

## FoxO3a Gene Down-regulation in Pathogenesis of Pediatric Acute Lymphoblastic Leukemia

### Abstract

**Introduction:** Acute lymphoblastic leukemia (ALL) is the most common malignancy found in the pediatrics with the peak prevalence between the ages of 2 and 5 years. The constitutive activation of PI3K/AKT pathway inhibits the tumor-suppressor role of *FoxO3a* (a member of the forkhead class O [FoxO] transcription factor family) in a variety of cancers and leads to tumorigenesis. This study aims to investigate the expression of *FoxO3a* in three different stages of pediatric ALL in mRNA level. **Subjects and Methods:** In this case-control study, 70 patients with childhood ALL and 70 healthy age- and gender-matched as the control group were enrolled. Real-time quantitative RT-polymerase chain reaction (qRT-PCR) was used to detect the mRNA expression level of *FoxO3a* in children with different stages of ALL and healthy children as a control group. **Results:** Data showed that the expression of *FoxO3a* mRNA was lower in newly diagnosed ALL patients compared to controls ( $P < 0.0001$ ), maintenance ( $P = 0.0342$ ), and relapse ( $P = 0.0006$ ) groups, while no difference was observed between other groups. In addition, T-ALL patients showed decreased expression of *FoxO3a* compared to Pre-B ALL ones ( $P < 0.0001$ ). **Conclusion:** The study results suggest that *FoxO3a* plays a tumor-suppressor role in ALL. Thus, its up-regulation seems to be a plausible therapeutic strategy for this type of tumor.

**Keywords:** *FoxO3a*, gene expression, pediatric acute lymphoblastic leukemia, PI3K/AKT pathway

### Introduction

Acute lymphoblastic leukemia (ALL), one of the four main types of leukemia, is a clonal hematological disorder which occurs due to an uncontrolled proliferation of undifferentiated or poorly differentiated lymphocytes in bone marrow that quickly spread into the blood.<sup>[1]</sup> ALL can occur at any age. However, it is the most common pediatric malignancy diagnosed in children under the age of 20 years.<sup>[2]</sup> ALL is divided into two main subtypes based on tumor cell immunophenotype and histology: Lymphoblasts of B- and T-lineage.<sup>[3]</sup> Since ALL is a multifactorial disease, it can arise from interactions between exogenous or endogenous exposures, genetic susceptibility, and chance.<sup>[2,4]</sup> Genetically, fundamental changes in proto-oncogenes and tumor-suppressor genes can cause leukemia, since these changes alter key regulatory processes, including self-renewal, proliferation, differentiation, and apoptosis in target cells.<sup>[5]</sup>

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Deregulation of the PI3K (Phosphoinositide 3-kinase)/Akt (Protein kinase B) signaling pathway is involved in tumorigenesis and chemotherapeutic resistance, and it has been investigated in a variety of cell lines.<sup>[6]</sup> This pathway triggers by phosphorylation cascade in which Akt is phosphorylated and leads to phosphorylation and activation or inactivation of several important genes.<sup>[7]</sup> *FoxO3a*, a member of the Forkhead family of transcription factors (*FoxO1*, *FoxO3a*, *FoxO4*, and *FoxO6* in human), is one of the direct downstream targets of the PI3K/Akt pathway, regulating the cell cycle, cell growth, apoptosis, DNA damage responses, and angiogenesis.<sup>[6,8]</sup> *FoxO3a* functions as a tumor-suppressor gene and expresses in various tissues such as B- and T-lineage cells.<sup>[9]</sup> This gene transcriptionally activates various target genes including *P27*, *P21*, *P130*, and *cyclin D1/2* (cell cycle regulation),<sup>[10]</sup> *Bim*, *Fas ligand*, *TRAIL*, *Puma* (apoptosis),<sup>[11]</sup> and *GADD45a* (DNA repair).<sup>[12]</sup> Another important role of *FoxO3a*, which has been reported recently, is the modulation of the response to physiologic oxidative stress leading to

