

Down-regulation of TCF8 is involved in the leukemogenesis of adult T-cell leukemia/lymphoma

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Adult T-cell leukemia/lymphoma (ATLL) is caused by latent human T-lymphotropic virus-1 (HTLV-1) infection. To clarify the molecular mechanism underlying leukemogenesis after viral infection, we precisely mapped 605 chromosomal breakpoints in 61 ATLL cases by spectral karyotyping and identified frequent chromosomal breakpoints in 10p11, 14q11, and 14q32. Single nucleotide polymorphism (SNP) array-comparative genomic

hybridization (CGH), genetic, and expression analyses of the genes mapped within a common breakpoint cluster region in 10p11.2 revealed that in ATLL cells, transcription factor 8 (*TCF8*) was frequently disrupted by several mechanisms, including mainly epigenetic dysregulation. *TCF8* mutant mice frequently developed invasive CD4⁺ T-cell lymphomas in the thymus or in ascitic fluid in vivo. Down-regulation of *TCF8* expression in ATLL

cells in vitro was associated with resistance to transforming growth factor β 1 (TGF- β 1), a well-known characteristic of ATLL cells, suggesting that escape from TGF- β 1-mediated growth inhibition is important in the pathogenesis of ATLL. These findings indicate that *TCF8* has a tumor suppressor role in ATLL. (Blood. 2008;112:383-393)

Introduction

Adult T-cell leukemia/lymphoma (ATLL) is a peripheral CD4⁺ T-cell malignancy caused by infection with human T-lymphotropic virus-1 (HTLV-1).¹ HTLV-1 infection is endemic in a number of well-defined geographic regions within Japan, and as many as 20 million individuals worldwide are estimated to harbor it.² ATLL occurs after a prolonged latency period of up to 50 years in approximately 5% of individuals who have been infected with HTLV-1 around the time of birth. HTLV-1 encodes a transactivator, *Tax*, which plays a key role in the polyclonal growth of infected T cells through the activation of various genes.³ However, recent studies have shown that *Tax* expression is undetectable in circulating ATLL cells, while a genetically and epigenetically defective provirus was observed in more than half of the ATLL patients examined.^{4,5} Considering the long latency period of ATLL, it has been proposed that at least 5 additional genetic or epigenetic events are required for the development of overt disease.^{1,6}

Nonrandom chromosomal translocations are considered to cause leukemic transformation, including structural and/or quantitative abnormalities of transcription factors such as *AML1*, *EVII*, and *MLL*.⁷ To identify disease-specific chromosomal translocations in ATLL, karyotypes of 107 ATLL cases determined by the G-banding method were reviewed in Japan.⁸ There was a high degree of diversity and complexity, and disease-specific translocations were not found; however, translocations involving 14q32

(28%) or 14q11 (14%) and the deletion of 6q (23%) were the most frequent chromosomal abnormalities.⁸ Recently, chromosome-based comparative genomic hybridization (CGH)⁹ and BAC array-based CGH showed complex chromosomal abnormalities with gains in 1q, 2p, 4q, 7p, and 7q, and losses in 10p, 13q, 16q, and 18p.¹⁰ To date, however, no gene involved in the development of ATLL has been isolated. Array CGH is useful for detecting genomic deletions or amplifications, but it cannot detect chromosomal translocations or inversions.

In this study, we searched for the existence of recurrent chromosomal rearrangements by multicolor spectral karyotyping (SKY) and high-resolution single nucleotide polymorphism (SNP) array-CGH (SNP array-CGH). We precisely mapped 605 chromosomal breakpoints in 61 ATLL cases. Breakpoints occurred most frequently in 10p11 and were mapped within a 1-Mb region in 10p11.2 with heterozygous deletions in all cases. A minimal common region of chromosome deletions, including a region of homozygous deletion, was mapped to a 2-Mb region. Genetic and expression analyses of the genes mapped within the deleted region revealed transcription factor 8 (*TCF8*) to be frequently altered in ATLL cells by several mechanisms, including mainly epigenetic dysregulation, suggesting that *TCF8* may be a candidate tumor suppressor gene. *TCF8* (GenBank accession number, NM 030751¹¹), *AREB6*, *ZFHEP*, *NIL-2A*, *ZFHXA*, *NIL-2-A*, *MGC133261*, or

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ZEB1 encodes a 2-handed zinc finger homeodomain protein,¹² which represents a key player in pathogenesis associated with tumor progression in solid cancers.^{13,14} In this study, we found that *TCF8* mutant mice frequently developed CD4⁺ T-cell lymphoma/leukemia half a year after birth. Furthermore, we showed that down-regulation of *TCF8* expression in ATLL cells in vitro was associated with TGF- β 1 resistance, a well-known characteristic of ATLL cells, suggesting that escape from TGF- β 1-mediated growth inhibition is one of the primary mechanisms in the pathogenesis of ATLL. These findings suggest that *TCF8* has an important tumor suppressor role in ATLL.

Methods

Patient samples

ATLL cells were collected from patients at the time of admission to hospital and before chemotherapy.¹⁵ Diagnosis of ATLL was made on the basis of clinical features, hematologic characteristics, serum antibodies against HTLV-1 antigens, and insertion of the HTLV-1 viral genome into leukemia cells by Southern blot hybridization. Using Shimoyama's criteria,¹⁶ all patients were diagnosed as acute-type ATLL. Mononuclear cells were obtained from heparinized blood or ascites by Histopaque density gradient centrifugation (Sigma-Aldrich, St Louis, MO). After separation, ATLL cell enrichment of more than 90% was confirmed by 2-color flow cytometric analysis. All samples were separated by Histopaque density gradient centrifugation, quickly frozen within 3 hours, and cryopreserved at -80°C . This study was approved by the Institutional Review Board of the Faculty of Medicine, University of Miyazaki. Informed consent was obtained from all blood and tissue donors in accordance with the Declaration of Helsinki.

Cell lines

Acute lymphoblastic leukemia (ALL) cell lines used in this study were described previously.¹⁵ Briefly, 4 of the cell lines, Jurkat, MOLT4, MKB1, and KAWAI, are HTLV-1-negative human T-cell acute lymphoblastic leukemia (T-ALL) cell lines.^{17,18} Three cell lines, KOB, SO4, and KK1, are interleukin 2 (IL2)-dependent ATLL cell lines.¹⁹ ED, Su9T, and S1T are IL2-independent ATLL cell lines.²⁰ MT2 and HUT102 are human T-cell lines transformed by HTLV-1 infection.²¹ CTLL2 is a murine IL2-dependent T-lymphoma cell line.²² All the cell lines were maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) and with or without IL2.

Cell culture and karyotype analysis

G-banding studies were performed as described previously.⁸ Briefly, leukemia cells were diluted in 10 mL RPMI1640 medium supplemented with 10% FCS at a final concentration of 10^6 cells/mL. The cells were cultured at 37°C for 24 to 48 hours in humidified air with 5% CO_2 , exposed to colcemid (0.05 mg/mL) for 60 minutes, processed in 0.075 M potassium chloride for 20 minutes, and fixed with methanol/glacial acetate (3:1). The chromosomes were treated with trypsin, stained with a Giemsa solution, and karyotyped according to the International System for Human Cytogenetic Nomenclature (ISCN 2005).²³ The remaining chromosome pellets were stored at -20°C for SKY and fluorescence in situ hybridization (FISH) analyses.

SKY and DAPI banding analysis

The strategy of combined spectral karyotyping (SKY) and 4,6-diaminido-2-phenylindole dihydrochloride (DAPI) banding analysis of chromosome abnormalities was published²⁴ and is briefly described as follows: The chromosomes prepared on a slide glass were denatured and hybridized with a cocktail probe mixture for 2 days at 37°C . The SKY probe mixture and hybridization reagents were purchased from Applied Spectral Imaging

(Vista, CA), and signal detection was performed according to the manufacturer's protocol. The chromosomes were counterstained with DAPI combined with an antifade solution (Vectashield; Vector Laboratories, Burlingame, CA). Images were acquired by an SD200 Spectracube (Applied Spectral Imaging) mounted on an Olympus BX50-RF (Olympus, Tokyo, Japan) using a custom-designed optical filter (SKY-1; Chroma Technology, San Diego, CA). With another special optical filter, the inverted DAPI images were captured in conjunction with spectral classifications as QFH band patterns for the identification of chromosomal breakpoints. For each case, 10 to 20 metaphase spreads were analyzed, and karyotypes were described according to the ISCN 2005.²³

FISH analysis

The plasmid library from sorted human chromosomes 10 (pBS10) was used as a whole chromosome painting (WCP) probe, labeled with digoxigenin-16-dUTP (Boehringer-Ingelheim, Ingelheim, Germany) by standard nick translation. BAC clones were labeled with biotin-16-dUTP (Sigma-Aldrich). Hybridization and signal detection were performed as described previously.²⁵ A minimum of 50 nuclei was examined for each FISH. FISH analysis was performed on metaphase and interphase chromosomes by 53 BAC clones mapped to the chromosome bands 10p11-12 in the human genome mapping of NCBI (build 36 version 1)²⁶ as probes.

