Table 3. Cox analysis of eruption type and T stage for clinical factors and OS

	Univariate		Multivariate (eruption type and clinical factors)		Multivariate (T stage and clinical factors)	
Clinical factor	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Clinical subtype						
Acute type	1		1		1	
Lymphoma type	0.5 (0.1-0.8)	.013	0.9 (0.3-2.5)	.852	0.9 (0.1-1.3))	.852
Chronic type	0.1 (0.3-1.1)	.082	0.4 (0.1-1.4)	.167	0.4 (0.3-2.4)	.140
Smoldering type	0.1 (0.1-0.2)	< .001	0.2 (0.8-0.6)	.002	0.2 (0.1-0.6)	.003
Patient-related factors						
Sex						
Male	1		1		1	
Female	1.0 (0.6-1.6)	.903	1.2 (0.7-2.1)	.576	1.2 (0.7-2.1)	.576
Age, y						
< 60	1		1		1	
≥ 60	1.0 (0.6-1.7)	.922	0.9 (0.5-1.6)	.658	0.9 (0.5-1.5)	.578
Complications at diagnosis						
Absent	1		1		1	
Present						
Diabetes mellitus	0.6 (0.2-2.0)	.427	0.5 (0.1-1.9)	.314	0.5 (0.1-1.8)	.274
Hypertension	0.4 (0.2-1.2)	.119	1.0 (0.3-3.9)	.958	1.1 (0.3-4.2)	.905
Stroke	1.8 (0.4-7.3)	.425	3.5 (0.6-19.4)	.161	2.9 (0.5-17.3)	.256
Opportunistic infection	1.0 (0.4-2.6)	.927	1.0 (0.3-3.1)	.958	1.1 (0.3-3.4)	.938
Hematologic factors						
WBC count, × 10^9/L						
< 12.0	1		1		1	
≥ 12.0	3.6 (2.1-6.2)	< .001	1.8 (0.6-5.2)	.279	1.7 (0.6-4.9)	.325
Total lymphocyte count, × 10^9/L						
< 6.5	1		1		1	
≥ 6.5	3.7 (2.0-6.5)	< .001	1.1 (0.5-2.6)	.803	1.1 (0.5-2.7)	.768
Laboratory factors						
LDH						
≤ NI	1		1		1	
> NI	3.0 (1.8-4.9)	< .001	1.2 (0.6-2.2)	.581	1.2 (0.6-2.2)	.599
Calcium						
≤ NI	1		1		1	
> NI	1.3 (0.8-2.1)	.381	1.0 (0.6-1.8)	.960	1.0 (0.6-1.8)	.914
Eruption type						
Patch	1		1			
Plaque	2.2 (0.5-10.9)	.321	1.4 (0.3-8.0)	.680		
Nodulotumoral	12.5 (2.7-57.1)	.001	8.8 (1.6-48.0)	.012		
Erythrodermic	68.4 (11.5-405.9)	< .001	21.2 (3.0-150.3)	.002		
Multipapular	4.8 (1.0-22.6)	.045	3.5 (0.6-20.1)	.159		
Purpuric	7.1 (1.1-45.7)	.039	6.8 (0.9-53.7)	.071		
T stage						
T1	1				1	
T2	4.0 (1.0-15.1)	.047			2.2 (0.5-9.8)	.304
T3	15.3 (4.4-52.8)	< .001			11.3 (2.8-46.0)	.001
T4	83.9 (17.8-394.6)	< .001			27.5 (5.0-151.8)	< .001
Multipapular type	5.8 (1.6-20.9)	.007			8.1 (1.4-47.1)	.045
Purpuric type	8.6 (1.7-44.5)	.010			8.1 (1.4-47.1)	.020

CI indicates confidence interval; and NI, normal index.

types were significantly higher than that of the patch type, and that skin eruption is an independent prognostic factor for ATLL.

Table 4. Univariate analyses of T stage compared with patients having no skin eruptions

	Univariate	
Clinical factor	**HR (95% CI)**	***P***
T stage		
T1	1	
T2	3.6 (0.9-13.6)	.059
T3	16.4 (4.9-55.1)	< .001
T4	127.4 (26.2-618.1)	< .001
No eruption	1.3 (0.3-4.8)	.670

It has been reported that the smoldering type of ATLL with skin eruptions, especially those of the nodulotumoral type, has a poorer prognosis than ATTL without skin involvement.[19] Another group of investigators reported that the MSTs of the nodulotumoral and maculopapular types were 26 and 80 months, respectively, which are significantly shorter than those in ATLL without cutaneous eruptions.[23] Our findings are in agreement with these observations, and further clarify the relationship between type of skin lesion and prognosis. Skin-targeted therapy using topical steroids, psoralen photochemotherapy, or narrow-band UVB therapy[19] may improve the prognosis of ATLL for patients with skin eruptions.

