IJBC 2016; 8(2): 47-51

Iranian Journal of Blood & Cancer

Journal Home Page: www.ijbc.ir

ORIGINAL ARTICLE

Expression Analysis of Foxo3a Gene in Pediatric Acute Lymphoblastic Leukemia in Southern Iranian Population

Malihe Mirzaei[1,2], Mahboobeh Nasiri[1,2*], Mehran Karimi[3]

1. Department of Biology, Islamic Azad University, Arsanjan Branch, Arsanjan, Iran
2. Young Researchers and Elite Club, Islamic Azad University, Arsanjan Branch, Arsanjan, Iran
3. Hematology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

ARTICLE INFO

Article History:
Received: 09.01.2016
Accepted: 02.04.2016

Keywords:
FoxO3a
Pediatric ALL
Gene expression
PI3K/AKT pathway

**Corresponding author:*
Mahboobeh Nasiri,
Address: Department of Biology, Arsanjan Branch, Islamic Azad University, Arsanjan, Iran
Tel: +98 917 3010601
Email: nasiri@iaua.ac.ir

ABSTRACT

Background: Acute lymphoblastic leukemia (ALL), the most common childhood cancer with a peak incidence in children from 2-5 years old, might be associated with poor prognosis and resistance to therapy in specific cytogenetic backgrounds. FoxO3a, a member of the forkhead class 'O' (FoxO) transcription factors, is a main downstream target of PI3K/AKT pathway which regulates different variety of biological processes and is overactivated in several human cancers. We aimed to evaluate the aberration of the FoxO3a gene in mRNA level in childhood ALL and compare them with healthy controls.
Methods: Real-time quantitative RT-PCR (qRT-PCR) was used to detect FOXO3a expression in 30 new cases of pediatric ALL and 30 age- and sex-matched healthy children as the control group.
Results: the expression level of the FoxO3a gene was significantly lower in ALL patients compared to healthy controls (P<0.0001), while no difference was observed between the two sub-types B- and T-ALL.
Conclusion: Our study suggested that decreased FoxO3a expression may play an important role in the development of pediatric ALL. FoxO3a could be considered as a molecular target of therapy in ALL malignancy.

Please cite this article as: Mirzaei M, Nasiri M, Karimi M. Expression Analysis of Foxo3a Gene in Pediatric Acute Lymphoblastic Leukemia in Southern Iranian Population. IJBC 2016; 8(2): 47-51.

Introduction

Leukemia is a malignancy of hematopoietic cell populations that include diverse and biologically distinct sub-groups.[1] Acute Lymphoblastic Leukemia (ALL) is one of the four major types of leukemia which is common in both children and adolescents; however, it is the most common pediatric malignancy diagnosed in children younger than 20 years.[2,3] Regarding the World Health Organization (WHO) definition, ALL is categorized in B-Lymphoblastic Leukemia (B-ALL) and T-Lymphoblastic Leukemia (T-ALL), originated from B- and T-Lineage lymphoid precursor cells, respectively.[4]

The disease pathogenesis results from blockade at any stages of normal lymphoid differentiation due to uncontrolled proliferation of lymphoid cells.[5] Lack of enough information on the precise origin of the leukemic cells, biological behavior of the hematopoietic primitive cells, mechanisms that damage the earliest steps of the lymphoid development as well as high genetic heterogeneity make ALL a condition full of ambiguity.[6,7]

From genetic point of view, proto-oncogenes and tumor suppressor genes are the most important genes involved in leukemogenesis,[8,9] which their alterations disrupt normal regulatory processes such as self-renewal, proliferation, differentiation and apoptosis in target cells.[10]

FoxOs (Forkhead box, class O) transcription factors function as a tumor suppressor gene and are important for stem cell maintenance.[11] They are key regulators of the cellular differentiation, growth, survival, cell cycle, metabolism, and cellular stress.[12] FoxO1, FoxO3a,

