Journal of NeuroVirology, 12: 171–177, 2006

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280600827336

Taylor & Francis
Taylor & Francis Group

Clinical symptoms and the odds of human T-cell lymphotropic virus type 1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) in healthy virus carriers: Application of best-fit logistic regression equation based on host genotype, age, and provirus load

Hirohisa Nose,[1] Mineki Saito,[1] Koichiro Usuku,[2] Amir H Sabouri,[1] Toshio Matsuzaki,[1] Ryuji Kubota,[3] Nobutaka Eiraku,[4] Yoshitaka Furukawa,[5] Shuji Izumo,[3] Kimiyoshi Arimura,[1] and Mitsuhiro Osame[1]

[1]Department of Neurology and Geriatrics, [2]Department of Medical Information Science, and [3]Center for Chronic Viral Diseases, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan; [4]Kagoshima University Health Service Center, Kagoshima, Japan; [5]Division of Blood Transfusion Medicine and Cell Therapy, Kagoshima University Hospital, Kagoshima, Japan

The authors have previously developed a logistic regression equation to predict the odds that a human T-cell lymphotropic virus type 1 (HTLV-1)–infected individual of specified genotype, age, and provirus load has HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) in southern Japan. This study evaluated whether this equation is useful predictor for monitoring asymptomatic HTLV-1–seropositive carriers (HCs) in the same population. The authors genotyped 181 HCs for each HAM/TSP-associated gene (tumor necrosis factor [TNF]-α −863A/C, stromal cell-derived factor 1 (SDF-1) +801G/A, human leukocyte antigen [HLA]-A*02, HLA-Cw*08, HTLV-1 *tax* subgroup) and measured HTLV-1 provirus load in peripheral blood mononuclear cells using real-time polymerase chain reaction (PCR). Finally, the odds of HAM/TSP for each subject were calculated by using the equation and compared the results with clinical symptoms and laboratory findings. Although no clear difference was seen between the odds of HAM/TSP and either sex, family history of HAM/TSP or adult T-cell lenkemia (ATL), history of blood transfusion, it was found that brisk patellar deep tendon reflexes, which suggest latent central nervous system compromise, and flower cell-like abnormal lymphocytes, which is the morphological characteristic of ATL cells, were associated with a higher odds of HAM/TSP. The best-fit logistic regression equation may be useful for detecting subclinical abnormalities in HCs in southern Japan. *Journal of NeuroVirology* (2006) **12,** 171–177.

Keywords: best-fit logistic regression equation; clinical symptoms; HAM/TSP; HTLV-1; HTLV-1 carriers

Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1) (Poiesz *et al*, 1980; Yoshida *et al*, 1982) infection is of particular interest to the field of immunology as well as neurology because HTLV-1 is never eliminated from the host in spite of a vigorous cellular and humoral immune response against the virus, but causes no disease in a majority of infected subjects (asymptomatic HTLV-1–seropositive

Address correspondence to Mineki Saito, MD, PhD, at present address, Department of Microbiology, Kanazawa Medical University, 1-1 Daigaku, Uchinada-machi, Ishikawa 920-0293, Japan. E-mail: mineki@kanazawa-med.ac.jp

The authors thank Ms. Y. Nishino and T. Muramoto of Kagoshima University for their excellent technical assistance. This work was supported by Grant in Aid for Research on Brain Science of the Ministry of Health, Labor and Welfare, Japan and grant 17590886 from the Ministry of Education, Science, Sports and Culture, Japan.

Received 28 December 2005; revised 14 April 2006; accepted 20 May 2006.