High-density SNP array comparative genomic hybridization (array-CGH) analysis

Total genomic DNA was digested with *Xba*I, ligated to an adaptor, and subjected to polymerase chain reaction (PCR) amplification using a single primer. After treatment with DNase I, 40 μg of the PCR products was labeled with a biotinylated nucleotide analog and hybridized the microarray. SNP genotypes were scored with the GTYPE 4.1 software (Affymetrix, Santa Clara, CA). Chromosome copy number and LOH were calculated with 2 programs, ACUE 2.1 (Mitsui Knowledge Industry, Tokyo, Japan, <http://bio.mki.co.jp/en/product/acue2/index.html>) and CNAG 2.0 (Affymetrix).²⁷ For data normalization, we used 6 normal reference samples. Genomic location of probes on the array was determined with the information in NCBI genome map build 35.1.²⁶

Mice

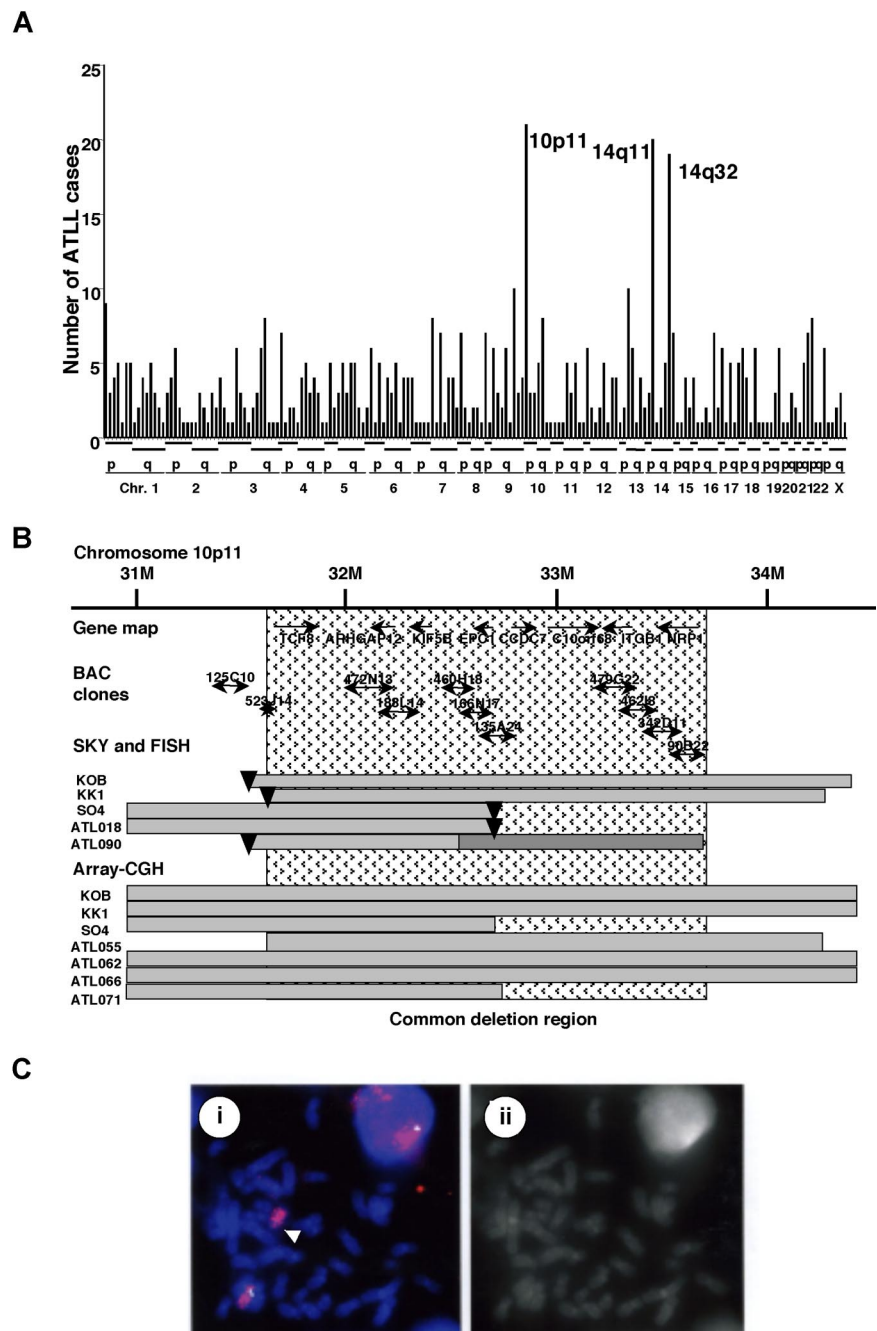
C57BL/6 and ICR mice were purchased from CLEA Japan (Tokyo, Japan) and maintained under specific pathogen-free conditions. The targeted allele of the δ *EF1* gene, the murine orthologue of *TCF8*, lacks only the COOH-proximal zinc finger cluster domain.²⁸ Approximately 20% of the homozygous *TCF8* mutant mice were born alive and grew up to adulthood, although it was reported to cause a defect in the thymic T-cell development.^{28,29} To produce viable homozygous *TCF8* mutant mice, we made their genetic backgrounds more heterogeneous by crossing the C57BL/6 background *TCF8* mutant mice with the ICR outbred strain or F1 (C57BL/6 \times C3H) mice.

Assay for cell proliferation

Control siRNA was purchased from Qiagen (Valencia, CA; AllStars Negative Control [ANC] siRNA) and the *TCF8* siRNA was from Ambion (Austin, TX; murine *TCF8*; 5'-CCUGUGGAUUUAUGAGUUA-3', human *TCF8* 5'-GGGUUACUUGUACACAGCU-3'). For the construction of vectors expressing *TCF8*, human *TCF8* cDNA was subcloned into pCMV26 (Sigma-Aldrich). The cells were transiently transfected using the Nucleofector Kit (Amaxa, Gaithersburg, MD) according to the manufacturer's recommendations. The transfection efficiency, evaluated by fluorescence microscopy of green fluorescent protein, was more than 80%. Twenty-four hours after transfection, the expression of *TCF8* protein in the cells was investigated by Western blotting, while for the cell proliferation studies, each transfectant was plated at a density of 4×10^3 cells per well in 96-well microtiter plates. The cells were treated with various concentrations of transforming growth factor (TGF- β 1; R&D Systems, Minneapolis, MN) for 72 hours and counted by the methyl thiazolyl tetrazolium (MTT) assay

Figure 1. Mapping of the deletions at 10p11.2.

(A) Mapping of the chromosomal breakpoints in whole chromosomes in acute-type ATLLs. An analysis of the chromosomal breakpoints was performed by spectral karyotyping (SKY), and all chromosomal breakpoints were mapped in each region of the chromosomes (x-axis), as indicated at the bottom. The y-axis shows the numbers of ATLL cases with the chromosomal breakpoints in each chromosomal region. Three regions, 10p11, 14q11, and 14q32, were frequently identified with chromosomal breakpoints. (B) Physical and transcriptional maps of the region containing the chromosomal deletion at 10p11. A FISH analysis was performed on metaphase and interphase chromosomes using 53 BAC clones mapped to the chromosome bands at 10p11-12 in the human genome map of NCBI (build 36 version 1²⁶) as probes. The bars indicate the region covering each BAC clone. Horizontal bars indicate the region with hemizygous deletions in each DNA sample from the ATLL cell lines or ATLL cells from patients, which were detected by SKY and FISH or array-CGH analyses. The inverted triangles indicate the regions of chromosomal breakpoints. Closed bars indicate the region of a homozygous deletion in a DNA sample from ATLL cells (ATL090). The hatch pattern represents the minimal heterozygous deletion at 10p11.2. *TCF8* through *NRP1* represent the names of the genes within the region in the human genome map of NCBI (build 36 version 1²⁶). (C) FISH validation of the RP11-188L14 probe to detect the hemizygous deletion of the chromosome10p11.2 in SO4 cell line. The RP11-188L14 probe was green (FITC) and the whole chromosome painting probe was red (TRITC). FISH with RP11-188L14 shows no signal on the abnormal chromosome 10 as indicated by the arrow (i), and a DAPI photograph corresponding to the FISH picture is shown on the right side (ii). Images were captured through the oil objective lens (100×) with a CCD camera (SenSys 0400-GI; Photometrics Ltd, Tucson, AZ). Subsequent image processing was performed with the Software IPLab version 2.4.0 (BD Biosciences Bioimaging, Rockville, MD).



using Tetra color one assay kit (Seikagaku Kogyo, Tokyo, Japan). Each experiment was performed 3 times, and typical results are shown.

