saturation of proteasome binding sites and tissue distribution occur after multiple dosing, and the CL value on day 11 may be a better representation of the true value.

It was also found that the blood 20S proteasome inhibition at each dose level recovered over time, but was prolonged compared with the temporal decrease in plasma bortezomib concentration. Similarly to CL, this could be due to the large distribution volume of bortezomib and its slow return from tissues to plasma.

Delayed elimination and enhanced proteasome inhibition were observed with repeated administration and dose increase, but no clear tendency in the incidence or degree of adverse reactions was observed. However, the PD results of the present study in Japanese patients demonstrate that the inhibition of 20S proteasome activity does not recover even after 72 h, which is specified as a minimum interval for bortezomib dosing. Accordingly, when bortezomib is used in clinical practice, it is important to determine the optimal dosage and determine whether it is appropriate to administer bortezomib while considering the balance between safety and efficacy.

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ONCOGENOMICS

Identification of the novel *AML1* fusion partner gene, *LAF4*, a fusion partner of *MLL*, in childhood T-cell acute lymphoblastic leukemia with t(2;21)(q11;q22) by bubble PCR method for cDNA

Y Chinen[1,2], T Taki[1], K Nishida[2], D Shimizu[2], T Okuda[2], N Yoshida[2], C Kobayashi[3], K Koike[3], M Tsuchida[3], Y Hayashi[4] and M Taniwaki[1,2]

[1]Department of Molecular Laboratory Medicine, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kamigyo-ku, Kyoto, Japan; [2]Department of Molecular Hematology and Oncology, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kamigyo-ku, Kyoto, Japan; [3]Department of Pediatrics, Ibaraki Children's Hospital, Futabadai, Mito, Japan and [4]Gunma Children's Medical Center, Shimohakoda, Hokkitsu, Shibukawa, Gunma, Japan

The *AML1* gene is frequently rearranged by chromosomal translocations in acute leukemia. We identified that the *LAF4* gene on 2q11.2–12 was fused to the *AML1* gene on 21q22 in a pediatric patient having T-cell acute lymphoblastic leukemia (T-ALL) with t(2;21)(q11;q22) using the bubble PCR method for cDNA. The genomic break points were within intron 7 of *AML1* and of *LAF4*, resulting in the in-frame fusion of exon 7 of *AML1* and exon 8 of *LAF4*. The *LAF4* gene is a member of the *AF4/FMR2* family and was previously identified as a fusion partner of *MLL* in B-precursor ALL with t(2;11)(q11;q23), although *AML1-LAF4* was in T-ALL. *LAF4* is the first gene fused with both *AML1* and *MLL* in acute leukemia. Almost all *AML1* translocations except for *TEL-AML1* are associated with myeloid leukemia; however, *AML1-LAF4* was associated with T-ALL as well as *AML1-FGA7* in t(4;21)(q28;q22). These findings provide new insight into the common mechanism of *AML1* and *MLL* fusion proteins in the pathogenesis of ALL. Furthermore, we successfully applied bubble PCR to clone the novel *AML1-LAF4* fusion transcript. Bubble PCR is a powerful tool for detecting unknown fusion transcripts as well as genomic fusion points.

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Correspondence: Dr T Taki, Department of Molecular Laboratory Medicine, Kyoto Prefectural University of Medicine Graduate School of Medical Science, 465 Kajii-cho Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan.
E-mail: taki-t@umin.net
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Introduction

A large number of leukemias have been found to be associated with specific chromosomal aberrations. Recent studies have demonstrated that several chromosomal rearrangements and molecular abnormalities are strongly associated with distinct clinical subgroups and can predict clinical features and therapeutic responses (Rowley, 1999; Taki and Taniwaki, 2006). Some genes have been associated with recurrent rearrangements and have many fusion partner genes, such as *MLL* at 11q23, *TEL (ETV6)* at 12p13 and *NUP98* at 11p15; *AML1* (*RUNX1*, *CBFA2*) at 21q22 is one of the most frequent targets of these chromosomal rearrangements in both acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) (Miyoshi *et al.*, 1991; Hayashi, 2000; Kurokawa and Hirai, 2003). To date, a number of in-frame fusion partners of *AML1* have been cloned: *YTHDF2* at 1p35 (Nguyen *et al.*, 2006), *ZNF687* at 1q21.2 (Nguyen *et al.*, 2006), *MDS1/EVI1* at 3q26 (Mitani *et al.*, 1994), *FGA7* at 4q28 (Mikhail *et al.*, 2004), *SH3D19* at 4q31.3 (Nguyen *et al.*, 2006), *USP42* at 7p22 (Paulsson *et al.*, 2006), *MTG8* (*ETO*, *CBFA2T1*) at 8q22 (Erickson *et al.*, 1992; Miyoshi *et al.*, 1993), *FOG2* at 8q23 (Chan *et al.*, 2005), *TRPS1* at 8q24 (Asou *et al.*, 2007), *TEL* (*ETV6*) at 12p13 (Golub *et al.*, 1995), *MTG16* at 16q24 (Gamou *et al.*, 1998) and *PRDX4* at Xp22 (Zhang *et al.*, 2004). Most *AML1* translocations, except for *TEL-AML1*, are associated with AML, involving the N-terminus Runt domain and lacking the C-terminus transactivation domain (Kurokawa and Hirai, 2003). AML1 fusion proteins are associated with leukemogenesis by dominantly interfering with normal *AML1*-mediated transcription and acting as a transcriptional repressor (Okuda *et al.*, 1998; Wang *et al.*, 1998). Clinically, patients with AML harboring t(8;21) in both children and adults show a high rate of complete remission, and its prognosis is considered better than that of patients with a normal karyotype or other chromosomal aberrations (Grimwade *et al.*, 1998).

In the present study, we analysed pediatric T-ALL with t(2;21)(q11;q22) and identified the *LAF4* gene,

which is one of the fusion partners of *MLL*, as a novel fusion partner of the *AML1* gene.

Results

Case report

A 6-year-old boy with a high leukocyte count (64 700 μl^{-1}), containing 84% blasts in peripheral blood and with a mediastinal mass, was diagnosed as having T-ALL. A bone marrow smear was hypercellular with 69% blasts and negative for myeloperoxidase. The leukemic cells, after gating of CD45-positive cells, were positive for CD5 (90.7%), CD7 (90.7%), CD58 (69.9%) and cytoplasmic CD3 (92.8%), and negative for HLA-DR, IgG, IgM, Igκ, Igλ, CD8, CD13, CD14, CD19, CD20 and CD33. He was treated on the Tokyo Children's Cancer Study Group (TCCSG) L04-16 extremely high-risk (HEX) protocol, including stem cell transplantation, because the response to initial 7-day prednisolone (60 $mg\,m^{-2}$) monotherapy was poor. He achieved complete remission after the induction phase. After the early consolidation phase and two courses of the consolidation phase, he received allogeneic bone marrow transplantation from an unrelated HLA-matched donor 4 months after diagnosis. He has been in complete remission for 17 months.

The patient's leukemic cells at diagnosis were analysed after written informed consent was obtained from his parents, and the ethics committee of Kyoto Prefectural University of Medicine approved this study.

Identification of the AML1-LAF4 *fusion transcript*

Cytogenetic analysis of the leukemic cells of the patient using routine G-banding revealed 47, XY, add(1)(p36), +der(2)t(2;21)(q13;q22), t(2;21)(q13;q22), −9, −9, +mar1, +mar2, and spectral karyotyping (SKY) analysis revealed 47, XY, der(1)t(1;17)(p36.1;q23), der(2)t(2;21)(q11.2;q22), +der(2)t(2;21)(q11.2;q22), del(5)(p15.1), del(9)(q22), del(9)(p13), der(21)t(2;21)(q11.2;q22) (Supplementary Figure S1). Since *AML1* is located at 21q22, we inferred that *AML1* was rearranged in this case. Fluorescence *in situ* hybridization analysis using *AML1*-specific BAC (bacterial artificial chromosome) clones showed split signals of *AML1* on two der(2)t(2;21)(q11.2;q22) and der(21)t(2;21)(q11.2;q22) chromosomes (Figure 1a).