FoxO4 and FoxO6 are four members of the FoxO transcription factor family in humans.[13] FoxO3a expresses in various tissues including B- and T-lineage cells.[12,14] Transcriptionally, FoxO3a activates several target genes such as apoptosis-related genes (Bim, FasL, TRAIL, PUMA) and cell cycle inhibitor genes (P27, P21).[15] FoxO3a is an important target of PI3K/AKT signaling pathway, which is hyperactivated in various type of cancers.[16] Hyperactivation of this pathway in leukemia leads to inactivation of FoxO3a in leukemic cells and eventually tumor growth.[17] This evidence emphasize on FoxO3a as a tumor suppressor role gene. Moreover, overexpression of FoxO3a in B and T cell lines induces cell cycle arrest in G1 phase and triggers apoptosis by induction of the cell cycle inhibitor protein, P27, and pro-apoptotic molecules FasL and Bim, respectively.[14]

So far, few reports have been published concerning the role of FoxO3a in childhood ALL. The expression profile of FoxO3a in childhood ALL has not yet been reported. Thus, we aimed to analyze the mRNA expression level of FoxO3a in children with ALL among the population in southern Iranian.

Patients and Methods

Patient Characteristics and Sample Collection

30 children aged 2-17 years referred to Amir Oncology Hospital, Shiraz, Iran, and diagnosed as new cases of acute lymphoblastic leukemia were included in the study. 30 healthy age- and sex-matched children without a history of any malignancies were enrolled as the control group. The accuracy of the diagnosis was confirmed using immunology and cytogenetic tests as well as monitoring the morphology of the cells. Patients who met the following criteria were excluded; a) age more than 20 years, b) presence of other hematological disorders, history of other malignancies or relapsed ALL, and c) patients under chemotherapy or radiotherapy. The study design was approved by the Ethics Committee of Islamic Azad University, Arsanjan Branch and written informed consent was obtained from the parents of all children who participated in the study.

RNA Extraction and Real-Time PCR Analysis

To determine the expression level of FoxO3a gene, as a candidate gene involving in the pathogenesis of ALL, total RNA from fresh blood samples was isolated using RNX-Plus solution (CinnaGen, Iran) according to the manufacturer's instructions and cDNA was prepared using RevertAid first-strand cDNA synthesis kit (Thermo Scientific Fermentas, USA) following the manufacturer's instructions. All primer pairs used in this study were designed by Allele ID v7.8 software. Primers were specific for mRNA and did not amplify genomic DNA. The primer sequences were as follows: Forward, 5-CGGACAAACGGCTC ACTCT-3 and reverse, 5-GGACCCGCATGAATCG ACTAT-3 for FoxO3a gene; and forward, 5-CCCGAAACGCCGAATATAAT-3 and reverse, 5-CTGGACTGTTCTTCAC TCTTG-3 for TBP gene. The cDNA were subjected to quantitative RT-PCR (qRT-PCR) analysis using a Rotor-Gene Q 2plex HRM Platform real-time PCR system (Corbett Life Science) to evaluate the relative expression levels of FoxO3a and TBP (as an endogenous control gene). Each 15µl reaction volume contained 7.5µl of 2x Evagreen mastermix (Yekta Tajhiz Azma, Iran), 1.25µl of cDNA, and 0.4µl (10pm) of each pair of oligonucleotide primers. All reactions were done in duplicate. The PCR cycling began with an initial step of 95˚C for 15 min followed by 35 cycles of 95˚C for 25 sec, 54˚C for 20 sec and 72˚C for 20 sec; then a melting curve analysis was performed. The threshold cycle (CT) values were determined using Rotor-gene Q sequence detection system. The relative expression levels of the target gene were normalized to that of the endogenous control gene, TBP. The data were analyzed using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method.

Statistical Analysis

Chi-square test was used to compare the nominal variables among ALL patients (cases) and healthy children (controls). Data were analyzed with GraphPad Prism statistical software (La Jolla, USA) using unpaired t-test to compare the difference in gene expression between cases and controls. P value of <0.05 was considered statistically significant.

