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

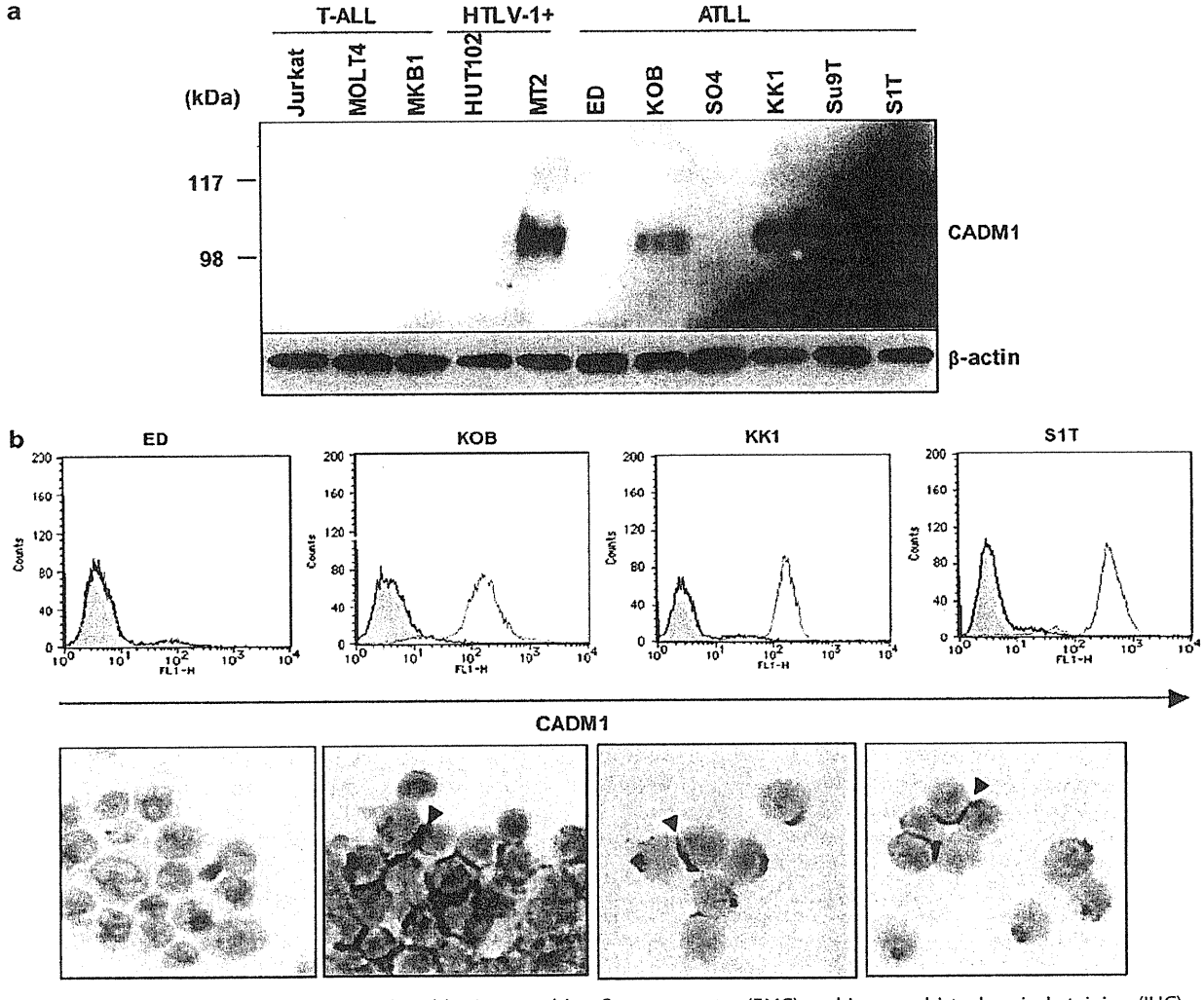

Figure 1. High CADM1 expression in ATLL analyzed by immunoblot, flow cytometry (FMC) and immunohistochemical staining (IHC). (**a**) Immunoblot analysis was performed on a series of T-lymphoid leukemia cell lines (three T-ALL, T-acute lymphoid leukemias; two HTLV-1 +, HTLV-1-infected cell lines; six 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.

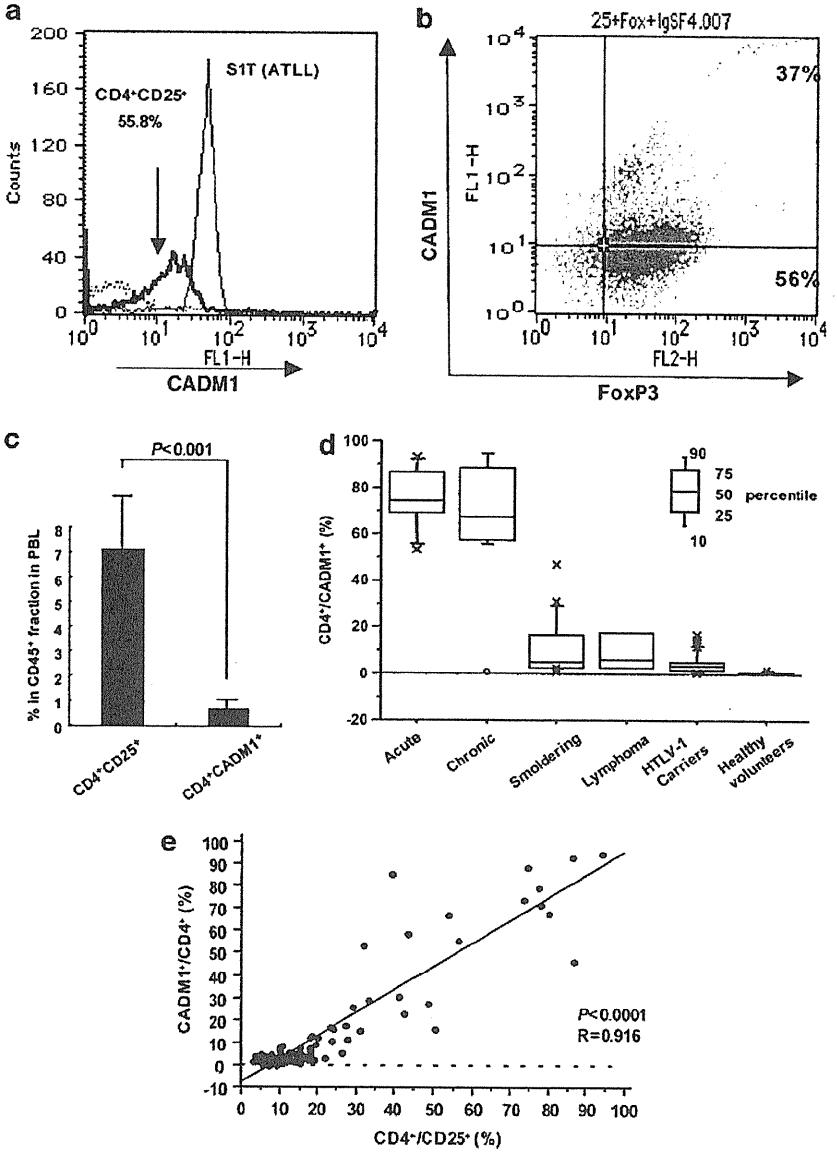

Figure 2. Flow cytometric analysis of CADM1 in T-reg lymphocytes, ATLL cells and HTLV-1-infected T cells. (**a**) Flow cytometric analysis of CADM1 expression in the $CD4^+CD25^+$ fraction from peripheral T lymphocytes. Each sample was stained with an Alexa 488-labeled anti-CADM1 antibody. The S1T-ATLL 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

