Abstract
T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive disease resulting from the accumulation of genetic changes that affect the development of T-cells. The precise role of lymphoid enhancer binding factor 1 (LEF1) in T-ALL has been controversial since both overexpression and inactivating LEF1 mutations have been reported to date. Here, we have investigated the potential gene targets of LEF1 in human T-cell leukemia cell line, Jurkat. We have successfully knocked down LEF1 in Jurkat cells using small interfering RNA (siRNA) technology and then compared the gene expression levels in the LEF1 knockdown cells with non-targeting siRNA transfected and non-transfected cells by employing microarray analysis. We identified DHRS2, a tumor suppressor gene, as the most significantly downregulated gene in LEF1 knockdown cells and further confirmed its downregulation by real time quantitative polymerase chain reaction (qRT-PCR) in mRNA and by western blotting in protein level. Our results revealed that DHRS2 is positively regulated by LEF1 in Jurkat cells which indicates the capability of LEF1 as a tumor suppressor, and together with previous reports, suggest that LEF1 exhibits a regulatory role in T-ALL via not only its oncogenic targets but also tumor suppressor genes.
**Introduction**

T-cell acute lymphoblastic leukemia (T-ALL) is a malignancy associated with a significant risk of relapse and poor prognosis [1]. T-ALL represents approximately 25% of the adult and 15% of pediatric ALL patients [2]. Although the prognosis of T-ALL has gradually improved over the years due to the modern treatment protocols, resistance and relapse still remain major challenges in the treatment. Thus, our understanding of molecular pathogenesis and classification of the patients can improve treatment outcomes and thereby increase success rate [3, 4]. Activating mutations in \textit{NOTCH1} or inactivating mutations in its negative regulator (\textit{FBXW7}) occur in about 60% of T-ALL cases [5–8]. Activation of \textit{NOTCH} signalling pathway cooperates with loss of p16/\textit{INK4A} and p14/\textit{ARF}. In addition, translocations in oncogenes, such as LIM-only domain (\textit{LMO}) genes, homeobox (\textit{HOX}) genes, \textit{MYC} and \textit{MYB}, frequently place these genes under the control of strong T cell specific enhancers, thus causing aberrant overexpression [2, 5].

Lymphoid enhancer binding factor 1 (\textit{LEF1}), a downstream transcriptional regulator of the Wnt/β-catenin pathway, regulates many cell cycle regulatory and cellular proliferation genes [9]. \textit{LEF1} can also modulate gene transcription independently [10]. Previous studies have shown that \textit{LEF1} plays a crucial role in normal hematopoiesis [9, 11]. Defective pro-B cell survival and proliferation have been shown in \textit{LEF1} knockout mice. Overexpression of \textit{LEF1} in bone marrow progenitors results in B lymphoblastic and acute myeloid lymphoma in recipient animals [11]. In leukemia and solid tumors, abnormal changes in \textit{LEF1} expression have been reported in several studies [12–15].

The findings on prognostic significance of \textit{LEF1} expression show inconsistency among previously reported studies. For example, \textit{LEF1} expression has been found to be associated with poor prognosis in adult precursor B cell acute lymphoblastic leukemia (preB-ALL) and chronic lymphocytic leukemia (CLL) [14, 16, 17] while overexpression of \textit{LEF1} has been determined as a favorable prognostic factor in childhood ALL, acute myeloid leukemia (AML) [13, 18–20].

Heretofore, many gene targets of \textit{LEF1} and their associated pathways have been identified. However, its precise role in T-ALL has not been clarified yet. While some studies have shown an increased expression of \textit{LEF1} in both premalignant thymocytes and T-ALL [16], others have reported the deletion of the \textit{LEF1} gene accompanied with \textit{NOTCH1}, \textit{PTEN} mutations, biallelic \textit{INK4A} \textit{ARF} (\textit{CDKN2A}) deletions, or activating \textit{PI3K} or \textit{AKT} gene mutations in T-ALL [16, 21, 22]. These contradictory findings necessitate further studies to understand the molecular mechanism of \textit{LEF1} in T-ALL.

