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 adult and 15% of pediatric ALL patients [2]. Although the prognosis of T-ALL has gradually improved over the years due to modern treatment protocols, resistance and relapse still remain major challenges in...
treatment. Thus, our understanding of molecular pathogenesis and the classification of patients can improve treatment outcomes and thereby increase success rates [3,4]. Activating mutations in NOTCH1 or inactivating mutations in its negative regulator (FBXW7) occur in about 60% of T-ALL cases [5,6,7,8]. Activation of the NOTCH signaling pathway cooperates with loss of p16/INK4A and p14/ARF. In addition, translocations in oncogenes, such as LIM-only domain (LMO) genes, homeobox (HOX) genes, MYC, and 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 (LEF1), a downstream transcriptional regulator of the Wnt/β-catenin pathway, regulates many cell cycle regulatory and cellular proliferation genes [9]. LEF1 can also modulate gene transcription independently [10]. Previous studies have shown that LEF1 plays a crucial role in normal hematopoiesis [9,11]. Defective pro-B cell survival and proliferation have been shown in LEF1 knockout mice. Overexpression of 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 LEF1 expression have been reported in several studies [12,13,14,15].

The findings on the prognostic significance of LEF1 expression show inconsistency among previously reported studies. For example, LEF1 expression has been found to be associated with poor prognosis in adult precursor B-cell acute lymphoblastic leukemia and chronic lymphocytic leukemia [14,16,17], while overexpression of LEF1 has been determined as a favorable prognostic factor in childhood ALL and acute myeloid leukemia [13,18,19,20].

Many gene targets of 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 LEF1 in both premalignant thymocytes and T-ALL [16], others have reported the deletion of the LEF1 gene accompanied with NOTCH1 and PTEN mutations, biallelic INK4A/ARF (CDKN2A) deletions, or activating PI3K or AKT gene mutations in T-ALL [16,21,22]. These contradictory findings necessitate further studies to understand the molecular mechanism of LEF1 in T-ALL.

In this study, we have investigated LEF1-regulated genes in Jurkat, a well-characterized human T acute lymphoblastic leukemia cell line that is widely used in a variety of studies to understand T-cell biology and T-cell signaling. The aim of our study was to identify potentially critical LEF1-regulated genes as well as related molecular signaling pathways using the Jurkat line 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, Ebsdorfergund, Germany), 100 U/mL penicillin, 100 mg/mL streptomycin (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) and 2 mM L-glutamine.

LEF1 Small Interfering RNA (siRNA) Transfection

Jurkat cells were transfected with 100 nM LEF1 siRNA (SMARTpool ON-TARGET plus siRNA, Dharmacon, Lafayette, CO, USA), which targets both long (transcript variant 1, NCBI ID: NM_016269.5) and short isoforms (transcript variants 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 h.

RNA Isolation

Total RNA was isolated from Jurkat cells using the RNasy Mini Kit (QIAGEN) in accordance with the manufacturer’s instructions. RNA concentrations were measured using a spectrophotometer (NanoDrop ND-1000, Thermo Scientific, Waltham, MA, 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, Mannheim, Germany) following the manufacturer’s manual. To quantify the gene expression, primers specific to the LEF1 gene, DHRS2 gene, and housekeeping TATA binding protein gene (TBP) were designed. qRT-PCR was performed using LightCycler 480 SYBR Green I Mix (Roche) and LightCycler 480 Instrument II (Roche) under the 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 (5’-3’) were as follows: TBP-forward: ACT TGA TTA GCA 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 the Affymetrix GeneChip® 3’ IVT Express Kit (Affymetrix, Santa Clara, CA, USA). Sample preparation was conducted in accordance with the manufacturer's protocol. Fragmented end-labeled cDNA was hybridized onto the Affymetrix GeneChip® HG-U133 Plus 2.0 Array according to Affymetrix's standard procedure. After hybridization, the chip was washed and scanned in the 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 are available in the NCBI Gene Expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/geo/) [23] under accession number GSE129917.

**Microarray Data Analysis**

Differential gene expression analyses were performed using the limma package in R. One-way ANOVA was applied to the RMA expression values in order to determine whether genes were differentially expressed between three groups. 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-values (FDR-corrected) of <0.05 and fold changes of >2 were considered significant. Morpheus (https://software.broadinstitute.org/morpheus) was used for the heatmap visualization of gene expression level differences. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) [25,26] web-based tool was used for the biological interpretation of differentially expressed genes. The identified genes were classified based on 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 LEF1 and 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 a 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 the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). A total of 15 μg of proteins were separated in 4%-12% Bis-Tris gels (Nupage Novex, Life Technologies, Bleiswijk, the 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 1 h at room temperature, western blotting was performed using primary antibodies against p53 (dilution, 1:100, DO-1 sc126, Santa Cruz), LEF1 (dilution, 1:250, sc8592, Santa Cruz), DHRS2 (dilution, 1:200, abcam, ab83254), and β-actin (1:1000, I-19R sc1616K, Santa Cruz) by overnight incubation at 4 °C. After a washing step, the HRP-conjugated secondary goat anti-mouse antibody for p53 (1:3,000, ab97023, abcam), rabbit anti-goat ab for LEF1 (1:2,000 abcam, ab6741), goat anti-rabbit for β-actin, and DHRS2 (1:5,000, Abbkine A21020-1, Abbkine Scientific, Redlands, CA, USA) were added and incubated for 1 h at room temperature. Bands were visualized by the WesternBright Sirius system (Advansista, Menlo Park, CA, USA) and analyzed using an 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**

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for data analyses. For both LEF1 and DHRS2, mRNA expression level differences between study groups were assessed by Student’s t-test. Values of p<0.05 were considered statistically significant.

