indicated that the provirus load in PBMCs from HCs in genetic relatives of patients with HAM/TSP in Kagoshima was significantly higher than that of non-HAM/TSP-related HCs, suggesting the importance of genetic background for developing HAM/TSP (Nagai *et al.*, 1998). In the Kagoshima population, an association between HLA-DRB1*0101, HLA-B*5401, HLA-A*02 and HLA-Cw*08 and the outcome of HTLV-1 infection has been reported, where HLA-A*02 and HLA-Cw*08 genes were each independently associated with a lower HTLV-1 provirus load and with protection from HAM/TSP, whereas HLA-DRB1*0101 and HLA-B*5401 were associated with an increased susceptibility to HAM/TSP (Jeffery *et al.*, 1999, 2000). The association of HLA-DRB1*0101 with disease susceptibility was only evident in the absence of the protective effect of HLA-A*02 (Jeffery *et al.*, 1999). These results are consistent with the hypothesis that a strong class I-restricted T-cell response is beneficial (Bangham, 2000). In another study, an association between HTLV-1 *tax* gene sequence variation and the risk of HAM/TSP was reported (Furukawa *et al.*, 2000). The *tax* subgroup A was more frequently observed in HAM/TSP patients than in HCs and this effect was independent of HLA-A*02. These reports suggested that both host genetic factors and HTLV-1 subgroup independently play a part in determining the risk of developing HAM/TSP.

HTLV-1 is also endemic in the Caribbean Basin (Blattner *et al.*, 1982), Africa (Biggar *et al.*, 1984), South America (Zamora *et al.*, 1990; Cartier *et al.*, 1993; Zaninovic *et al.*, 1994) and the Melanesian islands (Yanagihara *et al.*, 1990). The city of Mashhad in northeastern Iran has also been reported as an endemic centre for HTLV-1 (Safai *et al.*, 1996). In a recent study, the prevalence of HTLV-I infection was reported to be 0·77 % among blood-bank donors of Mashhad (Abbaszadegan *et al.*, 2003), but the prevalence and incidence of HAM/TSP are unknown in this population. Since there has been no report to compare the genetic risk factors for HAM/TSP among different ethnic populations, it was interesting to study whether genetic risk factors found in Kagoshima, Japan, were also valid for HAM/TSP development in the Mashhadi Iranian population. We therefore analysed the HTLV-1 provirus load, HTLV-1 *tax* subgroup and the allele frequencies of HLA-A*02, HLA-B*5401, HLA-Cw*08 and HLA-DRB1*0101 in Iranian HTLV-1-infected individuals using the same methods and techniques that were used in the Kagoshima studies (Nagai *et al.*, 1998; Jeffery *et al.*, 1999, 2000). The effect of host genetic factors and HTLV-1 *tax* subgroups on the risk of HAM/TSP development in different ethnic groups is discussed.

METHODS

Study populations. Peripheral blood samples were studied from 58 Iranian patients with HAM/TSP and 74 HCs from blood donors of the Blood Transfusion Center in the city of Mashhad and Neyshabour, both located in HTLV-1-endemic northeastern Iran. The study population from Kagoshima consisted of 222 patients with HAM/TSP and 184 HCs, all of whom were enrolled in the previous Kagoshima studies (Nagai *et al.*, 1998; Jeffery *et al.*, 1999, 2000; Furukawa *et al.*, 2000). The diagnosis of HAM/TSP was made according to the World Health Organization diagnostic criteria (Osame, 1990). Informed consent was obtained from all patients. This research was approved by the institutional review boards of the authors' institutions.

DNA preparation. All Japanese and Iranian blood samples were taken by vacuum tube pre-filled with the anticoagulant EDTA. Genomic DNA extraction procedures were different for each population. In the case of Kagoshima samples, fresh PBMCs were isolated by Histopaque-1077 (Sigma) density-gradient centrifugation and genomic DNA was extracted using a QIAamp Blood kit (Qiagen). For Iranian samples, for economical and technical reasons, fresh blood specimens were frozen immediately after collection and frozen whole-blood samples were transported to Kagoshima University on dry ice. Genomic DNA of nucleated blood cells was isolated from whole blood in Kagoshima University using the PureGene DNA Purification kit (Gentra Systems).

Provirus load measurement. To assay the HTLV-1 provirus load, we carried out a quantitative PCR using ABI Prism 7700 (PE Applied Biosystems) with 100 ng genomic DNA (equivalent to approx. 10^4 cells) from PBMCs (for Kagoshima samples) or nucleated blood cells (for Iranian samples) as reported previously (Nagai *et al.*, 1998). Using β-actin as an internal control, the amount of HTLV-1 provirus DNA was calculated using the following formula: copy number of HTLV-1 *tax* per 10^4 PBMCs (for Japanese samples) or nucleated blood cells (for Iranian samples) $=[(\text{copy number of } tax)/(\text{copy number of } \beta\text{-actin}/2)] \times 10^4$. All samples were tested in triplicate. The lower limit of detection was one copy of HTLV-1 *tax* per 10^4 PBMCs. In this study, we used the previously analysed provirus load data of Kagoshima samples from our database (Nagai *et al.*, 1998). All Iranian samples and some randomly selected Kagoshima samples were analysed using the same kit (AmpliTaq Gold and TaqMan probe; PE Applied Biosystems) and machine (ABI Prism 7700) at the same time. The same standard DNA for *tax* and β-actin was used throughout the study and there was no discrepancy between old and new data (not shown).

Sequencing of the HTLV-1 *tax* gene. Randomly selected Iranian samples from 10 HAM/TSP patients and 10 HCs were sequenced over almost the entire HTLV-1 *tax* gene (nt 7295–8356, nucleotide numbers correspond to those of the prototypic strain, ATK-1; Seiki *et al.*, 1983). PCR was done on extracted DNA to amplify provirus DNA, and nucleotide sequences were determined by direct sequencing in both directions. We amplified 100 ng DNA in 35 cycles of PCR, using an expanded high-fidelity PCR system (Boehringer Mannheim) and 1 μM primers (PXO1$^+$, 5′-TCGAAACAGCCCTGCAGATA-3′, nt 7257–7276, and PXO2$^+$, 5′-TGAGCTTATGATTTGTCTTCA-3′, nt 8447–8467). Each PCR cycle consisted of denaturation at 94 °C for 60 s, annealing at 58 °C for 75 s, extension at 72 °C for 90 s and a final extension at 72 °C for 10 min. Amplified DNA products were purified using a purification kit (QIAquick; Qiagen) and 0·1 μg PCR product was sequenced with a dye terminator DNA sequencing kit (Applied Biosystems) with 3·2 pmol each primer [PXI1$^+$, 5′-ATACAAAGTTAACCATGCTT-3′, nt 7274–7293; PXI2$^+$, 5′-GGCCATGCGCAAATACTCCC-3′, nt 7618–7637; PXI3$^+$, 5′-TTCCGTTCCACTCAACCCTC-3′, nt 8001–8020; PXI1$^-$, 5′-GGGTTCCATGTATCCATTTC-3′, nt 7644–7663, PXI2$^-$, 5′-GTCCAAATAAGGCCTGGAGT-3′, nt 8024–8043; and PXI3$^-$, 5′-AGACGTCAGAGCCTTAGTCT-3′, nt 8374–8393] in an automatic DNA sequencer (model 377; Applied Biosystems).

Restriction fragment length polymorphism (RFLP) analysis of the HTLV-1 *tax* gene. To determine the HTLV-1 *tax* gene subgroup (*tax* A or B) in Iranian samples, we carried out a PCR-RFLP

analysis as previously described (Furukawa *et al.*, 2000). For RFLP analysis, 4 μl PCR product was digested with 5 U *Acc*II (Takara) in 10 μl total volume at 37 °C for 1 h followed by electrophoresis on 2 % Nusieve agarose gel. The previously analysed *tax* subgroup data of Kagoshima samples (Furukawa *et al.*, 2000) were extracted from our database. Positive and negative controls of known Japanese samples of *tax* gene subgroups A and B, which were confirmed by direct sequence analysis, were included in all experiments.

HLA typing. PCR sequence-specific primer reactions were performed to detect HLA-A*02, HLA-B*5401, HLA-Cw*08 and HLA-DRB1*0101 as previously described (Bunce *et al.*, 1995; Olerup & Zetterquist, 1992). We used previously analysed HLA data of Kagoshima samples from our database (Jeffery *et al.*, 1999, 2000).

Statistical analysis. Statistical analysis was performed using the SPSS for Windows release 7.0, run on an IBM-compatible computer (Analytical Software, version 7). The χ^2 test, the Mann–Whitney U test and the odds ratio (OR) were used for statistical analysis. Values of $P<0{\cdot}05$ were considered statistically significant.

