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Evaluating the expression of key genes involved in resistance to oxidative stress in ALL patients

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Abstract

Background: Leukemia accounts for about 8% of all cancers and causes approximately 7% of mortalities due to malignancies. Acute lymphoblastic leukemia (ALL) is the most common childhood cancer and rare in older subjects. The aim of this study was to evaluate the expression of oxidative stress resistance genes including Catalase, manganese superoxide dismutase (MnSOD), Forkhead Box O3 (Foxo3a), and sirtuin-1 (SIRT1) in ALL patients that may be applied for therapeutic purposes in the future.

Materials and Methods: In this observational case-control study, blood samples were drawn from 60 newly diagnosed ALL patients and 10 healthy individuals as a control group. After RNA extraction and cDNA synthesis, real-time polymerase chain reaction (RT-PCR) amplification was performed using specific primers for evaluating the expression of Catalase, MnSOD, Foxo3a, and SIRT1 genes.

Results: The expression of all studied genes were significantly higher in ALL patients than in the control group; catalase gene, FOX gene, MnSOD gene, and SIRT1 gene were expressed 4 times ($p = 0.04$), 4.5 times ($p = 0.001$), 2.2 times ($p = 0.05$) and 4.8 ($p = 0.01$) times higher than healthy individuals in the control group respectively. However, no significant relationship between their expression and the stage of the disease and blast percentage was demonstrated ($P > 0.05$).

Conclusion: According to these results, the authors believe that the pathways involved in oxidative stress may be one of the most important causes of ALL disease's development and progression. In this regard, targeting the critical genes of these pathways can be considered a potential treatment with fewer side effects.

Keywords: Acute lymphoblastic leukemia, Catalase, Forkhead box O3, Oxidative stress

Introduction

Cancer results from uncontrolled cell division due to environmental factors and genetic disorders. More than half of all cancers occur in developing countries, such as South American and Asian countries (1). Leukemia comprises about 8% of all human cancers and around half of them are classified as acute leukemia (AL) which is a malignant disorder due to clonal expansion and maturation arrest of lymphoid and myeloid progenitors (2). Leukemia causes approximately 7% of mortalities due to malignancies and is the fifth leading cause of death in the world and the second in Iran (3). According to the World Health Organization (WHO) classification, there are several types of leukemia based on their cell lines, including acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), and chronic lymphocytic leukemia (CLL). Among these, ALL is the most common leukemia in childhood, which represents about 70-80% of acute leukemia in this age group (4). However, there is a second incidence peak of ALL among individuals aged above 60 years (5). It seems that leukemic clones derived from normal blast cells arising from one or more mutations in its genome. According to this concept, each cell of the mutated clone will probably have the same kind of genetic changes (5). Due

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to the advances in cytogenetic and molecular characterization of acute leukemias over the past two decades, genetic variations are now detectable in more than 80% of ALL cases (6). Various studies confirmed that free radicals play an important role in the pathogenesis of various diseases such as cancer, neurodegenerative diseases, diabetes, etc. (7). In fact, there is so much evidence suggesting that cancer cells produce more reactive oxygen species (ROS) than normal cells which expose them to oxidative stress. Studies have also demonstrated the crucial role of ROS in mediating the tumorigenic function of some oncogenes (8). Silent information regulator 1 (SIRT1) is involved in the regulation of various biological processes such as gene expression, apoptosis, cell survival, proliferation, differentiation, metabolism, and carcinogenesis. SIRT1 gene is overexpressed in many malignancies (9) that increases the proliferation and survival of cancer cells. SIRT1 overexpression has been reported in hematologic malignancies as well. Accordingly, inhibition of SIRT1 activity leads to a decrease in cancer cell growth and an increase in apoptosis (9-11). Recent researches in patients with AML indicated that SIRT1 inhibits the expression of FOXO-induced pro-apoptotic genes by de-acetylating key transcription factors such as FOXO. Additionally, it revealed that SIRT1 induces the expression of genes responsible for resistance to oxidative stress like free radical neutralizing enzymes (Catalase, MnSOD) via FOXO. SIRT1 activity increases the survival and proliferation of cancer cells by affecting the expression of key genes involved in resistance to oxidative stress (9-11). Among genes involved in oxidative stress resistance, investigators can focus on Forkhead box O3 (FOXO3a) transcription factor gene, manganese superoxide dismutase (MnSOD) gene and Catalase (CAT) gene. Manganese superoxide dismutase and catalase are neutralizing enzymes that play a crucial role in scavenging free radicals. Therefore, in this study, the authors evaluated the expression of FOXO3a, SIRT1, MnSOD, and Catalase genes, since they appear to be involved in the stress resistance in malignant cells. The aim of this study was to determine the expression of oxidative stress resistance genes (Catalase, MnSOD, Foxo3a, and SIRT1) in ALL patients. It may suggest the necessity of further studies about their function that will make them as a promising therapeutic tool in future.