carriers; HCs). Only approximately 2% to 3% develop adult T-cell leukemia (ATL) and another 2% to 3% develop chronic inflammatory diseases involving the central nervous system (HTLV-1–associated myelopathy/tropical spastic paraparesis; HAM/TSP) (Bangham, 2000), the eyes (Mochizuki *et al*, 1992; Nakao and Ohba, 1993), the lungs (Sugimoto *et al*, 1987; Matsuyama *et al*, 2003), the joints (Nishioka *et al*, 1989), or the skeletal muscles (Higuchi *et al*, 1993; Uchiyama, 1997; Saito *et al*, 2002). Therefore, evaluation of the individual risk for developing HTLV-1–associated diseases in each HC would certainly be of considerable importance in HTLV-1 endemic area. HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities (Nakagawa *et al*, 1996). Although the factors that cause these different manifestations of HTLV-1 infection are not fully understood, our previous population association study in Kagoshima, HTLV-1 endemic southern Japan, revealed that high provirus load (Nagai *et al*, 1998; Yoshida *et al*, 1989), certain human leukocyte antigen (HLA) (Jeffery *et al*, 1999, 2000) and non-HLA (Sabouri *et al*, 2004; Vine *et al*, 2002) genes are closely associated with HAM/TSP development. Namely, HLA-A*02 and -Cw*08 genes were associated with a lower HTLV-1 provirus load and with protection from HAM/TSP, whereas HLA-DRB1*0101 and B*5401 were associated with susceptibility to HAM/TSP (Jeffery *et al*, 1999, 2000). Because the function of class 1 HLA proteins is to present antigenic peptides to cytotoxic T lymphocytes (CTLs), these results imply that the efficient lysis of HTLV-1–expressing infected cells by HLA-A*02- or Cw*08-restricted CTLs reduce the risk of HAM/TSP, mainly through a reduction in provirus load. In the same cohort, we also determined the host genotype at over 100 single nucleotide polymorphisms (SNPs) in over 70 loci outside HLA class 1, and polymorphisms in at least 4 loci (tumor necrosis factor [TNF]-α, interleukin [IL]-15, SDF-1, and IL-10) were found to have statistically significant independent effects on the provirus load or the risk of HAM/TSP, or both (Sabouri *et al*, 2004; Vine *et al*, 2002). The TNF-α promoter -863 A allele predisposed to HAM/TSP (Vine *et al*, 2002), whereas SDF-1 +801A, IL-15 +191C (Vine *et al*, 2002), and IL-10 -592A alleles (Sabouri *et al*, 2004) conferred protection against HAM/TSP. In another study we reported the association between HTLV-1 *tax* gene sequence variation and the risk of HAM/TSP (Furukawa *et al*, 2000). The *tax* subgroup A was more frequently observed in HAM/TSP patients and this effect was independent of HLA-A*02. These results indicate that both host and viral genetic factors play a role in determining the risk of developing HAM/TSP. Based on these observations, we developed a best-fit logistic regression equation that can be used to predict the odds that an HTLV-1–infected individual of specified genotype (TNF-α -863A/C, SDF-1 +801G/A, HLA-A*02, HLA-Cw*08, HTLV-1 *tax* subgroup), age, and provirus load in Kagoshima has HAM/TSP (Vine *et al*, 2002). In this study, to validate whether this multivariate logistic equation can be useful to identify HAM/TSP-related symptom in HCs, we calculated the odds in 181 consecutive HCs and the individual odds of these HCs were compared with their clinical parameters and laboratory findings.

Table 1 Characteristics of 181 asymptomatic HTLV-1 carriers participated in the study

	HCs (n = 181)
Age	46.5 ± 12.7
Sex	
Male	95
Female	86
Serum anti-HTLV-I antibody titer*	
(Mean ± SD)	$\times 2932.6 \pm 6447.4$
(Median)	$\times 1024$
HTLV-I provirus load in PBMCs**	
(Mean ± SD)	240.8 ± 361.4
(Median)	82

*Anti-HTLV-1 antibodies were titrated by the particle agglutination method.
**HTLV-1 Tax copy number per 1×10^4 PBMCs.

Results

Demographic and clinical characteristics of healthy HTLV-1 carriers

A total of 181 consecutive HCs (95 men and 86 women) were completed the evaluation. Demographic and clinical characteristics of these HCs are given in Table 1. The age of the subjects enrolled ranged from 10 to 79 years with a mean age of 46.5 ± 12.7 years (men, 45.6 ± 13.2 years; women, 47.6 ± 12.2 years; mean ± SD). There were no abnormalities in complete blood cell count, electrolytes, glucose, renal and liver function tests, and the percentages of $CD4^+$, $CD8^+$, $CD8^+$ $CD3^+$, $CD16^+$, $CD56^+$ cells in peripheral blood mononuclear cells (PBMCs). The HTLV-1 provirus load of HCs was 240.8 ± 361.4 copies/10^4 PBMCs (mean ± SD). There was no significant difference between the sexes in HTLV-1 provirus load (men, 235.2 ± 336.0; women, 247.0 ± 389.6; mean ± SD) and anti-HTLV-1 antibody titer (men, $\times 2254.3 \pm 3644.6$; women, $\times 3682.6 \pm 8502.8$; mean ± SD).

DNA analyses and odds of developing HAM/TSP

The numbers of subjects with each genotype of HAM/TSP associated genes are shown in Table 2. Frequencies of HLA-A*02 was slightly lower and TNF-α -863A allele was slightly higher than HCs of our previous analysis (Vine *et al*, 2002). Of 181 HCs, 17 (9.4%) had tax subgroup A and 164 (90.6%) had *tax* subgroup B. The frequency of *tax* subgroup A in these HCs was similar to our previous findings (14 out of 200 HC; 7.0%) (Furukawa *et al*, 2000). Based on these data, we calculated the odds for developing HAM/TSP by using the best-fit logistic regression equation for the risk of HAM/TSP in the

Table 2 Frequencies of genotypes and alleles for the different polymorphisms of HAM/TSP associated genes in 181 asymptomatic HTLV-1 carriers participated in the study

Genes	*Allele*	*Number of HCs*	*Genotype*	*Number of HCs*
TNF-α −863	A	80 (22.1)*	AA	18 (9.9)
	C	282 (77.9)	AC	43 (23.8)
			CC	120 (66.3)
	Total	362	Total	181
SDF-1 +801	G	241 (66.6)	GG	80 (44.2)
	A	121 (33.4)	GA	81 (44.8)
			AA	20 (11.0)
	Total	362	Total	181
HLA-Cw*08	Positive	27 (14.9)		
	Negative	154 (85.1)		
	Total	181		
HLA-A*02	Positive	64 (35.4)		
	Negative	117 (64.6)		
	Total	181		
Tax subgroup	Subgroup A	17 (9.4)		
	Subgroup B	164 (90.6)		
	Total	181		

*Numbers in parentheses are percentage.