Results

Demographic features of ALL cases and controls are shown in table 1. Among them, 9 (30%) patients were diagnosed with T-cell ALL, and the rest (70%) with B-cell precursor ALL. 36.7% of all patients were female, and 63.3% were male. Although there was not a significant difference in frequency of blood groups between the patients and control group, the frequency of blood groups B and O were slightly higher among cases compared to the control group (table 2).

Foxo3a mRNA Expression Analyzed by qRT-PCR

The mRNA level of Foxo3a was measured by qRT-PCR in blood samples derived from ALL patients and

Table 1: Frequency distributions of selected features in ALL cases and controls

Features	Cases (n=30)	Controls (n=30)	P*
Age (range; year)	2-17	1-17	
Age			
≤5	10 (33)[a]	11 (37)	0.79
>5	20 (67)	19 (63)	
Gender			
Male	19 (63)	19 (63)	1
Female	11 (37)	11 (37)	

*Pearson chi-square; [a]Percentage of total within each group/ subgroup.

Table 2: The frequency of blood groups between cases and control

Groups \ Blood types	A^+	A^-	B^+	B^-	O^+	O^-	AB^+
Controls	9 (30)[a]	2 (6.7)	5 (16.7)	0 (0.0)	11 (36.7)	0 (0.0)	3 (10)
Cases	5 (16.7)	1 (3.3)	8 (26.9)	1 (3.3)	12 (40)	1 (3.3)	2 (6.7)
P value[*]	0.72						

[*]Pearson's chi-square; [a]Percentage of total within each group/ subgroup

healthy subjects. The Foxo3a mRNA expression level was significantly lower in ALL patients compared with the control group (P<0.0001). Quantitative RT-PCR showed more than 3-fold downregulation of Foxo3a gene (figure 1).

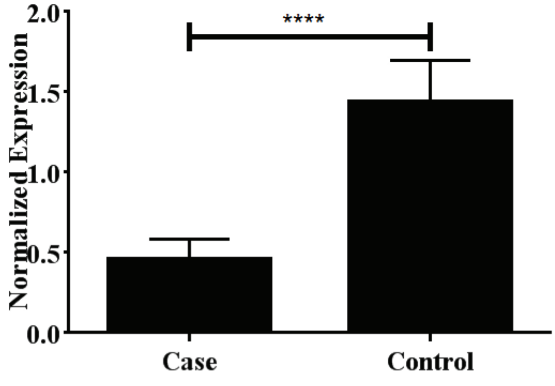

Figure 1: Real-time quantitative RT-PCR analysis of Foxo3a expression in blood samples from ALL patients and controls. The relative mRNA expression of Foxo3a was significantly lower in ALL patients compared with the healthy controls (****P<0.0001).

Furthermore, we analyzed the mRNA expression level of Foxo3a in 21 B-ALL patients compared with 9 T-ALL patients. Results showed more reduction in the FoxO3a expression, but this difference was not statistically significant between the two groups (P=0.23) (figure 2).

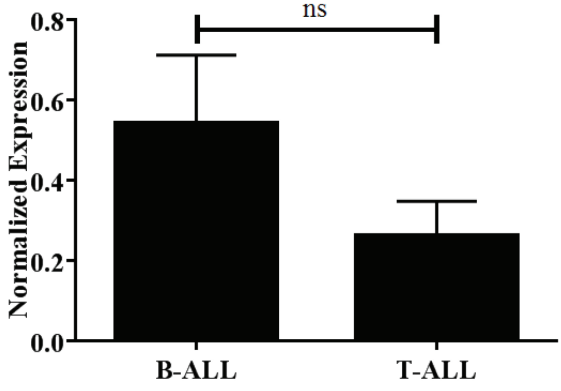

Figure 2: Real-time quantitative RT-PCR analysis of Foxo3a expression in blood samples from B-ALL and T-ALL patients. The relative mRNA expression of Foxo3a showed no significant difference between these two groups (P=0.23).

