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Smad3 and *Smad7* Genes Expression in TGF-β Signaling Pathway in Patients with Acute Lymphoblastic Leukemia

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A B S T R A C T

Introduction: Acute lymphoblastic leukemia (ALL) is an aggressive hematologic malignancy characterized by the clonal expansion of immature lymphoid blasts in the bone marrow, affecting both children and adults. The transforming growth factor-β/ Smad signaling pathway, a critical tumor-suppressor pathway, is frequently dysregulated in various cancers. This study aimed to investigate the expression levels of *Smad3* and *Smad7* in ALL patients to assess potential dysregulation.

Materials and Methods: We analyzed bone marrow and peripheral blood samples from 52 newly diagnosed ALL patients (including 20 pediatrics and 32 adults) and 15 healthy controls. Samples were assessed for morphology, immunocytochemistry, immunophenotyping, and molecular markers. Reverse transcription quantitative real-time polymerase chain reaction was used to evaluate *Smad3* and *Smad7* expression levels.

Results: Compared to the control group, ALL leukemia patients exhibited a statistically significant downregulation of *Smad3* and upregulation of *Smad7*. The oncogenic role of *Smad7* was more prominent in specific subtypes, with its expression being significantly higher in pediatric ALL compared to adult ALL, and in B-ALL compared to T-ALL.

Conclusions: Our findings suggest that decreased *Smad3* (a key receptor-regulated Smad) and elevated *Smad7* (an inhibitory Smad) may contribute to Transforming growth factor-β pathway resistance, potentially playing a role in ALL pathogenesis. This study highlights the involvement of Smad dysregulation in patients with ALL, offering insights into molecular mechanisms and potential therapeutic targets. Further research is warranted to validate these findings and explore clinical applications.

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Introduction

Acute lymphoblastic leukemia (ALL) is an aggressive hematologic malignancy characterized by the clonal expansion of immature lymphoid blasts in the bone marrow, affecting both children and adults [1]. ALL represents the most frequent cancer in children, becoming progressively less common with increasing age. Due to optimized, risk-adapted chemotherapy protocols developed over recent decades, approximately 90% of pediatric ALL patients now achieve long-term survival [2, 3]. However, outcomes in adults remain significantly worse, highlighting the need for continued therapeutic advancements [3]. Current treatment relies on conventional chemotherapy and radiotherapy, which carry substantial toxicity risks and may lead to life-threatening complications [2]. Consequently, research into novel therapeutic strategies remains a critical priority.

The etiology of ALL is multifactorial, involving genetic susceptibility, environmental exposures, and stochastic events [4]. Advances in genomic profiling have identified key mutations and structural alterations that drive leukemogenesis, offering potential biomarkers for risk stratification and personalized treatment [4]. Cytogenetic abnormalities, such as hyperdiploidy and the *ETV6/RUNX1* t(12; 21) translocation, serve as prognostic markers, distinguishing favorable from high-risk disease [5].

Transforming growth factor beta (TGF-β) is a pleiotropic cytokine that regulates essential cellular processes, including proliferation, differentiation, and apoptosis [6]. In hematopoiesis, TGF-β signaling maintains immune homeostasis and hematopoietic stem cell quiescence [7]. While TGF-β acts as a tumor suppressor in normal cells by inhibiting proliferation and inducing apoptosis, it paradoxically promotes tumor progression in advanced cancers [8, 9]. This dual role depends on cancer type, stage, and alterations in downstream signaling [10].

The canonical TGF-β pathway involves receptor-mediated activation of Smad proteins, which modulate gene expression [11-13]. Tumor-suppressive effects are primarily mediated through Smad-dependent signaling, whereas Smad dysfunction activates alternative pathways (e.g., NF-κB), contributing to oncogenesis [12]. Key regulators include receptor-activated Smads (R-Smads, e.g., *Smad3*), the co-Smad (*Smad4*), and inhibitory Smads (I-Smads, e.g., *Smad6/7*) [13]. Smad3, a critical transcriptional regulator, functions as a tumor suppressor, with its inactivation linked to malignant transformation [14, 15]. Conversely, *Smad7* overexpression disrupts TGF-β signaling by blocking R-Smad phosphorylation [14].

