

Investigating the Correlation Between TGF- β Gene Expression and Disease-Related Prognostic Factors in Bone Marrow Aspiration of Adults With Acute Lymphoblastic Leukemia

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Abstract- Acute lymphoblastic leukemia (ALL) is a malignant transformation and proliferation of lymphoid progenitor cells in the bone marrow, blood and extramedullary sites and the second most common acute leukemia in adults. While dose-intensification strategies have led to a significant improvement in outcomes for pediatric patients, the prognosis for the elderly remains very poor. Aberrant or excessive expression of cytokines may be related to the pathogenesis of acute leukemia. TGF- β is a cytokine that plays a role in regulating various cellular processes such as growth, proliferation, and apoptosis. We evaluated the expression of TGF- β mRNA in adults with ALL compared to the control and its relationship with disease-related prognostic factors. Bone marrow specimens were obtained from 90 newly-diagnosed adults with ALL and 33 healthy adults. After immunophenotyping by flow cytometry, RNA was extracted, and RQ-PCR was done. Our result showed that from all patients, 63 (70%) were identified as B-ALL and 27(30%) as T-ALL. TGF- β transcript levels in both T-ALL and B-ALL patients showed a significant decrease compared to the control group ($P<0.001$). However, the expression of the TGF- β transcripts was not different between the different immunophenotypic subtypes ($P=0.54$). The gene expression level of TGF- β was not correlated with age ($P=0.47$), gender ($P=0.29$), ALL subtypes ($P=0.54$), the percentage of bone marrow blasts ($P=0.92$) and peripheral blood leukocyte count ($P=0.38$) of ALL patients. In conclusion, since TGF- β has a tumor suppressor role, it seems that leukemic cells may use TGF- β down-regulation to be more freely proliferated and evolve the clone.

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Introduction

Acute lymphoblastic leukemia (ALL) is a group of blood neoplasms that are associated with increased proliferation and differentiation arrest of both B and T precursor cells (1). ALL is the most common cancer occurring in children, representing 23% of cancer diagnoses among children younger than 15 years (2). While 80% of ALL occurs in children, it represents a devastating disease when it occurs in adults. ALL is the second most common acute leukemia in adults, almost 25% of cases develop from precursors of the T-cell lineage (3) which is characterized by a higher age of

onset, male superiority and worse prognosis compared to B-ALL (4,5). The ALL prognosis is strongly related to the patient's age. 80-90% of children respond to therapy (6), but despite a high rate of response to induction chemotherapy, only 30-40% of adult patients with ALL will achieve long-term remission (2).

The mechanisms by which leukemic cells escape the processes of regulating and inhibiting growth are not fully understood. Blood cells and their precursors in the bone marrow are affected by environmental factors, including cytokines (7). Aberrant or excessive expression of cytokines may be related to the pathogenesis of acute leukemia (8). Cytokines produced by cancer cells and

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stromal cells (such as macrophages and dendritic cells) cause tumor growth, survival, and invasion by activating oncogenic signaling pathways (9). TGF-β is a cytokine that plays a role in regulating various cellular processes such as growth, proliferation, and apoptosis (10). Although TGF-β plays a major role as a tumor suppressor but also can facilitate tumor growth particularly in the later stages of disease (11). TGF-β is considered as the main anti-inflammatory and cell proliferation regulator cytokine at the beginning of malignancy; however, many advanced tumors are resistant to the inhibitory function of TGF-β and TGF-β can activate metastatic pathways (12).

Considering that ALL in adults have a worse prognosis than children and a recurrence of it even after bone marrow transplantation is high (2), this study aimed to evaluate the expression of TGF-β gene in adults with ALL compared to the control and its a relationship with disease-related prognostic factors.

Materials and Methods

Patients and samples

Bone marrow (BM) aspiration specimens were obtained from 90 newly-diagnosed adults with ALL and 33 age-matched healthy adults who donated for BM transplantation at Hematology-Oncology and Stem Cell Transplantation referral center of Shariati hospital, Tehran. The diagnosis was based on cytomorphologic and immunophenotypic features of BM leukemic cells. Patient's disease-related prognostic factors are listed in table 1. Informed consent was obtained from patients or their parents. The study was approved by the ethics committee of Kerman University of Medical Sciences (permit number: IR.KMU.REC.1396.1193).

Table 1. Patient's disease-related prognostic factors

Variables		Values
Gender, number (%)	Male	57 (63.3)
	Female	33 (36.7)
Median age, years (range)		28 (15-74)
Median WBC*, ×10 ⁹ /L (range)		16.4 (0.53-452)
Median BM* blasts, % (range)	T-ALL	27 (30)
	Pro B-ALL	11 (12.2)
	Early pre B-ALL	24 (26.7)
	Pre B-ALL	19 (21.1)
	Immature/Mature B-ALL	9 (10)

*WBC: White Blood Cell; BM: Bone Marrow

MNCs enrichment by density centrifugation

Normal and leukemic mononuclear cells were isolated from BM using Histopaque (Sigma, St. Louis, USA) density-gradient centrifugation, as described (13). The MNCs layer was then removed and washed twice with PBS prior to immunophenotyping and RNA extraction.