Discussion

ALL accounts for about 78% of all childhood leukemias.[18] Poor prognosis and resistance toward treatment are the key characteristics which represent some cases of ALL as an incurable cancer.[19] Poor prognosis for ALL reflects in part the lack of knowledge about the tumor basic biology. Present study for the first time, best of our knowledge, demonstrates a new molecule that its aberrant expression plays an important role in ALL pathogenesis among children. Decrease in mRNA level of the FoxO3a gene, as shown in our study confirmed the role of this transcription factor as a tumor suppressor gene in pathogenesis of the pediatric ALL. The result of our study was similar to those for breast, ovarian, prostate and gastric cancers.[13,20-22] In all these studies, it has been shown that overexpression of FoxO3a inhibits cell proliferation and then prevents tumor progression.

It has been demonstrated that PI3k/AKT signaling pathway is a central circuit in pathogenesis of acute leukemia.[23] Constitutive activation of this pathway has been demonstrated as a key pathogenic mechanism involved in AML development.[24] Similar to AML, PI3K/AKT activation is frequently found in B-ALL; however, its alterations is predominant in T-ALL in comparison with other leukemias.[25,26] One of the most favorable downstream effects of the activated PI3K/AKT pathway includes inactivation of FoxO3a through phosphorylation and restoration of this transcription factor.[27] Here, we also suggest the PI3k/AKT signaling pathway as a molecular mechanism which controls cell growth, apoptosis, development and progression of ALL via downregulation of FoxO3a.

One of the previous studies on the chemoresistance of T-ALL cells have shown the cytoplasmic localization of FoxO3a; therefore these cells inactivate FoxO3a in order to escape TRAIL and Noxa-induced apoptosis.[17] FoxO3a deficient mice showed reduced number of Pre-B cells and re-circulating B cells in bone marrow and peripheral blood, so FoxO3a makes a unique contribution to B cell development.[28] Conditional deletion of FoxO3a in mice affects lymphoproliferation and finally widespread organ inflammation. Mice with conditional deletion of FoxO1, FoxO3a and FoxO4 showed abnormalities in lymphoid development resulting in a long term defect in repopulation activity of the bone marrow stem cells.[12,29,30]

In summary, the current study provided information, for the first time, on essential role of FoxO3a in development of ALL disease. Understanding the precise role of FoxO3a in ALL will not only increase our knowledge of the biology of this malignancy but its upregulation may also allow development of a novel therapeutic strategy.

Acknowledgement

The authors appreciate the assistance of Dr. Majid Yavarian for technical and academic assistance. We

also thank Mohammad Moghadam for monitoring the laboratory procedures. The data was provided from the thesis of Ms. M. Mirzaei, MSc student of Molecular Genetics in Islamic Azad University, Arsanjan Branch.

Conflict of Interest: None declared.

