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Prognostic evidence of *LEF1* isoforms in childhood acute lymphoblastic leukemia

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Abstract

Introduction: The lymphoid enhancer factor 1 (LEF1) is a DNA-binding transcription factor that functions in the Wnt signaling pathway. Increased LEF1 activity is associated with progression of several types of cancer including leukemia. Here, we investigated *LEF1* isoform expression and genomic variations in acute lymphoblastic leukemia (ALL).

Methods: *LEF1* isoform expression was evaluated by quantitative real-time PCR in 87 newly diagnosed childhood ALL patients and controls. Moreover, Western blot analysis was performed for detection of LEF1 expression and the hotspot region of *LEF1* was screened by deep sequencing.

Results: The *LEF1* mRNA expression of B cell ALL patients was higher than the controls (*LEF1*-total $P = .011$, *LEF1*-long $P = .026$). Moreover, B-ALL samples showing higher total *LEF1* expression had significantly shorter relapse-free survival ($P = .008$) and overall survival ($P = .011$). Although full-length *LEF1* expression was similar to the controls in T-ALL, 50% (n = 15) of the ALL patients had increased full-length LEF1 protein expression. Imbalance between short- and full-length LEF1 isoforms may lead

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to cell survival in ALL. Beside the LEF1 activation, *LEF1* gene variations were rarely observed in our cohort.

Conclusion: The results indicate that the Wnt pathway may have a pathogenic function in a group of ALL patients and high *LEF1*-total expression might be a marker for shorter relapse-free survival time in B cell ALL.

KEYWORDS

ALL, biomarker, gene expression, LEF1, prognosis

1 | INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a progressive disorder of B cell or T cell progenitors. Development of risk-adapted therapy approaches, effectiveness of molecular-targeted agents, and large clinical trials have led to an increase in long-term survival rates of 90% in childhood ALL. Among these remarkable achievements, relapse is still observed in 15% of patients and treatment response after relapse remains poor. A proportion of leukemia stem cells harbor relapse-inducing potential in ALL.[1,2]

Wnt signaling pathway is an evolutionarily conserved pathway and plays an important role in the maintenance, activation, and proliferation of hematopoietic stem cells.[3] On the other hand, aberrant activation of Wnt signaling is involved in malignant hematopoiesis and promotes leukemia development and recurrence.[4-10] The presence of Wnt activation causes β-catenin to migrate into the nucleus and forms a complex with LEF/T cell factor DNA-binding proteins. This formation induces the transcription of many target genes.[9]

Lymphoid enhancer factor 1 (LEF1) plays a role in the normal function of stem cell, and aberrant expression of *LEF1* was observed in different cancers including hematological malignancies.[11,12] LEF1 binds to DNA in a sequence-specific manner, and although it cannot start the transcription by itself, it is an important member of a multiprotein enhancer complex. *LEF1* gene encodes several isoforms with distinct functions; the full-length isoform encodes the 54 kDa nuclear protein, where domains for β-catenin binding and transcription activation (CAD) are located. This isoform also promotes cell proliferation and inhibits differentiation via Wnt signaling. On the other hand, truncated isoform of *LEF1* lacks a β-catenin–binding domain and may function as an inhibitory isoform by interacting with cofactors that also bind to a full-length isoform.[13-15]

Normally, the full-length and short-length isoforms of *LEF1* are transcribed in sync and it is reported that this balance is lost in colon cancer and acute myeloid leukemia (AML).[14-17] Furthermore, recurrent mutations of *LEF1* are described in ALL and increased *LEF1* expression is reported as a poor prognostic factor, especially in adulthood ALL.[18-21]

Here, we aimed to evaluate *LEF1* gene variations, its isoform expressions, and their clinical correlations in a group of diagnostic and relapsed childhood ALL patients.

2 | MATERIAL AND METHODS

2.1 | Case-control selection

We studied 138 childhood diagnosed ALL patients (87 B-ALL and 51 T-ALL; 49 females, 89 males; median age: 6 years, min: 5 months-max: 17 years) and 14 matched relapse samples. All patients were evaluated according to the FAB (French-American-British) classification criteria. Bone marrow (BM) samples, with a minimum tumor load of 85% at diagnosis and >50% at relapse, were enrolled in this study. The patients were treated with the Turkish-BFM (Berlin-Frankfurt-Munster)-ALL or COG protocols between 1998 and 2011. After the first BM relapse, patients uniformly received treatment according to the ALL-REZ BFM 2002 relapse protocol.

The sorted thymocyte subsets (double-positive (DP) DP $CD3^+$ and $DP3^-$, single-positive (SP) SP $CD4^+$, SP $CD8^+$, n = 10) were used as controls for T-ALL,[22] healthy BM sample subsets (pro–B cells, pre–B cells, and immature B cells, n = 4) as controls for B-ALL, and total mononuclear cells from healthy BM (n = 5) as controls for B-ALL and T-ALL.[23] B-ALL (NALM6, FLEB-14, REH) and T-ALL cell lines (MOLT4, JURKAT, TALL-1, CEM, RPMI-8402) were also included in the study.

The institutional review board of Istanbul Medical Faculty, Istanbul University, approved the study, and all donors/ parents provided written informed consent.

2.2 | Gene expression analysis

Mononuclear cells of ALL patients and healthy volunteers were obtained by Ficoll-Hypaque gradient centrifugation. Total RNA was isolated from mononuclear cells and ALL cell lines by using RNeasy Mini Kit (Qiagen, GmbH, Germany). cDNA was synthesized from 1 μg of total RNA by random hexamer primers and MMLV reverse transcriptase (MBI Fermentas Life Sciences).