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maintenance of the hematopoietic stem cell (HSC) pool.<sup>[13]</sup> Phosphorylation of *FoxO3a* by Akt prevents its nuclear translocation, thereby activation of these target genes are inhibited.<sup>[14]</sup> Hyperactivation of PI3K/Akt pathway has been investigated in various types of cancers and leukemia is not the exception.<sup>[15]</sup> Due to hyperactivated PI3K/Akt pathway, *FoxO3a* is exported from the nucleus to the cytoplasm, where it interacts with 14-3-3 proteins, resulting in its proteasomal degradation and loss of function as a transcription factor.<sup>[16]</sup> Furthermore, it has been recognized that, in B- and T-cell lines, overexpression of *FoxO3a* leads to cell cycle arrest in G1 phase and induction of P27 (cell cycle inhibitor protein) and Fas ligand and Bim (pro-apoptotic molecules) which in turn trigger apoptosis.<sup>[17]</sup>

These findings strongly suggest that the loss of the transcriptional function of *FoxO3a* by hyperactivation of PI3K/Akt pathway is involved in the pathophysiology of leukemia. However, to the best of our knowledge, few reports have been published concerning the role of *FoxO3a* in pediatric ALL. Since the expression profile of *FoxO3a* in different stages of pediatric ALL has not yet been assessed, we aim to analyze the mRNA expression level of *FoxO3a* in three different groups of children in different stages of ALL in the south Iranian population.

## Subjects and Methods

### Patients' characteristics and sample collection

In this case-control study, seventy patients diagnosed with childhood ALL and 70 healthy age- and gender-matched ( $\pm 5$  years) children without a history of any malignancies as the control group were enrolled. Healthy controls were selected from the same geographic area, and none of them were relative to cases. Patients ranged in age from 1 to 20 years old. Based on the stage of malignancy, patients were divided into three categories: (a) 30 newly diagnosed ALL patients before therapy; (b) 20 ALL patients in the maintenance phase of chemotherapy; and (c) 20 ALL patients who showed relapse 1 year after they acquired the last treatment. The cases were recruited from referral Oncology Hospital in Shiraz, southern Iran between February 2015 and August 2015. The diagnosis was confirmed using immunology and cytogenetic tests as well as monitoring the morphology of the bone marrow cells. Patients who met the following criteria were excluded from sampling; (a) age more than 20 years and under 1 year; (b) The presence of other hematological disorders or history of other malignancies; and (c) Patients with other stages of treatment.

A designed questionnaire was administered for subjects to collect information, including parental alcohol consumption, cigarette smoking status, family history of blood disorders, and other cancers. The written informed consent was

obtained from parents of all children who participated in the study before sampling. This study was approved by the Ethics Committee of Islamic Azad University, Arsanjan Branch (Arsanjan, Iran).

### RNA extraction and real-time polymerase chain reaction analysis

Real-time polymerase chain reaction (PCR) was performed to determine the expression level of *FoxO3a* gene, as a candidate gene involving in the pathogenesis of ALL. Total RNA was isolated from Fresh blood samples using TRIzol solution (Invitrogen, USA) according to the manufacturer's protocol. RNase-Free DNase I was used to remove DNA contamination. RNA quality and concentration was assessed by absorbency at 260 nm and 280 nm using a Nanodrop spectrophotometer (ND-1000, Thermo Scientific, USA) to normalize the concentration of all RNA samples. Then, cDNA was synthesized from 5 ng of total RNA using the RevertAid first-strand cDNA synthesis kit (Thermo Scientific Fermentas, USA) following the manufacturer's instructions. Primer pairs were designed by Allele ID v7.8 software. Real-time PCR was performed using the Rotor-Gene Q 2plex HRM platform, real-time PCR system (Corbet life science) using the following primers for *FoxO3a* gene; Forward, 5'-CGGACAAACGGCTCACTCT-3' and reverse, 5'-GGACCCGCATGAATCGACTAT-3'; and forward, 5'-CCCAGAAACGCCGAATATAAT-3' and reverse, 5'-CTGGACTGTTCTTCACTCTTG-3' for TATA-binding protein gene (*TBP*) as an endogenous control gene. Each 15  $\mu$ l reaction volume contained 7.5  $\mu$ l of 2X SYBR Green master mix (Invitrogen, USA), 1  $\mu$ l of cDNA, and 0.4  $\mu$ l (10 pm) of each pair of oligonucleotide primers. All reactions were done in duplicate. The cycling parameters began with an initial step of 95°C for 10 min followed by 35 cycles of 95°C for 25 s, 56°C for 20 s and 72°C for 10 s; then, a melting curve analysis was performed. The threshold cycle (CT) values were measured during the exponential amplification phase by Rotor-gene Q sequence detection system. The expression data were normalized to the expression level of the housekeeping gene, *TBP* and the comparative threshold cycle ( $2^{-\Delta\Delta CT}$ ) method was used to analyze the data.