Kagoshima HTLV-1–infected cohort as previously described (Table 3) (Vine *et al*, 2002). The median odds in HCs was 0.36, which was significantly lower than that of HAM/TSP patients (median: 21.0) in our previous analysis ($P < .0001$, by Mann-Whitney U test). (Vine *et al*, 2002).

Receiver operator characteristic (ROC) curve analysis

The receiver operating characteristic (ROC) curve was used to compare the diagnostic accuracy among anti-HTLV-1 antibody titer, HTLV-1 provirus load, and the odds for HAM/TSP calculated by the best-fit logistic regression equation. The area under the curve (AUC) of the ROC was used to estimate the predictive value of each parameter. Judged by their areas, the accuracy of odds for HAM/TSP (0.95) is much higher than that of HTLV-1 provirus load (0.88) and anti-HTLV-1 antibody titer (0.86). The cut-off value to differentiate HAM/TSP and HCs was also determined from the ROC curve. We have chosen HAM/TSP odds "0.78" as a cut-off value (Figure 1), which maximizes sensitivity to diagnose HAM/TSP and minimizes false-positive rate to misdiagnose HCs as HAM/TSP. Using this value, sensitivity and specificity of the HAM/TSP odds to diagnose HAM/TSP are 92.1% and 86.5%, respectively. After dividing our present 181 HCs by this cut-off value, then clinical parameters and laboratory findings were compared.

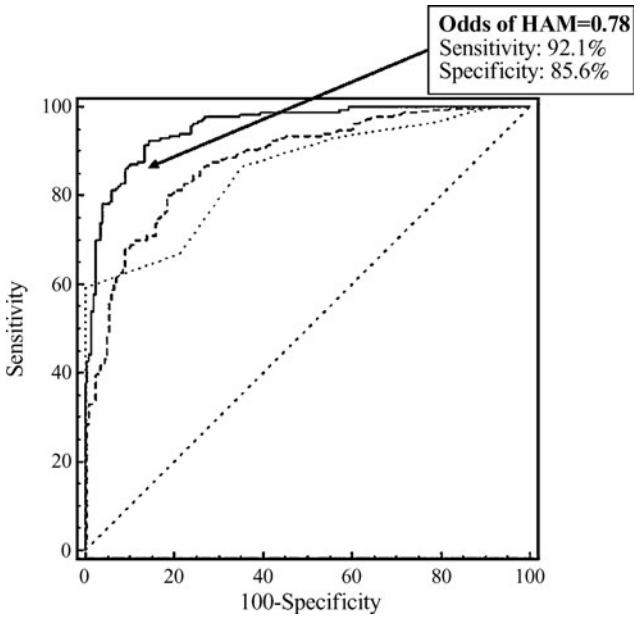

Figure 1 Receiver operating characteristic (ROC) curve of anti-HTLV-1 antibody titer, HTLV-1 provirus load, and the odds for HAM/TSP calculated by the best-fit logistic regression equation. ROC curve was constructed by plotting sensitivity against the false-positive rate (1-specificity) over a range of odds for HAM values or HTLV-1 provirus load or anti-HTLV-1 antibody titers by using our previously reported Kagoshima cohort data that consisted of 222 patients with HAM/TSP and 184 HCs (Vine *et al*, 2002). The cut off value to differentiate HAM/TSP and HCs was determined from the ROC curve as 0.78. Odds for HAM/TSP = 0.78 maximizes the sensitivity to diagnose HAM/TSP and minimizes the false-positive rate to misdiagnose HCs as HAM/TSP. Using this value, the sensitivity and specificity of the HAM/TSP odds required to diagnose HAM/TSP are 92.1% and 86.5%, respectively. ————: Odds for HAM/TSP; – – – – – –: $\log_{10}$ (HTLV-1 Tax copy number per 1×10^4 PBMCs); - - - - - - - - - -: serum anti-HTLV-I antibody titer.