LEF1 mRNA expressions were detected by quantitative real-time PCR (qRT-PCR) both in cell lines and in 87 out of 138 diagnosed samples of ALL patients (n = 45 B-ALL, n = 42 T-ALL) which had sufficient material for RNA isolation.

Thirty of the 42 T-ALL patients total *LEF1* expression was already evaluated in our previous study.[5] But herein, we evaluated both the

full-length *LEF1* isoform and the total expression of *LEF1* isoforms in 42 T-ALL cases.

LEF1 full-length isoform (*LEF1*-long) primers; forward: 5′-AATCATCCCGGCCAGCA-3′ and reverse: 5′-TGTCG TGGTAGGGCTCCTC-3′, and probe: 6-FAM-5′-ACACGAGGT GGCCAGACAAGCACAA-3′-TAMRA were used as designed by Wang et al.[24] Both short- and full-length isoforms (*LEF1*-total) were detected by the following primers, *LEF1*-total forward: 5′-TGCATCAGGTACAGGTCCAA-3′, *LEF1*-total reverse: 5′-CGTTGGGAATGAGCTTCG-3′ (covers exon 10-11/12 and detect *LEF1* transcript variants 1, 2 and 4) and UPL 31 TaqMan Probe by qRT-PCR and normalized to *CYPA* expression. qRT-PCR was performed with a LightCycler 480 Instrument (Roche Applied Sciences). In each run, a positive control and a negative control were used and the samples were run with duplicates. The relative expression of *LEF1* isoform transcripts was calculated by using the comparative cycle threshold (C_t) method.[25] Only the samples with <30 cycles of *CYPA* amplification were evaluated.

2.3 | Deep sequencing of *LEF1*

Genomic DNA was isolated from ALL mononuclear cells using QIAamp DNA Mini Kit (Qiagen, GmbH). Sufficient genomic DNA was available for 79 diagnosed ALL (n = 62 B-ALL, n = 17 T-ALL) and 14 matched relapsed ALL patients (n = 13 B-ALL, n = 1 T-ALL). The hotspot regions of *LEF1* (exons 2 and 3) were screened for mutation analysis.[18] The primers were designed with 10 base molecular identifier barcode sequences (MID) and validated by IRON-II (Interlaboratory Robustness of Next-Generation Sequencing) Consortium. FastStart High Fidelity PCR System (Roche Applied Science) was used to amplify the amplicons. The panels were designed as 96-well plates, allowing us to multiplex patients per sequencing lane using a GS Junior instrument (Roche Applied Science). The purification and amplicon library preparation protocols were already designed and optimized by the IRON-II study group. The amplicons were aligned to the NM_001130713.2 as reference sequencing after the data quality assessment. GS Amplicon Variant Analyzer software version 2.5.3 was used for variant detection, and candidate variants were validated by Sanger sequencing. For the variant interpretations of the clinical association and functional prediction, in silico tools, MutationTaster, PolyPhen and SIFT were used.[26-28]

2.4 | Copy number variation (CNV) analysis

Copy number variation (CNV) data were available for 14 matched diagnosed-relapsed (n = 10 B-ALL, n = 4 T-ALL) samples (Illumina HumanCytoSNP-12 BeadChip 300K). *LEF1* gene was covered by 17 probes in HumanCytoSNP-12 BeadChip (300K). The CNVs and loss of heterozygosity (LOH) were visualized in the Illumina GenomeStudio software (Illumina). Genotype calls, B allele frequency, and logR ratio were generated by default settings. DNA segment of copy number change was calculated by cnvPartition tool.[29] The threshold geometric mean CNV was ≥2.5 for amplification and ≤1.5 for deletion. Genes that overlapped with the CNV regions were reported according to UCSC genes (GRCh37/hg19).

2.5 | Protein detection assays

2.5.1 | Western blot

B-ALL (n = 16) and T-ALL (n = 13) patients' cells, thymocyte, and SP $CD8^+$ cells were available for protein analysis. Patients and control sample cells were lysed with radioimmunoprecipitation assay (RIPA) buffer and were separated by 10% SDS-PAGE gel. After the blotting step, polyvinylidene difluoride membranes (Bio-Rad) were blocked with 5% blocking buffer and subsequently incubated with anti-LEF1 antibody (1:5000, Rabbit monoclonal EP2030Y; Abcam). Then, the membranes were incubated with HRP-conjugated secondary antibody (1:3000, Anti-Rabbit IgG; Cell Signaling). After the detection of LEF1, membranes were incubated with a stripping buffer for 30 minutes (Thermo Scientific) and then incubated with β-actin (1:1000, β-actin antibody 4967; Cell Signaling). The same Anti-Rabbit IgG secondary antibody was used for the detection of β-actin. All the blots were developed by the enhanced chemoluminescence detection kit (Clarity Western ECL Substrate; Bio-Rad Laboratories, Inc), and semiquantitative analysis of the relative density of the bands in blots was performed by ImageJ.

2.5.2 | Immunofluorescence staining of the Thymus for LEF1

Paraffin-embedded healthy human thymus tissue was also used for LEF1 protein detection analysis in early T cells. Thymus paraffin sections were de-paraffinized with xylene and rehydrate in a graded series of ethanol. 1/200 Diluted Rabbit monoclonal EP2030Y LEF-1 antibody (Abcam) was applied to each section and incubated overnight. Goat Anti-Rabbit IgG was used as a secondary antibody (DyLight 488; Abcam). The imaging process was completed using a digital florescence microscope (Leica CTR 6000; Leica).

