

The evaluation of the TGF- β 1 and T β RII gene expression in patients with acute lymphoblastic leukemia

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Abstract

Background: Tumor suppressors are key molecules involved in the malignant process. TGF- β is one of the most important suppressor genes with a complex role in intracellular processes. Although TGF- β is a traditional tumor suppressor; recent evidence has shown its promoter role in solid tumors. However, it is not determined whether TGF- β has a tumor suppressor or tumor promoter role in hematologic malignancies. In this study we evaluated the expression of TGF- β and its receptor in patients with acute lymphoblastic leukemia, as an important hematologic malignancy.

Material and Method: In this study, the expression of TGF- β and T β RII was analyzed in 52 patients with acute lymphoblastic leukemia in comparison with 13 normal controls; all of them informed volunteers. The mononuclear cell was separated using Ficoll for RNA isolation. After synthesis of c-DNA, the gene expression was measured using cyber green RQ-PCR.

Results: Our results showed that the expression level of TGF- β (3.6 fold) and T β RII (7.7 fold) was significantly decreased in all patient groups in comparison with healthy controls. Reduction in TGF- β was significantly correlated to blast count; TGF- β reduction also had correlation with chromosomal translocation, however, we did not observe any correlation with other parameters such as age, gender and leukemic cell immunophenotype.

Conclusion: Altogether our findings suggest defeated TGF- β signaling in patients with acute lymphoblastic leukemia (ALL), and it seems that the targeting of TGF- β signaling component is one of the basic and essential mechanisms in cancer development. More research in this field can help us to design novel methods for ALL diagnosis, classification, monitoring and treatment.

Key words: acute lymphoblastic leukemia, gene, TGF- β , TGF- β RII

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Introduction

Acute lymphoblastic leukemia (ALL) is a malignant disorder of hematopoietic stem cells which is characterized by accumulation of immature and inefficient lymphoid precursors (lymphoblasts) in bone marrow and other lymphoid organs (1). Generally the risk classification of ALL patients is based on patient's age, primary WBC count, disease's immunophenotype, chromosomal abnormalities and treatment response rate (2). All of the above prognostic markers are highly dependent on substantial molecular defects of the leukemic cells. Routinely, prognostic categorization of patients based on the molecular features assists physicians greatly in choosing more successful treatment strategies (3).

Tumor suppressors and oncogenes are two types of key molecules involved in the malignant process (4,12). Oncogenes have traditionally attracted more attention in the scientific research. However, recent studies clearly proved the essential role of tumor suppressor defects in cancer development (13,18). In this regard, TGF- β signaling has been reported as a prominent tumor suppressor by several studies (19-21). TGF- β regulates the expression of a wide range of genes involved in critical cellular functions including cell cycle, cell differentiation, cell apoptosis, hematopoietic stem cell dormancy, extracellular matrix formation, genetic integrity and cell migration (22,27).

Naturally, TGF- β induces the expression of cell cycle inhibitors (such as P15, P21 and P53) and inhibits cell cycle inducers (such as c-myc and ID family of proteins) thereby it inhibits tumor formation and induces cell

differentiation at the same time (28). Different studies indicated TGF- β signaling abnormalities in tumors. In this way, the aberrant expression of TGF- β has been reported in different kinds of malignancies such as colon cancer, breast cancer and hematological neoplasms (29-32). Other studies demonstrated mutation in other elements of TGF- β signaling pathway including T β RII and SMAD proteins in human malignancies (33,34). In hematologic malignancies it has also been demonstrated that some fusion genes such as AML1-EV11 and TEL-AML1 inhibits TGF- β signaling pathway elements. All these studies proved tumor suppressor effects of TGF- β signaling (35-37).