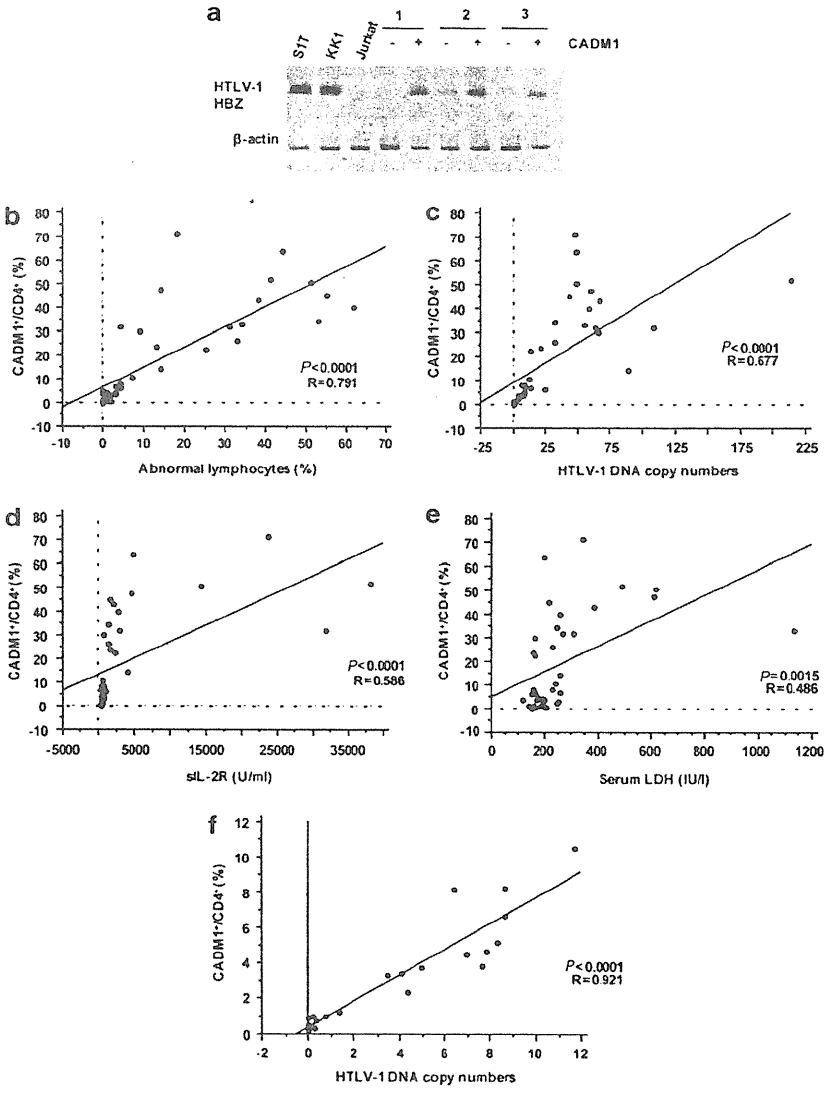

Figure 3. Correlation of the percentages of the CD4$^+$CADM1$^+$ fraction with the percentages of abnormal lymphocytes, HTLV-1 DNA copy number and the levels of soluble IL-2Rα and serum LDH in both various types of patients with ATLL and HTLV-1 carriers. (**a**) Identification of the HTLV-1 genome by 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

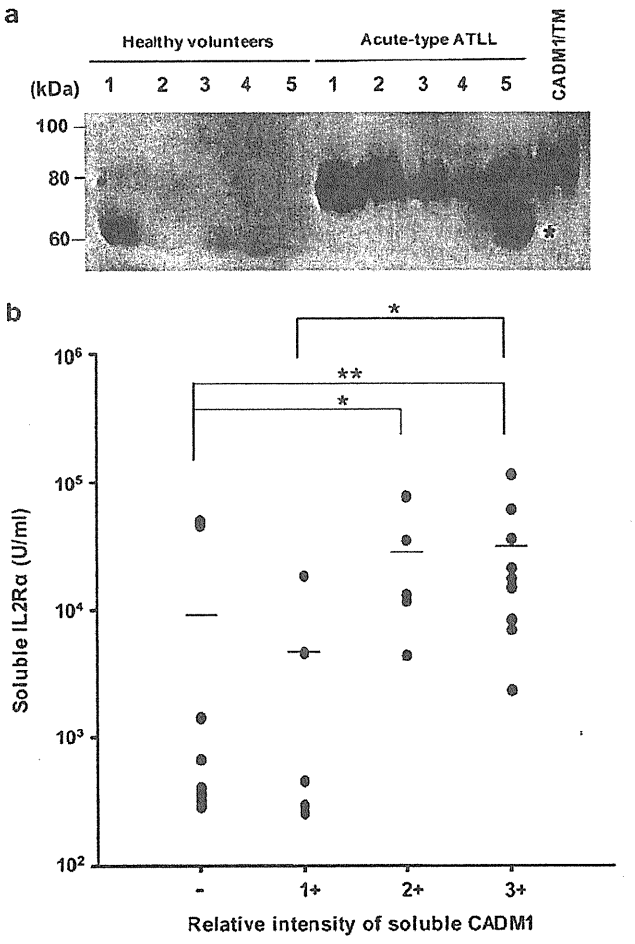

Figure 4. Identification of a soluble form of CADM1 in ATLL patients. (**a**) The soluble form of CADM1 in the peripheral blood from five healthy volunteers and five patients with acute-type ATLL was identified by immunoblot analysis using an anti-CADM1 antibody. The asterisk indicates an albumin band. 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