Table 3 Best-fit logistic regression equation for the risk of HAM/TSP in the Kagoshima HTLV-1–infected cohort ($n = 402$) (Vine *et al*, 2002)

Factor, condition	*ln (odds of HAM/TSP)*	*Odds ratio (P)*
Constant	−1.716	
Age	−(0.145 × age) + (0.003 × age^2)	
Provirus load	+(0.460 × load) + (0.487 × load^2)	
TNF-α −863A$^+$	+3.057 − (4.616 × load) + (1.476 × load ^{2})	
SDF-1 +801GA	−0.808	0.45 (0.042)
SDF-1 +801AA	−1.689	0.18 (0.003)
HLA-A*02$^+$	−0.638	0.53 (0.043)
HLA-Cw*08$^+$	−0.894	0.41 (0.046)
HTLV-1 subgroup B	−1.587	0.20 (0.017)

Example: An HTLV-1–infected individual in Kagoshima, 60 years old, with a $\log_{10}$ (provirus load) of 2.5 with the genotype TNF-α −863A$^+$, SDF-1 +801AA, HLA-A*02$^-$, HLA-Cw*08$^+$, HTLV-1 subgroup B has a predicted ln odds of HAM/TSP of $-1.716 - (0.145 \times 60) + (0.003 \times 60^2) + (0.46 \times 2.5) + (0.487 \times 2.5^2) + 3.057 - (4.616 \times 2.5) + (1.476 \times 2.5^2) - 1.689 - 0.894 - 1.587 = 1.14975$. That is, this HTLV-1–infected individual's odds of developing HAM/TSP = exp(1.14975) = 3.157403.

Physical and neurological findings

First, the odds in HCs were compared with their demographic data and clinical variables to assess whether there was any association between the odds and each clinical parameter. As shown in Table 4,

Table 4 Comparison between the odds of HAM and clinical findings

	High odds (≥0.78)	*Low odds (<0.78)*	*P value*	*OR*	*95% CI*
$n = 181$	$n = 69$	$n = 112$			
Age (years)	53.4 ± 10.8	42.3 ± 12.1	<.001	N/A	N/A
Sex (male/female)	33/36	62/50	.41	N/A	N/A
Anti-HTLV-1 antibodies*	5123.7 ± 9495.7	1558.1 ± 2585.7	<.001	N/A	N/A
HTLV-1 provirus load**	505.1 ± 463.0	78.0 ± 101.6	<.001	N/A	N/A
Brisk patellar tendon reflexes	24/45	22/90	.036	2.18	1.11–4.31
Absent superficial abdominal reflexes	29/40	32/80	.089	1.81	0.97–3.4
Increased urinary frequency (≥10 times/day)	17/51	17/95	.15	1.86	0.88–3.96
Increased nocturia (≥2 times/night)	10/58	12/100	.58	1.44	0.58–3.53
Skin lesion	6/63	9/103	.90	1.09	0.37–3.21
Superficial lymph nodes swelling	6/63	8/104	.93	1.24	0.41–3.73
History of blood transfusion	2/67	5/107	.60	0.64	0.12–3.39
Family history of HAM/TSP or ATL	14/51	25/82	.93	0.90	0.43–1.89

The values are shown as the mean ± SD. N/A: not applicable.
*Anti-HTLV-1 antibodies were titrated by the particle agglutination method.
**HTLV-1 tax copy number per 1×10^4 PBMCs.

when we select odds for HAM/TSP = 0.78 as a cut-off value, there was no clear difference between the sex, family history of HAM/TSP or ATL, history of blood transfusion, number of urinations per day, nocturia, superficial lymph node enlargement, skin lesion, absent superficial abdominal reflexes (SARs), and the odds for HAM/TSP. However, brisk patellar deep tendon reflexes (PTRs), which may suggest latent central nervous system compromise, were more frequently observed in the HCs with higher odds (≥0.78) than the HCs with lower odds (<0.78) ($P = .036$, by χ^2-test with Yates correction). Absent superficial abdominal reflexes also tend to be more frequent in healthy carriers with higher odds, but P value did not reach statistical significance ($P = .089$, by χ^2-test with Yates correction).

Laboratory findings

Although no clear association was seen between the odds of HAM/TSP and either the complete blood cell count, electrolytes, glucose, renal or liver function tests, or the percentages of $CD8^+$, $CD8^+$ $CD3^+$, $CD16^+$, $CD56^+$ cells in PBMCs, both the absolute number and the percentage of flower cell-like abnormal lymphocytes (Ably), which is a morphological characteristic of ATL cells, were more frequently observed in healthy carriers with higher odds (≥0.78) than the healthy carriers with lower odds (<0.78) ($P = .011$ and .010, respectively by Mann Whitney U test) (Table 5).

Discussion

We have previously developed a logistic regression equation based on age, HTLV-1 provirus load, and genotypes of HAM/TSP–associated genes (TNF-α −863A/C, SDF-1 +801G/A, HLA-A*02, HLA-Cw*08, HTLV-1 *tax* subgroup) to predict the odds that an HTLV-1–infected individual in Kagoshima has HAM/TSP (odds of HAM/TSP) (Vine *et al*, 2002). To compare the diagnostic value of this equation, HTLV-1 provirus load and anti-HTLV-1 antibody titer for predicting the risk that an HTLV-1–infected individual will develop HAM/TSP, we employed receiver operating characteristic (ROC) curve analysis.

