the immune system. For example, it has been proved that, aberrations in expression of Dicer could lead to defects in selection of thymic T cells, sensitivity of T cells to antigens, and maturation of B cells due to dysregulation in microRNAs levels (Gounaris-Shannon & Chevassut, 2013). It has also been indicated that, down-regulation of Dicer is a poor prognostic factor for ovarian (Merritt, et al., 2008) and lung (Chiosea, et al., 2007) cancers. Of course, there are some studies reported up-regulation of Dicer in adenocarcinoma, melanoma (Ma, et al., 2011) as well as prostate cancer (Chiosea, et al., 2006).

Genistein (GEN) a a type of herbal isoflavonoid derived from soybeans is known as a protein tyrosine kinase inhibitor and tumor suppressor (Tuli, et al., 2019). Moreover, genistein mediates the changes in histone acetylation and DNA methylation of different genes in cancerous cell lines(Li & Chen, 2011). Recent studies have provided convincing evidence regarding inhibitory effects of genistein on cancerous cells of different types of cancer such as the breast, prostate, stomach ,and ovaries (Huang, Wan, Luo, Huang, & Luo, 2014). Genistein is capable of inhibiting uncontrolled cell growth through inhibiting WNT pathways. It can also inhibit NF- κ B activity and Akt signaling pathway (Banerjee, Li, Wang, & Sarkar, 2008). The effect of genistein on many signaling pathways such as E-cadherin, MMP ,and NF- κ B has been well recognized (Wang, Li, & Chen, 2012).

According to above-mentioned reasons, the dysregulation of factors involved in biogenesis of microRNAs can have pathological effects in malignancies. Considering significance of ALL worldwide, importance of altered expression of miRNA, and existing gap in the literature, the present research was conducted to evaluate the effect of changes in the mRNA expression of Dicer and AGO2 on exacerbation of ALL. Besides, to evaluate effectiveness and toxicity of genistein on Acute Lymphoblastic Leukemia (ALL), it was also tried to clarify, whether its anti-cancerous effects could make significance changes in mRNA expression of factors involving in miRNAs biogenesis pathway. It is assumed that, finding another pathway influenced by the genistein can be helpful.

Methods

Editorial Policies and Ethical Consideration

This research project was ratified and approved by the Ethics and Human Rights Committee (EHRC) of Hormozgan University of Medical Sciences (HUMS), Bandar Abbas, Iran (HUMS.REC.1394.84).

In Vivo Study: Specimens

Following previous research of our team (Piroozian, et al., 2019), blood samples were collected from 40 patients with ALL (15 samples were added to previously obtained samples) and 35 healthy controls referred to the Pediatric Hospital and the Pathology Department of Shahid Mohammadi Hospital, Bandar Abbas, Iran, after obtaining an informed consent from the participants' parents. The disease was diagnosed in the patients by oncologists and pathologists using the outcomes of immunohistochemical tests, flow cytometry, and blood cell count along with peripheral blood smear test. None of included patients had undergone radiotherapy or chemotherapy. All the patients who had received any type of treatment before the study were excluded from the study. Healthy controls were selected with respect to their peripheral blood smear test and blood cell count, and they were matched based on age, gender, and even ethnicity. The subjects were included in the study from the population of Hormozgan province within 4 years. Furthermore, 40 subjects were assigned to the patient group (afflicted with ALL) and 35 healthy individuals were assigned to the control group. Disease stages were evaluated according to French-American-British (FAB) classification (L1, L2, and L3) and WHO classification (pro-B, common, and pre-B-cell). Flow cytometry, cytomorphology, cytochemistry, and immunohistochemically staining of bone marrow biopsies totally employed by pathologist for exact diagnosing of ALL. Patients were also classified into three groups: good, intermediate and poor – related to leukocyte count, bulky extramedullary disease, CD10 negativity, immature pro-B phenotype, and favorable treatment prognosis. Categorization based on age (10 months > age > 10 years, 10 months < age ≤ 10 years), first leucocyte count at diagnosis (WBC count < 50 000/μL or ≥ 50 000/μL), subtypes of ALL, gender, spread to certain organ sites (cerebrospinal fluid; testis in males), and response to induction therapy as indicated by the Minimal Residual Disease (MRD) leads to classify patients into very high risk, high risk, and standard risk groups. **Table-1** shows the subjects' information for this study. About 2 ml of blood samples of all included subjects was taken. Then, 200 μl of their blood was mixed with 600 μl of ZR whole – blood RNA (Cat. No.: R1020) and was subsequently stored at -80°C.

Table-1. Demographic data of patients with Acute Lymphoblastic Leukemia (ALL) and healthy controls (H.C).

Variable	ALL Patient (n = 40)	Healthy Control (n = 35)	p-value
Mean Age (± SD) (Min-Max)	6.70 (± 3.42) (2.0-13.5)	6.58 (± 3.38) (2.2–14.5)	0.931
Age ≤ 7	26 (65.0%)	23 (65.7%)	
Age > 7	14 (35.0%)	12 (34.3%)	
Gender			1.001
Female	17 (42.5%)	15 (42.8%)	
Male	23 (57.5%)	20 (57.2%)	
Mean % Blast Cells (± SD) (Min-Max)	74.53 (± 7.58) (62–89)	0	
% Blast < 75	14 (35.0%)	0	
% Blast ≥ 75	26 (65.0%)	0	
ALL FAB sub-classification			
L1	19 (47.5%)	0	
L2	21 (52.5%)	0	
L3	0	0	
ALL EGIL sub-classification			
B-I: Pro B-ALL	18 (45.0%)	0	
B-II: Common B-Cell	0	0	
B-III: Pre B-ALL	22 (55.0%)	0	
B-IV: mature B-ALL	0	0	
Favorite in Treat Prognosis			
Good	5 (12.5%)	0	
Intermediate	27 (67.5%)	0	
Poor	8 (20.0%)	0	
EGIL: European Group of Immunophenotyping Leukemia			
In vitro study: Cell culture of ALL cell lines			
Jurkat, Molt-4, Molt-17, and Nalm-6 cell lines were purchased from the cell bank of Pasteur Institute of Iran. The samples were cultured in RPMI 1640 medium (Invitrogen) containing 10% FBS, 100 U/ml of penicillin, 100 µg/mL of streptomycin, and 2 mM of L-glutamine (Invitrogen) and were incubated under desired conditions with 5% of CO ₂ and 95% of humidity at 37°C.			

MTT-Assay

MTT-Assay was used to evaluate sensitivity and cytotoxic effect of genistein (Sigma, Germany) on cells and cell viability. Genistein (Sigma: Cat. No. G6649) was solved into DMSO (with a stock concentration of 100 mM). 15000, 16000, 32000, and 4000 cells of Jurkat, Nalm-6, Molt-4, and Molt-17 were counted by Neubar lam and were cultured into a 96-well plate. Then, different concentrations of genistein were added to each well (after IC50 measurement, 15 μ M, 30 μ M, and 60 μ M concentrations of genistein were selected). The plates were incubated for 24 and 48 hours (one plate for 24 and one for 48 hours for each cell line). After incubation, 20 μ L of MTT solution was added to each well proceeded by incubation for 4 hours at 37°C. Then, they were centrifuged (for 10 minutes at the rate of 4000 g) and the supernatant was removed. Subsequently, 150 μ L of non-sterile, culture-specific DMSO was added to the formazan crystals at bottom of the plate, which was shaken slowly so that the crystals were solved in the liquid. The plates were later incubated for 10 minutes in a cell culture incubator and their absorption was read using an ELISA Reader device (Anthos 2020; England) at 495 nm. Cell viability percentage was measured according to the $OD(T)/OD(C) \times 100$ formula. OD(T) showed absorbance of the cells treated with genistein and OD(C) represented absorbance of non-treated cells.