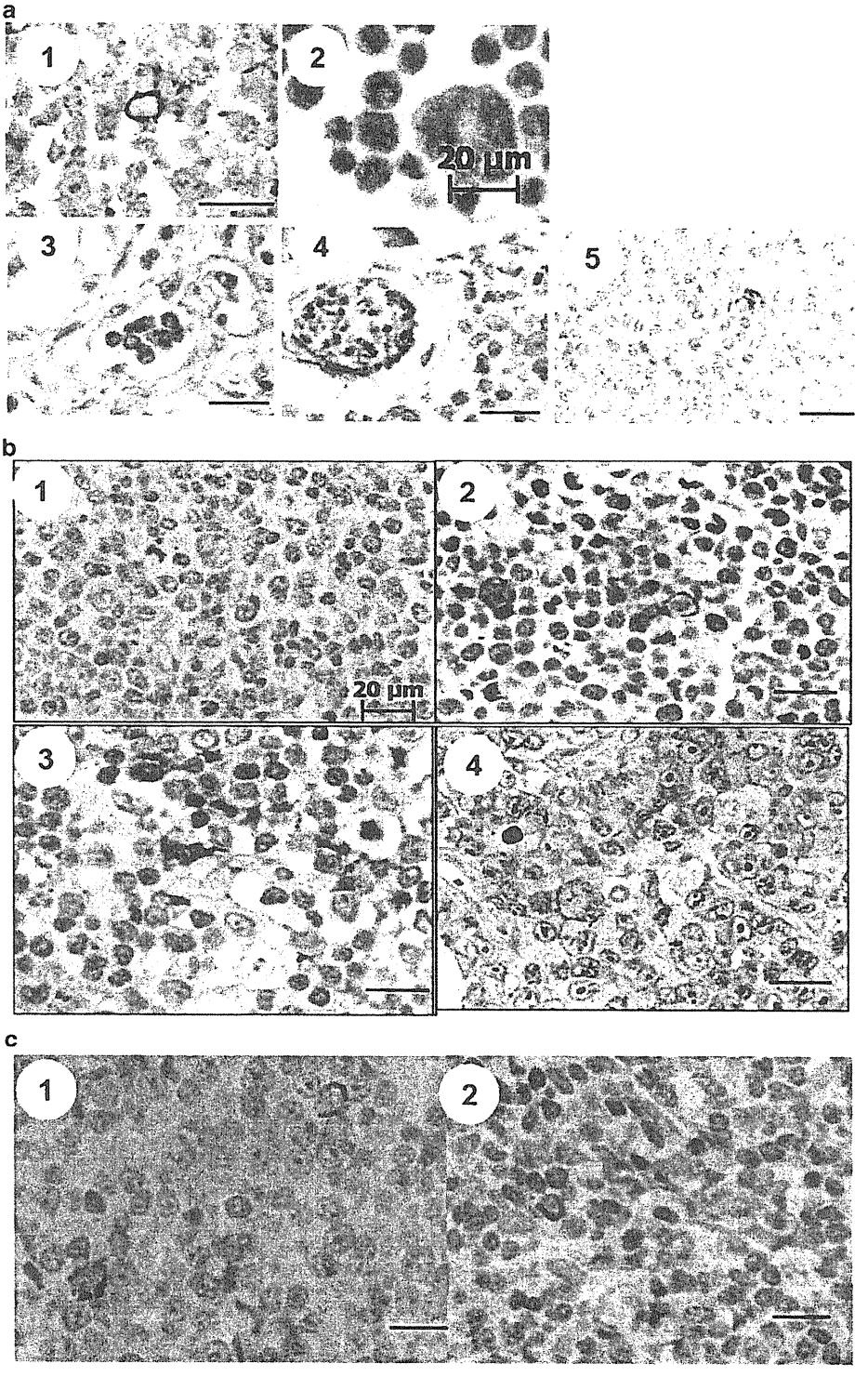

Figure 5. Expression of CADM1 in lymphoma-type ATLL. (**a**) Immunostaining of CADM1 in the S1T-ATLL cell line was used as a positive control (a1), and the ED-ATLL cell line was used as a negative control (a2) for CADM1 expression using an anti-CADM1 antibody (1-10C). Immunostaining of erythrocytes in 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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ORIGINAL REPORT

Defucosylated Anti-CCR4 Monoclonal Antibody (KW-0761) for Relapsed Adult T-Cell Leukemia-Lymphoma: A Multicenter Phase II Study

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ABSTRACT

Purpose
Adult T-cell leukemia-lymphoma (ATL) is usually resistant to conventional chemotherapies, and there are few other treatment options. Because CC chemokine receptor 4 (CCR4) is expressed on tumor cells from most patients with ATL, KW-0761, a humanized anti-CCR4 monoclonal antibody, which markedly enhances antibody-dependent cellular cytotoxicity, was evaluated in the treatment of patients with relapsed ATL.

Patients and Methods
A multicenter phase II study of KW-0761 for patients with relapsed, aggressive CCR4-positive ATL was conducted to evaluate efficacy, pharmacokinetic profile, and safety. The primary end point was overall response rate, and secondary end points included progression-free and overall survival from the first dose of KW-0761. Patients received intravenous infusions of KW-0761 once per week for 8 weeks at a dose of 1.0 mg/kg.

Results
Of 28 patients enrolled onto the study, 27 received at least one infusion of KW-0761. Objective responses were noted in 13 of 26 evaluable patients, including eight complete responses, with an overall response rate of 50% (95% CI, 30% to 70%). Median progression-free and overall survival were 5.2 and 13.7 months, respectively. The mean half-life period after the eighth infusion was 422 ± 147 hours (± standard deviation). The most common adverse events were infusion reactions (89%) and skin rashes (63%), which were manageable and reversible in all cases.

Conclusion
KW-0761 demonstrated clinically meaningful antitumor activity in patients with relapsed ATL, with an acceptable toxicity profile. Further investigation of KW-0761 for treatment of ATL and other T-cell neoplasms is warranted.

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INTRODUCTION

Adult T-cell leukemia-lymphoma (ATL) is an aggressive peripheral T-cell neoplasm caused by human T-cell lymphotropic virus type I. The disease is resistant to conventional chemotherapeutic agents, and there currently exist limited treatment options; thus, it has a poor prognosis.[1-4] A recent phase III trial for previously untreated patients with aggressive ATL (acute, lymphoma, or unfavorable chronic type) age 33 to 69 years demonstrated that a dose-intensified multidrug regimen, VCAP-AMP-VECP (vincristine, cyclophosphamide, doxorubicin, and prednisone; doxorubicin, ranimustine, and prednisone; and vindesine, etoposide, carboplatin, and prednisone), resulted in median progression-free (PFS) and overall survival (OS) of 7.0 and 12.7 months, respectively.[5] This remains unsatisfactory compared with responses in other hematologic malignancies. Allogeneic hematopoietic stem-cell transplantation has evolved into a potential approach to treating patients with ATL over the last decade. However, only a small fraction of patients with ATL have the opportunity to benefit from transplantation, such as those who are younger, have achieved sufficient disease control, and have an appropriate stem-cell source.[6,7] Therefore, the development of alternative treatment strategies for patients with ATL is an urgent issue.