2.6 | Statistical analysis

Patients were grouped into two groups according to their median value of *LEF1* isoform expressions. Patients with *LEF1* expression higher than the median value were classified as high *LEF1* expression (*LEF1*-total^{high}, *LEF1*-long^{high}), and all other patients were considered as low *LEF1* expression (*LEF1*-total^{low}, *LEF1*-long^{low}). Clinical, and biological features between the high and low LEF1 groups were compared using the chi-squared test or Fisher's exact test for categorical data, the nonparametric Mann-Whitney *U* test for continuous

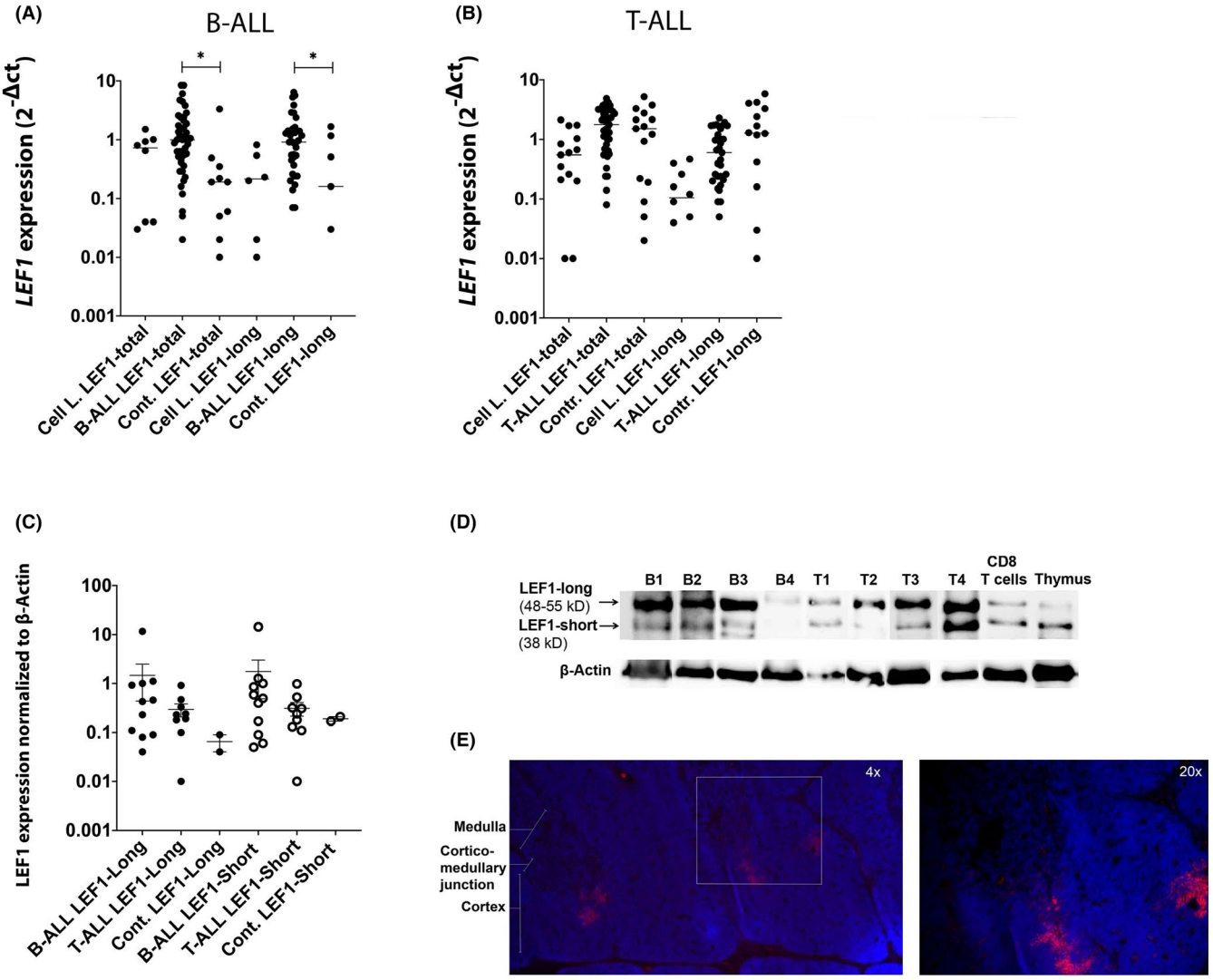


FIGURE 1 Expression levels of *LEF1* isoforms in ALL and controls. A-B, Relative *LEF1* expressions levels of total and full-length *LEF1* isoforms in B-ALL (A) and T-ALL (B). Expression levels are represented by median. *LEF1*-total presented high expression in patients with B-ALL (n = 45) compared with healthy controls (n = 10)($P = .011$), and the full-length isoform of *LEF1* showed a significant expression difference relative to control samples ($P = .026$). B) No differences were found in *LEF1* isoforms values between patients with T-ALL (n = 42) compared with healthy T cell subsets (n = 15) (*LEF1*-total vs controls, $P = .542$; *LEF1*-long vs controls, $P = .176$). C, Semiquantitative LEF1 expression in ALL patients and T cell controls. Data are shown as mean ± SEM. D, Western blot analysis of LEF1 in representative B-ALL (n = 4), T-ALL (n = 4) patients and controls (thymus and SP $CD8^+$ cells). E, The human thymus section from a child was stained for LEF1 (red) with DAPI (blue)

variables and Welch's *t* test for independent samples. Descriptive statistics given with the mean ± standard deviation, n (%), and median (minimum-maximum).

