We successfully identified ATLL cells in the peripheral blood and in lymphoma samples and detected the soluble form of CADM1 in the peripheral blood of ATLL patients using specific antibodies for CADM1. The CADM1 antibody may therefore represent a useful tool in the diagnosis of ATLL cells.

MATERIALS AND METHODS

Quantification of HTLV-1 proviral load

HTLV-1 proviral DNA load was determined by real-time PCR as previously described.[20] Briefly, genomic DNA from peripheral blood mononuclear cells (PBMCs) was extracted by proteinase K digestion and phenol/chloroform extraction and then subjected to a real-time TaqMan PCR assay using an ABI PRISM 7000 detection system (Perkin Elmer/Applied Biosystems, Forster City, CA, USA) with two sets of primers specific for the *pX* region of the HTLV-1 provirus and the human gene encoding the RNase P enzyme. The primers and the probe for the *RNase P* gene were purchased from Applied Biosystems; those for the *pX* region of the HTLV-1 provirus were described previously.[20] Genomic DNA of normal control PBMCs mixed with a plasmid DNA, which contained almost the whole genome of the HTLV-1 provirus (*Sac*I site of 5′-LTR to *Sac*I site of 3′-LTR), was used as a standard to quantify the proviral DNA copies. The copy number of the plasmid DNA was calculated based on the size and weight of the plasmid DNA, as measured by spectrophotometry. HTLV-1 proviral loads in some of the PBMC samples were measured by the Group of Joint Study on Predisposing Factors of ATL Development (JSPFAD, Japan) as described previously.[21] The amount of HTLV-1 proviral DNA was calculated as the copy number of HTLV-1 per 100 PBMC = ((copy number of *pX*)/(copy number of *RNase P*/2)) × 100.

RESULTS

Frequent expression of surface CADM1/TSLC1 among ATLL-derived cell lines

CADM1/TSLC1/IgSF4 was identified as a novel surface marker on ATLL cells by gene expression profiling using DNA microarray analysis and was found to be frequently expressed in leukemia cells from patients with acute-type ATLL.[17] We first analyzed the CADM1 protein levels in a panel of T-leukemia cell lines using a chicken anti-human CADM1 antibody (MBL, Nagoya, Japan). A 107 kDa band was clearly detected in whole-cell lysates from the KOB, KK1 and S1T cell lines (Figure 1a), which have been reported

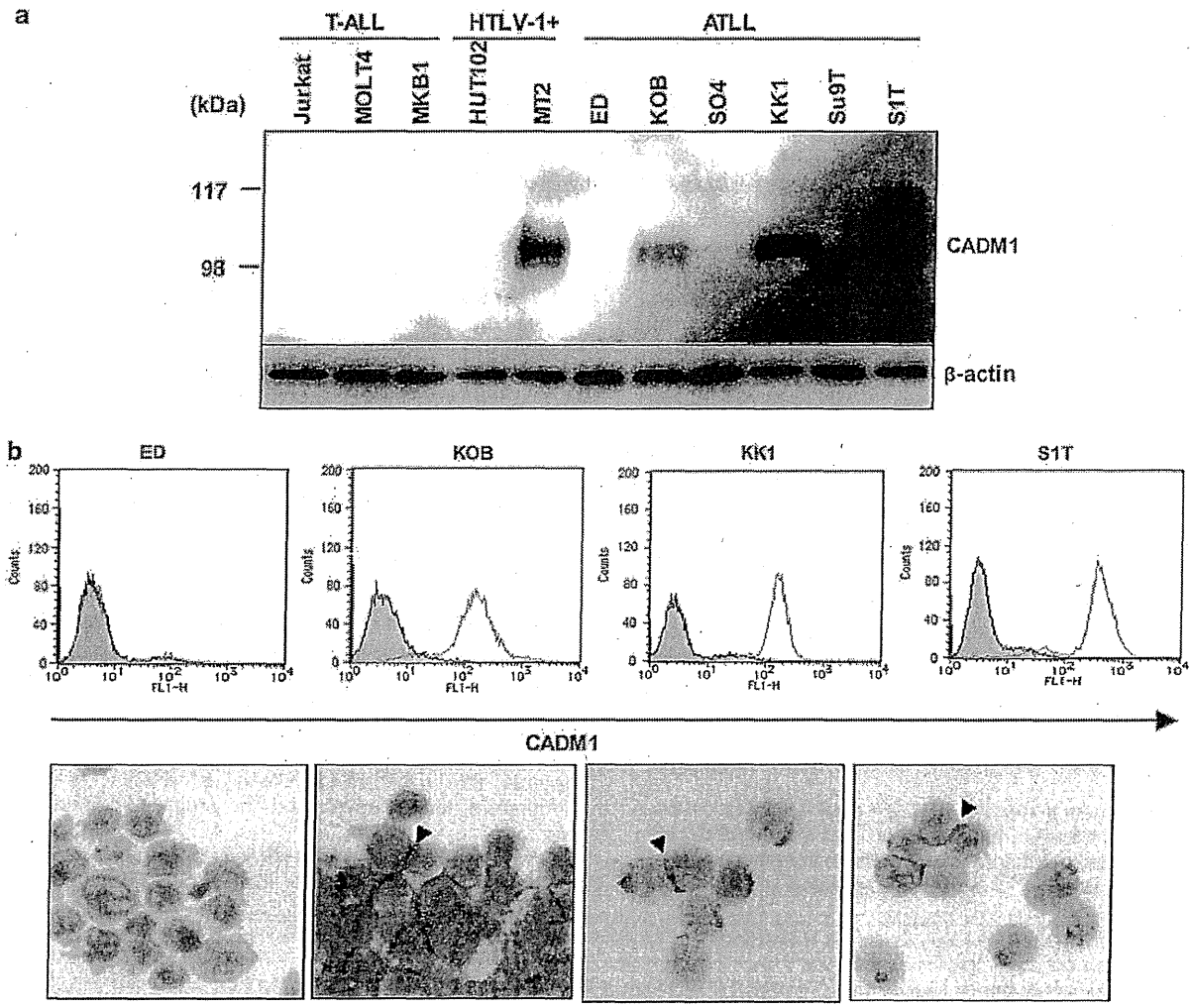

Figure 1. High CADM1 expression in ATLL analyzed by immunoblot, flow cytometry (FMC) and immunohistochemical staining (IHC). (**a**) Immunoblot analysis was performed on a series of T-lymphoid leukemia cell lines (three T-ALL, T-acute lymphoid leukemias; two HTLV-1 +, HTLV-1-infected cell lines; six ATLL, ATLL-derived cell lines) with a chicken anti-human CADM1 antibody. (**b**) A human anti-human CADM1 antibody (051–054), which was established by phage display, was used for FMC and IHC. The anti-CADM1 antibody was visualized by Alexa 488 in FMC and by horseradish peroxidase in IHC.

to express CADM1 according to reverse transcriptase PCR and northern blot analysis.[17] To confirm CADM1 expression on the cell surface of ATLL cells, we examined CADM1 membrane expression by flow cytometry with an Alexa 488-labeled human anti-CADM1 antibody generated by phage-display technology.[22] Four ATLL cell lines were used for flow cytometry: CADM1-negative ED and CADM1-positive KOB, KK1 and S1T cell lines. In all three CADM1-positive cell lines, the fluorescence intensity of CADM1 expression was two logs greater than that of the isotype immunoglobulin G control (Figure 1b, upper panels), while only background levels of fluorescence could be seen in the CADM1-negative ED-ATLL cell line, which had high levels of DNA methylation in the CADM1 promoter region.[17] To evaluate the subcellular distribution of CADM1, immunohistochemical staining was performed on the same cell lines using the anti-CADM1 antibody (Figure 1b, bottom panels). CADM1 was highly concentrated at the cell–cell contact sites in the three CADM1-positive cell lines, and no staining of CADM1 was detected in the ED cell line. These data suggest that CADM1 expression in ATLL cells may promote cell-to-cell contact.

Low levels of CADM1 expression in the T-reg fraction of peripheral lymphocytes

To examine the expression of CADM1 in peripheral blood T-lymphocytes of healthy volunteers, T-reg populations were analyzed for CADM1 expression because $CD4^+CD25^{high}$ T-reg cells are a potential source of ATLL cells.[5,6] Initially, the $CD4^+CD25^+$ cell fraction was separated from PBMCs of a healthy volunteer by the magnetic bead method and stained with an anti-CADM1 antibody. Almost 100% of the S1T-ATLL cell line was strongly stained with the anti-CADM1 antibody; however, 55.8% of the $CD4^+CD25^+$ cells were stained weakly in comparison with the high level of staining of S1T-ATLL cells (Figure 2a). To confirm whether the purified $CD4^+CD25^+$ cells expressing CADM1 were T-reg cells, the sorted $CD4^+CD25^+$ cells were stained for both FoxP3 (a master regulator in the development of T-reg cells) and CADM1. In all, 93% of the $CD4^+CD25^+$ double-positive cells in the peripheral blood were stained by the anti-FoxP3 antibody, while 37% of the cells were stained with both the anti-CADM1 and anti-FoxP3 antibodies (Figure 2b), suggesting that a fraction of the $CD4^+CD25^+FoxP3^+$ T-reg cells weakly expressed CADM1 on their cell surfaces.

We then determined the proportion of $CD4^+CADM1^+$ and $CD4^+CD25^+$ T cells in PBMCs from 10 healthy volunteers after selection with Cy5-labeled CD45 staining. On average, 7.3% of $CD45^+$ cells in PBMCs expressed CD4 and CD25, while only 0.6% of the cell population expressed CD4 and CADM1 (Figure 2c and representative fluorescence-activated cell sorting data are shown in Supplementary Figures 1a and b), indicating that the number of $CD4^+CADM1^+$ cells was significantly lower than the number of $CD4^+CD25^+$ cells in the PBMCs of healthy volunteers. To determine the percentage of $CD4^+CADM1^+$ cells in peripheral lymphocytes of various types of ATLL and HTLV-1 carriers, $CD45^+$ PBMCs from 40 patients diagnosed with various types of ATLL (7 acute-type, 4 lymphoma-type, 6 chronic-type and 23 smoldering-type), 51 HTLV-1 carriers and 10 normal volunteers were analyzed for the surface expression of CD4 and CADM1 by flow cytometry analysis, which was performed by double staining of CD12/CD19, CD3/CD8, CD4/CD25, CD23/CD5, CADM1/CD4, CD20/CD11c, CD16/CD56, CD30/CD7 and κ-chain/λ-chain. The median percentages of $CD4^+CADM1^+$ cells were 73.9% in acute cases, 72.4% in chronic cases (except for a patient with CD4-negative ATLL described below), 5.6% in lymphoma cases, 11.5% in smoldering cases, 4.4% in HTLV-1 carriers and 0.5% in normal volunteers (Figure 2d). In these subjects, the percentages of $CD4^+CD25^+$ cells were significantly correlated with those of $CD4^+CADM1^+$ cells ($R=0.907$, $P<0.0001$) (Figure 2e), suggesting that most of the ATLL cells were $CD4^+CD25^+CADM^+$. However, we also observed a cell surface profile of $CD3^+CD8^-$ (91.3%), $CD25^+CD4^-$ (81.5%) and $CD4^-CADM1^+$ (83.6%) in a case of chronic ATLL, suggesting that the surface markers of the ATLL cells represented $CD4^-CD8^-$ double-negative T lymphocytes that expressed CD25 and CADM1.

CADM1 expression in leukemia cells from ATLL patients and HTLV-1-infected cells from HTLV-1 carriers

To confirm that most of the HTLV-1-infected ATLL cells were indeed in the $CD4^+CADM1^+$ cell fraction, PBMCs from an HTLV-1 carrier and two ATLL patients with chronic or smoldering ATLL were isolated and separated into CADM1-positive and CADM1-negative cell fractions by anti-CADM1 antibody-conjugated magnetic beads. The cell fractions were then analyzed for the expression of CD4 and CADM1 by fluorescence-activated cell sorting analysis (Supplementary Figure 2). In these three patients, 3.4 to 31.4% of PBMCs were positive for CD4 and CADM1. After separation by the magnetic CADM1 antibody, 73.5 to 96.5% of the cells were $CD4^+CADM1^+$. To assess whether these $CD4^+CADM1^+$ cells indeed represented the HTLV-1-infected cell population, the HTLV-1 status was determined by PCR of the proviral DNA with primers against the *HBZ* region of the HTLV-1 genome. As shown in Figure 3a, the HTLV-1 genomic sequence was detected in the three CADM1-positive cell fractions, while weak or no signal was detected in the CADM1-negative cell fractions, indicating that the majority of HTLV-1-positive cells are present in the CADM1-positive cell fractions.