Because CC chemokine receptor 4 (CCR4) is expressed on tumor cells from most patients with ATL,[8,9] we postulated that it might represent a novel molecular target for immunotherapy. Accordingly, KW-0761, a next-generation humanized anti-CCR4 immunoglobulin G1 (IgG1) monoclonal antibody (mAb) with a defucosylated Fc region, which markedly enhances antibody-dependent cellular cytotoxicity (ADCC), was developed.[10,11] We demonstrated that robust ADCC by the defucosylated anti-CCR4 mAb against primary tumor cells from patients with ATL mediated by autologous effector cells was triggered both in vitro and in a humanized mouse model in vivo.[11-13] These promising preclinical results prompted us to conduct a phase I clinical trial of KW-0761 for patients with relapsed CCR4-positive peripheral T-cell lymphoma (PTCL), including ATL. This study demonstrated good tolerability, predictable pharmacokinetics, and preliminary evidence of potent antitumor activity and resulted in a recommended dose of 1.0 mg/kg for subsequent clinical trials.[14] Herein, we report the results of a multicenter phase II study designed to assess the efficacy, pharmacokinetic profile, and safety of KW-0761 monotherapy in patients with relapsed CCR4-positive aggressive ATL.

PATIENTS AND METHODS

Patients

Patients 20 years of age or older with CCR4-positive aggressive ATL (acute, lymphoma, or unfavorable chronic type)[1,4] who had relapsed after at least one prior chemotherapy regimen were eligible. The unfavorable chronic type of ATL was defined by the presence of at least one of the following three factors: low serum albumin, high lactate dehydrogenase, or high blood urea nitrogen concentration.[5] CCR4 expression was determined by immunohistochemistry or flow cytometry using a mouse anti-CCR4 mAb (KM2160)[8,14] and confirmed by a central review committee. All patients were required to have an Eastern Cooperative Oncology Group performance status of 0 to 2. Eligibility criteria also included the following laboratory values: absolute neutrophil count $\geq$ 1500/μL, platelet count $\geq$ 50,000/μL, hemoglobin $\geq$ 8.0 g/dL, AST $\leq$ 2.5 $\times$ the upper limit of the normal range (UNL), ALT [lteuq] 2.5 $\times$ UNL, total bilirubin $\leq$ 1.5 $\times$ UNL, serum creatinine $\leq$ 1.5 $\times$ UNL, corrected serum calcium $\leq$ 11.0 mg/dL, and arterial partial oxygen pressure $\geq$ 65 mmHg or arterial blood oxygen saturation $\geq$ 93%. Patients were excluded if they had an active infection, a history of organ transplantation, active concurrent cancers, CNS involvement, a bulky mass requiring emergent radiotherapy, or seropositivity for hepatitis B virus antigen, hepatitis C virus antibody, or HIV antibody.

Study Design

This study was a multicenter, single-arm, phase II trial. Objectives of the study were to evaluate the efficacy, pharmacokinetic profile, and safety of KW-0761 monotherapy. Patients received intravenous infusions of KW-0761 once per week for 8 weeks at a dose of 1.0 mg/kg.[14] Oral antihistamine and acetaminophen were administered before each KW-0761 infusion to prevent infusion reactions. The primary end point was overall response rate (ORR), and secondary end points included the best response by disease site, PFS, and OS. Objective responses were assessed after the fourth and eighth infusions of KW-0761 by an independent efficacy assessment committee according to the modified response criteria for ATL.[4] It was estimated that 25 patients would be required to detect a lower limit of the 95% CI exceeding the 5% threshold of ORR based on the assumptions that the minimum required ORR for a new drug for relapsed, aggressive ATL is 5%,[15] with an expected ORR for KW-0761 of 30%[14] with 90% power. Adverse events (AEs) were graded according to the National Cancer Institute Common Terminology Criteria for AEs, version 3.0. The presence of human anti-KW-0761 antibodies in the patients' plasma was examined using enzyme-linked immunosorbent assay. Blood samples collected at times strictly in accordance with the protocol were employed for the pharmacokinetic analysis. Samples were obtained from patients who had received at least one dose of KW-0761 up to all eight doses. When any event resulted in an alteration in the infusion protocol, only those samples taken before the alteration were used for the analysis. The following parameters were calculated for plasma KW-0761: maximum drug concentration and trough drug concentration of each KW-0761 administration, area under the blood concentration time curve from 0 to 7 days after the first and eighth doses, and half-life period ($t_{1/2}$) after the eighth dose. As an additional research parameter, we investigated blood T-cell subset distribution during and after KW-0761 treatment and compared these values with those of 10 healthy donors as controls (five men, five women; median age, 45 years; range, 41 to 57 years).

Statistical Analysis

Survival estimates were calculated using the Kaplan-Meier method. PFS was defined as the time from the first dose of KW-0761 to progression, relapse, or death resulting from any cause, whichever occurred first. OS was measured from the day of the first dose to death resulting from any cause. Regarding T-cell subset analysis, differences between the patients' values before KW-0761 treatment and those of the controls were examined using the Mann-Whitney U-test. Differences between KW-0761 pretreatment values and those at each time point after KW-0761 treatment were examined using the Wilcoxon signed-rank test. All analyses were performed with SPSS Statistics 17.0 (SPSS, Chicago, IL). In this study, $P < .05$ was considered significant.

Study Oversight

The study was sponsored by Kyowa Hakko Kirin Company (Tokyo, Japan). The academic investigators and the sponsor were jointly responsible for the study design. The protocol was approved by the institutional review board at each participating site, and all patients and controls provided written informed consent before enrollment according to the Declaration of Helsinki.

RESULTS

Patients

Of the 28 patients enrolled onto the study, 27 (12 men, 15 women) received at least one infusion of KW-0761. One patient was withdrawn for aggravation of the general condition before the administration of KW-0761. Demographics and clinical characteristics of the 27 patients are summarized in Table 1. Median age was 64 years (range, 49 to 83). The disease subtypes included 14 acute, six lymphoma, and seven unfavorable chronic type ATL. Of these 27 patients, 14 (52%) completed the schedule of eight planned infusions. Of the remaining 13 patients, 11 (41%) discontinued treatment because of disease progression, one (4%) because of skin rash, and another (4%) because of concurrent colon cancer, for which this patient was excluded from the efficacy evaluation.

Efficacy of KW-0761

Of 26 patients evaluable for efficacy, objective responses were noted in 13 patients (ORR, 50%; 95% CI, 30% to 70%), including eight complete responses (CRs). Responses according to disease site were 100% (13 of 13; all CRs) for blood, 63% (five of eight) for skin, and 25% (three of 12) for nodal and extranodal lesions. Responses according to disease subtype were 43% (six of 14) for acute, 33% (two of six) for lymphoma, and 83% (five of six) for unfavorable chronic type ATL. Responses according to number of prior chemotherapy regimens were 48% (10 of 21) in those who had one prior regimen and 60% (three of five) for those who had two or three prior regimens. Median PFS and OS were 5.2 and 13.7 months, respectively (Figs 1A, 1B).