RNA Extraction and cDNA Synthesis

For human samples, RNA was extracted from the whole peripheral blood using ZR whole -blood RNeasy mini kit (Cat. No. R1020, Zymo Research Co.) according to the manufacturer's protocol. On the other hand, the mRNAs were extracted from the control group, non-treatment group, the other groups treated with 15, 30, and 60 μ M of genistein at 24 and 48 hours from the 4 cell lines cultured in a 6-well microplate according to the manufacturers' instructions (Qiagen; Germany). Quality and quantity of extracted RNA were measured by running on 1% formamide agarose gel and using NanoDrop (BioRad; USA). A good qualified extracted RNA has 3 bands in gel electrophoresis including 5S, 18S, and 28S with the absorbance of 1.9–2 at a ratio of 280/260. In the present study, extracted RNAs had good qualities. An equal weight of RNA (1 μ g) was extracted from the samples and added to synthesis reaction tube of cDNA. Synthesis of cDNA was done using a mixture of random Hexamer and pol(T) primers in the RevertAid H -Minus First Strand cDNA Synthesis Kit (Cat.No. K1632, Fermentase,) according to the manufacturers' protocol.

Real-Time PCR

Table-2 shows sequences of Dicer, AGO2, and β -actin (as the housekeeping gene) primers and their annealing temperatures. 1 μ L of synthesized cDNA was added to the reaction tubes and the final volume of each reaction with the resultant master mix reached 20 μ L. The expression of β -actin as housekeeping gene was measured in order to normalize the expression level of Dicer and AGO2 genes. Serial dilutions of PCR products for mentioned genes were used for standardization of Real-time PCR according to the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara Bio Inc.; Japan) using the Rotor-Gene™ 6000 system (Corbett Research, Australia). Real-time PCR was accomplished for two times and in triplicate for every sample in each time. Consequently, average Ct was assessed from the 6 Cts for each sample.

Table-2. Primer sequences and real-time PCR conditions

Gene	Sequence of primers (5'→3')	Amplicon (bp)	Annealing Temp. (°C)
DICER	F- TGGGTCCTTTCTTTGGACTG	245	62
	R- CTGGTTTGCAGAGTTGACCA		
AGO-2	F- AGGCTGCTCTAACCCCTCTTG	114	60
	R- ACGCTGTTGCTGACACATC		
β-actin	F- GCCTTTGCCGATCCGC	90	59
	R- GCCGTAGCCGTTGTCTG		

Flow Cytometry

To evaluate apoptotic and necrotic effect of genistein on ALL cell lines, the flow cytometry was used. Translocation of phosphatidylserine (PS) residues from cytoplasmic face of the plasma membrane to the cell surface is an early event in apoptosis. Annexin-V has a high affinity for phosphatidylserine hence, it can be used as a marker for identification of apoptosis (Sheng, Zhang, Pu, Ma, & Li, 2002). One of the purposes of this research was to see whether GEN is capable of apoptosis induction in ALL cells. In this study, two cell lines including Jurkat and Molt-4 were assessed, which were treated with 3 doses of genistein (15, 30, and 60 μM) at 24 and 48 hours. At 24 and 48 hours, the FITC Annexin-V Apoptosis Detection Kit with 7-AAD (BioLegend; UK) was used according to manufacturers' protocol to wash and stain the cells. All these procedures were repeated for both cell lines at three times separately.

Statistical Analysis

SPSS-21, Microsoft Excel, and Prism-5 softwares were used. Kolmogorov-Smirnov test was used to check normal distribution of data for human samples. Consequently, proper parametric and non-parametric tests were done to compare the data. To check significant differences in mRNA expression of AGO2 and Dicer before and after the genistein treatment, Students' t-test, Mann-Whitney U-test, and Wilcoxon test were used. In addition, $2^{-\Delta\Delta CT}$ method was used to evaluate gene expression. Significance level was set at 0.05.

Results

In the present research, L2 (afflicting 52.5% of patients) was reported as the highest level of the disease among the subjects. The rest of them were classified as L1. Regarding favorite treatment prognosis, 12.5% of the patients suffered from a good prognosis, while 67.5% of them had intermediate prognosis, and 20% of them had a poor prognosis (**Table-1**). Unfortunately, two patients died because of a poor

prognosis. The percentage of blast cells lesser than 75% observed in 35% patients, and 65% of patients had above or equal to 75% blast cells.

mRNA Expression of Dicer

mRNA expression of the Dicer increased by 1.74 times in the patients in comparison with healthy controls ($p \leq 0.0001$) (Fig. 1). Moreover, a positive correlation was observed between up-regulation of Dicer and the disease stages especially from L1 to L2. mRNA expression of the Dicer increased from L1 to L2 in healthy controls. There was also a significant difference in the mRNA expression of the Dicer between patients with $\geq 75\%$ blast cells and patients with $< 75\%$ blast cells ($p = 0.024$) (the data not shown). On the other hand, alteration in mRNA expression of the Dicer was compared in patients with ALL sub-types whose disease was diagnosed according to The European Group for the Immunological **Classification** of Leukemias (**EGIL**) classification (**Table-3**). Dicer mRNA level increased significantly in all subtypes of ALL as compared with healthy controls ($p < 0.0001$). But No significant difference was observed between patients with pre-B-cells and pre-B-cells with high risk, as well as pro-B-cells ($p = 0.2340$) and pro-B-cells with high risk ($p = 0.1268$) (Fig. 2).

Table-3. Real-Time PCR results for Dicer and AGO2 mRNA expression according to sub-classification of ALL

Subtypes	Mean (\pm SD) (Min-Max)	<i>p</i> -value
Dicer mRNA expression in:		
Normal	1.00 (\pm 0.268) (0.69–1.42)	
Pro B-ALL & Std. Risk	1.76 (\pm 0.106) (1.12–2.05)	0.0086
Pre B-ALL & Std. Risk	1.51 (\pm 0.126) (0.74–1.45)	0.0241
Pro B-ALL & High Risk	2.19 (\pm 0.318) (1.58–3.14)	0.0002
Pre B-ALL & High Risk	1.96 (\pm 0.423) (0.88–2.81)	0.0023
Ago2 mRNA expression in:		
Normal	1.0 (\pm 0.191) (0.56–1.35)	
Pro B-ALL & Std. Risk	0.62 (\pm 0.172) (0.28–0.69)	0.0031
Pre B-ALL & Std. Risk	0.79 (\pm 0.149) (0.48–0.85)	0.0127
Pro B-ALL & High Risk	0.32 (\pm 0.039) (0.29–0.46)	< 0.0001
Pre B-ALL & High Risk	0.41 (\pm 0.095) (0.38–0.73)	0.0111

mRNA Expression of AGO2

The mRNA expression of AGO2 decreased significantly by 0.54 times in patients with ALL in comparison with controls ($p \leq 0.0001$) (Fig. 1). Besides, the AGO2 mRNA expression reduced from L1 to L2 in healthy controls. Moreover, there was no significant difference between AGO2 mRNA expression of patients with $\geq 75\%$ blast cells and patients with $< 75\%$ blast cells ($p = 0.742$). It was also revealed that, patients' age and gender did not significantly influence the change in mRNA expressions of Dicer and AGO2. AGO2 mRNA expression reduced significantly in the patients with subtypes of ALL compared to the healthy controls ($p \leq 0.0001$) (Table-3). But there was no significant difference in the AGO2 mRNA expression between patients with standard risk and those with high risk (Fig. 2).

MTT-Assay

To evaluate cytotoxic effect of genistein, MTT- assay was run for ALL cell lines at 24- and 48- hours treatment with genistein. Results indicated that, genistein had an inhibitory dose-related effect on cell growth. For instance, in Molt-17 an increase in the dosage was accompanied by a descending trend of cell survival at 24 hours until it reached 44%. At 48 hours, it showed further decrease until it reached 24% (Fig. 3). Generally, an increase in the dosage of genistein and time was accompanied by a descending trend of cell survival.