Despite extensive research on TGF-β in solid tumors, its role in leukemia, particularly ALL remains underexplored [16]. Given the limitations of current therapies and the need for targeted approaches, this study investigates the expression patterns of *Smad3* and *Smad7* in ALL patients to elucidate their potential contributions to leukemogenesis and therapeutic resistance.

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Materials and Methods

Study Participants

Peripheral blood and bone marrow samples were collected from 52 newly diagnosed ALL patients and 15 healthy volunteers after obtaining informed consent. Mononuclear cells were isolated from all samples using Ficoll-Paque density gradient centrifugation, snap-frozen, and stored at –80°C until further analysis.For B-ALL patients, leukemic bone marrow blasts constituted >80% of total cellularity, particularly after Ficoll separation. To minimize additional manipulation and maintain sample integrity, ALL samples were used directly after Ficoll purification without further processing. All samples underwent morphological examination and immunophenotyping for precise classification according to the World Health Organization classification of hematological malignancies. Informed consent was obtained from all participants or guardians, permitting the use of bone marrow and peripheral blood samples for diagnostic and research purposes. Data were anonymized to ensure confidentiality, and residual samples were used after clinical testing.

RNA Extraction

Total RNA was extracted from bone marrow mononuclear cells using TRIzol reagent (Invitrogen, Inc.) following the manufacturer's protocol.

Sample preparation: Bone marrow samples were immediately homogenized in 2 mL TRIzol and stored at -80 °C until RNA extraction.

Debris removal: A 1 mL aliquot of the buffy coat was centrifuged at 12,000 × g for 15 min at 4 °C to pellet debris and genomic DNA.

Phase separation: The supernatant was mixed with 200 µL chloroform, vortexed for 15 sec, incubated at room temperature for 2-3 min, and centrifuged at 12,000 × g for 10 min at 4 °C.

RNA Precipitation: The aqueous phase (500 µL) was combined with an equal volume of isopropanol, incubated at -20°C for 10 min, and centrifuged at 14,000 × g for 10 min at 4 °C.

RNA wash and resuspension: The RNA pellet was washed with 75% ethanol, centrifuged again at 14,000 × g for 10 min at 4 °C, air-dried, and dissolved in 40 µL DEPC-treated water.

Quality control: RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer, and integrity was verified via agarose gel electrophoresis.

cDNA synthesis

RNA was treated with amplification grade DNase I to eliminate any residual genomic DNA from the sample. cDNA synthesis was performed on 1 ug of RNA in a 20 µl sample volume using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit as recommended by the manufacturer. The RNA was incubated with 1 µL of oligo (dT) 12–18mers primers and water, nuclease-free to 12 µL for 5 min at 65 °C and then transferred onto ice. Then 8 µl of a master mix containing 4 µl of 5X reaction buffer, 2 µl of 10 mM dNTP Mix, 1 µl RiboLock RNase Inhibitor (20 U/µL) and 1 µL of RevertAid M-MuLV RT (200 U/µL). cDNA product could be directly used in PCR applications or stored at -

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20 °C for less than one week. For longer storage, -70 °C is recommended. The sets of primers were designed using the SeqBuilder program. Primer selection parameters were set to primer size: 20-26 nts; primer melting temperature: 60 to 64 °C; GC clamp: 1; and product size range: generally, 120-240 bp but down to 100 bp if no appropriate primers could be identified. (Genes and sequence of the primers was presented in table 1).

Reverse transcription quantitative real-time PCR (RT-qPCR)

RT-qPCR analysis after reverse transcription was performed. The expression levels for 2 genes, *smad3*, *smad7* were measured with a Rotor-Gene® Q — Pure Detection machine using the Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific) according to the manufacturer's instructions. To determine the expression level of genes in ALL patient, all samples were tested in duplicate, and the average values were used for quantification. Specificity of the amplifications was confirmed by melting curve analysis and comparisons to standard templates. Gene-specific primers and PCR conditions are summarized in Table 1. For each gene of interest, external standard curves were constructed using serial dilutions of linearized templates, prepared by amplification from cDNA templates, subcloning into a TA-cloning vector, and restriction digestion. Normalization of target transcript levels was performed using the housekeeping gene *ABL* as an endogenous control.

