Original Article

Evaluation of *FOXP1* gene expression in pediatric B-cell precursor acute lymphoblastic leukemia patients at remission induction therapy

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Abstract

Background: Transcription factors (TFs) play a key role in the development, therapy, and relapse of B-cell malignancies, such as B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Given the essential function of Forkhead box protein P1 (FOXP1) transcription factor in the early development of B-cells, this study was designed to evaluate *FOXP1* gene expression levels in pediatric BCP-ALL patients and NALM6 cell-line.

Materials and Methods: This case-control study was done on the NALM6 cell-line and bone marrow specimens of 23 pediatric BCP-ALL patients (median age: 7.5 years; range: 2.0 – 15.0 years) at different clinical stages including new diagnosis, 15th day after the treatment, and relapse. Also, 10 healthy children were included as the control group. *FOXP1* gene expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). The correlation analysis was performed between the *FOXP1* gene expression and patients' demographic and laboratory characteristics.

Results: The results showed that *FOXP1* gene expression was significantly downregulated in the NALM-6 cell-line (median=0.05, $P<0.001$) and patients at new diagnosis (median=0.06, $p<0.0001$), and relapse (median=0.001, $p<0.0001$) phases, compared to the control group (median=0.08). *FOXP1* gene expression on the 15th day of the treatment was significantly higher than its level at the new diagnosis stage ($p<0.001$). Moreover, *FOXP1* gene was significantly downregulated in the relapse phase compared to the new diagnosis. Patients whose number of bone marrow blasts on the 15th day of the treatment was below 5% had higher *FOXP1* gene expression at the diagnosis phase (Spearman's correlation, $P<0.05$, r=-0.485) and higher ratio of diagnosis/day 15 ($p<0.001$, Mann-Whitney U test).

Conclusions: *FOXP1* levels could be a potential biomarker of therapy response in remission induction therapy for pediatric BCP-ALL patients**.**

Keywords: FOXP1 protein, Neoadjuvant Therapy, Neoplasm, Residual, Precursor B-cell lymphoblastic leukemia

Introduction

Acute lymphoblastic leukemia (ALL) is the most common hematologic malignancy among pediatric patients, but it can also occur in adults with weaker prognosis. ALL is classified into two main subgroups: T-cell and B-cell acute lymphoblastic leukemia (T-ALL and B-ALL), based on the World Health Organization (WHO) classification. The criteria for this classification are based on the patient's molecular and pathogenetic changes. Accordingly, it would be possible to perform better in the diagnosis and treatment of malignancies by investigating the progression of the disease at the molecular level. Although chemotherapy has improved the treatment of this disease, it is better to provide novel therapeutic approaches due to the possibility of high recurrence and severe toxicity. Evaluations of molecular genetics and epigenetic abnormalities have led to a broader understanding of drug resistance and their adverse effects (1). Cell genetic program settings are performed using lineage-specific transcription factors. These factors are generally involved in the decision of several lineages that generally lead to a particular fate for the cell in the presence of

other transcription factors expressed with them. Therefore, genomic evaluation, along with a review of transcriptional profiles, provides a better insight into the pathogenesis and biology of the disease and determines the treatment goals associated with prognosis. As a result, these findings should lead to the development of a personalized approach to medicine with greater efficacy and less toxicity (2). In children with ALL, the measurable residual disease (MRD) is used to monitor the response to the treatment, prognosis, and risk stratification in the management of clinical conditions. Monitoring MRD is performed by precise methods, such as quantitative polymerase chain reaction (qPCR)-based techniques and multiparametric flow cytometry (MFC). qPCR is the gold standard method in ALL patients. Generally, MRD assessment is carried out on the 15th or 33rd days of treatment. Although PCR can detect 10^{-4} to 10^{-5} leukemic cells in ALL patients, it is just used in case of specific tumor DNA indices, including T receptor genes, rearranged immunoglobulins, and fusion genes, such as bcr / abl exist. Even though it is not always possible to distinguish the leukemic cells from normal hematopoietic progenitors, it can be performed using the current techniques. In other words, leukemic blasts exhibit a different gene expression pattern after genetic mutations, which differed from normal hematopoietic progenitors and they are detectable by flow cytometry. However, this technique has several limitations that affect its clinical application (3, 4). Forkhead family proteins are necessary for the development and organogenesis in human and mice (5, 6). Forkhead box protein P1 (FOXP1) is encoded by the *FOXP1* gene located at 3p14.1 position in human. FOXP1 belongs to the forkhead box protein subfamily and is expressed in many tissues (7, 8). Lack of *FOXP1* transcription factor gene causes genetic defects in the development of early B-cells, and it is associated with a defect in the production of μ immunoglobulin chain.