In this study, we have investigated \textit{LEF1} regulated genes in Jurkat, a well characterized human T acute lymphoblastic leukemia cell line that is widely used in variety of studies to understand T cell biology and T cell signalling. The aim of our study was to identify \textit{LEF1} regulated potentially critical genes as well as related molecular signalling pathways using Jurkat as model cells.

**Materials and Methods**

**Cell Culture**

Jurkat cells were cultured at 37°C with 5% CO₂ in RPMI-1640 medium (Lonza, Basel, Switzerland) containing 10% fetal bovine serum (Capricorn Scientific, Ebsdorfergrund, Germany), 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 2mM L-glutamine.

**LEF1 Small Interfering (siRNA) Transfection**

Jurkat cells were transfected with 100 nM \textit{LEF1} siRNA (Smartpool on-target plus siRNA,
Dharmacon) which targets both long (transcript variant 1, NCBI ID: NM_016269.5) and short isoforms (transcript variant 2, 3, 4; NCBI IDs: NM_001130713.2, NM_001130714.2, NM_001166119.1, respectively) of LEF1 or 100 nM non-targeting siRNA (Smartpool on-target plus siRNA, Dharmacon) with HiPerFect transfection reagent (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol and cultured for 24 and 48 hours.

**RNA Isolation**

Total RNA was isolated from Jurkat cells using the RNeasy Mini kit (Qiagen) in accordance with the manufacturer’s instructions. RNA concentrations were measured using a spectrophotometer (NanoDrop ND-1000, Thermo Scientific, IL, USA).

**Real Time Quantitative PCR (qRT-PCR)**

LEF1 siRNA knockdown and microarray results were confirmed by qRT-PCR. Reverse transcription was performed using random hexamers, total RNA and the Transcriptor First Strand cDNA Synthesis Kit (Roche Life Science, Germany) following the manufacturer’s manual. To quantitate the gene expression, primers specific to LEF1 gene, DHRS2 gene and housekeeping gene TATA binding protein (TBP) were designed. qRT-PCR was performed using LightCycler 480 SYBR Green I Mix (Roche, Germany) and LightCycler 480 Instrument II (Roche Life Science, Germany) under following PCR conditions: 95 °C for 5 min, 95 °C for 20 s, 64 °C for 20 s and 72 °C for 15 s (45 cycles). Forward and reverse primers were (5’-3’) as follows TBP-forward: ACT TGA CCT AAA GAC CAT TGC AC and TBP-reverse: CTT GAA GTC CAA GAA CTT AGC TGG; DHRS2-forward: CGA CTT CCT GGT GTG CAG and DHRS2-reverse: GTT CTC CAT GTA GGG CAG C; LEF1-forward TGG TGC AGC CAT CCC ATG and LEF1-reverse CTT GAT GGG ATA TAC AGG CTG ACC. Quantification was performed using the relative standard curve method. Each experiment was performed in triplicate. Gene expressions were normalized using the housekeeping gene TBP.

**Microarray**

Microarray experiments were performed using Affymetrix's GeneChip® 3' IVT Express Kit (Affymetrix, Santa Clara, CA). The sample preparation was conducted in accordance with the manufacturer’s protocol. Fragmented end-labelled cDNA was hybridized onto the Affymetrix's GeneChip® HG-U133 Plus 2.0 Array according to Affymetrix's standard procedure. After hybridization, the chip was stained and washed in a GeneChip Fluidics Station 450 (Affymetrix) and scanned by GeneChip Array scanner 3000 G7 (Affymetrix). Expression signals were extracted and normalized using the Expression Console (Affymetrix) applying the Robust Multichip Average (RMA) normalization method. The microarray expression data generated in this study is available at the NCBI’s Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) database [23] under accession number GSE129917.