RT-qPCR analysis of gene expression

The RT-qPCR data were analyzed using a comparative relative quantification method. In this method, the expression of a gene in a target sample was compared with its expression in a reference sample named calibrator. The fold change in gene expression was calculated using the comparative Ct (2−ΔΔCT) method. The ΔΔCT value was determined using the following formula: ΔΔCT= (CT, target − CT, housekeeping) test − (CT, target − CT. housekeeping) control.

In this study, the fold change in gene expression was normalized to the CT value of ABL used as endogenous control and was calculated in relation to the CT value of normal mononuclear cells used as calibrator. We designed and validated two specific primer pairs for the RT-qPCR analysis of *Smad3* and *Smad7*, key genes in the TGF-β pathway (Table 1). Primer specificity was confirmed by melting curve analysis.

Table 1. Sequences of primers for RT-qPCR for *smad3* and *smad7*

Genes	Primer sequences	Length of amplicon size bp/
Smad3	F: 5' AAGCTTTTCCAGGTTTTGTTG 3'	104 bp
	R: 5' CCTATGTTGGCCTGAGATGTTT 3'	
Smad7	F: 5' GGCTTTCAGATTCCCAACTTC 3'	94bp
	R: 5' AGCCTCCCCACTCTCGTCT 3'	

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Statistical analysis

Statistical analyses were performed using SPSS 26.0 (IBM Corp., USA) and GraphPad Prism 8.0 (GraphPad Software, USA), with data normality assessed via Q-Q plots and the Kolmogorov-Smirnov test. Due to the violation of the normality assumption, all continuous variables were analyzed with non-parametric methods. Differences in Smad gene expression between two independent groups were assessed with the Mann-Whitney U test. Relationships between continuous variables, such as *Smad3* and *Smad7* expression levels, were analyzed using Spearman's rank correlation coefficient.

Results

Patients' characteristics

The study comprised 52 patients (mean age: 26.4 ± 22.7 years; range: 1–89 years). The majority were adults (>14 years, n=32, 61.5%), with 20 pediatric patients (38.5%). Males accounted for 57.7% (n=30) of the study. Immunophenotyping analysis revealed that the vast majority of cases were of B-cell lineage (82.7%, n=43), with Pre-B cell and mature Pre-B cell being the most common subtypes (each n=14, 26.9% of total). T-cell lineage was identified in 9 patients (17.3%). Cytogenetic analysis was available for all patients, with the most frequent translocation being t(12; 21) (*ETV6-RUNX1*) (n=6, 11.5%), followed by t(9; 22) (*BCR-ABL1*) (n=5, 9.6%). A majority of patients (n=37, 71.2%) did not harbor any of the tested translocations. The baseline demographic and clinical characteristics of the 52 study participants are summarized in Table 2.

Table 2. Baseline demographic and clinical characteristics of patients with acute lymphoblastic leukemia at diagnosis (N = 52)

Characteristic	Category	N (%)
Age (years)	Pediatric (≤ 14)	20 (38.5)
	Adult (> 14)	32 (61.5)
Gender	Male	30 (57.7)
	Female	22 (42.3)
Immunophenotype	B cell lineage	43 (82.7)
	Pro-B cell	4 (7.7)
	Early Pre-B cell	11 (21.2)
	Pre B cell	14 (26.9)
	Mature Pre B cell	14 (26.9)
	T cell lineage	9 (17.3)
Cytogenetic translocation	t(12; 21) (ETV6-RUNX1)	6 (11.5)
	t(9; 22) (BCR-ABL1)	5 (9.6)
	t(1, 19)	3 (5.8)
	t(4, 11)	1 (1.9)
	No translocation	37 (71.2)

Note: Acute lymphoblastic leukemia was classified according to the World Health Organization classification.

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Expression analysis of *smad3* and *smad7* in ALL leukemia samples and normal controls

We investigated the involvement of the TGF-β signaling pathway in ALL by quantifying the expression of *Smad3* and *Smad7*, two key genes within the Smad family. Gene expression profiling was performed on 52 ALL leukemia samples and 15 healthy controls using RT-qPCR arrays. Hierarchical clustering analysis revealed distinct expression patterns that clearly separated ALL samples from controls (Fig. 1). Notably, *smad7* was significantly upregulated, whereas *smad3* was markedly downregulated in the ALL cohort compared to controls (Fig. 1), indicating a dysregulation of Smad signaling components in ALL leukemia patients that may contribute to leukemogenesis. Interestingly our results showed the *Smad7/smad3* expression ratio was 37.6-fold greater in ALL sample to the normal volunteers.