Materials and Methods

Sampling

In this observational case-control study, 60 subject samples in the Taleghani Hospital, Tehran according to $\alpha = 0.05$ and power of 95% were estimated. Sixty newly diagnosed ALL patients (age range of 2-70 years) were included and diagnostic criteria considered according to FAB categorization. Ten healthy individuals were studied as control group, and underlying diseases and cardiovascular diseases were the exclusion criteria for this group. For genetic analysis, 10 ml of the venues blood samples and bone marrow samples were drawn into EDTA tubes.

Real Time PCR

Total RNA was isolated from blood and bone marrow samples using the conventional TRIzol RNA extraction method. To evaluate the quality of the extracted RNA, samples of total RNA were electrophoresed on 1.5% agarose gel, which confirmed an acceptable quality of the extracted RNA. The quantity and purity of RNA were determined by Nanodrop. Complementary DNA (cDNA) was generated from total RNA with the ThermoScientific cDNA kit (ThermoScientific, Waltham, MA, USA). The cDNA synthesis was carried out in thermal-cycler; The CDNA synthesis profile consisted of 5 min at ٢٥°C followed by 60 min at 42° C and 5 min at ٧٠° C . Primer sequences and PCR reagent are shown in Table I. Profile consisted of 5 min at 95°C for activation of HotStar-Taq

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DNA polymerase followed by 40 cycles of denaturation (95°C for 15s), annealing (1 minute at 55°C for FOXO, 58 forSIRT, 59 for CAT, and 61 for MnSOD), and extension (72°C for 1 minute). Relative quantification and ΔΔ ct methods were used to evaluate the expression of Catalase, MnSOD, Foxo3a, and SIRT1 genes by Real-time PCR test. The amplification curve and melting curve files were exported by the software of the ABI Real-time PCR instrument (Applied Biosystems, Foster City, CA).

Ethical Consideration

All experimental procedure and informed consent form were approved by the ethics review committee of the Bushehr University of Medical Sciences under the ethical code of IR.BPUMS.REC.1398.141. The participants or their parents signed written informed consent forms.

Statistical Analysis

CT of the genes was determined and fold change in gene expression for patient subjects compared to the control group and was calculated according to the $2^{-\Delta\Delta CT}$ formula. SPSS 21 and Graph pad prism (GraphPad Software Inc. California) were applied for statistical analysis and drawing graphs and charts. P value<0.05 was considered as a significant difference.

In order to compare the mean of results between studied groups, the Kruskal-Wallis test was used and the ANOVA test was conducted for comparing each specific gene and potential significant difference.

Results

All studied samples were selected All studied samples were selected according to the inclusion and exclusion criteria mentioned previously. For each patient, demographic and laboratory parameters including age, stage of the disease, and blast percentage were recorded. The relationship between collected data and the expression of SIRT, MnSOD, FOXO3a, and Catalase genes was investigated. The mean CT values are demonstrated in Table II.

The results showed that all evaluated genes were over-expressed in the ALL group compared to the control group; catalase gene, FOX gene, MnSOD gene, and SIRT1 gene were expressed 4 times (p-value=0.04), 4.5 times (p-value=0.001), 2.2 times (p-value=0.05) and 4.8 (p-value=0.01) times higher than healthy individuals in the control group, respectively (Table III). Due to the normal distribution of data in both patient and control groups, the T-test was used for the MnSOD gene; but for CAT, SIRT1, FOXO3a genes, the Mann-Whitney U test was used to compare the difference between the means in two studied groups.

Comparison of expression levels of SIRT1, FOXO3a, MnSOD, and CAT genes in different subgroups based on their immunophenotype:

Among all four genes, only MnSOD gene showed the significant differences (p-value=0.017) in its expression between the different immunophenotype groups (p-value>0.05). Catalase (p-value=0.13), Sirt1 (p-value=0.61), and Foxo3 (p-value=0.09) (Figure 1).