The purpuric type of ATLL is one of the rarest skin eruptions of ATLL,[24] and has been reported to occur in 1.6% of ATLL patients with

Table 5. Cox multivariate analyses of clinical factors and OS compared with patients having no eruption

	Multivariate	
Clinical factor	**HR (95% CI)**	***P***
Clinical subtype		
Acute type	1	
Lymphoma type	2.4 (0.8-7.2)	.110
Chronic type	0.3 (0.1-0.9)	.036
Smoldering type	0.4 (0.1-1.1)	.068
Patient-related factors		
Sex		
Male	1	
Female	0.8 (0.4-1.4)	.440
Age, y		
< 60	1	
≥ 60	0.4 (0.2-0.8)	.012
Complications at diagnosis		
Absence	1	
Presence		
Diabetes mellitus	1.9 (0.7-4.8)	.188
Hypertension	0.6 (0.2-2.1)	.443
Stroke	2.6 (0.6-10.5)	.191
Opportunistic infection	1.1 (0.2-6.1)	.925
Hematologic factors		
WBC count, × 10^9/L		
< 12.0	1	
≥ 12.0	1.1 (0.3-3.5)	.864
Total lymphocyte count, × 10^9/L		
< 6.5	1	
≥ 6.5	2.0 (0.7-5.9)	.197
Laboratory factors		
LDH		
≤ NI	1	
> NI	1.2 (0.7-2.1)	.596
Calcium		
≤ NI	1	
> NI	1.1 (0.6-1.9)	.826
T stage		
T1	1	
T2	2.4 (0.6-9.7)	.227
T3	13.4 (3.3-53.9)	< .001
T4	60.8 (10.1-366.4)	< .001
No eruption	0.9 (0.2-3.6)	.847

CI indicates confidence interval; and NI, normal index.

skin lesions.[19] However, its incidence is higher than was previously thought, because we documented a 4.2% frequency in this study. The production of granzyme B by ATLL cells may lead to the destruction of vessels and the development of purpuric eruptions in these patients.[24] The prognosis for the purpuric type of skin lesion has not been investigated because of the rarity of this type. There have been 9 cases of the purpuric type reported in the literature.[24-31] When these are divided into the punctate and macular subtypes, the prognosis of the punctate purpuric subtype might be better than the macular purpuric subtype.[24-31] In our 5 purpuric cases, 2 cases of the punctuate purpuric subtype survived, with a 73.4-month mean survival time (the MST was not estimable), whereas 3 cases of the macular purpuric subtype died with 2.1 months of the MST. This suggests that the punctuate subtype has a good clinical prognosis, and the poor prognosis of the total purpuric type is derived from the macular subtype.

In addition to the purpuric type, the erythrodermic type is a rare skin manifestation in ATLL patients, with a prevalence of 3.5% reported in a previous study[19] and 4.2% in the present study. The majority of ATLL cases associated with the erythrodermic type of skin lesion are aggressive. In our study, all patients with erythrodermic lesions also belonged to the acute type and had the poorest prognosis among all skin eruption types. In patients with cutaneous T-cell lymphoma (CTCL), the erythrodermic type is typically termed SS and also has a poor prognosis.[16] In some erythrodermic CTCL patients, the decreased expression of intercellular adhesion molecule-1 by keratinocytes may lead to an inability of malignant T cells to enter the epidermis and infiltrate the blood and other organs.[32] This pathomechanism in erythrodermic CTCL can also be applied to ATLL, resulting in poor prognosis. Skin biopsy specimens of the erythrodermic type of ATLL revealed scant tumor cell infiltration into the epidermis.[33,34]

We applied MF/SS classification T stages to ATLL assessment, and demonstrated that the OS was worse as the T stage became more advanced. The results shown in Table 3 indicated that the prognosis of T1 stage was better than that for T2, suggesting that the difference in the body surface area of skin lesions is associated with the prognosis of ATLL. Moreover, the prognosis of T3 patients was poorer than those of T1 and T2, indicating that the depth of tumor-cell infiltration is associated with survival rate. T-stage classification accurately reflects the prognosis of ATLL and MF/SS. However, the multipapular and purpuric types are not applicable to T stage. We found that the multipapular type and T2 had similar outcomes, and that the purpuric type had a significantly poorer prognosis than T1. This may provide clinically useful information for patient management and choice of therapy. Moreover, our present study demonstrated that skin eruption is an independent prognostic factor for ATLL patients: the presence of skin eruptions may indicate poorer outcome compared with no eruptions. Therefore, evaluation of skin lesions and treatments targeting the skin may be important for improving clinical outcome.