Downregulated expression of *Smad3* in ALL leukemia samples

To investigate the potential role of *Smad3* as a tumor suppressor in ALL, we quantified its mRNA expression levels in diagnostic samples collected from 52 patients with ALL between July 2013 and June 2015. Using RT-qPCR, we observed a markedly reduced expression of *Smad3* mRNA in ALL patients compared to healthy controls. Statistical analysis using Mann-Whitney U test demonstrated a highly significant difference in *Smad3* expression between the two groups ($p = 0.00$), confirming a consistent and substantial downregulation across all patient samples. The observed decrease in *Smad3* expression was approximately **7**.1-fold, suggesting a critical role in the disease pathology (Fig. 1).

Upregulated expression of *Smad7* in ALL leukemia samples

To assess the expression levels of *Smad7* in ALL leukemia samples, we performed RT-qPCR analysis on samples from 52 ALL leukemia patients. The results revealed a statistically significant upregulation of *Smad7* mRNA expression in ALL cells compared to healthy controls ($p = 0.03$). Notably, a majority of patient samples exhibited markedly high levels of *Smad7*, while controls showed consistently low expression. This aberrant expression profile indicates that *Smad7*, a known inhibitory Smad (I-Smad), may contribute to the impaired TGF-β signaling observed in ALL pathogenesis. Quantitative fold-change analysis further demonstrated an approximately 5.3-fold increase in *Smad7* expression in ALL samples relative to controls ($p= 0.003$), supporting a robust and consistent upregulation across all patients analyzed.

Comparative analysis of TGF-β pathway disruption in pediatric and adult ALL patients

We next sought to determine if the degree of TGF-β pathway disruption differed between pediatric and adult ALL. we performed gene expression analysis on pediatric and adult ALL patient study's, alongside normal control samples, using focused RT-qPCR arrays. The study comprised 15 normal controls, 20 pediatric ALL, and 32 adult ALL samples (demographics detailed in Table 2).

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A comparative analysis demonstrated that the dysregulation was more pronounced in the pediatric cohort. The overexpression of *Smad7* was significantly higher in pediatric patients compared to adults (p = 0.02) (Fig. 3). A concomitant trend towards greater downregulation of *Smad3* was also observed in pediatric ALL; however, this difference did not reach statistical significance (p = 1.0) when compared to the adult cohort. Collectively, these findings point to a more profound disruption of the TGF-β pathway in pediatric ALL, characterized by a significant overrepresentation of the inhibitory *Smad7* and a reduction in the effector *Smad3*. This pronounced dysregulation suggests a potential mechanistic link to the distinct clinical behavior and prognosis associated with pediatric ALL.

Differential *Smad3* and *Smad7* gene expression in T-ALL and B-ALL subtypes

Our cohort comprised primary ALL samples stratified by immunophenotype, with B-ALL representing the majority (approximately 80%) of cases, and T-ALL constituting a smaller subset (approximately 15%). Within the B-ALL group, the early pre–B-cell subtype was predominant, accounting for approximately two-thirds of the pediatric cases. Comparative analysis revealed a stark contrast in the expression of TGF-β pathway genes between these immunophenotypes. The expression of *Smad7* was significantly elevated in B-ALL compared to T-ALL (p = 0.001). In contrast, *Smad3* expression levels did not differ significantly between the two groups (Fig. 3).

Analysis of the relationship between *Smad3* and *Smad7* expression

To determine if the dysregulation of *Smad3* and *Smad7* was interrelated, a Spearman correlation analysis was performed on the combined cohort of ALL samples (N=52). The analysis revealed a weak, non-significant positive correlation between *Smad3* and *Smad7* expression levels (p = 0.193, ρ = 0.170). This finding indicates that the downregulation of *Smad3* and the upregulation of *Smad7* occur independently within the TGF-β pathway in ALL (Fig. 4).