To isolate fusion transcripts of *AML1*, we performed the bubble PCR method for cDNA (Figure 2) and obtained various-sized products (Figure 3a). Four different-sized products were sequenced and two products contained *AML1* sequences fused to unknown sequences. Basic local alignment search tool (BLAST) search revealed that the unknown sequences were part of the *LAF4* gene and both products had the same in-frame junctions (Figure 3b). *LAF4* was located on chromosome 2q11.2–12, which was compatible with the result of spectral karyotyping analysis. We next performed reverse transcription-PCR to confirm *AML1-LAF4* fusion transcripts, and obtained three different-sized *AML1-LAF4* fusion products, including only one in-frame product (Figures 3c and d); however, reciprocal *LAF4-AML1* fusion transcripts were not generated (Figure 3c). Type 2 transcript is an out-of-frame fusion and generated premature termination in exon 9 of *LAF4* (Figure 3d). On the other hand, type 3 transcript is an in-frame fusion of exon 7 of *AML1* and exon 8 of *LAF4*, the same as the type 1 transcript; however, the type 3 transcript contained an 85-bp intronic sequence between exons 9 and 10 of *LAF4*, which might be due to splicing error, and appeared as a premature termination codon within the intronic sequences (Figure 3d). *AML1-LAF4* fusions were also confirmed by fluorescence *in situ* hybridization analysis (Figure 1b).

Detection of AML1-LAF4 *genomic junctions*

Southern blot analysis using a cDNA probe within exon 7 of *AML1* detected a rearranged band derived from an

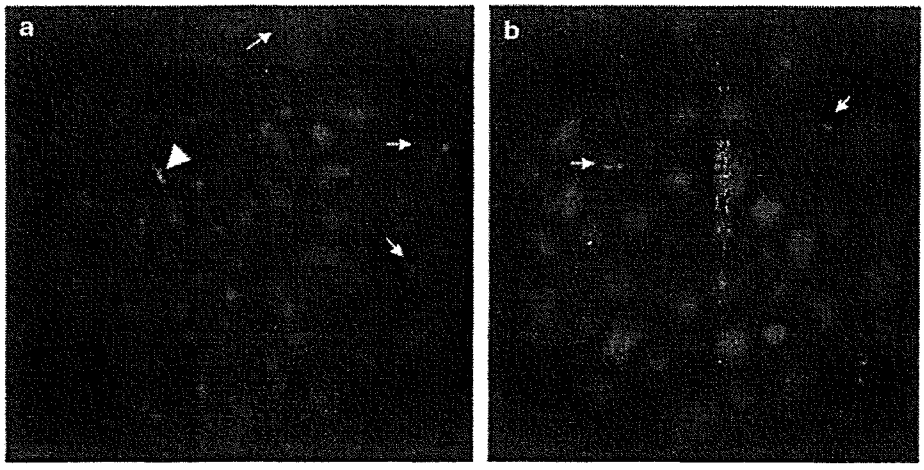

Figure 1 Fluorescence *in situ* hybridization analysis of the leukemic metaphase. (**a**) Both RP11-272A3 (green, 3′ side of *AML1*) and RP11-994N6 (red, 5′ side of *AML1*) were hybridized to normal chromosome 21 (arrowhead), RP11-272A3 to der(21)t(2;21)(q11.2;q22) (arrow, green signal) and RP11-994N6 to two der(2)t(2;21)(q11.2;q22) chromosomes (arrows, red signal). (**b**) Two fusion signals of RP11-994N6 (5′ of *AML1*, red signals) and RP11-527J8 (3′ of *LAF4*, green signals) were detected on two der(2)t(2;21)(q11.2;q22) chromosomes (arrows).

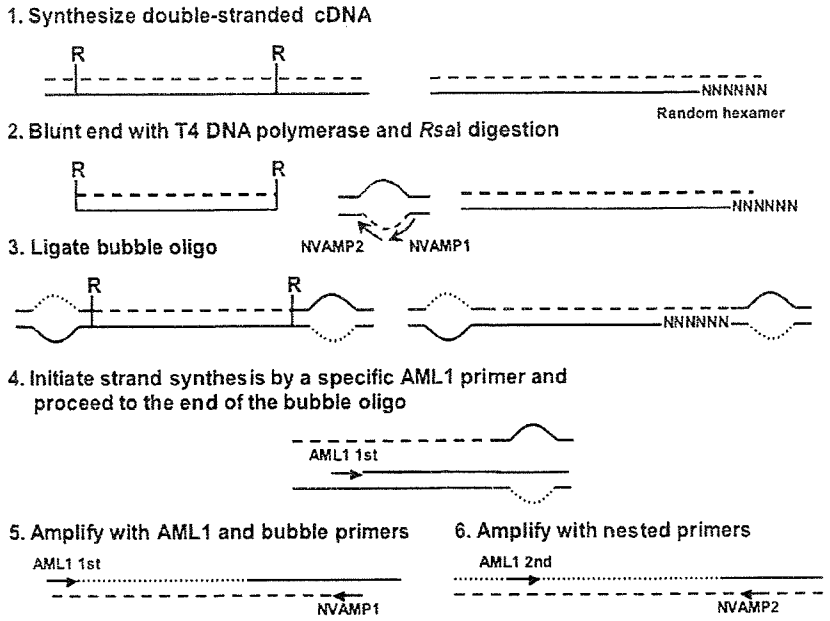

Figure 2 Outline of bubble PCR for cDNA. Bubble PCR primers (NVAMP-1 and NVAMP-2) can only anneal with one complementary sequence for bubble oligo synthesized with *AML1* primer, but not bubble oligo itself; therefore, this single-stranded bubble provides the specificity of the reaction.

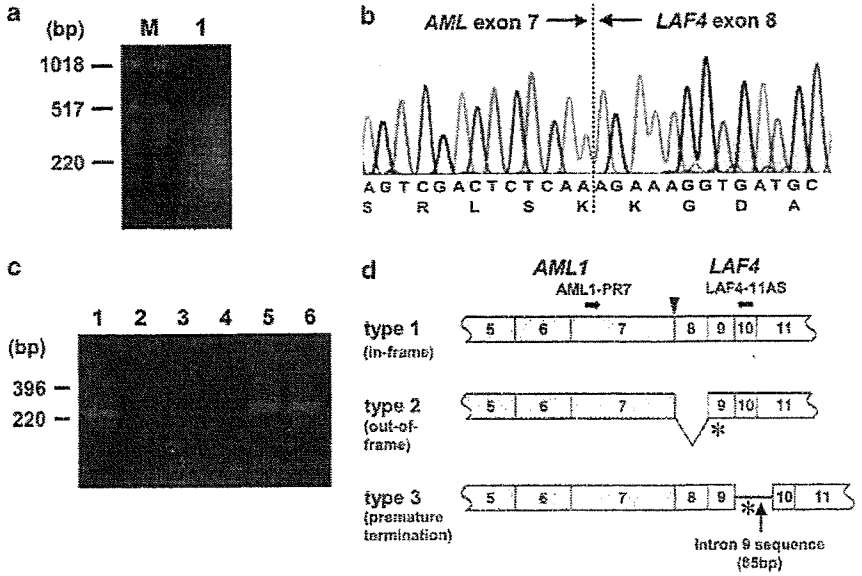

Figure 3 Identification of *AML1-LAF4* fusion transcript. (**a**) Bubble PCR products by nested PCR using AML1-5S and NVAMP1 for first PCR, and AML1-E6S and NVAMP2 for second PCR (lane 1). M, size marker. (**b**) Sequence analysis of *AML1-LAF4* fusion transcript. The single letter amino-acid sequences surrounding the fusion point are shown at the bottom of the figure. (**c**) Detection of *AML1-LAF4* fusion transcripts by reverse transcription-PCR. Primers were AML1-PR7 and LAF4-11AS (lanes 1 and 3), AML1-PR8 and LAF4-PR5 (lanes 2 and 4), and β-actin, respectively. Lanes 1, 3 and 5, patient's leukemic cells; lanes 2, 4 and 6, normal peripheral lymphocytes. (**d**) Three fusion transcripts of *AML1-LAF4* are schematically depicted. Gray/dotted boxes denote predicted *AML1* exons and white boxes represent predicted *LAF4* exons. Type 3 contains the *LAF4* intron 9 splicing donor site. AML1-PR7 and LAF4-11AS indicate the primers used for reverse transcription-PCR. Asterisk shows the termination codon.