RESULTS

Differences in HTLV-1 provirus load between HAM/TSP patients and asymptomatic carriers is significantly lower in Iranian HTLV-1-infected individuals than in Japanese

We used the previously analysed provirus load data of Kagoshima samples from our database (Nagai *et al.*, 1998); all Iranian samples were newly analysed. The median age of HAM/TSP patients in both Kagoshima (57·3 years, range 15–80 years, 70·4 % female) and Iran (49·7 years, range 24–80 years, 72·1 % female) was greater than that of HCs in Kagoshima (39·4 years, range 16–64 years, 52·7 % female) and Iran (41·4 years, range 22–73 years, 38·3 % female), respectively. There was no significant difference in age between the control groups (HCs) of the two populations. All HCs in each group originated from unrelated blood donors. Since we extracted Japanese genomic DNA samples from PBMCs but Iranian samples from whole blood, direct comparison of HTLV-1 provirus load between the two populations was inappropriate. Since the main target of HTLV-1 infection is human T cells, whole blood-derived DNA contains more uninfected nucleated cells than PBMCs, and therefore the provirus load data in Iranians was likely to be underestimated if we used β-actin as an internal control. Thus, we compared the HTLV-1 provirus load between HAM/TSP patients and asymptomatic carriers within each population. As shown in Fig. 1, although the HTLV-1 provirus load of Iranian HAM/TSP patients was significantly higher than that of Iranian HCs ($P=0{\cdot}009$, Mann–Whitney U test), as reported in Japanese patients (Nagai *et al.*, 1998), the differences in median provirus load between Iranian HAM/TSP patients and HCs (twofold greater in the HAM/TSP patients than in the HCs) was much smaller than that of Japanese subjects (13-fold). Interestingly, although provirus load data were probably underestimated in Iranian samples compared with Japanese samples, the HTLV-1 provirus load in Iranian HCs was still significantly higher than that of Japanese HCs ($P=0{\cdot}004$, Mann–Whitney U test).

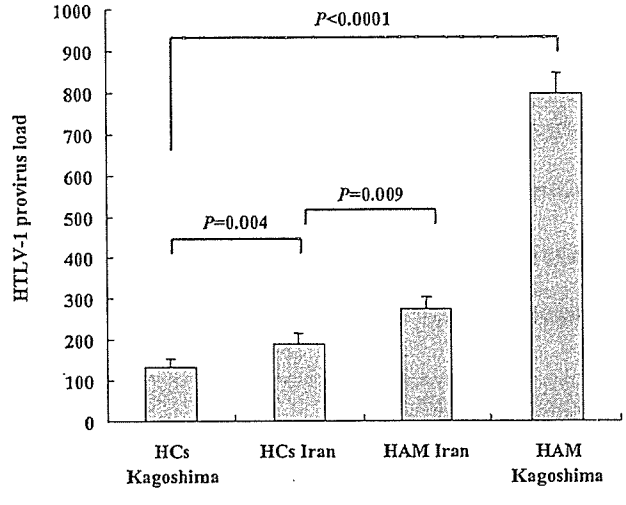

Fig. 1. HTLV-1 provirus load of Japanese and Iranian HTLV-1-infected individuals. Mean HTLV-1 copy numbers per 10^4 PBMCs for Japanese samples and per 10^4 nucleated cells for Iranian samples determined by quantitative PCR are shown. The HTLV-1 provirus load of Iranian HAM/TSP patients was significantly higher than that of Iranian HCs ($P=0{\cdot}009$, Mann–Whitney U test). The difference in median provirus load between Iranian HAM/TSP patients and HCs was much smaller than that of Japanese (Kagoshima) subjects, since HTLV-I provirus load in Iranian HCs is significantly higher than in Japanese HCs ($P=0{\cdot}004$). Error bars indicate SEM.

HLA-A*02 and HLA-Cw*08 are not associated with a lower risk of HAM/TSP and a lower provirus load in Iranian HTLV-1-infected individuals

To examine whether the previously reported associations between class I and class II HLA alleles and HAM/TSP prevalence in Kagoshima was also valid for HAM/TSP development in the Iranian population, we genotyped HLA-DRB1*0101 and HLA-A*02, HLA-B*5401 and HLA-Cw*08 by PCR-based DNA typing in 132 Iranian HTLV-1-infected individuals (58 HAM/TSP and 74 HCs). All Japanese HLA data had been previously analysed and were extracted from our database (Jeffery *et al.*, 1999, 2000). As shown in Table 1, the genotype frequency of HLA-A*02 and HLA-Cw*08 in Kagoshima subjects was significantly lower among the cases of HAM/TSP compared with HCs ($P=0{\cdot}0006$ and 0·0196, respectively). In contrast, the genotype frequency of HLA-A*02 and HLA-Cw*08 was not significantly different between Iranian HAM/TSP and HCs ($P=0{\cdot}346$ and 0·940, respectively). Also, whereas HLA-A*02 and HLA-Cw*08 were associated with a lower median provirus load in Kagoshima subjects ($P=0{\cdot}0003$ for A*02 and $P=0{\cdot}009$ for HLA-Cw*08; Mann–Whitney U test), this effect was not observed in Iranian subjects

Table 1. HLA-A*02 and HLA-Cw*08 are not associated with a lower risk of HAM/TSP in Iranian HTLV-1-infected individuals

Population (no. HAM/HCs)	HLA allele	HAM/TSP	HCs	χ^{2}*	*P*	OR†	95 % CI
Iranian (58/74)	HLA-A*02^{+}	21 (36·2 %)	20 (27·0 %)	0·887	0·346	1·53	0·73–3·22
	HLA-A*02^{-}	37 (63·8 %)	54 (73·0 %)				
Japanese (222/184)‡	HLA-A*02^{+}	67 (30·2 %)	87 (47·3 %)	11·784	0·0006	0·48	0·32–0·72
	HLA-A*02^{-}	155 (69·8 %)	97 (52·7 %)				
Iranian (58/74)	HLA-Cw*08^{+}	9 (15·5 %)	10 (13·5 %)	0·006	0·940	1·18	0·44–3·11
	HLA-Cw*08^{-}	49 (84·5 %)	64 (86·5 %)				
Japanese (222/184)‡	HLA-Cw*08^{+}	24 (10·8 %)	36 (19·6 %)	5·45	0·0196	0·50	0·29–0·87
	HLA-Cw*08^{-}	198 (89·2 %)	148 (80·4 %)				

*With Yates correction.

†OR used the approximation of Woolf (1955).

‡Japanese data were extracted from a database from previous analyses (Jeffery *et al.*, 1999, 2000).

($P=0·071$ for A*02 and $P=0·75$ for HLA-Cw*08; Mann–Whitney U test; Table 2), indicating that a protective effect of HLA-A*02 and HLA-Cw*08 was not observed in Iranian HTLV-1-infected individuals. As expected, HLA-B*5401, which is known to be almost exclusively found in East Asian populations, was not found in the Iranian subjects analysed.

HLA-DRB1*0101 increases the odds of HAM/TSP development in both Japanese and Iranian HLA-A*02-negative, but not in HLA-A*02-positive, HTLV-1-infected individuals

In contrast to HLA-A*02, HLA-DRB1*0101 was associated with susceptibility to HAM/TSP in both Japanese ($P=0·049$) and Iranian ($P=0·035$) populations (Table 3). This effect was observed only in the HLA-A*02-negative subjects but not in the HLA-A*02-positive subjects in both populations (Table 3). Although possession of HLA-DRB1*0101 was associated with a significantly lower provirus load in the Japanese HAM/TSP patients (Table 4, $P=0·024$) but not in HCs, HLA-DRB1*0101 was not associated with a difference in the provirus load in the Iranian HTLV-1-infected HAM/TSP patients and HCs (Table 4).