Next, the percentages of $CD4^+CADM1^+$ cells were compared with those of abnormal lymphocytes or with the DNA copy numbers of HTLV-1 in PBMCs of patients with various types of ATLL, which included 6 acute-type, 8 chronic-type and 6 smoldering-type of ATLL, and 20 HTLV-1 carriers (Figures 3b and c). The percentages of $CD4^+CADM1^+$ cells showed a high degree of correlation with those of abnormal lymphocytes ($R=0.791$, $P<0.0001$) and with the HTLV-1 DNA copy numbers ($R=0.677$, $P<0.0001$) in these patient samples. Notably, in two samples from chronic- and smoldering-type ATLL patients, the number of $CD4^+CADM1^+$ cells was less than one-half of the number of HTLV-1 DNA copies (32.0% vs 107.97 copies and 30.0% vs 65.76 copies), which may be due to multiple copies of proviral DNA in the cells. In addition, the percentages of $CD4^+CADM1^+$ cells were correlated with the levels of sIL-2Rα ($R=0.586$, $P<0.0001$) and with the levels of LDH ($R=0.486$, $P=0.0015$) (Figures 3d and e). Consistent with earlier studies, both serum sIL-2Rα and LDH levels were correlated with the HTLV-1 DNA copy numbers ($R=0.705$; $P<0.0001$ and $R=0.44$; $P=0.0045$, respectively) in this study (data not shown).

To further evaluate the diagnostic efficacy of measuring CADM1-positive cells to detect HTLV-1-infected cells, the copy number of the HTLV-1 provirus in PBMCs of carriers was compared with the percentages of $CD4^+CADM1^+$ cells and the serum levels of sIL-2Rα and LDH. The percentage of $CD4^+CADM1^+$ cells showed a significant correlation with the HTLV-1 DNA copy number ($R=0.921$, $P<0.0001$) (Figure 3f), while there was a poor correlation between HTLV-1 copy number and the levels of sIL-2Rα and LDH (data not shown). A correlation between the percentage of $CD4^+CADM1^+$ cells and abnormal lymphocytes was also observed in the HTLV-1 carriers ($R=0.819$, $P<0.0001$), although abnormal lymphocytes and $CD4^+CADM1^+$ cells were very rare in these subjects (Supplementary Figure 3). On the basis of these data, in addition to the determination of copy numbers of HTLV-1 proviral DNA, quantification of $CD4^+CADM1^+$ cell number by flow cytometry may be useful for monitoring the number of HTLV-1-infected cells in the peripheral blood of ATLL patients and HTLV-1 carriers.

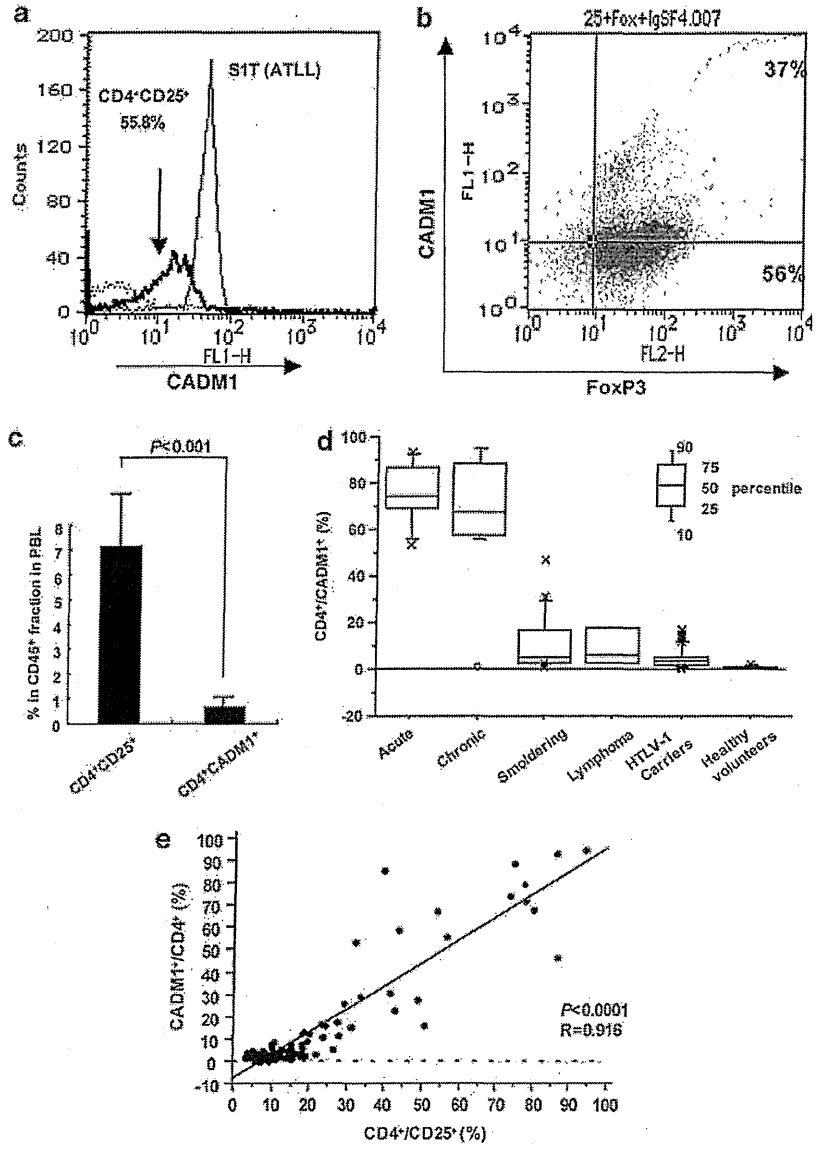

Figure 2. Flow cytometric analysis of CADM1 in T-reg lymphocytes, ATLL cells and HTLV-1-infected T cells. (**a**) Flow cytometric analysis of CADM1 expression in the $CD4^+CD25^+$ fraction from peripheral T lymphocytes. Each sample was stained with an Alexa 488-labeled anti-CADM1 antibody. The S1T-ATLL cell line with high CADM1 expression was used as a positive control. (**b**) The $CD4^+CD25^+$ fraction from peripheral lymphocytes was stained by the Alexa 488-labeled anti-CADM1 and PE-labeled anti-FoxP3 antibodies. (**c**) Comparison of percentages between the $CD4^+CD25^+$ and $CD4^+CADM1^+$ cell fractions in the $CD45^+$ fraction of peripheral blood lymphocytes. (**d**) Box plots are shown for the percentages of the $CD4^+CADM1^+$ cell fractions in $CD45^+$ peripheral blood lymphocytes from patients with various types of ATLL, HTLV-1 carriers and healthy volunteers. The data from a CD4-negative ATLL case are indicated by a white circle. (**e**) Comparison between $CD4^+CADM1^+$ and $CD4^+CD25^+$ cell fractions in $CD45^+$ peripheral blood lymphocytes from patients with various types of ATLL, HTLV-1 carriers and healthy volunteers. Spearman correlation coefficients were calculated to assess the association between $CD4^+CADM1^+$ and $CD4^+CD25^+$ cell fractions.

The soluble form of CADM1 is detected in the serum of ATLL patients

A soluble isoform of CADM1 consisting of the extracellular domain was recently isolated in murine mast cells.[23] We determined whether the soluble form of CADM1 was present in the serum of ATLL patients by western blot using a chicken anti-human CADM1 antibody. As a positive control, soluble CADM1 was produced by transfection of 293 cells with a construct encoding a soluble form of CADM1 (1 to 374 aa). The soluble CADM1 band (72 kDa) and the recombinant soluble form of CADM1 were clearly detected in the sera of five patients with acute-type ATLL but not in the sera of five healthy volunteers (Figure 4a). We screened the sera of 5 healthy controls and 25 ATLL patients (14 acute-type, 7 lymphoma-type, 2 smoldering-type and 2 HTLV-1 carrier) for the presence of soluble CADM1. We detected different levels of soluble CADM1 among these ATLL patients by western blot (data not shown). In addition, we compared the levels of soluble CADM1 in the serum and the percentages of $CD4^+CADM1^+$ cells in the peripheral blood (Supplementary Figure 4) and confirmed that high levels of soluble CADM1 are present in the serum of patients who had high numbers of $CADM1^+$ cells in the peripheral blood. As serum levels of soluble IL-2Rα are correlated with the prognosis

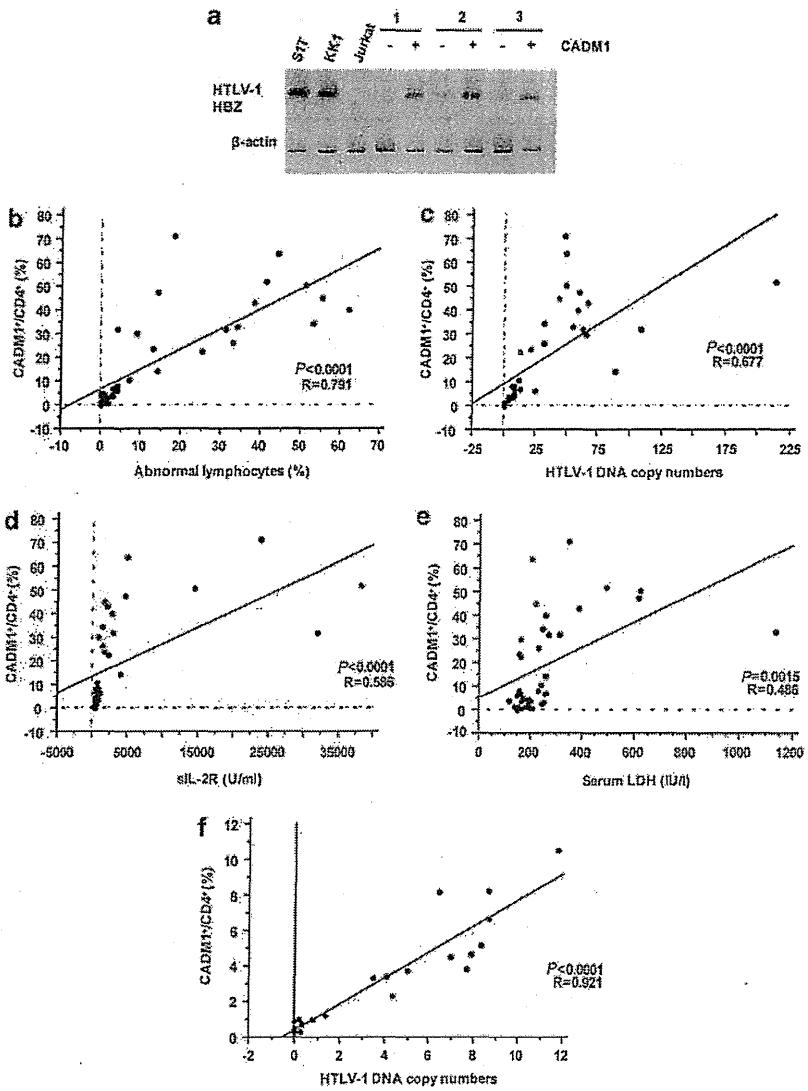

Figure 3. Correlation of the percentages of the $CD4^+CADM1^+$ fraction with the percentages of abnormal lymphocytes, HTLV-1 DNA copy number and the levels of soluble IL-2Rα and serum LDH in both various types of patients with ATLL and HTLV-1 carriers. (**a**) Identification of the HTLV-1 genome by PCR amplification after separation by CADM1-magnetic beads. After separation of the peripheral blood of three ATLL patients by magnetic beads, genomic DNA was extracted from both the CADM1 and non-CADM1 fractions and amplified by specific PCR primers for HTLV-1 *HBZ*. Two ATLL cell lines (S1T and KK1) were used as positive controls, and a T-ALL cell line (Jurkat) was used as a negative control for the HTLV-1 *HBZ*. Lane 1, smoldering ATLL; lane 2, chronic ATLL; lane 3, HTLV-1 carrier. (**b-e**) The percentage of the $CD4^+CADM1^+$ fraction was compared with the percentage of abnormal lymphocytes, the HTLV-1 DNA copy number and the levels of soluble IL-2Rα and serum LDH in both various types of patients with ATLL and HTLV-1 carriers. In (**d**), data from one acute-type patient were not included in the analysis because of the extremely high levels of soluble IL-2Ra ($CD4^+CADM1^+$, 32.9%; IL-2Ra, 96 900 U/ml). (**f**) The percentage of the $CD4^+CADM1^+$ fraction was compared with the HTLV-1 DNA copy number in HTLV-1 carriers.

of ATLL patients, we compared the serum levels of soluble CADM1 and soluble IL-2Rα in individual cases. As shown in Figure 4b, significantly higher levels of soluble CADM1 were detected in the serum of ATLL patients who had increased levels of soluble IL-2Rα; thus, serum CADM1 levels may be a diagnostic tool for the prediction of disease progression in ATLL.