Table 1. Patient Demographics and Clinical Characteristics (n = 27)*

Characteristic	No.	%
Age, years		
Median	64	
Range	49-83	
≥ 65	13	48
Sex		
Male	12	44
Female	15	56
ECOG performance status†		
0	15	56
1	7	26
2	5	19
Disease subtype		
Acute	14	52
Lymphoma	6	22
Chronic	7	26
Prior chemotherapy regimens, No.		
1	22	82
2	3	11
3	2	7

Abbreviation: ECOG, Eastern Cooperative Oncology Group.
*Of 28 patients enrolled, 27 received at least one infusion of KW-0761.
†ECOG performance status scores range from 0 (normal activity) to 5 (death), with higher scores indicating more severe disability.

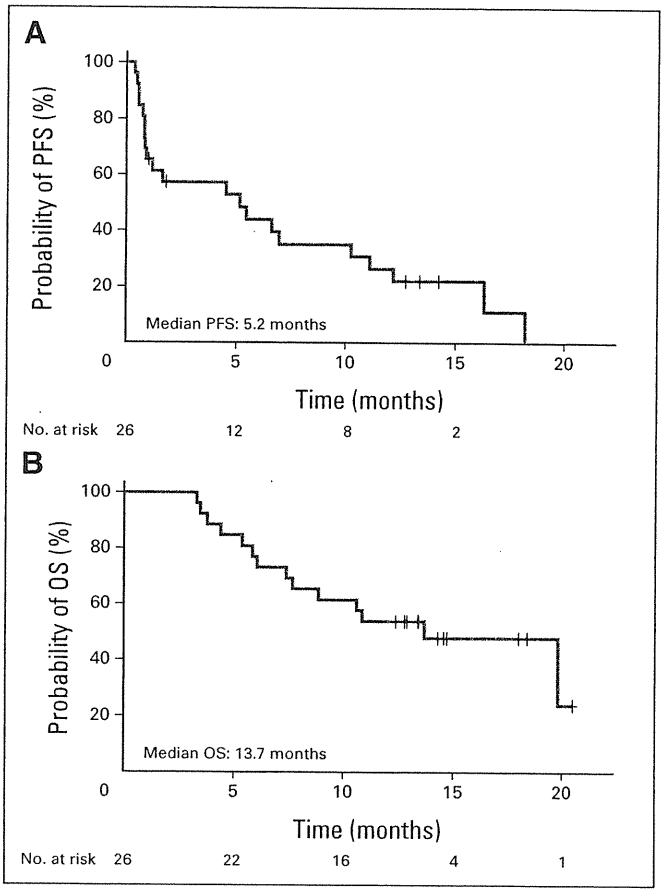

Fig 1. Kaplan-Meier curves of estimated (A) progression-free survival (PFS; median, 5.2 months) and (B) overall survival (OS; median, 13.7 months).

Pharmacokinetics

KW-0761 plasma concentrations over eight infusions, once per week, at 1.0 mg/kg are shown in Figure 2. Mean maximum drug concentration and trough drug concentration (± standard deviation) of the eighth infusion were 42.9 ± 14.2 μg/mL and 33.6 ± 10.6 μg/mL, respectively. Mean area under the blood concentration time curve from 0 to 7 days after the eighth infusion was 6,297 ± 1,812 μg × hours/mL. The mean $t_{1/2}$ after the eighth infusion was 422 ± 147 hours.

AEs

Table 2 lists AEs that occurred in at least 15% of patients or at grades 3 to 4, which were determined as possibly, probably, or definitely KW-0761 related. The most common nonhematologic AE was an infusion reaction (89%). In addition, 80% or more of the following recorded AEs occurred along with an infusion reaction: fever, chills, tachycardia, hypertension, nausea, and hypoxemia (Table 2). These events occurred primarily at the first infusion, becoming less frequent with subsequent treatments. The infusion reactions and component events were transient, and all patients recovered, although some needed systemic steroids. Skin rashes were observed as another frequent nonhematologic AE (63%), mostly occurring after the fourth or subsequent infusions. Of the 14 patients who developed grade 2 or higher skin rashes, objective responses were noted in 13 patients (93%), including eight CRs. On the other hand, of the 12 patients who developed no or grade 1 skin rashes, no objective responses were observed. A typical clinical course of the rash is depicted in Appendix Figures A1A and A1B (online only). The skin rash observed in this patient appeared after the seventh infusion, and the corresponding skin biopsy revealed mild perivascular CD8-positive cells dominating an inflammatory reaction, with an absence of ATL cells. The skin rash recovered on application of topical steroid. Of the 17 patients who developed skin rashes, one developed Stevens-Johnson syndrome, which was determined as possibly KW-0761 related, although that patient also received trimethoprim/sulfamethoxazole, fluconazole, and acyclovir for prevention of infection according to the protocol. This patient stopped those preventive agents and was treated with

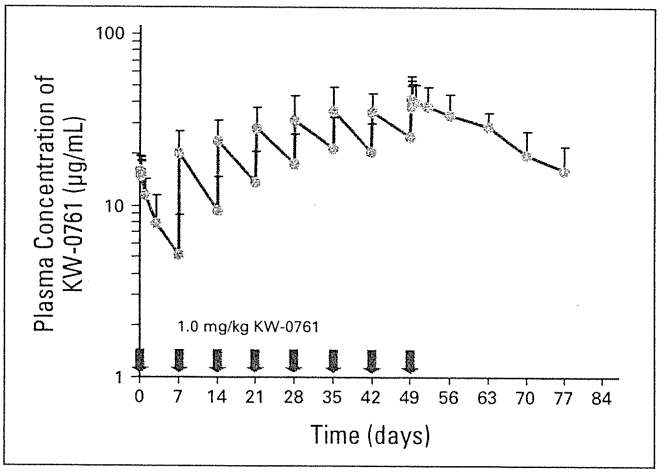

Fig 2. Pharmacokinetics of KW-0761. Mean KW-0761 plasma concentrations during and after 1.0 mg/kg KW-0761 infusions once per week for 8 weeks. Bar indicates upper limit of standard deviation.