All Iranian HTLV-1 isolates have 10 nt substitutions in the *tax* region including all the *tax* subgroup A substitutions

Based on the LTR gene sequence, HTLV-1 can be classified into three types: Melanesian, Central African and cosmopolitan types, while cosmopolitan types can be further classified into subtypes A, B and C (Miura *et al.*, 1994). There are two distinct subtypes in Japan; the most frequently observed (nearly 80 %) Japanese subtype belongs to cosmopolitan subtype B, while a minor subtype (20 %), which seems to cluster in the southern islands of Kyushu and the Ryukyu Islands, belongs to cosmopolitan subtype A. A previous report suggested that, although Mashhadi HTLV-1 isolates belonged to cosmopolitan subtype A, this strain formed a tight cluster that was distinct from the other isolates of cosmopolitan subtype A from Japan, India, the Caribbean Basin and South America (Yamashita

Table 2. HLA-A*02 and HLA-Cw*08 are not associated with a lower provirus load in Iranian HTLV-1-infected individuals

Population	HLA allele	Provirus load (mean ± SE)*	Provirus load (median)*	No. subjects	*P*†
Iranian	HLA-A*02^{+}	262·1 ± 34·5	190·0	41	0·071
	HLA-A*02^{-}	209·6 ± 24·9	120·0	91	
Japanese‡	HLA-A*02^{+}	366·8 ± 43·4	118·5	156	0·0003
	HLA-A*02^{-}	525·6 ± 41·5	266·0	250	
Iranian	HLA-Cw*08^{+}	198·2 ± 42·8	131·0	19	0·75
	HLA-Cw*08^{-}	233·6 ± 22·9	147·0	113	
Japanese‡	HLA-Cw*08^{+}	300·7 ± 56·4	120·0	60	0·009
	HLA-Cw*08^{-}	492·0 ± 34·5	234·0	346	

*Provirus load is the HTLV-1 *tax* copy number per 10^{4} PBMCs for Japanese samples and per 10^{4} nucleated cells for Iranian samples by quantitative PCR.

†Two-tailed Mann–Whitney U test.

‡Japanese data were extracted from a database of previous analyses (Nagai *et al.*, 1998; Jeffery *et al.*, 1999, 2000).

Table 3. HLA-DRB1*0101 increases the odds of HAM/TSP development in Japanese and Iranian HLA-A*02-negative, but not in HLA-A*02-positive, HTLV-1-infected individuals

Population	Subjects	HAM/TSP (*n*)		HCs (*n*)		χ^2*	*P*	OR†	95% CI
		DRB1$^+$	DRB1$^-$	DRB1$^+$	DRB1$^-$				
Iranian	All	18	40	12	62	3·3	0·035	2·33	1·01–5·34
	A*02$^-$	13	24	9	45	3·1	0·038	2·71	1·01–7·24
	A*02$^+$	5	16	3	17	0·1	0·376	1·77	0·36–8·65
Japanese‡	All	34	161	20	163	2·8	0·049	1·72	0·95–3·12
	A*02$^-$	27	107	10	83	2·9	0·044	2·09	0·96–4·57
	A*02$^+$	7	54	10	80	0·005	0·47	1·03	0·37–2·89

*Reported as one-tailed with Yates correction.

†OR used the approximation of Woolf (1955).

‡Japanese data were extracted from a database of previous analyses (Jeffery *et al.*, 1999).

Table 4. HLA-DRB1*0101 associated with lower HTLV-1 provirus load in Japanese but not in Iranian HAM/TSP patients

The DRB1-positive Japanese HAM/TSP patients developed HAM/TSP with a significantly lower provirus load than DRB1-negative HAM/TSP patients, but this effect was not observed in Iranian HAM/TSP patients.

Population	HLA allele	HAM/TSP			HCs		
		Median provirus load*	No. subjects	*P*†	Median provirus load*	No. subjects	*P*†
Iranian	DRB1$^-$	193·0	40	0·31	115·0	62	0·34
	DRB1$^+$	357·0	18		104·0	12	
Japanese‡	DRB1$^-$	602·6	161	0·024	34·7	163	0·33
	DRB1$^+$	331·1	34		49·0	20	

*Provirus load is HTLV-1 *tax* copy number per 10^4 PBMCs for Japanese samples and per 10^4 nucleated cells for Iranian samples by quantitative PCR.

†*P* level reported using two-tailed Mann–Whitney U test.

‡Japanese data were extracted from the database of previous analyses (Nagai *et al.*, 1998; Jeffery *et al.*, 1999).

et al., 1995). A previous report indicated that the *tax* subgroup A was more frequently observed in HAM/TSP patients in the Kagoshima cohort and that this effect was independent of HLA-A*02 (Furukawa *et al.*, 2000). The higher HAM/TSP risk *tax* subgroup A corresponds to the cosmopolitan subtype A, and the lower HAM/TSP risk *tax* subgroup B corresponds to the cosmopolitan subtype B according to the LTR sequence (Furukawa *et al.*, 2000). We sequenced almost the entire *tax* region of HTLV-1 provirus (nt 7295–8356) from 20 different Iranian subjects (10 HAM/TSP and 10 HCs) by direct sequencing in both directions. As shown in Table 5, all Iranian HTLV-1 sequences (EMBL/GenBank/DDBJ accession no. AB181224) differed at 10 nt compared with the Japanese prototypic

Table 5. Nucleotide variations specific to Iranian HTLV-1

Amino acid changes in *tax* A resulting from the nucleotide substitution are shown. Nucleotide numbers correspond to those of the prototypic strain, ATK-1 (Seiki *et al.*, 1983). N, No change.

Subgroup	Nucleotide variation (nucleotide position and amino acid change)									
	7622 M→V	7811 I→V	7855 N	7897 N	7959 A→V	7991 N→H	8208 S→N	8313 G→E	8314 N	8344 N
ATK-1 (=*tax* B)	A	A	T	C	C	A	G	G	C	A
tax A				T	T		A			C
Iranian *tax*	G	G	C	T	T	C	A	A	G	C

Table 6. HLA-A*02 is associated with a lower risk of HAM/TSP development only in *tax* subgroup B subjects in Kagoshima

Japanese data were extracted from a database of previous analyses (Jeffery *et al.*, 1999; Furukawa *et al.*, 2000).

tax subgroup	HLA allele	HAM/TSP	HCs	χ^2*	*P*	OR†	95 % CI
tax A	HLA-A*02$^+$	16 (57 %)	6 (60 %)	0·047	0·829	0·89	0·20–3·87
	HLA-A*02$^-$	12 (43 %)	4 (40 %)				
tax B	HLA-A*02$^+$	51 (26 %)	81 (47 %)	15·5	<0·0001	0·41	0·26–0·63
	HLA-A*02$^-$	143 (74 %)	93 (53 %)				

*With Yates correction.
†OR used the approximation of Woolf (1955).

ATK-1 strain (Seiki *et al.*, 1983). Among these, nt 7897, 7959, 8208 and 8344 were exactly the same as those in *tax* subgroup A. In addition to these four residues, the Iranian *tax* sequences had 6 nt differences, which encoded four additional amino acid differences from Japanese *tax* subgroup A. We further performed PCR-RFLP analysis to determine the HTLV-1 *tax* subgroup (*tax* A or B) of all of the remaining Iranian samples and found that all Iranian HTLV-1 isolates had *tax* subgroup A substitutions.

HLA-A*02 is associated with a lower risk of HAM/TSP and a lower provirus load only in HTLV-1-infected individuals with *tax* subgroup B in Kagoshima subjects

As the majority of HTLV-1 isolates observed in the Kagoshima population were *tax* subgroup B, we examined further whether the effect of HLA-A*02 on the risk of HAM/TSP and HTLV-1 provirus load was observed only in HTLV-1 *tax* subgroup B-infected individuals in Kagoshima subjects. Japanese *tax* subgroup data were extracted from our existing database (Furukawa *et al.*, 2000). As shown in Table 6, the effects of HLA-A*02 on the risk of HAM/TSP and provirus load were not observed in HTLV-1 *tax* subgroup A-infected subjects in Kagoshima. We next sought a possible interaction between HLA-A*02 and HTLV-1 provirus load among HTLV-1 *tax* subgroup A-infected subjects in Kagoshima (Table 7). HLA-A*02 was associated with a lower provirus load only in the *tax* subgroup B subjects in Kagoshima, but not in the *tax* subgroup A subjects in either Japan or Iran.