References

1. Ayremlou P, Razavi SM, Solaymani-Dodaran M, Vakili M, Asadi-Lari M. Demographic and Prognostic Factors of 455 Patients With Acute Leukemia Admitted to Two Referral Hospitals in Tehran-Iran During Ten Years (2001-2011). Iran J Cancer Prev. 2012; 5(3):157-63. doi: 10.1080/01635581.2015.989368. PubMed Central PMCID: PMC4294538.
2. Xie Y, Davies SM, Xiang Y, Robison LL, Ross JA. Trends in leukemia incidence and survival in the United States (1973–1998). Cancer. 2003;97(9):2229-35. doi: 10.1002/cncr.11316. PubMed PMID: 12712476.
3. Inaba H, Greaves M, Mullighan CG. Acute lymphoblastic leukaemia. The Lancet. 2013; 381(9881):1943-55. doi: 10.1016/S0140-6736(12)62187-4. PubMed PMID: 23523389.
4. Chiaretti S, Zini G, Bassan R. Diagnosis and Subclassification of Acute Lymphoblastic Leukemia. Mediterr J Hematol Infect Dis. 2014; 6(1): e2014073. doi: 10.4084/MJHID.2014.073. PubMed Central PMCID: PMC4235437.
5. Conter V, Rizzari C, Sala A, Chiesa R, Citterio M, Biondi A. Acute Lymphoblastic Leukemia. Orphanet Encyclopedia. December 2004. https://www.orpha.net/data/patho/Pro/en/AcuteLymphoblasticLeukemia-FRenPro3732.pdf
6. Mullighan CG. The molecular genetic makeup of acute lymphoblastic leukemia. Hematology Am Soc Hematol Educ Program. 2012;2012(1):389-96. doi: 10.1182/asheducation-2012.1.389. PubMed PMID: 23233609.
7. Purizaca J, Contreras-Quiroz A, Dorantes-Acosta E, Vadillo E, Arriaga-Pizano L, Fuentes-Figueroa S, et al. Lymphoid progenitor cells from childhood acute lymphoblastic leukemia are functionally deficient and express high levels of the transcriptional repressor Gfi-1. Clin Dev Immunol. 2013; 2013:349067. doi: 10.1155/2013/349067. PubMed PMID: 24198842.
8. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. N Engl J Med. 2004; 350(15):1535-48. PubMed PMID: 15071128.
9. Fröhling S, Döhner H. Chromosomal abnormalities in cancer. N Engl J Med. 2008; 359(7):722-34. doi: 10.1056/NEJMra0803109. PubMed PMID: 18703475.
10. Pui CH. Acute lymphoblastic leukemia: introduction. Semin Hematol. 2009;46(1):1–2. doi:10.1053/j.seminhematol.2008.09.011. PubMed PMID: 19100362.
11. Ferber EC, Peck B, Delpuech O, Bell GP, East P, Schulze A. FOXO3a regulates reactive oxygen metabolism by inhibiting mitochondrial gene expression. Cell Death Differ. 2012;19(6):968-79. doi: 10.1038/cdd.2011.179. PubMed PMID: 22139133.
12. Zhang X, Tang N, Hadden TJ, Rishi AK. Akt, FoxO and regulation of apoptosis. Biochim Biophys Acta. 2011;1813(11):1978-86. doi:10.1016/j.bbamcr.2011.03.010. PubMed PMID: 21440011.
13. Yang XB, Zhao JJ, Huang CY, Wang QJ, Pan K, Wang DD, et al. Decreased expression of the FOXO3a gene is associated with poor prognosis in primary gastric adenocarcinoma patients. PLoS One. 2013; 8(10):e78158. doi:10.1371/journal.pone.0078158. PubMed PMID: 24194912.
14. Kerdiles YM, Beisner DR, Tinoco R, Dejean AS, Castrillon DH, DePinho RA, et al. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. Nat Immunol. 2009; 10(2):176-84. doi: 10.1038/ni.1689. PubMed PMID: 19136962.
15. Nho RS, Hergert P. FoxO3a and disease progression. World J Biol Chem. 2014; 5(3):346-54. doi: 10.4331/wjbc.v5.i3.346. PubMed PMID: 25225602.
16. Marinkovic D, Zhang X, Yalcin S, Luciano JP, Brugnara C, Huber T, et al. Foxo3 is required for the regulation of oxidative stress in erythropoiesis. J Clin Invest. 2007;117(8):2133-44. doi:10.1172/JCI31807DS1. PubMed PMID: 17671650.
17. Ausserlechner MJ, Salvador C, Deutschmann A, Bodner M, Viola G, Bortolozzi R, et al. Therapy-resistant acute lymphoblastic leukemia (ALL) cells inactivate FOXO3 to escape apoptosis induction by TRAIL and Noxa. Oncotarget. 2013;4(7):995-1007. doi: 10.18632/oncotarget.953. PubMed PMID: 23828551.
18. Rasool M, Farooq S, Malik A, Shaukat A, Manan A, Asif M, et al. Assessment of circulating biochemical markers and antioxidative status in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) patients. Saudi J Biol Sci. 2015;22(1):106-11. doi: 10.1016/j.sjbs.2014.09.002.
19. Mendivil-Perez M, Velez-Pardo C, Jimenez-Del-Rio M. TPEN induces apoptosis independently of zinc chelator activity in a model of acute lymphoblastic leukemia and ex vivo acute leukemia cells through oxidative stress and mitochondria caspase-3-and AIF-dependent pathways. Oxid Med Cell Longev. 2012;2012:313275. doi: 10.1155/2012/313275. PubMed PMID: 23320127.
20. Lin H, Dai T, Xiong H, Zhao X, Chen X, Yu C, et al. Unregulated miR-96 induces cell proliferation in human breast cancer by downregulating transcriptional factor FOXO3a. PloS one. 2010; 5(12):e15797. doi: 10.1371/journal.pone.0015797. PubMed PMID: 21203424.
21. Arimoto-Ishida E, Ohmichi M, Mabuchi S, Takahashi T, Ohshima C, Hayakawa J, et al. Inhibition of phosphorylation of a forkhead transcription factor sensitizes human ovarian cancer cells to cisplatin. Endocrinology. 2004;145(4):2014-22. doi: 10.1210/en.2003-1199. PubMed PMID: 14701673.
22. Kikuno N, Shiina H, Urakami S, Kawamoto K, Hirata H, Tanaka Y, et al. Knockdown of astrocyte-elevated gene-1 inhibits prostate cancer progression through