approximately 11 kb *Bgl*II germline fragment on chromosome 21 (data not shown). To isolate the fusion point of chromosomes 2 and 21, we next performed bubble PCR on genomic DNA and detected nested PCR products using primers AML1-GNM8-2S and NVAMP2 (Figure 4a). Sequence analysis of the subcloned PCR product revealed the genomic junction of 5′-*AML1-LAF4*-3′ (Figures 4c and d), and the result

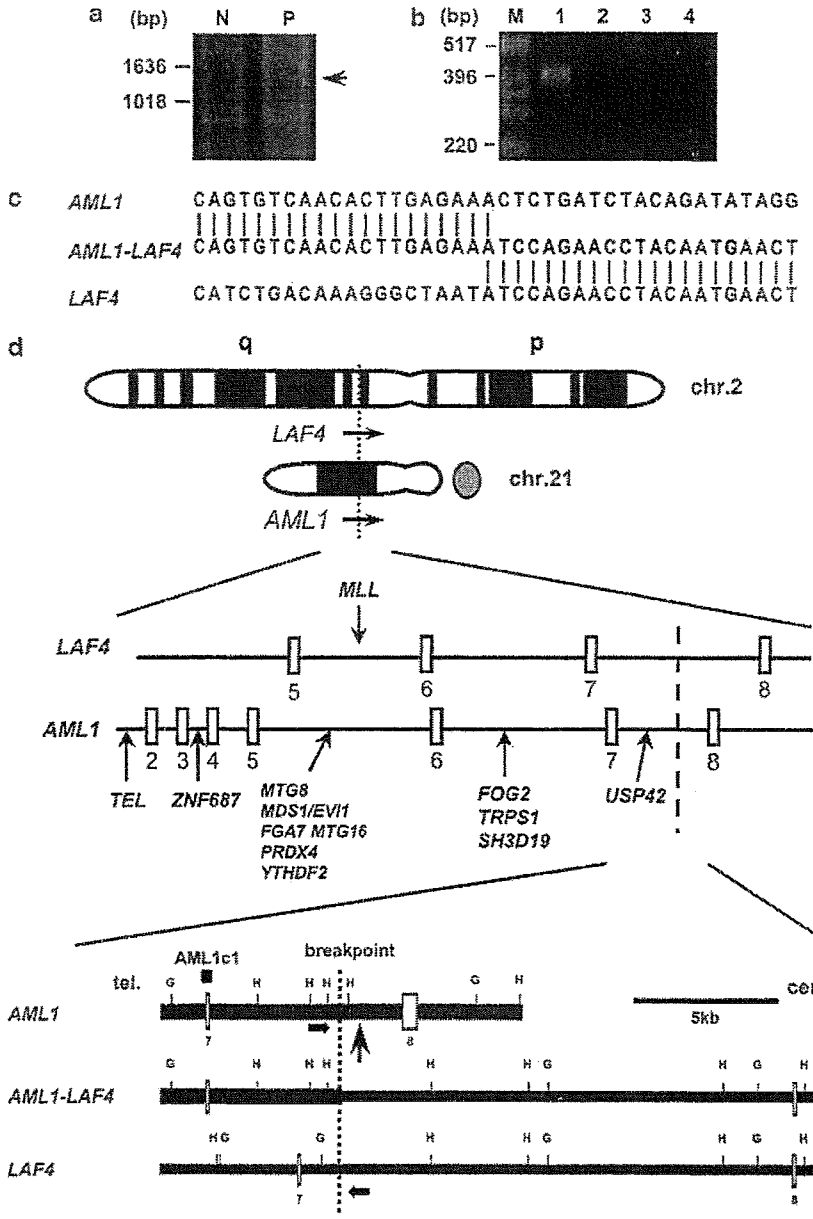

Figure 4 Cloning of the genomic junction of *AML1* and *LAF4*. (**a**) Bubble PCR for genomic DNA. N, normal human lymphocytes; P, patient's leukemic cells. (**b**) Detection of the genomic fusion point of *AML1-LAF4* by PCR. Primers were AML1-GNM8-4S and LAF4-GNM11-2AS (lanes 1 and 3), and LAF4-GNM11-2S and AML1-GNM8-2AS (lanes 2 and 4). Lanes 1 and 2, patient's leukemic cells; lanes 3 and 4, normal peripheral lymphocytes. M, size marker. (**c**) Sequences of breakpoints in the patient's leukemic cells. (**d**) Physical map of the breakpoint regions. Open vertical boxes represent defined exons in each gene. Horizontal arrows show the primers used. Restriction sites are indicated by capital letters: G, *Bgl*II; H, *Hin*dIII. AML1c1 indicates the position of the cDNA probes for Southern blot analysis. A vertical arrow shows *AML1-USP42* breakpoint.

was confirmed by PCR analysis using primers AML1-GNM8-4S and LAF4-GNM11-2AS (Figure 4b); however, no 5′-*LAF4-AML1*-3′ product was generated, suggesting interstitial deletion near genomic break points (Figure 4b). These sequences near the break points did not contain any lymphoid heptamer/nonamer sequences, *Alu* sequences or consensus topoisomerase II cleavage sites.

Discussion

In this study, we identified that *LAF4* was fused to *AML1* in pediatric T-ALL with t(2;21)(q11;q22). Other regions with chromosomal aberrations in this patient were not considered to be associated with recurrent cytogenetic changes involving T-ALL, except for the deletion of the short arm of chromosome 9. Spectral

karyotyping analysis detected del(9)(p13), and additional analysis of genome array (Human Mapping 50 K Hind Array, Affymetrix, Tokyo, Japan) revealed homozygous deletion of 4.5 Mb within the 9p21 region, including the *CDKN2A/p16/p14* locus (data not shown), which is frequently deleted in T-ALL (Ohnishi *et al.*, 1995).

Although the patient showed a complex chromosomal abnormality, t(2;21)(q11;q22) can form regular head-to-tail fusion transcripts of both *AML1* and *LAF4*, because the transcription direction of *AML1* and *LAF4* is telomere to centromere. Furthermore, fluorescence *in situ* hybridization analysis revealed two der(2)t(2;21)(q11.2;q22) creating 5′-*AML1-LAF4*-3′, suggesting that 5′-*AML1-LAF4*-3′ is critical for leukemogenesis.

LAF4 was previously reported to be a fusion partner of *MLL* in pediatric B-precursor ALL with t(2;21)(q11;q23) (von Bergh *et al.*, 2002; Bruch *et al.*, 2003; Hiwatari *et al.*, 2003). *LAF4* is the first gene fused to both *AML1* and *MLL*, and both *AML1-LAF4* and *MLL-LAF4* contained the same domains of *LAF4* (Figure 5). During the preparation of this manuscript, we found another pediatric T-ALL patient with *AML1-LAF4* reported in the Meeting Abstract (Abe *et al.*, *Blood* (ASH Annual Meeting Abstracts) 2006; **108**: 4276), suggesting that t(2;21)(q11;q23) is a recurrent cytogenetic abnormality and that the *AML1-LAF4* fusion gene is associated with the T-ALL phenotype. Both putative fusion proteins of AML1-LAF4 observed in two patients contained the Runt domain of AML1, and the transactivation domain, nuclear localization sequence and C-terminal homology domain of LAF4, although the fused exon of *LAF4* differed in the two cases. Several studies have reported that the fusion partners of *MLL* fused with different genes such as *MLL-AF10* and *CALM-AF10*, *MLL-CBP* and *MOZ-CBP* or *MLL-p300* and *MOZ-p300* (Ida *et al.*, 1997; Taki *et al.*, 1997; Chaffanet *et al.*, 2000). Comparison of the structure and function between *AML1-LAF4* and *MLL-LAF4* will facilitate our understanding of the molecular mechanisms underlying *AML1*- and *MLL*-related leukemia.