Results

Identification of a common hemizygous deletion in 10p11 in ATLL by mapping chromosomal breakpoints

We recently studied recurrent chromosomal rearrangements in adult T-cell leukemia lymphoma (ATLL) cells from 61 patients by spectral karyotyping (T.H. et al, manuscript in preparation). In examining the molecular changes in ATLL cells, 605 chromosomal breakpoints in 61 cases were identified and precisely mapped by DAPI banding analysis. The frequency of the breakpoints was counted in each region of the chromosomes, with an average of

around 10 translocations in each case (Figure 1A). Most of the chromosomal translocations were unbalanced, and a few recurrent reciprocal translocations were found. Chromosomal breakages were most frequently identified at 10p11 (21 [34.6%] of 61 cases), and they were also frequently represented at 14q11 and 14q32 regions (Figure 1A). Based on the data of SKY, these 3 events occurred almost independently; however, almost 50% of the cases with 14q32 abnormality demonstrated a 10p11.2 abnormality, suggesting that both events are interrelated chromosomal abnormalities. The 10p11 regions were translocated to more than 10 different partner chromosomal regions, such as 21q22, 13q14, and 14q32.

Therefore, we precisely mapped the chromosomal breakpoints at 10p11 in 3 ATLL cell lines (KK1, KOB, and SO4) and 2 primary ATLL cases (ATL018 and ATL090) by FISH. We identified der(10)t(10,22)(p11.2;q13.1) in KK1, der(10)t(10,14)(p11.2;q11.2)

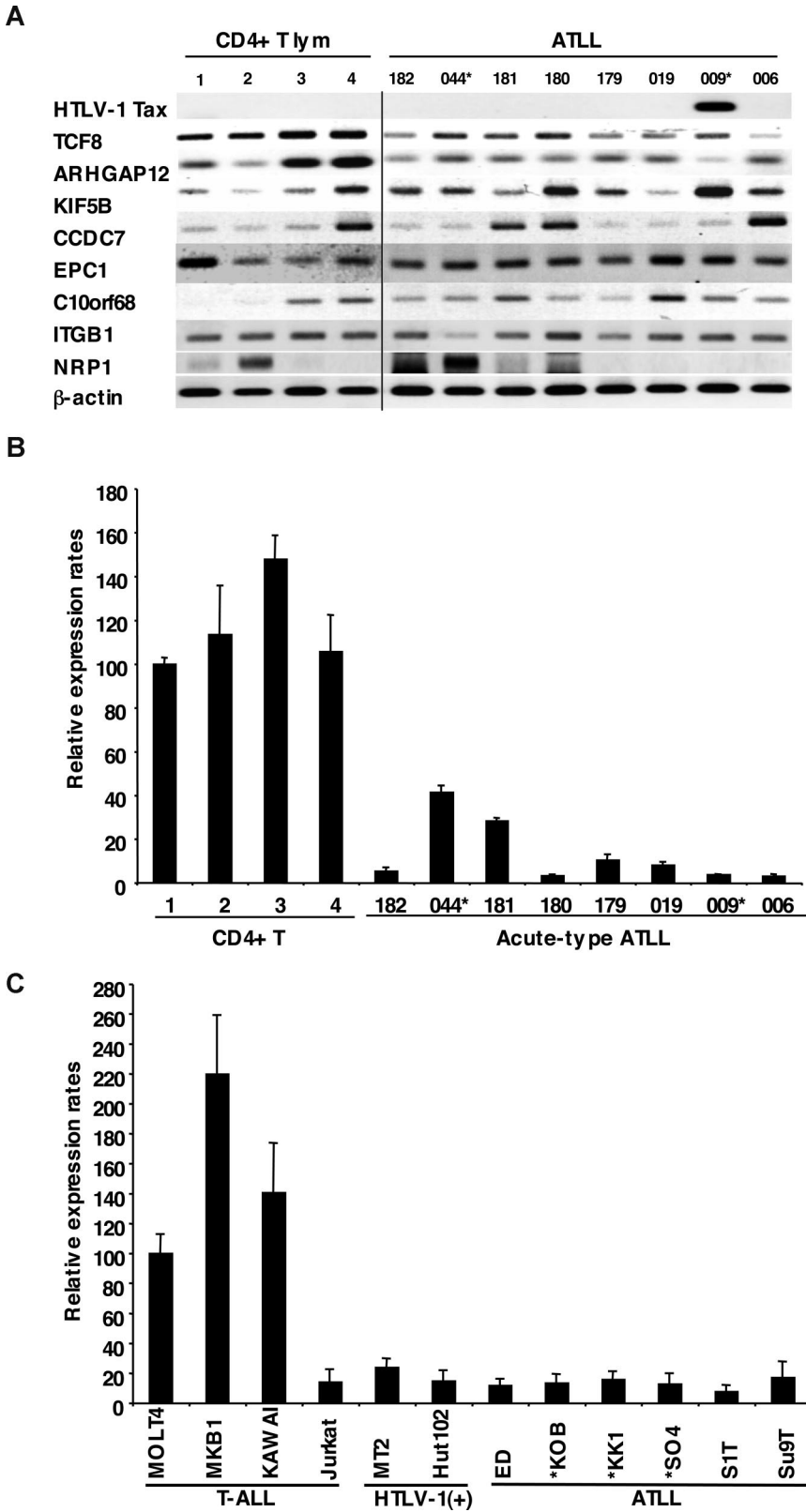


Figure 2. Down-regulated expression of *TCF8* in ATLL cells. (A) The expression profiles of the genes mapped within the deletion region at 10p11.2. Semiquantitative reverse-transcription PCR (RT-PCR) was performed to determine the expression of the genes mapped within the deletion region. *TCF8*, *ARHGAP12*, *KIF5B*, *CCDC7*, *EPC1*, *C10orf68*, *ITGB1*, and *NRP1* showed a single band of amplified cDNA from CD4⁺ T lymphocytes from healthy volunteers as controls and from ATLL cells from the patients. A band of HTLV1 Tax was amplified from only 1 of 8 ATLL cells. A vertical line has been inserted to indicate a repositioned gel lane. (B) Quantitative RT-PCR analysis of *TCF8* mRNA in 4 samples of CD4⁺ T lymphocytes from healthy volunteers and 8 samples of ATLL cells from the patients. The data were normalized to β -actin mRNA and calibrated to the *TCF8*/ β -actin ratio (Δ CT) in the case of healthy volunteer no. 1, as a relative expression rate of 100. The data are the mean and standard deviation of $2^{-\Delta\Delta Ct}$ in a duplicate assay. Two patients (indicated by *) have the chromosome 10p11.2 abnormalities. (C) Quantitative RT-PCR analysis of *TCF8* mRNA in various types of T lymphoblastic leukemia cell lines. MOLT4, MKB1, KAWAI, and Jurkat are T-lymphoid leukemia cell lines; MT2 and HUT102 are HTLV-1-infected cell lines; and ED, KOB, KK1, SO4, S1T, and Su9T are ATLL cell lines. Three ATLL cell lines (indicated by *) showed the deletion of chromosome 10p11.2 with *TCF8*.