High expression of CADM1 in ATLL-derived lymphomas

To examine the expression of CADM1 in tissue sections from lymphoma-type ATLL, formalin-fixed lymphoma samples from different types of malignant lymphomas were immunostained with the anti-CADM1 antibody. For these studies, we used a monoclonal antibody (1-10C) raised against the recombinant extracellular domain of the CADM1 protein. To confirm the reactivity of the anti-CADM1 antibody in formalin-fixed ATLL cells, cell pellets from various leukemia cell lines were fixed in 10% formalin, embedded in paraffin and stained for CADM1. The anti-CADM1 antibody specifically stained the surface of the CADM1-positive S1T-ATLL cell line but did not react with the CADM1-negative ED-ATLL and all non-ATLL cell lines (Figure 5a, panels 1 and 2, and Supplementary Figure 5a). Western blot analysis confirmed the lack of CADM1 expression in these cell lines (Figure 1a and Supplementary Figure 5b). We next performed immunostaining of lymph node biopsies from ATLL patients with malignant lymphoma using the anti-CADM1 antibody. As positive controls, we used erythrocytes and peripheral nerve tissue (Figure 5a, panels 3 and 4).[17,18] In addition, we examined CADM1

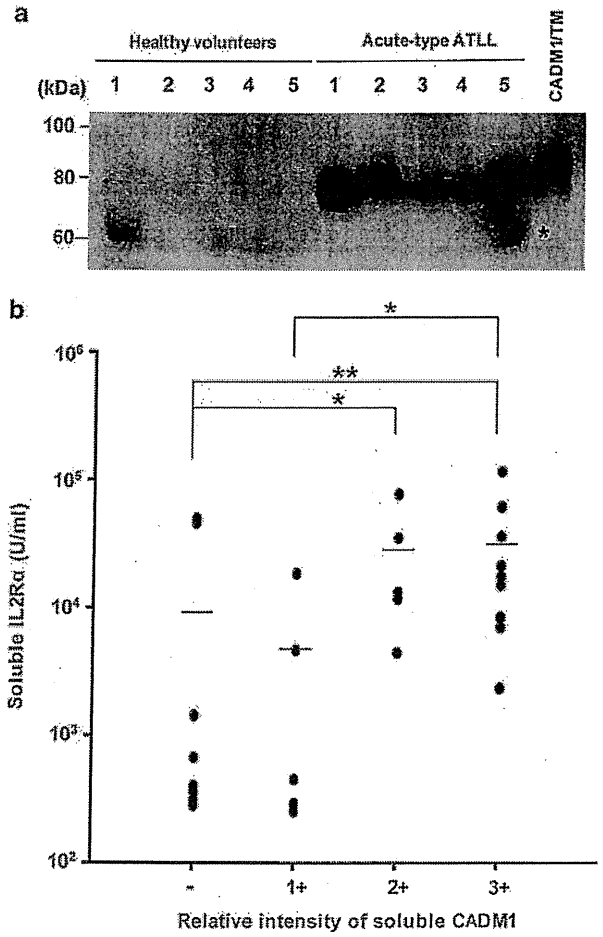

Figure 4. Identification of a soluble form of CADM1 in ATLL patients. (**a**) The soluble form of CADM1 in the peripheral blood from five healthy volunteers and five patients with acute-type ATLL was identified by immunoblot analysis using an anti-CADM1 antibody. The asterisk indicates an albumin band. Truncated CADM1 with an extracellular domain was purified from the culture supernatant of 293 cells after transfection of the CADM1 expression plasmid as a positive control. (**b**) The relative band intensity of CADM1 by immunoblot was compared with the level of sIL-2Rα in various serum samples from healthy volunteers, HTLV-1 carriers and ATLL patients. The band intensity was measured by the Image Gauge software (Fujifilm, Tokyo, Japan). The signal intensities were classified as either high (3+), medium (2+), low (1+) or undetectable (-). Asterisks indicate a significant difference between the band intensities of the groups (*$P<0.001$, **$P<0.0001$).

expression in three cases of lymph nodes with reactive follicular and/or paracortical hyperplasia (reactive lymph nodes) and found that most of the lymphocytes in the reactive lymph nodes were negatively stained and <1% of the cells were positively stained (Figure 5a, panel 5). The staining pattern of the CADM1-positive cells in the reactive lymph nodes mainly shows a uniform cytoplasmic pattern rather than the specific membranous staining that was seen in ATLL cells (as shown below and in Figure 1b). The CADM1-positive cells in reactive lymph node possibly correspond to histiocytes, including dendritic cells because a subset of T-cell zone dendritic cells was reported to express CADM1 (Necl-2) within the lymph node.[24,25] We examined 90 tissue samples from patients with various types of lymphoma, including 36 patients with ATLL and 54 with non-ATLL lymphomas, using erythrocytes and nerve fascicles as positive controls. Of the non-ATLL samples, 29 cases were T- or NK-cell lymphomas, 37 cases were B-cell lymphomas and 2 cases were null-cell lymphomas. Using a four-grade scale to score CADM1 immunohistochemical staining (0 to 3+, Figure 5b), we found that 92% of ATLL lymphomas were positive for CADM1, and 50% of them were heavily stained and were scored 2+ or higher (Table 1). Of note, a few lymphoma cells showed diffuse cytoplasmic staining in addition to membrane staining with CADM1. Among the non-ATLL lymphomas, a few CADM1-positive cells were observed, the morphology of which was small to medium in size with normochromatic round to ovoid nuclei and lacking nuclear atypia (Figure 5c). Based on the morphology and the CADM1-staining patterns, the CADM1-positive cells in the non-ATLL lymphomas were not considered as lymphoma cells but may correspond to histiocytes, including dendritic cells, because these cells were similar to the CADM1-positive cells found in reactive lymph nodes (Figure 5a, panel 5 and Figure 5c). Based on these results, a high degree of cell membrane staining for CADM1 with a score of 2+ may provide high specificity in the diagnosis of ATLL, and combined staining with CADM1 and other T-cell-specific markers may be necessary for a more accurate diagnosis of lymphoma-type ATLL.

DISCUSSION

In this study, we made a series of antibodies against CADM1 to be used as diagnostic tools for ATLL, such as for the identification and separation of ATLL and HTLV-1-infected cells, the detection of the soluble form of CADM1 in peripheral blood and the pathological identification of lymphoma-type ATLL after formalin fixation. Expression of CADM1 by flow cytometry was clearly detected on the surface of ATLL cells and HTLV-1-infected T lymphocytes, which was confirmed by detection of the HTLV-1 genome after separation by magnetic beads with a CADM1 antibody. The percentage of $CD4^+CADM1^+$ cells in the peripheral blood correlated highly with the DNA copy number of HTLV-1 in lymphocytes from HTLV-1 carriers and ATLL patients. In particular, we identified the soluble form of the CADM1 protein in the peripheral blood of HTLV-1 carriers and ATLL patients. The definitive diagnosis of ATLL is based on the confirmation of ATLL cells in the peripheral blood or in lymphoma tumors by detection of HTLV-1 genomic integration; therefore, measurement of serum levels of soluble CADM1 protein as well as detection of $CD4^+CADM1^+$ cells in the blood, when used in conjunction with other standard diagnostic methods, would be useful for identifying and monitoring disease progression in HTLV-1 carriers with increased accuracy and may aid in the early diagnosis and measurement of treatment effects for ATLL.

It has been proposed that HTLV-1 infects various types of cells, including T-reg cells and subsets of T helper cells (Th2 and Th17), in a cell-to-cell manner.[26–29] There is also evidence that ATLL cells act as T-reg cells that express CD4, CD25 and FoxP3 and are thought to contribute to the immune suppression of ATLL patients;[6] however, it was reported that CADM1 is expressed at low levels on resting naive T cells, and its expression is further downregulated 14 h following TCR activation.[30] Therefore, we determined the expression of CADM1 in the T-reg cell fraction of the peripheral blood of healthy volunteers. The results showed that a subset of the T-reg fraction weakly expressed CADM1, suggesting that CADM1 is not a major marker for the T-reg fraction and that CADM1 expression on ATL cells may reflect the fact that ATL cells originate from T-reg cells. As ATLL cells that constitutively express CD25 exhibited heterogeneous Foxp3 expression patterns,[5] a part of ATLL is likely derived from $FoxP3^+$ T-reg cells. In another report, a population of $FoxP3^+$ cells distinct from ATLL cells was shown to have a regulatory function and was found to impair the cell-mediated immune response to HTLV-1 in patients with ATLL.[31] Although we do not know whether the population of T-reg cells with weak expression of CADM1 in the

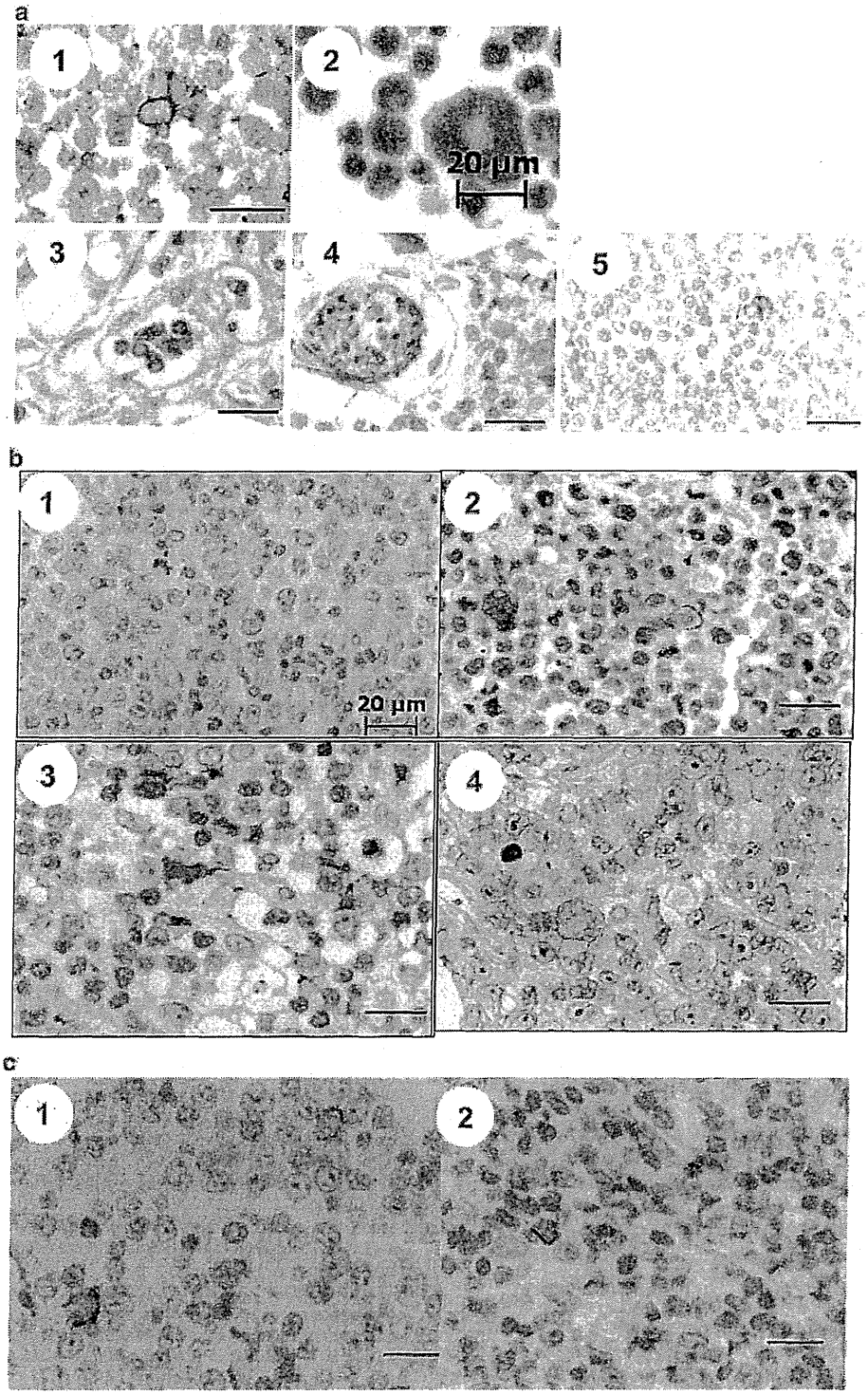

Figure 5. Expression of CADM1 in lymphoma-type ATLL. (**a**) Immunostaining of CADM1 in the S1T-ATLL cell line was used as a positive control (a1), and the ED-ATLL cell line was used as a negative control (a2) for CADM1 expression using an anti-CADM1 antibody (1-10C). *Immunostaining of erythrocytes in the blood vessels* (**c**)*, peripheral nerve cells (a3) and reactive lymph nodes (a4) using the same antibody.* Scale bar, 20 μm. (**b**) The immunoreactivity for CADM1 was scored using a standardized four-tiered scoring scale as follows: staining in >30% of cells was scored as 3+ (b4); staining in >5% but <30% of cells was scored as 2+ (b3); staining in <5% of cells was scored as 1+ (b2); and a lack of staining was scored as 0 (b1). These images were taken from immunostained ATLL lymphoma sections. Scale bar, 20 μm. (**c**) Representative CADM1 immunostaining in B-cell (c1) and NK-cell (c2) lymphomas. Scale bar, 20 μm.