### Statistical analysis

Probable risk factors for ALL and other categorical variables were compared between ALL patients (cases) and healthy children (controls) using Chi-square test (version 16.0, SPSS Inc., Chicago, IL, USA). The expression data expressed as mean  $\pm$  standard deviation, for comparisons between two groups, unpaired 2-tailed students *t*-test; and to compare more than two groups, ANOVA followed by the Bonferroni multiple comparison *post hoc* tests were applied using GraphPad Prism7 software (La Jolla, USA). The value of  $P < 0.05$  was considered statistically significant.

**Results**

The frequencies of demographic and clinical features of ALL cases and controls are presented in Table 1. The cases and controls were matched for age and gender ( $P = 0.12$  and  $P > 0.99$ , respectively). Significantly, greater number of parents in case group were identified with smoking ( $P = 0.001$ ) and drinking habit ( $P = 0.033$ ); moreover, the history of blood disorders and other malignancies in the first- and second-degree relatives ( $P = 0.025$ ), as well as other malignancies in first- to third-degree relatives were higher in cases compared to controls ( $P = 0.002$ ). Immunophenotyping revealed that 51.4% of the ALL patients were Pre-B ALL, 17.1% were B-ALL and 31.4% were T-ALL. The proportions of patients based on the lymphoblast type were as follows: 74.3% L1, 18.6% L2, and 7.1% L3 types.

There was no significant difference in the frequency of blood groups between the cases and control subjects, but the frequency of B and O blood groups was higher among cases compared to the control group [Table 2].

Quantitative RT-PCR were performed for expression profiling of FoxO3a gene in blood samples of 30 newly diagnosed ALL patients, 20 patients in the maintenance phase of treatment, 20 relapse ALL patients and 70 healthy controls. The FoxO3a expression was significantly lower in newly diagnosed ALL patients compared to the control group (3.5-fold down-regulation,  $P < 0.0001$ ), the maintenance group (2-fold down-regulation,  $P = 0.0342$ ), and the relapse group (3.5-fold down-regulation,  $P = 0.0006$ ), but the differences in expression level of FoxO3a were not statistically significant between control/maintenance, control/relapse, and maintenance/relapse groups [Figure 1].

In further analysis, the mRNA expression level of FoxO3a was evaluated in comparison to each two groups of ALL regarding the types of the lymphoblasts. The mRNA expression level of FoxO3a was significantly lower in T-ALL patients compared to the Pre-B-ALL ones (3.4-fold down-regulation,  $P < 0.0001$ ). No significant difference was seen between T-ALL/B-ALL and/or B-ALL/Pre-B ALL [Figure 2].

**Discussion**

Tumor progression depends on different types of factors including heterogeneous interactions of genetic and environmental stimuli.<sup>[18]</sup> The constitutive activation of multiple signal transduction pathways enhances the survival and proliferation of leukemic cells, among them, PI3K/AKT signaling pathway has a critical role in tumor development which target FoxO3a tumor-suppressor gene.<sup>[19]</sup> Since this transcription factor regulates a wide range of target genes implicated in cell-cycle inhibition, resistance to oxidative stress, and apoptosis, loss of its functions has been observed in a number of human