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Authorship

Contribution: Y.S. collected and analyzed the data and wrote the manuscript; R.H. analyzed the data; K.H. collected the data; S.O., H.F., S.Y., S.F., M.T., R.K., M.Y., D.N., K.S., R.Y., T.S., T.M., K.I., M.K., and M.N. diagnosed and treated ATLL patients; and Y.T. organized the study.

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Correspondence: Yu Sawada, MD, Department of Dermatology, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan; e-mail: longago@med.uoeh-u.ac.jp; or Ryosuke Hino, MD, PhD, Department of Dermatology, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan; e-mail: hinoti@med.uoeh-u.ac.jp.

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Detection of HTLV-1 by means of *HBZ* gene *in situ* hybridization in formalin-fixed and paraffin-embedded tissues

Kei Shimizu-Kohno,[1,2] Yorifumi Satou,[3] Fumiko Arakawa,[1] Junichi Kiyasu,[1] Yoshizo Kimura,[1] Daisuke Niino,[1] Yasuo Sugita,[1] Fumihiko Ishikawa,[4] Masao Matsuoka[3] and Koichi Ohshima[1,5]

[1]Department of Pathology, School of Medicine, Kurume University, Kurume; [2]Department of Neurology, Hematology, Metabolism, Endocrinology and Diabetology, School of Medicine, Yamagata University, Yamagata; [3]Laboratory of Virus Control, Institute for Virus Research, Kyoto University, Kyoto; [4]Research Unit for Human Disease Models, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan

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Adult T-cell leukemia/lymphoma (ATLL) is a T-cell malignancy associated with HTLV-1. The HTLV-1 provirus genome has the pX region that encodes *tax* and HTLV-1 basic leucine zipper factor (*HBZ*). Previous studies have reported that the *tax* gene is expressed in few ATLL cases, but the *HBZ* gene in all ATLL cases. In this study, we used *HBZ* gene *in situ* hybridization (HBZ-ISH) for detection of the *HBZ* gene in formalin-fixed paraffin-embedded tissues. This method showed that all cases (n = 19) were positive for the ATLL cell line (MT-1, MT-2, and MT-4) and ATLL mouse model (HBZ-Tg mice and NOD/SCID/β2-microglobulin^{null} mice with ATLL transplanted), and the *HBZ* gene was also detected in all human ATLL cases (n = 16). The percentage of positive cells in HBZ-ISH was 5–70%. Immunohistochemical staining for Tax protein showed positivity in seven of 11 cases in NOD/SCID/β2-microglobulin^{null} mice with ATLL transplanted and in six of eight human ATLL cases, but the percentage of positive cells was very low (range, 1–5%). Although HBZ-ISH is unsuitable to detect HTLV-1 clonality, this method is convenient and can be useful for the histological diagnosis of ATLL in HTLV-1 sero-indeterminate patients. (*Cancer Sci* 2011; 102: 1432–1436)

Adult T-cell leukemia/lymphoma (ATLL) is a human T-cell malignancy derived from CD4+ T-cells and induced by HTLV-1 infection.[1–3] The HTLV-1 genome encodes common structural and enzymatic proteins (Gag, Pol, and Env) and the regulatory and accessory proteins (Tax, Rex, p12, p13, p21, and p30). Among these viral proteins, the Tax protein, encoded by pX in a double splicing manner, is thought to be mainly implicated in the oncogenesis of ATLL through indirect and direct interactions between Tax and cellular molecules. However, ATLL cells often contain genetic and epigenetic alterations of the 5′-LTR of the HTLV-1 provirus, resulting in the loss of Tax expression, as Tax expression is the major target of cytotoxic T lymphocytes. Takeda *et al.*[4] reported that tax transcripts were detected in only 40% of all ATLL cases.