DISCUSSION

Currently, several different approaches including family-based linkage and population-based case–control studies have been used to identify genetic susceptibility to numerous infectious pathogens such as malaria, mycobacteria, hepatitis viruses and human immunodeficiency virus (Hill, 1998). The candidate gene approach (case–control studies) can only utilize known genes and will not identify unknown genes, but genome-wide linkage studies have less power than candidate gene studies to pick up genes that have only a small or moderate effect on disease risk; therefore the two approaches are complementary. Although our Kagoshima cohort of HAM/TSP is the world's largest, only 300 HAM/TSP patients were available for analysis. Also, extensive studies in one ethnic population may not disclose the marker-disease distance or exclude a possible spurious association due to admixture. Studies in different ethnic populations may thus provide useful information about marker-disease distance, as well as confirming the reliability of results from our previous association studies. In this study, we compared the risk factors for developing HAM/TSP in two ethnic groups living in quite different environments, namely, Kagoshima in southwest Japan and Mashhad in northeast Iran. It is

Table 7. HLA-A*02 is associated with a lower provirus load only in *tax* subgroup B subjects in Kagoshima

Japanese data were extracted from a database of previous analyses (Nagai *et al.*, 1998; Jeffery *et al.*, 1999; Furukawa *et al.*, 2000).

tax subgroup	HLA allele	Provirus load (mean ± SE)*	Provirus load (median)*	No. subjects	*P*†
tax A	HLA-A*02$^+$	635·0 ± 169·3	389·0	22	0·98
	HLA-A*02$^-$	586·4 ± 164·9	356·5	16	
tax B	HLA-A*02$^+$	328·5 ± 41·6	99·0	132	0·0001
	HLA-A*02$^-$	520·0 ± 42·7	266·0	236	

*Provirus load is the HTLV-1 *tax* copy number per 10^4 PBMCs by quantitative PCR.
†Two-tailed Mann–Whitney U test.

almost certain a priori that there will be significant differences between populations in the genetic contribution to susceptibility to HAM/TSP, since HLA-B*5401 is prevalent in Japan and elsewhere in East Asian populations, but is virtually absent from many other populations. Since HLA-B*5401 has an important influence on the risk of disease in Kagoshima (Jeffery *et al.*, 2000), its presence in the population is certain to influence the risk associated with other HLA alleles, and the absence of HLA-B*5401 in other populations with endemic HTLV-1 infection will alter the relative importance of other genes to the risk of developing HAM/TSP.

We first examined the HTLV-1 provirus load in Iranian HAM/TSP patients and HCs, since one of the major risk factors for developing HAM/TSP is the provirus load (Nagai *et al.*, 1998). The median HTLV-1 provirus load of Iranian HAM/TSP patients was twofold greater in HAM/TSP patients than in HCs, whereas that of Japanese HAM/TSP patients was 13-fold greater than in HCs. Interestingly, despite differences in the methods of DNA extraction between the two study groups (whole blood-derived DNA for Iranian samples vs PBMC-derived DNA for Japanese samples), the HTLV-1 provirus load in Iranian HCs was still significantly higher than Japanese HCs ($P=0{\cdot}004$, Mann–Whitney U test). This may be the main cause of the smaller observed ratio of median provirus load between HAM/TSP patients and HCs in the Iranian study group. To investigate the reason for this difference between the two populations, we further analysed the frequencies of certain HLA alleles and the HTLV-1 *tax* subgroup in the Iranian population.

In the Kagoshima population, possession of either of the HLA class I genes HLA-A*02 or HLA-Cw*08 was associated with a statistically significant reduction in both HTLV-1 provirus load and the risk of HAM/TSP (Jeffery *et al.*, 1999, 2000). However, in Mashhadi Iranian subjects, both HLA-A*02 and HLA-Cw*08 had no effect on either the risk of HAM/TSP or provirus load. In contrast, HLA-DRB1*0101 was associated with increased susceptibility to HAM/TSP both in Kagoshima ($P=0{\cdot}049$) and Iran ($P=0{\cdot}035$). In HAM/TSP, $CD4^+$ cells are the predominant cells present early in the active lesions (Umehara *et al.*, 1993) and are also the HTLV-1-infected cells in the inflammatory spinal cord lesions (Moritoyo *et al.*, 1996). Moreover, HLA-DRB1*0101 restricts $CD4^+$ T-cell immunodominant epitopes of HTLV-1 *env* gp21 (Yamano *et al.*, 1997; Kitze *et al.*, 1998). Therefore, it is possible that HLA-DRB1*0101 is associated with susceptibility to HAM/TSP via an effect on $CD4^+$ T-cell activation and subsequent bystander damage in the central nervous system (Ijichi *et al.*, 1993; Bangham, 2000). However, since possession of HLA-DRB1*0101 was associated with a significantly lower provirus load in the Japanese HAM/TSP patients but not in the Iranian HAM/TSP patients, the underlying mechanism involving HLA-DRB1*0101 may not be the same between Iranian and Japanese HTLV-1-infected individuals. Differences in other genetic factors, including non-HLA genes, may also be important for explaining the observed differences between the populations.

Another possible explanation of the observed differences in the present study is that certain HLA genotypes are associated with different effects on different subtypes of the virus. In human papilloma virus (HPV) infection, the association of the DRB1*1501–DQB1*0602 haplotype with HPV-related cervical carcinoma was reported to be specific for the viral type HPV-16, suggesting that specific HLA haplotypes may influence the immune response to specific virus-encoded epitopes and affect the risk of viral disease (Apple *et al.*, 1994). To test this possibility, we sequenced almost the entire region of the *tax* gene in 20 Mashhad Iranian HTLV-1-infected individuals (10 HAM/TSP and 10 HCs) and compared the sequence with that of two Japanese strains, *tax* subgroups A and B. Although we could not identify any amino acid differences in the Tax11–19 immunodominant epitope between the Iranian and Japanese *tax* subgroups A and B, we found that Iranian HTLV-1 possessed 10 different nucleotides in the *tax* region compared with Japanese *tax* subgroup B. Among these, nt 7897, 7959, 8208 and 8344 were identical to *tax* subgroup A. Therefore, Iranian *tax* sequences have four additional different amino acids compared with Japanese *tax* subgroup A and six additional different amino acids compared with Japanese *tax* subgroup B. These findings suggest that both the lack of consistency of host genetic influences and the smaller difference in median provirus load between HAM/TSP patients and HCs in Iran may be due in part to different strains of HTLV-1. Our present observation that HLA-A*02 was associated with a lower provirus load only in the *tax* subgroup B-infected subjects in Kagoshima, but not in *tax* subgroup A-infected subjects, is consistent with this hypothesis. Further studies to examine functional differences between Iranian and Japanese HTLV-1 Tax proteins will provide important information to clarify this point.

The interaction between different genes and/or environmental factors is also likely to contribute to the observed differences between the two populations. For example, co-infection with *Strongyloides stercoralis* (Gabet *et al.*, 2000) can affect the HTLV-1 provirus load. In Japan, *S. stercoralis* infection is endemic in the southwestern islands Amami and Ryukyu, but is rarely reported on the mainland including Kagoshima (Arakaki *et al.*, 1992). However, there are no data on the prevalence of *S. stercoralis* infection in Mashhad, Iran, and therefore future epidemiological studies are necessary to clarify this possibility.

It seems likely that the same evolutionary selection pressures that induce polymorphisms in 'infection-resisting genes' have contributed to marked allele-frequency differences at the same loci. When geographical variation in pathogen polymorphism is superimposed on this host genetic heterogeneity, considerable variation in detectable allelic associations is likely to result in the different

populations. In other words, genetic resistance to infectious diseases that is formed by complex host genetic effects is complicated further by pathogen diversity and environmental factors. Considering this background of complexity, the most practical approach to finding reliable results may be first to identify disease-associated genes in a single large population, and secondly to analyse subsequently whether a similar effect is found in other ethnic populations, as we have shown in this study.

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ApaI polymorphism of vitamin D receptor gene is associated with susceptibility to HTLV-1-associated myelopathy/tropical spastic paraparesis in HTLV-1 infected individuals

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Abstract

HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is one outcome of human T-cell lymphotropic virus type-1 (HTLV-1) infection. It remains unknown why the majority of infected people remain healthy, whereas only approximately 2–3% of infected individuals develop the disease. The active form of vitamin D has immunomodulatory effects, and allelic variants of the vitamin D receptor (VDR) appear to be associated with differential susceptibility to several infectious diseases. To investigate whether VDR single nucleotide polymorphisms (SNPs) are associated with the development of HAM/TSP, we studied four VDR SNPs in a group of 207 HAM/TSP patients and 224 asymptomatic HTLV-1 seropositive carriers (HCs) in Kagoshima, Japan, by using PCR-RFLP analysis. We found that *ApaI* polymorphism of VDR is associated with the risk of HAM/TSP, although this polymorphism did not affect the provirus load of HTLV-1 in either HAM/TSP patients or HCs.