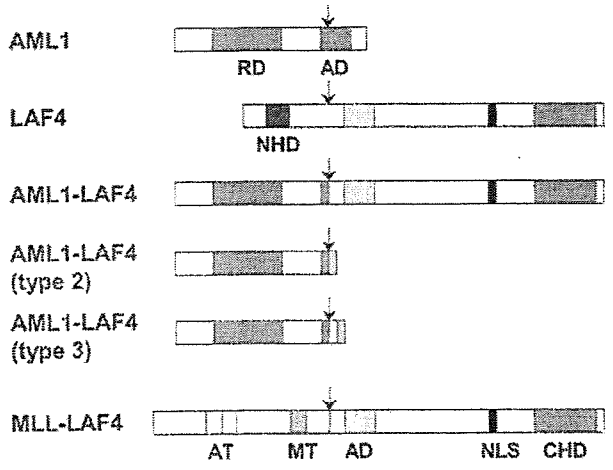

Figure 5 Schematic representation of putative AML1, LAF4 and AML1-LAF4 fusion proteins. Putative MLL-LAF4 fusion protein is also indicated for comparison. Arrows, break points or fusion points; AD, transactivation domain; AT, AT hooks; CHD, C-terminal homology domain; DNA, methyltransferase homology region; RD, RUNT domain; MT, DNA methyltransferase homology region; NLS, nuclear localization sequence.

The only *AML1* fusion partners in T-ALL are *LAF4* and *FGA7*. It is not known how *FGA7* is associated with T-ALL leukemogenesis, because *FGA7* does not show any significant sequence homology to any known protein motifs and/or domains (Mikhail *et al.*, 2004). Both patients with *AML1-LAF4* and *MLL-LAF4* fusions were diagnosed as having ALL, but they have different lymphoid lineages. *MLL-LAF4* is associated with B-lineage ALL; however, *AML1-LAF4* generates T-ALL. Our previous study showed that *LAF4* was expressed not only in B-lineage ALL but also in T-lineage ALL cell lines (Hiwatari *et al.*, 2003). *LAF4* showed strong sequence similarity to *AF4* (Ma and Staudt, 1996), which has a role in the differentiation of both B and T cells in mice (Isnard *et al.*, 2000). Furthermore, it was reported that *AML1* also plays an important role in T- and B-cell differentiation, because *AML1*-deficient bone marrow increased defective T- and B-lymphocyte development (Ichikawa *et al.*, 2004). These findings support that both *AML1* and *LAF4* are associated with T-ALL, respectively. Further functional analysis of the *AML1-LAF4* fusion gene will provide new insights into the leukemogenesis of *AML1*-related T-ALL. Recently, it has been reported that C-terminal truncated AML1-related fusion proteins play critical roles in leukemogenesis (Yan *et al.*, 2004, Agerstam *et al.*, 2007), suggesting that the two additional types of fusion transcripts observed in our patient (types 2 and 3 in Figures 3d and 5) have an additional function in leukemogenesis other than that of the entire AML1-LAF4 fusion protein.

In this study, we first applied the panhandle PCR method, which is usually used for cloning the fusion partners of *MLL* or *NUP98* (Megonigal *et al.*, 2000; Taketani *et al.*, 2002); however, no fusion transcripts could be obtained. Therefore, we searched for another method to clone the fusion transcripts and adapted the bubble PCR method for cDNA cloning. To date, bubble PCR has been performed for cloning unknown genomic fusion points but not fusion cDNAs (Zhang *et al.*, 1995). Using double-stranded cDNA, we could apply the bubble PCR method for cloning fusion cDNA with fewer nonspecific products. The bubble PCR primer can only prime DNA synthesis after a first-strand cDNA has been generated by an *AML1*-specific primer because of the bubble-tag with an internal non-complementary region (Zhang *et al.*, 1995). Although bubble PCR for genomic DNA generated one or two amplification products (Smith, 1992), bubble PCR for cDNA generated a complex set of amplification products that appeared as a smear by SYBR green staining, suggesting that a random hexamer generated various double-stranded cDNA containing the *AML1* sequence. This means that various fusion points can be estimated, even if after bubble oligo ligation was generated. Furthermore, bubble PCR for cDNA could amplify in both 5′–3′ and 3′–5′ directions of the gene or transcript, and easily

Table 1 Comparison between bubble PCR and panhandle PCR

Characteristics	*Bubble PCR*	*Panhandle PCR*
Available orientation of fusion transcript	5′–3′, 3′–5′	Only 5′–3′
AML1-specific random hexamer[a]	Not necessary	Necessary
Self-annealing	Not necessary	Necessary
Number of required polymerase reaction	2	4
Number of final products	Many (smear)	A few
Nonspecific product	Few	Few
Number of extra sequences other than targeted sequences in cloned product	50–60 bp	> 100 bp
Search for other targeted exons	Easy	Hard[b]

[a]30-mers *AML1*-specific oligonucleotide with random hexamer (AML1-N). [b]Necessary to use another *AML1*-specific random hexamer if the target exons are 5′ region of the initial target.

handle any exons fused to unknown partners for amplification. Once-ligated cDNAs are also available for cloning any genes, other than *AML1*, as the target. We demonstrated the efficiency and specificity of bubble PCR for cDNA (Table 1 and Supplementary Figure S2).

To date, a great number of fusion genes associated with chromosomal translocations have been cloned, although these fusion genes are found as a minor part of various malignancies. Recently, high frequencies of mutations in *NOTCH1* in T-ALL (James *et al.*, 2005), *NPM* in AML with normal karyotype (Weng *et al.*, 2004) and *JAK2* in myeloproliferative disorders (polycythemia vera, essential thrombocythemia and idiopathic myelofibrosis) (James *et al.*, 2005) have been reported, and these mutations are considered to be a good target for therapy. These genes were first identified as associated with chromosomal translocations in a small subset of specific phenotypes of hematologic malignancies (Ellisen *et al.*, 1991; Morris *et al.*, 1994; Lacronique *et al.*, 1997). These findings suggest that continuing attempts to identify genes associated with chromosomal translocations can be expected to provide further insights into the significance of various gene alterations in cancer and the development of novel-targeted therapies (Taki and Taniwaki, 2006). The bubble PCR method for cDNA will contribute to identifying numerous novel translocation partners more easily and further functional analysis of chimeric transcripts.

Materials and methods

Spectral karyotyping analysis
Spectral karyotyping analysis was performed with a Sky-Painting kit (Applied Spectral Imaging, Migdal Ha'Emek, Israel). Signal detection was performed according to the manufacturer's instructions.

Fluorescence in situ *hybridization analysis*
Fluorescence *in situ* hybridization analysis of the patient's leukemic cells using *AML1*-specific BAC clones (RP11-272A3, 3′ of *AML1* and RP11-994N6, 5′ of *AML1*) was carried out as described previously (Taniwaki *et al.*, 1994). Fusion of *AML1* and *LAF4* was analysed with the patient's leukemic cells using RP11-994N6 (5′ of *AML1*) and RP11-527J8 (3′ of *LAF4*).

Bubble PCR for cDNA
We modified the original bubble PCR method to apply for cDNA cloning (Figure 2; Supplementary Figure S2) (Smith, 1992; Zhang *et al.*, 1995).

Poly(A)$^+$ RNA was extracted from the patient's leukemic cells using a QuickPrep Micro mRNA Purification Kit (GE Healthcare, Buckinghamshire, UK). Two hundred nanograms of poly(A)$^+$ RNA was reverse transcribed to cDNA in a total volume of 33 μl with random hexanucleotide using the Ready-To-Go You-Prime First-Strand Beads (GE Healthcare). Double-stranded cDNAs were synthesized from 10 μl of single-stranded cDNA with a phosphorylated random hexanucleotide, blunt ended with T4 DNA polymerase, digested with *Rsa*I endonuclease and ligated with bubble oligo. *Rsa*I, a 4-bp blunt-ended cutter, was chosen to shorten the bubble oligo-ligated fragments, so that almost all bubble oligo-ligated fragments would be easy to clone by standard PCR reaction. This suggests that poor-quality samples are also suited to this method, although it is unsuitable for cloning long products.

The sequences of the primers used are listed in Supplementary Table S1 and their positions in the *AML1* gene are shown in Supplementary Figure S2. Nested PCR was performed using primers NVAMP-1 (bubble oligo) and AML1-5S (exon 5) for first round PCR, and NVAMP-2 (bubble oligo) and AML1-E6S (exon 6) for nested PCR. NVAMP1 and NVAMP2 can only anneal to the newly synthesized unique sequence of the bubble oligo by AML1-5S.