Although, these studies indicated tumor suppressor role for TGF- β , recent studies unexpectedly showed tumor promoter function for this signaling pathway in some types of cancers which indicates context dependent function of TGF- β in different kinds of malignancies (32). This complexity is greatly dependent on factors such as the type of malignancy, tumor environment, types of genetic abnormalities, stage of malignancy and the rate of tumor progression (38-40). The tumor promoter function of TGF- β has been mainly explained by alternative signaling. In normal tissues, classic TGF- β signaling occurs through SMAD pathway of proteins. TGF- β signaling by SMAD activates the transcription of several target genes with tumor suppressor activity (41). However, during alternative TGF- β signaling in tumor promoter context, TGF- β signaling doesn't occur through SMADs pathway any more. In this condition, alternative signaling by MAP kinases, PI3K/AKT, GTPase Rho-like leads to malignant cells proliferation and survival (42,43).

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TGF- β has an essential role in the functional regulation of blood cells such as monocytes, macrophages, neutrophils, platelets and even hematopoietic stem cells; moreover, these cells are also the essential sources of TGF- β secretion (44). However, most studies on the role of TGF- β signaling have been performed on solid tumors and the role of TGF- β signaling in hematologic malignancies has been less considered. This study attempts to evaluate the expression of TGF- β and its receptor (T β RII) in acute lymphoblastic leukemia patients to better clarify the possible role of this cytokine in malignant lymphoblasts.

Methods

Patient samples

The present study was performed on the bone marrow (BM) and peripheral blood (PB) samples obtained from newly diagnosed ALL patients and 13 normal control subjects, with informed consent. Consent letter was approved by the local Ethics Committee. The patients were referred to Mofid, Taleghani and Emam Khomeini Hospital, Tehran, Iran with diagnoses based on clinical features and laboratory tests including: morphological assessment, immunophenotyping (according to the FAB classification system) and molecular studies. The median age of individuals in this study was 18 years, with a range of 1-89 years and mean age of 26 years. Samples were taken from 22 female and 30 male subjects.

RNA isolation, cDNA synthesis, quantitative realtime PCR

Total cellular RNA was extracted from MNCs using RNeasy minikit (Qiagen, Germany). The quality and quantity of extracted RNA was measured by NanoDrop (Thermo Scientific, Wilmington, North Carolina, USA) (OD 260/280 nm ratio >1.8). Subsequently, 2 μ L (0.5 mg) RNA was used for cDNA synthesis in a final volume of 20 μ L using a Thermo Scientific kit (Qiagen, Hudson, NH, USA). c-DNA synthesis was checked by ABL primer as housekeeping gene. An aliquot of 1/10th of the resulting cDNA from control and patient (1 μ L) was used as substrate for qRT-PCR amplification. Primers specific to TGF- β 1 and T β RII and ABL1 (housekeeping gene) were designed using oligo7 software [Table 1] using data obtained from NCBI databases and designed primers were evaluated for specificity by NCBI primer BLAST. Consequently, the expression of TGF- β 1 and T β RII and ABL1 mRNA was analyzed by qRT-PCR (Rotor Gene 6000, Bosch, Qiagen, Germany). qRT-PCR reaction components for each gene were composed of 1 μ L of template cDNA, 1 μ L primer (forward and reverse), 7 μ L of RealQ Plus 2x Master Mix GreenLow ROX (Ampliqon, Denmark), and 6 μ L water for a total reaction volume of 15 μ L. For each qRT-PCR reaction, standard curve was considered using five consecutive 1:10 dilutions of cDNA sample (1, 0.1, 0.01, and 0.001). The thermal cycler conditions for each reaction (TGF- β 1 and T β RII) consist of initial holding at 95°C for 10 minutes, second phase as denaturation at 95°C for 10 s including 40 cycles, annealing/extension at

65°C for 15 s and final extension at 72°C for 10 minutes. All experiments were performed in duplicate and the relative quantification of mRNA expression for each sample (fold change = FQ) was calculated using the Livak method (2- $\Delta\Delta\text{ct}$). (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008).