Table 1. Immunohistochemical staining of CADM1 in various types of lymphomas, including ATLL

	Case numbers	Staining scores				Positive rates (%)	
		Negative	1+	2+	3+	⩾1+	⩾2+
ATLL	36	3	15	14	4	33/36 (92)	18/36 (50)
Non-ATLL	54	37	16	1	0	17/54 (31)	1/54 (1.8)
T/NK	15	12	3	0	0	3/15 (20)	0/15 (0)
B	37	23	13	1	0	14/37 (38)	1/37 (2.7)
Null	2	2	0	0	0	0/2 (0)	0/2 (0)

Abbreviations: ATLL, adult T-cell leukemia/lymphoma; CADM1, cell adhesion molecule 1. The immunoreactivity for CADM1 was scored using a standardized four-tiered scoring scale as follows: staining in >30% of cells was scored as 3+; staining in >5% but <30% of cells was scored as 2+; staining in <5% of cells was scored as 1+; lack of staining was scored as 0.

PBMCs of healthy volunteers is the cellular origin for ATLL cells, CADM1 is thought to be one of the major markers for the various types of ATLL cells. In fact, we observed strong expression of CADM1 in rare cases of ATLL characterized by the $CD4^+CD8^+$, $CD4^-CD8^+$ or $CD4^-CD8^-$ phenotypes (data not shown); therefore, the $CADM1^{high}$ population of T-lymphocytes in peripheral blood can be considered ATLL cells.

The question of why CADM1 is strongly expressed on the surface of various types of ATLL remains unclear. Previously, we investigated whether the expression of CADM1 was induced by HTLV-1/Tax expression and found that Tax protein expression did not activate the expression of CADM1 in JPX-9 cells (data not shown). We also introduced a Tax expression vector into MOLT4 and 293T cells and determined the expression level of CADM1. We found that Tax could not induce CADM1 expression in these cells, suggesting that Tax expression is not related to the high expression of CADM1. As HBZ is known to be constitutively expressed in both HTLV-1-infected cells and ATLL cells and can modulate transcription of cellular genes,[16] it is possible that HBZ activates CADM1 expression. We also speculate that $CADM1^{high}$ expression in ATLL cells may be associated with transcriptional abnormalities in ATLL cells through the accumulation of genomic or epigenomic alterations. In this study, we found a good correlation between HTLV-1 copy numbers and the percentages of $CD4^+CADM1^+$ cells in the peripheral blood of HTLV-1 carriers, suggesting that HTLV-1 carriers with high percentages of $CD4^+CADM1^+$ cells could be associated with progressive genetic alterations and might be at high risk for developing ATLL.

Recent studies have shown that a few markers, such as CCR4 and CD70, are unique ATLL surface markers.[32,33] Although the proportion of $CD4^+CCR4^+$ cells and $CD4^+CD70^+$ cells in the PBMCs from healthy individuals were found to be approximately 5%,[27,33] the proportion of $CD4^+CADM1^+$ cells was <1% (Figure 2); therefore, measurement of $CADM1^+$ T cells is particularly efficient in the diagnosis of HTLV-1 infection in individuals who carry a small number of HTLV-1-infected cells. We have demonstrated previously that CADM1 has important functions in increasing cell adhesion and mediating progression to organ invasion.[19] In this study, we succeeded in isolating a low percentage of both HTLV-1-infected cells from the PBMCs of HTLV-1 carriers with high HTLV-1 copy numbers and ATLL cells from patients with ATLL. The sorted HTLV-1-infected cells and ATLL cells could become useful tools for transcriptional and/or genomic analysis that may be used to compare their results with those of PBMCs from either healthy volunteers or peripheral leukemia cells from patients with ATLL. The results may provide important information on the expression patterns and/or genomic abnormalities that occur at the early stages of HTLV-1 infection and/or ATLL development.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Review Article

CADM1/TSLC1 is a Novel Cell Surface Marker for Adult T-Cell Leukemia/Lymphoma

Shingo Nakahata and Kazuhiro Morishita

CADM1/TSLC1 (Cell adhesion molecule 1/Tumor suppressor in lung cancer 1) is a cell adhesion molecule that was originally identified as a tumor suppressor in lung cancer. *CADM1/TSLC1* expression is reduced in a variety of cancers via promoter methylation, and this reduction is associated with poor prognosis and enhanced metastatic potential. In contrast, we observed that *CADM1/TSLC1* is highly and ectopically expressed in all primary adult T-cell leukemia/lymphoma (ATLL) cells and in most human T-cell leukemia virus type (HTLV)-1-infected T-cell and ATLL cell lines. No expression, however, was detected in $CD4^+$ T cells or in several other non-HTLV-1-infected leukemia cells. Moreover, we identified that high CADM1/TSLC1 expression plays an important role in enhanced cell-cell adhesion to the vascular endothelium, tumor growth and the ability of ATLL cells to infiltrate organs. We developed various antibodies as diagnostic tools to identify $CADM1^+$ ATLL cells. Using flow cytometry, we determined that CADM1/TSLC1 is present on the surface of ATLL cells. The percentage of $CD4^+$ $CADM1^+$ cells in the peripheral blood of HTLV-1 carriers and ATLL patients was highly correlated with the DNA copy number of HTLV-1 in lymphocytes. In particular, we identified the soluble form of CADM1/TSLC1 in the peripheral blood of HTLV-1 carriers and ATLL patients. Therefore, measurements of soluble CADM1/TSLC1 serum levels and the detection of $CD4^+$ $CADM1^+$ cells in the blood, when combined with standard diagnostic methods, would be useful for identifying and monitoring disease progression in HTLV-1 carriers. Such tests would provide increased accuracy and may aid in early diagnosis and in determining the effects of ATLL treatments. [*J Clin Exp Hematopathol 52(1) : 17-22, 2012*]

Keywords: CADM1/TSLC1, cell adhesion molecule, cell surface marker, invasion, adult T-cell leukemia/ lymphoma

INTRODUCTION

The *CADM1* (*Cell adhesion molecule 1, Tumor suppressor in lung cancer 1*; *TSLC1*, *IgSF4*, *Necl2*, *Syncam* or *SgIGSF*) gene encodes an immunoglobulin (Ig) superfamily cell adhesion molecule (IgCAM). *CADM1* is a well-known tumor suppressor gene in a variety of human cancers, particularly those of epithelial cell origin, including liver, pancreatic and prostate cancers. This gene is generally inactivated in these carcinomas via 2 mechanisms; promoter methylation and/or loss of heterozygosity at the gene locus.[1] The *CADM1* gene encodes a 442-amino acid class I membrane protein and contains three Ig loops in the extracellular domain, a transmembrane domain and a short cytoplasmic domain.[2] This primary structure is also observed in IgCAM protein family members, which are referred to as nectins. Although nectins associate with afadin, CADM1/Necl2 (Nectin-like protein 2) does not.[3] However, several CADM1/TSLC1-interacting proteins have been identified, such as DAL-1/4.1B of the protein 4.1 family, the members of which are known to be spectrin-actin-binding proteins.[4] CADM1/TSLC1 forms homodimers through *cis* interactions, and these interactions contribute to cell-cell interactions at the lateral membranes of polarized epithelial cells.[5] Class I-restricted T cell-associated molecule (CRTAM), a two Ig domain-bearing surface receptor, was also identified as a CADM1/TSLC1 ligand.[6] CADM1/TSLC1 interacts with CRTAM to promote natural killer (NK) cell cytotoxicity, interferon-γ secretion by $CD8^+$ cells *in vitro* and NK cell-mediated rejection of tumors that express CADM1/TSLC1 *in vivo*.[6] It is proposed that the disruption of cell adhesion via the loss of *CADM1/TSLC1* leads to cancer cell invasion or metastasis.[1] The tumorigenic potential of *CADM1/TSLC1*, which is located on chromosome 11q23, was first reported by Murakami *et al.*[7,8] This gene was identified as a tumor suppressor in human non-small-cell lung cancers (NSCLCs) based on combinatorial analyses of yeast artificial chromosome transfers into human NSCLC cells and a tumorigenicity assay of these modified lines in

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nude mice.[7,8] Subsequently, a number of studies reported the loss of CADM1/TSLC1 expression in malignant carcinomas. For example, promoter methylation of *CADM1/TSLC1* was demonstrated in 44% of NSCLCs, 27% of pancreatic cancers, 29% of hepatocellular carcinomas, and 32% of prostate cancers.[8-11] Thus, it was concluded that *CADM1/TSLC1* was a tumor suppressor. On the contrary, our laboratory has reported that CADM1/TSLC1 is overexpressed in adult T-cell leukemia/lymphoma (ATLL) cells and plays a role in oncogenesis.[12] Here, we summarize the findings and significance of this area of research.

IDENTIFICATION OF *CADM1/TSLC1* AS A POSSIBLE MARKER FOR ATLL

ATLL is an aggressive and fatal $CD4^+$ T-cell malignancy that is caused by infection with human T-cell leukemia virus type 1 (HTLV-1).[13,14] HTLV-1 is endemic in the Caribbean basin, southern Japan, central and western Africa. After a long latency period, a fraction of carriers develop ATLL.[15] The lifetime incidence of ATLL among HTLV-1 carriers is estimated to be 2.1% in women and 6.6% in men.[16] Despite improved therapies, ATLL still has a very poor prognosis.

The potent oncoprotein Tax is encoded in the pX region of HTLV-1. Tax activates the transcription of HTLV-1 and cellular genes by cooperating with cellular transcription factors. Tax alters many transcriptional pathways, activating cyclic adenosine monophosphate response element binding protein, activator protein-1, and nuclear factor-κB. Tax also represses p53 and interferes with several cell cycle regulators, including cyclins and CDK inhibitors (p15 and p16).[17] These multiple functions of Tax are believed to be involved in the immortalization of HTLV-1-infected cells.

In contrast, Tax expression is undetectable in approximately 60% of leukemias.[18] There are 3 proposed mechanisms for the inactivation of Tax expression in ATLL cells. First, genetic changes (nonsense mutations and deletion) of the *Tax* gene have been described.[18] Second, the deletion of the 5′ long terminal repeat (LTR) that contains the viral promoter has been implicated in the inactivation of Tax expression.[19] Third, the 5′-LTR can be hypermethylated, leading to promoter inactivation.[20] Because Tax is the major target of cytotoxic T-lymphocytes, a disruption or decrease in Tax expression can facilitate the escape of ATLL cells from the host cytotoxic T-lymphocytes, contributing to the development of ATLL. Alternatively, the 3′ LTR may remain unmethylated and intact in ATLL cells.[21] *HBZ* is transcribed from the minus strand of the provirus using the 3′ LTR as a promoter. It has been reported that *HBZ* is expressed in ATLL cells and promotes ATLL cell proliferation.[22] Considering the long latency period of ATLL, it has been proposed that at least five additional genetic or epigenetic events are required for the development of overt disease.[23]

Although no specific chromosomal abnormalities have been identified in ATLL,[24,25] human leukemias are often associated with primary genetic alterations, usually non-random reciprocal chromosomal translocations.[26] It has been reported that the tumor suppressor genes *p53, p15*, and *p16* are disrupted in aggressive ATLL via chromosomal loss or promoter methylation.[27] However, because ATLL-related genomic alterations are enormously diverse and complex, the molecular basis of the multistep process of leukemogenesis in ATLL remains unclear.