We used poly(A)$^+$ RNA in bubble PCR for cDNA with the expectation that this approach could amplify fewer transcripts; however, total RNA is also suitable for this method.

Bubble PCR for genomic DNA
Bubble PCR for genomic DNA was performed as described previously (Smith, 1992; Zhang *et al.*, 1995). Primers were as follows: NVAMP-1 and AML1-GNM8S for first round PCR, and NVAMP-2 and AML1-GNM8-2S for second round PCR (Supplementary Table S1).

Reverse transcription–PCR and genomic PCR analyses
Reverse transcription–PCR and genomic PCR analyses were performed as described previously. After 35 rounds of PCR (30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C), 5μl of PCR product were electrophoresed in a 3% agarose gel. Primers were as follows: AML1-PR7 and LAF4-11AS, and AML1-PR8 and LAF4-PR5 for reverse transcription-PCR; and AML1-GNM8-4S and LAF4-GNM11-2AS, and LAF4-GNM11-2S and AML1-GNM8-2AS for genomic PCR (Supplementary Table S1).

Nucleotide sequencing
Nucleotide sequences of PCR products and, if necessary, subcloned PCR products were analysed as described previously (Hiwatari *et al.*, 2003).

Southern blot analysis
High-molecular-weight DNA was extracted from the patient's leukemic cells by proteinase K digestion and phenol/chloroform extraction. DNA (10 μg) was digested with *Bgl*II, subjected to electrophoresis on 0.7% agarose gel and transferred to a nylon membrane. Blots were hybridized to probes that were labeled by the Dig-labeled PCR method according to the manufacturer's instructions (Roche Applied Science, Tokyo, Japan). Probes

were 112bp *AML1* cDNA fragments (AML1c1, nucleotides 1233–1344; GenBank accession no. NM_001754).

Abbreviations

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia.

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

*Tomonori Hidaka,[1,2] *Shingo Nakahata,[1] *Kinta Hatakeyama,[3] Makoto Hamasaki,[1,4] Kiyoshi Yamashita,[2] Takashi Kohno,[5] Yasuhito Arai,[6] Tomohiko Taki,[7] Kazuhiro Nishida,[7] Akihiko Okayama,[8] Yujiro Asada,[3] Ryoji Yamaguchi,[9] Hirohito Tsubouchi,[2,10] Jun Yokota,[5] Masafumi Taniwaki,[7] Yujiro Higashi,[11] and Kazuhiro Morishita[1]

[1]Division of Tumor and Cellular Biochemistry, Department of Medical Sciences, [2]Department of Internal Medicine II, University of Miyazaki, Miyazaki; [3]First Department of Pathology, Faculty of Medicine, University of Miyazaki, Miyazaki; [4]Miyazaki Prefectural Industrial Foundation, Miyazaki; [5]Biology Division, National Cancer Center Research Institute, Tokyo; [6]Cancer Genome Project, National Cancer Center Research Institute, Tokyo; [7]Department of Hematology and Oncology, Kyoto Prefectural University of Medicine, Kyoto; [8]Department of Rheumatology, Infectious Diseases and Laboratory Medicine, University of Miyazaki, Miyazaki; [9]Department of Veterinary Pathology, University of Miyazaki, Miyazaki; [10]Department of Digestive and Life-style related Disease, Kagoshima University Graduate School of Medicine and Dental Sciences, Kagoshima; and [11]Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan

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 β1 (TGF-β1), a well-known characteristic of ATLL cells, suggesting that escape from TGF-β1–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, EVI1,* 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 030751[11]), *AREB6, ZFHEP, NIL-2A, ZFHX1A, NIL-2-A, MGC133261,* or

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*T.H., S.N., and K.H. contributed equally to this paper.

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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-β1 resistance, a well-known characteristic of ATLL cells, suggesting that escape from TGF-β1–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 published[24] 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 × 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′-CCUGUGGAUUAUGAGUUCA-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-β1; R&D Systems, Minneapolis, MN) for 72 hours and counted by the methyl thiazolyl tetrazolium (MTT) assay

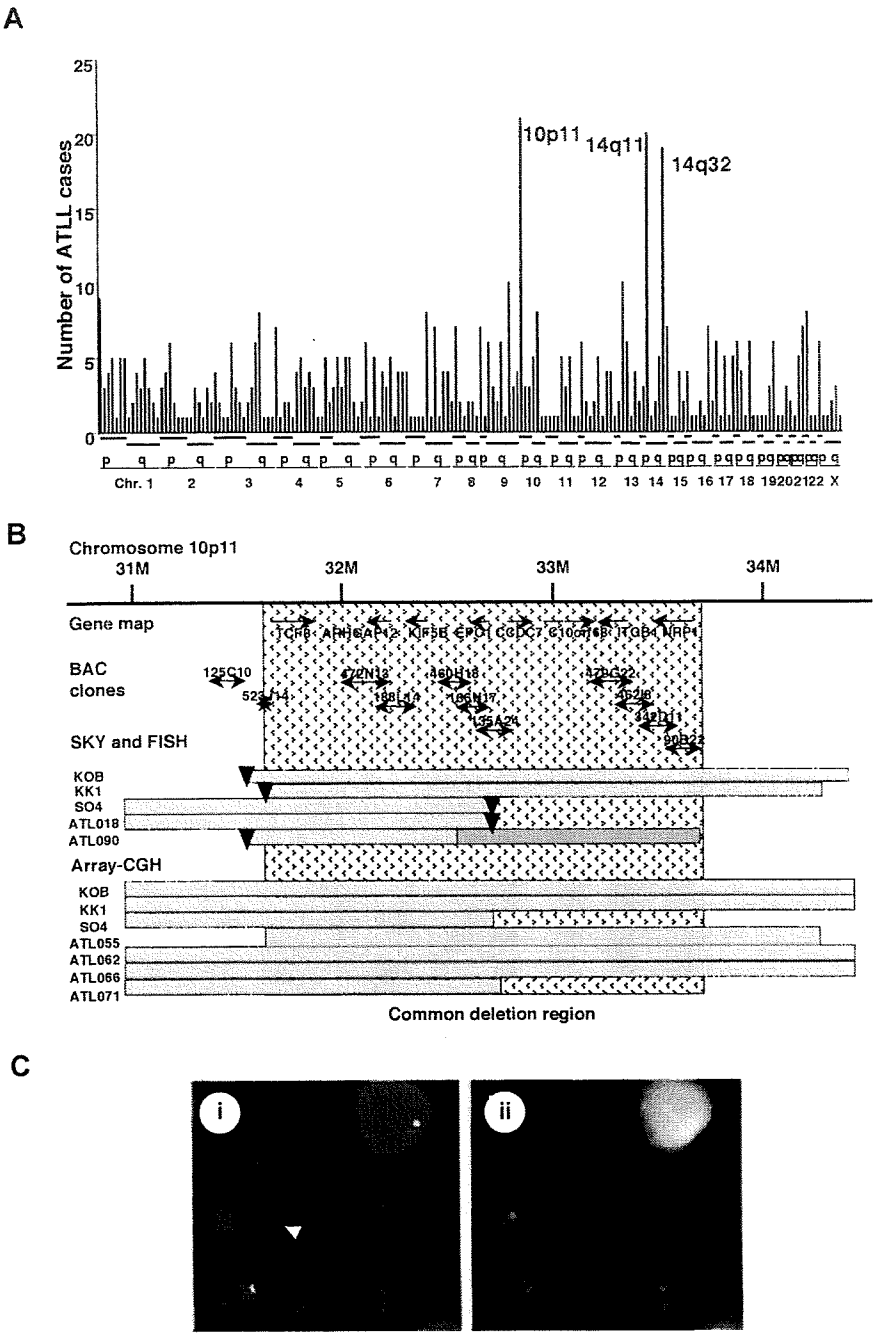

Figure 1. Mapping of the deletions at 10p11.2. (A) Mapping of the chromosomal breakpoints in whole chromosomes in acute-type ATLLs. An analysis of the chromosomal breakpoints was performed by spectral karyotyping (SKY), and all chromosomal breakpoints were mapped in each region of the chromosomes (x-axis), 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 1[26]) 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 1[26]). (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)