Recent studies of the minus strand viral gene, HTLV-1 basic leucine zipper factor (*HBZ*) found that it is expressed in leukemic cells of all ATLL cases and plays a role in ATLL oncogenesis.[5,6] Satou *et al.*[6] generated transgenic mice containing the *HBZ* gene under control of a murine CD4-specific promoter/enhancer/silencer (HBZ-Tg mice). HBZ-Tg mice spontaneously developed systemic dermatitis, alveolitis, and lymphoma as they aged and expressed the *HBZ* gene in all murine CD4$^+$ cells. Moreover, Kawano *et al.*[7] developed a novel xenogeneic engraftment model in which primary adult T-cell leukemia (ATL) cells are transplanted i.v. into neonatal NOD/SCID/β2-microglobulin^{null} (NOD/SCID/β2m^{null}) mice. Engrafted ATL cells were dually positive for human CD4 and CD25, and displayed patterns of HTLV-1 integration identical to those of donors. When NOD/SCID/β2m^{null} newborns were used as recipients, clonal expansion of primary ATL cells was evidently achieved in a xenogeneic transplantation setting. Engrafted mice showed monoclonal or polyclonal proliferation of ATL cells in blood and lymph nodes, evidenced by clinical features specific to each subtype of transplanted ATL.

In situ hybridization (ISH) is one of the most important techniques for visualization of gene expression at the cellular level in various tissues. For use with this technique, peptide nucleic acid (PNA) probes have recently been developed.[8] These molecules are DNA mimics in which the negatively charged sugar-phosphate backbone is replaced by an achiral, neutral polyamide backbone formed by repetitive units of N-(2-aminoethyl) glycine. Peptide nucleic acid can hybridize with complementary nucleic acid targets according to the Watson–Crick base-pairing rules. Compared with traditional DNA probes and due to the uncharged backbone, PNA probes have superior hybridization characteristics, showing rapid and stronger binding to complementary targets and an absence of electrostatic repulsion.[9,10]

Although the diagnosis of HTLV-1 infection is based on the initial specific antibody detection by particle agglutination assay or ELISA and subsequent confirmation by Western blot analysis or indirect immunofluorescent assay, these methods for detection of HTLV-1 usually require fresh or frozen specimens. In HTLV-1 sero-indeterminate cases, diagnosis of ATLL needs to establish histological detection of HTLV-1. However, because expression of the *HTLV-1* gene and protein is uncertain, it is difficult to detect the *HTLV-1* gene and proteins with conventional methods such as immunohistological staining or ISH.[11,12] As the aim of our study was to detect HTLV-1 in formalin-fixed and paraffin-embedded lymph node tissues of ATLL patients, we used the ISH-targeted *HBZ* gene and a PNA probe.

Materials and Methods

Tissue samples. The characteristics of the formalin-fixed and paraffin-embedded biopsy tissues selected for this study are summarized in Table 1. The following cell lines were used: (i) MT-1, MT-2, and MT-4, with HTLV-1 infected cell lines used as a positive control;[13,14] and (ii) Jurkat, T-cell acute lymphoblastic leukemia (T-ALL) cell line, with the uninfected cell line used as a negative control. These cultured cell lines were fixed with formalin and agar, then embedded in paraffin. Patients with ATLL (n = 16) and diffuse large B-cell lymphoma (DLBCL)

[5]To whom correspondence should be addressed.
E-mail: ohshima_kouichi@med.kurume-u.ac.jp

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Table 1. Summary of samples and results of immunohistochemistry and *HBZ* gene *in situ* hybridization (HBZ-ISH)

Case	Tissue	CD20	CD3	CD4	CD25	HBZ-ISH (%)	Tax (%)
Cell line							
MT-1 (ATLL)	NA	–	+	+	+	70	90
MT-2 (ATLL)	NA	–	+	+	+	40	90
MT-4 (ATLL)	NA	–	+	+	+	80	90
Jurkat (T-ALL)	NA	–	+	–	+	0	0
HBZ-Tg mice							
T1	Spleen	–	+	+	ND	30	0
T2	Spleen	–	+	+	ND	40	0
T3	LN	–	+	+	ND	35	0
T4	LN	–	+	+	ND	30	0
T5	LN	–	+	+	ND	30	0
T6	LN	–	+	+	ND	60	0
T7	LN	–	+	+	ND	20	0
T8	Spleen	–	+	+	ND	50	0
NOD/SCID/β2m^{null} mice with ATLL transplanted							
N1	Liver	–	+	+	+	50	5
N2	LN	–	+	+	+	30	1
N3	Lung	–	-	+	+	10	0
N4	Lung	–	-	+	+	10	0
N5	Lung	–	+	+	+	10	1
N6	Lung	–	-	+	+	10	1
N7	LN	–	+	+	+	30	5
N8	Liver	–	+	+	+	40	1
N9	LN	–	+	+	+	30	0
N10	Spleen	–	+	+	+	15	0
N11	Spleen	–	+	+	+	60	1
Human ATLL							
H1	LN	–	+	+	+	20	1
H2	LN	–	+	+	+	10	1
H3	LN	–	+	+	+	40	1
H4	LN	–	+	+	+	30	1
H5	LN	–	+	+	+	15	ND
H6	LN	–	–	+	+	20	1
H7	LN	–	+	+	+	30	0
H8	LN	–	+	–	+	40	1
H9	LN	–	+	+	–	5	0
H10	LN	–	–	+	+	20	ND
H11	LN	–	+	–	–	5	ND
H12	LN	–	+	ND	ND	60	ND
H13	LN	–	+	ND	ND	40	ND
H14	LN	–	+	+	+	70	ND
H15	LN	–	+	+	+	70	ND
H16	LN	–	+	+	+	20	ND
Human DLBCL							
D1	LN	+	–	–	–	0	0
D2	LN	+	–	ND	ND	0	0
D3	LN	+	–	–	–	0	0
D4	LN	+	–	–	+	0	0
D5	LN	+	–	ND	–	0	0