Keywords: Vitamin D receptor; Single nucleotide polymorphism; HAM/TSP; HTLV-1; Provirus load

1. Introduction

Human T-cell lymphotropic virus type-1 (HTLV-1) [1,2] infection is closely associated with a slowly progressive neurological disease called HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [3,4]. Infection with HTLV-1 is estimated to affect 10–20 million people worldwide [5]. However, only a minority of infected individuals develops HAM/TSP, by mechanisms incompletely understood [6]. Since it has been reported that the subtype of the viral transactivator Tax is associated with the risk of developing HAM/TSP [7], many other reported findings suggest that host factors are most important to determine the risk of HAM/TSP. Our case/control studies in Kagoshima strongly support this hypothesis. In the Kagoshima population, possession of the HLA-class I genes HLA-A*02 and Cw*08 was associated with a statistically significant reduction in both HTLV-1 provirus load and the risk of HAM/TSP, whereas possession of HLA-class I HLA-B*5401 and class II HLA-DRB1*0101 predispose to HAM/TSP [8,9]. Further analysis to look at non-HLA host genetic factors revealed that the TNF-α promoter-863 A allele predisposed to HAM/TSP, whereas SDF-1 +801A 3′ UTR, and IL-15 191 C alleles conferred protection against this disease

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[10], suggesting that non-HLA gene polymorphism also affects the risk for developing HAM/TSP.

It is well known that the active metabolite of vitamin D, 1,25-dihydroxyvitamin D3 (1,25-$[OH]_2D_3$), is involved in the maintenance of mineral homeostasis [11]. The effect of 1,25-$[OH]_2D_3$ is mediated by its receptor, which is the ligand-dependent transcription factor, and the vitamin D receptor (VDR) gene consists of nine exons with a number of polymorphisms [12]. To date, over 30 studies to test an association between polymorphisms of VDR and osteoporosis were reported, with about half confirming the association and the remainder failing to confirm [13]. Not only for mineral homeostasis, 1,25-$[OH]_2D_3$ is also implicated in the regulation of the immune system [11]. The immunomodulatory actions of the 1,25-$[OH]_2D_3$ are mediated by interaction with VDR, which is expressed in resting and activated lymphocytes [14]. Therefore, VDR may also interact to determine the risk of infectious diseases. Some studies have revealed a close association between VDR polymorphisms and the outcome of certain infectious diseases such as tuberculosis [15], hepatitis B virus (HBV) [15] and leprosy [16]. Most importantly, reports by Barber et al. and Nieto et al. showed that individuals with the VDR *BsmI* BB and *FokI* heterozygotes, respectively, were associated with rapid progression to AIDS among HIV-1 seropositive intravenous drug users [17,18], suggesting that particular polymorphisms in the VDR contribute to the host immune reaction against viral infection. Since the most commonly studied VDR polymorphisms include a *Fok*I restriction fragment-length polymorphism (RFLP) in exon 2 (alleles F/f or nucleotides C/T), *Bsm*I (B/b or nucleotides T/C) and *Apa*I (A/a or nucleotides T/G) variants in intron 8, and a *Taq* I (T/t or nucleotides T/C) variant in exon 9, with lowercase alleles indicating the presence of restriction sites, we genotyped these four SNPs and analyzed the effect of each SNP on the risk of HAM/TSP.

2. Patients and methods

2.1. Study population

The study population consisted of 207 patients with HAM/TSP and 224 asymptomatic HTLV-1 seropositive carriers (HCs), all residing in HTLV-1 endemic Kagoshima Prefecture in Southern Japan. The diagnosis of HAM/TSP was done in accordance with World Health Organization criteria [19]. Clinical characteristics of the patients are shown in Table 1. All samples were taken with the consent of the patients.

2.2. Isolation and cryopreservation of PBMCs and DNA extraction

Fresh peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation using a Histopaque-1077 instrument (Sigma, Tokyo, Japan) and washed three times with phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS). Isolated PBMCs were cryopreserved in liquid nitrogen until use. Genomic DNA was extracted from PMBCs using a QIAamp blood kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

2.3. PCR-RFLP analysis

Fifty nanograms of genomic DNA was amplified by PCR in a total volume of 25 μl using the primer and PCR conditions described by Harris et al. [20] for the *FokI*, by Wilkinson et al. [21] for the *TaqI* and *BsmI*, and Niino et al. [22] for the *ApaI* polymorphism of VDR. The primer sequences were as follows: 5′ -AGC TGG CCC TGG CAC TGA CTC TGC TCT-3′ and 5′ -ATG GAA ACA CCT TGC TTC TTC TCC CTC-3′ for *FokI*; 5′ -GGG ACG ATG AGG GAT GGA CAG AGC-3′ and 5′ -GGA AAG GGG TTA GGT TGG ACA GGA-3′ for *TaqI*; 5′ -AAC TTG CAT GAG GAG GAG CAT GTC-3′ and 5′ -GGA GAG GAG CCT CTG TCC CAT TTG-3′ for *BsmI*; 5′ -GTC GCT GAG GGA TGG-3′ and 5′ -GTC GGC TAG CTT CTG GAT-3′ for *ApaI*. After PCR amplification, the 15 μl of PCR product was digested for 12 h with an excess of restriction enzyme. Finally, digested PCR products were electrophoresed through a 2% agarose gel and visualized with ethidium bromide.

2.4. Quantification of HTLV-1 provirus load, CSF neopterin and anti-HTLV-1 antibody titers

To examine the HTLV-1 provirus load, we carried out a quantitative PCR method using ABI Prism 7700™ (PE-

Table 1
Clinical characteristics of HAM/TSP patients and asymptomatic HTLV-1 carriers (HCs)

	Age	Male/female	Anti-HTLV-1 antibodies[a]	HTLV-1 provirus load[b]	Neopterin in CSF[c]
HAM/TSP (n=207)	57.0±12.1[d]	62/145	×26,458±41,433	719.3±709.2	112.7±112.6
HCs (n=224)	39.8±13.1	109/115	×1478±1453	131.9±243.0	N/A

N/A: not applicable.
HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis.
HCs: asymptomatic HTLV-1 seropositive carriers.
[a] Anti-HTLV-1 antibodies were titrated by the particle agglutination method. 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.
[b] Tax copy number per 1×10^4 PBMCs.
[c] Neopterin levels were evaluated by HPLC with fluorometric detection methods.
[d] The values are shown as the mean±S.D.

Applied Biosystems) with 100 ng of genomic DNA (roughly equivalent to 10^4 cells) from PBMC samples as reported previously [23]. 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 (pX) per 1×10^4 PBMC=[(copy number of pX)/(copy number of β-actin/2)]$\times10^4$. All samples were performed in triplicate. Neopterin levels were evaluated by high-performance liquid chromatography (HPLC) with fluorimetric detection methods [24]. Serum and CSF antibody titers to HTLV-1 were determined by a particle agglutination method (Serodia-HTLV-1®, Fujirebio). 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.

2.5. Statistical analysis

Comparisons of genotype frequency between HAM/TSP patients and HCs were calculated by the chi-squared test. For multiple comparisons of the HTLV-1 provirus load measured in HAM/TSP and HCs individuals, subdivided according to their *ApaI* genotype, we used one-factor ANOVA when variance of each group was equal by Bartlett test. If variance of each group was different, Kruskal–Wallis test was employed. Linkage disequilibrium (LD) was calculated between SNPs as previously described [25] by using SNPAlyze software ver. 3.2 (Dynacom, Mobara, Japan), which provides a *D* prime (*D′*) value between 0 and 1. A *D′* value of 0 indicates no LD between the two markers and a *D′* value of 1 indicates complete LD between two markers. Significance was considered at $p<0.05$.

3. Results

3.1. Vitamin D receptor gene polymorphism in HAM/TSP patients and asymptomatic HTLV-1 carriers

Initially, we genotyped 118 patients with HAM/TSP and 129 HCs for each SNP. There were no significant differences in the distribution of the *FokI*, *TaqI* and *BsmI* genotypes and allele frequencies between 118 HAM/TSP patients and 129 HCs (Table 2). In contrast, the *ApaI* genotypes (AA, Aa, aa) showed a significant difference in frequency ($\chi^2=8.04$ on 2 *df*, $p=0.018$). We therefore further analyzed a total of 207 cases of HAM/TSP and 224 HCs for *ApaI* polymorphism and identified a significant association between AA genotype and reduced risk of HAM/TSP ($\chi^2=10.48$ on 2 *df*, $p=0.0012$, Odds ratio=0.28, 95%CI=0.13–0.63). In both HAM/TSP patients and HCs, the genotype frequencies were distributed according to the Hardy–Weinberg equilibrium. Previously reported allele and genotype frequencies of *ApaI* polymorphism from a Japanese normal control population showed similar results with the HCs group of our present study [22].