Table 5 Comparison between the odds of HAM and laboratory findings

	Odds of HAM/TSP		*P value*
	≥0.78	*<0.78*	
	$n = 68$	$n = 110$	
WBC counts	5430.9 ± 1491.4	5560.9 ± 1333.7	0.36
Lymphocyte counts	1640.1 ± 708.1	1721.5 ± 537.0	0.39
Lymphocyte %	30.4 ± 9.8	31.8 ± 8.6	0.27
Abnormal lymphocyte* counts	51.7 ± 79.0	19.0 ± 39.4	0.011
Abnormal lymphocyte %	0.88 ± 1.23	0.36 ± 0.77	0.010
Atypical lymphocyte** counts	32.6 ± 53.2	36.8 ± 63.2	0.66
Atypical lymphocyte %	0.65 ± 1.02	0.65 ± 1.23	1.00
	Cluster of differentiation		
	$n = 65$	$n = 104$	
$CD4^+$ counts	820.0 ± 410.1	820.1 ± 266.7	0.34
$CD4^+$ %	49.1 ± 8.0	47.3 ± 6.9	0.28
$CD8^+$ counts	497.3 ± 233.7	532.0 ± 191.2	0.12
$CD8^+$ %	30.5 ± 7.1	30.8 ± 7.3	0.71
CD4/8 ratio	1.73 ± 0.62	1.65 ± 0.56	0.71
	$n = 59$	$n = 101$	
$CD4^+CD3^+$ counts	797.7 ± 428.8	753.0 ± 249.7	0.59
$CD4^+CD3^+$ %	45.4 ± 7.9	43.3 ± 7.7	0.21
$CD8^+CD3^+$ counts	411.1 ± 241.4	427.1 ± 168.2	0.95
$CD8^+CD3^+$ %	23.8 ± 6.5	24.5 ± 6.5	0.82
$CD4^+CD3^+/CD8^+$ $CD3^+$ ratio	2.13 ± 0.89	1.92 ± 0.70	0.20
$CD16^+CD56^+CD3^-$ counts	228.7 ± 117.7	257.6 ± 166.5	0.64
$CD16^+CD56^+CD3^-$ %	15.1 ± 7.7	14.7 ± 6.9	0.64
$CD16^+CD56^+CD3^+$ counts	35.8 ± 44.7	47.3 ± 57.8	0.56
$CD16^+CD56^+CD3^+$ %	2.25 ± 2.83	2.80 ± 3.39	0.42
	Blood chemistry		
	$n = 66$	$n = 109$	
LDH	363.2 ± 114.9	349.8 ± 92.4	0.25

Cell counts are per/mm^3.
*Abnormal lymphocyte: flower cell (ATL cell)-like lymphocytes (see Materials and Methods).
**Atypical lymphocyte: a reactive lymphocyte due to antigenic stimulation with increased size and presence of active DNA synthesis, i.e., lobulated or indented nucleus with slightly finer chromatin, and the cytoplasm vary in color being basophilic, dark blue, plasmacytic to pale gray.

The ROC curve analysis was also used to identify a threshold at which sensitivity is highest at the lowest possible false-positive rate for each valuable. Our results clearly suggested that "odds for HAM/TSP" is better parameter for predicting disease than both HTLV-1 provirus load and anti-HTLV-1 antibody titer. The ROC curve of the odds of HAM/TSP showed an area under the curve (AUC) of 0.95, and best cut-off value being 0.78. After dividing HCs into the higher odds group and the lower odds group by this cut-off value (0.78), we have compared different clinical and laboratory parameters between two groups.

The comparison between the odds for HAM/TSP and clinical parameters revealed that brisk PTR were more frequently observed in the HCs with higher odds ($\geq$0.78) than the HCs with lower odds (<0.78) ($P = .036$, by χ^2-test with Yates correction), although their neurological signs were subtle and none had any motor signs. In contrast, there was no statistically significant association between the odds of HAM/TSP and either sex, family history of HAM or ATL, history of blood transfusion, number of urinations per day, nocturia, superficial lymph node enlargement, skin lesion. Because deep tendon reflexes (DTRs) test the integrity of the neurological system such as neuromuscular junction, peripheral nerve, nerve root, spinal cord and certain supraspinal centers, these reflexes are routinely used by clinicians to evaluate the nervous system for anatomical diagnosis. Hyperactive DTRs suggest central nervous system compromise. Therefore, increased frequencies of brisk PTRs in HCs with higher odds suggest that the calculated odds of HAM/TSP could be used as an indicator of HAM/TSP-related symptom. However, limitations of DTR are its qualitative nature of the assessments based upon subjective grading, and limited inter-rater reliability. Therefore, we next compared the objective laboratory data between HCs with higher odds ($\geq$0.78) and the HCs with lower odds (<0.78).