in KOB, der(10)t(2,10)(p23;p11.2) in SO4, t(10;21)(p11.2;q11.2) in ATL018, and t(10;13)(p11.2;q14) in ATL090 (Table S1, available on the Blood website; see the Supplemental Materials link at the top of the online article). Using 53 BAC clones on 10p as DNA probes for FISH (Table S2), the chromosomal breakpoints in these 5 cases were mapped to a 1-Mb region at 10p11.2 (Figure 1B). It

was noted that the genomic deletions surrounding the chromosomal breakpoints were detected by a FISH analysis (Figure 1C) and heterozygous deletions of the 10p11.2 region with translocations were found in all 5 samples (Table S2; Figure 1B). Heterozygous deletions of approximately 2 to 8 Mb in the 10p11.2 region with translocations were found in all 5 samples. In addition, no FISH

Table 1. Summary of the genetic and epigenetic abnormalities in ATLL cell lines

Cell line	Cell type	10p abnormalities	Point mutation	Treatment		
				5-Aza-dC, fold \pm SD	TSA, fold \pm SD	5-Aza-dC+TSA, fold \pm SD
MOLT4	T-ALL	None	None	1.25 \pm 0.46	1.65 \pm 0.21	1.65 \pm 0.22
Jurkat	T-ALL	None	None	11.17 \pm 0.59*	10.04 \pm 1.74*	3.07 \pm 0.54*
MT2	HTLV-1 (+)	None	None	3.38 \pm 1.17*	8.25 \pm 1.76*	4.58 \pm 0.90*
HUT102	HTLV-1 (+)	None	255A>C Asn78Thr	4.39 \pm 0.49*	6.94 \pm 0.16*	8.03 \pm 1.05*
ED	ATLL	None	None	4.32 \pm 1.57*	7.48 \pm 0.91*	3.93 \pm 0.21*
KOB	ATLL	10p del	None	3.26 \pm 1.12*	2.32 \pm 0.81	2.08 \pm 0.54
KK1	ATLL	10p del	None	9.92 \pm 0.45*	6.76 \pm 0.17*	9.72 \pm 0.35*
SO4	ATLL	10p del	None	1.40 \pm 0.33	1.57 \pm 0.18	0.59 \pm 0.06
S1T	ATLL	None	None	3.66 \pm 0.21*	3.95 \pm 0.75*	2.32 \pm 1.63
Su9T	ATLL	None	None	3.91 \pm 0.45*	9.66 \pm 2.38*	1.42 \pm 0.21

Data are means plus or minus SD.

* $P < .05$ versus control (Dunnett test).

signals were detected in the 1-Mb region from RP11-135A24 to RP11-462L8 in ATLL090, suggesting that a 10p11 region-specific homozygous deletion had occurred in this case (Figure 1B). Therefore, a minimal common region of chromosome deletions, including a region of homozygous deletion in ATLL090, was mapped to a 2-Mb region from PR11-523J14 to RP11-342D11 (Figure 1B).

To confirm these results, we performed SNP array-CGH using DNA from 8 ATLL-related cell lines including KK1, KOB, SO4, and an additional 10 samples from acute-type ATLL patients. Deletions in 10p11.2, including the 2-Mb deletion region, were noted in 3 cell lines: KK1, KOB, and SO4, and an additional 4 patient samples (Figure 1B; Table S3). Using SNP array-CGH, the telomeric deleted regions in chromosome 10p11.2 in KOB and KK1 covered a wider area than those detected by FISH analysis, and each deleted region in the 3 cell lines and 4 patients samples covered the common deletion region. To combine these data, the same minimal common region of chromosome deletions, including regions of homozygous deletion in ATLL090, was mapped to a 2-Mb region from PR11-523J14 to RP11-342D11 (Figure 1B), suggesting that a tumor suppressor gene possibly exists in this 2-Mb region in 10p11.2.

Down-regulation of *TCF8* mRNA in ATLL cells

We examined the mRNA expression profiles of all 12 genes within the commonly deleted region in 10p11.2, which were identified by NCBI and Celera gene maps (Rockville, MD). Since mRNA samples from the ATLL patients used for the deletion mapping were not available, we initially used the mRNA expression profiles of the other 8 leukemia cell samples from acute-type ATLL patients by semiquantitative reverse-transcription PCR (RT-PCR), which had been previously identified by DNA microarray.¹⁵ Two leukemia samples from patients with ATLL had chromosome 10p11.2 abnormalities: t(10;15) (p11.2;q26) in ATLL044 and monosomy 10 in ATLL009 (Table S1). Expression levels of 8 genes (*TCF8*, *ARHGAP12*, *KIF5B*, *CCDC7*, *EPC1*, *C10orf68*, *ITGB1* and *NRP1*) and HTLV-1 *Tax* as well as β -actin are shown in Figure 2A. The results showed that levels of *TCF8* mRNA in ATLL cells had a tendency to be lower than those in CD4⁺ T lymphocytes from healthy volunteers, even though only 2 of 8 patients had chromosome 10p11.2 abnormalities. Other genes did not show any differences in expression level between the 2 groups. Expression profiles of the leukemia cells using a DNA microarray gave the same results (Figure S1), and quantitative real-time RT-PCR also showed that

the expression level of *TCF8* mRNA in ATLL cells was significantly lower than that in CD4⁺ T lymphocytes (Figure 2B).

To confirm these results, 12 T-ALL cell lines containing 6 ATLL cell lines (ED, KOB, KK1, SO4, S1T, and Su9T), 2 HTLV-1-infected T-cell lines (MT2 and HUT102), and 4 HTLV-1-uninfected T-ALL cell lines (Jurkat, MOLT4, MKB1, and KAWAI) were used for an expression study. Three cell lines (KOB, KK1, and SO4) revealed the deletion of chromosome 10p11.2 with *TCF8*. Although no other genes except *TCF8* showed any change in expression level in these cell lines (Figure S2), the expression level of *TCF8* was specifically down-regulated in all of the ATLL cell lines along with Jurkat cells by quantitative real-time RT-PCR (Figure 2C). These data suggest that *TCF8* transcription might be down-regulated by epigenetic inactivation in most ATLL-related cell lines with Jurkat cells.

Increased expression of *TCF8* by 5-aza-2'-deoxycytidine or trichostatin A in ATLL cell lines

To clarify whether DNA methylation and/or histone deacetylation of the *TCF8* gene promoter were involved in the transcriptional repression of *TCF8* in ATLL cell lines with Jurkat cells, 10 cell lines (2 T-ALL, 2 HTLV-1-infected, and 6 ATLL-derived cell lines) were cultured with (1) 10 μ M 5-aza-2'-deoxycytidine (5-aza-dC), a DNA demethylating agent, for 72 hours, (2) 1.2 μ M trichostatin A (TSA), an inhibitor of histone deacetylase, for 48 hours, or (3) 1.2 μ M TSA for 48 hours following culture with 10 μ M 5-aza-dC for 24 hours. After treatment with 5-aza-dC, *TCF8* expression was up-regulated in 8 of 10 cell lines (Jurkat, MT2, HUT102, ED, KOB, KK1, S1T, and Su9T), with more than 3-fold activation ($P < .05$) as detected by real-time RT-PCR (Table 1). After treatment with TSA for 48 hours, the levels of *TCF8* mRNA increased in 7 of 10 cell lines (Jurkat, MT2, HUT102, ED, KK1, S1T, and Su9T), also with more than 3-fold activation ($P < .05$). In addition, combination therapy induced *TCF8* mRNA expression in 5 cell lines by more than 3-fold. Therefore, *TCF8* mRNA expression was activated in 7 of 8 ATLL-related cell lines along with Jurkat cells by either 5-aza-dC or TSA treatment, suggesting that the down-regulation of *TCF8* in most of the ATLL cell lines except SO4 cells with a chromosome 10p hemizygous deletion was dependent on epigenetic abnormalities.