ATLL, adult T-cell leukemia/lymphoma; DLBCL, diffuse large B-cell lymphoma; HBZ-Tg mice, HBZ transgenic mice; LN, lymph node; NA, not applicable; ND, not done; T-ALL, T-cell acute lymphoblastic leukemia.

(n = 5) for negative control were identified in the lymph node registry files of the Department of Pathology, Kurume University (Kurume, Japan). The specimens of HBZ-Tg mice (n = 8) and NOD/SCID/β2m^{null} mice with ATLL transplanted (n = 11) were supplied by Dr. Masao Matsuoka (Institute for Virus Research, Kyoto University, Kyoto, Japan) and Dr. Fumihiko Ishikawa (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan). In all cases, Southern blot analysis was used to examine clonal integration of HTLV-1 proviral DNA. Informed consent was obtained from all patients.

The specimens were fixed with buffered formalin, embedded in paraffin, and stained with H&E. The paraffin-embedded samples were used for immunohistochemical analysis of L26 (CD20) for B cells (DakoCytomation, Glostrup, Denmark), UCHL-1 (CD45RO) and CD3 for T cells (DakoCytomation), CD4, CD8, CD15, CD30 (DakoCytomation) and Tax.[15]

Peptide nucleic acid probes. The sequence of oligonucleotides for *HBZ* used in this study was CCA TCA ATC CCC AAC TCC TG (nucleotide positions 664–645). The synthesized PNA probe was made to order by Bio-Synthesis (Lewisville, TX, USA).

Tissue preparation and pretreatment for ISH. Formalin-fixed and paraffin-embedded tissue blocks were cut into 4-μm-thick sections, and the sections were mounted on silane-coated clean

glass slides. After deparaffinization in xylene and graded ethanol, the slides were immersed in distilled water then treated with 0.1–1.0 μg/mL proteinase K (Dako, Santa Fe, CA, USA) for 5 min at 37°C. The proteinase K was washed out in distilled water. Finally, the sections were immersed in 95% and 100% ethanol, and air-dried.

Peptide nucleic acid ISH combined with EnVision kit. The *HBZ*-specific target probe was then added in 50 μL fresh hybridization buffer at a concentration of 5.19 nM/μL to each of the slides. Coverslips were placed over them. Hybridization was carried out in a humidified slide chamber on a slide warmer at 55°C for 2 h. After hybridization, the sections were washed once in Tris buffer, followed by rigorous washing in distilled water for 10 min, twice. The sections were immersed in Peroxiblock (Dako) for 10 min to block endogenous peroxidase activities. Next, they were incubated with preamplifier probes for 60 min at room temperature, and washed in three changes of PBS for 5 min. The tissue sections were then incubated with amplifier probes for 30 min at room temperature and washed in two changes of PBS. Signals were developed with a 3,3′-diaminobenzidine tetrahydrochloride (Dohjin, Kumamoto, Japan) chromogen solution, followed by hematoxylin counterstaining, then observed under a light microscope.

Reverse transcription–polymerase chain reaction. cDNA was reverse transcribed from approximately 4.5 μg total RNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Little Chalfont, UK) and primed with an oligo(dT) oligonucleotide (GE Healthcare). Three microliters of cDNA was subjected to PCR reaction using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). The oligonucleotide primers were as follows: for *HBZ*, 5′-AAA CGC ATC GTG ATC GGC AGC GAC-3′ (sense) and 5′-CTT CCA ACT GTC TAG TAT AGC CAT CA-3′ (antisense); for *tax*, 5′-CTC TGG GGG ACT ATG TTC GGC C-3′ (sense) and 5′-GTA CAT GCA GAC AAC GGA GCC T-3′ (antisense); and for β-actin, 5′-CAA GAG ATG GCC ACG GCT GCT-3′ (sense) and 5′-TCC TTC TGC ATC CTG TCG GCA-3′ (antisense). Product sizes were 294 bp for *HBZ*, 373 bp for *tax*, and 275 bp for β-actin. Amplification conditions consisted of denaturation at 95°C for 30 s (10 min for the first cycle), annealing at 60°C for 30 s, and extension at 72°C for 1 min (10 min for the last cycle) for 35 cycles for *HBZ* and *tax*, and 30 cycles for β-actin. The amplified products were evaluated in 2% agarose gel and visualized by means of ethidium bromide staining under UV light. The quality of cDNA was monitored using RT-PCR with β-actin primers. cDNAs yielding a 275-bp product for β-actin mRNA without contamination with the 370-bp genomic amplification product were used for this experiment.