In addition, its deficiency leads to a reduction in the expression of recombinant activator genes 1 and 2 (Rag1/2) and decreases the expression of all B-cell genes in fetal liver B220$^+$cells, along with a pause in the transfer from pro-B to pre-B-cell. FOXP1 is involved in the control of the recombinant variable- (diversity) -joining of the immunoglobulin heavy chain (9, 10). Direct relationship between Ikaros and FOXP1 causes the regulation of G protein-coupled receptor G2A in B-cells and ALL. The disruption of this interaction might change the expression of G2A and cause G2 arrest in B-ALL (11). This study aimed to evaluate the *FOXP1* gene expression levels at different clinical stages and investigate the correlation between *FOXP1* gene expression and MRD in pediatric BCP-ALL patients.

Materials and Methods
Cell-line and patient's samples
Bone marrow samples were collected from 23 pediatric BCP-ALL patients at different clinical stages including new diagnosis (day 0), 15th day of the treatment (day 15), and relapse from Amir Oncology hospital, Shiraz. The samples of days 0 and 15 were obtained from the same patient. Patients with standard-risk BCP-ALL were considered in this study. Patients administered one or two courses of induction therapy with vincristine 1.5 mg/m2 (max 2 mg) IV, dexamethasone 6mg/m2/day IV or PO, pegylated L-asparaginase 2500 units/m2/IV / 2 hours IV or IM or L-asparaginase 6000 units/m2 IM (maximum 10000 unit). Written informed consent was given by the parents /legally authorized representatives of all participants included in the study. After obtaining consent from healthy volunteers and patients, normal cells were also collected from the peripheral blood of healthy volunteers. The Ethics Committee of Shiraz University of Medical Sciences (1396-01-10-15829) approved this case-control study. In addition to patient's sample, B-cell precursor cell-line, NALM-

6, was also purchased from ATCC. Cell culture materials were obtained from Gibco-Invitrogen-USA and SIGMA. NALM-6 cells were cultured in CM10 medium containing 90% RPMI 1640, 10% fetal bovine serum (FBS) plus 1% penicillin-streptomycin solution containing 100 U/ml penicillin and 100μg streptomycin, and 2mM L-glutamine. They were incubated at 37°C and atmospheric conditions containing 5% carbon dioxide and 95% moisture content in order to provide the cells with suitable conditions, the cells were passaged once they achieved 80% confluency. The trypan blue exclusion assay was performed to keep the cells' viability above 95%. Exact diagnosis in all patients was made based on morphological evaluation, immunocytochemistry, immunohistochemistry, and flow cytometry results.

Morphological evaluation
The morphological investigation of each sample was accomplished at least once by a pathologist or oncologist. To take bone marrow biopsy samples, we applied a local anesthesia by a hematologist to numb the skin over the hip bone before the needle had penetrated into the marrow space. Patients were categorized according to the French-American-British (FAB) and WHO criteria at the diagnostic phase. For each sample, Wright-Giemsa staining on the aspiration samples of the bone marrow was evaluated. In each field, the bone marrow aspiration smear was estimated to be about 500 cells, and the samples with inadequate bone marrow content were excluded from the study.

Flow cytometry for diagnosis and detection of MRD
Immunophenotype analysis was performed using a panel of antibodies against CD45, HLA-DR, CD19, CD10, CD13, CD33, CD117, CD34, CD14, CD64, CD5, CD7, CD2, CD20, CD123, CD36, and CD15. A typical lyse/wash method was used for lysing the erythrocytes after incubation