Statistical Analysis

Data analysis was performed using the SPSS Statistics (V 16.0) and GraphPad Prism (V 6.07) software. The results were represented in form of Mean+SEM. Shapiro Wilk

and the Kolmogorov-Smirnov tests were used to evaluate the normal distribution of TGF- β 1 and T β R11 expression in ALL patients and control group. The t-test was also used to determine whether there was a significant difference in TGF- β 1 and T β R11 expression between ALL patients and the normal controls. The Pearson's chi-squared test was used to analyze the correlation between TGF- β 1 and T β R11 expression. The ANOVA test was applied to evaluate the differential expression of TGF- β 1 and T β R11 according to FAB classification. ($p = 0.05$ was considered as significance level).

Table 1: The sequence of forward and reverse primers for TGF- β genes, TGF- β R11 and ABL1 gene is shown with the length of each primer with unit of nucleotide.

Gene	Premier	Sequence	Length
TGF- β	Forward	AAGGACCTCGGCTGGAAGTG	bp 20
	Reverse	CCCGGGCCATGCTGGTTGTA	bp 20
TGF- β R11	Forward	GGTTTTAGTTATCTCCAGTCCA	23 bp
	Reverse	GGGGTCCAGGTAGGCAGTG	19 bp
ABL1	Forward	AGTCTCAGGATGCAGGTGCT	20 bp
	Reverse	TAGGCTGGGGCTTTTTGTAA	20 bp

Results

The expression rate of TGF- β and TGF- β R11 in case and control groups:

The results showed that the expression of TGF- β (1.19 ± 0.07) and TBR11 (1.01 ± 0.16) in ALL patients was significantly lower compared to normal controls with P value < 0.0015 (mean TGF- β in patients: 0.63 ± 0.08 and mean TGF- β R11 in patients: 1.01 ± 0.16). TGF- β expression was 3.6 fold and TBR11 expression was 7.7 fold lower than normal controls. There was no significant difference between gene expression levels of TGF- β and TGF- β R11 in men and women (P value = 0.69 and 0.62 respectively). The patients were divided into two age classes of younger than 16 and older than 16 years. The expression rate of genes in the two age groups were evaluated, the results showed that there was no significant difference between the two age groups (P value = 0.58 and 0.24)

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The expression rate of TGF- β and T BR11 in case groups regarding to, translocation and immunophenotype classification:

Regarding existence of chromosomal translocation, patients were divided into two groups (trans positive and trans negative); our results showed no significant changes in TGF- β and T β R11 expression between these groups (P value = 0.34 and 0.28). On the other hand, TGF- β and T β R11 expression level was evaluated according to type of translocation including t(12:21), t(9:22), t(1:19) and t(4:11). The results showed that the expression level of TGF- β was not significantly different among patients with t(9:22), t(1:19) and t(4:11) translocations (P value = 0.92), while the expression rate of T β R11 was significantly higher in patients with t(12:21) (P value = 0.04) in comparison with other translocations. Our patients were also categorized into B lineage and T lineage ALL. There was no significant difference between TGF- β and T β R11 expression in these two groups. Patients were also categorized into 5 groups including T-ALL, Pro B ALL, Early pre B-ALL, Pre B-ALL and B-ALL. The expression level of TGF- β and T β R11 was evaluated and the results showed no significant difference in TGF- β and T β R11 expression level between different leukemic phenotypes (P value = 0.18 and 0.41)