Results

Histology. Histological studies of HBZ-Tg mice and NOD/SCID/β2m^{null} mice with ATLL transplanted showed a pleomorphic lymphoma in the lymph nodes (Fig. 1a,b). The lymph nodes of human ATLL showed a diffuse proliferation of atypical lymphoid cells, varying in size and form with the appearance of cerebriform giant cells (Fig. 1c).

Immunohistochemical staining identified these tumor cells of HBZ-Tg mice as mouse CD3$^+$ (data not shown) and mouse CD4$^+$ T cells (Fig. 1d), and tumor cells of NOD/SCID/β2m^{null} mice with ATLL transplanted as human CD3$^+$ (Fig. 1e) and human CD4$^+$ cells (data not shown). The lymph node tissues of these mice were therefore diagnosed as malignant T-cell lymphoma. Immunohistochemical staining showed that the human ATLL cells expressed the CD3$^+$ (Fig. 1f) and/or CD4$^+$, CD20$^-$ T cell phenotypes (Table 1).

Reverse transcription–polymerase chain reaction and immunohistochemical staining of tax. We used RT-PCR to identify the expression of *HBZ* and *tax* in human ATLL cases (n = 6), ATLL cell line (MT-4), non-specific lymphoadenitis cases without HTLV-1 (n = 2), and T-ALL cell line (Jurkat). The RT-PCR products for *HBZ* were found in all ATLL patients and MT-4, but not in non-specific lymphadenitis cases or Jurkat. The density of RT-PCR bands for *tax* was low in five of the six ATLL cases and in one case *tax* could not be detected (Fig. 2).

Immunohistochemical staining of Tax protein showed a few lymphoma cells to be positive, whereas no Tax signals were detected in some cases (Table 1). These results suggest that the presence of *tax* does not prove the existence of HTLV-1.

***HBZ* gene *in situ* hybridization.** We carried out ISH with the PNA probe targeting the *HBZ* gene in formalin-fixed and paraffin-embedded tissues. A positive reaction was detected, but not in all of the cells in the ATLL cell lines (MT-1, MT-2, and MT-4). Moreover, all HBZ-Tg mice (n = 8), NOD/SCID/β2m^{null} mice with ATLL transplanted (n = 11), and human ATLL (n = 16) showed expression of the *HBZ* gene. The percentage of