Relapse-free survival (RFS) was calculated as the time of achieving complete remission (CR) until the time of relapse, any death, or last contact. Overall survival (OS) was calculated as the time of diagnosis until the date of death, or last contact.

Survival rate estimations were done by the Kaplan-Meier method and the Cox regression analysis. The Kaplan-Meier survival analysis was performed according to the median cutoff. Differences were compared with the two-sided log-rank test. The statistical analyses were done by SPSS for Windows 21.0 (IBM SPSS Data Editor Inc) and GraphPad Prism 8 (GraphPad Software).

3 | RESULTS

3.1 | *LEF1* expression in ALL patients, cell lines, and controls

To quantify the mRNA expression of *LEF1* isoforms (*LEF1*-total, *LEF1*-long) in children with ALL (BM samples from 45 pre–B-ALL and 42 T-ALL), control samples and cell lines were examined by qRT-PCR. The mRNA expression levels of *LEF1* isoforms were not significantly different between B-ALL and T-ALL patients (T-ALL vs B-ALL; *LEF1*-long $P = .249$, *LEF1*-total $P = .473$).

The expression level of *LEF1*-total in B-ALL patients was significantly higher than B cell controls ($P = .011$), and the

TABLE 1 *LEF1* variations in ALL patients

Sample ID	Sample time	Phenotype	Position	Amino acid change	Variation type	Variant frequency (%)	Read depth	Known in database	Mutation taster	*PolyPhen*	*SIFT*
P99D	Diagnosis	pre-B-ALL	NM_001130714.2:c.253G>A	Asp85Asn	Missense	46.4	2461	rs61752607	Disease Causing	Benign	Tolerated
P99R	Relapse	pre-B-ALL	NM_001130714.2:c.253G>A	Asp85Asn	Missense	40.75	2265	rs61752607	Disease Causing	Benign	Tolerated
P12D	Diagnosis	pre-B-ALL	NM_001130714.2:c.280+29T>A	NA	Intronic	48.65	1739	rs146768776	Polymorphism	NA	NA
P116D	Diagnosis	pre-B-ALL	NM_001130714.2:c.280+122T>A	NA	Intronic	2.45	531	Novel	Polymorphism	NA	NA
P94D	Diagnosis	T-ALL	NM_001130714.1:c.414+728C>G	NA	Intronic	3	340	Novel	Polymorphism	NA	NA

Abbreviation: NA, not available.

full-length isoform of *LEF1* also showed a significant expression difference relative to control samples ($P = .026$; Figure 1A). On the other hand, B-ALL cell lines showed a similar expression profile for *LEF1* isoforms according to controls (*LEF1*-total, B-ALL cell line vs controls, $P = .348$; *LEF1*-long, B-ALL cell line vs controls, $P = .907$).

T-ALL patients had similar expression levels for *LEF1* expression relative to controls (*LEF1*-total vs controls, $P = .542$; *LEF1*-long vs controls, $P = .176$). T-ALL cell lines had lower *LEF1*-total expression ($P = .005$), but similar *LEF1*-long expression relative to controls ($P = .087$; Figure 1B).

We investigated LEF1 protein isoform expression by Western blot in 29 ALL patients (Figure 1C,D). Thymocyte and SP $CD8^+$ cells highly expressed LEF1 and had a relatively higher expression of short isoform of LEF1 compared with long isoform. About 86% of the patients (n = 25) both expressed LEF1-long and LEF1-short isoforms. Semiquantitative LEF1-long and LEF1-short isoform expressions in T-ALL patients did not show a statistically significant difference relative to controls (Student's *t* test, LEF1-long $P = .235$, LEF1-short $P = .620$). However, half of the patients who expressed LEF1 had an increased level of LEF1-long relative to the LEF1-short isoform. The rest of the ALL patients showed non- or low-expression for long and short isoforms of LEF1. LEF1 expression was enriched at the cortex and corticomedullar part of the thymus where T cell maturation occurs (Figure 1E).

In our cohort, 53% of ALL patients (n = 36) had aberrant myeloid marker expression (>20% positivity of CD13 and/or CD14 and/or CD33 and/or MPO). ALL patients with aberrant myeloid expression showed similar *LEF1*-total and *LEF1*-long expression levels compared to the patients without myeloid marker expression (*LEF1*-$\text{total}^{\text{high}}$ vs *LEF1*-$\text{total}^{\text{low}}$, B-ALL $P = .990$, T-ALL $P = .099$; *LEF1*-$\text{long}^{\text{high}}$ vs *LEF1*-long^{low}, B-ALL $P = .15$, T-ALL $P = .320$).

3.2 | *LEF1* variations in the diagnose and relapsed ALL

We analyzed SNP array data for *LEF1* CNV in 14 matched diagnosed-relapsed ALL samples and did not detect any focal deletions or focal copy number alterations. *LEF1* gene is located at 4q25, which is a variable region and six of ALL patients showed CNV variations for the region. Three of the B-ALL patients had copy number amplification at diagnosis and two of them also had it at relapse time. One B-ALL patient had monoallelic deletion at diagnosis but not at relapse time, and one T-ALL patient had monoallelic deletion both at diagnosis and relapse time. One B-ALL patient showed copy neutral loss of heterozygosity (cnLOH) at diagnosis and relapse time for the corresponding region.

We performed amplicon deep sequencing for hotspot regions of *LEF1* in 93 samples (n = 79 diagnose, n = 14 relapse ALL). To be able to detect the subclones, the sequencing depth was set to 500X. In total, four patients out of 79 ALL cases accumulated *LEF1* variants. All the detected variations are listed in Table 1.