Comparison of expression levels of SIRT1, FOXO3a, MnSOD, and CAT genes based on patient ages:

Patients were divided into two age subgroups (<30 years and> 30 years). For making this comparison, the Kruskal-Wallis test was used for CAT, SIRT1, and FOXO3 genes and the ANOVA test was applied for the MnSOD gene as described before. Among all four genes, only catalase gene showed the significant differences in its expression between the age groups (p-value=0.02), MnSOD (p-value=0.88), Sirt1 (p-value=0.76), and Foxo3 (p-value=0.90) (Figure 2).

Comparison of expression levels of SIRT1, FOXO3a, MnSOD, CAT genes based on blast percentage:

In this study, patients were divided into four groups based on their initial blast percentage, including; group 1 with 20-40% blast, group 2 with 40-60% blast, group 3 with 60-80% blast, and group 4

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with more than 80% blast. In order to make this comparison, the Kruskal-Wallis test was used for CAT, SIRT1, and FOXO3 genes that had abnormal distribution, and the ANOVA test was applied for the MnSOD gene, which had a normal distribution. No significant difference was observed in the expression of any of the genes among the four groups (p-value>0.05), MnSOD (p-value=0.38), Sirt1 (p-value=0.11), Foxo3 (p-value=0.45), and catalase (p-value=0.23) (Figure 3).

Table I: Primer's sequence

Gene		Sequences	Product	Tm
Sirt1	F	GTTGACTGTGAAGCTGTACGAGGA	100	60.5
	R	ATGGGGTATGGAACTTGGAATTAGTG		60.8
FOXO3a	F	TGCTCACTTCGGACTCAC	137	56.6
	R	GGACATCATCGGATCATTGC		56.7
MnSOD	F	CTCCCCGACCTGCCCTACGACTAC	374	67.6
	R	AAACCAAGCCAACCCCAACCTGAG		65.6
CAT	F	GCGGAGATTCAACACTGCCAATG	79	62.8
	R	CTGTTCCTCATTCAGCACGTTCAC		62.3
ABL	F	AGTCTCAGGATGCAGGTGCT	290	61
	R	TAGGCTGGGGCTTTTTGTAA		62

Table II: Mean of CT

	Minimum CT	Maximum CT	Mean CT	Std. Deviation
Catalase in control group	18.13	32.88	21.0247	2.19519
Catalase in patient group	18.12	21.08	20.0640	.98368
foxo in control group	15.46	36.02	19.7213	2.55835
foxo in patient group	15.93	37.55	18.3800	6.36625
MnSOD in control group	14.02	25.79	21.5132	2.30145
MnSOD in patient group	15.42	22.24	19.8682	2.16628
SIRT in control group	19.48	37.22	31.5285	3.71889
SIRT in patient group	22.18	35.23	25.7980	3.68769

Table III: Comparison of expression levels of SIRT1, FOXO3a, MnSOD, CAT genes (2-ΔΔCT)

	Mean ΔCT Patient group	Mean ΔCT control group	CTΔΔ	$2^{-\Delta\Delta CT}$
Catalase	-1.6	-3.645	-2	4
FOXO	-5	-7.2	-2.2	4.59
MnSOD	-2.93695	-4.077	-1.17	2.25
SIRT	-5.812	-8.089	-2.27	4.8

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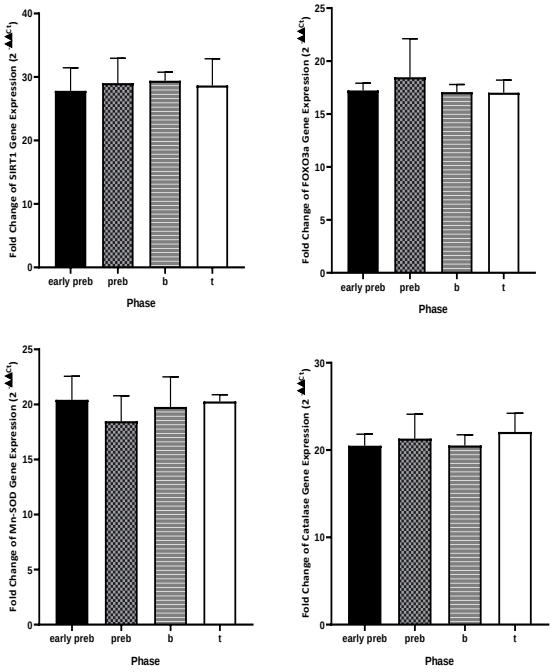