To identify specific genetic markers for ATLL, we previously described the gene expression profiles of ATLL cells and identified highly expressed genes therein. These analyses were performed using cells from patients with acute-type ATLL using a GeneChip microarray, which contained oligonucleotide hybridization probes for more than 12,000 genes.[12] After determining the expression profiles from the panels of ATLL patients, we identified three genes that were up-regulated more than 30-fold in ATLL cells, including *CADM1/TSLC1*, *caveolin 1* (*CAV1*), and *prostaglandin D2 synthase*. Unexpectedly, *CADM1/TSLC1* was overexpressed in all primary ATLL cells and in most HTLV-1-infected T-cells and ATLL cell lines. As described above, CADM1/TSLC1 is a member of the Ig superfamily of cell adhesion molecules and participates in cell-cell interactions. Previous reports have demonstrated that CADM1/TSLC1 is expressed in nearly all organs but not in lymphoid tissues,[2] suggesting a functional role in epithelial cell adhesion. In our studies of a series of hematopoietic cells, CADM1/TSLC1 was weakly expressed in erythrocytes, to a lower degree by neutrophils, monocytes, and B cells and was not expressed in T cells. The activation of T cells with PHA or an anti-CD3 and anti-CD28 antibody mixture did not induce CADM1/ TSLC1 expression, indicating that the CADM1/TSLC1 expression in ATLL cells did not result from the activation of normal T cells. Moreover, CADM1/TSLC1 expression was not detected in 34 HTLV-1-uninfected leukemia cell lines, consisting of 17 myelocytic/monocytic, 3 megakaryocytic, 2 erythrocytic, 4 B-lymphocytic, and 8 T-lymphocytic leukemia cell lines.[12] These findings suggest that TSLC1 is specifically expressed in ATLL and HTLV-1-infected T cells.

PHYSIOLOGICAL ROLES OF CADM1/TSLC1 IN ATLL CELLS

One characteristic feature of ATLL cases is the invasion of the lymph nodes, skin, or various other organs by malignant cells. When the ED-ATLL cell line, which expresses very low CADM1/TSLC1 levels, was stably transfected with a CADM1 vector and assayed for self-aggregation ability, ED/CADM1 cells were found to form aggregates within 30 minutes. In contrast, parental ED cells or ED/neo cells exhibited little aggregation in this time period,[12] suggesting that

CADM1/TSLC1 mediates the intercellular adhesion of ATLL cells via homophilic interactions. However, the potential *in vivo* pathologic significance of this self-aggregation of ATLL cells is unclear. The initial step in the invasion of various human organs by ATLL cells is their interaction with vascular endothelial cells.[28] Therefore, we examined the possible involvement of CADM1/TSLC1 in the adhesion of ATLL cells to human umbilical vein endothelial cells (HUVECs) *in vitro*.[12] When fluorescence-labeled ED cells and their derivatives were seeded onto HUVECs, incubated for 30 minutes, and then washed with medium, the numbers of attached ATLL cells was significantly (3-fold) higher in ED/CADM1 cells compared with parental ED or ED/neo cells. These results suggest that ectopic CADM1/TSLC1 expression in ATLL cells may promote their invasion of various organs via an interaction with the surface molecules of vascular endothelial cells.

CRITICAL ROLE OF *CADM1/TSLC1* IN THE GROWTH AND ORGAN INFILTRATION OF ATLL CELLS

We examined the role of CADM1/TSLC1 in the growth and infiltration of leukemia cells using C57BL/6J and NOD-SCID/γc^{null} (NOG) mice.[29] First, a murine IL-2-independent T-lymphoma cell line (EL4) was intraperitoneally injected into syngeneic C57BL/6J mice as a model for ATLL. The EL4/CADM1 mice died significantly earlier than the control mice. Massive tumor metastasis was evident in the livers of the mice that were injected with EL4/CADM1 cells. These results indicate that CADM1/TSLC1 overexpression in T-lymphoma cells aggressively promotes the development of leukemia/ lymphoma. Next, ATLL-derived ED cells were subcutaneously injected into NOG mice. The ED/CADM1 cell lines caused greater formation of larger tumors than did the ED/Neo and parental cell lines. Clinical signs of being near death (e.g., piloerection, weight loss, and cachexia) at the time of sacrifice were more prevalent in those mice that were injected with the ED/CADM1 cell line. These results suggest that CADM1/TSLC1 expression in ATLL cells enhances *in vivo* tumor growth in NOG mice. Because the mice died within 4 weeks due to heavy tumor burden following subcutaneous inoculation of leukemia cells, ED/CADM1 or ED/Neo cells were intravenously injected into NOG mice to investigate their capacity to invade various organs. Macroscopically, all of the mice injected with ED/CADM1 cells (six/six) exhibited severe liver invasion with ovarian swelling. None of the mice injected with ED/Neo cells exhibited liver invasion but did demonstrate ovarian involvement. Microscopically, all of the mice that were inoculated with ED/CADM1 cells exhibited severe and massive liver and lung invasions. Alternatively, only one of the six mice that were inoculated with ED/Neo cells exhibited a sizable liver metastasis. Thus, CADM1/TSLC1 overexpression in ATLL cells may enhance organ invasion, particularly that of the liver and lung. Finally, we have shown that primary ATLL cells with high CADM1/TSLC1 expression levels can efficiently grow and infiltrate various organs in NOG mice.[29] Primary CADM1$^+$ ATLL cells, with various CADM1/TSLC1 expression levels, from five acute-type and five chronic-type ATLL patients were subcutaneously inoculated into the postauricular region of NOG mice. All of the mice exhibited clinical signs of being near death (e.g., piloerection, weight loss, and cachexia) 6 to 8 weeks following the inoculation. These mice also exhibited enlarged lymph nodes, spleens, lungs, and livers. Microscopically, the ATLL cells invaded various organs to different degrees in all of the ATLL-bearing NOG mice. The dispersion diagram for the levels of invasion and the cellular CADM1/TSLC1 expression levels revealed a correlation coefficient of 0.714, suggesting that there was a moderate correlation between the invasive capability of the cells and the CADM1/TSLC1 expression level. Thus, CADM1/TSLC1 aided in the formation of a rapidly growing large tumor and massive infiltration of ATLL cells into various organs in NOG mice. Because CADM1/TSLC1 is expressed in various ATLL types, including smoldering and chronic types, this gene may be a promising target for the development of a novel ATLL therapy. The NOG mouse model system described here may provide a novel means by which to understand and further investigate the importance of CADM1 in ATLL progression.

CLINICAL SIGNIFICANCE OF *CADM1/TSLC1* EXPRESSION IN ATLL

The determination of clonal HTLV-1 proviral integration by Southern blot analysis is the gold standard for a definitive ATLL diagnosis. In addition, the presence of leukemia cells with multi-lobulated nuclei (referred to as "flower cells") in the peripheral blood is a morphologic characteristic feature of ATLL. Hypercalcemia and high levels of either serum lactate dehydrogenase (LDH) or soluble IL-2Ra (sIL-2Rα) have been demonstrated to be unfavorable markers for ATLL; however, these markers are not specific for the diagnosis of ATLL.[30,31] Recently, we generated a series of antibodies against CADM1/TSLC1 to be used as diagnostic tools for ATLL. These antibodies can be used for the identification and separation of ATLL and HTLV-1-infected cells, the detection of the soluble form of CADM1/TSLC1 in the peripheral blood and the pathological identification of lymphoma-type ATLL following formalin fixation.[32,33]

CADM1/TSLC1 expression in leukemia cells from ATLL patients and from HTLV-1-infected cells from viral carriers

ATLL cells exhibit an activated helper T-cell profile (i.e.,

$CD3^+$, $CD4^+$, $CD8^-$, and $CD25^+$). It was reported that 10 of 17 ATLL cases (59%) expressed forkhead box P3, the expression of which is characteristic of $CD4^+$ and $CD25^+$ regulatory T (T-reg) cells.[34] These data suggest that certain ATLL cases originate from T-reg cells. Interestingly, we observed, using flow cytometric analysis, that a subset of the T-reg fraction weakly expressed CADM1/TSLC1, suggesting that CADM1/TSLC1 is not a major marker for the T-reg fraction and that CADM1/TSLC1 expression on ATLL cells may reflect their T-reg cell origin.[33] Among $CD45^+$ cells in PBMCs from healthy volunteers, 7.3% of $CD45^+$ cells also expressed CD4 and CD25, while only 0.6% of the cell population expressed CD4 and CADM1/TSLC1. These results indicate that the number of $CD4^+CADM1^+$ cells was significantly lower than the number of $CD4^+CD25^+$ cells in healthy volunteer PBMCs. The median percentages of $CD4^+CADM1^+$ cells were 73.9% in acute cases, 72.4% in chronic cases, 5.6% in lymphoma cases, 11.5% in smoldering cases and 4.4% in HTLV-1 carriers. The percentages of $CD4^+CD25^+$ cells were significantly correlated with those of $CD4^+CADM1^+$ cells ($R = 0.907$, $P < 0.0001$), suggesting that the majority of the ATLL cells were $CD4^+CD25^+CADM^+$. The percentages of $CD4^+CADM1^+$ cells exhibited a high degree of correlation with both the percentage of abnormal lymphocytes ($R = 0.791$, $P < 0.0001$) and with the HTLV-1 DNA copy number ($R = 0.677$, $P < 0.0001$) in various ATLL types. In addition, the percentages of $CD4^+CADM1^+$ cells were correlated with sIL-2Rα and LHD levels ($R = 0.586$, $P < 0.0001$ and $R = 0.486$, $P = 0.0015$, respectively). To further evaluate the diagnostic efficacy of $CADM1^+$ cell numbers in detecting HTLV-1-infected cells, the HTLV-1 provirus copy number was compared with the percentages of $CD4^+CADM1^+$ cells and sIL-2Ra and LDH serum levels in carrier PBMCs. The percentage of $CD4^+CADM1^+$ cells exhibited a significant correlation with the HTLV-1 DNA copy number ($R = 0.921$, $P < 0.0001$), whereas a poor correlation was observed between the HTLV-1 copy number and sIL-2Ra and LDH levels. Based on these data, in addition to the determination of HTLV-1 proviral DNA copy number, the quantification of $CD4^+$ $CADM1^+$ cell number by flow cytometry may be useful with respect to monitoring the number of HTLV-1-infected cells in the peripheral blood of ATLL patients and HTLV-1 carriers.