Table 2. Adverse Events (n = 27)*

Adverse Event	Grade (No. of patients) 1	2	3	4	All Grades No. of Patients	%	Infusion Reaction Related (No. of patients) All Grades	≥ Grade 2
Nonhematologic								
Infusion reaction	1	22	1	0	24	89		
Fever	20	2	0	0	22	82	18	2
Rash	3	9	5	0	17	63	1	0
Chills	14	2	0	0	16	59	16	2
ALT	5	4	2	0	11	41		
AST	3	5	2	0	10	37		
Tachycardia	9	0	0	0	9	33	9	0
Hypertension	6	2	0	0	8	30	8	1
Albuminemia	7	1	0	0	8	30		
ALP	4	2	0	0	6	22		
Weight gain	5	0	0	0	5	19		
Nausea	4	1	0	0	5	19	5	1
Hyponatremia	5	0	0	0	5	19		
Hypoxemia	0	2	3	0	5	19	4	4
Hypotension	2	2	0	0	4	15	3	1
Pruritus	0	3	1	0	4	15		
γ-GTP	0	1	3	0	4	15		
Hypophosphatemia	0	4	0	0	4	15		
Hyperuricemia	4	0	0	0	4	15		
Hypercalcemia	1	1	0	1	3	11		
Hypokalemia	1	0	2	0	3	11		
Erythema multiforme†	0	0	1	0	1	4		
Hyperglycemia	0	0	1	0	1	4		
Tumor lysis syndrome	0	0	1	0	1	4		
Metabolic/laboratory, other‡	4	7	3	0	14	52		
Hematologic								
Lymphopenia§	0	6	9	11	26	96		
Leukocytopenia	3	7	8	0	18	67		
Thrombocytopenia	7	2	3	2	14	52		
Neutropenia	5	4	5	0	14	52		
Hemoglobin	4	3	1	0	8	30		

Abbreviations: ALP, alkaline phosphatase; BUN, blood urea nitrogen; CRP, C-reactive protein; GTP, glutamyl transpeptidase.

*Of 28 patients enrolled, 27 received at least one infusion of KW-0761. Listed are adverse events determined as possibly, probably, or definitely KW-0761 related that occurred in at least 15% of patients or were of grade 3 to 4 severity.

†One patient diagnosed as having Stevens-Johnson syndrome.

‡Other metabolic and laboratory test abnormalities included hypoproteinemia, BUN elevation, CRP, glycosuria, hypochloremia, and hyperammoniemia.

§Lymphopenia included decrease of abnormal lymphocytes.

systemic steroids, but improvement required the passage of 4 months. Lymphopenia, including a decrease in the number of ATL cells, occurred in 26 (96%) of the 27 patients. Grades 3 to 4 thrombocytopenia was observed in five patients (19%) but was not associated with bleeding, and grade 3 neutropenia also occurred in five patients but did not lead to a febrile episode. The latter two hematologic AEs improved in all patients. None of the patients developed detectable anti–KW-0761 antibody.

T-Cell Subset Analysis

The numbers of circulating blood CD4+ CCR4+, CD4+ CD25+ FOXP3+, CD4+ CCR4−, and CD4− CD8+ cells from KW-0761–treated patients and those from the 10 controls are presented as box and whisker plots in each graph (Appendix Figs A2A to A2D, online only). The numbers of CD4+ CCR4+ and CD4+ CD25+ FOXP3+ cells in patients with ATL before treatment were significantly higher than those in the controls but were significantly reduced after the first KW-0761 infusion. The reduction lasted for at least 4 months after the eighth infusion (Appendix Figs A2A, A2B; online only). The numbers of CD4+ CCR4−, and CD4− CD8+ cells in patients with untreated ATL were significantly lower than those in the controls. KW-0761 treatment led to a transient further reduction of those cells; however, recovery took place by the fifth infusion (Appendix Figs A2C, A2D; online only).

DISCUSSION

In the present multicenter phase II study, KW-0761 monotherapy demonstrated significant responses in patients with relapsed ATL with an acceptable toxicity profile. An ORR of 50% and median PFS and OS values of 5.2 and 13.7 months, respectively, were observed. Because the lower limit for an ORR with a 95% CI was 30%, this study met the primary end point. These results suggest an improvement over what has been achieved with other agents in relapsed ATL.[15] Cladribine was associated with an ORR of 7% (one of 15 patients),[16] and irinotecan hydrochloride treatment had an ORR of 38% (five of 13 patients) with a median duration of response of 31 days.[17] Antiviral therapy consisting of a combination of zidovudine and interferon, which has been proposed as a standard first-line therapy in leukemic subtypes of ATL,[18] was initially reported as having a median OS of 3.0 months in 19 patients with acute or lymphoma type ATL.[19] In addition, White et al[20] reported three objective responses lasting longer than 1 month with zidovudine plus interferon in 18 patients with ATL, of whom 15 had received prior therapy. Those observations collectively suggest that KW-0761 may offer an advantage over or provide an additional therapeutic option to the currently available therapy for relapsed ATL, although there were no direct comparisons.

On examining the results of ATL treatment according to disease site, disease in blood seemed to be more sensitive to KW-0761 than at other disease sites. Currently, we are unable to fully explain this difference; however, factors such as the KW-0761 delivery or the amount of ADCC effector cells such as natural killer (NK) cells and monocytes/macrophages in each disease site may be important.

Pharmacokinetic analyses demonstrated that the $t_{1/2}$ after the eighth administration of KW-0761 was nearly the same as that of circulating endogenous human IgG1, indicating good stability of this antibody in vivo. In addition, no anti-KW-0761 antibody was detected, suggesting that the antigenicity of this novel defucosylated mAb is not likely to be a problem clinically, consistent with findings in our preceding phase I study.[14]

The infusion reactions observed in the present study may also provide novel insights into problems associated with antibody therapy. It is generally recognized that complement plays a major role in infusion reactions,[21] but this mechanism cannot apply to KW-0761, because the agent is unable to mediate complement-dependent cytotoxicity.[11] Therefore, the infusion reactions observed here may have a different mechanism compared with those of other antibody therapies, such as rituximab. KW-0761 has a defucosylated Fc region, which markedly enhances ADCC because of increased binding affinity to the

Fcγ receptor on effector cells. Defucosylated IgG1 is a more potent activator of NK cells than nondefucosylated IgG1 during ADCC.[22] We surmise that the infusion reactions to KW-0761 were mainly induced by cytokines and related cytotoxic molecules released from highly activated NK cells.