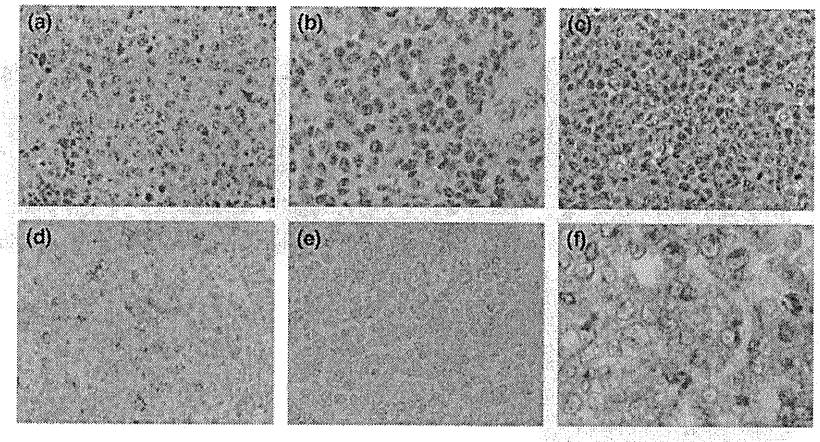

Fig. 1. Histological studies and *HBZ* gene *in situ* hybridization. (a,d) Lymph node sections from HBZ transgenic (HBZ-Tg) mice. (b,e) Lymph node from NOD/SCID/β2-microglobulin^{null} (β2m^{null}) mice with adult T-cell leukemia/lymphoma (ATLL) transplanted. (c,f) Lymph node from patient with ATLL. (a–c) H&E staining. (d) Immunostaining of mice CD4 for T cells. (e,f) Immunostaining of human CD3 for T cells. Histological studies of HBZ-Tg mice and NOD/SCID/β2m^{null} mice with ATLL transplanted showed a pleomorphic lymphoma in the lymph nodes (a,b). Immunohistochemical staining indicated that these tumor cells of HBZ-Tg mice were mice CD3+ (data not shown) and mice CD4$^+$ T cells (d), and the tumor cells of NOD/SCID/β2m^{null} mice with ATLL transplanted were human CD3$^+$ (e) and human CD4$^+$ (data not shown).

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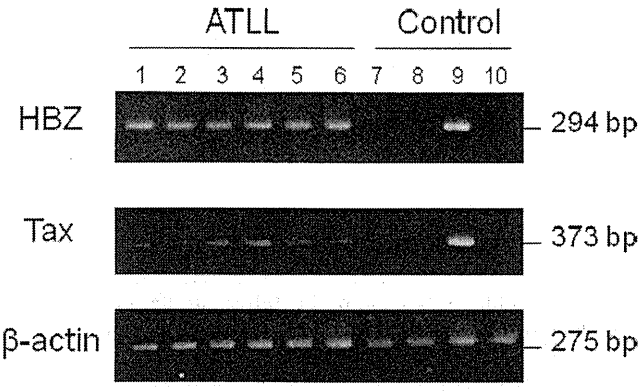

Fig. 2. Expression of mRNA for *HBZ* and *tax* in RT-PCR. Lanes 1–6, patients with adult T-cell leukemia/lymphoma (ATLL); lanes 7 and 8, patients with non-specific lymphadenitis without HTLV-1; lane 9, ATLL cell line (MT-4); lane 10, T-cell acute lymphoblastic leukemia (T-ALL) cell line (Jurkat). The RT-PCR products for *HBZ* were found in ATLL patients and the ATLL cell line, but not in non-specific lymphadenitis cases and the T-ALL cell line. The density of RT-PCR bands for *tax* in ATLL patients was low. β-actin was used as an internal standard. β-actin product could be seen in all cases used in this experiment.

positive cells out of all cells ranged from 5% to 70%. The HBZ-ISH signals localized to the nuclei of lymphoma cells (Fig. 3a–c), but were not detected in the T-ALL cell line (Jurkat) or human DLBCL (n = 5), used as the negative control (Fig. 3d).

Discussion

Adult T-cell leukemia/lymphoma is a human T-cell malignancy associated with HTLV-1. The diagnosis of ATLL can be established on the basis of clinical and laboratory features. HTLV-1 serology is mandatory examination. Southern blot analysis for detection of the monoclonal integration of the HTLV-1 provirus genome is especially necessary for the diagnosis of ATLL, but this method usually requires fresh or frozen specimens and takes a lot of time.

It is important that ATLL should be differentiated from other mature T-cell malignancies because the clinical outcome of each subtype is different.[16] The main subtypes are the cerebriform variants of T-cell prolymphocytic leukemia, mycosis fungoides and Sezary syndrome, peripheral T-cell lymphoma, unspecified (PTCL-U), and occasionally Hodgkin's lymphoma and angioimmunoblastic T-cell lymphoma. The differential diagnosis of ATLL and PTCL-U is often very difficult because of similar morphological and phenotypic features, even though the etiology of ATLL consists of infection by the HTLV-1 virus. The existence of HTLV-1 is thus essential for the differential diagnosis of ATLL and PTCL-U.

The HTLV-1 provirus genome has the *gag*, *pol*, and *env* genes, flanked by an LTR. The pX region is a unique structure of HTLV-1, found between *env* and the 3′-LTR. The plus strand of the pX region encodes the regulatory proteins Tax, Rex, p12, p13, p21, and p30, and the minus strand encodes HBZ. Tax was found to be an important factor in the pathogenesis of ATLL. However, Takeda *et al.*[4] reported that Tax expression was absent in approximately 60% of ATLL cases, and that the frequency of *tax* gene transcription was very low and could not be detected by Western blot analysis, but could be detected by RT-PCR. In addition, the 5′-LTR is frequently methylated or deleted, whereas the 3′-LTR remains intact in ATLL cells.[17,18] In our study, a few lymphoma cells were positive for immunohistochemical staining of Tax protein but Tax signals were not detected in some cases (Table 1). Furthermore, the density of RT-PCR bands for *tax* in ATLL patients was very low (Fig. 2). These results show a low frequency of *tax* detected in tumor cells, so that the presence of *tax* will not prove the existence of HTLV-1. Thus, because expression of the *HTLV-1* gene and protein is uncertain in ATLL, it cannot be the target for histological methods such as immunohistological staining or ISH. If this gene and protein are targeted, therefore, sensitivity may be too low. Ohshima *et al.*[11] reported using ISH targeting HTLV-1 mRNA, as found in the *pX*, *env*, and *gag* regions, and immunohistological staining targeting peptide amino acids such as gag p19, env gp46, and pX40 tax in the lymph nodes of ATLL cases. The reported sensitivity was 45% and the percentage of positive cells was very low.