Flow Cytometry Results and Apoptosis Induction by the Genistein

The graphs are related to the groups treated with genistein, control groups and those treated with doxorubicin (the anti-cancerous medication inducing apoptosis) as negative and positive controls, respectively. Finally, early and late apoptosis and necrosis were estimated in studied groups. An increased dose of genistein in Jurkat and Molt-4 caused an increase in total apoptosis in the patients compared to the controls. This trend was obvious in both 24- and 48-hr intervals, and it was higher in early apoptosis than the late one. No statistically significant difference was found in the necrotic cells between the group treated with genistein and the control group in both cell lines. However, the anti-cancerous doxorubicin was shown to significantly increase count of necrotic cells in both cell lines. Genistein, especially in higher doses helped to increase apoptosis in Molt-4 and Jurkat cell lines. For instance, Fig. 4 shows the effect of 48- hr treatment with different doses of genistein on apoptosis of Jurkat cell line as obtained by flow cytometry (Fig. 4).

Dicer mRNA Expression after Treatment with Genistein

Dicer mRNA was expressed in all the 4 cell lines (Fig. 5). There was a significant increase in the expression of Dicer in Molt-17 cell line (as a mild ALL cell line) of the patients than the control group ($p \leq 0.002$). Expression of Dicer was about 1.5 and 1.8 times at 30 and 60 μM of genistein. The same increasing trend of Dicer expression concurrent with increasing dose was observed in Nalm-6 cell line, which was also statistically significant ($p \leq 0.04$). A significant correlation was also observed between the mRNA expression of Dicer in Molt-4 cells and the increase in genistein dose in the control group within 48 hours ($p = 0.004$). Despite an increase in the expression of Dicer in both 24- and 48-hr treatments, but no significant difference was observed between these two intervals, particularly at a dose of 60 μM (Fig. 5).

There was no statistically significant correlation between altered expression of Dicer in Jurkat cell line in the genistein-treated groups and the control group ($p \geq 0.05$) (Fig. 5).

AGO2 mRNA Expression after Treatment with Genistein

AGO2 gene was expressed with nearly the same degree in all the 4 cell lines. As shown in Fig. 6, an increasing in AGO2 expression in all the 4 cell lines was accompanied by increasing in the dose and time of exposure to genistein. Overall, the increase in the dose of genistein was accompanied by a higher expression of AGO2 in all the 4 cell lines to a similar degree in the patients in comparison with the controls.

Discussion

Acute Lymphoblastic Leukemia (ALL) is a lymphoid progenitor cells malignancy and the most common type of cancer in children (Tomizawa & Kiyokawa, 2017). MicroRNAs have been confirmed to play a significant role in regulation of hematopoiesis (Slavov, Gimenes Teixeira, & Rego, 2010). Several microRNAs have been found to be up- or down-regulated in patients with ALL (Calin, et al., 2004; Garzon, et al., 2008; Luan, Yang, & Chen, 2015; Wallaert, et al., 2017). Therefore, the genes involved in biogenesis of microRNAs are likely to play a key role in development of ALL. Besides, genistein, a soy isoflavone has been proved to have anticancer effects in various cancers. It seems that, biological effect of genistein is related to several physiological pathways (Malla & Ramalingam, 2018). Due to importance of ALL worldwide, significance of dysregulation of microRNA expression and also inhibitory role of genistein in some cancers, the current study was conducted to evaluate the effect of genistein on 4 ALL cell lines, *in-vitro*. Not only apoptosis induction and toxicity effect of genistein were assessed on ALL cell lines, but also the effect of genistein was determined on probable changes in expression of two of these key genes, namely Dicer and AGO2 contributing in biogenesis of microRNAs in cytoplasm. Therefore, in this paper, both *in-vivo* and *in-vitro* studies were performed.

In our previous work, we showed significant changes in level of these two genes in blood samples of the patients with ALL (Piroozian, et al., 2019). In the present research, first an *in-vivo* study was performed to assess the mRNA level of Dicer and AGO2 in the other group of patients with ALL in comparison with healthy subjects. To clarify dysregulation of expression in Dicer and AGO2 in patients compared to healthy controls, we decided to investigate whether genistein could control proliferation of ALL cells via Dicer and AGO2 levels (*in-vitro* study). Consequently, results of this study can probably reveal another pathway for anti-proliferative and tumor-suppressive activity of genistein. To the best of our knowledge, there is no study on the effects of genistein on Dicer and AGO2 mRNA expressions. Thus, the present study is a pioneer study in evaluating the effect of genistein on Dicer and AGO2 expressions in patients with ALL.

Recent investigations indicated that, genistein can inhibit uncontrolled cellular growth in cancers through inhibiting signaling pathways such as WNT, and Akt (Thangavel, Puga-Olguín, Rodríguez-Landa, & Zepeda, 2019; Wang, et al., 2012). These findings are consistent with results of the present research that

showed anti-proliferative effect of this compound. It has also been reported that, in the case of neuroblastoma, genistein treatment caused an increase in the expression of cell mortality factors such as Fas, FADD, and TRADD. This increased expression finally leads to activation of caspase-8 (George, Banik, & Ray, 2010) which, in turn, can trigger apoptosis. Results of this study highlighted the effect of genistein on inducing apoptosis in ALL cell lines. Several investigations reported inhibitory effect of genistein on cancerous cells in the breast, prostate, stomach, and ovaries (Banerjee, et al., 2008), confirming strong inhibitory effect of genistein on cancerous cells. Generally, there are two forms of cell mortality, *i.e.*, apoptosis and necrosis. Those cells that die through apoptosis are naturally removed from the body, but necrosis occurs as a result of accidents, trauma, or self-immunity diseases such as lupus erythematosus. The cells that die through necrosis activate the immune system, which recognizes them as strangers and thus results in occurrence of inflammation in the body (Mattson & Bazan, 2012). Before taking medicines that cause cell mortality, we need to consider the fact that, whether they cause apoptosis or necrosis. As observed in the flow cytometry results, cell mortality caused by genistein is insignificant through necrosis. It is as low as or lower than necrosis in the control or non-treated groups. It produces less necrosis in cells than doxorubicin, which was used in the control group for apoptosis and as a positive control in flow cytometry. In other words, it seems that, genistein can induce apoptosis of cancerous cells and does not produce an immune response and inflammation in the body. Besides apoptosis, which can influence growth rate, there are factors involved in the cell cycle pathway and DNA replication (Hu, et al., 2019; Zhang, Bao, & Yang, 2019). Previous studies proved strong inhibitory effect of genistein on PTK activity, which is related to epidermal growth factor receptor (EGFR) (Russo, et al., 2016). It has also been confirmed that, genistein had an inhibitory effect on the topoisomerase enzyme (Bandelet & Osheroff, 2007). These findings are consistent with the hypothesis presented in the present research that, genistein probably influences on the cell cycle and cell proliferation. MTT-Assay and flow cytometry results showed that, genistein had an inhibitory dose-related and time-related effect on growth rate and mortality rate of ALL cells by induction of apoptosis. Our results briefly showed that, genistein had anti-proliferative effect against ALL cells through increasing mortality rate via induction of apoptosis and decreased growth rate.

Our *in-vivo* study showed that, expression of AGO2 mRNA decreased in the patients compared to healthy controls. Interestingly, down-regulation of AGO2 was found to be correlated with aggressive subtype of ALL (from standard risk to high risk). Findings of the study showed an increase in the concentration of genistein in 4 cell lines that helped to significantly raise the expression of AGO2 in the patients compared to the control group, suggesting tumor-suppressive role for AGO2 in ALL. Our results are compatible with several recent studies. It has been confirmed that, epidermal growth factor receptor (EGFR) phosphorylates the AGO2 at Tyr 393 in response to hypoxic stress, decreasing the binding of Dicer to AGO2 and prevents microRNAs biogenesis, leading to invasiveness under hypoxia, and it is associated with poor overall survival in breast cancer patients (Shen, et al., 2013). In another study, inhibitory effect of genistein has been shown on the PTK related to EGFR (Russo, et al., 2016). In line with our data, thus, it can be inferred that, genistein can inhibit EGFR and therefore can reduce phosphorylation of the Tyr protein of AGO2. It can finally increase tendency of AGO2 toward Dicer and correct processing of microRNAs to inhibit cancer (Russo, et al., 2016).

