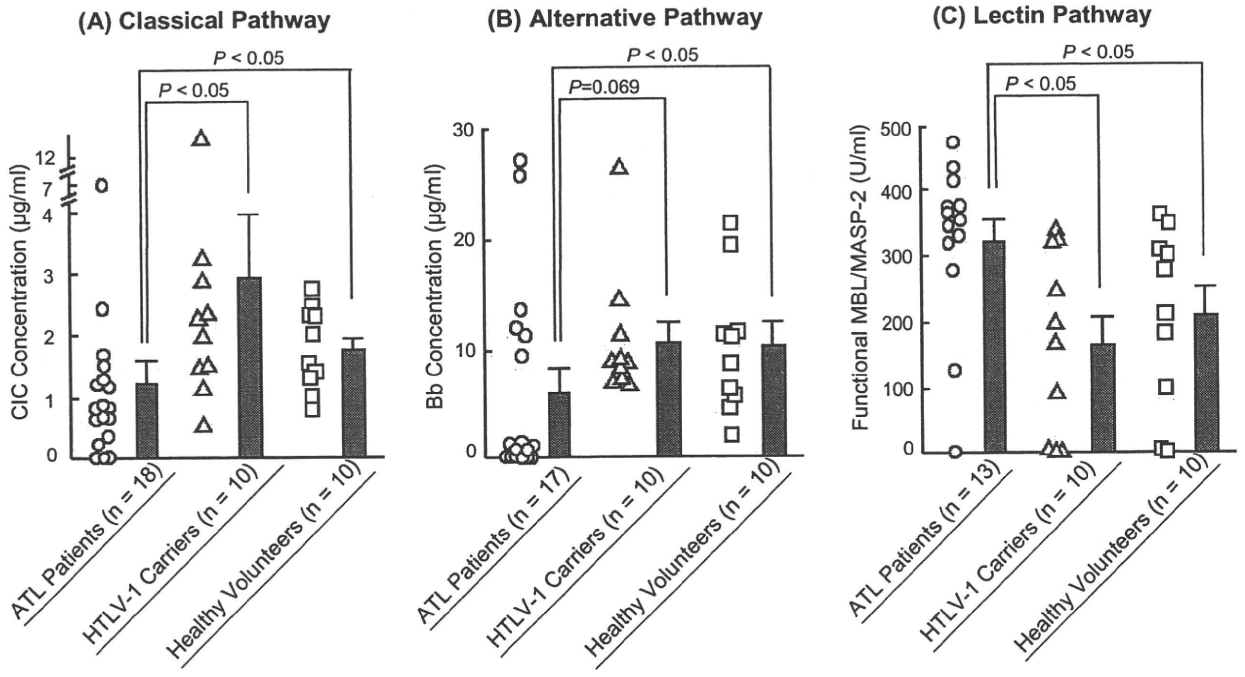

Fig. 6. Expression of markers specific to complement pathways in ATL patients. The concentration of CIC (A), Bb (B), and functional MBL/MASP-2 (C) in sera from ATL patients (○), HTLV-1 carriers (△), and healthy volunteers (□) was examined by C1q solid-phase enzyme immunoassay, Bb fragment enzyme immunoassay, and Hbt human MBL-C4 activation complex test kit, respectively. Columns represent the means ± SE. In the comparison of HTLV-1 carriers (△) and healthy volunteers (□), ATL patients (○) had elevated levels of functional MBL/MASP-2 (C), while low levels of CIC (A) and Bb (B).

The level of CIC as a marker of the classical pathway was lower in ATL. This was unexpected because CIC has been reported to be increased in various malignancies, including leukemia and lymphoma [46]. This difference between ATL and other malignancies is possibly associated with the metabolism of CIC. CIC is removed by phagocytosis of monocytes and macrophages [46,47]. Also, the production of phagocytosis-inducing factor (PIF) has been reported to be induced in T-cells transformed with HTLV-1 and ATL cells [48].While CIC was not affected in HTLV-1 carriers, maybe owing to the small number of HTLV-1-infected cells (Fig. 6A), the production of PIF may be increased with the appearance and abnormal proliferation of a malignant cell (ATL cell), and then CIC may be removed by the induced phagocytosis, resulting in a low level.

Similarly to CIC, Bb concentration as a marker of the alternative pathway was also lower in ATL patients (Fig. 6B). Bb is a cleavage product of factor B, which also requires non-cleaved C3 [23,24]. The increase of iC3b and C3f (Figs. 2 and 5), which are produced by C3 cleavage, is possibly accompanied by the decrease of non-cleaved C3. For this decrease, factor B may not be cleaved into Bb, leading to a low level of Bb in ATL.

Investigation into the relationship between the complement system and ATL development may lead to discovering diagnostic markers and novel target molecules for ATL chemotherapy. Studies are in progress, focusing on diagnosis using components of the complement system and triggers that activate the lectin pathway in ATL.

Conflict of interest statement

Disclosures: Yo-ichi Ishida – Grant/Research Support: the Collaboration of Regional Entities for the Advancement of Technological Excellence (CREATE) from the Japan Science and Technology Agency.

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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 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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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