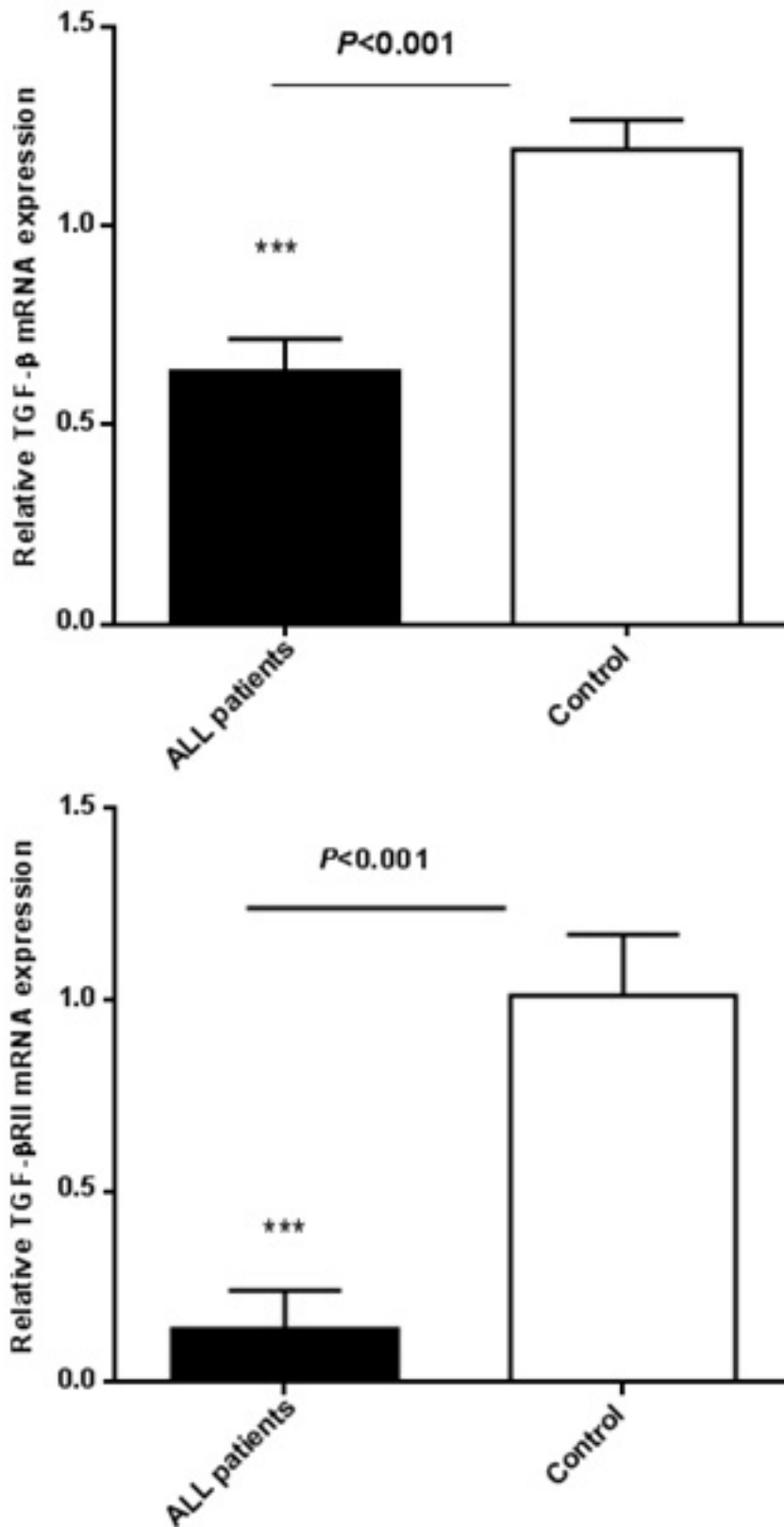
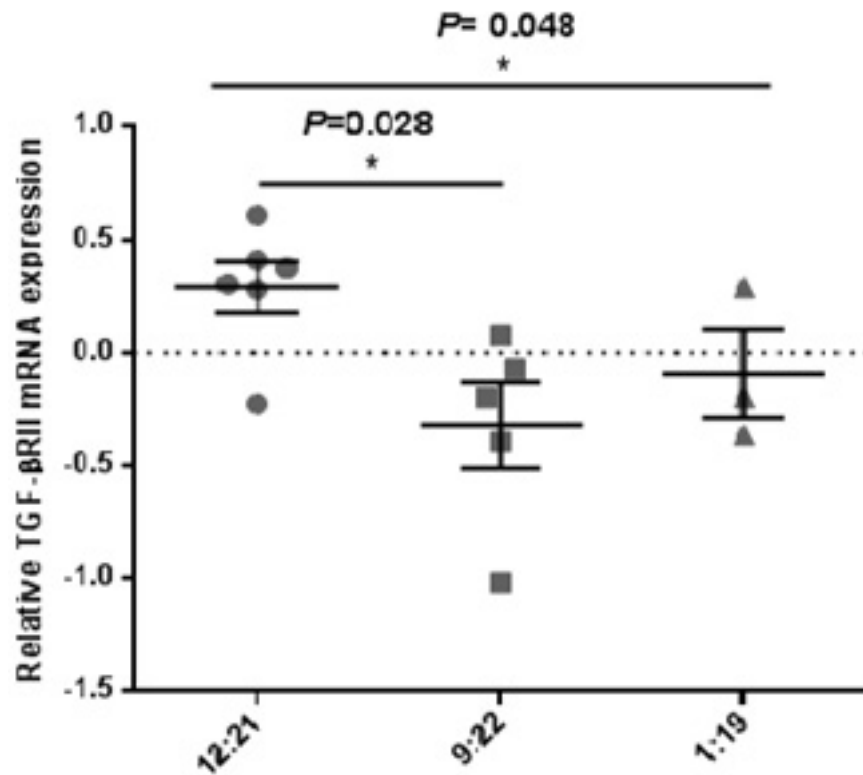
Figure 1: The expression level of TGF- β and TGF- β RII in case and control groups