upregulation of FOXO3a activity. Oncogene. 2007; 26(55):7647-55. doi:10.1038/sj.onc.1210572. PubMed PMID: 17563745.
23. Fransecky L, Mochmann LH, Baldus CD. Outlook on PI3K/AKT/mTOR inhibition in acute leukemia. Mol Cell Ther. 2015;3:2. doi: 10.1186/s40591-015-0040-8. PubMed PMID: 26056603.
24. Kubota Y, Ohnishi H, Kitanaka A, Ishida T, Tanaka T. Constitutive activation of PI3K is involved in the spontaneous proliferation of primary acute myeloid leukemia cells: direct evidence of PI3K activation. Leukemia. 2004;18(8):1438-40. doi:10.1038/sj.leu.2403402. PubMed PMID: 15175626.
25. Gomes AM, Soares MV, Ribeiro P, Caldas J, Póvoa V, Martins LR, et al. Adult B-cell acute lymphoblastic leukemia cells display decreased PTEN activity and constitutive hyperactivation of PI3K/Akt pathway despite high PTEN protein levels. haematologica. 2014; 99(6):1062-8. doi: 10.3324/haematol.2013.096438. PubMed PMID: 24561792.
26. Silva A, Yunes JA, Cardoso BA, Martins LR, Jotta PY, Abecasis M, et al. PTEN posttranslational inactivation and hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability. J Clin Invest. 2008;118(11):3762-74. doi: 10.1172/JCI34616. PubMed PMID: 18830414.
27. Brandts CH, Sargin B, Rode M, Biermann C, Lindtner B, Schwäble J, et al. Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. Cancer Res. 2005;65(21):9643-50. doi: 10.1158/0008-5472. PubMed PMID: 16266983.
28. Hinman RM, Nichols WA, Diaz TM, Gallardo TD, Castrillon DH, Satterthwaite AB. Foxo3−/− mice demonstrate reduced numbers of pre-B and recirculating B cells but normal splenic B cell subpopulation distribution. Int Immunol. 2009;21(7):831-842. doi: 10.1093/intimm/dxp049. PubMed Central PMCID: PMC2699488.
29. Wang Y, Zhou Y, Graves DT. FOXO transcription factors: their clinical significance and regulation. Biomed Res Int. 2014;2014:925350. doi: 10.1155/2014/925350.
30. So CW, Cleary ML. MLL-AFX requires the transcriptional effector domains of AFX to transform myeloid progenitors and transdominantly interfere with forkhead protein function. Mol Cell Biol. 2002;22(18):6542-52. doi: 10.1182/blood-2012-09-456665. PubMed PMID: 12192052.