The present study demonstrated that compared with the levels in the controls, KW-0761 led to a significant and lasting decrease in the number of CD4+ CCR4+ but not CD4+ CCR4− or CD4− CD8+ cells in patients with ATL. Consistent with the fact that CCR4 is expressed not only on T-helper type 2 cells but also on regulatory T (Treg) cells,[23-26] KW-0761 treatment also resulted in a significant and lasting decrease in CD4+ CD25+ FOXP3+ cells, including both ATL cells and endogenous non-ATL Treg cells.[27-29] Reduction or suppression of Treg cells is expected to be a potentially promising strategy for boosting antitumor immunity in patients with cancer, as observed in studies with ipilimumab,[30-33] although ipilimumab and KW-0761 have different targets; the former suppresses Treg cell function, and the latter decreases their number. Hence, KW-0761 could also lead to activation of antitumor immunity, which might also contribute to its potent anti-ATL response. Because ipilimumab causes immune-related AEs such as diarrhea and colitis, we were especially vigilant in monitoring for this type of AE. Because CCR4 contributes to lymphocyte skin-specific homing,[34] it was not surprising that skin rashes, which could be an immune-related AE, were frequently observed in the present KW-0761 study. Skin rashes, including the most severe case of Stevens-Johnson syndrome, the causal association of which with concomitant medications other than KW-0761 could not be excluded, proved to be manageable, and patients improved in all cases, although some needed systemic or topical steroid treatment. The observed better responses to KW-0761 in patients with grade 2 or higher skin rashes were highly impressive. However, the underlying mechanisms for this finding are not clear; thus, further detailed investigation is warranted. All of the 14 patients who developed grade 2 or higher skin rashes received five or more KW-0761 infusions according to the protocol, whereas only three of the 12 patients who developed no or grade 1 skin rashes received five or more KW-0761 infusions. This suggests the possibility that skin rashes were associated with the number of KW-0761 infusions. The Cochran-Mantel-Haenszel test stratified by the number of KW-0761 infusions ($\leq$ four v $\geq$ five) indicated a significant association between clinical response and skin rashes (no or grade 1 v grades 2 to 4; $P = .009$). However, the sample size is insufficient to draw such a conclusion.

Following on a phase III study (JCOG9801 [Japan Clinical Oncology Group 9801]) for untreated aggressive ATL,[5] the present promising results for KW-0761 monotherapy prompted us to conduct a subsequent randomized trial of VCAP-AMP-VECP chemotherapy with or without KW-0761 for previously untreated ATL (Clinicaltrials.gov: NCT01173887). CCR4 is also expressed on tumor cells from a subgroup of PTCL other than ATL, which also has an unfavorable prognosis.[2,35,36] Thus, we are currently conducting a phase II study of KW-0761 monotherapy for relapsed CCR4-positive PTCL (Clinicaltrials.gov:NCT01192984). In addition, Duvic et al[37] recently reported a phase I/II study of KW-0761 for refractory cutaneous T-cell lymphoma. They found that KW-0761 was well tolerated at doses of 0.1 to 1.0 mg/kg, and a promising ORR of 39% (15 of 38 patients) was achieved, although expression of CCR4 on lymphoma cells was not included as one of the eligibility criteria (Clinicaltrials-.gov: NCT00888927). Furthermore, clinical trials of KW-0761 for patients with Hodgkin's lymphoma may be worth trying, because it has been reported that Hodgkin's lymphoma tumor cells produce CCR4 ligand molecules, and migratory CCR4-expressing Treg cells prevent a host immune attack on tumor cells, thereby creating an immunologically favorable environment for the tumor cells.[38]

Although this phase II study offers a novel promising treatment option (KW-0761) for patients with relapsed ATL, some limitations should be discussed. First, the present phase II study was relatively small, with consequent limitations on drawing definitive conclusions about the efficacy and safety profile of KW-0761. Second, patients received different prior systemic chemotherapy regimens, which could affect the results of the present study. Finally, the enrolled patients all had aggressive ATL, but three clinical subtypes (acute, lymphoma, and unfavorable chronic type) were included. Although there may be no significant differences in susceptibility to conventional chemotherapies between these subtypes, the heterogeneity of the enrolled patients might have affected the results.

In conclusion, this multicenter phase II study demonstrated that KW-0761 monotherapy showed clinically meaningful antitumor activity in patients with relapsed ATL, with an acceptable toxicity profile. Further investigation of KW-0761 for ATL and other T-cell neoplasms is warranted on the basis of the present results.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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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′-GGCTAGCGCTACGGGAAAAG-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.

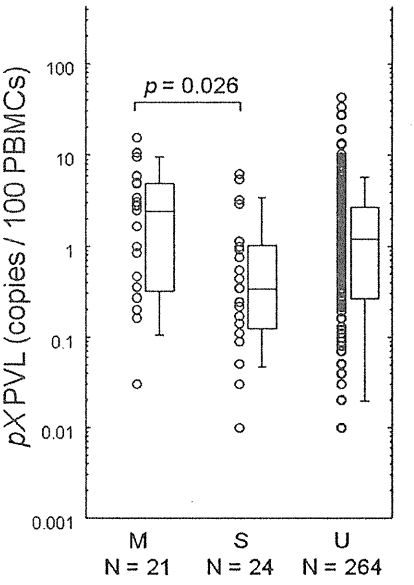

Figure 1. *PX* PVLs in HTLV-1 carriers with different infection routes M: Carriers with infection from mothers; S: Carriers with infection from spouses; U: Carriers with undetermined infection routes.

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.

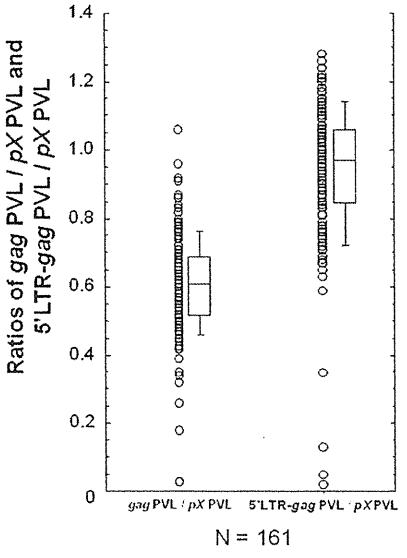

Figure 2. The ratios of PVLs for the 5′LTR-*gag* or *gag* regions to PVL for the *pX* region in 161 asymptomatic HTLV-1 carriers, whose *pX* PVLs were equal to or greater than 1 copy/100 PBMCs.