**Table 1: Demographic characteristics of the study**

Variables	groups		P
	Cases (n=70), n (%)	Controls (n=70), n (%)	
Age (years)			
≤5	22 (31.4)	31 (44.3)	0.12*
>5	48 (68.6)	39 (55.7)	
Gender			
Male	41 (58.6)	41 (58.6)	>0.99
Female	29 (41.4)	29 (41.4)	
Parental smoking status			
Never	41 (58.6)	59 (84.3)	0.001
Ever	29 (41.4)	11 (15.7)	
Parental drinking status			
Never	56 (80)	65 (92.9)	0.033
Ever	14 (20)	5 (7.1)	
Blood cancers or disorders history (1 and 2 degree family)			
Negative	54 (77.1)	64 (91.4)	0.025
Positive	16 (22.9)	6 (8.6)	
Other cancer history (1-3 degree family)			
Negative	47 (67.1)	63 (90)	0.002
Positive	23 (32.9)	7 (10)	
Immunophenotype			
Pre-B ALL	36 (51.4)	-	
B-ALL	12 (17.1)	-	
T-ALL	22 (31.4)	-	
Lymphoblast type			
L1	52 (74.3)	-	
L2	13 (18.6)	-	
L3	5 (7.1)	-	

\*Pearson Chi-square test,  $P < 0.05$  considered significant. Frequency percentages are in parenthesis. ALL – Acute lymphoblastic leukemia

**Table 2: Distribution of blood group frequencies in patients and controls**

Types	Blood groups							
	A <sup>+</sup>	A <sup>-</sup>	B <sup>+</sup>	B <sup>-</sup>	O <sup>+</sup>	O <sup>-</sup>	AB <sup>+</sup>	AB <sup>-</sup>
Controls (%)	18 (25.7)	9 (12.9)	8 (11.4)	1 (1.4)	19 (27.1)	3 (4.3)	9 (12.9)	3 (4.3)
Cases (%)	13 (18.6)	2 (2.9)	19 (27.1)	3 (4.3)	25 (35.7)	3 (4.3)	3 (4.3)	2 (2.9)

P: 0.065, Pearson  $\chi^2$ ;  $P < 0.05$  considered significant

cancers and leads to tumorigenesis.<sup>[20]</sup> Conditional deletion of FoxO1, FoxO3a, and FoxO4 in the adult hematopoietic system leads to loss of HSC maintenance.<sup>[21]</sup> It has been found that FoxO3a deficient mice faced with impaired long-term reconstruction of HSCs, Moreover, it was shown that the number of HSC in aging mice whose FoxO3a gene was knocked out was increased in comparison with wild-type littermate controls.<sup>[22]</sup> On the other hand, FoxO transcription factors have a key role in lineage development,

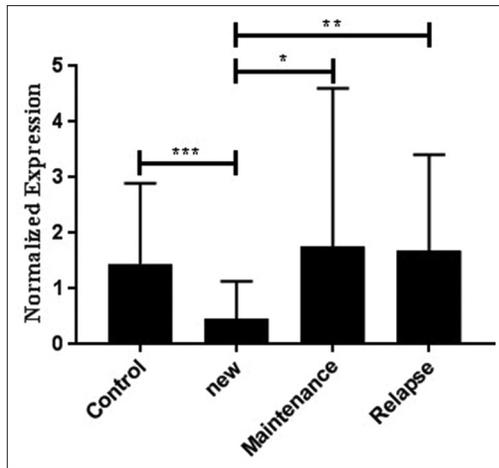


Figure 1: Comparison of the mRNA expression level between controls, new cases, and maintenance and relapse cases using quantitative reverse transcriptase-polymerase chain reaction. The expression level of *FoxO3a* gene was significantly lower in newly diagnosed cases compared to controls [\*\*\* for the  $P$  value  $< 0.001$ , while \* and \*\* for  $P < 0.05$ ] ( $P < 0.0001$ ) and other cases in different stage of treatment ( $P < 0.05$ )