Conclusions

According to our findings obtained from *in-vivo* study, down-regulation of Dicer was expected as a result of the effect of genistein in ALL cell lines, but our results revealed an increasing trend in expression level of Dicer in both patients as well as cell lines after genistein treatment. It seems that, genistein is capable of exerting its inhibitory and inductive effect on apoptosis through increasing Dicer expression. This is consistent with some other researches on the P53 tumor suppressor gene on Dicer. It can be concluded that, increased expression of Dicer under the effect of genistein helps to inhibit cancerous cells via P53 (Kurzynska-Kokorniak, et al., 2015). It has also been observed that, Dicer depletion can stimulate aggressive behavior of cells in mice via activation of the EGF receptor and growth factor receptors, Met. Furthermore, it has been indicated that ,mutant p53 can decrease expression of Dicer through TAp63 (Muller, Trinidad, Caswell, Norman, & Vousden, 2014) and loss of Dicer promotes DNA damage in the epidermis of mice. Besides, both Dicer and p53 together suppress mammalian skin carcinogenesis (Lyle, et al., 2014). It seems that, genistein can have a lethality effect and inhibitory role in cell cycle (besides causing apoptosis and ceasing the cell cycle) through changing expression of AGO2 and Dicer in ALL.

Evaluation of AGO2 and Dicer mRNA expression in human samples confirmed a significant dysregulation in mRNA expression of these genes especially in correlation with ALL progression from lower to higher subtypes, suggesting the potential biomarker role for prognosis and diagnosis of ALL. In this research, for the first time, it was reported that, genistein has an inhibitory effect on ALL through inducing apoptosis and possibly ceasing the cell cycle via changing expression of Dicer and AGO2. Such effects can lead to changes in mRNA expression in Dicer and AGO2 and then on FGFR, immigration ,and angiogenesis pathways (MacFarlane, Gu, Casson, & Murphy, 2010) or changes on P53 (Kurzynska-Kokorniak, et al., 2015). It can be inferred that, genistein can inhibit EGFR and thus increase tendency of AGO2 and Dicer, which in turn contributes in proper maturation of miRNAs and a proper cellular regulation. In this way, cancer can be inhibited. In other words, Dicer and AGO2 can be considered as the key factors involved in formation and development of ALL. Alterations in the level of these genes were accompanied by a change in apoptosis of ALL cells. Aberrations in level of these genes may have a pathological effect on developing of the disease via damaging the cell cycle, controlling of tumorigenesis, and apoptosis. Genistein is a non-toxic, anti-cancerous herbal compound with a dose-related anti-cancerous effect. Accordingly, it can be claimed that, anti-cancerous effects of genistein are mediated through changes in Dicer and AGO2 expression. Overall, further in-depth investigations are needed to elaborate on a more certain hypothesis related to this issue.

Abbreviations

ALL, Acute lymphoblastic leukemia; AGO2, argonaute RISC catalytic component 2; DGCR8, DGCR8 microprocessor complex subunit; WNT, Wingless-related integration site; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; E-cadherin, extracellular cadherin; MMP, Matrix metalloproteinase; RISCs, RNA-Induced Silencing Complexes; FBS, fetal bovine serum; FITC, fluorescein

isothiocyanate-conjugated; MTT-Assay, *3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* for Assay; PS, phosphatidylserine; 7-AAD, 7-Aminoactinomycin D; DMSO, Dimethyl sulfoxide

Declarations

Ethics approval and consent to participate

This research project was ratified and approved by the Ethics and Human Rights Committee (EHRC) of Hormozgan University of Medical Sciences (HUMS), Bandar Abbas, Iran (HUMS.REC.1394.84).

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to two reasons. First, we should have made a supplementary file with up to six figures. Second, we put important information in half of the others but they are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was financially supported by the Research Vice Chancellor of Hormozgan University of Medical Sciences, Bandar Abbas, Iran.

Authors' contributions

F. P. and K. M. Z. designed the experiments, drafted sections of the manuscripts. F. P. and H. B. V. performed cell culture, MTT-Assay and analyzed the IC50. F. P. and A. S. performed Flow Cytometry. F. P. and H. B. V. performed Real time-PCR. K. M. Z. was responsible for the supervision of project. F. P., A. B. N., and M. A. analyzed data. F. P. and K. M. Z. wrote the manuscript and data analysis. K. M. Z. approved final version of article. All authors read and confirmed the final version of manuscript.

Acknowledgments

The researchers would like to highly appreciate Research Vice Chancellor of Hormozgan University of Medical Sciences support. The authors would like to thank the colleagues especially ALL research team in the department at Molecular Medicine Research Center (MMRC) for their helpful participation and comments.

References

- Bandeled OJ, Osheroff N. Bioflavonoids as poisons of human topoisomerase II α and II β . *Biochemistry*. 2007;46(20):6097-108.
- Banerjee, S., Li, Y., Wang, Z., & Sarkar, F. H. (2008). Multi-targeted therapy of cancer by genistein. *Cancer letters*, 269(2), 226-242.
- Calin, G. A., Liu, C.-G., Sevignani, C., Ferracin, M., Felli, N., Dumitru, C. D., . . . Dono, M. (2004). MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. *Proceedings of the National Academy of Sciences*, 101(32), 11755-11760.
- Carroll, W. L., Loh, M., Biondi, A., & Willman, C. (2011). *The Biology of acute lymphoblastic leukemia Childhood Leukemia* (pp. 29-61): Springer.
- Chiosea, S., Jelezcova, E., Chandran, U., Acquafondata, M., McHale, T., Sobol, R. W., & Dhir, R. (2006). Up-regulation of dicer, a component of the MicroRNA machinery, in prostate adenocarcinoma. *The American journal of pathology*, 169(5), 1812-1820.
- Chiosea, S., Jelezcova, E., Chandran, U., Luo, J., Mantha, G., Sobol, R. W., & Dacic, S. (2007). Overexpression of Dicer in precursor lesions of lung adenocarcinoma. *Cancer research*, 67(5), 2345-2350.
- Drobna, M., Szarzyńska-Zawadzka, B., & Dawidowska, M. (2018). T-cell acute lymphoblastic leukemia from miRNA perspective: Basic concepts, experimental approaches, and potential biomarkers. *Blood reviews*, 32(6), 457-472.
- Garzon, R., Volinia, S., Liu, C.-G., Fernandez-Cymering, C., Palumbo, T., Pichiorri, F., . . . Nakamura, T. (2008). MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. *Blood*, 111(6), 3183-3189.
- Gebert, L. F., & MacRae, I. J. (2019). Regulation of microRNA function in animals. *Nature reviews Molecular cell biology*, 20(1), 21-37.
- George, J., Banik, N. L., & Ray, S. K. (2010). Genistein induces receptor and mitochondrial pathways and increases apoptosis during BCL-2 knockdown in human malignant neuroblastoma SK-N-DZ cells. *Journal of neuroscience research*, 88(4), 877-886.
- Gounaris-Shannon, S., & Chevassut, T. (2013). *The role of miRNA in haematological malignancy. Bone marrow research*, 2013.
- Hu, X., Wu, X., Liu, H., Cheng, Z., Zhao, Z., Xiang, C., . . . Qing, Y. (2019). Genistein-induced DNA damage is repaired by nonhomologous end joining and homologous recombination in TK6 cells. *Journal of Cellular Physiology*, 234(3), 2683-2692.