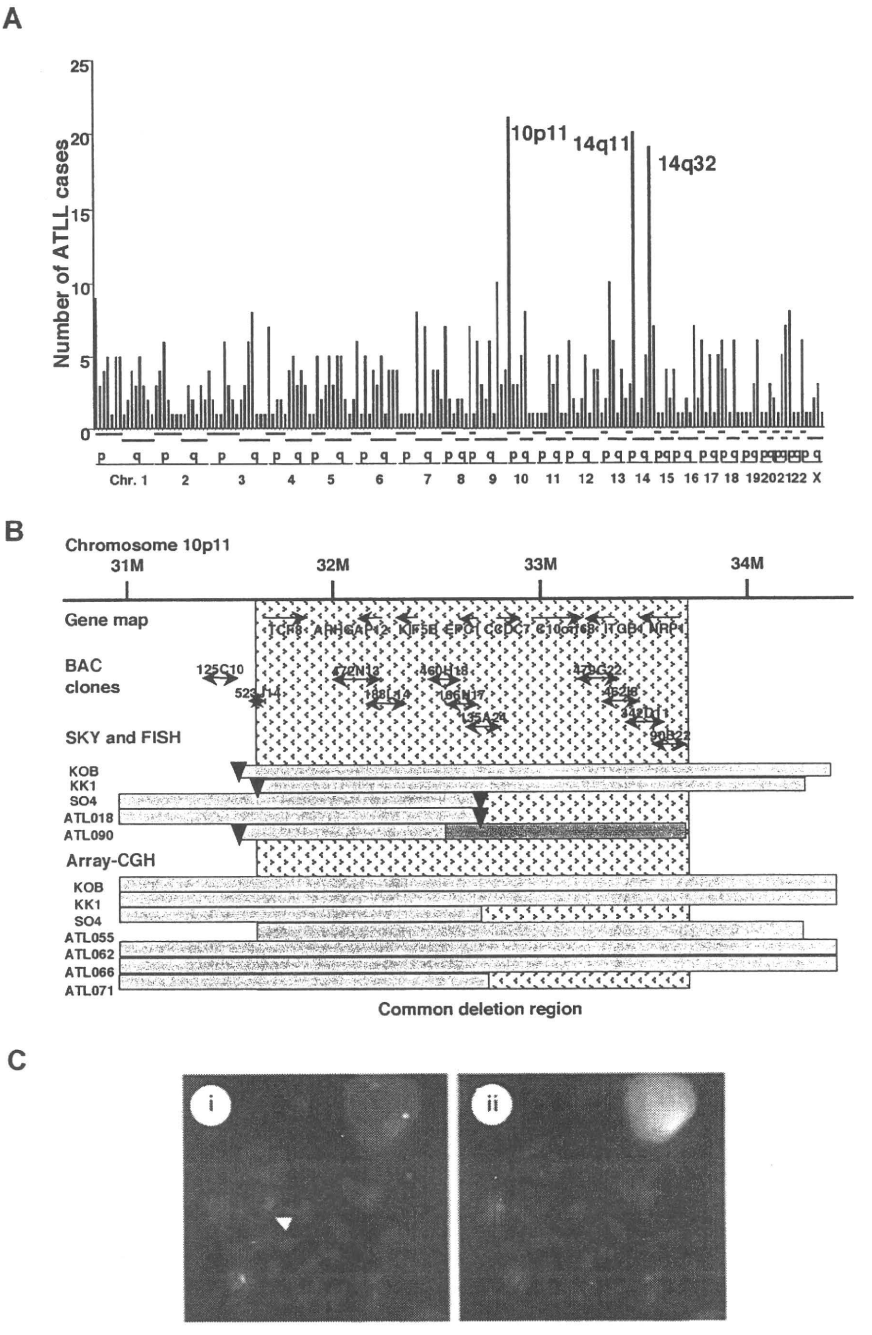

Figure 1. Mapping of the deletions at 10p11.2. (A) Mapping of the chromosomal breakpoints in whole chromosomes in acute-type ATLLs. An analysis of the chromosomal breakpoints was performed by spectral karyotyping (SKY), and all chromosomal breakpoints were mapped in each region of the chromosomes (x-axis),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

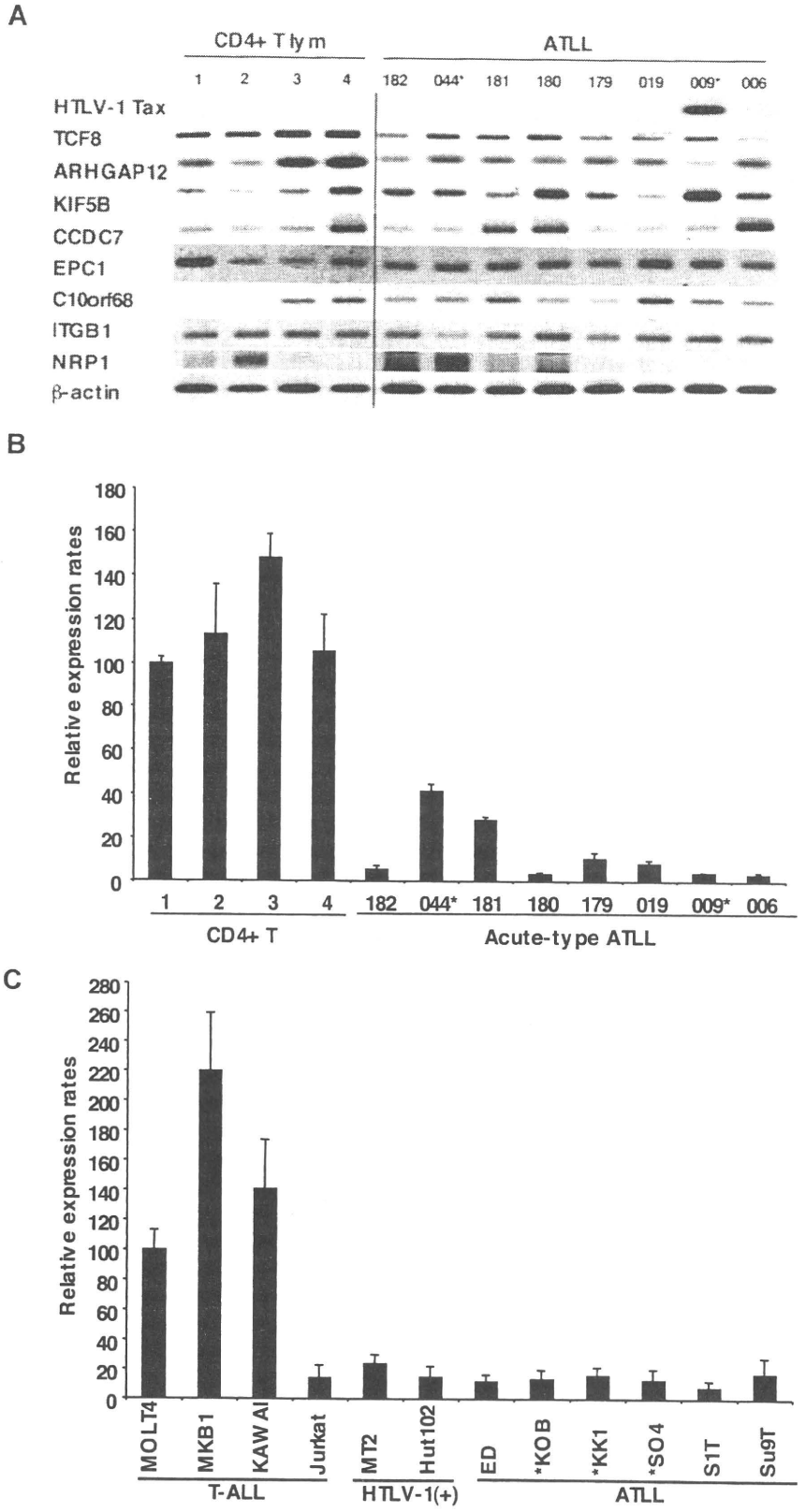

Figure 2. Down-regulated expression of *TCF8* in ATLL cells. (A) The expression profiles of the genes mapped within the deletion region at 10p11. Semiquantitative reverse-transcription PCR (RT-PCR) was performed to determine the expression of the genes mapped within the deletion region. *TCF8, ARHGAP12, 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