**Microarray data analysis**

Differential gene expression analyses were performed using limma package in R. A one-way ANOVA was applied to the RMA expression values in order to determine whether genes were differentially expressed between three groups. A multiple-testing correction was applied to the p values of the F-statistics to adjust the false discovery rate [24]. Expression level differences with p-value (FDR corrected) < 0.05 and a fold change > 2 were considered significant. Morpheus (https://software.broadinstitute.org/morpheus) was used for the heatmap visualization of gene expression level differences. The web-based tool Database for Annotation, Visualization, and Integrated Discovery (DAVID) [25, 26] was used for the biological interpretation of differentially expressed genes. The identified genes were classified based on the Gene Ontology Resource [27] annotations and associated pathways were determined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) [28].

**Protein isolation and western blotting**
Western blotting was performed to detect \textit{LEF1} and \textit{DHRS2} protein expression in the cells. All protein samples were prepared from a pool of siRNA treated culture cells (three wells) which were homogenized and treated with RIPA lysis buffer system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) on ice. β-actin was used as an internal control. The protein concentrations were quantified using Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL, USA). A total of 15µg proteins were separated in 4-12% bis-tris gels (Nupage Novex, Life Technologies, Bleiswijk, Netherlands) and then transferred onto a nitrocellulose membrane using i-Blot Gel transfer Stacks (Novex, Life Technologies). After incubation with blocking buffer (5% BSA) for one hour at room temperature, western blotting was performed using primary antibodies against \textit{p53} (dilution, 1:100, DO-1 sc126 Santa Cruz), \textit{LEF1} (dilution, 1:250, sc8592 Santa Cruz), \textit{DHRS2} (dilution, 1:200, abcam, ab83254) and \textit{b-actin} (1:1000, I-19R sc1616K, Santa Cruz) by overnight incubation at 4°C. After washing step, the HRP-conjugated secondary goat anti-mouse antibody for \textit{p53} (1:3,000, ab97023, abcam), rabbit anti-goat ab for \textit{LEF1} (1:2,000 abcam, ab6741), goat anti-rabbit for \textit{b-actin} and \textit{DHRS2} (1:5,000, Abbkine A21020-1, Redlands, CA, USA) were added and incubated for 1 h at room temperature. Bands were visualized by the WesternBright Sirius (Advansista, Menlo Park, CA, USA) and analyzed using the imaging system (Wealtec Keta, Wealtec Bioscience Co., Ltd., New Taipei City, Taiwan). For protein quantification, densitometric analyses were done using Image J software (http://rsbweb.nih.gov/ij/index.html).

**Statistical Analysis**

The Statistical Package for Social Sciences (SPSS version 17.0, SPSS Inc, Chicago, IL, USA) was used for data analyses. For both \textit{LEF1} and \textit{DHRS2}, mRNA expression level differences between study groups were assessed by the Student’s T test. P values of <0.05 were considered statistically significant.

**Results**

In order to assess the efficiency of \textit{LEF1} suppression after the transfection of Jurkat cells with \textit{LEF1} siRNA, we have determined mRNA levels of \textit{LEF1} by qRT-PCR. 24h after transfection, we observed an approximately 74.7% reduction in \textit{LEF1} siRNA transfected (si\textit{LEF1}) cells, compared to non-targeting siRNA transfected (siNT) cells (Figure 1). We have measured and compared gene expression levels between si\textit{LEF1}, siNT and non-transfected (NTC) Jurkat cells by microarray analysis which revealed differentially expressed genes (DEGs), potential targets of \textit{LEF1}. The most significant 10 DEGs list included histone genes and \textit{DHRS2} (Figure 2). The GO enrichment analysis of the significantly downregulated genes in si\textit{LEF1} cells showed the distribution of the most abundant categories (Table 1). After GO enrichment analysis, we searched for the associated pathways for the DEGs using KEGG and found that metabolic pathways, pathways in cancer, viral carcinogenesis, transcriptional dysregulation in cancer, mitogen-activated protein kinase (MAPK) signalling and PI3K-Akt pathway were among aberrantly expressed signalling pathways in \textit{LEF1} downregulated cells (Table 2).