In all the laboratory parameters tested, only flower cell–like abnormal lymphocytes (Ably), both in their absolute number and frequency, were more frequently observed in HCs with higher odds ($\geq$0.78) than the HCs with lower odds (<0.78), with statistically significant level ($P = .011$ and .010, respectively by Mann Whitney U test). Because the odds for HAM/TSP is strongly correlated with provirus load, our result is consistent with previous studies, which demonstrated the presence of circulating Ably in HCs and a correlation between Ably frequency and HTLV-1 provirus load (Hisada *et al*, 1998; Tachibana *et al*, 1992). It may therefore be possible that the higher odds of HAM/TSP is associated with "genetically determined" efficient proliferation of HTLV-1–infected cells *in vivo*. However, neither the absolute number nor the frequency of $CD4^+CD3^+$ T cells, which is the main reservoir of HTLV-1 provirus, was significantly greater in HCs with higher odds ($\geq$0.78) than in the HCs with lower odds (<0.78) ($P = .59$ and .21, respectively, by Mann Whitney U test). This result suggests the presence of clonal outgrowth of HTLV-1–infected cells and skewed T cell repertoire, which is probably due to a long history of constant antigenic exposure, in $CD4^+CD3^+$ T cells of HCs with higher odds. Indeed, a previous report indicated that HTLV-1 infection is characterized by perturbation in T cell receptor (TCR) Vβ usage and CDR3 size distributions in both $CD8^+$ and $CD4^+$ T cells with clonal expansions (Eiraku *et al*, 1998). Therefore, HCs with higher odds of HAM/TSP may have more sustained clonal expansions and immune activation than HCs with lower odds, and these condition may also induce higher Ably level. If this is the case, Ably will be a good marker for the efficient clonal expansion of HTLV-1–infected T-cells and increase the risk of HAM/TSP. It would be informative, in a further study, to test whether clonal proliferation of infected $CD4^+$ T cells as well as TCR repertoire is related to the odds of HAM/TSP.

In conclusion, our study shows the possibility that our best-fit logistic regression equation could be useful for detecting HAM/TSP-related symptoms within HCs in Kagoshima cohort. This provides important indications for the management of HCs in an endemic area. It is possible that selective antiretroviral therapy as well as the therapeutic agents designed to reduce the effects of proinflammatory cytokines will reduce the risk for developing HAM/TSP in individuals with a higher odds for HAM/TSP. Further follow-up study is warranted to confirm the present findings.

Materials and methods

Study population

This study includes 190 consecutive HTLV-1–infected asymptomatic individuals who attended the Kagoshima University Hospital HTLV-1 Carrier Consultation Clinic between February 1999 and November 2004. Participation was voluntary and written informed consent was obtained from each subject upon entry into the study. This study was approved by the ethics committee of the Kagoshima University Graduate School of Medical and Dental Sciences. Nine cases were diagnosed as ATL by examination and blood tests and were therefore excluded from study. All cases were Japanese and resided in Kagoshima Prefecture, an HTLV-1 endemic region in southern Japan. On the first visit, all study participants interviewed by one of the three consultant neurologists who were certified by the board of Japanese Society of Neurology, then received a physical and neurological examination as well as blood tests. The following demographic data and clinical variables were assessed: sex, past history of blood transfusion, family history of hematological malignancies, family history of HAM/TSP, deep tendon reflexes, superficial abdominal reflexes, pathological reflexes, number of urinations per day, nocturia, superficial lymph node enlargement, skin lesion, HTLV-1 provirus load, anti-HTLV-1

antibody titer, complete blood cell count, differential leukocyte count, electrolytes, glucose, renal and liver function tests, and percent of $CD4^+$, $CD8^+$, $CD8^+$ $CD3^+$, $CD16^+$, $CD56^+$ cells in peripheral blood mononuclear cells (PBMCs).

Provirus load measurement and anti-HTLV-1 antibody titers

To assay the HTLV-1 provirus load, we carried out a quantitative polymerase chain reaction (PCR) method using ABI Prism 7700 (PE-Applied Biosystems) with 100 ng of genomic DNA (roughly equivalent to 10^4 cells) extracted from PBMCs using a QIAamp blood kit (Qiagen), according to the manufacturer's instructions (Nagai *et al*, 1998). Using β-actin as an internal control, the amount of HTLV-1 provirus DNA was calculated by the following formula: copy number of HTLV-1 *tax* per 1×10^4 PBMCs = [(copy number of *tax*)/(copy number of β-actin/2)] $\times$ 10^4. All samples were tested in triplicate. The lower limit of detection was one copy of HTLV-1 *tax* per 10^4 PBMCs. Serum antibody titers to HTLV-1 were determined by a particle agglutination method (Serodia-HTLV-1; Fujirebio). Namely, the antibody titers were achieved by performing a serial dilution of the patient serum and noting the highest dilution at which agglutination is still present.