Figure 1. Comparison of expression of CAT, MnSOD, FOXO3, SIRT1 genes in different

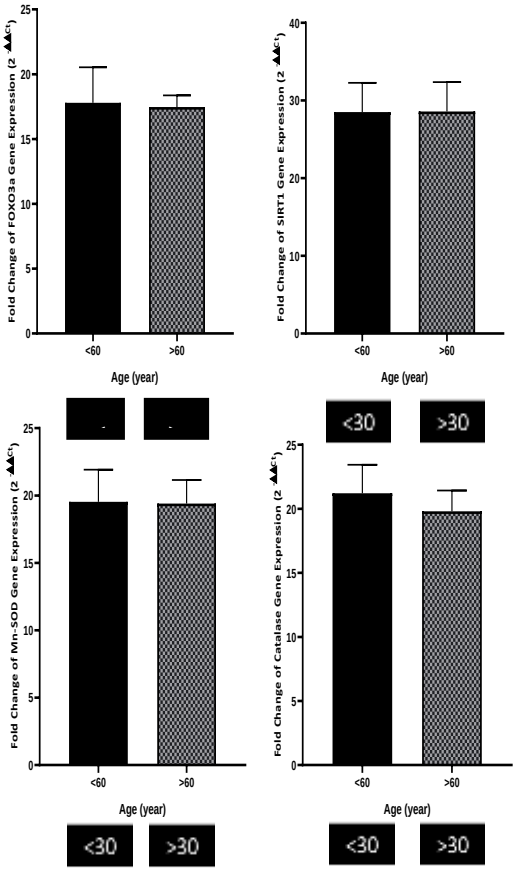

Figure 2. Comparison of expression of SIRT1, FOXO3a, MnSOD, CAT genes in two age groups including under and over 30 years old.

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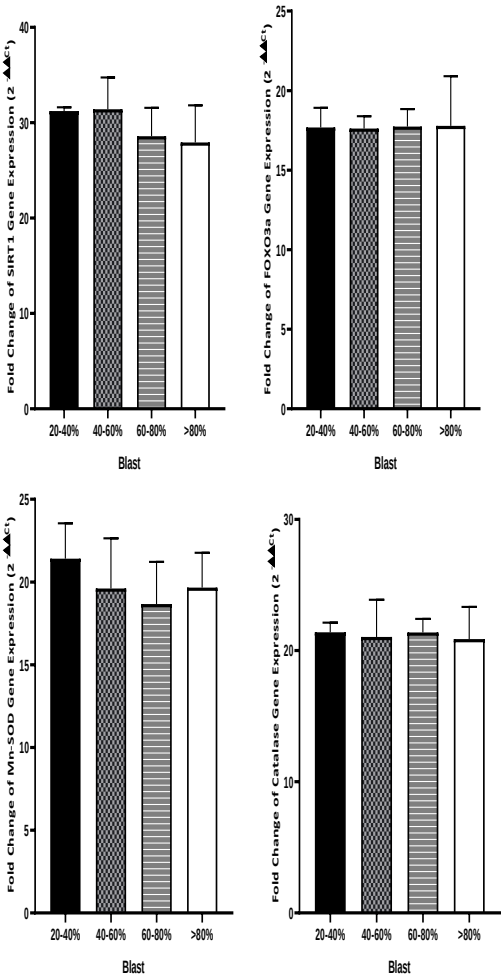

Figure 3.Correlation between SIRT1, FOXO3a, MnSOD and CAT gene expression levels with blast percentage of the patients. The X axis represents the blast percentage and the Y axis represents the expression of the desired gene.

Discussion

ALL is a type of leukemia that occurs due to an increase in cell proliferation as well as maturation arrest. Bone marrow normally generates hematopoietic stem cells that eventually grow into mature and functional blood cells. Unfortunately, the outcome of adults ALL is relatively unfavorable than pediatric ALL (12). Despite the significant advances in diagnosis and treatment including immunological targeted therapies, mortality rates remain unacceptably high in adult patients(12). Various studies indicated the complete remission rate of about 52% to 49%. Regarding the importance of the adult ALL and its high mortality rate, studying its pathophysiology and identifying the underlying genes involved in its pathogenesis can be critical.