- Huang, W., Wan, C., Luo, Q., Huang, Z., & Luo, Q. (2014). Genistein-inhibited cancer stem cell-like properties and reduced chemoresistance of gastric cancer. *International journal of molecular sciences*, 15(3), 3432-3443.
- Iacobucci, I., & Mullighan, C. G. (2017). Genetic basis of acute lymphoblastic leukemia. *Journal of Clinical Oncology*, 35(9), 975.
- Kurzynska-Kokorniak, A., Koralewska, N., Pokornowska, M., Urbanowicz, A., Tworak, A., Mickiewicz, A., & Figlerowicz, M. (2015). The many faces of Dicer: the complexity of the mechanisms regulating Dicer gene expression and enzyme activities. *Nucleic acids research*, 43(9), 4365-4380.
- Kwon, S. C., Baek, S. C., Choi, Y.-G., Yang, J., Lee, Y.-s., Woo, J.-S., & Kim, V. N. (2019). Molecular basis for the single-nucleotide precision of primary microRNA processing. *Molecular cell*, 73(3), 505-518. e505.
- Li, Q., & Chen, H. (2011). Epigenetic modifications of metastasis suppressor genes in colon cancer metastasis. *Epigenetics*, 6(7), 849-852.
- Li, X., Luo, X., Han, B., Duan, F., Wei, P., & Chen, Y. (2013). MicroRNA-100/99a, deregulated in acute lymphoblastic leukaemia, suppress proliferation and promote apoptosis by regulating the FKBP51 and IGF1R/mTOR signalling pathways. *British journal of cancer*, 109(8), 2189.
- Luan, C., Yang, Z., & Chen, B. (2015). The functional role of microRNA in acute lymphoblastic leukemia: relevance for diagnosis, differential diagnosis, prognosis, and therapy. *OncoTargets and therapy*, 8, 2903.
- Lyle, S., Hoover, K., Colpan, C., Zhu, Z., Matijasevic, Z., & Jones, S. N. (2014). Dicer cooperates with p53 to suppress DNA damage and skin carcinogenesis in mice. *PloS one*, 9(6), e100920.
- Ma, Z., Swede, H., Cassarino, D., Fleming, E., Fire, A., & Dadras, S. S. (2011). Up-regulated Dicer expression in patients with cutaneous melanoma. *PloS one*, 6(6), e20494.
- MacFarlane, L.-A., Gu, Y., Casson, A. G., & Murphy, P. R. (2010). Regulation of fibroblast growth factor-2 by an endogenous antisense RNA and by argonaute-2. *Molecular Endocrinology*, 24(4), 800-812.
- Malla, A., & Ramalingam, S. (2018). Health Perspectives of an Isoflavonoid Genistein and its Quantification in Economically Important Plants Role of Materials Science in Food Bioengineering (pp. 353-379): Elsevier.
- Mattson, M. P., & Bazan, N. G. (2012). Apoptosis and necrosis *Basic Neurochemistry* (pp. 663-676): Elsevier.
- Merritt, W. M., Lin, Y. G., Han, L. Y., Kamat, A. A., Spannuth, W. A., Schmandt, R., . . . Nick, A. M. (2008). Dicer, Drosha, and outcomes in patients with ovarian cancer. *New England Journal of Medicine*, 359(25), 2641-2650.

- Muller, P. A., Trinidad, A. G., Caswell, P. T., Norman, J. C., & Vousden, K. H. (2014). Mutant p53 regulates Dicer through p63-dependent and-independent mechanisms to promote an invasive phenotype. *Journal of Biological Chemistry*, 289(1), 122-132.
- Piroozian, F., Bagheri Varkiyani, H., Koolivand, M., Ansari, M., Afsa, M., AtashAbParvar, A., & MalekZadeh, K. (2019). The impact of variations in transcription of DICER and AGO2 on exacerbation of childhood B-cell lineage acute lymphoblastic leukaemia. *International journal of experimental pathology*.
- Pokornowska, M., Milewski, M. C., Ciechanowska, K., Szczepańska, A., Wojnicka, M., Radogostowicz, Z., . . . Kurzynska-Kokorniak, A. (2019). The RNA–RNA base pairing potential of human Dicer and Ago2 proteins. *Cellular and Molecular Life Sciences*, 1-14.
- Russo, M., Russo, G. L., Daglia, M., Kasi, P. D., Ravi, S., Nabavi, S. F., & Nabavi, S. M. (2016). Understanding genistein in cancer: The “good” and the “bad” effects: A review. *Food chemistry*, 196, 589-600.
- Shen, J., Xia, W., Khotskaya, Y. B., Huo, L., Nakanishi, K., Lim, S.-O., . . . Chen, C.-H. (2013). EGFR modulates microRNA maturation in response to hypoxia through phosphorylation of AGO2. *Nature*, 497(7449), 383.
- Sheng, G.-Q., Zhang, J.-R., Pu, X.-P., Ma, J., & Li, C.-L. (2002). Protective effect of verbascoside on 1-methyl-4-phenylpyridinium ion-induced neurotoxicity in PC12 cells. *European journal of pharmacology*, 451(2), 119-124.
- Simioni, C., Martelli, A. M., Zauli, G., Melloni, E., & Neri, L. M. (2019). Targeting mtor in acute lymphoblastic leukemia. *Cells*, 8(2), 190.
- Slavov, S., Gimenes Teixeira, H., & Rego, E. (2010). The role of micro-ribonucleic acids in normal hematopoiesis and leukemic T-lymphogenesis. *Brazilian Journal of Medical and Biological Research*, 43(7), 619-626.
- Thangavel, P., Puga-Olguín, A., Rodríguez-Landa, J. F., & Zepeda, R. C. (2019). Genistein as Potential Therapeutic Candidate for Menopausal Symptoms and Other Related Diseases. *Molecules*, 24(21), 3892.
- Tomizawa, D., & Kiyokawa, N. (2017). *Acute Lymphoblastic Leukemia Hematological Disorders in Children* (pp. 33-60): Springer.
- Tuli, H. S., Tuorkey, M. J., Thakral, F., Sak, K., Kumar, M., Sharma, A. K., . . . Bishayee, A. (2019). Molecular Mechanisms of Action of Genistein in Cancer: Recent Advances. *Frontiers in Pharmacology*, 10, 1336.
- Wallaert, A., Van Looche, W., Hernandez, L., Taghon, T., Speleman, F., & Van Vlierberghe, P. (2017). Comprehensive miRNA expression profiling in human T-cell acute lymphoblastic leukemia by small RNA-sequencing. *Scientific reports*, 7(1), 7901.
- Wang, H., Li, Q., & Chen, H. (2012). Genistein affects histone modifications on Dickkopf-related protein 1 (DKK1) gene in SW480 human colon cancer cell line. *PloS one*, 7(7), e40955.

Wiemels, J. (2012). Perspectives on the causes of childhood leukemia. *Chemico-biological interactions*, 196(3), 59-67.

Zhang, Q., Bao, J., & Yang, J. (2019). Genistein-triggered anticancer activity against liver cancer cell line HepG2 involves ROS generation, mitochondrial apoptosis, G2/M cell cycle arrest and inhibition of cell migration. *Archives of medical science: AMS*, 15(4), 1001.