Unmethylated putative *TCF8* promoter in ATLL cell lines

Next, we determined the methylation status of the *TCF8* promoter by bisulfite sequencing. A CpG island containing 50 CpGs was

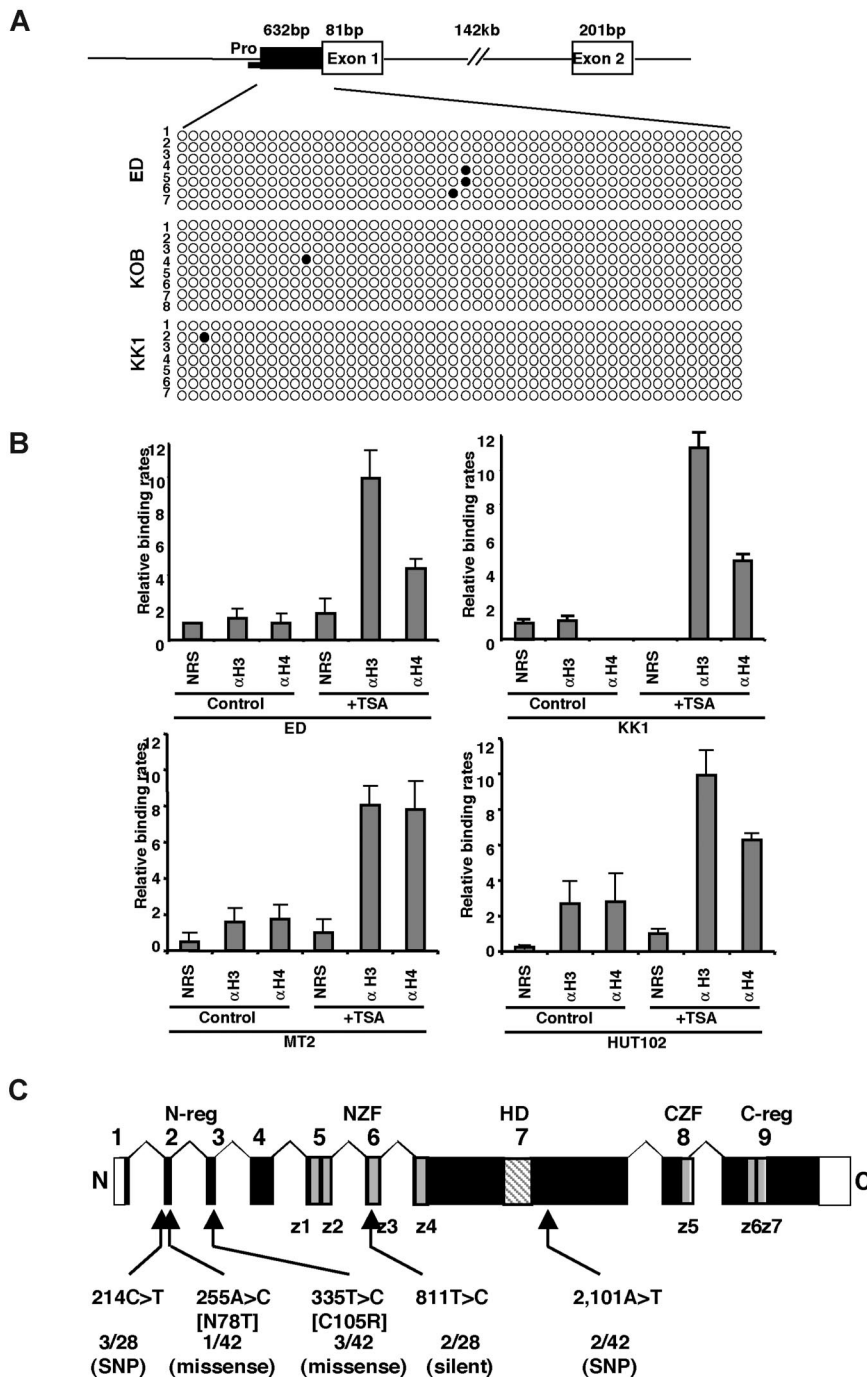


Figure 3. Genetic and epigenetic abnormalities of the *TCF8* gene in ATLL cells. (A) Bisulfite genomic sequencing of the *TCF8* promoter region in 3 ATLL cell lines: ED, KOB, and KK1. PCR products amplified from bisulfite-treated DNA were subcloned, and 8 clones in each cell line were sequenced. ○ indicate unmethylated CpGs (Thy), and ● indicate methylated CpGs (Cyt). The sequenced region contains 50 CpGs in 632 bp, just upstream from exon 1. Pro indicates a region of the *TCF8* promoter for chromatin immunoprecipitation. (B) Specific DNA binding of acetylated histone H3 or H4 to the *TCF8* promoter region detected by chromatin immunoprecipitation (ChIP). Two genomic DNA fragments containing every possible DNA-binding site, *TCF8* promoter, or β -actin promoter were amplified from the genomic DNA of fixed ATLL-related cell lines (MT2, HUT102, ED, and KK1) after immunoprecipitation with normal rabbit serum (NRS) or with antiacetylated histone H3 or H4 antibodies (α H3 or α H4). Quantitative PCR data calibrated to the *TCF8* promoter/ β -actin ratio are shown in the NRS as a relative expression rate of 1. Data are the means plus or minus standard deviation of $2^{-\Delta\Delta Ct}$ in a duplicate assay. Cell lines were cultured in RPMI1640 medium containing 10% FCS with (+ TSA) or without (control) 1.2 μ M TSA. (C) Genomic structure of the *TCF8* gene with a missense mutation and single nucleotide polymorphisms. Locations of the mutations and the single nucleotide polymorphisms relative to the exons encoding the functional domains are shown. *TCF8* encodes a homeodomain (HD) flanked by 2 zinc-finger clusters (z1 to z4 and z5 to z7) (NZF indicates N-terminal zinc finger repeats, CZF; C-terminal zinc finger repeats). The N-terminal transcriptional regulatory domain (N-reg) could bind to p300/CBP and the C-terminal transcriptional regulator domain (C-reg) is the region where acidic amino acids are clustered just after the last zinc-finger domain. Values represent the number of mutated cases per total number of tested cases. SNP indicates single nucleotide polymorphism. White boxes represent noncoding regions in exons 1 and 9.

amplified from a 632-bp region of the putative *TCF8* promoter adjacent to exon 1 using 2 pairs of PCR primers and bisulfite-treated genomic DNA from 3 ATLL cell lines: ED, KOB, and KK1. However, the *TCF8* promoter was not methylated in any of the 3 ATLL cell lines in which *TCF8* expression was induced by 5-aza-dC (Figure 3A), suggesting that the CpG island was not a direct target for DNA methylation in ATLL cells. Moreover, *TCF8* mRNA was up-regulated in various ATLL cell lines after treatment with hydralazine, which was reported to decrease DNA methyltransferase expression (Figure S3). This observation suggests that a transactivating regulator of *TCF8* may be modulated by methylation or the other regulatory elements are located outside the *TCF8* promoter. Such enhancer-related methylation events have been described for the imprinting of *H19* and *Igf2*, *p21WAF1* regulation

by *p73*, and *Apaf-1*.³⁰⁻³³ Therefore, further analyses will be needed to determine the exact regulatory element near the *TCF8* gene or to find a transactivating regulator of *TCF8*, which is directly methylated in ATLL cells.

Histone deacetylation is directly involved in down-regulation of *TCF8* mRNA expression in ATLL cells

To confirm the correlation between reduced *TCF8* mRNA expression and histone deacetylation, *TCF8* expression and histone acetylation status were analyzed in the ATLL-related cell lines (MT2, HUT102, ED, and KK1) by chromatin immunoprecipitation (ChIP) after treatment with or without TSA. After treatment with TSA for 48 hours, the chromosomal DNA precipitated by antiacetylated histone H3 or H4

antibody was amplified with 2 sets of primers for the *TCF8* promoter region or for the human β -actin promoter region (Figure 3B). Band intensities of the *TCF8* promoter region in 4 cell lines were amplified 3- to 6-fold after treatment with TSA, indicating that histone deacetylation of the *TCF8* promoter region was directly involved in the down-regulation of *TCF8* mRNA expression in ATLL cells.

Identification of missense mutations in *TCF8* in ATLL cells

We then searched for somatic *TCF8* mutations in DNA samples from 34 patients with acute-type ATLL and 10 T-cell leukemia cell lines. Genomic PCR did not detect any homozygous deletions in any of the 9 coding exons of *TCF8* in these samples. We detected 5 types of nucleotide substitutions, and all were heterozygous (Figure 3C). The 255A>C substitution in HUT102, creating a missense mutation (Asn78Thr) in exon 2, and the 335T>C substitution in the leukemia cells from 3 ATLL patients, creating a missense mutation (Cys105Arg) in exon 3, were likely to be somatic mutations (Table S4), since they were not detected in noncancerous cells from 95 Japanese volunteers.