The absence of a significant inverse relationship is particularly notable, as it deviates from the expected feedback inhibition model. This finding strongly suggests that the dysregulation of these two critical nodes—the downregulation of the tumor-suppressive effector *Smad3* and the upregulation of the inhibitory *Smad7*—are independent events in leukemogenesis. This decoupling implies the involvement of distinct oncogenic drivers, such as separate transcriptional regulators or genomic alterations, that independently target each gene, thereby leading to a compounded inactivation of the TGF-β pathway's tumor-suppressive arm.

Discussion

Our investigation reveals a profound and coordinated disruption of the TGF-β signaling pathway in ALL, characterized by the concurrent downregulation of the effector *Smad3* and upregulation of the inhibitory *Smad7*.

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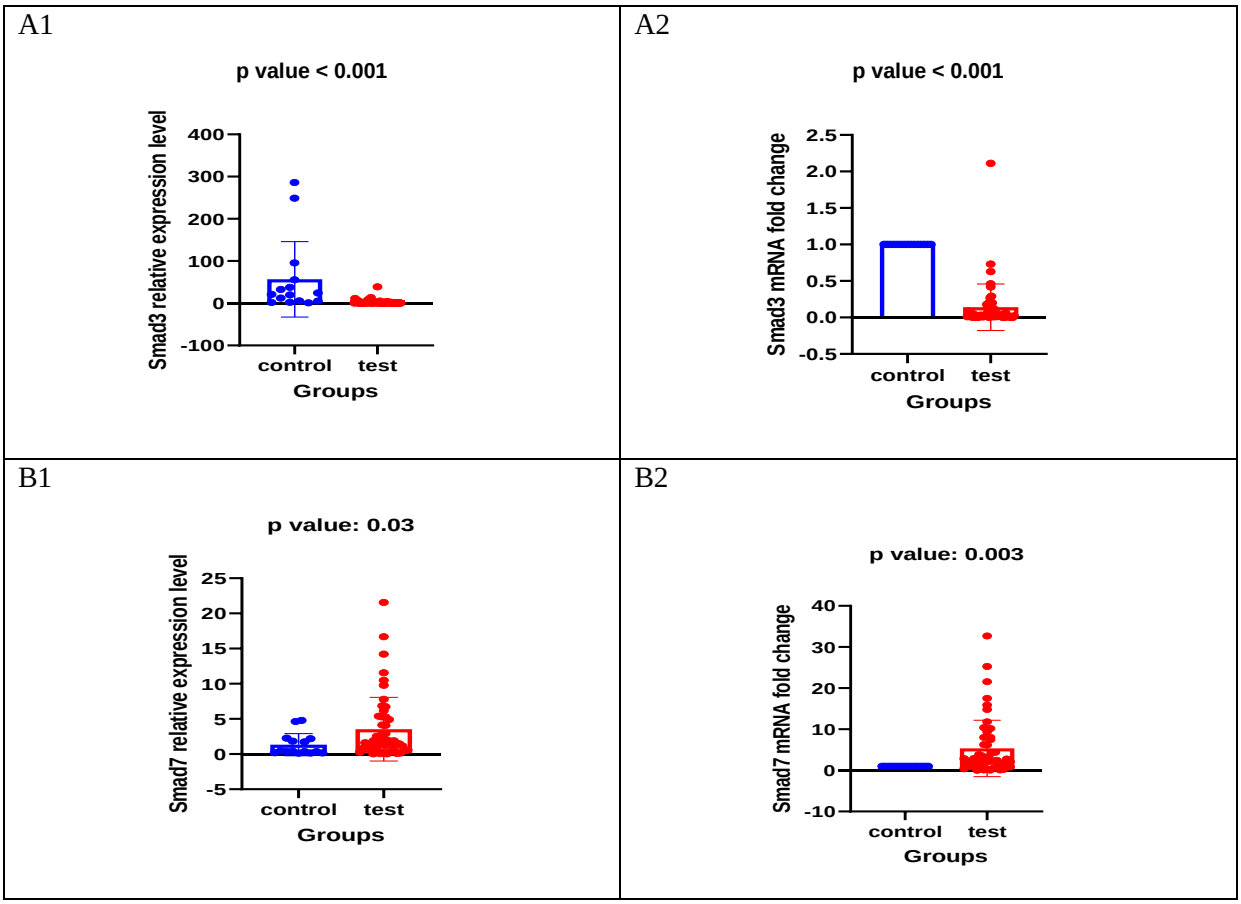