Figures

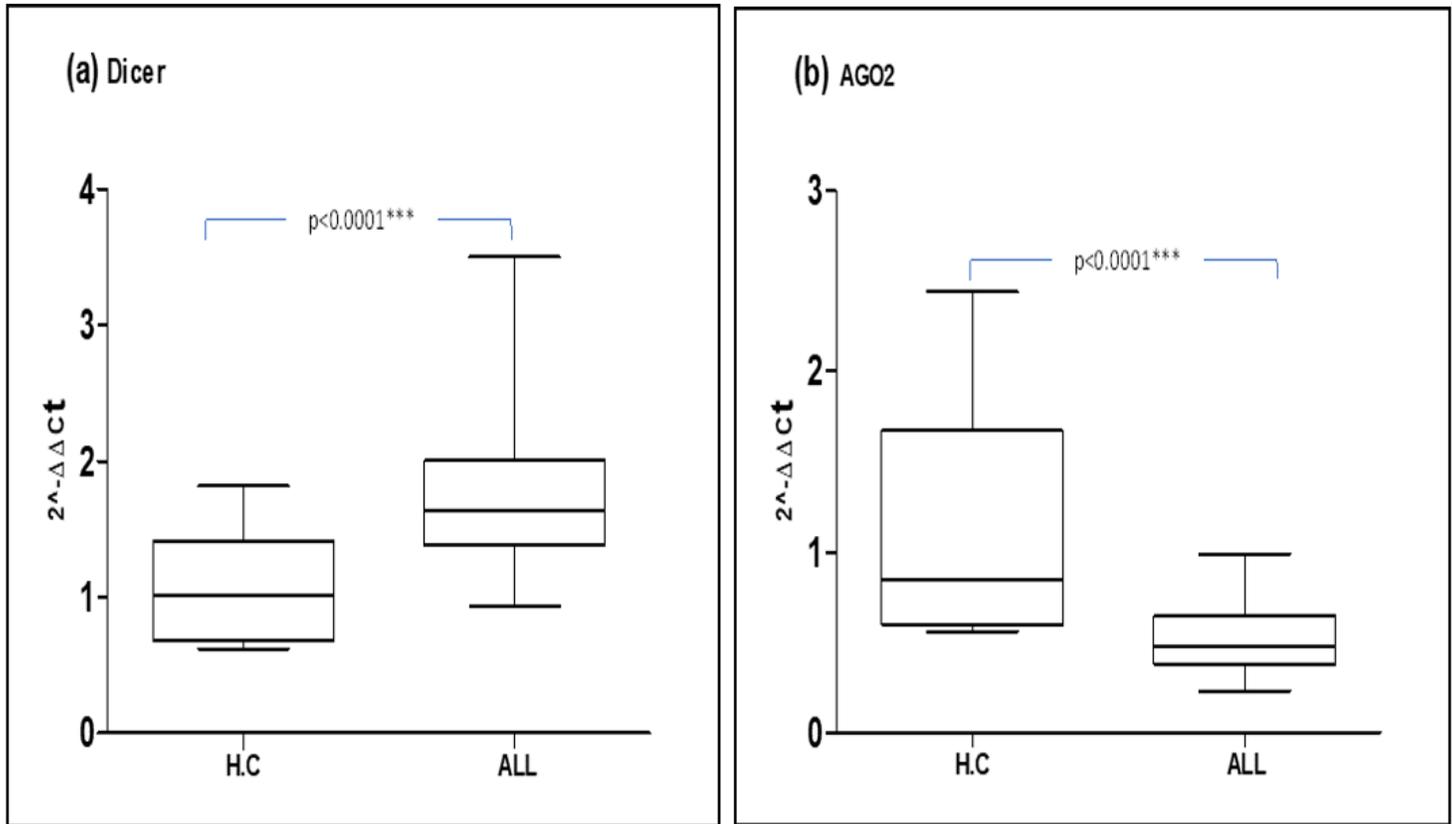


Figure 1

The comparison of mRNA expression of Dicer and AGO2 between patients with Acute Lymphoblastic Leukemia (ALL) and healthy controls (H.C).

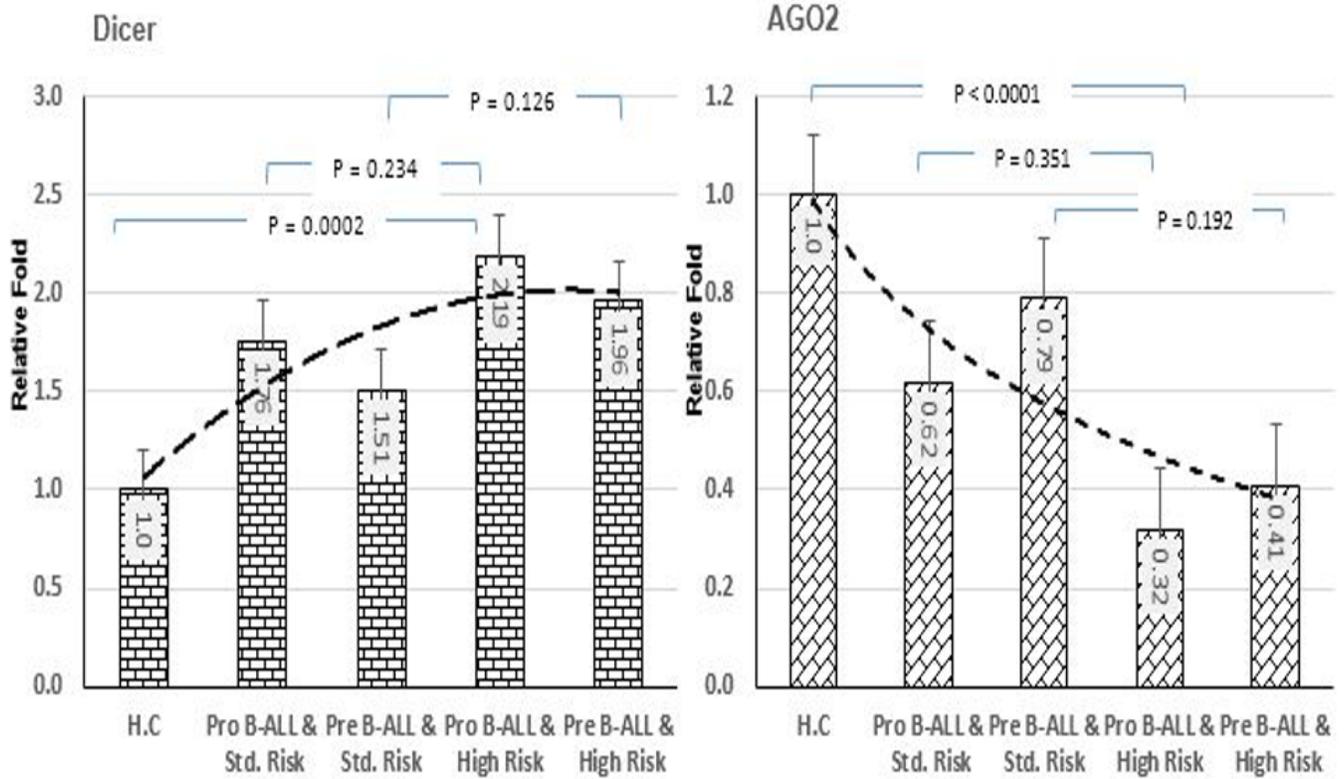


Figure 2

Dicer and AGO2 mRNA expression in patients with different ALL subtypes as compared to healthy controls (HC)