Detection of the soluble form of CADM1/TSLC1 in the serum of ATLL patients

A soluble CADM1/TSLC1 isoform consisting of the extracellular domain was recently isolated in murine mast cells.[35] Using western blot analysis, we observed a 72-kDa soluble CADM1/TSLC1 protein in the sera of 5 patients with acute-type ATLL but not in the sera of 5 healthy volunteers. The analysis of the sera of 5 healthy controls and of 25 ATLL patients (14 acute-type, 7 lymphoma-type, 2 smoldering-type and 2 HTLV-1 carriers) revealed that high levels of soluble CADM1/TSLC1 were present in the serum of patients who had high numbers of $CADM1^+$ cells in the peripheral blood. Furthermore, when comparing the soluble IL-2Ra and CADM1/TSLC1 serum levels in individual cases, significantly higher levels of soluble CADM1/TSLC1 were detected in the serum of ATLL patients who had increased levels of soluble IL-2Ra ; thus, serum CADM1/TSLC1 levels may be predictive of disease progression in ATLL.[33]

High CADM1/TSLC1 expression in ATLL-derived lymphomas

We examined immunohistochemical staining of 90 tissue samples from patients with various types of lymphoma, including 36 patients with ATLL and 54 with non-ATLL lymphomas. These latter cases included T- or NK cell lymphomas, B-cell lymphomas, and null-cell lymphomas. Using a four-grade scale to score CADM1 immunohistochemical staining (0 to 3+), we observed that 92% of ATLL lymphomas were positive for CADM1/TSLC1.[33] Of these, 50% stained heavily for CADM1 and scored 2+ or higher. Specific membranous staining was typically observed in ATLL cells. Among the non-ATLL lymphomas, a small number of CADM1-positive cells (fewer than 5% ; score 1+) were observed. These cells were small to medium-sized and contained non-atypical, normochromatic round/ovoid nuclei. Based on these morphological and CADM1-staining analyses, the CADM1-positive cells in the non-ATLL lymphomas were not considered to be lymphoma cells but possibly histiocytes, including dendritic cells. This was suspected because these cells were similar to the CADM1-positive cells that were observed in reactive lymph nodes. Based on these results, a high degree of cell membrane staining for CADM1/TSLC1 with a score of 2+ may be highly specific for a diagnosis of ATLL. Furthermore, combined staining with CADM1/TSLC1 and other T-cell-specific markers may be necessary for a more accurate diagnosis of lymphoma-type ATLL.[33]

The question of why CADM1/TSLC1 is strongly expressed on the surface of various ATLL types remains unclear. We previously examined whether CADM1/TSLC1 expression is induced by HTLV-1/Tax expression, demonstrating that Tax protein expression did not activate CADM1/TSLC1 expression in JPX-9 cells. We also introduced a Tax expression vector into MOLT4 and 293T cells and determined the subsequent CADM1/TSLC1 expression levels. With these analyses, we demonstrated that Tax was not able to induce CADM1/TSLC1 expression in these cells, suggesting that Tax expression is unrelated to high CADM1/TSLC1 expression. Because HBZ is known to be constitutively expressed in both HTLV-1-infected and ATLL cells and can modulate host transcription,[22] it is possible that HBZ activates CADM1/TSLC1 expression. We also speculate that

high CADM1 expression in ATLL cells may be associated with transcriptional abnormalities in ATLL cells via the accumulation of genomic or epigenomic alterations. The positive correlation between HTLV-1 copy number and the percentage of $CD4^+CADM1^+$ cells in the peripheral blood of HTLV-1 carriers suggests that, if they also exhibit high percentages of $CD4^+CADM1^+$ cells, these individuals may have developed more extensive genetic alterations and may be at high risk for developing ATLL.

Recent studies have shown that certain markers, such as CCR4 and CD70, are unique ATLL surface markers.[36,37] Whereas the proportion of $CD4^+CCR4^+$ and $CD4^+CD70^+$ cells in the PBMCs of healthy individuals were observed to be approximately 5%,[37,38] the proportion of $CD4^+CADM1^+$ cells in this population was less than 1%; therefore, the measurement of $CADM1^+$ T cells is particularly efficient in the diagnosis of HTLV-1 infection in individuals who carry a small number of HTLV-1-infected cells. We have shown that CADM1/TSLC1 has important functions in increasing cell adhesion and in mediating cancer progression to organ invasion.[29] In addition, we succeeded in using anti-CADM1-coated magnetic beads to isolate low percentages of HTLV-1-infected cells from the PBMCs of HTLV-1 carriers with high HTLV-1 copy numbers, and ATLL cells from ATLL patients.[33] Sorted HTLV-1-infected and ATLL cells may be useful tools for transcriptional and/or genomic analyses. The results of such tests could be compared between the PBMCs of healthy volunteers and peripheral leukemia cells from ATLL patients, potentially providing important information with respect to the expression pattern and genomic abnormalities that occur at the early stages of HTLV-1 infection and/or ATLL development.

A recent study demonstrated that CADM1/TSLC1 directly associates with the PDZ domain of T-lymphoma invasion and metastasis 1 (Tiam1). This interaction induces the formation of lamellipodia by activating Rac in both HTLV-I-transformed cell lines and ATLL cell lines.[39] These results indicate that Tiam1 integrates signals from CADM1/TSLC1 to regulate the actin cytoskeleton through Rac activation, potentially leading to tissue infiltration of leukemic cells in ATLL patients. The elucidation of the different downstream cascades that are activated by CADM1/TSLC1 in epithelial cells and T-lymphocytes would provide important insights into the roles of CADM1/TSLC1 in tumorigenesis.

CONCLUSION

In this manuscript, we describe how CADM1/TSLC1 is highly expressed in the majority of ATLL cells and in a subset of peripheral blood cells from HTLV-1 carriers. These data suggest that CADM1/TSLC1 is potentially a prediagnostic indicator of ATLL development in high-risk HTLV-1 carriers. Therefore, we are currently developing a diagnostic kit to detect soluble CADM1/TSLC1 protein in the peripheral blood. Furthermore, we are attempting to apply this diagnostic kit to various types of ATLL patient samples, including HTLV-1 carriers. Moreover, CADM1/TSLC1 may be a novel molecular target for the treatment of ATLL. Dr. Kurosawa's group (Fujita Health University, Japan) has already developed several types of anti-human CADM1/TSLC1 antibodies using the phage-display technique and has observed that certain clones exhibited cytotoxic activity against ATLL cells (manuscript in preparation). Moreover, recombinant shuttle viruses are being developed that will target ATLL cells by binding to CRTAM and/or CADM1/TSLC1. Finally, small molecules that interfere with the cell adhesion characteristics of ATLL cells will be valuable for blocking their organ-invading ability. Thus, CADM1/TSLC1 is a clinically useful molecule for detecting and for targeting ATLL cells.

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Proviral loads of human T-lymphotropic virus Type 1 in asymptomatic carriers with different infection routes

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High human T-lymphotropic virus Type 1 (HTLV-1) proviral DNA load (PVL) has been reported to be one risk factor for the development of adult T-cell leukemia/lymphoma (ATL). ATL is also believed to develop in HTLV-1 carriers who acquire infection perinatally. ATL cells have been reported to frequently harbor defective provirus. In our study, PVLs for three different regions of HTLV-1 provirus (5′LTR-*gag*, *gag* and *pX*) were measured in 309 asymptomatic carriers with different infection routes. PVLs for the *pX* region in 21 asymptomatic carriers with maternal infection was significantly higher than in 24 carriers with spousal infection. Among 161 carriers with relatively high *pX* PVLs (equal to or greater than 1 copy per 100 peripheral blood mononuclear cells), 26 carriers (16%) had low *gag* PVL/*pX* PVL (less than 0.5) and four (2%) had low 5′LTR-*gag* PVL/*pX* PVL (less than 0.5). Low *gag* PVL/*pX* PVL ratio, which reflects deficiency and/or polymorphism of HTLV-1 proviral DNA sequences for the *gag* region, was also associated with maternal infection. These data suggest that HTLV-1 carriers with maternal infection tend to have high PVLs, which may be related to provirus with deficiency and/or the polymorphism of proviral DNA sequences. In addition, there is a possibility that this ratio may be used as a tool to differentiate the infection routes of asymptomatic HTLV-1 carriers, which supports the need for a large scale study.

Human T-lymphotropic virus Type 1 (HTLV-1) is the causative agent of adult T-cell leukemia/lymphoma (ATL) and a progressive neurological disease known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[1–4] Major routes of HTLV-1 infection have been reported as mother to child infection at infancy, sexual contact between spouses and blood transfusion.[5–7] The majority of HTLV-1 carriers are asymptomatic, and only a fraction of carriers develop ATL after a long latent period.[8,9] It has been reported that approximately 4% of HTLV-1 carriers develop ATL eventually.[10] Studies of the mothers of patients with ATL have reported most of them to be HTLV-1 carriers.[11,12] Therefore, ATL is believed to develop in HTLV-1 carriers who acquire infection perinatally. However, there has been no method of identifying the infection route of HTLV-1 positive individuals without information on family HTLV-1 status.

When an individual is infected by HTLV-1, the virus randomly integrates into the genome of affected T-cells in the form of provirus.[13] HTLV-1 infection drives the proliferation of T-cells, leading to the clonal expansion of HTLV-1 infected cells.[14–16] Recently, it was reported that HTLV-1 clonal expansion *in vivo* is favored by orientation of the provirus in the same sense as the nearest host gene.[17] We have reported that the clonality of HTLV-1 infected cells in adult seroconverters who were newly infected from HTLV-1 carrier spouses is more heterogeneous and less stable than that of long-term carriers who acquired infection from their mothers at infancy.[18] The selective maintenance of certain clones is supposed in the latter. Recently, we reported that clonal expansion of HTLV-1 infected cells was found in a certain population of asymptomatic carriers and that these carriers had high proviral DNA loads (PVLs).[19] High PVLs have been reported to be a risk factor for developing ATL.[20,21] In another study, we analyzed the PVLs of 13 pairs of HTLV-1 seroconverters and their spouses.[22] Although seroconverters and their spouses shared the same HTLV-1, PVLs in both individuals in a couple were not always equivalent. These findings suggested that host-related factors play an important role to determining the PVL in each carrier. However, it was

Key words: HTLV-1, defective virus, infection route, proviral DNA loads

Abbreviations: ATL: adult T-cell leukemia/lymphoma; HTLV-1: human T-lymphotropic virus type 1, LTR: long-terminal repeat, PBMCs: peripheral blood mononuclear cells, PCR: polymerase chain reaction; PVLs: proviral DNA loads

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not clear in that study whether HTLV-1 carriers who acquired infection from their mothers at infancy have more PVLs than the carriers who acquired infection from their spouses in adulthood.

Defective provirus has frequently been detectable in patients with ATL.[23–27] The complete HTLV-1 provirus is approximately 9 kb and contains the coding regions for core protein (*gag*), protease (*pro*), polymerase (*pol*), envelope protein (*env*), regulatory proteins, such as Tax and Rex, and some accessory molecules between 5′ and 3′ long-terminal repeats (LTRs).[8,28] Tamiya *et al.*[23] reported two types of genome deletion in defective provirus. One form retains both LTRs and lacks internal sequences, such as the *gag* and *pol* regions. The other form has the 3′ LTR, and the 5′ LTR and its flanking internal sequences are preferentially deleted. HTLV-1 infected cells harboring the latter defective virus were frequently found in patients with ATL.[26] Both types of defective provirus were suspected of being harbored by the clonally expanded HTLV-1 infected cells in asymptomatic carriers.[19] The polymorphism of the proviral genome was also found in asymptomatic carriers in that study; however, we could not show how commonly the deficiency or polymorphism of the proviral genome was detectable.

These questions prompted us to investigate HTLV-1 PVLs in asymptomatic carriers with different infection routes. In addition, to clarify whether the defective provirus and/or polymorphism of the proviral genome affected PVLs, we tested PVLs for three different regions (5′LTR-*gag*, *gag* and *pX*) of provirus in each individual and compared them among the carriers with different infection routes in our study.

Material and Methods

Samples

Samples of peripheral blood mononuclear cells (PBMCs) were obtained from 309 HTLV-1 carriers (103 men and 206 women, median age: 67 years), who had no symptoms or laboratory data suggesting HTLV-1 related disease, in the Miyazaki Cohort Study.[29] Infection routes were investigated by family HTLV-1 status and history of HTLV-1 seroconversion.[18,22] An HTLV-1 carrier with HTLV-1 positive mother/HTLV-1 negative spouse or with HTLV-1 positive siblings/HTLV-1 negative spouse or with HTLV-1 seroconverter was defined as infected by his/her mother. An HTLV-1 carrier who was a HTLV-1 seroconverter with HTLV-1 positive spouse or with HTLV-1 negative mother/HTLV-1 positive spouse was defined as infected by his/her spouse. Carriers with history of blood transfusion were excluded from the analysis of family status. As a result, 21 and 24 carriers were defined as infected by their mothers and by their spouses, respectively. Infection routes could not be determined in 264 carriers. Informed consent was obtained from the study participants and the study protocol was approved by the institutional review board at University of Miyazaki.