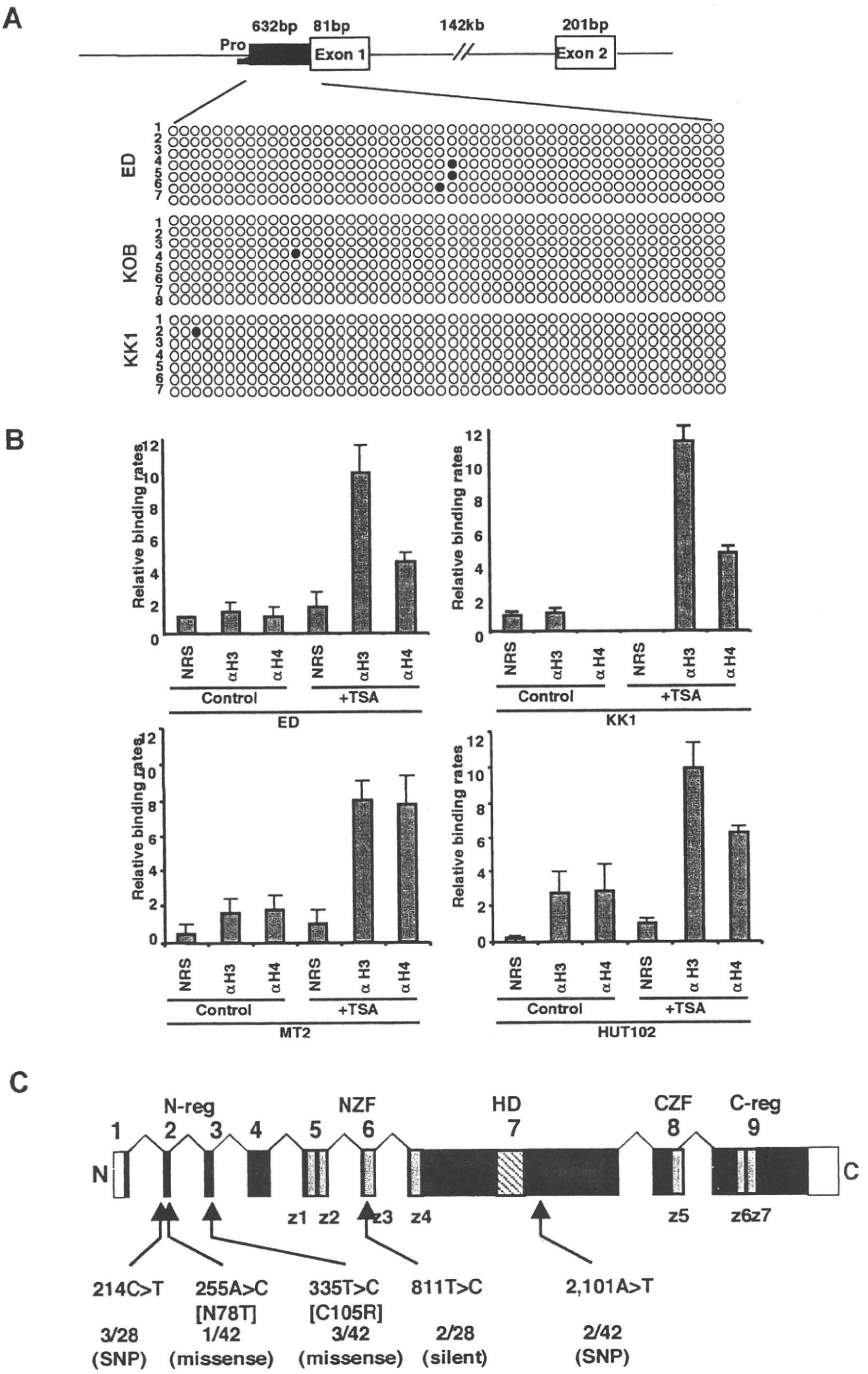

Figure 3. Genetic and epigenetic abnormalities of the *TCF8* gene in ATLL cells. (A) Bisulfite genomic sequencing of the *TCF8* promoter region in 3 ATLL cell lines: ED, KOB, and KK1. PCR products amplified from bisulfite-treated DNA were subcloned, and 8 clones in each cell line were sequenced. ○ indicate 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 *δEF1* (mouse homologue of *TCF8*) gene-targeted mutant mice, which lack the COOH-proximal zinc finger clusters ($\delta EF1^{\Delta C\text{-}fin}$ 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