3.2. AA genotype is associated with a lower risk for HAM/TSP only in female gender and FokI heterozygotes

Interestingly, the protective effect of *ApaI* AA genotype was observed only in the female subjects (72 HAM/TSP patients and 59 HCs, $\chi^2=7.11$ on 2 *df*, $p=0.029$) but not in the male subjects (38 HAM/TSP patients and 50 HCs, $\chi^2=4.31$ on 2 *df*, $p=0.116$). Because a series of the three polymorphisms (*BsmI*, *ApaI* and *TaqI* SNPs) in the 3′

Table 2
Frequencies of genotypes and alleles for the different polymorphisms in the vitamin D receptor gene

SNP	Allele	HAM/TSP	HCs	*P* value[a]	Genotype	HAM/TSP	HCs	*P* value[b]
ApaI	A	105 (25.4)[c]	143 (31.9)	0.034*	AA	8 (3.9)	28 (12.5)	0.0053*
	a	309 (74.6)	305 (68.1)		Aa	89 (43.0)	87 (38.8)	
					aa	110 (53.1)	109 (48.7)	
	total	414	448		total	207	224	
FokI	F	141 (59.7)	157 (60.9)	0.80	FF	44 (37.3)	50 (38.8)	0.97
	f	95 (40.3)	101 (39.1)		Ff	53 (44.9)	57 (44.2)	
					ff	21 (17.8)	22 (17.1)	
	total	236	258		total	118	129	
BsmI	B	28 (11.9)	32 (12.4)	0.85	BB	2 (1.7)	1 (0.8)	0.70
	b	208 (88.1)	226 (87.6)		Bb	24 (20.3)	30 (23.3)	
					bb	92 (78.0)	98 (76.0)	
	total	236	258		total	118	129	
TaqI	T	208 (88.1)	228 (88.4)	0.93	TT	92 (78.0)	100 (77.5)	0.78
	t	28 (11.9)	30 (11.6)		Tt	24 (20.3)	28 (21.7)	
					tt	2 (1.7)	1 (0.8)	
	total	236	258		total	118	129	

HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis.
HCs: asymptomatic HTLV-1 seropositive carriers.
[a] *P* values are calculated by χ^2-test with 2×2 contingency table.
[b] *P* values are calculated by χ^2-test with 2×3 contingency table.
[c] Numbers in parentheses are percentage.
* $P<0.05$.

Table 3
Linkage disequilibrium (LD) between four vitamin D receptor polymorphisms in HTLV-1 infected subjects

VDR SNP	*FokI*-HAM	*BsmI*-HAM	*TaqI*-HAM
ApaI-HAM	0.23444	**0.93886**	**1.0000**
	FokI-HAM	0.58781	0.44849
		BsmI-HAM	**0.91828**
			TaqI-HAM
ApaI-HCs	0.05065	**0.87419**	**0.93298**
	FokI-HCs	0.59933	0.41685
		BsmI-HCs	**0.88339**
			TaqI-HCs
ApaI-All	0.03603	**0.90706**	**0.96805**
	FokI-All	0.59305	0.43561
		BsmI-All	**0.90042**
			TaqI-All

HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis.
HCs: asymptomatic HTLV-1 seropositive carriers.
LD was calculated between SNPs, which provides a *D* prime (*D'*) value between 0 and 1.
D' values between two SNPs in each group were shown.
No LD=0, complete LD=1, strong LD=0.7–1.0 (values in bold).

UTR of the VDR gene have been shown to be in strong LD with one another in Western countries [26], we analyzed the presence of the LD in our subjects. As a result, the *BsmI*, *ApaI* and *TaqI* polymorphisms are in strong LD whereas the *FokI* polymorphism at the translation initiation site in exon2 of the VDR gene was not in significant linkage with the other polymorphisms (Table 3). Since previous report by Nieto et al. showed clear association between combined genotypes for *FokI* and *BsmI* polymorphisms and a faster progression to AIDS among HIV-1 seropositive intravenous drug users, despite no significant linkage between two polymorphisms in their population [18], we also tested whether the *FokI* genotype affect the observed protective effect of *ApaI* genotype against HAM/TSP development. When we analyzed the distribution of the *ApaI* polymorphism in the 274 HTLV-1 infected individuals with the non-Ff genotype (homozygous FF and ff), we observed that there were no significant differences in the distribution of the *ApaI* genotypes and allele frequencies between 130 HAM/TSP patients and 144 HCs. In contrast, in individuals with the Ff genotype (heterozygous, 53 HAM/TSP patients and 57 HCs), the risk ratios for progression to HAM/TSP in HTLV-1 infected individuals without AA genotype (Aa, aa and Aa+aa) was significantly higher than HTLV-1 infected individuals with AA genotype (Table 4). Therefore, the protective effect of *ApaI* AA genotype was observed only in the *FokI* heterozygotes (Ff) but not in homozygotes (FF and ff).

3.3. *Vitamin D receptor gene ApaI polymorphism is not a significant predictor of the HTLV-1 provirus load in HAM/TSP patients and asymptomatic HTLV-1 carriers*

To test whether VDR gene *ApaI* polymorphism is a significant predictor of the HTLV-1 provirus load, we measured the provirus load of HTLV-1 and compared it with *ApaI* genotypes (AA, Aa, aa) in HAM/TSP patients and HCs. Our data indicated that there was no association between *ApaI* genotypes and HTLV-1 provirus load (Table 5), CSF neopterin levels, as well as serum HTLV-1 antibody

Table 4
Vitamin D receptor *ApaI* allele/genotype frequencies in relation to *FokI* genotypes in HTLV-1 infected individuals

FokI genotype	*ApaI* allele	HAM/TSP	HCs	OR (95% CI)	*P* value
Homozygous (FF and ff)	A	41 (31.5)	49 (34.0)	0.89 (0.54–1.48)	0.66
	a	89 (68.5)	95 (66.0)	1.12 (0.68–1.86)	0.66
	total	130	144		
Heterozygous (Ff)	A	25 (23.6)	37 (32.5)	0.64 (0.35–1.17)	0.14
	a	81 (76.4)	77 (67.5)	1.56 (0.86–2.83)	0.14
	total	106	114		
FokI genotype	*ApaI* genotype	HAM/TSP	HCs	OR (95% CI)	*P* value
Homozygous (FF and ff)	AA	3 (4.6)	7 (9.7)	1 (reference)	
	Aa	35 (53.8)	35 (48.6)	2.33 (0.56–9.76)	0.24
	aa	27 (41.5)	30 (41.7)	2.10 (0.49–8.94)	0.31
	Aa+aa	62 (95.4)	65 (90.3)	2.23 (0.55–8.99)	0.25
	total	65	72		
Heterozygous (Ff)	AA	1 (1.9)	10 (17.5)	1 (reference)	
	Aa	23 (43.4)	17 (29.8)	13.53 (1.58–116.0)	0.0044*
	aa	29 (54.7)	30 (52.6)	9.67 (1.16–80.4)	0.014*
	Aa+aa	52 (98.1)	47 (82.5)	11.06 (1.36–89.7)	0.0062*
	total	53	57		

HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis.
HCs: asymptomatic HTLV-1 seropositive carriers.
Numbers in parentheses are percentage.
OR: odds ratio; 95% CI: 95% confidence interval.
* $P<0.05$.

Table 5
Vitamin D receptor *ApaI* polymorphism and HTLV-1 provirus load

	AA	Aa	aa	*P* value
HAM (207)	541.6±222.2 (8)	745.8±79.6 (89)	710.7±65.2 (110)	0.727[a]
HCs (224)	103.8±32.4 (28)	129.7±31.5 (87)	140.9±20.4 (109)	0.799[b]
All patients combined (431)	201.1±61.4 (36)	441.3±48.9 (176)	427.1±39.3 (219)	0.718[b]

HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis.
HCs: asymptomatic HTLV-1 seropositive carriers.
The values are shown as the mean tax value (tax copies/10^4 PBMCs)±S.E.
[a] *P* value by one-factor ANOVA.
[b] *P* value by Kruskal–Wallis test.

titers (data not shown) in our population. Also, the clinical course and disability of HAM/TSP were not specifically associated with the polymorphism and serum VEGF levels in HAM/TSP patients (data not shown).

4. Discussion

HTLV-1 infection is of particular interest to the field of immunology as well as neurology because it persists at a remarkably high level despite a vigorous cellular and humoral immune response and causes inflammatory demyelinating disease only in a minority of infected people. The outcome of HTLV-1 infection depends on both host genetic and viral factors. However, although different virus strains (denoted HTLV-1 subgroups) can influence the risk of developing HAM/TSP [7], the impact of HTLV-1 viral sequence variation in determining the risk of developing HAM/TSP in Kagoshima is not sufficient to predict disease. Our recent observations, as well as many reported findings, strongly suggest that the outcome of HTLV-1 infection mainly depends upon a host of genetic factors [27].