Figure 2: The expression level of T β RII in case groups regarding, translocation and immunophenotype classification



The expression rate of T β RII was significantly higher in patients with t(12:21) (P value=0.04) in comparison with other translocations. Our patients were also categorized into B lineage and T lineage ALL.

The correlation between the expression rate of TGF- β and T β RII:

Our results showed a positive and significant correlation between TGF- β and T β RII expression (p value= 0.015, $r=0.33$) (Chart 1) in our patients. Also there was a significant correlation between blast count and the expression rate of TGF- β and T β RII (P value=0.043, $r=-0.288$ and p value 0.74, respectively) (Chart 2), however there was no significant correlation between TGF- β and T β RII expression with age of patients (p value=0.15, $r=0.293$ and p value=0.089, $r=0.293$, respectively).

Chart 1: regression between TGF- β with T β RII

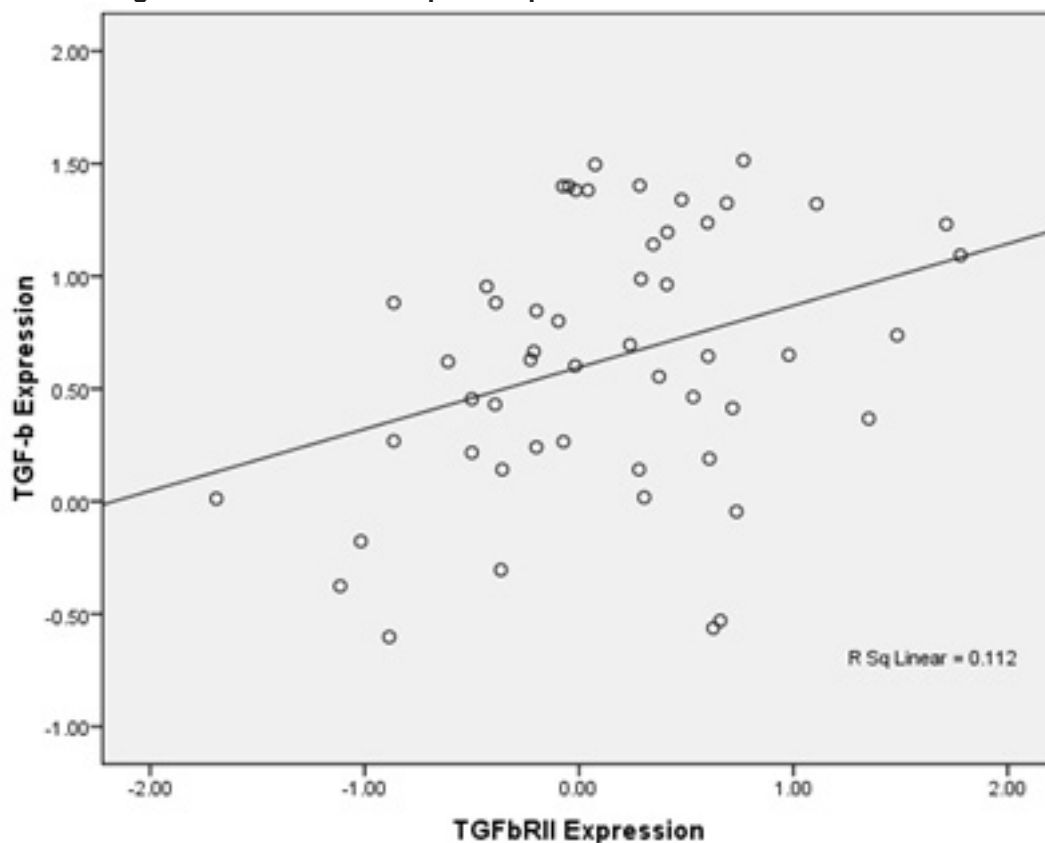
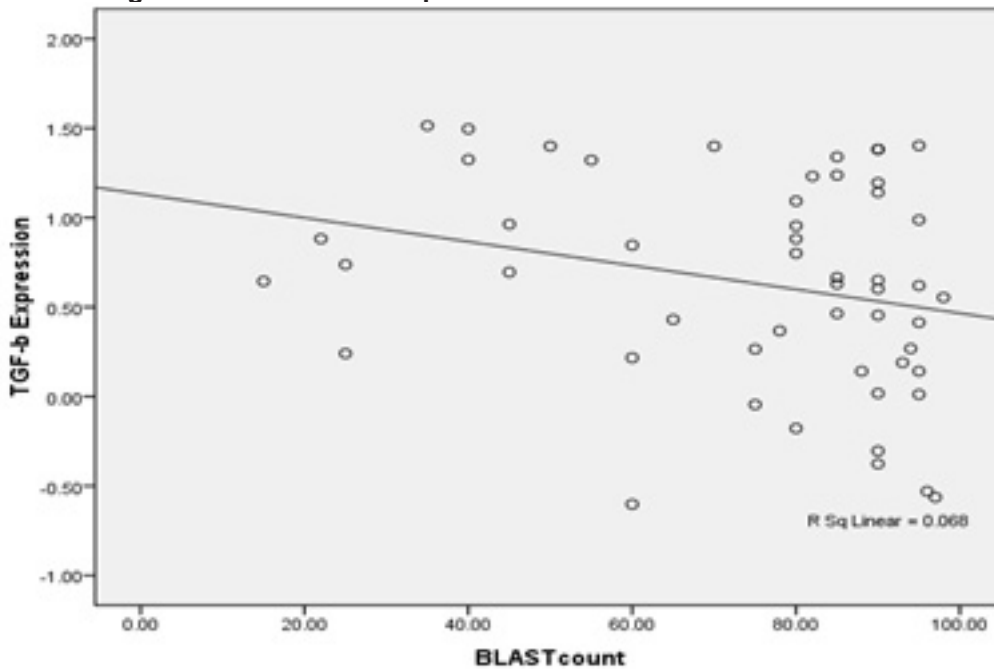