The results of genomic and expression analysis indicate that the *TCF8* gene is altered by several mechanisms, including hemizygous deletion, epigenetic dysregulation, and intragenic mutations. Regarding the ATLL-related cell lines, 3 of 9 showed hemizygous deletions of 10p11.2; 8 of 9 showed epigenetic dysregulation of the *TCF8* gene; and 1 of 9 showed an intragenic mutation (Table 1). Therefore, *TCF8* is a strong candidate tumor suppressor gene for ATLL leukemogenesis and is initially inactivated by unbalanced translocations with heterozygous deletion in the 10p11.2 region in ATLL cells.

Development of CD4⁺ T-cell lymphoma in *TCF8* mutant mice

To determine whether down-regulation of the *TCF8* gene could be a causative event for leukemogenic conversion of T lymphocytes to leukemia-lymphoma cells, we investigated δ *EF1* (mouse homologue of *TCF8*) gene-targeted mutant mice, which lack the COOH-proximal zinc finger clusters (δ *EF1* ^{Δ C-*fm*} allele) and were reported to have a defect in the thymic T-cell development.^{28,29} Since 20% of the homozygous *TCF8* mutant mice were born alive, we made their genetic backgrounds more heterogeneous by crossing the C57BL/6 background with the ICR or F1 (C57BL/6 \times C3H) mice. Homozygous mice on a mixed genetic background were born with almost normal Mendelian frequencies (wild-type: heterozygote:homozygote = 60:91:42). After 4 months, almost half of the *TCF8* homozygous mutant mice experienced enlargement of the abdomen due to ascites (27 [64.3%] of 42 mice), and many mice developed lymphomas with a median onset of disease of 30 weeks after birth and an earliest onset at 95 days after birth (Figure 4A). Half of the mice died within a year, and 84% of them developed fatal T-cell lymphomas. In *TCF8* homozygous mutant mice, 2 types of lymphomas were observed: (1) peripheral lymphomas with or without ascites, and (2) thymic tumors. Typical pathological findings of 15 mice (no. 6 to no. 21) are shown in Table S5. In the peripheral lymphoma group, a large amount of bloody or milky ascitic fluid had collected in approximately 60% of the mice with invasion of various organs (Figure 4B,C). Numerous lymphoma cells with medium to large, cleaved or noncleaved nuclei were observed in the ascitic fluid (Figure 4D). Lymphoma cells had invaded various lymph nodes, including the thoracic, peripancreatic, mesenteric, perirenal, mesenteric, and other peripheral lymph nodes (Figure 4E). Fluorescence-activated cell sorter

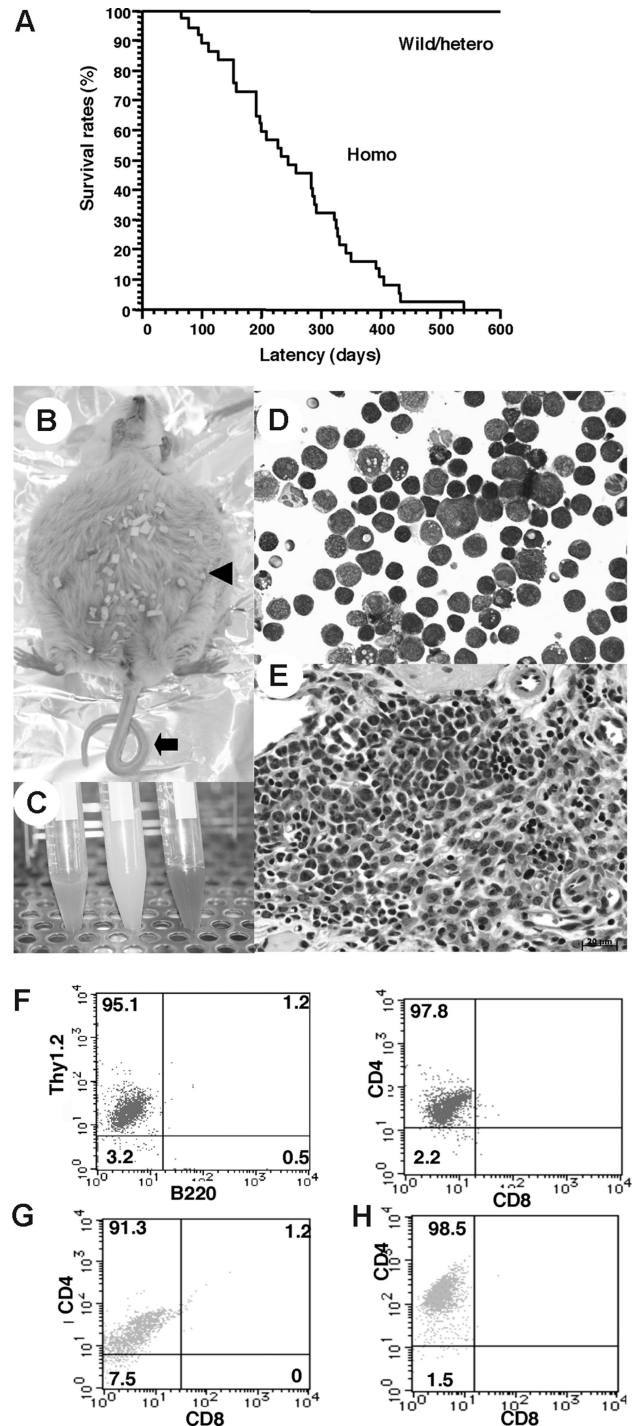


Figure 4. Survival rates and pathologic findings in *TCF8* mutant mice. (A) The survival rates of a cohort of wild-type (wild), *TCF8* heterozygous (hetero), and *TCF8* homozygous (homo) mutant mice were followed over the indicated period using Kaplan-Meier plots. (B) Gross photograph of *TCF8* mutant mice with ascites (▲) and curled tail (●). Approximately 30% of *TCF8*-homozygous mutant mice showed curled tail. (C) Bloody or milky ascites was pooled. (D) May-Giemsa staining of tumor cells in ascites. Original magnification $\times 400$. (E) Many lymphoma cells with medium- to large-sized nuclei infiltrated in the mesentery. Cells were examined using an Axioskop 2 plus inverted microscope (Carl Zeiss, Rugby, United Kingdom) and digital images were acquired using AxioCam camera and AxioVision 2.05 software (Carl Zeiss). Original magnification $\times 400$. (F) Tumor cells from ascitic fluids were analyzed by staining with a combination of monoclonal antibodies, either Thy1.2-PE with B220-FITC (left) or CD4-PE with CD8-FITC (right) and FACS. (G,H) The tumor cells that invaded liver (G) or spleen (H) were analyzed by staining with a combination of monoclonal antibodies, CD4-PE with CD8-FITC.