with monoclonal antibodies for 10 min at 4°C. A population of blasts was selected on SSC/CD45 and SSC/FSC scattergrams. Negative controls were assessed by IgG1FITC/IgG1PE/IgGPerCp. All antibodies were obtained from eBioscience (San Diego, CA). FACSCanto II instruments (BD Biosciences, San Diego, CA) were used to obtain the data. In short, ALL leukemic cells were different from normal lymphoid precursors for several reasons: 1) increased expression levels of antigens such as HLA-DR, CD19, CD10, CD20, and CD123, which were normally expressed on the cells; or altered pattern of CD45/side scatter, 2) aberrant expression of myelomonocytic markers on the myeloid blasts (CD4, CD64, CD36, CD15), myeloid antigens (CD13, CD34, CD117), and T cell lymphoid progenitors (CD2, CD5, CD7). For 20 samples, the MRD level was evaluated. These patients had more than 20% blasts at the diagnosis phase and it was expected that the blast would drop below 5% on the day 15. On day 15 of the treatment, if more than 5% blast was observed with two or more aberrant markers, it was considered to be a positive MRD.

Cytogenetic Analysis
The chromosomal evaluation was performed on diagnostic bone marrow aspirate samples on G-banded metaphase cells, which were cultured using standard methods. Briefly, the samples were cultured in RPMI 1640 basal medium containing 10% fetal calf serum (Gibco-Invitrogen-USA) 72 h at 37°C, and then treated with 0.1 microgram/ ml of colcemid (Gibco-Invitrogen-USA) in order to stop the cells in the metaphase of mitosis. After harvesting with hypotonic solution (0.068 mol/L KCL) and fixing with acetic acid /methanol (1/3), we spread the chromosomes and stained them using standard G-banding technique. The median number of metaphases was 20 (range: 10–50), which was analyzed by CytoVision® chromosomal karyotyping automatic

system (Genetix Company- USA). The karyotype was documented according to the International System for Human Cytogenetic Nomenclature (ISCN 2013). A successful cytogenetic analysis requires the detection of at least 2 or more cells with the same structural change or chromosomal gain, and 3 or more cells with the same chromosomal loss in at least 20 metaphases. The patients' karyotypes were subdivided into groups based on WHO classification [2008].

Molecular Analysis

RNA was extracted from each bone marrow aspirate sample. qRT-PCR was performed for the detection of t(9;22)(q34;q11.2), *BCR-ABL1* fusion gene (resulting in 210 kDa protein major breakpoint region and 190 kDa protein minor breakpoint region), t(4;11)(q21;q23) with KMT2A-AFF1, t(1;19)(q23;q13) with TCF3-PBX1, and t(12;21)(p13;q22) with *ETV6-RUNX1* fusion genes. Co-amplification of *ABL* gene was carried out in order to check the quality of RNA and absence of inhibitors. RNA from a well -documented positive case was also amplified simultaneously as a positive control.

Mononuclear cells isolation, RNA extraction and cDNA synthesis

Mononuclear cells were isolated from diagnostic bone marrow specimens and peripheral blood samples using ficoll solution (Courtaboeuf, France, Paris). Total RNA was extracted using TRIzol-based method (Thermo Fisher Scientific, MA). Concentration of the extracted RNA was determined by NanoDrop spectrophotometer (Thermo Scientific NanoDrop2000, Finland). Then, 1 µg of total RNA was applied to complementary DNA (cDNA) synthesis using PrimeScript™ RT reagent Kit (Takara, Tokyo, Japan) following the manufacturer's protocol.

Real-time PCR assay

To evaluate the expression of *FOXP1* gene, we calculated the quantitative real-time polymerase chain reaction (qRT-PCR), using specific gene primers (Table I) and SYBR Green qPCR MasterMix (Yekta Tajhiz Azma, Iran) according to the manufacturer's instructions in a Rotor-Gene Q system (Qiagen, Hilden, USA). To check the possible contamination, we included no-template controls (NTC) in every run. Each sample was analyzed in triplicate, and comparative Ct ($2^{-\Delta\Delta Ct}$) method was used to determine the relative expression level of *FOXP1* gene. *ACTB* gene was used as endogenous reference.

Statistical analysis

Mann-Whitney U test was used to evaluate the significance of the differences between *FOXP1* gene expression levels and internal control gene (*ABTB*). Wilcoxon sign rank test was applied to examine the significance of the differences between *FOXP1* gene expression levels before and after induction therapy. Spearman's rank correlation was used to investigate the association between continuous variables. P-values > 0.05 were considered significant. Statistical analysis was applied using SPSS software (version 25).