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

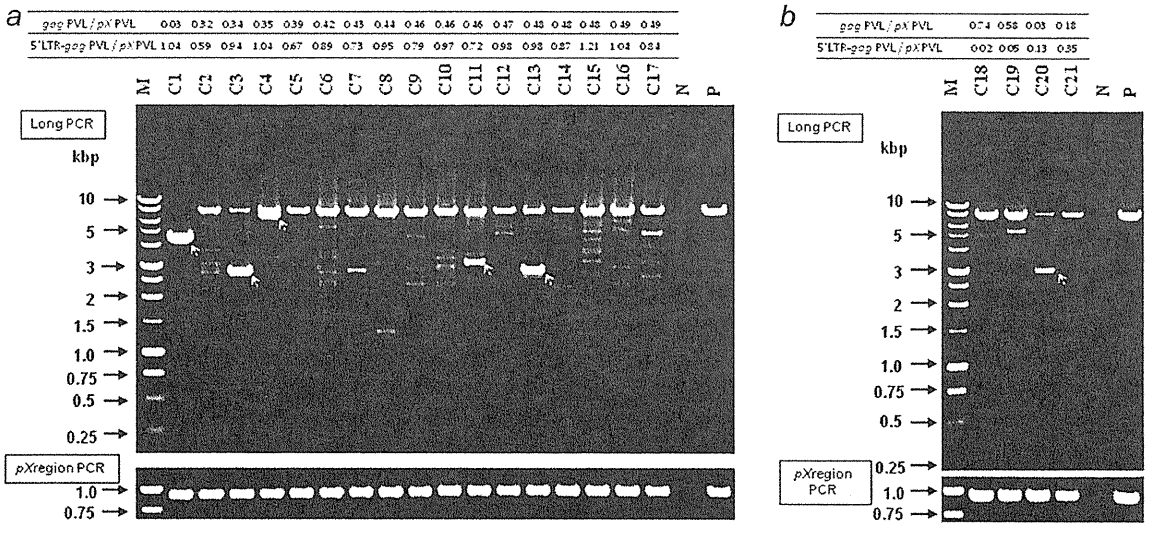

Figure 3. Detection of defective provirus by long PCR. (*a*) Asymptomatic HTLV-1 carriers with low *gag* PVL/*pX* PVL ratios less than 0.5. (*b*) Asymptomatic HTLV-1 carriers with low 5′LTR-*gag* PVL/*pX* PVL ratios less than 0.5. Arrows indicate PCR products for HTLV-1 provirus lacking large internal 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*).

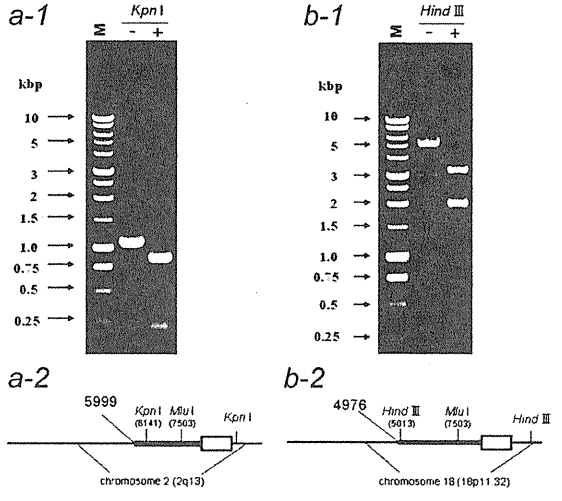

Figure 4. Detection of provirus with deletion of 5′LTR and its internal flanking sequence by IL-PCR. (*a-1*) Long PCR products from an asymptomatic HTLV-1 carrier, C20, with or without *Kpn* I digestion. (*a-2*) Scheme of the structure of defective provirus in C20. (*b-1*) Long PCR products from an asymptomatic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

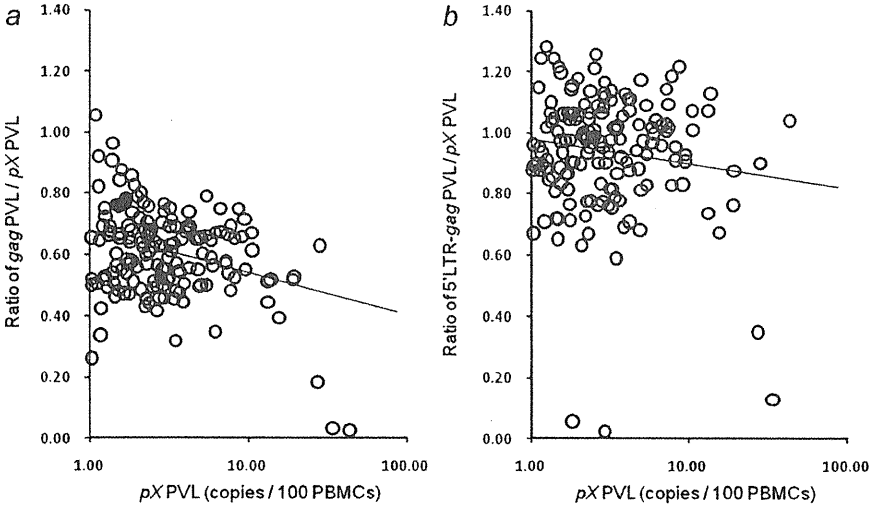

Figure 5. Relations of *pX* PVL and *gag* PVL/*pX* PVL or 5′LTR-*gag* PVL/*pX* PVL ratios in 161 asymptomatic carriers. (*a*) Relation of *pX* PVL and *gag* PVL/*pX* PVL. (*b*) Relation of *pX* PVL and 5′LTR-*gag* PVL/*pX* PVL.

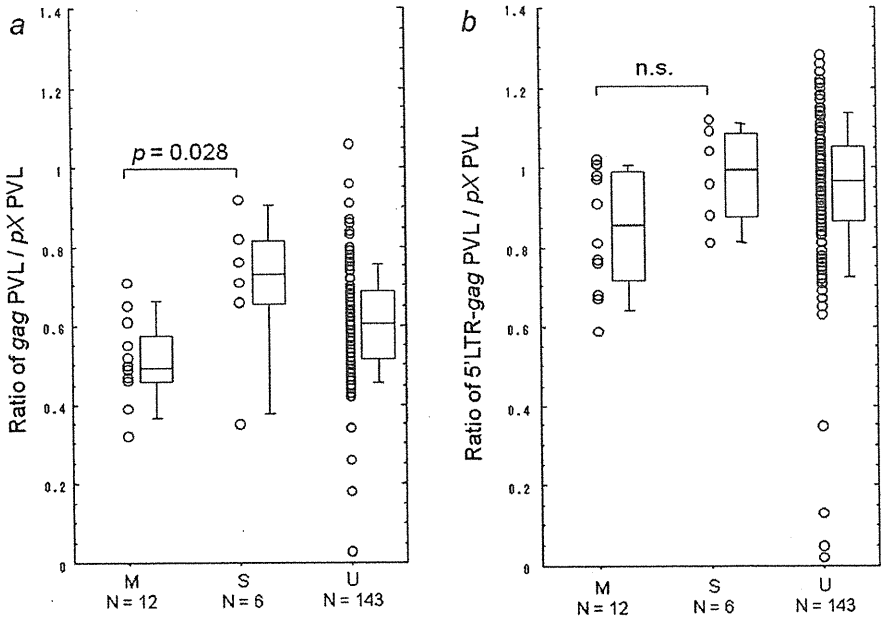

Figure 6. The ratios of *gag* PVL/*pX* PVL or 5′LTR-*gag* PVL/*pX* PVL in HTLV-1 carriers with different infection routes in 161 asymptomatic carriers. (*a*) The ratio of *gag* PVL/*pX* PVL. (*b*) The ratio of 5′LTR-*gag* PVL/*pX* PVL. M: Carriers with infection from mothers; S: Carriers with infection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