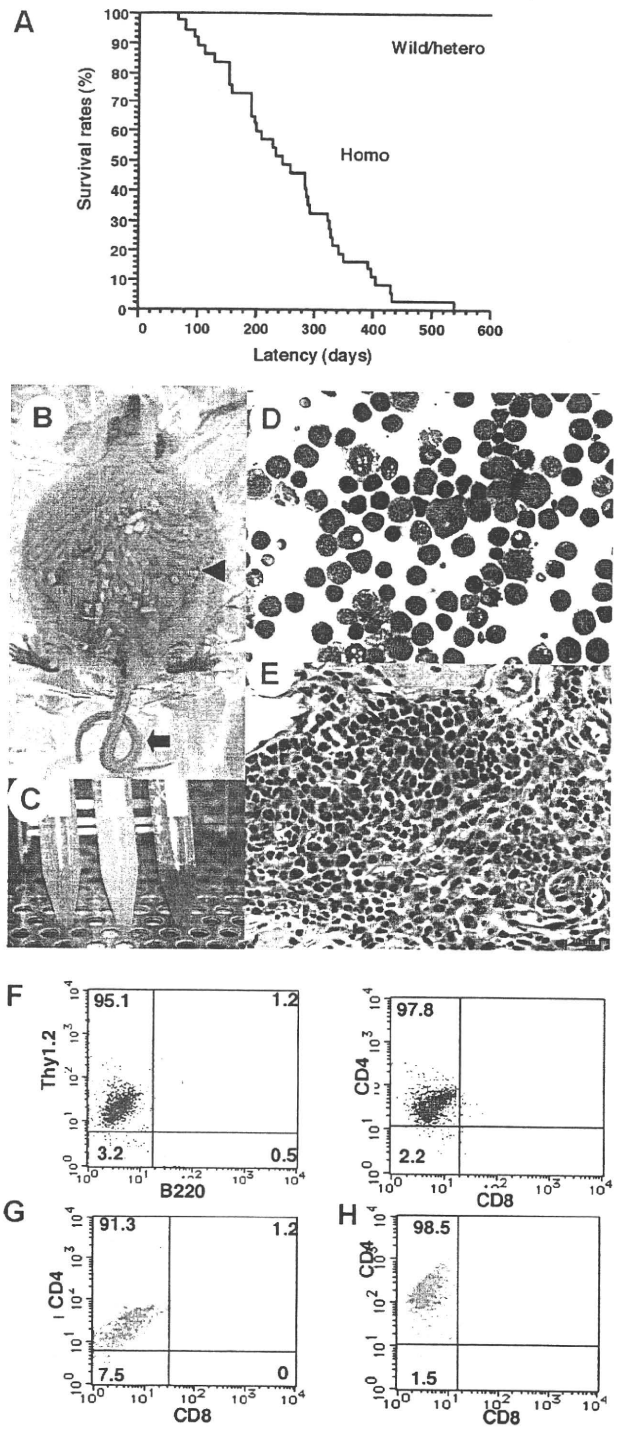

Figure 4. Survival rates and pathologic findings in *TCF8* mutant mice. (A) The survival rates of a cohort of wild-type (wild), *TCF8* heterozygous (hetero), and *TCF8* homozygous (homo) mutant mice were followed over the indicated period using Kaplan-Meier plots. (B) Gross photograph of *TCF8* mutant 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.

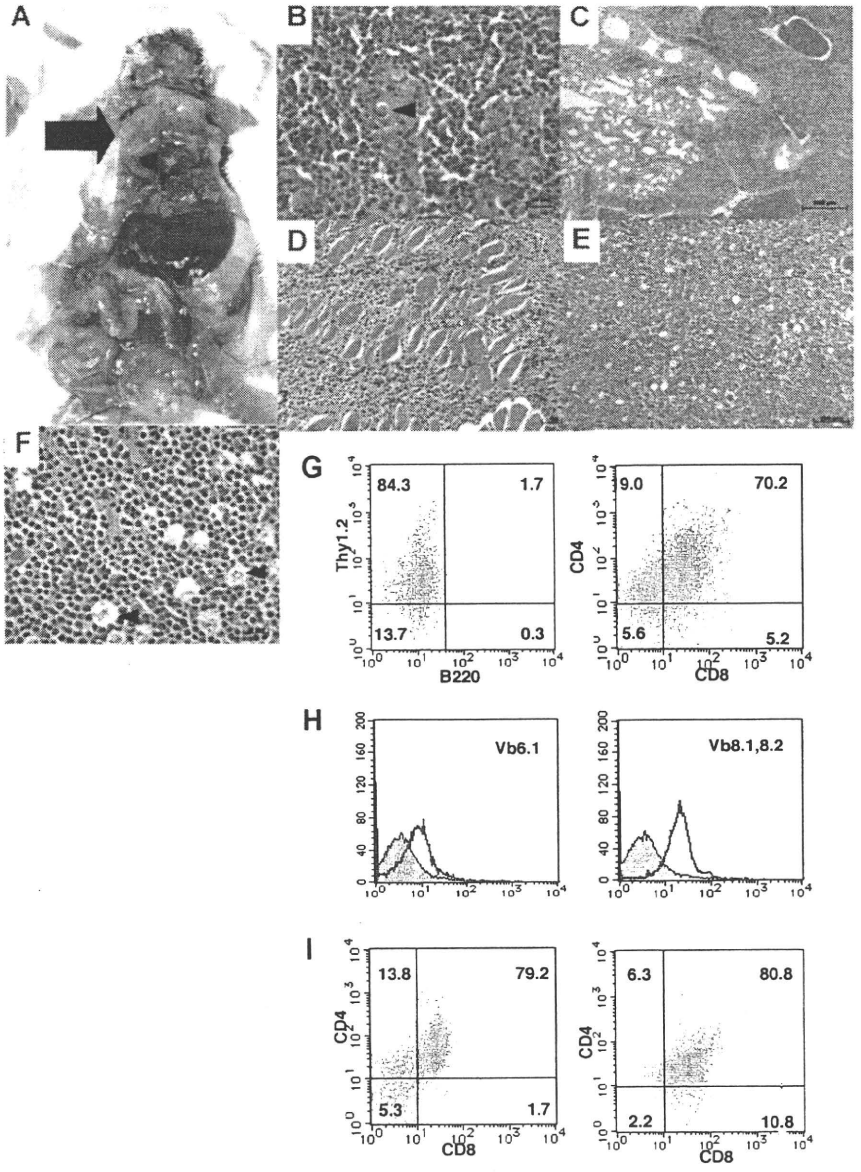

Figure 5. Pathological findings of *TCF8* mutant mice. (A) Gross autopsy of *TCF8* mutant mice with thymic tumors. A large thymic tumor (➡) was observed at the mediastinum of the dissected mouse. (B) Hematoxylin and eosin staining of tumor sections from the mouse as indicated. The normal thymic cellular 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