Nowadays, due to the severe side effects of chemotherapy regimens and multi-drug resistance in hematopoietic cell lineages, extensive efforts are being made to understand the molecular mechanisms involved in the development of the malignancy and drug resistance of malignant cells as well as to find key points for inducing selective apoptosis. The results of these studies can lead to the development

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of more effective compounds that have a greater potential for cytotoxicity and can overcome drug resistance (13, 14). Therefore, in the present study, the authors investigated the correlation between changes in the expression of SIRT, Foxo, MnSOD, and CAT genes in ALL paients regarding to stages and some laboratory parameters.

SIRT1 over-expression has been reported in hematologic malignancies. Studies showed that SIRT1 suppression decreases the proliferation and increases the apoptosis in malignant cells (15, 16). In the present study, the authors assessed the SIRT1 gene expression in the ALL group and compared it with the control group. Findings showed a significant increase in gene expression (4.8-fold) in the patients. Nosho et al. (17) and Hofman et al. demonstrated that SIRT gene expression increased in various types of cancers (18); their findings were consistent with the results of the current study. Ghotaslou et al. conducted a study in 2015 on forty-eight CML patients to assess SIRT1 gene expression. Their results indicated an increase in SIRT1 gene expression; moreover, they found that imatinib resistant patients have higher expression levels of SIRT1 gene than imatinib sensitive patients (19). Another study in 2015 found that an increased expression of SIRT1 in both mRNA and protein levels occurs within the leukemic cell population which subsequently increases cell survival, cell proliferation, and drug resistance. The results of all these studies were also consistent with the current study.

In the present study, the results indicated that similar to SIRT1 gene, the expression of FOXO3a gene significantly increased in the ALL group compared to the control group. Yasuhiko Sakoe et al. observed a similar result in a 2010 study. They showed the increased expression of FOXO3a in acute promyelocytic leukemia (APL), which inhibits one of the major pathways in inducing apoptosis (20). In this study, FOXO3a expression was also assessed in different subgroups of ALL. Although its expression was generally elevated in the ALL group, there was no significant difference in the expression of this gene between the four blast sub categories, probably due to the low sample size (21). Pot et al. showed that using some dephosphorylation compounds induces the nuclear translocation of FOXO transcription factor and over-expression of the FOXO gene, which leads to poor prognosis of AML (9). However, Hu et al. showed that FOXO overexpression in the breast cancer cell line inhibited cancer progression and reduced tumor size (22). Yang et al. in 2009 obtained similar results in breast cancer (23). However, these researchers also assessed the effect of FOXO gene on AML patients and revealed that the increased level of phosphorylated FOXOs reduced survival rate in the AML patients. The activation location or host cells of FOXO3a transcription factor (either in the nucleus or in the cytoplasm) may determine the tumor suppressive role of this protein or its tumorigenicity (23).

According to the results, MnSOD expression rate was significantly higher in the ALL patients compared to the control individuals. Fahimi et al. (2017) revealed an increased expression of MnSOD in colon tumor tissues compared with normal ones (24). Ser et al. (2016) found that the MnSOD expression increased in the early stages of cancer development. As cancer progresses, the MnSOD gene expression and protein production will be elevated. For instance, in grade 3 breast cancer, the expression of MnSOD was considerably upregulated (25). However, in the present study, no significant correlation was observed between different stages of the disease and the MnSOD gene expression, probably owing to the small sample size in each group. The results in this study indicated that catalase expression was significantly increased in the ALL group. Lobna et al. also showed an increase in catalase and MnSOD gene expression in ALL patients (26). On the other hand,

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Battis et al. (2008) found different results, where the CAT activity was lower in ALL patients compared with the normal group. Present findings suggesting a potential role for oxidative stress genes as MnSOD in the progression of leukemogenesis in ALL patients. However, investigations on greater number of involved genes such as the NRF2 gene and another family of Sirtuin (SIRT) can reveal this hypothesis.

Conclusion

In this study, the authors showed that the oxidative stress pathways could be essential in the development and progression of leukemic cells, in ALL. Therefore, targeting key genes involved in these pathways can be a promising strategy to develop more effective drugs with fewer side effects.

Conflict of intrests

The authors declare no conflict of interest.

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