Laboratory methods

Complete blood cell count, differential leukocyte count, electrolytes, glucose, renal and liver function tests, and the percentages of $CD4^+$, $CD8^+$, $CD8^+$ $CD3^+$, $CD16^+$, $CD56^+$ cells in PBMCs were measured on all fresh samples at the Kagoshima University Hospital Clinical Laboratory. Peripheral blood smears were obtained by smearing one drop of fresh blood onto a glass slide. All the slides were fixed by methanol and stained with Giemsa, and read by observers who were blinded to clinical information. The identification of flower cell (ATL cell)–like abnormal lymphocytes (Ably) followed the criteria by Sacher *et al* (1999). Namely, we classified the cells as Ably when they fulfilled the following criteria: the absence of azurophil granules; the presence of nuclear folding or lobulation; and at least two of the following characteristics: nuclear chromatin condensation, nuclear to cytoplasmic ratio of >80%, and/or cell size >1.5 times that of small lymphocytes. The number of abnormal lymphocytes and atypical lymphocytes in Table 5 were calculated as follows: (1) a trained medical technologist blind to subject HTLV serostatus performed three 100–white cell differential counts on a total of 300 leukocytes, then percentage of Ably or atypical lymphocytes was obtained. (2) The findings obtained by a trained medical technologist were reviewed by a board-certified hematologist to confirm the findings. (3) Using percentage of Ably or atypical lymphocytes and absolute WBC counts, the number of abnormal lymphocytes and atypical lymphocytes were calculated.

Restriction fragment length polymorphism (RFLP) analysis of the HTLV-1 tax gene

To identify the HTLV-1 *tax* gene subgroup (*tax* subgroup A or B), we carried out a PCR-RFLP analysis as previously described. (Furukawa *et al*, 2000). For RFLP analysis, 4 μl of the PCR product was digested with 5 U of *AccII* (Takara, Tokyo, Japan) in a 10-μl volume at 37°C for 1 h followed by electrophoresis on 2% Nusieve agarose gel. Positive and negative controls of known samples of *tax* gene subgroups A and B, confirmed by direct sequencing analysis, were included in all the experiments.

HLA typing

PCR sequence-specific primer reactions were performed to detect HLA-A*02 and HLA-Cw*08 as previously described (Bunce *et al*, 1995; Olerup and Zetterquist, 1992).

Receiver operator characteristic (ROC) curve analysis

Receiver operator characteristic (ROC) curve was constructed by plotting sensitivity against the false-positive rate (1-specificity) over a range of values of either the odds of HAM/TSP or the HTLV-1 provirus load or the anti-HTLV-1 antibody titers. These curves were constructed with data from our previously reported Kagoshima cohort, which consisted of 222 patients with HAM/TSP and 184 HCs (Vine *et al*, 2002). The area under the curve (AUC) of the ROC was used to estimate the predictive value of each parameter. The AUC is classified as low if the area is between 0.5 and 0.7; as moderate, if between 0.7 and 0.9; and as high, if greater than 0.9. The cut-off value to differentiate HAM/TSP and HCs was also determined from the ROC curve.

Statistical analysis

The chi-squared test, the Mann-Whitney *U* test, and the odds ratio were used for statistical analysis. Significance was considered at $P < 0.05$.

References

Bangham CR (2000). The immune response to HTLV-I. *Curr Opin Immunol* **12:** 397–402.

Bunce M, O'Neill CM, Barnardo MC, Krausa P, Browning MJ, Morris PJ, Welsh KI (1995). Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens* **46:** 355–367.

Eiraku N, Hingorani R, Ijichi S, Machigashira K, Gregersen PK, Monteiro J, Usuku K, Yashiki S, Sonoda S, Osame M, Hall WW (1998). Clonal expansion within CD4+ and CD8+ T cell subsets in human T lymphotropic virus type I-infected individuals. *J Immunol* **161:** 6674–6680.

Furukawa Y, Yamashita M, Usuku K, Izumo S, Nakagawa M, Osame M (2000). Phylogenetic subgroups of human T cell lymphotropic virus (HTLV) type I in the tax gene and their association with different risks for HTLV-I-associated myelopathy/tropical spastic paraparesis. *J Infect Dis* **182:** 1343–1349.

Higuchi I, Montemayor ES, Izumo S, Inose M, Osame M (1993). Immunohistochemical characteristics of polymyositis in patients with HTLV-I-associated myelopathy and HTLV-I carriers. *Muscle Nerve* **16:** 472–476.

Hisada M, Okayama A, Shioiri S, Spiegelman DL, Stuver SO, Mueller NE (1998). Risk factors for adult T-cell leukemia among carriers of human T-lymphotropic virus type I. *Blood* **92:** 3557–3561.