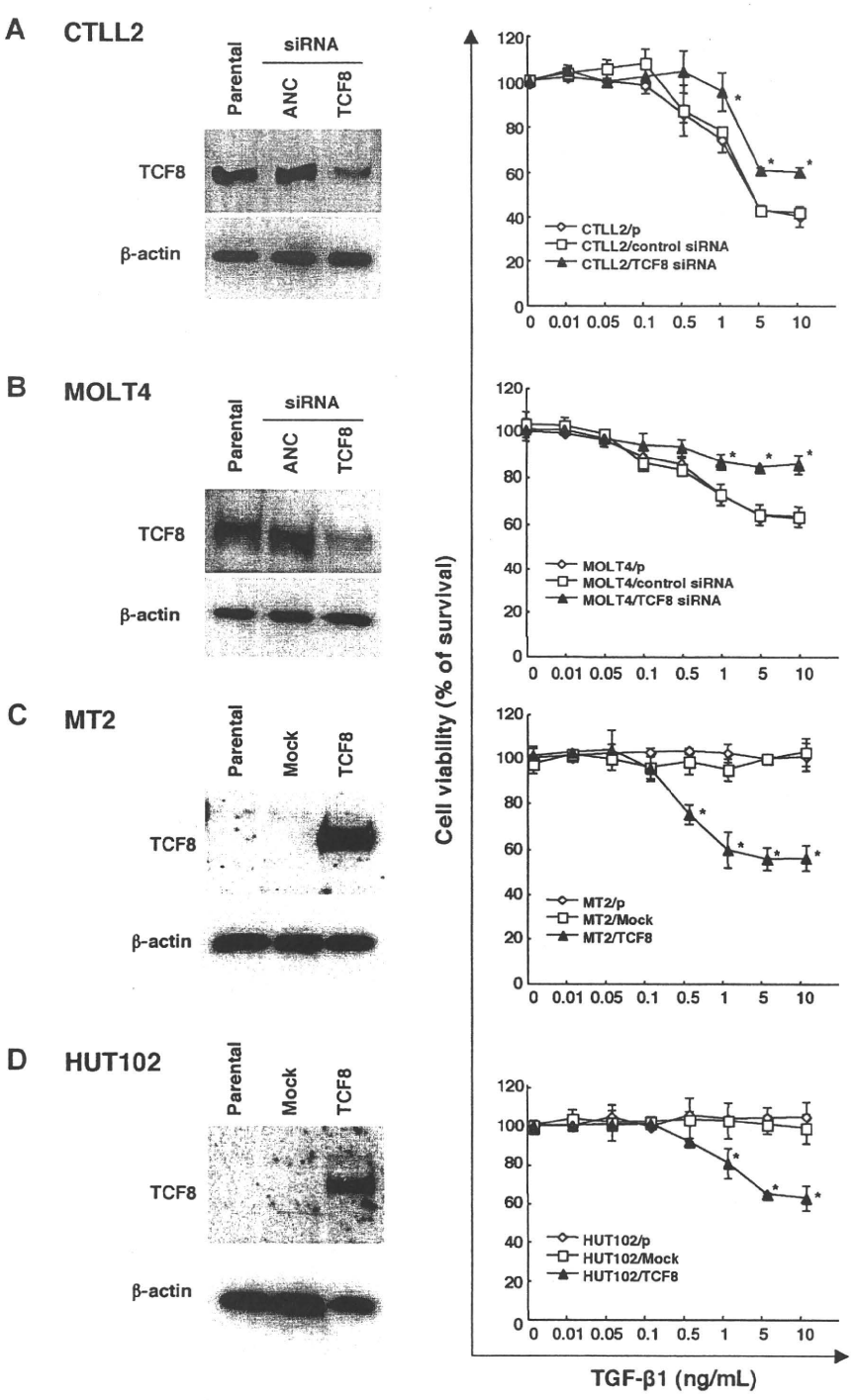

Figure 6. TGF-β1 responsiveness in various leukemia cell lines with the up- or down-regulation of TCF8 expression. (A,B) The down-regulation of the TCF8 protein by *TCF8* siRNA. The CTLL2 (A) and MOLT4 (B) cell lines were transfected with either *TCF8* or the AllStars Negative Control (ANC) siRNAs and 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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◆母子

HTLV－I　母子感染予防について

富山大学産科婦人科学教授　齋藤 滋

HTLV-Iとは

HTLV-Iは血液中のCD4陽性T細胞に感染するウイルスで、多くの感染者はキャリアとして健康に天寿を全うしますが、生涯に約５%の方が成人T細胞白血病（ATL）を発症します。また頻度はATLの約1/30ですが、HTLV-I関連脊髄症（HAM）を発症し歩行障害や排尿障害を引き起こします。現在日本では約108万人のキャリアがいると推定されています。従来は九州・沖縄にキャリアが集中していましたが、人の移動が盛んになったためキャリアは全国に拡がっています。

主な感染経路は母乳を介した母子感染

HTLV-I感染経路は母乳を介した母子感染、性交感染、輸血を介した感染が知られています。輸血感染は血液センターでHTLV-I抗体検査を施行後、ほとんどありません。感染からATL発病まで長期間かかるためATL発症者は母子感染例に限られます。このためATLの撲滅のためには母子感染を防ぐことが何より重要です。

どのような検査でキャリアと判るか

妊娠30週頃までに採血をし、血清中のHTLV-I抗体検査（PA法もしくはCLEIA法）で判定します。重要なことに、この検査では0.5%程度に偽陽性（HTLV-Iに感染していないのに検査が陽性となってしまうこと）が生じます。したがって、この時点でキャリアと告知することは避けなければなりません。必ず確認検査であるWestern blot（WB）法を行ってから、キャリアかどうかの判断をします。しかし、WB法を行なっても判定保留となることが2～20%あります。このような場合、保険収載されてはいませんがPCR法を行い、キャリアかどうかの判断をする場合もあります。ただしPCR法も絶対的なものではありません。

母子感染予防対策は

HTLV-Iキャリア妊婦が4か月間以上の母乳哺育をした場合、15～20%の乳児への感染が認められます。人工乳で育てた場合、感染率は母乳哺育の約1/6の3%に減少します。現在、もっとも確実なHTLV-I母子感染予防対策は人工乳哺育です。

しかし、母乳哺育には多くのメリットがあります。十分な症例数とはいえませんが、3か月未満の短期母乳や、母乳をいったんフリーザーで凍らせてから解凍して哺乳びんで与える凍結母乳哺育でも、母子感染率は人工乳と差がないとの研究報告もあります。

母乳哺育を一方的に禁止するのではなく、母乳を介してHTLV-Iが15～20%母子感染すること、人工乳哺育、短期母乳哺育や凍結母乳のメリット、デメリットを十分に説明したうえで、妊婦に栄養方法を選択してもらうことが重要です。

なお、お子さんの感染については3歳時に小児科で検査することができます。

基礎情報　**成人T細胞白血病（ATL）**

- HTLV-Ⅰウイルスへの感染が引き起こす白血病（血液のがん）のひとつ
- 母乳を介して感染し、多くは40歳以降で発病。発症する率は約５%だが、発症した場合、根本的な治療法はなく死亡率が高い。
- 感染している母親が母乳を４か月以上授乳したとき、乳児への感染率は15～20%。
- 授乳方法によって乳児への感染率が大幅に下がるというデータも。妊婦健診時の血液検査が求められている
- 近年、感染者が関東圏で増加している

Information

財団法人母子衛生研究会と母子保健事業団はマタニティマークの普及に取り組んでいます

妊婦さんはマタニティマークをバッグにつけることによって周囲の人に妊娠していることをさりげなく示すことができ、周囲も妊婦さんに対して「公共交通機関で席を譲る」「周囲で喫煙を控える」「飲酒を勧めない」などの配慮がしやすくなり、妊産婦にやさしい環境づくりを推進することにつながります。

財団法人母子衛生研究会と母子保健事業団は今後も、マタニティマークの普及に取組んでいく予定です。

母子保健情報

ヒト白血病ウイルスーⅠ型(HTLV−Ⅰ)の母子感染に関する情報の提供について

厚生労働省雇用均等・児童家庭局母子保健課

ヒト白血病ウイルスーⅠ型（HTLV−Ⅰ）の母子感染について理解を深めていただくため、厚生労働省のホームページに関係資料が掲載されましたので、情報提供いたします。
（http://www.mhlw.go.jp/bunya/kodomo/boshi-hoken16/index.html）

成人T細胞白血病（ATL）（※1）やHTLV－Ⅰ関連脊髄症（HAM）（※2）の原因であるヒト白血病ウイルス－Ⅰ型（HTLV－Ⅰ）（※1）の主な感染経路については、母乳等を介した母子感染であることや、母乳の授乳期間が長くなれば児のHTLV－Ⅰ感染率が上昇すると言われています。