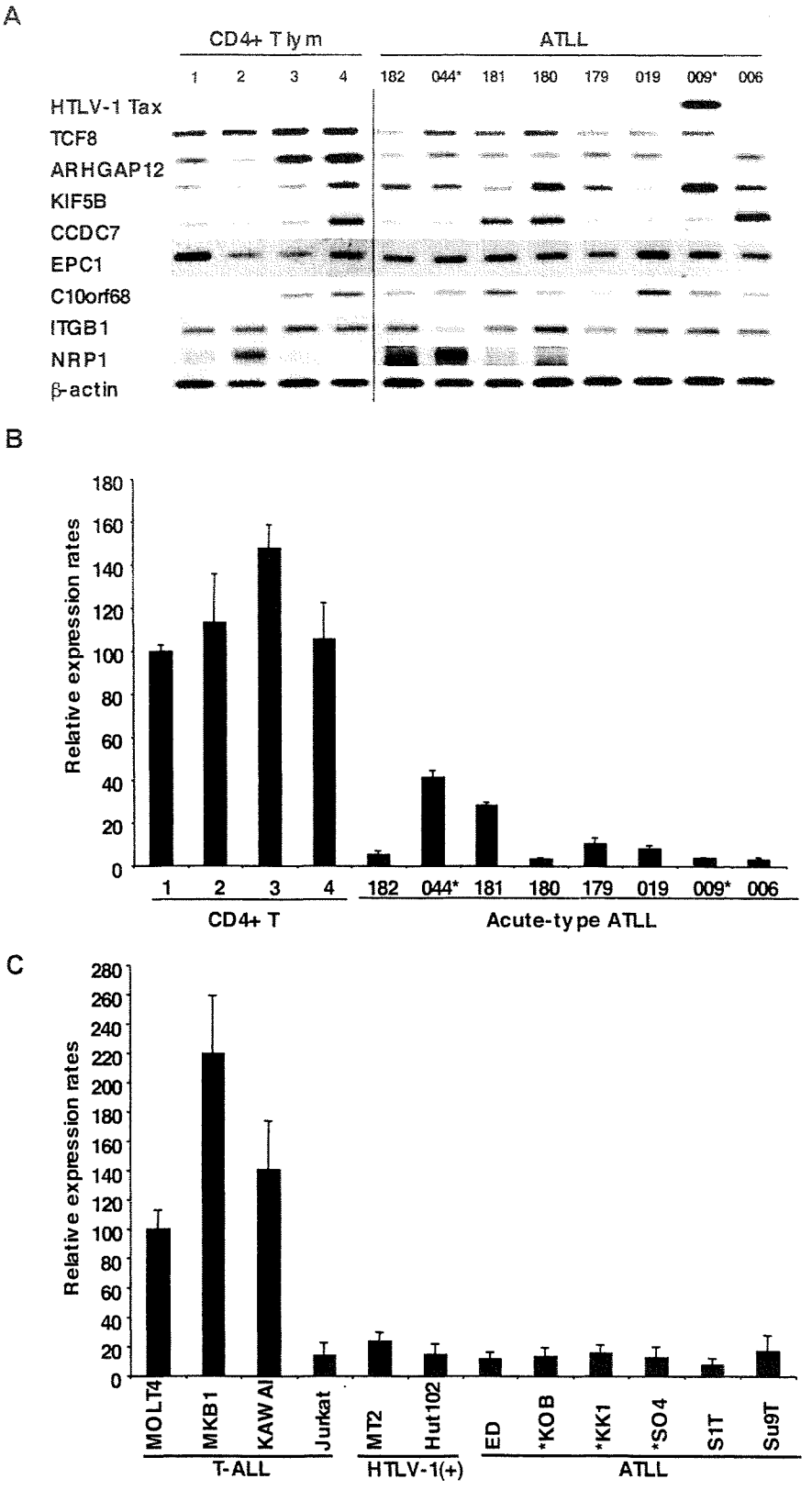

Figure 2. Down-regulated expression of *TCF8* in ATLL cells. (A) The expression profiles of the genes mapped within the deletion region at 10p11. Semiquantitative reverse-transcription PCR (RT-PCR) was performed to determine the expression of the genes mapped within the deletion region. *TCF8, ARHGAP12, 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 (ΔCT) 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 ± SD	TSA, fold ± SD	5-Aza-dC+TSA, fold ± SD
MOLT4	T-ALL	None	None	1.25 ± 0.46	1.65 ± 0.21	1.65 ± 0.22
Jurkat	T-ALL	None	None	11.17 ± 0.59*	10.04 ± 1.74*	3.07 ± 0.54*
MT2	HTLV-1 (+)	None	None	3.38 ± 1.17*	8.25 ± 1.76*	4.58 ± 0.90*
HUT102	HTLV-1 (+)	None	255A>C Asn78Thr	4.39 ± 0.49*	6.94 ± 0.16*	8.03 ± 1.05*
ED	ATLL	None	None	4.32 ± 1.57*	7.48 ± 0.91*	3.93 ± 0.21*
KOB	ATLL	10p del	None	3.26 ± 1.12*	2.32 ± 0.81	2.08 ± 0.54
KK1	ATLL	10p del	None	9.92 ± 0.45*	6.76 ± 0.17*	9.72 ± 0.35*
SO4	ATLL	10p del	None	1.40 ± 0.33	1.57 ± 0.18	0.59 ± 0.06
S1T	ATLL	None	None	3.66 ± 0.21*	3.95 ± 0.75*	2.32 ± 1.63
Su9T	ATLL	None	None	3.91 ± 0.45*	9.66 ± 2.38*	1.42 ± 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 ATL090, 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 ATL090, 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 ATL090, 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 ATL044 and monosomy 10 in ATL009 (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 μM 5-aza-2′-deoxycytidine (5-aza-dC), a DNA demethylating agent, for 72 hours, (2) 1.2 μM trichostatin A (TSA), an inhibitor of histone deacetylase, for 48 hours, or (3) 1.2 μM TSA for 48 hours following culture with 10 μM 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