TABLE 2 Clinical and molecular features with respect to LEF1-total and LEF1 full-length expression in B-ALL and T-ALL

Phenotype	B-ALL						T-ALL					
Characteristic	LEF1-total low (%)	LEF1-total high (%)	*P*	LEF1-long low (%)	LEF1-long high (%)	*P*	LEF1-total low (%)	LEF1-total high (%)	*P*	LEF1-long low (%)	LEF1-long high (%)	*P*
Age												
<1 y or >10 y	6 (25)	8 (38)	.344	14 (70)	13 (68)	.915	8 (38)	11 (52)	.352	6 (35)	8 (47)	.486
1-10 y	18 (75)	13 (62)		6 (30)	6 (32)		13 (62)	10 (48)		11 (65)	9 (53)	
Gender												
Female	10 (42)	11 (52)	.472	12 (57)	9 (47)	.429	2 (10)	8 (38)	**.03**	2 (12)	7 (41)	.118
Male	14 (58)	10 (48)		8 (40)	10 (53)		19 (90)	13 (62)		15 (88)	10 (59)	
WBC/L												
≤50 000	20 (83)	15 (71)	.476	15 (75)	14 (74)	1.000	5 (25)	8 (47)	.161	4 (27)	7 (43)	.320
>50 000	4 (17)	6 (29)		5 (25)	5 (26)		15 (75)	9 (53)		11 (73)	9 (56)	
Hb												
<10	11 (55)	12 (67)	.463	10 (59)	12 (80)	.265	5 (33)	6 (40)	.705	6 (55)	5 (39)	.431
>10	9 (45)	6 (33)		7 (41)	3 (20)		10 (67)	9 (60)		5 (45)	8 (61)	
Plt												
<20 000	3 (14)	3 (17)	.531	3 (17)	2 (13)	.958	1 (6)	0 (0)	.282	0 (0)	0 (0)	.673
20 000-100 000	14 (67)	9 (50)		11 (61)	10 (67)		9 (53)	11 (79)		8 (67)	10 (77)	
>100 000	4 (19)	6 (33)		4 (22)	3 (20)		7 (41)	3 (21)		4 (33)	3 (23)	
CNS involvement												
Yes	0	0		0	0		3 (14)	6 (29)	.259	2 (12)	6 (35)	.105
No	24 (100)	21 (100)		18 (100)	21 (100)		18 (86)	15 (71)		15 (88)	11 (65)	
Translocation												
Ph or MLL translocation	2 (8)	3 (14)	.652	2 (10)	2 (10)	1.000	2 (9)	3 (14)	1.000	3 (18)	1 (6)	.601
No translocation	22 (92)	18 (86)		18 (90)	17 (90)		19 (91)	18 (88)		14 (82)	16 (94)	
Risk groups												
Standard	5 (38)	4 (25)	.563	4 (33)	3 (23)	.586	1 (5)	2 (10)	.830	1 (7)	0 (0)	.872
Medium	4 (31)	8 (50)		4 (33)	7 (54)		5 (25)	5 (25)		3 (20)	5 (29)	
High	4 (31)	4 (25)		4 (33)	3 (23)		14 (70)	13 (65)		11 (73)	12 (71)	
Treatment response day 33												
Response	15 (94)	10 (91)	1.000	14 (93)	8 (89)	1.000	11 (69)	16 (76)	.716	9 (60)	14 (88)	.113
No response	1 (6)	1 (9)		1 (7)	1 (11)		5 (31)	5 (24)		6 (40)	2 (12)	

(Continues)

TABLE 2 (Continued)

Phenotype	B-ALL						T-ALL				
Characteristic	LEF1-total low (%)	LEF1-total high (%)	*P*	LEF1-long low (%)	LEF1-long high (%)	*P*	LEF1-total low (%)	LEF1-total high (%)	*P*	LEF1-long low (%)	LEF1-long high (%)	*P*
Relapse												
Yes	1 (4)	6 (29)	**.039**	2 (10)	4 (21)	.407	19 (91)	15 (71)	.238	1 (6)	5 (29)	.175
No	23 (96)	15 (71)		18 (90)	15 (79)		2 (9)	6 (29)		16 (94)	12 (71)	
Outcome												
Alive	23 (96)	15 (71)	**.039**	15 (75)	17 (89)	.407	15 (71)	13 (62)	.513	11 (65)	12 (71)	1.000
Dead	1 (4)	6 (29)		5 (25)	2 (11)		6 (29)	8 (38)		6 (35)	5 (29)	

Abbreviations: CNS, central nervous system; Hb, hemoglobin; *P*, *P*-value, bold, >.05 significance; Ph, Philadelphia chromosome; Plt, platelet; WBC, white blood cell count at diagnosis.

In one B-ALL patient (P#99), a missense Asp85Asn single nucleotide polymorphism (rs61752607) in exon 2 was detected both at diagnosis and relapse time. The rs61752607 variation was also confirmed by Sanger sequencing. P#99 was a 15-year-old boy who was classified in the intermediate-risk group at diagnosis and developed early relapse, and died 11 months after the diagnosis.

Pre–B-ALL patients; P#12 had intronic variation NG_015798.1 (LEF_v001):c.280+29T>A (rs146768776), P#116 had a novel intronic variation NG_015798.1(LEF_v001):c.280+122T>A. Both of the patients had a response to induction therapy and had at least 5-year RFS. T-ALL patient P#94 harbored a novel intronic variation NG_015798.1(LEF_v001):c.414+728C>G, who was classified in the high-risk group, had high WBC at diagnosis and developed early relapse. She died with respiratory insufficiency.