また、平成21年度厚生労働科学研究費補助金（厚生労働科学特別研究事業）「HTLV－Ⅰの母子感染予防に関する研究」（研究代表者：齋藤滋国立大学法人富山大学大学院医学薬学研究部教授）において、HTLV－Ⅰ抗体が陽性であることが判明した妊婦については、人工栄養による育児によって、児のHTLV－Ⅰの母子感染のリスクが一定程度低減できることなどが報告されています。

厚生労働省のホームページには、この研究報告書の全文の他、研究報告書から抜粋した「ATLとHTLV－ⅠのQ&A」やATLに関する妊婦向け普及啓発用に作成されたポスター「ATLどんな病気？」等が掲載されています。

妊娠中のHTLV－I検査と母子感染予防

富山大学産科婦人科学教授　齋藤（さいとう）　滋（しげる）

1990年に厚生省心身障害研究「成人T細胞白血病（ATL）の母子感染防止に関する研究班」報告書（重松班）では、HTLV-Iキャリアは九州・沖縄地区に多く、母子感染経路は母乳が主体であるが長期母乳投与の減少等乳児栄養法の変化により、放置しても感染者は自然に減少するとするシミュレーションも紹介されました。そのため、全国的な母子感染予防対策は特に必要とせず、九州・沖縄地区のみのHTLV-I母子感染予防対策を行なえば良いとの見解でした。しかし、2010年に厚生労働科学特別研究事業「HTLV-I母子感染予防に関する研究班」（齋藤班）ではHTLV-I母子感染予防対策が大きく見直されましたので紹介します。

問　HTLV-Iと成人T細胞白血病（ATL）、HTLV-I関連脊髄症（HAM）との関連性については？

答　HTLV-Iは成人T細胞白血病（ATL）、HTLV-I関連脊髄症（HAM）などの疾患を引き起こすレトロウイルスで、CD4陽性T細胞に感染し、通常はキャリアでも全く健康ですが、長い潜伏期を経て、その一部にATLやHAMを発症します。ATLは化学療法が奏効せず、極めて予後不良な疾患ですが、重要なことはATL発症者のほとんどが母子感染例であることです。このためHTLV-I母子感染予防はATLの撲滅という面からみても重要です。

問　20年前の報告書と今回の報告書で、どこが変わったのですか？

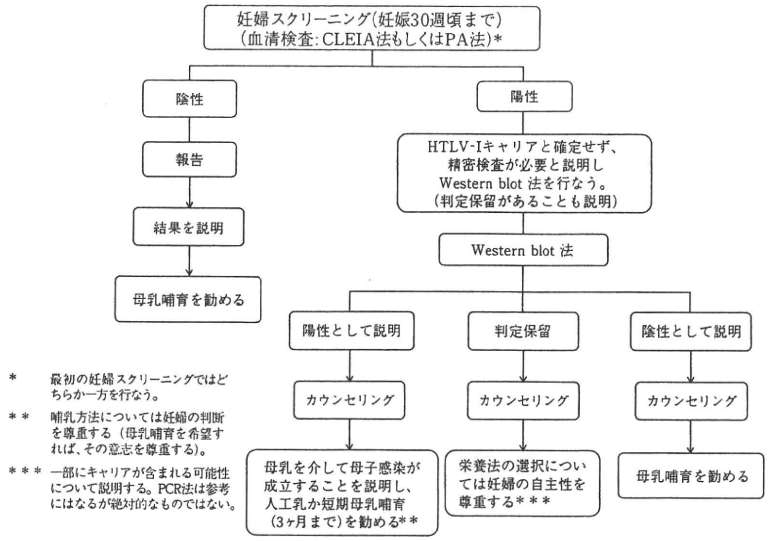
答　2つの大きな変更点があります。1つ目は20年前の報告書ではHTLV-Iキャリアは九州・沖縄地区に多いため、妊婦に対する全国一律のHTLV-I抗体検査や対策は必要ないと考えられていましたが、2008年の厚労省研究「HTLV-I感染及び関連疾患の実態調査と総合対策」（山口班）報告では、全国のHTLV-Iキャリア数は20年前の120万人から108万人へと減少はしているが、キャリアが全国に拡散する傾向が明らかとなり、新たな対策が必要であると提言されています。この提言をうけて、2010年の厚労研究班ではHTLV-I母子感染のスクリーニング検査を全国で行ない、母子感染を予防することを検討する時機に来ていると提言しています。この報告書を受けて、2011年4月に発刊予定の産婦人科診療ガイドラインでも妊娠時に行なう検査として、妊婦HTLV-I抗体検査を推奨レベルCからBに変更することが予定されています。

他の変更点として、HTLV-Iスクリーニング（PA法もしくはCLEIA法）を行なうと約0.5%（0.05%～0.59%）に偽陽性となることが判明しました。すなわちHTLV-Iに感染していなくても約0.5%の割合で検査が陽性となってしまいます。この検査のみでは真のキャリアか偽陽性かは判りません。このため真の陽性者を検出するためには確認検査であるWestern Blot（WB）法を行なう必要があります（図1）。なお、確認試験を行なっても判定保留が2～20%存在しますのでWBを行なう際には判定保留があることをあらかじめ説明して下さい。一次スクリーニングは自費ですが、確認試験として行なうWB法は保険診療で行なうことができます。WB法でも判定保留となった場合、PCR法は自費診療となりますが参考になります。しかし、絶対的なものではありません。すなわちPCR法陽性ではキャリアと診断できますが、感染細胞が少ないとキャリアであってもPCR法で陰性となってしまうことがあります。

問　HTLV-Iのスクリーニングの時期ならびに結果説明をどのように行なえば良いでしょうか？

答　血清検査（PA法もしくはCLEIA法）は妊娠30週頃までに行なって下さい。一次検査が陽性の際、確認試験が必要ですし、また陽性者には母乳哺育についても考慮する時間が必要なため分娩直前の検査では間に合わないからです。一次検査で陽性となっても偽陽性が含まれるため、この時点でHTLV-Iキャリアであると告知しないで下さい。精密検査が必要と説明の上、WB法を施行して下さい（図1）。WB法陰性者に対しては陰性と説明し、母乳哺育を指導して下さい。WB法陽性者に対しては、HTLV-Iキャリアであることを説明し、カウンセリングや哺乳法（後述）について詳しい説明が必要となります。日本でのHTLV-Iキャリアは約108万人で決してまれではないこと、将来のATL発症率（40歳を過ぎると年間にキャリア1,000人に1人、生涯発症率5%）、HAMの発症（30～50歳が多く年間にキャリア3万人に1人の発症）などを示し、母乳を介して母子感染が生じることなどの知識を提供します。不安をかきたてないような細心の注意が必要です。HTLV-I患者の会に尋ねたところ、妊娠時にHTLV-Iキャリアと告知されることは非常