in this case, conditional deletion of *FoxO1*, *FoxO3*, and *FoxO4* leads to increasing the level of myeloid cells and decreasing the number of peripheral blood lymphocytes.<sup>[21]</sup> Mutation of *FoxO3a* in hematopoietic progenitor cells of mice results in decreased in the formation of both myeloid and erythroid colonies supporting its role in lineage development.<sup>[23]</sup> It is revealed that the stronger PI3K activity in acute myeloid leukemia (AML) cells was associated with a higher rate of spontaneous proliferation of the cells;<sup>[24]</sup> Furthermore, it has been made obvious that the tumor suppressive function of *FoxO3a* is inactivated in AML cells due to its cytoplasmic localization.<sup>[25]</sup> It has been revealed that inactivation of *FoxO3a* leads to B-cell resistance to apoptosis and suggests that it might constitute a therapeutic target in this disease.<sup>[26]</sup> Down-regulation of *FoxO3a* gene has been seen in several types of tumors, including breast, ovarian, prostate, and gastric cancers, besides it has been shown that over-expression of *FoxO3a* inhibits cell proliferation and prevents tumor progression in these types of cancers.<sup>[27-30]</sup> Most of these studies were carried out in nonlymphoid cell lines, and hence, its relevance to B-and T-cells is not clear. This pursued us to analyze the mRNA expression level of *FoxO3a* gene in both B-ALL and T-ALL patients in three different stages. We found, using real-time PCR (qRT-PCR), a decreased in the expression of *FoxO3a* in the newly diagnosed ALL patients in comparison with control, maintenance, and relapse groups. Between-group comparisons revealed no statistical differences, suggesting that the chemotherapy affects the expression of *FoxO3a*. Concerning the ALL type (Pre-B ALL, B-ALL and T-ALL), T-ALL patients showed 3.4-fold down-regulation in the mRNA expression level of *FoxO3a* compared to the Pre-B ALL patients. These results are consistent with the findings of Ausserlechner *et al.* who reported that therapy-resistant

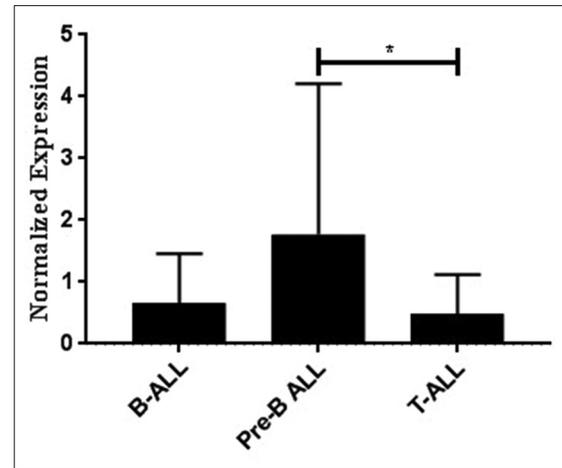


Figure 2: Intercomparison of *FoxO3a* gene expression between pre-B, B-and T-ALL using quantitative reverse transcriptase-polymerase chain reaction. *FoxO3a* gene expression was significantly lower in T-ALL patients compared to pre-B ALL cases\* ( $P < 0.0001$ )

T-ALL patients showed cytoplasmic localization of *FoxO3a* and also it is indicated that T-ALL cells in these patients inactivate *FoxO3a* to escape apoptosis induction by *TRAIL* and *NOXA* genes.<sup>[15]</sup> On the other hand, constitutive hyperactivation of PI3K/AKT pathway due to decreased PTEN activity has been seen in adult B-cell ALL cells.<sup>[31]</sup> Dewar *et al.* have shown that *FoxO3a* is a good biomarker for BCR-ABL-mediated leukemogenesis. They also found proteasomal inhibition of *FoxO3a* by bortezomib may be a promising therapeutic option in Philadelphia-positive ALL, where *FoxO3a* is down-regulated.<sup>[32]</sup> Tang *et al.* in 2016 showed that butien inhibited cell proliferation and induced cell cycle arrest in ALL via *FoxO3a/p27kip1* pathway.<sup>[33]</sup> These reports indicate that maintenance of proper *FoxO3a* levels is critical in preventing leukemogenesis.

## Conclusion

*FoxO3a* was found for the first time to have decreased expression in newly diagnosed pediatric ALL, and thus, it plays a tumor suppressor role in ALL. Increasing the activity of the *FoxO3a* transcription factor seems to be a plausible new therapeutic strategy for this type of tumor. Our result suggests that the *FoxO3a* can be a potential therapeutic target in pediatric ALL.

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## Conflicts of interest

There are no conflicts of interest.

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