Fig. 1. Reverse transcription quantitative real-time polymerase chain reaction analysis of *smad3* and *smad7* mRNA expression in the test and control groups. Statistical significance was determined using the Mann-Whitney U test. A1: Statistical comparison of *Smad3* relative expression levels between control and test groups ($p < 0.001$). A2: *Smad3* mRNA fold change between control and test groups ($p < 0.001$). B1: Statistical comparison of *Smad7* relative expression levels between control and test groups ($p = 0.03$). B2: *Smad7* mRNA fold change between control and test groups ($p = 0.003$).

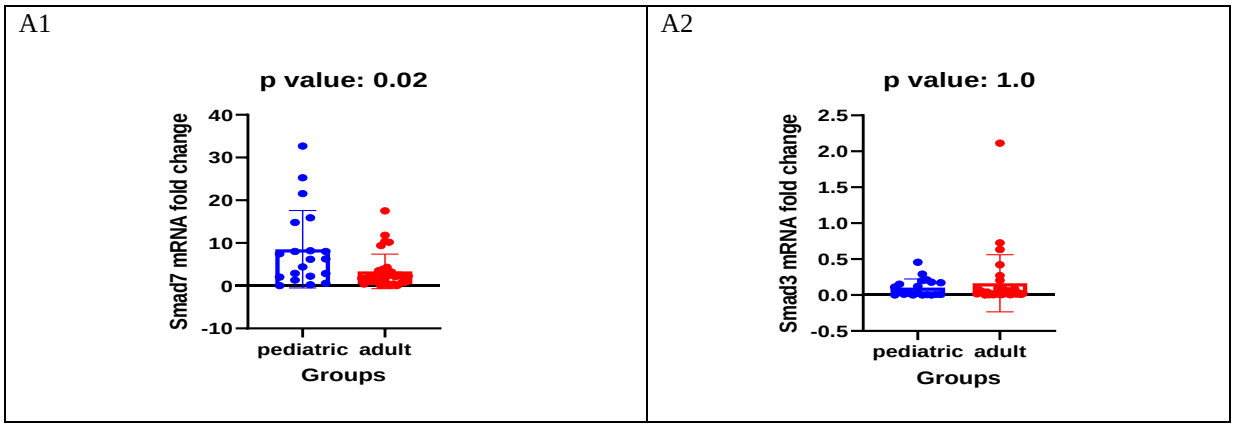


Fig. 2. Reverse transcription quantitative real-time polymerase chain reaction analysis of *smad3* and *smad7* mRNA expression in the pediatric and adult groups. A1: *Smad7* mRNA fold change between pediatric and adult groups ($p = 0.02$). A2: *Smad3* mRNA fold change between pediatric and adult groups ($p = 1.0$).

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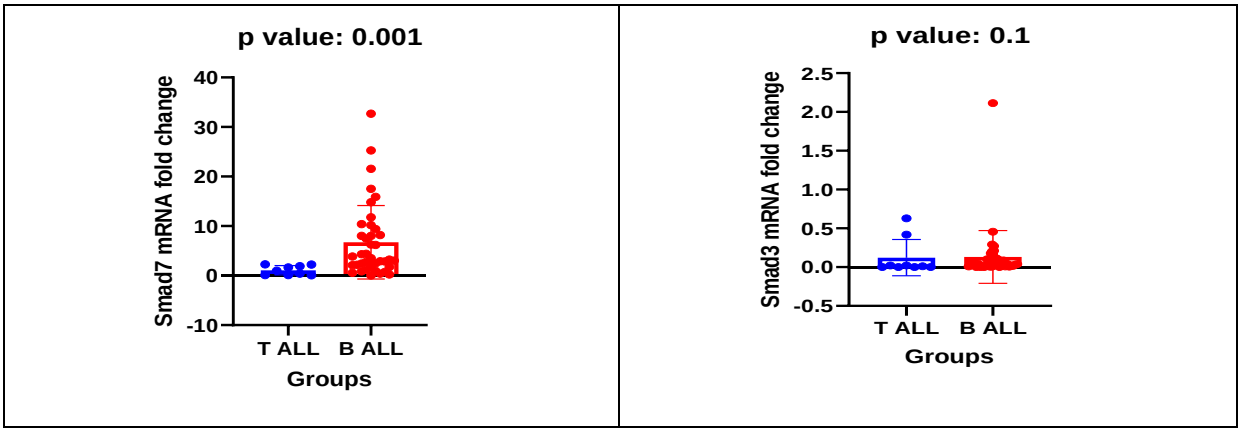