Flow cytometric analysis

After separation, the mononuclear cells were stained with a panel of fluorescent-conjugated monoclonal antibodies (DAKO, Glostrup, Denmark) including CD2, CD3, CD5, CD10, CD19, CD20, CD34, CD45, HLA-DR, TdT, CD13, CD14, CD33 and isotype controls in accordance with the manufacturer's instructions as described previously (14). Flomax software (Partec, Nuremberg, Germany) was used for data analysis. The criterion for surface marker positivity was an expression by at least 20% of the leukemia blast cell population.

RNA extraction and cDNA synthesis

Total RNA was separated from mononuclear cells after lysis with TRizol reagent (Invitrogen, Paisley, UK). After evaluating the quantity, integrity, and purity of the isolated RNA, 500 ng of total RNA was converted to cDNA using the PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). For reverse transcription, the reaction mixture was incubated for 15 minutes at 37° C and 5 minutes at 85° C.

Real-time quantitative PCR

Real-time quantitative PCR (RQ-PCR) was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, USA) using TGF-β and ABL1 (reference gene) specific primers (Table2). Each reaction mixture consisted of 1 µg of cDNA template, 0.7 µl (350 pM) each of forward and reverse primers, 10 µl of high ROX SBYR green mix adjusted with nuclease-free water to a final volume of 20 µl. It was carried out at 95° C for 15 min, followed by 40 cycles at 95° C for 15 s, 60° C for 15 s, 72° C for 15s. Specificity of primer pairs was checked both by checking the RQ-PCR amplified products on 2% agarose gel and performing an in silico PCR.

Statistical analysis

Mann-Whitney U test and Kruskal-Wallis was applied for comparison between the two and multiple groups, respectively. Spearman correlation coefficient was applied to study the correlations between different values. The relative amounts of TGF-β transcripts were determined using the 2^{-ΔCt} formula. The SPSS software

v.23 (SPSS, Chicago, IL, USA) and the GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, CA, USA) were used for analysis. *P* less than 0.05 was considered

significant.

Table 2. Primer sequences used for RQ-PCR

Gene	Genebank accession number	Primer sequence (5'-3')	Annealing temp. (°C)	Product size (bp)
<i>TGF-β</i>	NM_000660	F: AAGGACCTCGGCTGGAAGTG R: CCCGGTTATGCTGGTTGTA	60	137
<i>ABL1</i>	NM_005157	F:TGGAGATAACACTCTAAGCATAACTAAAGGT R:GATGTAGTTGCTTGGGACCCA	60	124

Results

Immunophenotyping of leukemic cells

From 90 Iranian ALL patients, 63 (70%) were identified as B-ALL, 27 (30%) as T-ALL. Further classification of B-ALL patients included pro-B (n=11), Early pre-B (n=24), pre-B (n=19) and immature/mature-B (n=9) (Table 1).

TGF-β mRNA expression

The relative expression of TGF-β mRNA in ALL patients, varying from 0.88 to 142 (median 8.5), were significantly downregulated compared with normal controls (median 55, range 16.4-136) (*P*<0.001, Figure 1). TGF-β transcript levels in both T-ALL and B-ALL patients showed a significant decrease compared to the control group (*P*<0.001, Figure 2A). However, the expression of the TGF-β transcripts was not different between the different immunophenotypic subtypes (*P*=0.54, Figure 2B).

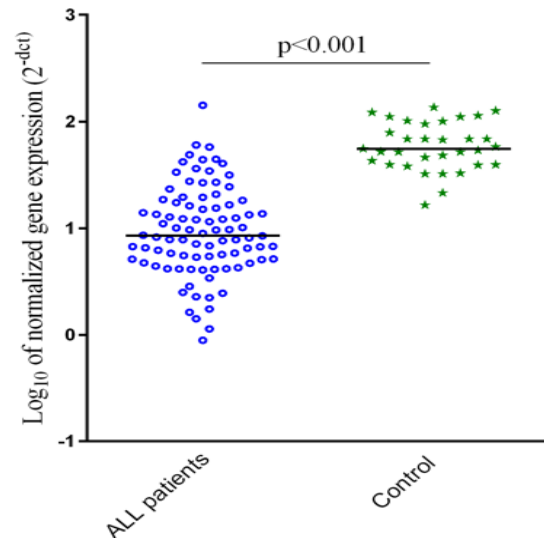


Figure 1. Relative expression levels of TGF-β in ALL patients compared with healthy control. The solid line represents the median of 2-dct of each gene expression