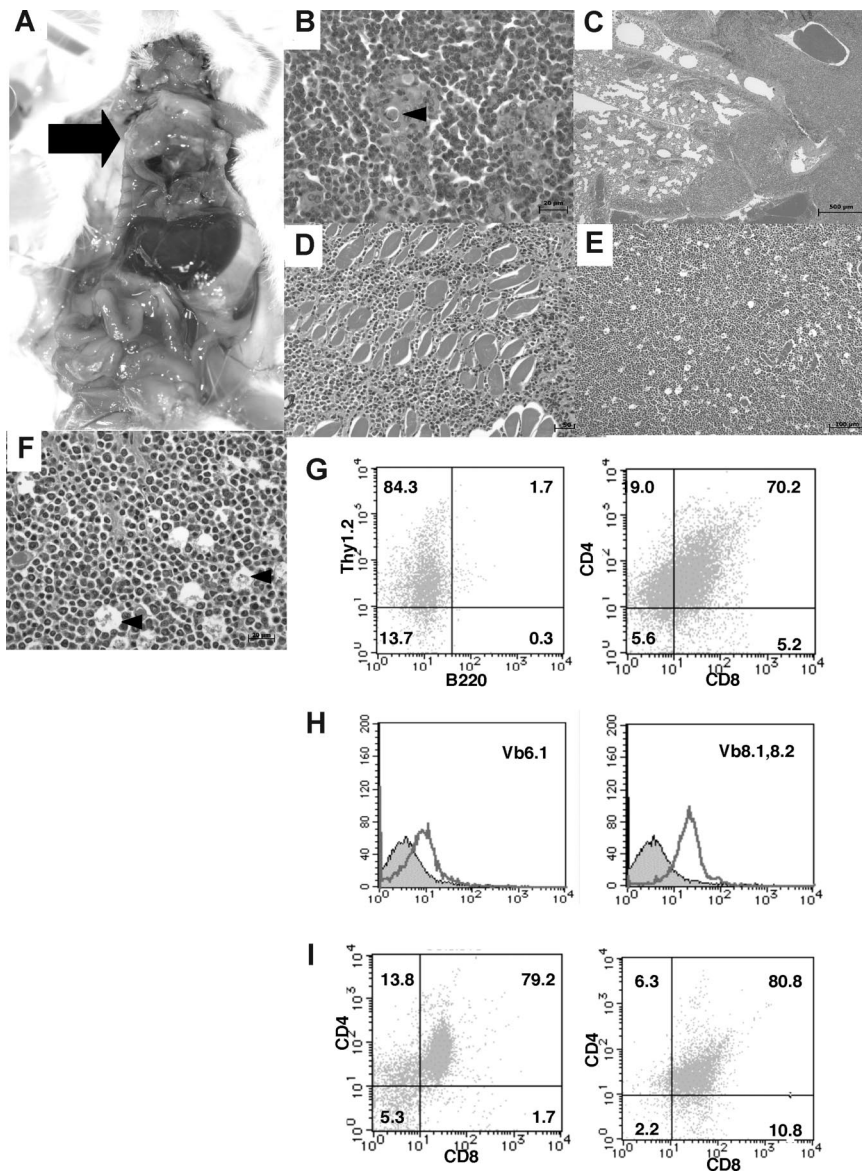


Figure 5. Pathological findings of *TCF8* mutant mice. (A) Gross autopsy of *TCF8* mutant mice with thymic tumors. A large thymic tumor (black arrow) was observed at the mediastinum of the dissected mouse. (B) Hematoxylin and eosin staining of tumor sections from the mouse as indicated. The normal thymic cellular architecture in the *TCF8* mutant mice is replaced by mononuclear fields of large, highly mitotic lymphoblasts with small Hassall bodies (black arrowhead). The scale bar indicates 20 μm . Original magnification $\times 400$. (C) The tumor cells invaded the lung, vascular tissues, and heart in the mouse. The scale bar indicates 500 μm . Original magnification $\times 25$. (D) The tumor cells invaded the muscular tissues of the chest wall. The scale bar indicates 50 μm . Original magnification $\times 200$. (E) Hematoxylin and eosin staining of peripheral lymph nodes. Tumor cells showed a diffuse proliferation of monomorphic lymphoma cells, focally mixed with tingible body macrophages ("starry-sky appearance") (black arrowhead). The scale bar indicates 100 μm . Original magnification $\times 100$. (F) Hematoxylin and eosin staining of peripheral lymph nodes. The scale bar indicates 20 μm . Original magnification $\times 400$. (G) Tumor cells from the thymic tumor were analyzed by staining with a combination of monoclonal antibodies, either Thy1.2-PE with B220-FITC (left) or CD4-PE with CD8-FITC (right) and FACS. (H) The tumor cells of the CD3⁺B220⁻ population did not express V β 6.1 TCR (left), but showed weak expression of V β 8.1-8.2 TCR (right). (I) Tumor cells from the liver (left) or spleen (right) were analyzed by staining with a combination of monoclonal antibodies, CD4-PE and CD8-FITC.

(FACS) analysis of the tumor cells showed that most of the lymphoma cells in the ascitic fluid and those that had invaded various organs were CD4⁺ SPT cells (Figure 4F-H).

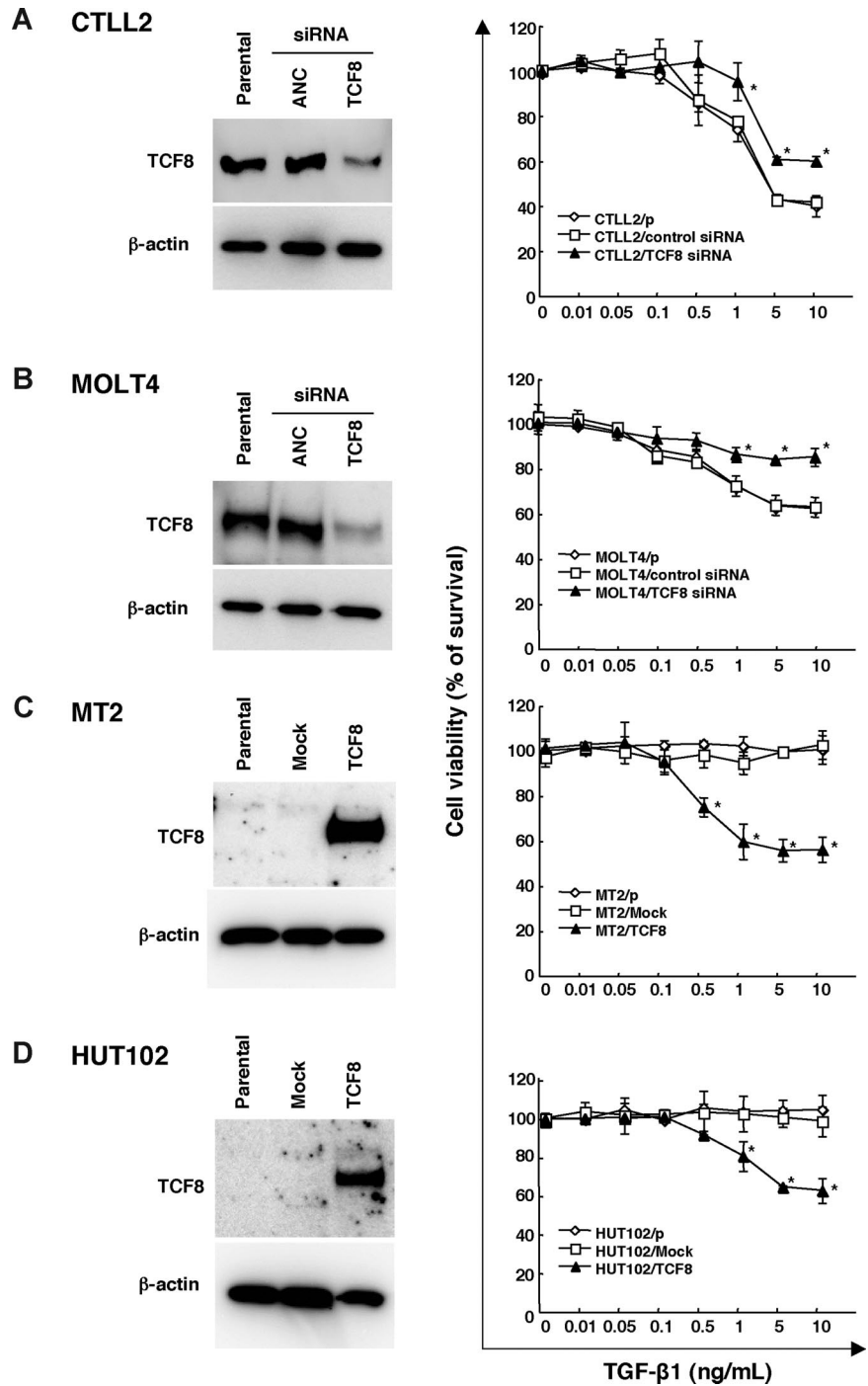
In the thymic tumor group, 16% of the homozygous mutant mice had large thymic tumors with a diameter of 1 to 3 cm (Figure 5A). Histologic analysis of the thymic tumors revealed that lymphoblastic lymphoma cells had densely proliferated in the cortex and medulla of the thymus (Figure 5B). Thymic lymphoma cells continuously invaded the lungs, chest wall, and pericardium (Figure 5C,D). In the peripheral lymph nodes, there was diffuse proliferation of monomorphic lymphoma cells focally mixed with tingible body macrophages, giving a "starry-sky" appearance (Figure 5E,F). In the thymic tumor group, surface marker analysis of thymic lymphoma cells revealed CD4⁺CD8⁺ DP T lymphoma cells (Figure 5G), which were negative for V β 6.1 TCR and weakly positive for V β 8.1-8.2 TCR with a single peak (Figure 5H). In this mouse, the mononuclear cells that invaded the liver, as well as a majority of the cells that invaded the spleen, were DP T lymphoma cells (Figure 5I). Therefore, in the same mouse, both tumor cells derived from the thymus and those that had invaded the organs were revealed to be DP T lymphoma cells. Moreover, the remaining

mice with large thymic tumors showed the same CD4⁺CD8⁺ DP T lymphoma cells. In total, 84% of the T-cell tumors in *TCF8* homologous mutant mice could be classified as CD4⁺ SP T-cell lymphoma, and 16%, as CD4⁺CD8⁺ DP thymic T-cell lymphoma. CD8⁺ SPT lymphomas were never observed. Thus, the histopathological and cellular findings revealed that CD4⁺ T-cell lymphoma/leukemia developed in most *TCF8* mutant mice.