Fig. 3. Reverse transcription quantitative real-time polymerase chain reaction analysis of *smad3* and *smad7* mRNA expression in the T ALL and B ALL groups. (A) *Smad7* mRNA fold change between T ALL and B ALL groups (p = 0.01). (2A) *Smad3* mRNA fold change between T ALL and B ALL groups (p = 0.1).
ALL= Acute lymphoblastic leukemia

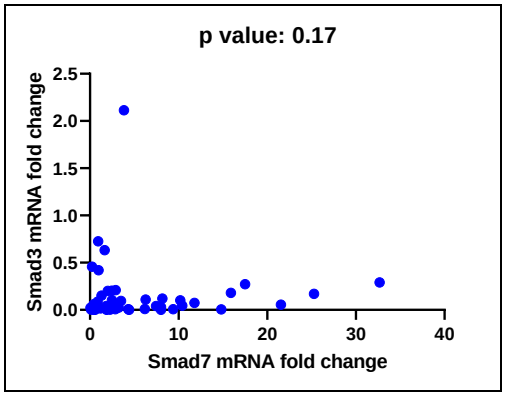


Fig. 4. Analysis of the correlation between *Smad3* and *Smad7* gene expression in acute lymphoblastic leukemia samples

This aberrant expression profile effectively creates a "double hit" against the pathway's tumor-suppressive function. The downregulation of *Smad3* was pronounced, showing an approximately 7.1-fold decrease in ALL patients compared to healthy controls, while *Smad7* was significantly overexpressed by approximately 5.3-fold. The collective impact of these changes is starkly illustrated by a 37.6-fold increase in the *Smad7/Smad3* expression ratio in leukemic cells, suggesting a severe imbalance favoring pathway inhibition. Furthermore, we identified that this pathway disruption is not uniform across ALL subtypes. The dysregulation was significantly more pronounced in pediatric ALL, with *Smad7* overexpression being notably higher than in adult cases. Additionally, a striking immunophenotypic specificity was observed, with *Smad7* expression being significantly elevated in B-ALL compared to T-ALL. Importantly, correlation analysis revealed no significant relationship between *Smad3* and *Smad7* expression levels, deviating from the canonical TGF-β feedback loop and indicating that their dysregulation occurs through independent molecular mechanisms.

TGF-β signaling pathway plays a complex, dual role in cancer, acting as both a tumor

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suppressor and a promoter of metastasis in a context-dependent manner [17]. In pediatric ALL, the precise role and regulation of this pathway remain incompletely characterized [18]. Our study provides novel insights into its dysregulation, revealing a consistent and pronounced imbalance between two key pathway mediators: significant downregulation of the effector *Smad3* and concurrent upregulation of the inhibitory *Smad7*.

Our data demonstrate a profound disruption of the TGF-β signaling axis in ALL. The marked downregulation of Smad3 (approximately 7.1-fold) and upregulation of Smad7 (approximately 5.3-fold) collectively represent a potent mechanism to abrogate the tumor-suppressive arm of the pathway. The dramatic 37.6-fold increase in the *Smad7/Smad3* expression ratio underscores a severe imbalance that likely cripples canonical TGF-β signaling. This "double-hit" model—simultaneously losing a key signal transducer and gaining a potent inhibitor—provides a robust explanation for the escape from TGF-β-mediated growth inhibition in leukemic blasts. A critical finding was the absence of a significant correlation between *Smad3* and *Smad7* expression levels. This deviation from the canonical negative feedback loop suggests their dysregulation arises from independent upstream mechanisms.