Real-time polymerase chain reaction

PVLs for three different proviral regions (5′LTR-*gag*, *gag* and *pX*) were determined by real-time polymerase chain reaction (PCR) using Light Cycler 2.0 (Roche Diagnostics, Mannheim, Germany). Genomic DNA was isolated from PBMCs of asymptomatic HTLV-1 carriers by sodium dodecyl sulfate-proteinase K digestion, followed by phenol-chloroform extraction and ethanol precipitation. Approximately 100 ng genomic DNA was used as the template. The nucleotide position number of HTLV-1 provirus was according to Seiki *et al.*[30] (accession no. J02029). The primers and probes for real-time PCR were designed to minimize the differences of the melting points 5′LTR-*gag*, *gag* and *pX* and were as follows: 5′LTR-*gag*: the forward primer (5′LTR-SDS-F 5′-AAGTACCGGCGACTCCGTTG-3′: positions 700–719), the reverse primer (HTLV-gag-LTR-R2 5′-GGCTAGCGGTACGGGAAAAG-3′: positions 854–835) and the FAM-labeled probe (5′-FAM-CGTCCGGGATACGAGCGCCCCTT-TAMRA-3′: positions 788–810); *gag*: the forward primer (HTLV-gag-F5 5′-ACCCTTCCTGGGCCTCTATC-3′: positions 1,602–1,621), the reverse primer (HTLV-gag-R5 5′-TCTGGCAGCCCATTGTCAAG-3′: positions 1,695–1,676) and the FAM-labeled probe (HTLV-gag-P5 5′-FAM-ACCACGCCTTCGTAGAACGCCTCAAC-TAMRA-3′: positions 1,644–1,669); *pX*: the forward primer (HTLV-pX2-S 5′-CGGATACCCAGTCTACGTGTT-3′: positions 7,359–7,379), the reverse primer (HTLV-pX2-AS 5′-CAGTAGGGCGTGACGATGTA-3′: positions 7,458–7,439) and the FAM-labeled probe (HTLV-pX2-Probe 5′-FAM-CTGTGTACAAGGCGACTGGTGCC-TAMRA-3′: positions 7,386–7,408).[18,26] A coding region for albumin (*Alb*) was used to measure the copy number of human genome. The primers and the probe for the *Alb* were as follows: The forward primer (Alb-S2 5′-TGTCATCTCTTGTGGGCTGT-3′), the reverse primer (Alb-AS2 5′-GGTTCTCTTTCACTGACATCTGC-3′) and the FAM-labeled probe (Alb-probe 5′-FAM-CCTGTCATGCCCACACAAATCTCTCC-TAMRA-3′). A plasmid containing PCR products for HTLV-1 5′LTR-*gag*, *gag*, *pX* regions and *Alb* was constructed using pGEM T-Easy Vector (Promega Corporation, Madison, WI) and was used as a control template for real-time PCR. PVLs of each region of HTLV-1 provirus were measured in a duplicate manner and were shown as copies per 100 PBMCs.

Detection of provirus with deletion of HTLV-1 internal sequence by long PCR

To detect the provirus with large deletion of HTLV-1 internal sequence, long PCR, which amplifies provirus maintaining both 5′ and 3′ LTR, was performed as described previously.[19] The primers were as follows: 5′LTR (HTLV-0647F 5′-GTTCCACCCCTTTCCCTTTCATTCACGACTGACTGC-3′: positions 647–682) and 3′LTR (HTLV-8345R 5′-GGCTCT AAGCCCCCGGGGGATATTTGGGGCTCATGG-3′: positions

8,345–8,310).[26] Long PCR was performed using LA Taq Hot start version (Takara Bio, Shiga, Japan). Genomic DNA containing 200 copies of HTLV-1 provirus for the *pX* region was used for this assay. To ensure that the same amount of provirus was used in each reaction, PCR for the *pX* region was performed as an internal control. Primers for this PCR were as follows: the forward primer (HTLV-7396F 5′-GGCGACTGGTGCCCCATCTCTGGGGGACTATGTTCG-3′: positions 7,396–7,431) and the reverse primer described above (HTLV-8345R). The PCR products were electrophoresed on 0.8% agarose gel and visualized by ethidium bromide staining.

Detection of provirus with deletion of 5′LTR and its flanking internal sequence by inverse long PCR

As described in results, both *gag* PVL/*pX* PVL ratio and 5′LTR-*gag* PVL/*pX* PVL ratio were low at less than 0.5 in two carriers (C20 and 21) and they were suspected of having provirus with deletion of 5′LTR and its flanking internal sequence. Inverse long PCR (IL-PCR) was used to amplify the genomic DNA adjacent to the 3′LTR of HTLV-1 provirus according to the method described previously with slight modifications.[15] In brief, the genomic DNA was digested with *Kpn* I, *Hind* III, *Sal* I or *Spe* I, and then self-ligated by T4 ligase following digestion with *Mlu* I. Amplification of the resultant DNA was performed using the LA Taq Hot start version. The primers used in this analysis were as follows; a forward primer in the U5 region of the LTR (5′-TGCCTGACCCTGCTTGCTCAACTCTACGTCTTTG-3′: positions 8,856–8,889) and a reverse primer, HTLV-7002R (5′-AGTATTTGAAAAGGAAGGAAGAGGAGAAGGCA-3′: positions 7,002–6,971). Subcloning of the amplified fragments of IL-PCR were subjected to sequencing assay according to the protocol of the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using ABI Prism 310 DNA Sequencer (Applied Biosystems) and the human genomic sequence downstream of the HTLV-1 provirus was obtained. The human genomic sequence upstream of the provirus was assumed based on this information by BLAT search (http://genome.ucsc.edu/cgi-bin/hgBlat).[31] The primers for human genomic sequence upstream of the provirus were designed and long PCR was performed using a forward primer (5′-GTGATCCATGGTGTTTGTCCACCTGAAAGC-3′) and a reverse primer HTLV-7002R in C20, and a forward primer (5′-TCCAAGTGGGATGTCACGGCCACTTCTC-3′) and a reverse primer HTLV-7002R in C21. To determine the upstream junction sequence between host genome and provirus, the PCR products were subjected to direct sequencing using the Big Dye Terminator v1.1 Cycle Sequencing Kit.

Statistical Analysis

Mann-Whitney's U test was used to compare *pX* PVLs, *gag* PVL/*pX* PVL or 5′LTR-*gag*/*pX* PVL ratios among the groups of asymptomatic HTLV-1 carriers with different infection routes. Spearman's correlation coefficient by rank was used to determine the relationship between *pX* PVL and *gag* PVL/*pX* PVL or 5′LTR-*gag* PVL/*pX* PVL ratio.

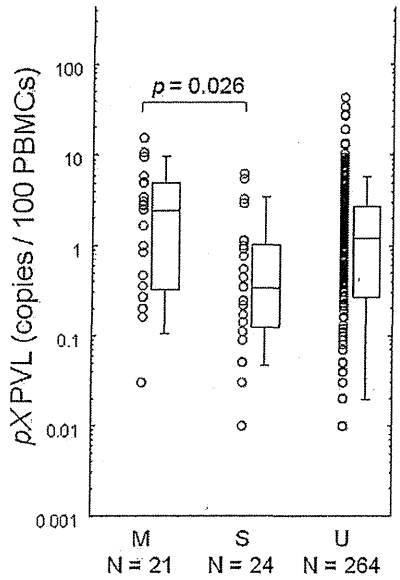

Figure 1. *PX* PVLs in HTLV-1 carriers with different infection routes M: Carriers with infection from mothers; S: Carriers with infection from spouses; U: Carriers with undetermined infection routes.

Results

pX PVLs in HTLV-1 carriers with different infectious routes

PVLs for the 5′LTR-*gag*, *gag* and *pX* regions in each individual were measured in 309 asymptomatic HTLV-1 carriers. Because the *pX* region has been reported to be conserved in the HTLV-1 provirus, *pX* PVL was considered to represent total PVLs.[23,25] As shown in Figure 1, median *pX* PVL (2.49 copies/100 PBMCs) in 21 asymptomatic carriers, who were infected by their mothers, was significantly higher than that (0.34 copies/100 PBMCs) in 24 carriers who were infected by their spouses ($p = 0.026$). Median *pX* PVL in 264 asymptomatic carriers, whose infection routes were undetermined, was between these values (1.24 copies/100 PBMCs).

PVLs for 3 different proviral regions (5′LTR-gag, gag and pX) of HTLV-1

To determine whether PVLs for three different proviral regions (5′LTR-*gag*, *gag* and *pX*) of HTLV-1 were equal in asymptomatic carriers, PVLs for the 5′LTR-*gag* and *gag* regions were measured and compared to PVLs for the *pX* region. Because 100 ng of genomic DNA, which is derived approximately 15,000 PBMCs, was used for the template for real time-PCR, 148 carriers with *pX* PVL, which was less than 1 copy/100 PBMCs, were not provided for further analysis to avoid unstable result due to the small number of proviral copies in each reaction. The results of our study were

shown as the ratio of PVLs for the 5′LTR-*gag* or *gag* regions to PVL for the *pX* region in each individual (Fig. 2). The median 5′LTR-*gag* PVL/*pX* PVL ratio of 161 HTLV-1 carriers tested was 0.97. Therefore, HTLV-1 proviral sequence for 5′LTR-*gag* PVL was considered to be conserved in the majority of asymptomatic carriers. The median *gag* PVL/*pX* PVL ratio, however, was 0.61.

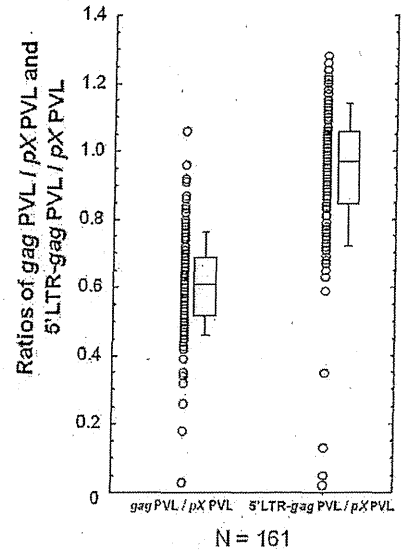

Figure 2. The ratios of PVLs for the 5′LTR-*gag* or *gag* regions to PVL for the *pX* region in 161 asymptomatic HTLV-1 carriers, whose *pX* PVLs were equal to or greater than 1 copy/100 PBMCs.

Detection of provirus with deletion of HTLV-1 internal sequence by long PCR

To determine whether the provirus with deletion of HTLV-1 internal sequence accounted for low *gag* PVL/*pX* PVL ratio, long PCR was performed. For this analysis, we chose 26 carriers with low *gag* PVL/*pX* PVL ratios of less than 0.5; however, adequate DNA sample for long PCR was available in only 17 of the 26 subjects. All subjects except C1 showed a band of 7.7 kb, which was considered to be derived from complete provirus, and some additional smaller bands suggesting defective provirus (Fig. 3*a*). C1 showed only a dense band of 4.5 kb. C1 was analyzed in our previous study and a large deficiency (3.2 kb, positions 1,203–4,368) of internal sequence was shown.[19] Additional four carriers (C3, 4, 11 and 13) showed dense bands equal to or stronger than the band for complete provirus (arrows in Fig. 3*a*). Cloning and DNA sequencing of these dense bands showed large deficiencies of internal sequences (4.9 kb, positions 1,368–6,286 in C3; 0.9 kb, positions 1,413–2,284 in C4; 4.8 kb, positions 1,009–5,763 in C11 and 4.8 kb, positions 1,133–5,974 in C13).

Four carriers (C18–21) had low 5′LTR-*gag* PVL/*pX* PVL ratios of less than 0.5. Long PCR of C18 and 19 showed dense bands of 7.7 kb, which were considered to be derived from complete provirus, and some additional smaller bands (Fig. 3*b*). Polymorphism of proviral DNA sequence of the sites for primers and/or probe for 5′LTR-*gag* PVL was suspected in these two cases, and cloning and DNA sequencing of the PCR products were performed. The polymorphisms of DNA sequence for the annealing site of the forward primer (708 G > A and 709 C > G in C18; 712 C > T in C19) were consistently found, and these polymorphisms were

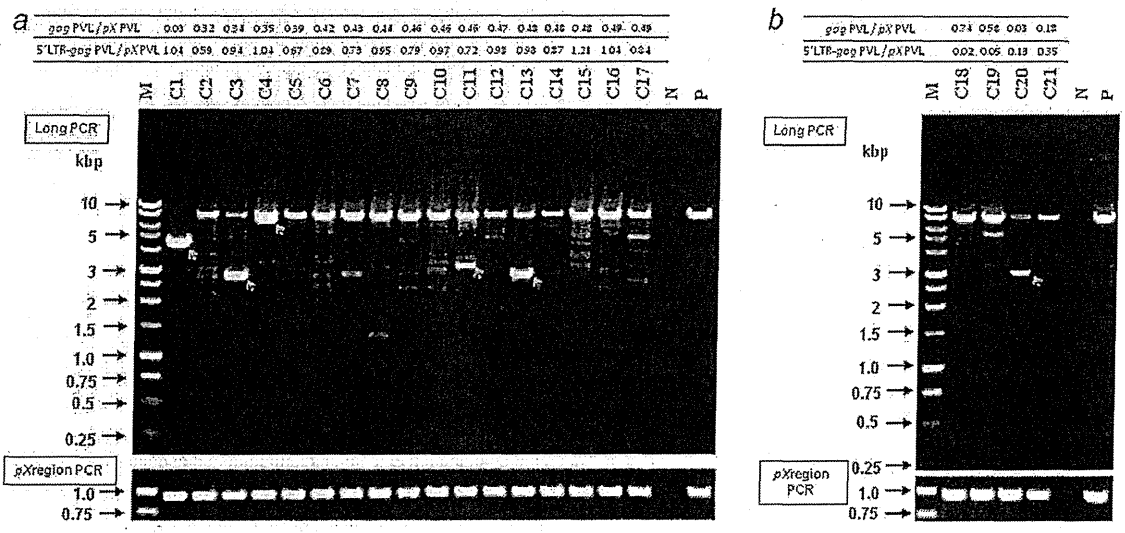

Figure 3. Detection of defective provirus by long PCR. (*a*) Asymptomatic HTLV-1 carriers with low *gag* PVL/*pX* PVL ratios less than 0.5. (*b*) Asymptomatic HTLV-1 carriers with low 5′LTR-*gag* PVL/*pX* PVL ratios less than 0.5. Arrows indicate PCR products for HTLV-1 provirus lacking large internal sequence. M: Molecular weight marker; N: HTLV-1-negative subject; P: HTLV-1-positive cell line, ED-40515(-).

considered to account for the decreased efficacy of real time-PCR for 5′LTR-*gag* PVL.