Results

ALL patients, control group, and their demographic characteristics

The samples were collected from 23 pediatric BCP-ALL patients, of whom 15 were newly diagnosed and 9 were in the relapse phase. There were 16 male patients (69.5 %). The median age was 7.5 (range: 2.0-15.0) years. Moreover, 10 healthy controls with a mean age of 5.6 years were included in this study.

FOXP1 gene expression is dysregulated in ALL patients' samples at different clinical stages and NALM6 cell-line

FOXP1 gene expression was significantly downregulated in the NALM-6 cell-line (median=0.05, P<0.001) and patients at new diagnosis (median=0.06, p<0.0001)

and relapse (median=0.001, p<0.0001) phases, compared to the control group (median=0.08). *FOXP1* gene expression on the 15th day of the treatment was significantly higher than its level at new diagnosis stage (p<0.001). Moreover, *FOXP1* gene was significantly downregulated in the relapse phase compared to new diagnosis. (Figure 1).

Correlation of *FOXP1* gene expression with patients' clinical findings at the diagnosis phase

No significant correlation was found between the expression of *FOXP1* gene and patients' laboratory characteristics including WBC count, hemoglobin level, platelet count, diagnostic blast percentage, cytogenetic data, molecular analysis, and extramedullary disease (p values>0.05). There was also no significant relationship between the *FOXP1* gene expression and aberrant expression of markers in the flow cytometry at diagnosis (p>0.05).

Correlations of *FOXP1* gene expression levels at diagnosis and 15th day of the treatment with response to the therapy.

The expression of *FOXP1* gene before and after treatment and its relationship with clinical characteristics of patients was considered as an indicator for the response to the therapy. The number of blasts on the 15th day of the therapy was inversely correlated with the *FOXP1* levels at diagnosis (Spearman's correlation, P<0.05, r=-0.485) and on day 15 of treatment (Spearman's correlation, P<0.05, r= -0.777). This study also considered the ratio of *FOXP1* gene expression levels at diagnosis phase to its levels on the 15th day of the treatment. A significantly high ratio was observed in patients with blast percentage less than 5% on the 15th day of the treatment (P<0.001, Figure 2). A significant association was observed between the expression of *FOXP1* gene before and after treatment (P<0.001, Figure 1). No significant association was observed between the expression of *FOXP1* gene on

the day 15 and WBC count, platelets, and hemoglobin levels (p values>0.05). Expression level of *FOXP1* gene on the 15th day of the treatment was not correlated with abnormal expression of markers in the flow cytometry.
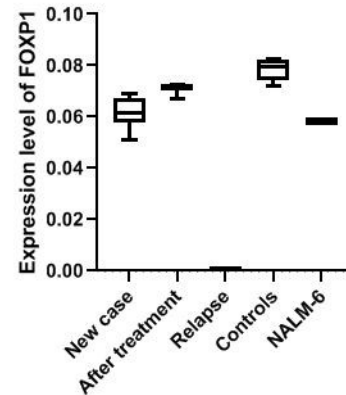

Figure 1. The levels of *FOXP1* gene expression in NALM6 cell-line and patients' samples in different clinical stages including new cases, 15th day of the treatment, and relapse compared to the control group. The expression levels of *FOXP1* were determined by quantitative real-time polymerase chain reaction method. Beta-Actin expression was used as an internal control (error bars are standard deviations). There was no association between the *FOXP1* gene expression levels and patients' demographic findings including gender and age measured at any time before and after the treatment.
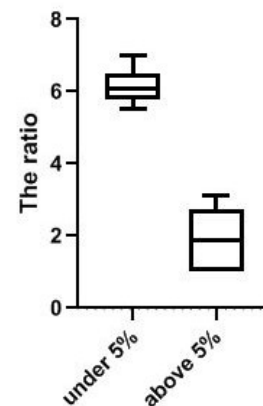

Figure 2. Boxplot chart indicates the ratio of *FOXP1* gene expression levels at the diagnosis phase to its levels on the 15th day of the treatment with percentage of blasts on the day 15 below and above 5% (Mann-Whitney test, P<0.001).