We have verified our microarray results by comparison of \textit{DHRS2} gene expressions among si\textit{LEF1}, siNT and NTC cells by qRT-PCR. 24 h after transfection, compared to siNT cells, 84% decrease has been observed in mRNA levels of \textit{DHRS2} in si\textit{LEF1} cells (Figure 3). Protein level verification of microarray and qRT-PCR results has been conducted by western blotting. Protein levels of \textit{LEF1} and \textit{DHRS2} were determined to investigate the \textit{LEF1} and \textit{DHRS2} genes downregulation in si\textit{LEF1} cells compared to siNT and NTC cells. \textit{LEF1} protein levels were almost undetectable 24h after transfection (Figure 4) and reduced by 1.8-fold 48h after transfection in si\textit{LEF1} cells compared to siNT cells (Figure 4). The protein level of \textit{DHRS2} were 2.1-fold reduced in si\textit{LEF1} cells compared to siNT cells 24h after transfection and the suppression persisted 48h after transfection (Figure 4). \textit{LEF1} and \textit{DHRS2} protein
levels obtained by western blotting were quantified by normalizing the protein expression levels to B-Actin expression (Figure 5).

**Discussion**

Although there have been many studies on T-ALL, the underlying molecular mechanisms of this disease have yet to be revealed. In this study, we examined the potential role of the transcription factor LEF1 in T-ALL by determining its target genes and regulation mechanisms. We have compared the gene expression levels of siLEF1, siNT and NTC Jurkat cells by microarray analysis in order to identify DEGs, which are potential targets of LEF1 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE129917). One of the most enriched pathways for downregulated genes was “Pathways in cancer-hsa05200” which is consistent with the association of LEF1 expression with a variety of cancers. The most significant 10 DEGs included DHRS2 (HEP27) and histone genes (Figure 2). As LEF1 is known to regulate cell cycle regulators and cellular proliferation genes, the accompanying downregulation of histone genes in LEF1 knockdown cells reflects the relationship between LEF1 and cellular proliferation. We have further focused on DHRS2 which is a member of the short-chain dehydrogenase/reductase enzyme family that have activity toward steroids, retinoids, prostaglandins and xenobiotics [29, 30]. Thus, to verify our microarray results, we analyzed the expression levels of LEF1 and DHRS2 in siLEF1, siNT and NTC cells using qRT-PCR. Additionally, protein levels of these two genes were evaluated by western blotting. Both RNA and protein level analyses confirmed our microarray results. We also searched the GEO database and found that DHRS2 gene is upregulated in colon cancer cells treated with adenoviral LEF1 expression vector (GEO accession number: GSE3229) which is consistent with our results.

DHRS2 is suggested to be a tumor suppressor gene in different tumor types, including nasopharyngeal carcinoma [31, 32], gastrointestinal stromal tumors [33, 34], and metastatic lung adenocarcinomas [35], esophageal squamous cell carcinoma [30] and renal cancer [36]. Previous reports showed that DHRS2 enzyme interacts with MDM2, a protein responsible for the negative regulation of p53 tumor suppressor gene [37–39]. Similarly, it is also known that one of the alternatively spliced transcripts of CDKN2 (ARF) antagonizes MDM2 dependent p53 degradation [40]. Furthermore, LEF1 inactivation has been associated with biallelic INK4a/ARF deletions in T-ALL [21]. Additionally, it has been reported that overexpression of β-catenin, a coactivator of LEF1, results in p53 accumulation through upregulation of ARF [41, 42] and N-terminal of LEF1 (ΔNLef1) which acts as a tumor promoter by preventing accumulation of p53 in human and mouse sebaceous tumors and ARF downregulation is likely to be responsible for this mechanism [43]. Thus, it may be possible that the activation of p53 accumulation by β-catenin and LEF1 depend on not only ARF but also DHRS2 upregulation. However, further functional studies are needed to investigate these relationships and understand the molecular mechanism.