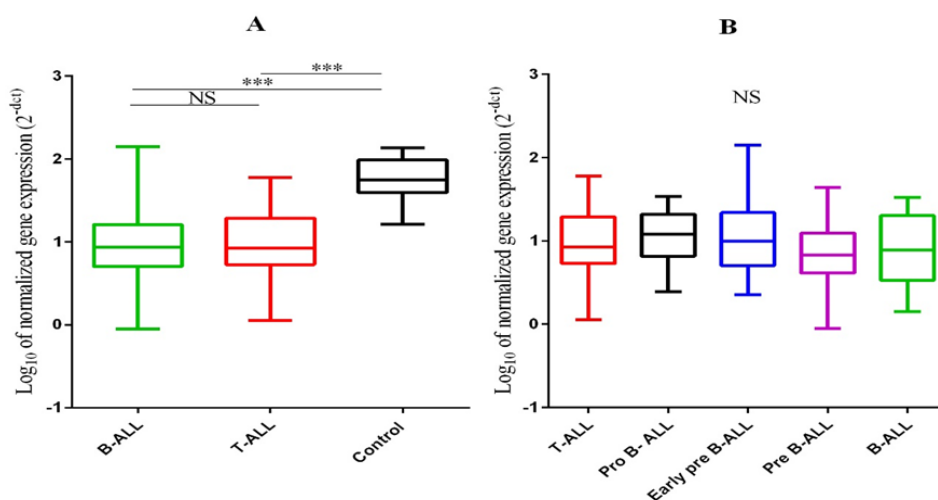


Figure 2. Relative expression levels of TGF-β (A) in B-ALL and T-ALL compared to control (B) in different immunophenotypic subtypes of ALL (NS: NOT Significant; ****P*<0.001)

Correlation of TGF- β expression with some disease-related prognostic factors

The gene expression level of TGF- β was not correlated with age ($P=0.47$), gender ($P=0.29$), ALL subtypes ($P=0.54$), the percentage of BM blasts ($P=0.92$) and peripheral blood leukocyte count ($P=0.38$) of ALL patients as a disease-related prognostic factor.

Discussion

It is obvious that TGF- β is an important pathway involved in different type of cancers. As showed by Wolfrain and his co-workers, in childhood T-cell ALL, Smad3 protein is absent or decreased despite normal levels of Smad3 mRNA and it's been discovered that Smads are downstream targets of TGF- β (15) but our information about adults with B-cell or T-cell ALL is much less, and we don't know exactly the level of TGF- β in these diseases. Lucas and his colleagues stated that TGF- β pathway serves as a primary tumor suppressor in CD8+ T cell tumorigenesis as they demonstrated that inactivation of TGF- β signaling had been seen in HTLV-1–induced adult acute T-cell leukemia (16). TGF- β plays its tumor suppressor role by increasing cell cycle inhibitors and decreasing the factors involved in increased proliferation and thus induces differentiation by inhibiting proliferation and cell cycle progression. Therefore, deactivating or decreasing its expression or disturbance in its signaling pathway reduces the inhibitory and anti-tumor pressure of TGF- β from leukemic cells and allows them to be more freely proliferated and evolve the clone (17). Our results showed that the level of TGF- β is decreased in adults with ALL in both B and T groups. We evaluated expression of TGF- β gene in adults ALL and unlike Douglas and Lagneaux studies that discussed which in chronic lymphocytic leukemia (CLL), cells became resistant to the growth-inhibitory and proapoptotic effects of TGF- β despite expressing high levels of TGF- β (18,19), our analysis showed that TGF- β mRNA levels in both B-ALL and T-ALL group decreased compared to control. The lack of response of CLL cells to TGF- β has been attributed to decreased cell-surface expression of TGF- β receptors, especially T β RI (19) but we didn't examine this matter in our research.

As Rowe discussed in his review about prognostic factors of ALL in adults, age has been recognized as probably the most important prognostic factor in ALL and not just in prognosis between childhood and adult

ALL, but also affects the prognosis among adults. Most clinical studies have used the age of 35 or 40 years as a prognostic cut-off point among young adults less than 60 years of age and patients over 60 years have a particularly poor prognosis. The white blood cell (WBC) count at diagnosis is been considered as another important prognostic factor or at least a surrogate for other prognostic factors. An arbitrary WBC cut-off of $30 \times 10^9/l$ for B-lineage or $100 \times 10^9/l$ for T-lineage has often been used in clinical studies. Gender has been reported to be an independent prognostic factor too, which male patients are having a poorer outcome than female patients (20). There was no correlation in our study between TGF- β mRNA expression and prognostic factors mentioned above. Historically, immunophenotype has been considered as one of the prognostic factors. In B-lineage ALL patients, those with a pro-B ALL have an inferior outcome compared to early pre B-ALL and pre-B ALL (21). Patients with T-ALL had a significantly lower 5-year survival than patient with B-ALL (22). However, in a more recent study, immunophenotype was not found to be an independently significant prognostic factor and cytogenetic and molecular classifications are a more critical prognostic factor than immunophenotype (20). In our research, there was no significant difference in TGF- β expression between B-ALL subtypes or even between B-ALL and T-ALL groups.

In conclusion, since TGF- β has a tumor suppressor role, it seems that leukemic cells may use TGF- β down-regulation to be more freely proliferated and evolve the clone. Therefore the TGF- β signaling pathway should be further explored in adults with ALL to reveal disease biology and potential therapeutic targets.

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