3.3 | *LEF1* expression and prognosis in ALL patients

Patients were grouped as low and high *LEF1*-total and *LEF1*-long as described above. The median level of relative *LEF1*-total expression is 1.0 in B-ALL and 1.9 in T-ALL. The median level of relative *LEF1*-long isoform expression is 0.91 in B-ALL and 0.63 in T-ALL. The B-ALL patients with high *LEF1*-total expression had an increased relapse risk ($P = .039$) compared with the *LEF1*-total^{low} patients. There were no significant associations between *LEF1* isoforms expression and other clinical variables such as age, gender, WBC, platelet count, hemoglobin count, treatment response, and the presence of *BCR-ABL1* or *MLL* translocations. The clinical features of the patients with low and high *LEF1* isoforms expression are summarized in Table 2. Twelve T-ALL patients had central nervous system (CNS) involvement, but none of the B-ALL patients had this. CNS involvement did not show any differences between high- and low-expression groups of *LEF1* isoforms (*LEF1*-total $P = .259$, *LEF1*-long $P = .105$).

In our cohort, the median OS was 36 months and RFS was 20 months. Survival analysis was performed for *LEF1* isoforms expression in B-ALL and T-ALL separately. Based on median relative expression thresholds, *LEF1*-total^{high} B-ALL patients had significantly shorter RFS ($P = .008$) and OS ($P = .011$) than *LEF1*-total^{low} patients (Figure 2). B-ALL patients with *LEF1*-long^{high} expression did not show any differences for RFS and OS than the *LEF1*-long^{low} group (RFS $P = .279$, OS $P = .240$; Figure S1).

The prognostic significance of *LEF1*-total expression in B-ALL was examined by univariate and multivariable analyses (Table 3). In univariate analysis, *LEF1*-total^{high} patients showed a higher risk of relapse or death compared with *LEF1*-total^{low} patients for RFS ($P = .033$) and OS ($P = .037$). The following variables were included in the multivariable model: age, wbc count, *BCR-ABL1* or *MLL* translocation status, treatment response at day 33, risk groups, and *LEF1*-total or *LEF1*-long expression. In multivariable model, no significant differences for RFS and OS in B-ALL were observed between patients with high and low *LEF1*-total (RFS $P = .622$, OS $P = .957$) or *LEF1*-long expressions (RFS $P = .238$, OS $P = .548$).

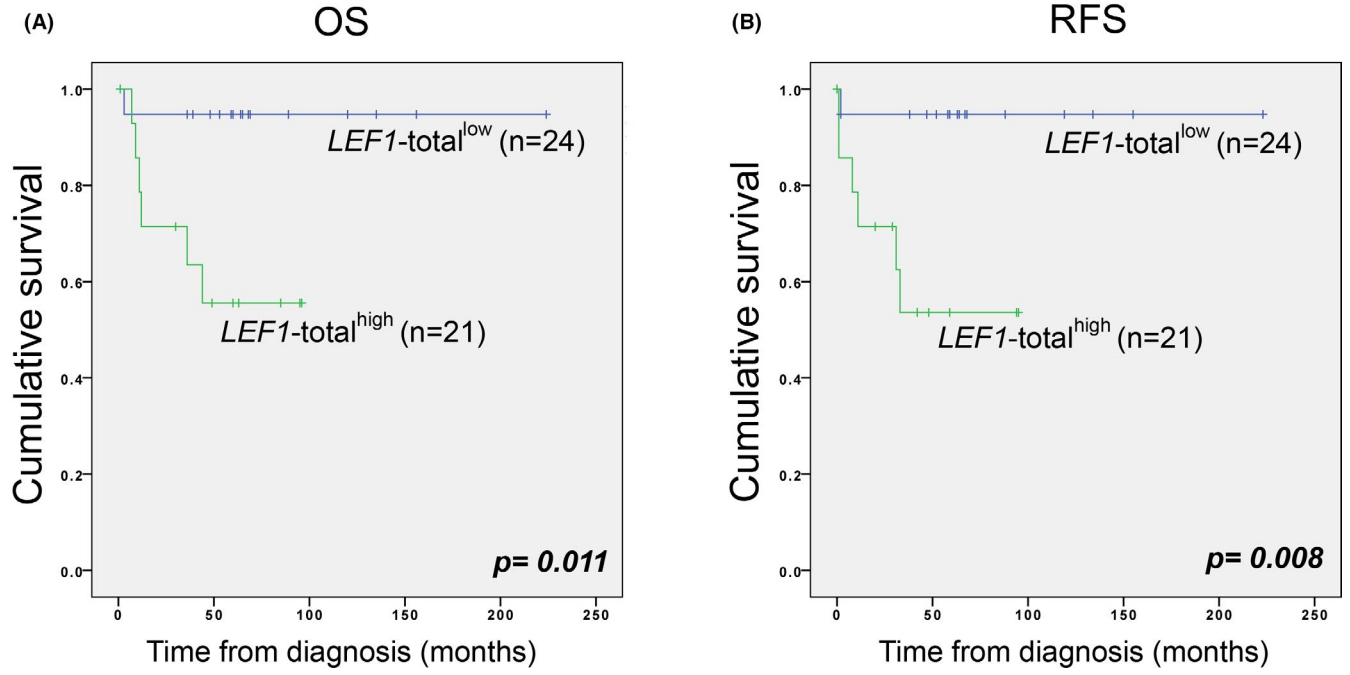


FIGURE 2 Survival curves of childhood B-ALL patients with high and low *LEF1-total* expression. Kaplan-Meier plots of *LEF1-total* expression with overall survival (A) and event-free survival (B)

When we examined *LEF1* expression in the prognostic risk groups of the patients (standard risk (SRG), intermediate risk (MRG) and high risk (HRG)), the Kaplan-Meier survival analysis did not reveal any significant differences in RFS and OS between B-ALL patients with *LEF1*^{high} and *LEF1*^{low} expression (*LEF1*-total, MRG OS $P = .132$, RFS $P = .115$; HRG OS $P = .07$, RFS $P = .07$; *LEF1*-long, MRG: OS $P = .435$, RFS $P = .489$; HRG OS $P = .695$, RFS $P = .695$; Figure S2, S3).