図1　HTLV-Ⅰスクリーニングの進め方



にショックであり、感染から40年以上経過したHTLV-Ⅰキャリアから年間およそ1,000人に1人の割合でATLを発症するとの説明の方が、生涯発病率5％という説明より精神的ストレスが和らぐとの意見もありました。また家族への説明は妊婦本人が希望した時のみ行なって下さい。なおHTLV-Ⅰは母乳、性交、輸血以外には感染しないので、家族内への感染については心配することがないことを説明することも重要です。

問　HTLV-Ⅰキャリア妊婦に対しての母乳哺育はどうすれば良いのでしょうか？

答　1990年の報告書では主として後方視的検討で、それ以降の研究では前方視的検討でHTLV-Ⅰ母子感染が研究されました。いずれの研究においても母乳哺育とくに4カ月以上の長期母乳哺育ではHTLV-Ⅰ母子感染率が高率（約17.7％）となり人工乳哺育による母子感染率（約3％）の約6倍となります。このため最も確実なHTLV-Ⅰ母子感染防止は人工乳を選択することですが、人工乳を選択しても原因は不明ですが約3％の母子感染は成立します。また症例数が少なく十分な結論を得るには至っていませんが、3カ月までの短期母乳哺育や一旦凍結した母乳を解凍してから投与する凍結母乳保育でも母子感染を人工乳と同じレベルにまで低下させるという報告もあります。

母乳中には消化器感染、呼吸器感染を防ぐIgAが大量に含まれており、その他重要な生理活性物質も含まれています。また母子の愛着形成にも母乳哺育は重要です。したがってHTLV-Ⅰキャリア妊婦に対して、一方的に断乳を勧めるのではなく、リスクとベネフィットを説明の上、栄養法については妊婦本人の意思を尊重するようにして下さい。

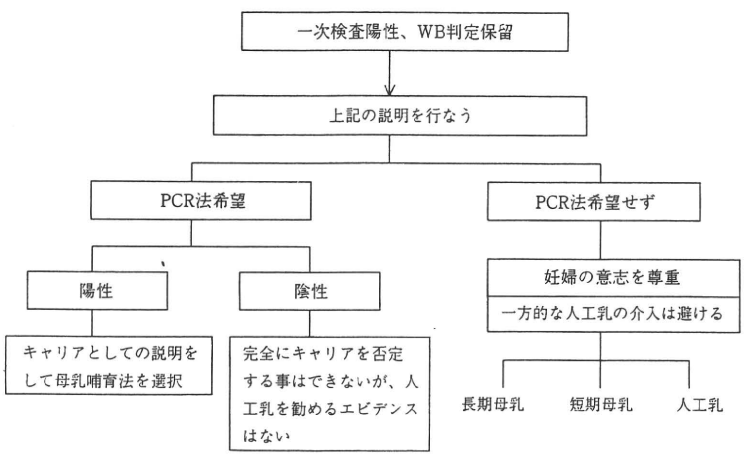
問　確認試験（WB法）で判定保留となった場合の対応は？

答　WB法が判定保留となった場合、WB法を再検しても同様の結果となる事が多く、確定診断は困難です。図2に示すように判定保留となった場合HTLV-Ⅰキャリアと断定できないこと、一部の症例でキャリアも存在するが、判定保留者の中にどのくらいの割合でキャリアが含まれているのかは現状では不明であること、判定保留者に含まれるキャリアからの母乳を介した感染率については全くデータがないこと、保険収載されていないがPCR法を施行する方法もあることを説明して下さい。PCR法陽性であればキャリアとして説明して下さい。PCR法陰性者に対しては、現時点では人工乳をすすめるエビデンスはなく、積極的な人工哺育への介入は望ましくありません。PCRを希望しない方には一方的な人工乳の介入は避け、リスクとベネフィットを説明した上で、妊婦の意志を尊重して下さい。

問　子供の感染の有無はいつ検査をしたら良いですか？

答　3歳すぎの抗体検査（PA法、CLEIA法）でHTLV-Ⅰ母子感染が成立したかどうか判ります。出生後半年くらいは母親の移行抗体が存在しますので血清検査ではキャリアかどうか判りません。できるだけ早く結果を知りたい場合1歳すぎに抗体検査を受けて下さい。この場合、3歳以降に再度抗体検査を受けていただき陰性であれば母子感染はなかったと判断できます。

図2



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眼内炎症と恒常性維持

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要 約

種々の原因で生じる眼内炎症の発症機構の解明は長い間未解決であったが1977年にWackerらが網膜の可溶性成分から実験動物にぶどう膜炎を惹起する網膜自己抗原(S抗原)の分離・精製に成功し，実験的自己免疫性ぶどう膜炎(EAU)を確立してから，ぶどう膜炎の発症機構と眼内の免疫機構の研究が急速に進展した．EAUで得られた知識と研究方法はヒトの眼内炎症にも応用され，多くの疾患の発症機序の解明と治療法の開発がなされた．それらの研究を通して，視覚に関与する眼内の重要な組織を炎症から守るために眼内には特殊な局所免疫機構が存在し，眼内の恒常性を維持し高度視機能の保持に役立っていることも明らかになりつつある．本稿では，眼内炎症の発症機構と防御機構におけるT細胞の役割に焦点を絞り，ぶどう膜炎の動物モデルとヒトの疾患における発症分子機構について論じ，さらに，眼内炎症を防御する眼内局所免疫機構について概説する．

1．ぶどう膜炎動物モデルにおける眼内炎症発症機構

T細胞が欠如したヌードラットを用いた実験，ならびに，S抗原感作リンパ球による移入実験により，EAUの発症には網膜自己抗原に感作された$CD4^+$ Tリンパ球が重要であることが明らかにされた．また，Tリンパ球を選択的に抑制する免疫抑制薬のシクロスポリンは従来の免疫抑制薬に比べEAU発症抑制と治療効果が高いことが示され，Behçet病などの難治性ぶどう膜炎の治療に臨床応用された．すなわち，自己免疫性ぶどう膜炎の発症機構で主役を演じるのはTリンパ球，特に活性化$CD4^+$ Tリンパ球であることがEAUを用いた研究で明らかになった．

2．ヒトのぶどう膜炎における眼内炎症発症機構

我々は，日本人に関係の深い二つの疾患，すなわちVogt—小柳—原田病とヒトT細胞白血病ウイルス1型(human T-cell leukemia virus type 1：HTLV-1)ぶどう膜炎の発症機構の研究を通して，ヒトの眼内炎症の発症機構にも活性化$CD4^+$ Tリンパ球が重要であることを明らかにした．