**Results**

In order to assess the efficiency of LEF1 suppression after the transfection of Jurkat cells with LEF1 siRNA, we determined the mRNA levels of LEF1 by real-time polymerase chain reaction (qRT-PCR). Twenty-four hours after transfection, we observed an approximately 74.7% reduction in LEF1 siRNA-transfected (siLEF1) cells compared to non-targeting siRNA-transfected (siNT) cells (Figure 1).
We measured and compared gene expression levels between siLEF1, siNT, and non-transfected (NTC) Jurkat cells by microarray analysis, which revealed differentially expressed genes (DEGs), potential targets of LEF1. The most significant 10 DEGs included histone genes and DHRS2 (Figure 2). The GO enrichment analysis of the significantly downregulated genes in siLEF1 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 the KEGG and found that metabolic pathways, pathways in cancer, viral carcinogenesis, transcriptional dysregulation in cancer, mitogen-activated protein kinase signaling, and the PI3K-Akt pathway were among the aberrantly expressed signaling pathways in LEF1-downregulated cells (Table 2).

We verified our microarray results by comparison of DHRS2 gene expressions among siLEF1, siNT, and NTC cells by qRT-PCR.

Table 1. Top 10 most enriched GO terms for downregulated genes in LEF1 knockdown cells.

| Category           | Term                                           | Count | %    | p-value   | FDR    |
|--------------------|------------------------------------------------|-------|------|-----------|--------|
| GOTERM_MF_DIRECT   | Protein binding                                | 117   | 52.9 | 4.9E-4    | 6.6E-1 |
| GOTERM_CC_DIRECT   | Nucleus                                        | 87    | 39.4 | 9.9E-7    | 1.2E-3 |
| GOTERM_CC_DIRECT   | Nucleoplasm                                    | 57    | 25.8 | 1.7E-7    | 2.2E-4 |
| GOTERM_MF_DIRECT   | DNA binding                                    | 42    | 19.0 | 2.9E-7    | 3.9E-4 |
| GOTERM_MF_DIRECT   | Protein heterodimerization activity            | 30    | 13.6 | 1.4E-14   | 1.9E-11|
| GOTERM_CC_DIRECT   | Nucleosome                                     | 23    | 10.4 | 3.2E-24   | 4.0E-21|
| GOTERM_BP_DIRECT   | Negative regulation of transcription from RNA polymerase II promoter | 19 | 8.6 | 5.7E-4 | 9.2E-1 |
| GOTERM_BP_DIRECT   | Nucleosome assembly                             | 17    | 7.7  | 1.1E-13   | 1.8E-10|
| GOTERM_CC_DIRECT   | Nuclear nucleosome                              | 13    | 5.9  | 1.3E-14   | 1.7E-11|
| GOTERM_CC_DIRECT   | Nuclear chromatin                               | 10    | 4.5  | 1.6E-4    | 2.0E-1 |

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

| Pathway id | Pathway name                              | No. of genes |
|------------|-------------------------------------------|--------------|
| hsa01100   | Metabolic pathways                        | 51           |
| hsa05034   | Alcoholism                                | 42           |
| hsa05322   | Systemic lupus erythematosus              | 39           |
| hsa05200   | Pathways in cancer                        | 36           |
| hsa05168   | Herpes simplex virus 1 infection          | 31           |
| hsa05203   | Viral carcinogenesis                      | 31           |
| hsa05202   | Transcriptional misregulation in cancer   | 26           |
| hsa04010   | MAPK signaling pathway                    | 26           |
| hsa04151   | PI3K-Akt signaling pathway                | 25           |
| hsa04217   | Necroptosis                               | 20           |
Twenty-four hours after transfection, compared to siNT cells, an 84% decrease was observed in mRNA levels of DHRS2 in siLEF1 cells (Figure 3).

Protein level verification of microarray and qRT-PCR results was conducted by western blotting. Protein levels of LEF1 and DHRS2 were determined to investigate the LEF1 and DHRS2 genes’ downregulation in siLEF1 cells compared to siNT and NTC cells. LEF1 protein levels were almost undetectable 24 h after transfection (Figure 4) and were reduced by 1.8-fold 48 h after transfection in siLEF1 cells compared to siNT cells (Figure 4). The protein level of DHRS2 was 2.1-fold reduced in siLEF1 cells compared to siNT cells 24 h after transfection and the suppression persisted 48 h after transfection (Figure 4).

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 further focused on DHRS2, which is a member of the short-chain dehydrogenase/reductase enzyme family that has 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 the DHRS2 gene is upregulated in colon cancer cells treated with the 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], metastatic lung adenocarcinomas [35], esophageal squamous cell carcinoma [30], and renal cancer [36]. Previous reports showed that the DHRS2 enzyme interacts with MDM2, a protein responsible for the negative regulation of the p53 tumor suppressor gene [37,38,39]. 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 the 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 depends 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 exon 6 of the 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 effects 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 was detected in 11% of adult T-ALL cases [21]. These contradictory observations might result from the altered LEF1 effects due to 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 the Jurkat human T-cell leukemia cell line. Based on these results, one may speculate that the inactivation of LEF1 may be causing the prevention of the tumor suppressor effect of DHRS2 in T cells and contributing to leukemogenesis.

Conclusion
In this study, we demonstrate that LEF1 positively regulates DHRS2 gene expression in the Jurkat human T-cell leukemia cell line and thus provide new insight into the 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, was 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.

Informed Consent: This study does not involve human subjects and, thus, informed consent is not required.

Authorship Contributions
Study Design: S.S.E, N.A.; Ç.G.; Processing: Z.E., M.S., B.S.; Analysis or Interpretation: S.S.E, C.G.E., B.S., N.A.; Writing: S.S.E, Ç.G.E.

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

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