Our finding of consistent *Smad3* downregulation aligns with and expands upon its established role as a tumor suppressor in hematopoiesis. Wolfraim et al. first identified *Smad3* deficiency as a hallmark of pediatric T-ALL, where haploinsufficiency accelerated leukemogenesis in murine models [19], while their work focused on T-ALL, our study extends this paradigm to a broader ALL context, suggesting *Smad3* downregulation is a unifying feature across immunophenotypes. This is further supported by findings in myeloid malignancies; for instance, Mahdloo et al. reported specific *Smad3* downregulation in CML [20]. The contrast with studies in AML, where high *Smad3* can be associated with poor prognosis [22], and CLL, where levels are stable [23], highlights the critical, lineage-specific functions of *Smad3*, acting primarily as a tumor suppressor in lymphoid leukemogenesis. Similarly, Shehata et al. identified *Smad7* and BAMBI (a TGF-β pseudo-receptor) as co-overexpressed in AML, correlating with inferior survival, further supporting the concept that TGF-β suppression is a common leukemogenic mechanism across subtypes [24].

The significant upregulation of *Smad7* in our cohort positions it as a key oncogenic driver in ALL by disrupting TGF-β-mediated growth control. This aligns with mechanistic studies across other leukemias. For example, in adult T-cell leukemia, Nakahata et al. demonstrated that *Smad7* overexpression contributes to the epigenetic silencing of TGF-β target genes [21]. Furthermore, our subgroup analysis revealed that *Smad7* overexpression is significantly more pronounced in pediatric ALL compared to adult cases and is markedly higher in B-ALL than in T-ALL. This subtype-specificity underscores its prominent role in the biology of the most common forms of ALL and may partly explain the distinct clinical behavior of these subtypes. The mechanistic link in pediatric ALL is strengthened by the work of Nabhan et al., who

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showed that downregulation of miR-181a leads to *Smad7* overexpression [25].

Our finding of more pronounced dysregulation in pediatric and B-ALL patients provides a compelling mechanistic link to their distinct disease biologies. Our study establishes the concurrent downregulation of *Smad3* and upregulation of *Smad7* as a central event in ALL pathogenesis, effectively creating a "double-hit" inactivation of the TGF-β tumor-suppressor pathway. The independence of these events and their subtype-specific intensity offer new layers of understanding for ALL biology.

This study is limited by its retrospective design and focus on mRNA expression without protein-level validation. Future work should integrate proteomic analyses, chromatin accessibility assays, and functional studies to delineate whether *Smad3/Smad7* alterations are drivers or bystanders of leukemogenesis. Additionally, investigating cross-talk between TGF-β and other pathways (e.g., PI3K, JAK/STAT) could uncover synergistic vulnerabilities. Also, Future research should focus on elucidating the precise upstream mechanisms responsible for this dysregulation and exploring therapeutic strategies, such as *Smad7* inhibition or epigenetic modulators, to reactivate this critical tumor-suppressive pathway.

Conclusion

This research concludes that a specific cellular pathway (TGF-β), which normally acts as a brake on cancer, is shut down in ALL through two simultaneous hits: the loss of a key signaler (*Smad3*) and the gain of a powerful inhibitor (*Smad7*). Critically, these two changes happen independently, suggesting multiple ways the cancer attacks this pathway. This disruption is especially strong in childhood and a common form of ALL (B-ALL). The study establishes this as a fundamental cause of the disease and recommends future work to develop drugs that can reverse this process.

Ethical Considerations

The Ethics Committee of Shahid Beheshti University of Medical Sciences approved the study.

Funding Statement

The authors have no funding to disclose.

Conflict of Interest

The authors declare no conflict of interest.

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We thank the members of department of hematology and blood banking of Shahid Beheshti University of Medical Sciences. The authors drafted this manuscript using ChatGPT-4 developed by OpenAI, 2025 for text refinement, then rigorously reviewed and edited all content. AI served only as a drafting aid—not for research, analysis, or creative input. The authors retain full responsibility for the work's accuracy and integrity.

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Authors' Contributions

M.A, M.H: Conceptualization, S.E, H.A, M.A: Methodology, M.H, S.E, M.A: Investigation, S.E, H.A: Resources and data curation, S.E, H.A, M.A: writing—original draft preparation, S.E, M.H, M.A: Writing—review and editing, M.A, S.E, H.A: Supervision. All authors have provided their consent for the publication of this manuscript.

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