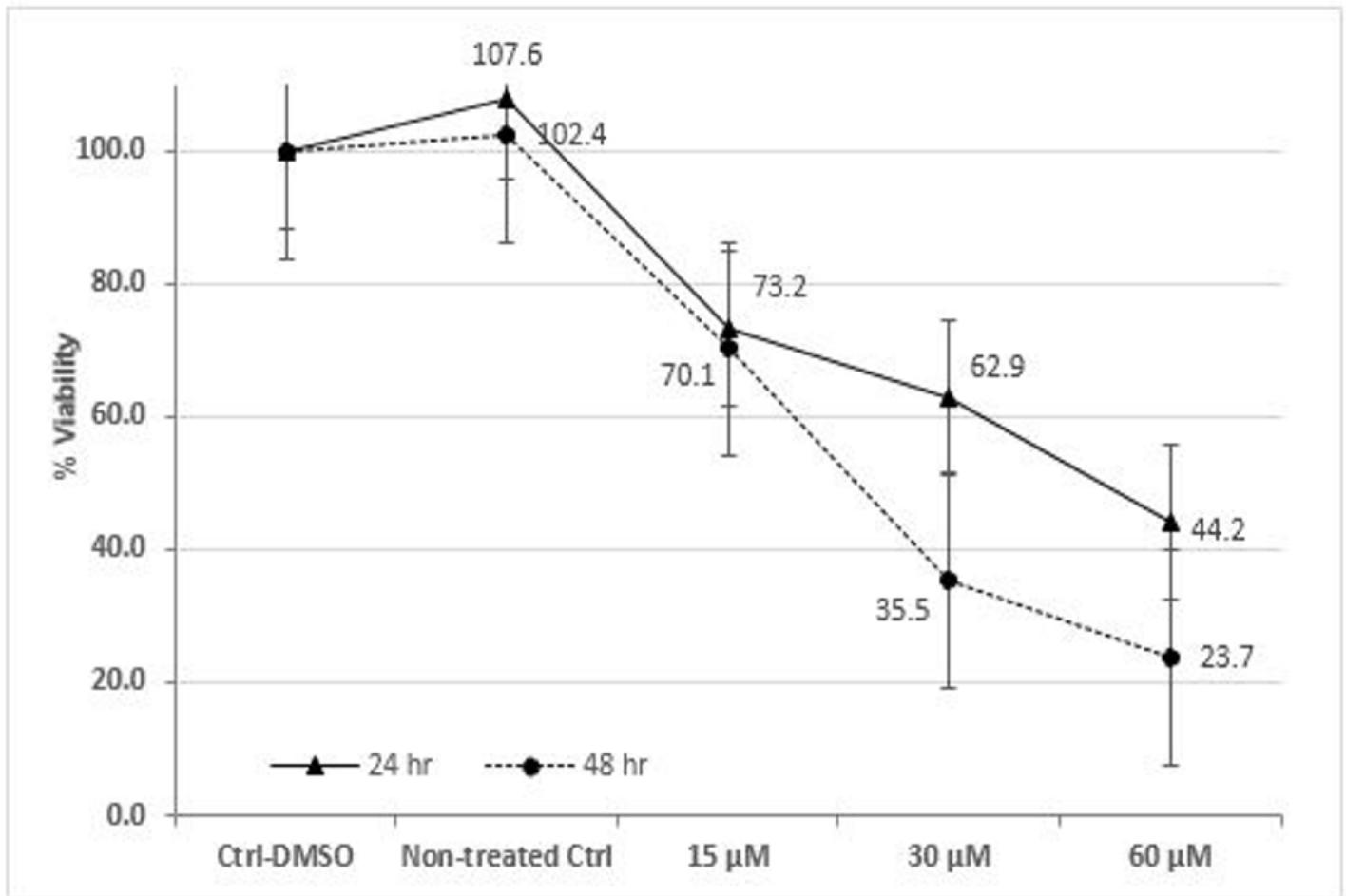


Figure 3

MTT-assay results in Molt-17 cell line in various concentrations of genistein in 2 days' culture (The impact of time- and dose- exposure).

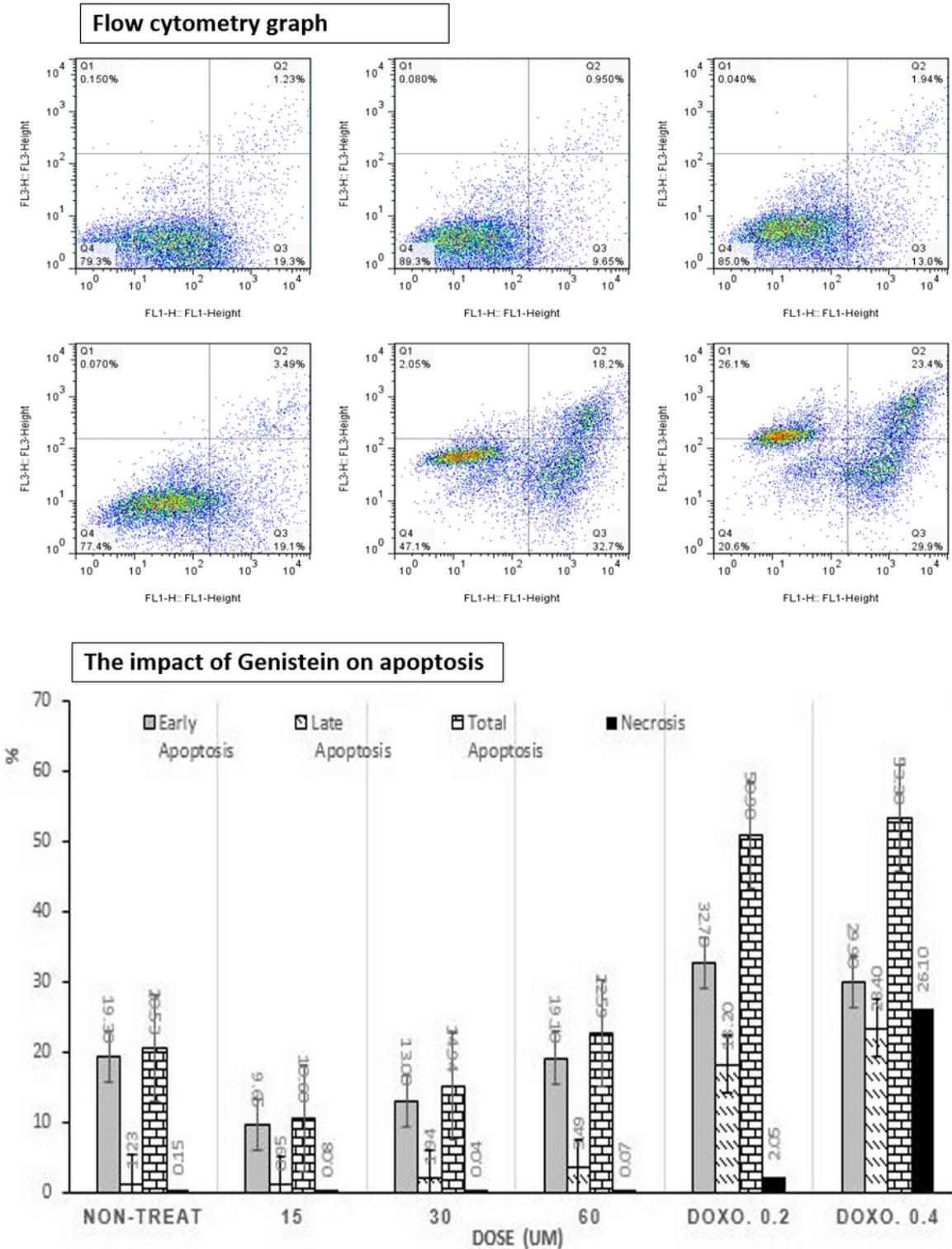


Figure 4

The impact of 48 hr treatment with different dose of genistein on Apoptosis of Jurkat cell line obtained by flow cytometry. The flow cytometry graph - above; from the left to the right: (1) control group (non-treated), (2) treated with 15 μ M genistein concentration, (3) treated with 30 μ M genistein concentration – bottom; from the left to the right: (1) treated with 60 μ M genistein concentration, (2) treated with 0.2 μ M concentration of doxorubicin, (3) treated with 0.4 μ M concentration of doxorubicin. The impact of

genistein on apoptosis; The impact of 48 hr treatment with different dose of genistein on Apoptosis of Jurkat cell line.