Table I: The forward and reverse primer sequences used for quantitative real-time polymerase chain reaction assay

Gene	Forward/Reverse	Sequence
FOXP1	Forward	5′-ATAAGAACGCAGAAGTTA-3′
	Reverse	5′-GTGAAGACTAAGATTATGAC-3′
ACTB	Forward	5′-ATCGTGCGTGACATTAAGGAG-3′
	Reverse	5′-GAAGGAAGGCTGGAAGAGTG-3′

Discussion

ALL is the most common hematologic malignancy among children. In general, the prognosis of childhood B-ALL is good and its rate of treatment is 80- 90% (12). Transcription factors can play key roles in the progression, treatment, and relapse of cancers. Recently, it has been found that genetic alternations in some transcription factors are associated with progression of ALL (13). In this study, the expression of *FOXP1* gene in BCP-ALL patients was investigated in three phases of new diagnosis, 15th day of the treatment, and relapse. FOXP1 is a transcription factor involved in immune regulation and carcinogenesis and has anti-proliferative effects on several types of cells. This factor is required in order to produce pre-B-cells efficiently and regulate the expression of recombination-activating genes (*RAG*) (14-17). Recently, novel *ABL*1 fusion genes associated with t(3;9) (p12;q34) producing *FOXP1-ABL1* fusion gene has been identified based on molecular analysis in BCP-ALL patients (18). The adult patients with B-ALL have also been identified with t(3;9) (p13;p13), which has been involved in *FOXP1-PAX5* fusion gene. This fusion induces leukemogenesis by stopping the mature B-cells development (19-22). In another study, the function of *FOXP1-PAX5* was evaluated in mice, and it was observed that this protein stopped lymphopoiesis from pro-B to pre-B-cell transition (23). However, probably due to the low prevalence of t(3;9) (at diagnosis: P=0.216, on day 15: P=0.978) in the current study, there was no significant correlation between *FOXP1* and *PAX5* gene.

Since a chromosomal aberration involving *FOXP1* has been reported in ALL (18), the expression level of *FOXP1* in NALM6 cell-line and pediatric BCP-ALL patients was evaluated for the first time. *FOXP1* gene expression was significantly downregulated in the new diagnosis phase compared to the controls (p<0.0001). Dysregulated expression of *FOXP1* gene in different clinical stages of pediatric BCP-ALL suggests that it might have a role in the disease progression. Its abnormal expression in various tumors of B-cells is especially related to adverse prognosis and lower survival in diffuse large B-cell lymphoma (DLBCL) patients (24, 25). *FOXP1* gene is also overexpressed in patients affected with follicular lymphomas (26). Similar to this study, Banham et al. found that there was no association between the *FOXP1* gene expression and cytogenetic abnormalities in DLBCL patients (27). The number of blasts on the 15th day of the therapy was inversely correlated with *FOXP1* levels at diagnosis (P<0.05, r= -0.485) and on day 15 (P<0.05, r= -0.777). *FOXP1* can have an anti-tumor or oncogenic function. Therefore, it can be an important therapeutic target in cancers. *FOXP1* is expressed in different lymphomas, such as DLBCL, and its expression is unusual and has an oncogenic role in these lymphomas (28). Resultant anti-tumor pattern of *FOXP1* gene in this study was in harmony with the results achieved by Razyi et al, in which tumor suppressive role of *FOXP1* in Jurkat cells has been reported. (29) Observation of a significantly high ratio of *FOXP1* expression levels at diagnosis and on day 15 in patients with blast percentage less

than 5% on day 15 indicated that good therapeutic response was related to an increase in the *FOXP1* gene expression in later phases of therapy. The mechanism of these clinical manifestations has not been still revealed. According to the Kim et al.'s study, *FOXP1* gene may be effective in chemoresistance (30). In another study carried out by Mottok et al, the high expression of *FOXP1* gene was associated with significantly shorter failure-free survival (FFS) in patients treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP), but not in patients treated with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) (26). Therefore, according to Mottok et al.'s study, vincristine had no effect on the expression of this gene (26). *FOXP1* expression was increased on the 15th day of the treatment (p<0.001, Wilcoxon sign rank test, Figure 1), in comparison with new diagnosis phase. Therefore, these drugs might have effects on the expression of this gene.

Conclusion
FOXP1 might be a tumor suppressor gene and is related to MRD in pediatric BCP-ALL patients. Further detailed investigations on a larger cohort of childhood ALL patients are required to validate the current results.

Acknowledgement

Conflict of interest
The authors declare no conflict of interest.

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