p53 mutations are known to be frequent in T-ALL [44, 45]. In Jurkat cells, a heterozygous, stop-gained mutation in exon6 of p53 gene (R196* or rs397516435) considered to be important in leukemogenesis or in the tumorigenic progression of leukemic T cells has been reported [46]. Thus, as Jurkat cells are p53 mutant, we could not detect p53 in western blotting analysis. Our findings imply that DHRS2-mediated p53 accumulation does not occur in p53 mutant Jurkat cells and overexpression of LEF1 may show oncogenic effect via overexpression of its downstream target, MYC which is known to play a major role in T-ALL [6, 47]. It has been reported that LEF1 is overexpressed in 30% of adult T-ALL patients [16]. On the other hand, LEF1 microdeletion detected %11 of adult T-ALL [21]. These contradictory observations might result from the altered LEF1 effects due to the cooperative tumorigenic genetic events. It is known that both oncogenes and tumor suppressor genes are targeted by LEF1 which suggests that cooperative genetic events in its downstream genes may
determine the final outcome of LEF1 action. Our results suggest that DHRS2 is one of the tumor suppressor targets of LEF1 in human T-cell leukemia cell line Jurkat. Based on these results one may speculate that the inactivation of LEF1 may be causing the prevention of tumor suppressor effect of DHRS2 in T cells and contributing to leukomogenesis. In this study, we demonstrate that LEF1 positively regulates DHRS2 gene expression in human T-cell leukemia cell line Jurkat and thus provide new insight into LEF1-p53 link in T-cell leukemogenesis. Our findings suggest a tumor suppressive role for LEF1 by the regulation of the downstream DHRS2-p53 signaling pathway which explains the molecular mechanism behind the observation of LEF1 induced p53 accumulation. This study supports the growing evidence that LEF1 plays a regulatory role in T cell proliferation and differentiation and its dysregulation contributes to the development of T-ALL. The main limitations of our study are that it was performed by using only one cell line, not validated in T-ALL patients and requires further functional investigations to confirm the implications of its results including the potential role of DHRS2 in T-ALL and its interactions with LEF1.

Ethics
Ethics Committee Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Authorship Contributions

Conflict of Interest:
The authors declare no conflicts of interest.

Financial Disclosure:
This work was supported by the Scientific Research Projects Coordination Unit of Istanbul University (Grant No. 3092).

References


**Figure 1.** Expression of LEF1 siRNA transfected Jurkat cells by quantitative PCR. NTC: Non-transfected cells, siNT: Non-targeting siRNA transfected cells, siLEF1: LEF1 siRNA transfected cells. **P = 0.013**

**Figure 2.** Heatmap of the most significant 10 DEGs. siNT: Non-targeting siRNA transfected cells, siLEF1: LEF1 siRNA transfected cells.

**Figure 3.** Expression of DHRS2 siRNA transfected Jurkat cells by quantitative PCR. NTC: Non-transfected cells, siNT: Non-targeting siRNA transfected cells, siLEF1: LEF1 siRNA transfected cells.
Figure 4. LEF1 and DHRS2 protein levels in siLEF1, siNT and NTC cells 24h and 48h after transfection. NTC: Non-transfected cells, siNT: Non-targeting siRNA transfected cells.
siLEF1: LEF1 siRNA transfected cells.

**Figure 5.** LEF1 and DHRS2 protein levels normalized by using B-Actin protein expression level. a) 24h after transfection, b) 48h after transfection. NTC: Non-transfected cells, siNT: Non-targeting siRNA transfected cells, siLEF1: LEF1 siRNA transfected cells.
Table 1. Top 10 most enriched GO terms for downregulated genes in LEF1 knockdown cells.

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Table 2. Top 10 KEGG pathways according to the number of associated DEGs.

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