In addition to its role in calcium and skeletal homeostasis, 1,25-$[OH]_2D_3$ plays an important role in immune system modulation [11]. The 1,25-$[OH]_2D_3$ promotes monocyte differentiation, stimulates cell-mediated immunity, and inhibits lymphocyte proliferation and secretion of cytokines such as interleukin (IL)-2, granulocyte-macrophage colony-stimulating factor and interferon-γ from T cells, and IL-12 from macrophages and B cells [28–31]. The inhibiting effect of vitamin D on the immune response appears to target Th1 cells by preventing their activation and cytokine production [28]. Interestingly, previous studies indicated that the Th1 cell response is predominant in HAM/TSP [32,33] and 1,25-$[OH]_2D_3$ has the potential to suppress cell proliferation through binding to the VDR overexpressed in HTLV-1 infected T cells [34,35]. Therefore, it is interesting to test whether VDR gene polymorphism is associated with the risk for developing HAM/TSP.

In the present study, the *ApaI* polymorphism of VDR showed a significant difference in allele frequency, and AA genotype was associated with the reduced risk of HAM/TSP in HTLV-1 infected individuals, whereas there were no significant differences in the distribution of the *FokI*, *TaqI* and *BsmI* genotypes and allele frequencies. Interestingly, the protective effect of *ApaI* AA genotype was observed only in females, which is one of the risk factor for developing HAM/TSP. The HTLV-1 provirus load of female patients with HAM/TSP was significantly higher than that of male patients [23] and the ratio of male to female HAM/TSP patients is about 1:2, as shown in the present study. However, there was no significant difference between any VDR genotypes and HTLV-1 provirus load in either HAM/TSP patients or HCs. Also, there were no correlations between CSF neopterin levels or serum anti-HTLV-1 antibody titers. Furthermore, the clinical course and disability of HAM/TSP were not associated with any VDR polymorphisms in HAM/TSP patients. This was strikingly different from the HLA-A*02 [8] and Cw*08 [9], which were associated with both the risk of HAM/TSP and lower provirus load in HCs. Since *ApaI* polymorphism is located in intron 8 and is not affecting any splicing site and/or transcription factor binding site [36], it is unlikely that this polymorphism is directly associated with the functional difference of VDR itself. Linkage disequilibrium with truly functional polymorphism elsewhere in the VDR gene or other gene(s) may be associated with the susceptibility to HAM/TSP via gender-specific mechanism other than an apparent effect on provirus load. In our population, significant LD was found among the *BsmI*, *ApaI* and *TaqI* polymorphisms located in the 3′ UTR of the VDR gene, but *FokI* polymorphism was not in LD with these three polymorphisms. However, the *FokI* genotype affects the observed protective effect of *ApaI* genotype against HAM/TSP, as observed in HIV-1 infected intravenous drug users for progression to AIDS [18]. In HTLV-1 infected individuals with the *FokI* Ff genotype, the risk ratios for progression to HAM/TSP without *ApaI* AA genotype (Aa, aa and Aa+aa) was significantly higher than HTLV-1 infected individuals with AA genotype, whereas there were no significant differences in the distribution of the *ApaI* genotypes and allele frequencies between 130 HAM/TSP patients and 144 HCs which were non-Ff genotype (homozygous FF and ff). These findings provide strong evidence to suggest that genetic variations at the VDR locus may affect the outcome of HTLV-1 infection. Recent transmission-disequilibrium test on family-based study also showed a significant association of tuberculosis with SNP combinations *FokI–ApaI* by the increased transmission to affected offspring [37]. It is possible that the presence of unidentified, associated functional alleles that lies on this haplotype background influence the susceptibility to HAM/TSP, although further studies are needed to clarify this point.

In conclusion, our results indicate that VDR *ApaI* polymorphism is a novel non-HLA host genetic factor to evaluate the risk of HAM/TSP. The functional significance

of this observation may reveal immunotherapeutic strategies that would retard the development of HAM/TSP in the future.

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A prospective uncontrolled trial of fermented milk drink containing viable *Lactobacillus casei* strain Shirota in the treatment of HTLV-1 associated myelopathy/tropical spastic paraparesis

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Abstract

Ten patients with human T-cell lymphotropic virus type-1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) were treated in an uncontrolled preliminary trial by oral administration of viable *Lactobacillus casei* strain Shirota (LcS) containing fermented milk. HTLV-1 provirus load, motor function, neurological findings, and immunological parameters were evaluated after 4 weeks. Although LcS did not change the frequencies or absolute numbers of all the examined cell surface phenotypes of peripheral blood mononuclear cells, NK cell activity was significantly increased after 4 weeks of oral administration of LcS preparation. Improvements in spasticity (modified Ashworth Scale scores) and urinary symptoms were also seen after LcS treatment. No adverse effect was observed in all the 10 patients throughout the study period. Our results indicated that LcS may be a safe and beneficial agent for the treatment of HAM/TSP; therefore randomized controlled studies are warranted.

Keywords: Immunomodulation; *Lactobacillus casei* strain Shirota; NK cell activity; HAM/TSP; HTLV-1

1. Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities [1–3]. Although the precise mechanism causing HAM/TSP is still obscure, virus–host immunological interactions are considered to be the most important cause of this disease, because (1) the median HTLV-1 provirus load is more than 10 times higher in HAM/TSP patients than in healthy HTLV-1 carriers (HCs), and high provirus load is also associated with an increased risk of progression to disease [4]; (2) the titer of anti-HTLV-1 antibody often reaches a very high level in HAM/TSP patients, and correlates well with a HTLV-1 provirus load [4]; (3) in HAM/TSP patients, large populations of activated T cells both in peripheral blood mononuclear cells (PBMCs) [5] and cerebrospinal fluid (CSF) [6], and spontaneous proliferation of PBMCs in vitro [7–9] have been reported; (4) HTLV-1-specific $CD8^+$ cytotoxic T lymphocytes (CTL) are abundant and activated in PBMCs in HAM/TSP patients [10], and these CTLs are preferentially accumulated in CSF cells [11,12]; and (5) it

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has been shown that HTLV-1 Tax_{11-19}-specific $CD8^+$ T cells have the potential to produce proinflammatory cytokines [13], whereas possession of the HLA-A*02 allele was associated with protection against HAM/TSP as well as a lower provirus load [14]. To regulate such immunologic processes, some therapeutic trials with agents such as prednisolone [15,16], plasma exchange [17], and interferon (IFN)-α [18–22], have been conducted and reported to be effective.

Lactobacillus casei strain Shirota (LcS), a type of lactic acid bacteria, was originally isolated from the human intestine and has been used commercially for a long time to produce fermented milk [23]. It was previously reported that LcS activates macrophages [24], NK cells [25], and cytotoxic T cells [26], and also exhibits a marked inhibitory effect against tumors [23,27], type II collagen-induced arthritis in DBA/1 mice [28], and infectious pathogens such as *Listeria monocytogenes* [29], herpes simplex virus [30], and influenza virus [31]. Adverse effects and toxic reactions to LcS are rarely reported. On the other hand, previous reports indicated that the NK cell activity and the percentages of NK cell subsets was significantly decreased in HAM/TSP patient compared with that in uninfected normal controls [32,33]. In our previous study, we also reported that one of the inhibitory NK cell receptor, NKG2A, was significantly decreased in frequency in HAM/TSP patients but not in HCs [34]. Since inhibitory NK cell receptors such as NKG2A can down-regulate antigen-mediated T-cell effector functions including cytotoxic activity and cytokine release, decreased NK cell function and NKG2A+ T cells may be associated with impaired regulation of T cell-mediated antiviral immune responses in HAM/TSP patients. These observations therefore raise the possibility that LcS could improve the symptoms of HAM/TSP via activating NK cell subsets.

To conduct a preliminary investigation of the efficacy of LcS for HAM/TSP–and to see if controlled trials are warranted–we evaluated in an uncontrolled trial with 10 HAM/TSP patients. After daily oral administration of LcS preparation that contains approximately 8×10^{10} viable LcS for 4 weeks, the effects of spasticity, motor disabilities, urinary symptoms and immunological parameters were evaluated.

2. Patients and methods

2.1. Study population

Our study constituted a prospective open evaluation of LcS over a 4-week period. Ten patients with clinical diagnoses of HAM/TSP in accordance with World Health Organization criteria [35] were enrolled in this study. All patients gave informed consent.

2.2. Patients and cells

Clinical characteristics of the patients are shown in Table 1. The duration of illness ranged from 7 to 24 years.