The Kaplan-Meier survival analysis revealed no significant difference in RFS and OS for *LEF1*-total^{high} vs *LEF1*-total^{low} (RFS $P = .993$, OS $P = .958$) or *LEF1*-long^{high} vs *LEF1*-long^{low} (RFS $P = .478$, OS $P = .467$) in T-ALL patients (Figure S4). T-ALL patients with increased *LEF1* expression levels also did not show any survival differences with intermediate- or high-risk groups (*LEF1*-total^{high} vs *LEF1*-total^{low}, MRG RFS $P = .870$, OS: 0. 948, HRG RFS $P = .47$, OS: 0.19; *LEF1*-long^{high} vs *LEF1*-long^{low}, MRG RFS $P = .728$, OS $P = .625$, HRG RFS $P = .937$, OS $P = .925$) (Figure S5, S6). Univariate and multivariate analyses of *LEF1* expression for T-ALL are given in Table S1.

In our T-ALL cohort, six patients had *NOTCH1/ FBXW7* mutations. However, we did not observe any correlation between *LEF1* isoform expressions and *NOTCH1/ FBXW7* mutations.

4 | DISCUSSION

Active Wnt signaling leads β-catenin to transfer to the nucleus and forms a complex with transcription factors LEF/TCF to activate Wnt target genes such as *c-MYC* and *Cyclin D1*.[30,31] In previous studies, aberrant *LEF1* expression and deregulated Wnt signaling were demonstrated in ALL.[5,10,18-20] In our study, we evaluated *LEF1* isoforms expression levels in ALL patients and compared with controls. Our results indicate that *LEF1* isoforms expressions were significantly higher in B-ALL patients (*LEF1*-long $P = .026$, *LEF1*-total $P = .011$). In our methodological design, we detected isoforms 1, 2, and 4 in *LEF-1* total analysis, but not isoform 3 that may cause a bias when compared to the previous studies. On the other hand, *LEF1*-total expression in B-ALL was found in line with previous reports, but not in T-ALL patients.[20,32] In our previous study, we examined *LEF1*-total expression in a larger cohort and detected *LEF1*-total overexpression in a subset of T-ALL[5] which is not completely present in our current study due to the limited biological material. In the study of Jia et al, higher *LEF1*-total expression was detected in ALL patients compared with healthy BM samples.[32] In our study in addition to healthy BM samples, stage-specific thymic subsets and total thymus tissue were used as T cell controls that showed high expression level of *LEF1*-total and T cell development is a multistep process and LEF1 expression was restricted in cortical and corticomedullar areas of the thymus. Besides, BM samples expressed *LEF1* isoforms at a basal level, not as high as thymocytes; therefore, this may explain the differences in the two study results for T-ALL, and also, we should consider the lack of *LEF1* isoform 3 expressions' contribution in LEF-total group.

There are four alternative *LEF1* isoforms with distinct functions. Full-length *LEF1* isoform acts as an effector of the Wnt pathway on the other hand, N-terminally truncated isoform may function in a dominant-negative manner. The change in the fraction of a full-length isoform of *LEF1* compared with a short isoform is thought to increase oncogenesis.[13,14] LEF1 overexpression is a marker for colon cancer and aberrant activation of full-length LEF1 isoform was reported in cancer studies.[14,15,17,24] Most of the studies that investigate *LEF1* expression in ALL had examined the *LEF1*-total expression, but which of these isoforms is expressed in ALL is not well known. Therefore, we also analyzed full-length *LEF1* isoform expression in ALL. Compared to the controls, the

TABLE 3 Univariate and multivariate analysis for RFS and OS in B-ALL patients

	RFS						OS					
	Univariate analysis		Multivariate analysis				Univariate analysis		Multivariate analysis			
Variable	*P*-value	HR(95%)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)
Age (<1 y or >10 y vs 1-10 y)	.280	2.284 (0.511-10.217	.813	0 (0-3.997e51)	.798	0 (0-3.979e33)	0.296	2.221 (0.497-9.925)	.498	5.320 (0.042-666.843)	.070	93.573 (0.691-12664.983)
Gender (female vs male)	.598	1.496 (0.335-6.692)	-	-	-	-	0.542	1.594 (0.356-7.125)	-	-	-	-
WBC count (>50 000 vs ≤50 000)	.790	3.834 (0.855-17.190)	.781	500 682.812 (0-6.467e45)	.440	3072.432 (0-2.169e12)	0.100	3.511 (0.785-15.704)	.658	0.542 (0.036-8.160)	.449	0.316 (0.016-6.225)
Translocation (presence vs absence)	**.017**	6.448 (1.395-29.804)	.771	437.939 (0-2.656e20)	.574	45.137 (0-26246278.68)	**0.033**	5.137 (1.140-23.157)	.080	43.690 (0.641-2979.494)	**.033**	366.370 (1.627-82483.510)
Treatment response day 33 (presence vs absence)	.466	2.229 (0.259-19.194)	.949	0.012 (0-7.653e56)	.996	0.793 (0-8.133e38)	0.512	2.052 (0.239-17.618)	.213	0.070 (0.001-4.619)	.087	0.018 (0-1.784)
Risk Group (standard vs median or high)	.227	1.942 (0.662-5.699)	.822	985.988 (0-1.358e29)	.597	56.826 (0-181545507.1)	0.259	1.852 (0.635-5.408)	.642	0.488 (0.024-9.989)	.171	0.201 (0.02-1.995)
LEF1-total (low vs high)	**.033**	10.050 (1.202-84.035)	.622	2.860e13 (0-8.400e66)	-	-	**0.037**	9.528 (1.143-79.448)	.957	333 462.294 (0-1.215e207)	-	-
LEF1-long (low vs high)	.296	0.417 (0.081-2.153)	-	-	.238	733 821 974.3 (0-3.895e23)	0.258	0.387 (0.075-2.000)	-	-	0.548	0.520 (0.062-4.392)
Overall model *P*-value			**.014**		**.014**				**.020**		0.111	

Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival; RFS, relapse-free survival; WBC, white blood cell,
Bold values indicate p values ≤0.05.

B-ALL patients had increased *LEF1*-long expression ($P = .026$); on the other hand, the T-ALL patients showed similar mRNA expression levels for *LEF1*-long ($P = .176$). Western blot results showed that ALL patients and control samples express both full-length and short isoforms of LEF1. Interestingly, almost half of the ALL patients predominantly expressed full-length LEF1 isoform while LEF1-short isoform expression was down-regulated or remained similar to the controls. Total thymus and SP $CD8^+$ cells were the only controls available for protein assays, and also, Wu et al showed low-level LEF1 expression in healthy B cell samples.[33] In line with previous studies, thymus samples and SP $CD8^+$ T cells exclusively expressed a short isoform of LEF1.[15,34] Leukemic cell growth is associated with canonical WNT signaling, and we showed the predominant expression of full-length LEF1 isoform in ALL samples.

We also further analyzed additional genetic factors for *LEF1* deregulation. We analyzed CNV data of 14 matched diagnosed-relapsed ALL and performed mutation analysis for the hotspot region of *LEF1* in 93 samples. In our cohort, we detected three intronic variations and one common B-ALL patient had a Asp85Asn variation. He had high WBC at diagnosis (576.000/L), relapsed, and died 3 months after induction therapy remission.

The clinical relevance of the *LEF1* variant is not clear yet. In earlier studies, recurrent *LEF1* microdeletions were reported in 11% of pediatric T-ALL and in 1.6% of pediatric B-ALL.[18,35] We did not detect any focal deletions or focal copy number alterations for *LEF1* in our cohort. Guiterez et al also observed *LEF1* inactivation in a subset of childhood T-ALL, which is correlated with *NOTCH1* activating mutations.[18] However, we did not observe such correlation in our cohort. Our results support that the transcriptional activity of the *LEF1* gene takes a more important role in pediatric ALL than the mutations. Although there is not clear evidence about epigenetic deregulation of the *LEF1* gene in leukemia, it is also possible that abnormal transcriptional activation could be due to an epigenetic event.

The identification of prognostic markers in ALL allows improvement of risk-adapted treatment strategies and is important for the development of new therapies. Accordingly, several studies have investigated *LEF1* expression and its prognostic impact in ALL. However, the results are controversial. In our study, a statistically significant relation was observed between the *LEF1*-total expression and decreased OS and RFS in B-ALL (RFS $P = .011$, OS $P = .008$). In contrast to our findings, in the study by Jia et al, high *LEF1* expressing cases showed longer survival time in childhood ALL. However, in the same study, they did not detect any prognostic relevance of *LEF1* expression when they analyzed B and T-ALL patients separately.[32] On the other hand, high *LEF1* expression is associated with adverse prognosis in adult B-ALL and CLL.[20,33] In the study of Kühnl et al, high *LEF1*-expressing adult B-ALL patients showed significantly shorter RFS and high *LEF1* expression was associated with decreased RFS in standard-risk patients.[20] Similarly, high *LEF1* expression was reported as a poor prognostic factor in cytogenetically normal AML.[36,37] Our data also demonstrated that high *LEF1* expression is associated with increased relapse risk in B-ALL. Wnt signaling is known to take part in the self-renewal of leukemic stem cells, and high LEF1 expression might contribute to survival advantages of stem cells in ALL. Herein, we did not detect survival differences between T-ALL patients with high and low expression of *LEF1* based on median cutoff level in line with the study of Gutierrez et al.[18] Median expression level of *LEF1*-long in T-ALL controls is higher than T-ALL patients. On the other hand, the number of patients was small, and further validation is required.

In conclusion, we revealed that ALL cells, which have to proliferate fast, strongly express the full-length LEF1 in contrast to controls. Furthermore, aberrant *LEF1* expression was identified as a poor prognostic factor in a group of childhood ALL. *LEF1* gene variations were rarely observed, and our findings support that LEF1 high expression may contribute to the formation of ALL.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

MS and OHN designed the study; YE and IC performed the mRNA expression analysis; YE performed protein assays, and CNV and statistical analyses; YE, SF, and FK performed sequencing analysis; OHN and YE performed thymic subset isolation; YE and MS wrote the manuscript; KK prepared the figures; KK, YE, and OT performed the statistical analysis; and all authors participated in collecting clinical and/or molecular data, helped analyze and interpret data, and reviewed and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

Data available in article supplementary material. The data that supports the findings of this study are available in the supplementary material of this article

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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