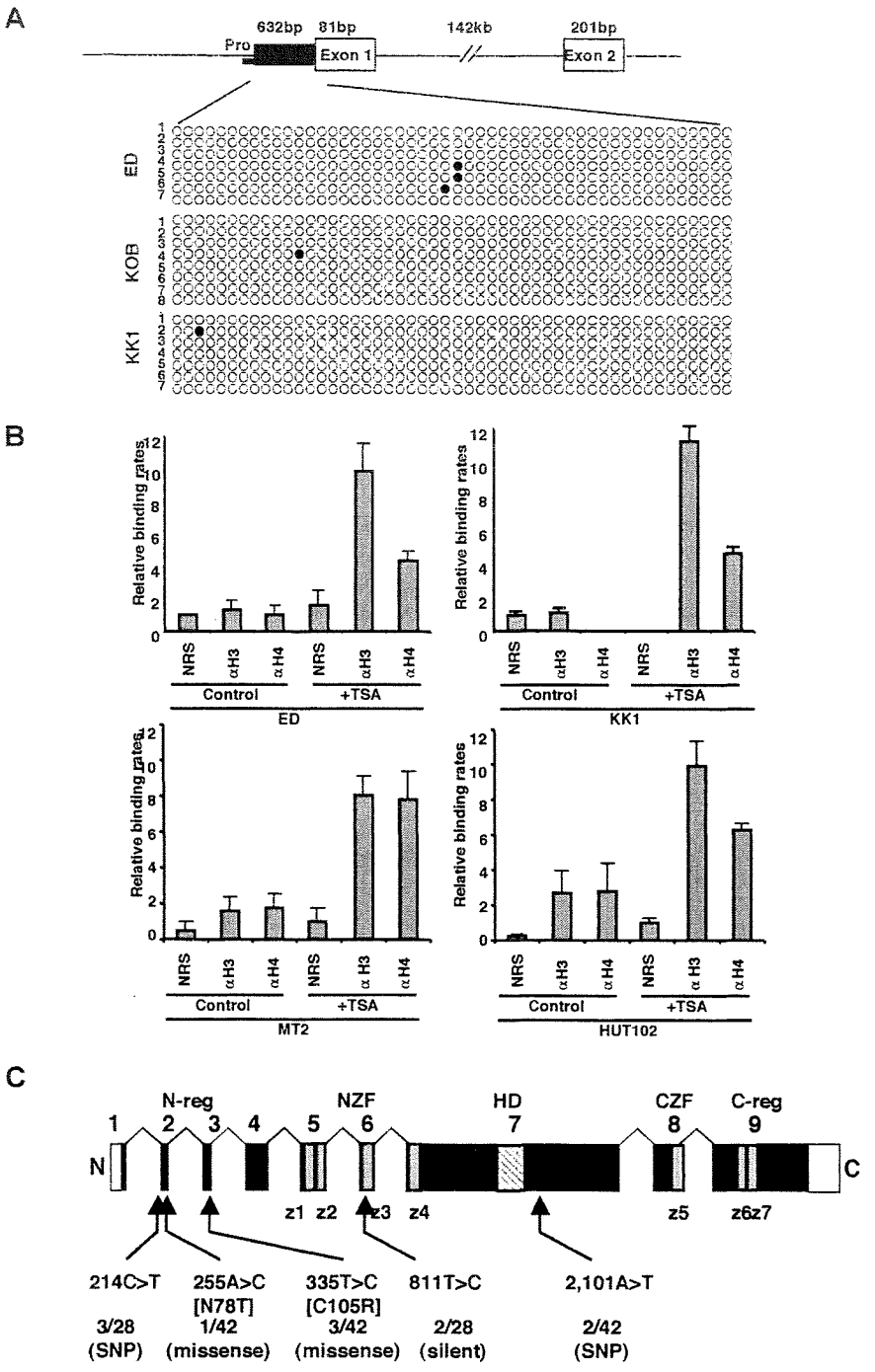

Figure 3. Genetic and epigenetic abnormalities of the *TCF8* gene in ATLL cells. (A) Bisulfite genomic sequencing of the *TCF8* promoter region in 3 ATLL cell lines: ED, KOB, and KK1. PCR products amplified from bisulfite-treated DNA were subcloned, and 8 clones in each cell line were sequenced. ○ indicate 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 (αH3 or αH4). 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 μM 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$EF1^{\Delta C\text{-}fin}$ 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 × 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

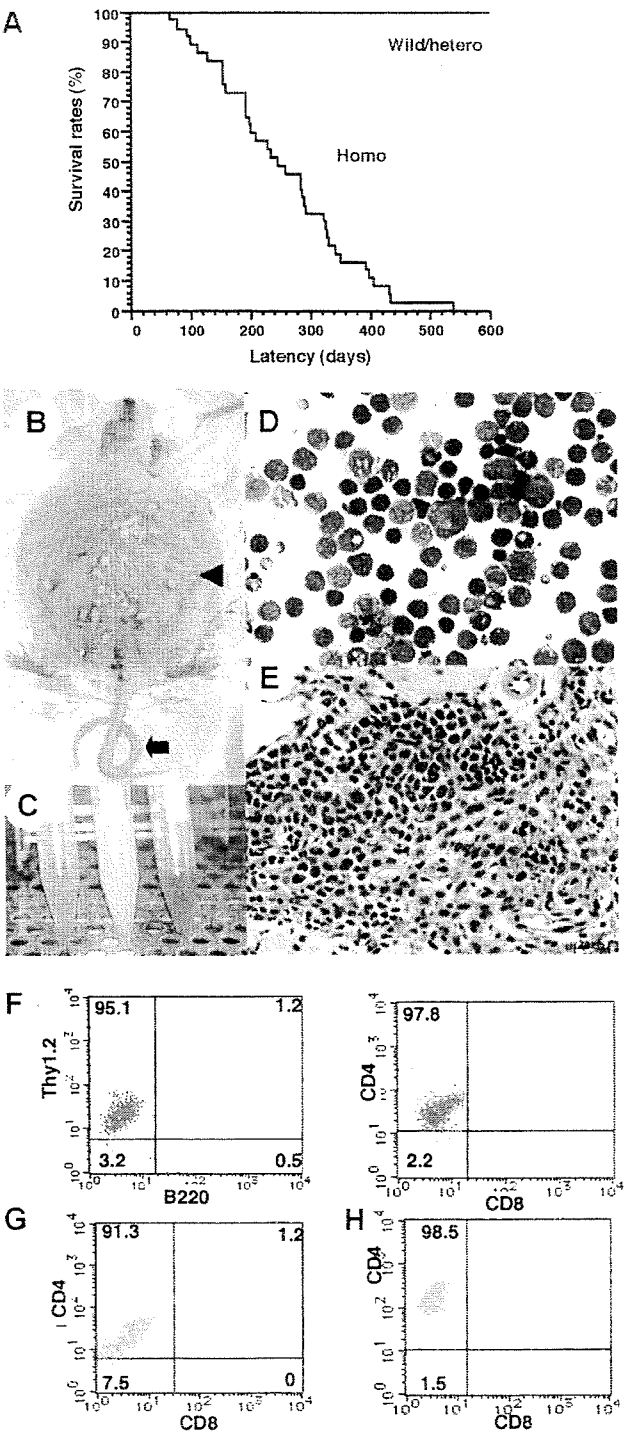

Figure 4. Survival rates and pathologic findings in *TCF8* mutant mice. (A) The survival rates of a cohort of wild-type (wild), *TCF8* heterozygous (hetero), and *TCF8* homozygous (homo) mutant mice were followed over the indicated period using Kaplan-Meier plots. (B) Gross photograph of *TCF8* mutant mice with ascites (◀). Approximately 30% of *TCF8*-homologous mutant mice showed curled tail (◆). (C) Bloody or milky ascites was pooled. (D) May-Giemsa staining of tumor cells in ascites. Original magnification ×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 aquired using AxioCam camera and AxioVision 2.05 software (Carl Zeiss). Original magnification ×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.