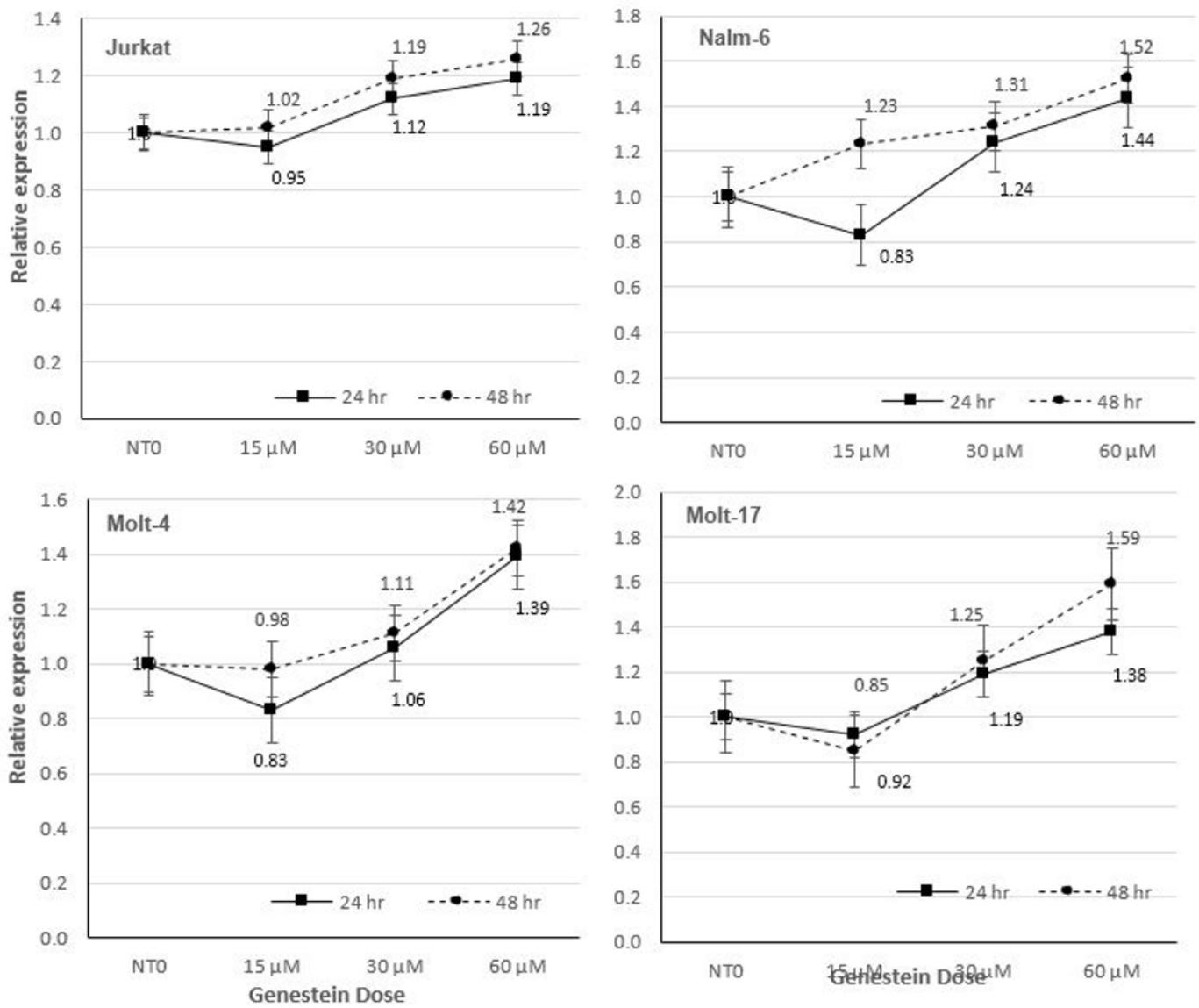


Figure 5

Behavior of Dicer mRNA expression after different doses treatment of genistein at 24- and 48- hours; Jurkat, Nalm-6, Molt-4, and Molt-17 cell lines. NT0: non-treated.

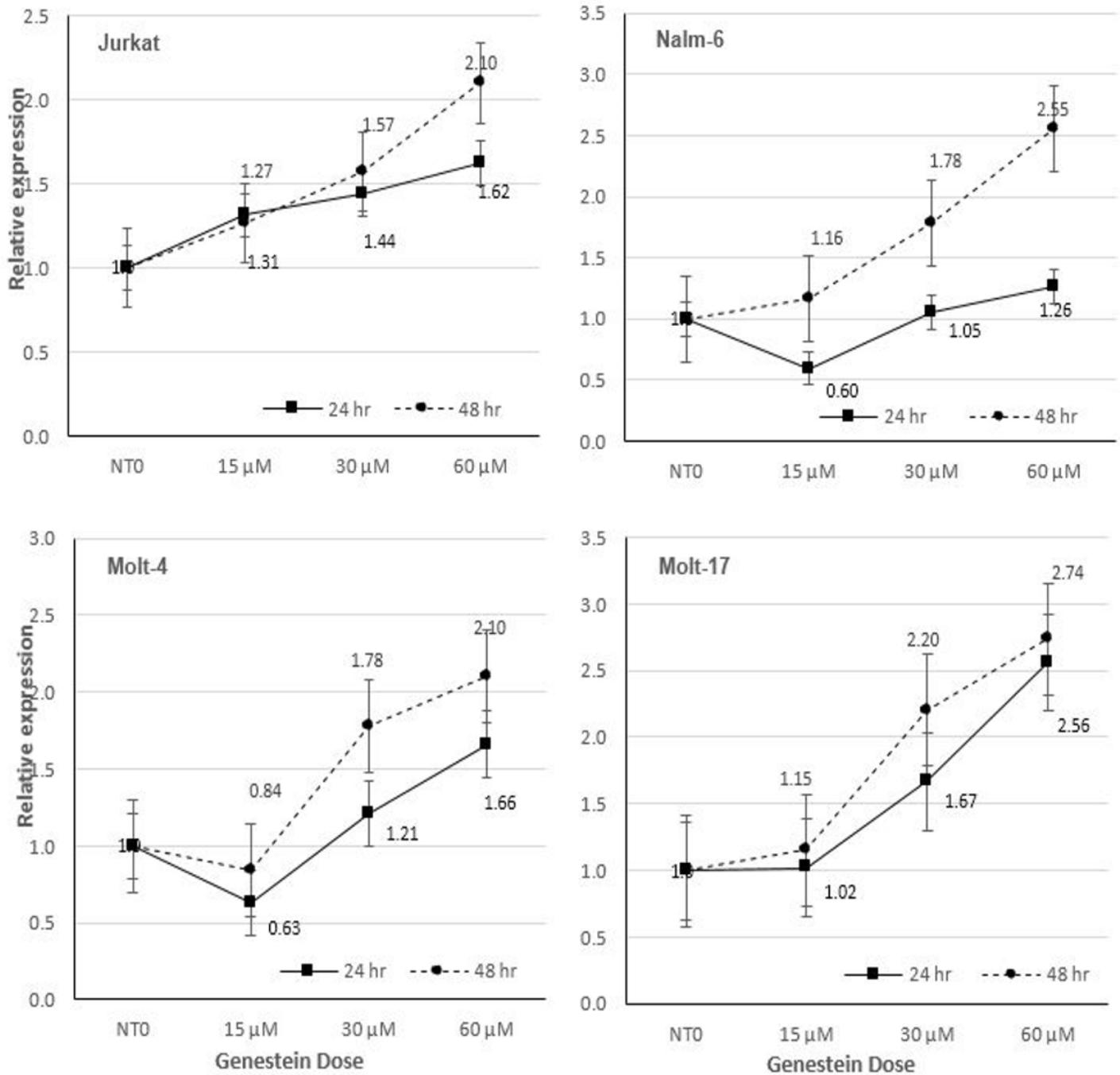


Figure 6

Behavior of AGO2 mRNA expression after different doses treatment of genistein at 24- and 48- hours; Jurkat, Nalm-6, Molt-4, and Molt-17 cell lines. NT0: non-treated.