Detection of provirus with deletion of 5′LTR and its flanking internal sequence by IL-PCR

Both *gag* PVL/*pX* PVL ratio and 5′LTR-*gag* PVL/*pX* PVL ratio were low at less than 0.5 in the additional two carriers (C20 and 21). Long PCR showed a weak band of 7.7 kb for complete provirus and a stronger band of 2.9 kb in C20 (Fig. 3*b*). In the case of C21, only a weak band for complete band was observed (Fig 3*b*). These data suggested defective provirus, which had not been detected by long PCR, existed in C20 and C21. Because these proviruses were suspected of lacking 5′LTR and its flanking internal sequence, we attempted to identify them by IL-PCR. First, the genomic DNA of C20 and C21 were digested with *Kpn* I, *Hind* III, *Sal* I or *Spe* I, and resultant DNA was provided for IL-PCR as a template. In C20, approximately 1.1 kb of PCR product was obtained in digestion with *Kpn* I alone (Fig. 4*a-1*). No IL-PCR product was obtained using other restriction enzymes (data not shown). When this PCR product was digested with *Kpn* I, two major bands appeared, as expected (Fig. 4*a-1*). Cloning and sequencing revealed that this product consisted of HTLV-1 provirus (*Kpn* I site at position: 6,141 to the end of 3′LTR) and its flanking genomic DNA of human chromosome 2 (2q13). Based on the information obtained, a forward primer to anneal the upstream human genome adjuncted to the provirus was prepared and clone-specific PCR was performed. Cloning and sequencing of this clone-specific PCR product revealed that it lacked 5′LTR and its internal flanking sequence (until position 5,999; Fig. 4*a-2*). In the case of C21, IL-PCR product was obtained in digestion with *Hind* III alone. Following the same procedure as in C20, it was revealed that a provirus integrated in human chromosome 18 (18p11.32), and that it lacked 5′LTR and its internal flanking sequence (until position 4,976) (Figs. 4*b-1* and 4*b-2*).

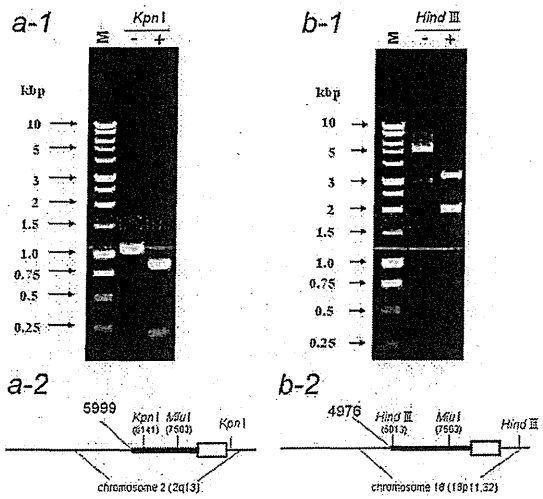

Figure 4. Detection of provirus with deletion of 5′LTR and its internal flanking sequence by IL-PCR. (*a-1*) Long PCR products from an asymptomatic HTLV-1 carrier, C20, with or without *Kpn* I digestion. (*a-2*) Scheme of the structure of defective provirus in C20. (*b-1*) Long PCR products from an asymptomatic HTLV-1 carrier, C21, with or without Hind III digestion. (*b-2*) Scheme of the structure of defective provirus in C21.

Relationship between pX PVL and gag PVL/pX PVL or 5′LTR-gag/pX PVL ratios

To determine whether the HTLV-1 PVLs correlated with the number of provirus with deficiency and/or polymorphism of the *gag* or 5′LTR-*gag* regions, the relationship between *pX* PVL and *gag* PVL/*pX* PVL or 5′LTR-*gag*/*pX* PVL ratios was analyzed. As shown in Figure 5*a*, there was a negative

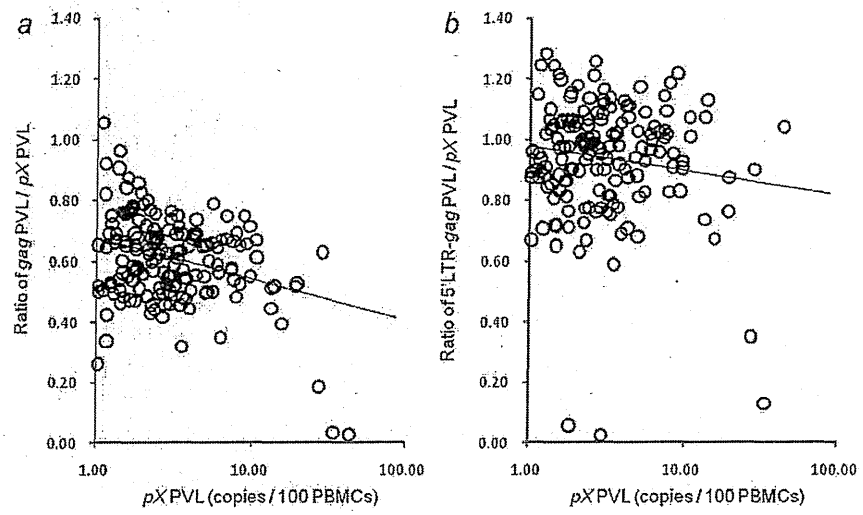

Figure 5. Relations of *pX* PVL and *gag* PVL/*pX* PVL or 5′LTR-*gag* PVL/*pX* PVL ratios in 161 asymptomatic carriers. (*a*) Relation of *pX* PVL and *gag* PVL/*pX* PVL. (*b*) Relation of *pX* PVL and 5′LTR-*gag* PVL/*pX* PVL.

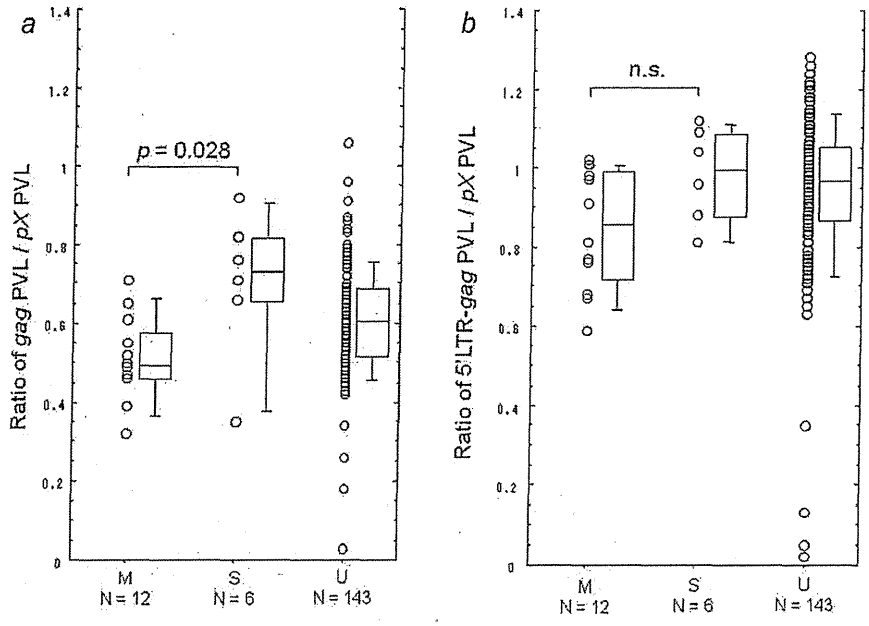

Figure 6. The ratios of *gag* PVL/*pX* PVL or 5′LTR-*gag* PVL/*pX* PVL in HTLV-1 carriers with different infection routes in 161 asymptomatic carriers. (*a*) The ratio of *gag* PVL/*pX* PVL. (*b*) The ratio of 5′LTR-*gag* PVL/*pX* PVL. M: Carriers with infection from mothers; S: Carriers with infection from spouses; U: Carriers with undetermined infection routes.

correlation between *pX* PVL and the *gag* PVL/*pX* PVL ratio ($r = -0.46$, $p = 0.02$). Therefore, HTLV-1 infected cells harboring provirus with deficiency and/or polymorphism of the *gag* region were considered to be more prevalent in asymptomatic carriers with high PVL. In the case of 5′LTR-*gag*/*pX* PVL ratio, the trend was not obvious (Fig. 5*b*) ($r = -0.20$, $p = 0.94$). However, variability of the 5′LTR-*gag*/*pX* PVL ratio was greater than that of *gag* PVL/*pX* PVL ratio. This may have been the result of technical inadequacies in the measurement of 5′LTR-*gag* PVL.

The ratios of gag PVL/pX PVL and 5′LTR-gag PVL/pX PVL in HTLV-1 carriers with different infection routes

Next, the relationships between infection routes and the *gag* PVL/*pX* PVL or 5′LTR-*gag*/*pX* PVL ratios were analyzed. The median ratio of *gag* PVL/*pX* PVL in 12 HTLV-1 carriers with maternal infection (0.50) was significantly lower than that in six carriers with spousal infection (0.74) ($p = 0.028$) (Fig. 6*a*). The median *gag* PVL/*pX* PVL ratio of 143 carriers with undetermined infection route (0.62) was between these. The 5′LTR-*gag* PVL/*pX* PVL ratio did not reveal a significant difference between the carriers with maternal infection and spousal infection (Fig. 6*b*). Therefore, the carriers with maternal infection were considered to have a greater number of HTLV-1 infected cells harboring provirus with deficiency and/or polymorphism of the *gag* region. In addition, when a *gag* PVL/*pX* PVL ratio of 0.65 was used as cut-off value, 11 of 12 (92%) carriers with maternal infection, against only one of six (17%) carriers with spousal infection, showed lower values.

Discussion

First, HTLV-1 PVLs in asymptomatic carriers with different infection routes were analyzed. *PX* PVL in 21 asymptomatic carriers with maternal infection was significantly higher than that in 24 carriers with spousal infection. These results agreed with data reported by Roucoux *et al.*[32] showing that PVLs in index HTLV-1 positive carriers were higher than those of their newly infected partners. Asymptomatic carriers whose infection routes were undetermined showed values between these. Previously, we analyzed the PVLs of HTLV-1 seroconverters and their spouses and showed that PVLs were not equivalent between them.[22] Because HTLV-1 in a seroconverter and in his/her spouse is identical, the host factor was considered important in the determination of HTLV-1 PVL. The results of our study suggest that infection route and/or time of infection are factors in the determination of PVL in HTLV-1 carriers. We also reported that HTLV-1 carriers who developed ATL had high PVLs even before they developed the disease.[20] Recently, Iwanaga *et al.*[21] also tested the PVLs of 1,218 HTLV-1 carriers and found that HTLV-1 carriers that developed ATL had high PVLs. These data suggest that high HTLV-1 PVL is a risk factor for developing ATL. In our study, HTLV-1 carriers with maternal infection tended to have high PVLs. This may account for why perinatal infection is a risk factor of ATL at least in part.

Because the frequent detection of defective provirus in patients with ATL has been reported, we examined provirus with deficiencies and/or polymorphism of proviral sequence in asymptomatic HTLV-1 carriers. The *pX* region has been