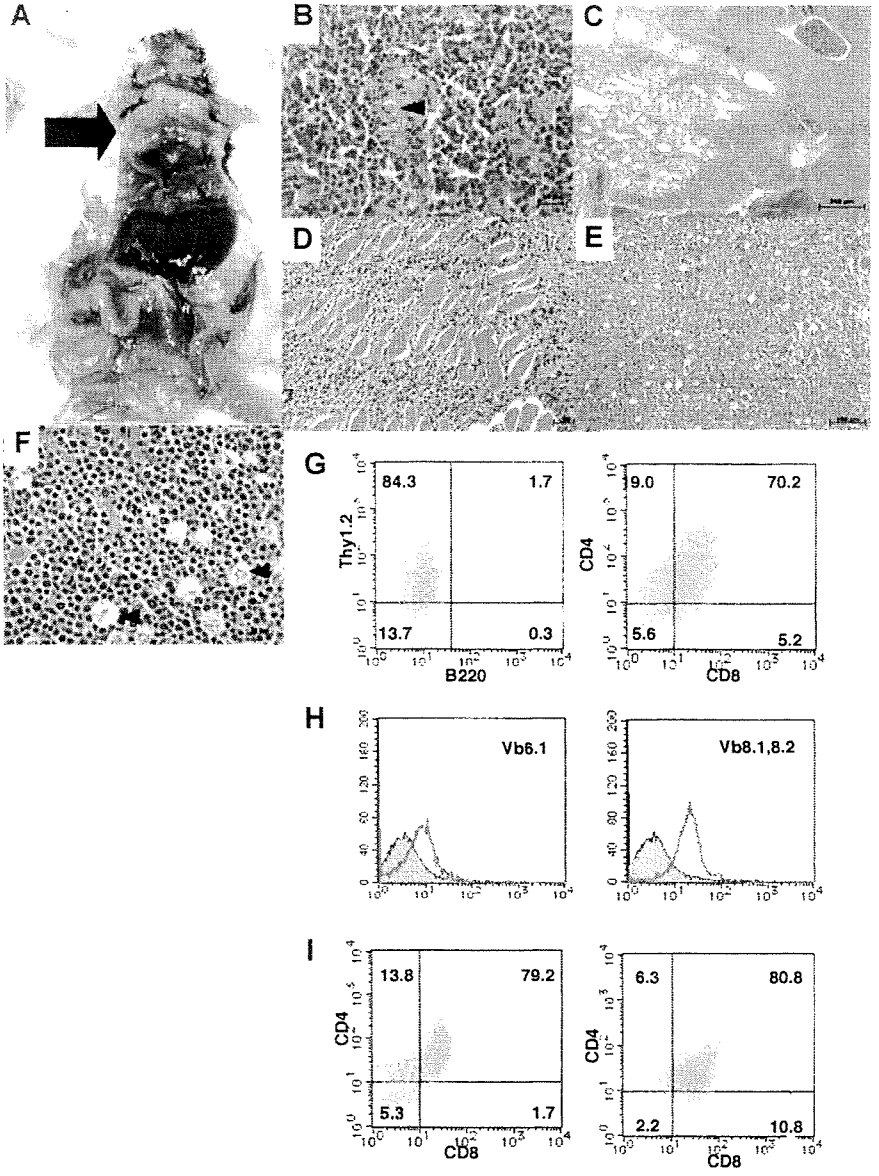

Figure 5. Pathological findings of *TCF8* mutant mice. (A) Gross autopsy of *TCF8* mutant mice with thymic tumors. A large thymic tumor (➡) was observed at the mediastinum of the dissected mouse. (B) Hematoxylin and eosin staining of tumor sections from the mouse as indicated. The normal thymic cellular architecture in the *TCF8* mutant mice is replaced with monotonous fields of large, highly mitotic lymphoblasts with small Hassall bodies (◀). The scale bar indicates 20 μm. Original magnification ×400. (C) The tumor cells invaded the lung, vascular tissues, and heart in the mouse. The scale bar indicates 500 μm. Original magnification ×25. (D) The tumor cells invaded the muscular tissues of the chest wall. The scale bar indicates 50 μm. Original magnification ×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") (◀). The scale bar indicates 100 μm. Original magnification ×100. (F) Hematoxylin and eosin staining of peripheral lymph nodes. The scale bar indicates 20 μm. Original magnification ×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 Vβ6.1 TCR (left), but showed weak expression of Vβ8.1-8.2 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 $CD4^+$ SP T 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 Vβ6.1 TCR and weakly positive for Vβ8.1-8.2 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. $CD8^+$ SP T 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-β1 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-β1, even with high levels of TGF-β1 expression,[35-37] suggesting that ATLL cells may have developed several mechanisms of resistance to escape the antiproliferative and inactivating signal mediated by TGF-β1, including Tax through activation of the JNK/c-Jun pathway[38,39] or MEL1S expression.[37] Since TCF8 is reported to synergize with Smad

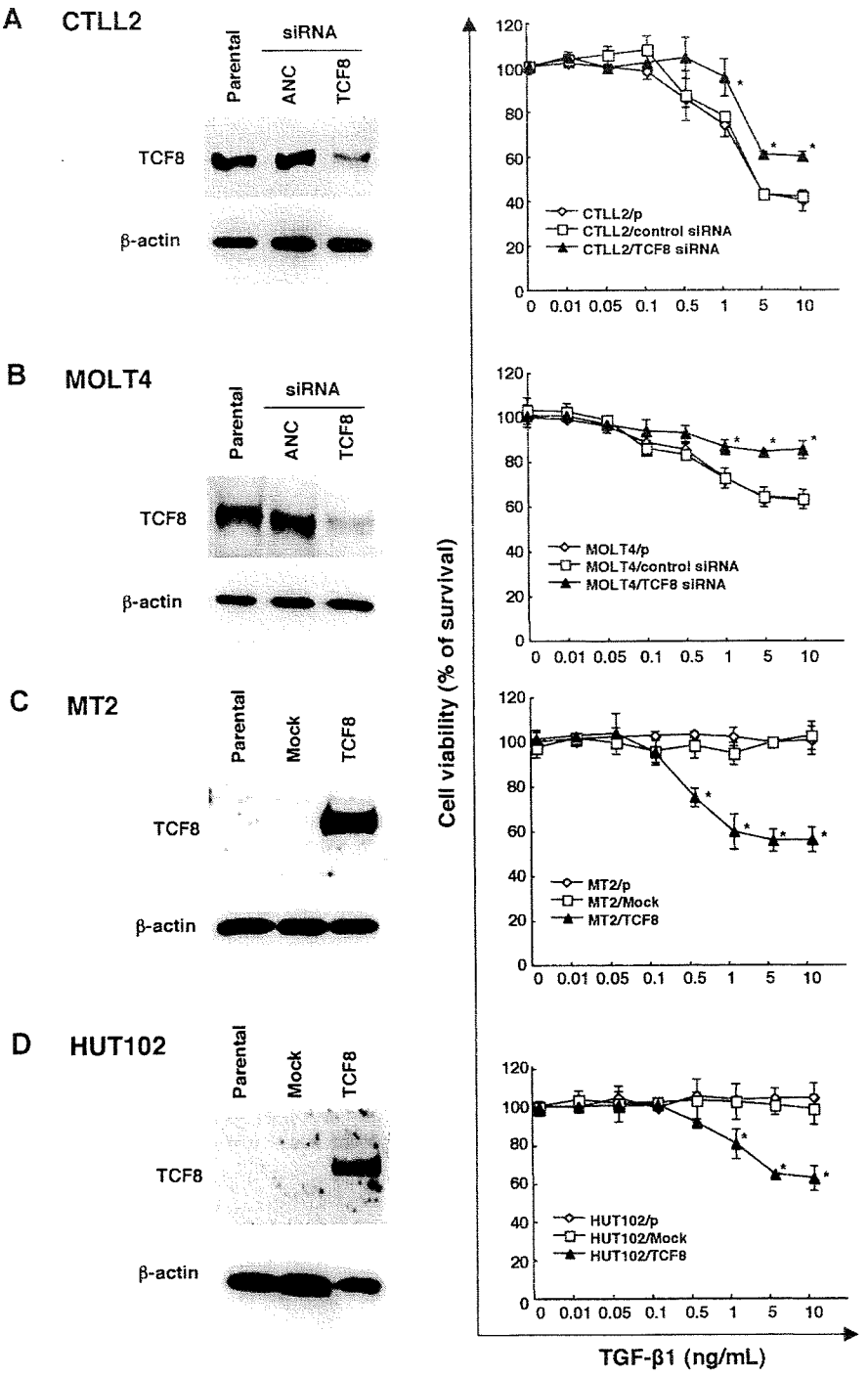

Figure 6. TGF-β1 responsiveness in various leukemia cell lines with the up- or down-regulation of TCF8 expression. (A,B) The down-regulation of the TCF8 protein by *TCF8* siRNA. The CTLL2 (A) and MOLT4 (B) cell lines were transfected with either *TCF8* or the AllStars Negative Control (ANC) siRNAs and 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-β1 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-β1–free culture (right panel). A ◇ represents parental cells, □ represents cells treated with ANC siRNA, and ▲ 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-β1 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-β1–free culture (right panel). Parental cells (◇), mock vector-transfected cells (□), and TCF8 expression plasmid-transfected cells (▲). 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-β1 signal transduction,[40,41] we investigated whether the down-regulation of TCF8 expression was associated with resistance to TGF-β1–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-β1 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-β1 treatment in a dose-dependent manner, whereas the parental and transfectants with mock plasmid did not die with TGF-β1 treatment at all (Figure 6C,D). These results indicate that down-regulation of TCF8 expression is one of the mechanisms of TGF-β1 resistance in ATLL cells, suggesting that $CD4^+$ T lymphoma cells might escape from negative selection due to reduced TGF-β1 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 $\delta EF1^{\Delta C\text{-}fin}$ 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+ SP T cells was significantly reduced relative to the number of CD4+ SP T 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+ SP T cells or DP T cells after 6 months. Interestingly, TGF-β1 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-β1 responsiveness. Moreover, *TGF-β1*–deficient mice had an increased number of CD4+ SP T cells and a decreased number of CD8+ SP T 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-β1*–deficient mice, we concluded that leukemogenesis in *TCF8* mutant mice was partly dependent on resistance to TGF-β1.

TCF8 is an E-box–binding transcription factor reported to regulate many genes. We found that the transcription of *CD4, α4 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 investigation 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 DP T cells, which invaded various organs, such as the liver, spleen, and lungs. In ATLL, the tumor cells were mainly CD4+ SP T 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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Correspondence: Kazuhiro Morishita, Division of Tumor and Cellular Biochemistry, Department of Medical Sciences, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki, Japan, 889-1692; e-mail: kmorishi@med.miyazaki-u.ac.jp.

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