1）Vogt—小柳—原田病(原田病)

我々は患者の眼内浸潤細胞からT細胞クローンを樹立する方法を確立し，この細胞を用いて原田病の発症機構を解析した．原田病患者の眼局所にはメラノサイト関連抗原のtyrosinaseに特異的に反応して炎症性サイトカインを産生する$CD4^+$ Tリンパ球が存在するが，Behçet病など他のぶどう膜炎ではそのようなTリンパ球は存在しない．また，原田病患者が自己抗原であるtyrosinaseに感作される機序として，tyrosinaseが外来抗原のサイトメガロウイルスのペプチドと構造類似性をもつこと，原田病患者の眼内および末梢血中のTリンパ球はサイトメガロウイルスとtyrosinaseの両方に感作されていて免疫学的交差反応を起こすことが明らかとなった．このことから，原田病ではサイトメガロウイルス感染でそのペプチドに感作された$CD4^+$ Tリンパ球が脈絡膜や髄膜，内耳，皮膚などに存在するメラノサイトのtyrosinaseペプチドと交差反応を起こすことにより，これらの組織に炎症が生じると考えられる．

2）HTLV-1ぶどう膜炎

我が国の九州南部に多いHTLV-1キャリアに生じるぶどう膜炎は，成人T細胞白血病やHTLV-1関連脊髄麻痺と並び，ヒトレトロウイルスのHTLV-1により生じる独立した疾患である．本症患者の眼局所浸潤細胞を解析することで発症機構の解明がなされ，HTLV-1感染$CD4^+$ Tリンパ球が眼内に多数集積すること，その眼内浸潤細胞から樹立したHTLV-1感染$CD4^+$ Tリンパ球は種々の炎症性サイトカインを大量に産生することなどが明らかになり，これらにより眼内炎症が生じると考えられる．

3．眼内局所防御機構

上記のように眼内に侵入してきた活性化$CD4^+$ Tリンパ球により眼内に一連の免疫反応と炎症が生じるが，その一方で，眼内は“immune privilege site”と呼ばれる特殊な免疫環境となっていて，眼内に侵入してきた活性化$CD4^+$ Tリンパ球の働きを抑制する免疫機構が存在する．我々は外来抗原が眼内に侵入する際に通過する血液眼関門を構築する眼色素上皮細胞に着目し，虹彩色素上皮，毛様体色素上皮，網膜色素上皮のTリンパ球に対する免疫抑制作用について研究した．そして，これ

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らの眼色素上皮細胞は活性化 $CD4^+$ T リンパ球の増殖反応とサイトカイン産生を非特異的に抑制すること，しかし，その抑制の仕方は前眼部と後眼部では大きく異なり，虹彩色素上皮は T リンパ球と細胞接触することで，一方，網膜色素上皮は可溶性因子により T リンパ球を抑制すること，さらに，これらの色素上皮は眼内に侵入してきた活性化 T リンパ球を制御性 T 細胞に変化させて眼内における免疫抑制作用の増幅を図る特異な機構をもつこと，そして，これらの眼局所防御機構で働く機能分子と遺伝子を明らかにした．これらの機能分子と遺伝子の相互作用により眼内で生じる制御性 T 細胞は，ヒトのぶどう膜炎の新しい治療法に発展する可能性を秘めているものといえる．(日眼会誌 113：344—378，2009)

キーワード：ぶどう膜炎，発症機構，実験的自己免疫性ぶどう膜炎，活性化 $CD4^+$ T リンパ球，Vogt—小柳—原田病，HTLV-1 ぶどう膜炎，T 細胞クローン，眼色素上皮細胞，眼局所免疫機構，形質転換増殖因子 β，制御性 T 細胞

A Review

Intraocular Inflammation and Homeostasis of the Eye

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Abstract

The pathogenic mechanisms of intraocular inflammation had not been well studied until Wacker and his colleagues found retinal soluble antigen (S antigen) and established experimental autoimmune uveitis (EAU), an animal model for autoimmune uveitis. Using this animal model, great progress in understanding the immunopathogenic mechanisms of uveitis was achieved not only in EAU, but also in many inflammatory disorders in humans. Intraocular inflammation is mediated by activated $CD4^+$ T cells. However, the eye has a unique regional immune system which protects intraocular tissues from these pathogenic activated $CD4^+$ T cells and contributes to the homeostasis of the intraocular microenvironment. In the present review article, the role of T cells in immunopathogenic mechanisms of ocular inflammatory disorders as well as in the regional defense system of the eye is highlighted.

1. Immunopathogenic mechanisms of EAU

Experiments using athymic nude rats as well as adoptive transfer of EAU by S-antigen-sensitized T lymphocytes into naïve Lewis rats disclosed that T lymphocytes, particularly $CD4^+$ T lymphocytes, play a central role in the immunopathogenic mechanisms of EAU. In addition, immunopharmacological studies showing the intense effects of cyclosporine on EAU with selective immunosuppression to T lymphocytes allowed us to use clinically the agent to treat patients with refractory uveitis of non-infectious origins, such as Behcet's disease.

2. Immunopathogenic mechanisms of uveitis in human

Two clinical uveitis entities commonly seen in Japan, i. e. Vogt-Koyanagi-Harada (VKH) disease and human T-cell leukemia virus type 1 (HTLV-1) uveitis, were studied for their pathogenic mechanisms.

(1) VKH disease

We established T cell clones from infiltrating cells in the eyes of VKH patients using limiting dilution methods. $CD4^+$ T cell clones from VKH disease, but not from other uveitis entities, responded to tyrosinase, a melanocyte-associated antigen, and produced inflammatory cytokines, and the response was specific to tyrosinase. Furthermore, DataBank analysis disclosed that tyrosinase had a structural homology with an exogenous antigen, a glycoprotein peptide of cytomegalovirus (CMV). $CD4^+$ T lymphocytes from VKH patients, but not from other diseases, which responded to both tyrosinase and CMV peptide. This indicates that molecular mimicry between CMV peptide and tyrosinase plays an important role in the immunopathogenic mechanisms by which $CD4^+$ T lymphocytes are sensitized to autoantigen of tyrosinase and cause inflammation in VKH disease.

(2) HTLV-1 uveitis

Similar to adult T cell leukemia and HTLV-1 associated myelopathy, uveitis in asymptomatic carriers of HTLV-1, prevalent in southern Kyushu, is a distinct clinical entity associated with HTLV-1, a human retrovirus. We analyzed ocular infiltrating cells and found that (a) HTLV-1-infected $CD4^+$ T lymphocytes were significantly accumulated in the eye, and (b) HTLV-1-infected $CD4^+$ T lymphocytes produced a large amount of various inflammatory cytokines. Thus, $CD4^+$ T lymphocytes play a central