Chart 2: regression between TGF- β with blast count

Discussion and Conclusion

Despite all advances in deciphering basic mechanisms involved in ALL leukemogenesis, still ambiguities exist in relation to tumor formation and development (45). While proliferation inducing factors work unrestrainedly in cancer cells, tumor suppressors generally stop their function. Albeit, tumor suppressor defects have an essential role in tumorigenesis, they have attracted less attention (46). There are several potent tumor suppressors, among them TGF- β is one of the most important (47-49). However recent studies have shown that there is also a tumor promoter function for TGF- β signaling in some neoplasms as well (50), thus we decided to evaluate the expression of TGF- β and T β RII as two key molecules involved in TGF- β signaling, in patients with ALL. Our data demonstrated a significant reduction in the expression of TGF- β (3.6 fold) and T β RII (7.7 fold) in our patients compared with normal controls and most cases represented a simultaneous reduction in TGF- β and T β RII expression. Statistical analysis using Pearson test indicated a positive and significant correlation between TGF- β and T β RII expression. On the other hand, we did not observe any correlation in TGF- β or T β RII expression with clinical data such as age, gender and disease immunophenotype. These data suggest that TGF- β signaling may be a defected pathway in ALL patients and malignant cells inactivate TGF- β signaling to escape from anti-tumor effects of this pathway. Such failures in TGF- β signaling can remove negative pressure of TGF- β signaling on the cell cycle and permits neoplastic cells to enter further and faster into the proliferation phase (46, 48). In agreement with our findings, Renald's showed reduced T β RII expression in patients with T cell sezar syndrome (51). Moreover, Swati Biswas et al also demonstrated that T β RII mutations disabled TGF- β signaling and is an important factor in the initiation and progression of colon cancer (52). Nikolaos Soulitzis et al and Dos Reis et al proved that patients with prostatic cancer have reduced TGF- β expression as well (53,54).

The involvement of other TGF- β signaling elements such as SMADs proteins has been observed in other studies, for example, Lin et al have shown that PML-RAR α fusion gene inhibits the activation of SMAD2/3 and prevents TGF- β signaling to the nucleus. After ATRA therapy PML-RAR α will be degraded and malignant cells respond to TGF- β signaling by their differentiation (55). Jakubowiak et al's study showed that AM1-ETO, another fusion gene in AML, also prevent TGF- β signaling through SMAD3 inhibition (56). A significant reduction in smad3 expression was also reported by Lawrence et al in T ALL patients (57). All these studies along with our observation strongly suggest tumor suppressor role for TGF- β signaling and TGF- β signaling is inactivated as a possible mechanism for tumor escape from regulatory pressure. Although the natural role of TGF- β pathway indicates tumor suppressor activities, recent studies unexpectedly reported its role in tumor promotion as well; in this regard, Yong Wu, et al study represented over-expression of T β RII in acute myeloid leukemia. In these cases, over-expression of aberrant isoforms of TGF- β RII inhibits normal TGF- β signaling in patients with AML (58). Hui-Jun Zhang et al reported epithelial-mesenchymal transition (EMT) which is a feature of advanced stage of cancer, during TGF- β over-expression in lung carcinoma (59). Mele et al in their study showed the role of this cytokine in colon cancer metastasis also (60). Although in normal condition TGF- β stops cell cycle at G1 stage to inhibit cell proliferation and induces cell differentiation simultaneously. In the mentioned cases, TGF- β over-expression was unable to prevent disease initiation but it unexpectedly acts along with tumor promoter factors. However we did not observe such a finding in ALL patients.

In conclusion; according to our evaluation, TGF- β seems to act as a tumor suppressor in ALL patients because it showed lower expression in ALL patients in comparison with normal controls. It is possible that leukemic cells use TGF- β signaling down regulation for ease in their growth

and survival. Actually, TGF- β acts as a double edged sword in malignancies by contradictory signaling through alternative and classic pathways. The reason for the mentioned contradiction probably lies in natural differences in various tumors context and remarkable genetic and epigenetic heterogeneity between different kinds of malignancies. In this study, although it was not unexpected to see a significant difference in TGF- β expression between adults and children, between male and females and finally between different ALL subtypes, there was not any significant differences. It suggests TGF- β signaling deficiency may act as a general mechanism in tumor formation and promotion in ALL patients. Since simultaneous decreased expression level in both TGF- β and T β RII was seen, it is supposed that tumoral cells benefit from deactivation of multiple target genes in this pathway. Finally, due to the critical role of TGF- β pathway in cell regulatory mechanisms, the evaluation of the significance of TGF- β signaling elements in disease risk stratification, choosing therapeutic options and patient monitoring is highly recommended in future work.

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