Jeffery KJ, Siddiqui AA, Bunce M, Lloyd AL, Vine AM, Witkover AD, Izumo S, Usuku K, Welsh KI, Osame M, Bangham CR (2000). The influence of HLA class I alleles and heterozygosity on the outcome of human T cell lymphotropic virus type I infection. *J Immunol* **165:** 7278–7284.

Jeffery KJ, Usuku K, Hall SE, Matsumoto W, Taylor GP, Procter J, Bunce M, Ogg GS, Welsh KI, Weber JN, Lloyd AL, Nowak MA, Nagai M, Kodama D, Izumo S, Osame M, Bangham CR (1999). HLA alleles determine human T-lymphotropic virus-I (HTLV-I) proviral load and the risk of HTLV-I-associated myelopathy. *Proc Natl Acad Sci USA* **96:** 3848–3853.

Matsuyama W, Kawabata M, Mizoguchi A, Iwami F, Wakimoto J, Osame M (2003). Influence of human T lymphotrophic virus type I on cryptogenic fibrosing alveolitis—HTLV-I associated fibrosing alveolitis: proposal of a new clinical entity. *Clin Exp Immunol* **133:** 397–403.

Mochizuki M, Yamaguchi K, Takatsuki K, Watanabe T, Mori S, Tajima K (1992). HTLV-I and uveitis. *Lancet* **339:** 1110.

Nagai M, Usuku K, Matsumoto W, Kodama D, Takenouchi N, Moritoyo T, Hashiguchi S, Ichinose M, Bangham CR, Izumo S, Osame M (1998). Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: high proviral load strongly predisposes to HAM/TSP. *J NeuroVirol* **4:** 586–593.

Nakagawa M, Nakahara K, Maruyama Y, Kawabata M, Higuchi I, Kubota H, Izumo S, Arimura K, Osame M (1996). Therapeutic trials in 200 patients with HTLV-I-associated myelopathy/tropical spastic paraparesis. *J NeuroVirol* **2:** 345–355.

Nakao K, Ohba N (1993). Clinical features of HTLV-I associated uveitis. *Br J Ophthalmol* **77:** 274–279.

Nishioka K, Maruyama I, Sato K, Kitajima I, Nakajima Y, Osame M (1989). Chronic inflammatory arthropathy associated with HTLV-I. *Lancet* **1:** 441.

Olerup O, Zetterquist H (1992). HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* **39:** 225–235.

Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC (1980). Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci U S A* **77:** 7415–7419.

Sabouri AH, Saito M, Lloyd AL, Vine AM, Witkover AW, Furukawa Y, Izumo S, Arimura K, Marshall SE, Usuku K, Bangham CR, Osame M (2004). Polymorphism in the interleukin-10 promoter affects both provirus load and the risk of human T lymphotropic virus type I-associated myelopathy/tropical spastic paraparesis. *J Infect Dis* **190:** 1279–1285.

Sacher RA, Luban NL, Ameti DI, Friend S, Schreiber GB, Murphy EL (1999). Low prevalence of flower cells in U.S.A. blood donors infected with human T-lymphotrophic virus types I and II. *Br J Haematol* **105:** 758–763.

Saito M, Higuchi I, Saito A, Izumo S, Usuku K, Bangham CR, Osame M (2002). Molecular analysis of T cell clonotypes in muscle-infiltrating lymphocytes from patients with human T lymphotropic virus type 1 polymyositis. *J Infect Dis* **186:** 1231–1241.

Sugimoto M, Nakashima H, Watanabe S, Uyama E, Tanaka F, Ando M, Araki S, Kawasaki S (1987). T-lymphocyte alveolitis in HTLV-I-associated myelopathy. *Lancet* **2:** 1220.

Tachibana N, Okayama A, Ishihara S, Shioiri S, Murai K, Tsuda K, Goya N, Matsuo Y, Essex M, Stuver S, *et al* (1992). High HTLV-I proviral DNA level associated with abnormal lymphocytes in peripheral blood from asymptomatic carriers. *Int J Cancer* **51:** 593–559.

Uchiyama T (1997). Human T cell leukemia virus type I (HTLV-I) and human diseases. *Annu Rev Immunol* **15:** 15–37.

Vine AM, Witkover AD, Lloyd AL, Jeffery KJ, Siddiqui A, Marshall SE, Bunce M, Eiraku N, Izumo S, Usuku K, Osame M, Bangham CR (2002). Polygenic control of human T lymphotropic virus type I (HTLV-I) provirus load and the risk of HTLV-I-associated myelopathy/tropical spastic paraparesis. *J Infect Dis* **186:** 932–939.

Yoshida M, Miyoshi I, Hinuma Y (1982). Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc Natl Acad Sci U S A* **79:** 2031–2035.

Yoshida M, Osame M, Kawai H, Toita M, Kuwasaki N, Nishida Y, Hiraki Y, Takahashi K, Nomura K, Sonoda S, *et al* (1989). Increased replication of HTLV-I in HTLV-I-associated myelopathy. *Ann Neurol* **26:** 331–335.