Down-regulation of *TCF8* expression is associated with TGF- β 1 resistance in ATLL cells

The TGF- β superfamily is known to inhibit the lineage commitment of double-positive (DP) cells toward CD4⁺ T-cell differentiation.³⁴ Interestingly, the ATLL cells were found to be resistant to growth inhibition by TGF- β 1, even with high levels of TGF- β 1 expression,³⁵⁻³⁷ suggesting that ATLL cells may have developed several mechanisms of resistance to escape the antiproliferative and inactivating signal mediated by TGF- β 1, including Tax through activation of the JNK/c-Jun pathway^{38,39} or MEL18 expression.³⁷ Since *TCF8* is reported to synergize with Smad

Figure 6. TGF- β 1 responsiveness in various leukemia cell lines with the up- or down-regulation of TCF8 expression. (A,B) The down-regulation of the TCF8 protein by *TCF8* siRNA. The CTLL2 (A) and MOLT4 (B) cell lines were transfected with either *TCF8* or the AllStars Negative Control (ANC) siRNAs and then were incubated for 24 hours. The levels of TCF8 protein were examined in each cell line by Western blotting (left panel). After transfection with siRNAs, the cells were treated with the indicated concentrations of TGF- β 1 for 72 hours. The degree of proliferation of each cell line was examined by MTT assay. The results are shown as percentages of the values obtained from the control TGF- β 1-free culture (right panel). A \diamond represents parental cells, \square represents cells treated with ANC siRNA, and \blacktriangle represents cells treated with *TCF8* siRNA. Student *t* test ($P < .05$) was used for the statistical analysis. (C,D) The enforced expression of TCF8 in HTLV-1-infected cell lines. The TCF8 protein levels were examined in each MT-2 (C) and HUT102 (D) cell transfected with a mock or TCF8 expression plasmid after 24 hours by Western blotting (left panel). The cells were treated with the indicated concentrations of TGF- β 1 for 72 hours and the proliferation of each was examined by MTT assay. The results are shown as percentages of the values obtained from the control TGF- β 1-free culture (right panel). Parental cells (\diamond), mock vector-transfected cells (\square), and TCF8 expression plasmid-transfected cells (\blacktriangle). All data are the means plus or minus standard deviation in a duplicate assay. Student *t* test ($P < .05$) was used for the statistical analysis.



proteins to activate TGF- β 1 signal transduction,^{40,41} we investigated whether the down-regulation of TCF8 expression was associated with resistance to TGF- β 1-mediated growth inhibition in ATLL cells. Thereafter, either *TCF8* or ANC siRNA was introduced into a murine IL2-dependent T-lymphoma cell line, CTLL2, and human T-ALL cell line, MOLT4. Western blot analyses revealed the TCF8 expression in the siRNA-treated cells to be less than half of that in the control cells, while the viable cell curves of both cell lines treated with *TCF8* siRNA exhibited a significantly higher resistance to TGF- β 1 than those of the control cells (Figure 6A,B). Next, the TCF8 expression plasmid was transiently introduced into 2 HTLV-1-infected T-cell lines (MT2 and HUT102) and up to 40% of the TCF8 transfectants died after

TGF- β 1 treatment in a dose-dependent manner, whereas the parental and transfectants with mock plasmid did not die with TGF- β 1 treatment at all (Figure 6C,D). These results indicate that down-regulation of TCF8 expression is one of the mechanisms of TGF- β 1 resistance in ATLL cells, suggesting that CD4⁺ T lymphoma cells might escape from negative selection due to reduced TGF- β 1 responsiveness.

Discussion

We demonstrated that in ATLL cells, the *TCF8* gene was mainly epigenetically inactivated in a majority of ATLL cells. In addition,

TCF8 (or $\delta E F 1^{\Delta C-fm}$ homozygous) mutant mice frequently developed CD4⁺ T-cell lymphoma and/or leukemia after a few months. These findings indicate that *TCF8* has a tumor suppressor role in ATLL. Since the heterozygous *TCF8* mutant mice did not develop any tumors and the level of *TCF8* expression in some ATLL cells was approximately 30% to 40% of that observed in the control CD4 lymphocytes, *TCF8* may therefore be involved in only some and not all of ATLL development. On the other hand, it is reported that *TCF8* overexpressed in colorectal or breast cancer cells induces epithelial-mesenchymal transition (EMT) with the development of metastatic properties such as migration and invasion in vitro and in vivo.⁴² Therefore, *TCF8* has dual functions in cancer progression, which are dependent on the type of the tumors, such as *WT1* or *TSLC1* tumor suppressor genes.^{43,44}

It was previously reported that *TCF8* mutant mice had a defect in T-cell development in the first week of life.²⁸ At the early stage of development, intrathymic c-kit⁺ T precursor cells in these mice were depleted to just 1% of the level in normal mice, and the number of CD8⁺ SPT cells was significantly reduced relative to the number of CD4⁺ SPT cells. These observations indicate that *TCF8* is involved in the regulation of T-cell development at multiple stages. Lymphoma cells in *TCF8* mutant mice showed either CD4⁺ SPT cells or DP T cells after 6 months. Interestingly, TGF- β 1 was important for regulating T-cell development in the thymus and for negative selection at the late stage of differentiation of DP T cells to CD4⁺ SP cells.³⁴ Recently, DNA microarray analysis identified a higher level of *TCF8* expression in DP thymocytes to CD4⁺ SP T cells,⁴⁵ suggesting that *TCF8* enhanced negative selection due to TGF- β 1 responsiveness. Moreover, TGF- β 1-deficient mice had an increased number of CD4⁺ SP T cells and a decreased number of CD8⁺ SPT cells.^{46,47} By correlating the development of CD4⁺ SP T-lymphoma cells in *TCF8* mutant mice with the increase in the number of CD4⁺ T cells in TGF- β 1-deficient mice, we concluded that leukemogenesis in *TCF8* mutant mice was partly dependent on resistance to TGF- β 1.

TCF8 is an E-box-binding transcription factor reported to regulate many genes. We found that the transcription of *CD4*, $\alpha 4$ *integrin*, and *GATA-3*, which was reported to be suppressed by *TCF8*,⁴⁸ was up-regulated in ATLL cells (data not shown). It was therefore suggested that impaired regulation of *TCF8* expression in ATLL induced the increase in expression of *CD4* and *GATA3*, which was crucial for the establishment of the ATLL phenotype in CD4⁺ SP helper T lymphocytes. Moreover, *TCF8* was reported to regulate p73, CCNG2, or p130.^{49,50} Since these genes are very important for cell-cycle progression and apoptosis, further investi-

gation is needed to determine which ones are directly related to leukemogenesis among those regulated by *TCF8*.

The phenotypes of T-cell lymphomas in *TCF8* mutant mice were very similar to those of ATLL patients. In *TCF8* mutant mice, the tumor cells were mainly CD4⁺ SP or DPT cells, which invaded various organs, such as the liver, spleen, and lungs. In ATLL, the tumor cells were mainly CD4⁺ SPT cells that also invaded various organs. One difference, however, was that thymic lymphomas developed in the *TCF8* mutant mice, which has not been reported in ATLL cases. Another difference is that lymphoma cells in *TCF8* mutant mice did not have multilobulated nuclei. Such nuclei result from alterations in the PI3-kinase signaling cascades,⁵¹ suggesting that down-regulation of *TCF8* expression is not related to the PTEN signaling pathway and that other mutations are necessary for the development of ATLL. This is the first report illustrating the importance of the disruption of *TCF8* in leukemogenesis of ATLL.

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Authorship

Contribution: T.H., S.N., and K.H. designed and performed experiments, analyzed data, and drafted the paper; M.H. performed experiments; T.K., Y.A., T.T., K.N., and M.T. performed experiments and data analysis; Y.A. and R.Y. performed the histopathology; K.Y., A.O., and H.T. collected case material and supervised the project; J.Y. and Y.H. supervised the project and drafted the paper; K.M. designed the experiments, analyzed data, and drafted the paper.

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