A recent and remarkable finding in HTLV-1 biology is the characterization of HBZ, which is encoded by the minus strand of a provirus. The HBZ protein suppresses the Tax-mediated transactivation of viral transcription from the 5′-LTR, whereas HBZ RNA promotes ATL cellular proliferation.[19–21] In a previous study, *HBZ* transcripts were detected in all ATL cells, whereas *tax* mRNAs were present in only 40% of cases.[5,22] Furthermore, in the study presented here, all ATLL cases examined expressed *HBZ*. Therefore, evidence of the presence of *HBZ* proves the existence of HTLV-1. This study showed the

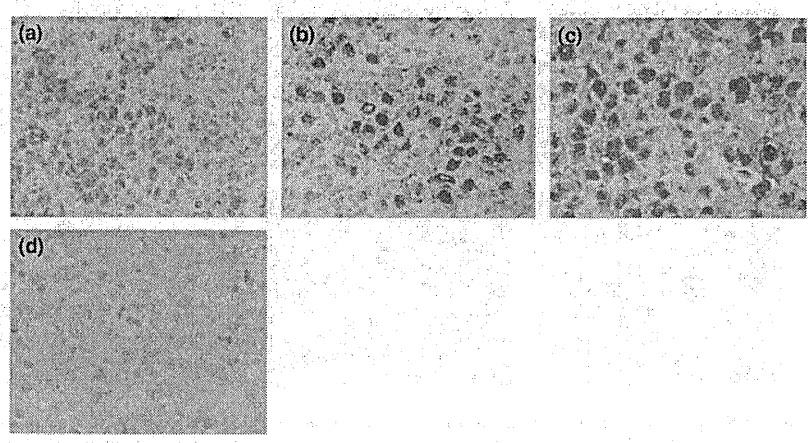

Fig. 3. *HBZ* gene *in situ* hybridization (HBZ-ISH). (a) Lymph node sections from HBZ transgenic mice. (b) Lymph node from NOD/SCID/β2-microglobulin^{null} mice with adult T-cell leukemia/lymphoma (ATLL) transplanted. (c) Lymph node from patient with ATLL. (d) Lymph node from patient with diffuse large B-cell lymphoma (DLBCL). The HBZ-ISH signals localized to the nuclei of lymphoma cells (a–c), but were not detected in human DLBCL (d) used as the negative control.

existence of HTLV-1 by using the HBZ-ISH method for analyzing formalin-fixed and paraffin-embedded tissues.

In a previous study,[12] the PCR/ISH and RT-PCR/ISH methods were useful to detect HTLV-1 positive cells in HTLV-1 sero-indeterminate patients. In ATLL cases, the percentage of positive cells by PCR/ISH and RT-PCR/ISH was not less than those by HBZ-ISH. Although the method of PCR/ISH and RT-PCR/ISH is complicated and frequently fails, HBZ-ISH is easy and failure is rare. Moreover, the character of the PNA probe allows: (i) a resistance to any protease and nuclease; (ii) a higher stability at all pH and salt concentrations; and (iii) a higher affinity for RNA and DNA. Therefore, the HBZ-ISH method is better to detect HTLV-1 positive cells in the same situation.

Although HBZ-Tg mice expressed the *HBZ* gene in all $CD4^+$ cells, in this study, some cells showed negative signals for HBZ-ISH. The reasons for this discrepancy could be that: (i) the ratio of $CD4^+$ cells differs in each mouse; (ii) *HBZ* expression is not constant; or (iii) the virus RNA was destroyed by formalin fixing. In human ATLL cases, differences in sensitivity may be due to the level of HBZ expression from cell to cell.

In conclusion, HBZ-ISH carried out in accordance with the technique described here constitutes a reliable method of HTLV-1 detection, and can be used for the diagnosis of ATLL. Although the method of HBZ-ISH is unsuitable to detect HTLV-1 clonality, this method can be useful for the diagnosis of ATLL with HTLV-1 sero-indeterminate patients.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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