Table 1
Summary of demographic and clinical characteristics for 10 patients with human T lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP)

Patient	Age, years	Sex	Disease duration, years	HTLV-1 antibody titer	Provirus load before Tx	Provirus load after Tx[a]	Concomitant Tx[b]	MAS before Tx	MAS after Tx[c]	OMDS before Tx	OMDS after Tx[d]	UDS before Tx	UDS after Tx[e]	Overall evaluation of efficacy[f]
HAM1	34	F	19	×131072	1757	1397	VC 375mg/day	2	1	4	3	2	0	good
HAM2	62	M	14	×32768	634	777	PSL 5mg/day, VC 375mg/day	+1	0	6	6	3	0	good
HAM3	50	F	17	×2048	907	779	None	2	1	5	4	3	0	good
HAM4	45	F	15	×16384	2942	471	PSL 5mg/day, VC 375mg/day	+1	0	5	3	6	3	excellent
HAM5	60	F	7	×8192	204	194	PSL 5mg/day, VC 375mg/day	3	4	4	4	1	0	fair
HAM6	47	M	18	×2048	716	849	PSL 10mg/day, VC 375mg/day	2	1	6	6	5	4	fair
HAM7	46	M	24	×16384	278	361	None	3	1	4	3	2	0	good
HAM8	55	F	10	×65536	2882	524	None	2	1	4	3	6	3	good
HAM9	41	F	17	×65536	1073	1263	None	3	0	2	2	3	1	good
HAM10	57	F	13	×32768	245	387	None	2	1	4	4	2	1	Fair

LcS, *Lactobacillus casei* strain Shirota; OMDS, Osame Motor Disability Score; UDS, urinary disturbance score; MAS, modified Ashworth scale.
[a] At 4 weeks after the first administration of LcS; for the changes in HTLV-1 provirus load, $p=0.401$, by the Wilcoxon signed rank test.
[b] VC, vitamin C; PSL, predonisolone.
[c] At 4 weeks after the first administration of LcS; for the change in MAS, $p=0.015$, by the Wilcoxon signed rank test.
[d] At 4 weeks after the first administration of LcS; for the change in OMDS, $p=0.157$, by the Wilcoxon signed rank test.
[e] At 4 weeks after the first administration of LcS; for the change in UDS, $p=0.0085$, by the Wilcoxon signed rank test.
[f] The evaluation was mainly based on improvement in motor dysfunction, urinary disturbances, and neurologic signs, as judged by the attending neurologists (see Patients and methods).

In these patients, motor disability scores rated from 0 to 13 (as described in Table 2), and ranged from 2 to 6 (Table 1). Fresh PBMCs were isolated on a Histopaque-1077 (Sigma) density gradient centrifugation, washed twice in RPMI 1640 with 5% heat-inactivated fetal calf serum (FCS), then stored in liquid nitrogen until use.

2.3. Treatment protocol

All patients were given LcS preparation, which contains approximately 4×10^{10} viable LcS, orally twice a day for 4 weeks. The drugs other than LcS such as muscle relaxants or vitamins were continued on the condition that the dosage regimen was kept constant before, during, and after treatment. Four patients (HAM2, HAM4, HAM5 and HAM6) received low-dose (5–10 mg daily) oral prednisolone throughout the study period. None of the patients—with the exception of these four—had received any immunomodulators.

2.4. Concomitant therapy

The drugs other than LcS such as vitamin C or low dose (5–10 mg daily) oral *prednisolone* were continued on condition that the dosage regimen was kept constant before, during, and after therapy. Four patients (HAM2, HAM4, HAM5 and HAM6) out of 10 had received low dose oral prednisolone throughout study period. None of the patients except for these four had received any immunomodulators (Table 1).

2.5. Clinical evaluation

Assessments were performed on subjective symptoms, degree of spasticity, disability grading, sphincter disturbance and adverse effects before and 1 month after administration of LcS. The clinical findings of each patient were assessed by the blinded consultant neurologists, i.e. the examining neurologist for post-clinical scores was blinded to the pre-clinical scores. Spasticity of the lower limbs was graded with the modified Ashworth scale (MAS) [36]. Motor dysfunction was evaluated based on the Osame Motor Disability Score (OMDS), which grades motor dysfunction from 0 (normal walking and running) to 13 (complete bedridden) (see Table 2). The severity of the three main symptoms of urinary disturbance, i.e. increased frequency of urination, feeling of residual urine, and urinary incontinence were scored using the following scale: 0=normal; 1=slight; 2=moderate; 3=severe. The urinary disturbance score (UDS) represents the sum of all three symptoms. An overall evaluation of efficacy (improvement of dysfunction) based mainly on the improvement of motor dysfunction, as well as changes in urinary disturbances and neurological signs, was also evaluated according to the following scale, which had been previously used in the multicenter, randomized, double blind, and controlled study for IFN-α [21]: (1) excellent: patients showing two or more grades of improvement on the OMDS; (2) good: patients with one grade of improvement on the OMDS, or patients with an improvement of motor dysfunction with no change on the OMDS, but apparent improvement on more than two items other than motor dysfunction; (3) fair: patients exhibiting an improvement of motor dysfunction with no change on the OMDS, or patients with no improvement in motor dysfunction but with an improvement on one or more of the other items examined; (4) poor: patients with no improvement; and (5) none: patients with continuing clinical deterioration. In this study, the entire HTLV-1 provirus load data was measured after 4 weeks of LcS administration were completed, by an investigator who was not involved in the patients' clinical care. Clinical neurologists did not have access to provirus load data.

Table 2
Osame Motor Disability Score (OMDS) for HAM/TSP

Grade	Disability
0	Normal gait and running
1	Normal gait but runs slow
2	Abnormal gait (staggering or spastic)
3	Abnormal gait and unable to run
4	Needs support (handrails) while using stairs but limited to 10 m
5	Needs one-hand support in walking
6	Needs two-hand support in walking
7	Needs two-hand support in walking but is limited to 10 m
8	Needs two-hand support in walking but limited to 5 m
9	Unable to walk even with support but can crawl in hands and knees
10	Crawls with hands
11	Unable to crawl but can turn sideways in bed
12	Unable to crawl but can move the toes
13	Completely bedridden

2.6. Lymphocytes phenotyping by flow cytometric analysis

After thawing, cells were washed three times with phosphate-buffered saline (PBS) and fixed in a PBS containing 2% paraformaldehyde (Sigma) for 20 min, and then resuspended in PBS at 4 °C. Fixed cells were washed with PBS containing 7% of normal goat serum (Sigma). Cells were then incubated for 15 min at room temperature with various combinations of fluorescence-conjugated monoclonal antibodies (mAb) as follows: fluorescein isothiocyanate (FITC)-labeled anti-CD27 (M-T271; Pharmingen, CA), FITC-labeled anti-CD3 (UCHT1; Beckman Coulter, CA), FITC-labeled anti-pan γδ T cell (Immu 510; Beckman Coulter, CA), Cy-Chrome-labeled anti-CD45RA (HI100; Pharmingen), phycoerythrin (PE)-labeled anti-CXCR3 (1C6/CXCR3; Pharmingen), PE-labeled anti-CD16 (3G8; Beckman Coulter), PE-labeled anti-NKG2A (Z199; Beckman Coulter), phycoerythrin-Cy5

(PC5)-labeled anti-CD4 (13B8.2; Beckman Coulter), PC5-labeled anti-CD56 (N901; Beckman Coulter), and energy-coupled dye (ECD)-labeled anti-CD8 (SFCI21ThyD3; Beckman Coulter). Isotype-matched mouse immunoglobulins were used as a control. The phenotype was determined by flow cytometry (EPICS® XL; Beckman Coulter, Tokyo, Japan) in the lymphocyte gate, based on forward versus side scatter.

2.7. NK cell-mediated cytotoxic assays

The erythroleukemia cell line K562 was maintained in RPMI 1640 medium, and supplemented with 10% FBS. Target cells (1×10^6 viable K562 cells) were labeled with $Na_2^{51}CrO_4$ (100 μCi) for 1 h at 37 °C, washed, and resuspended at a concentration of 5×10^4 cells/ml in a culture medium. Effectors and labeled targets were incubated in triplicates in 0.2 ml volume at E/T ratio of 50:1 in round-bottom 96-well plates, and incubated for 4 h. Percentage of lysis was determined by the following formula: % specific lysis $= 100 \times (\text{exp} - \text{spont})/(\text{max} - \text{spont})$, where experimental (exp) release represents the radioactivity from the experimental wells, maximum (max) release represents counts from detergent-lysed targets, and spontaneous (spont) release represents background release from wells with targets alone.

2.8. Quantification of HTLV-1 provirus load and anti-HTLV-1 antibody titers

To examine the HTLV-1 provirus load, we carried out a quantitative PCR using ABI Prism 7700™ (PE-Applied Biosystems) with 100 ng of genomic DNA (roughly equivalent to 10^4 cells) from PBMCs as reported previously [12]. We chose the pX gene of HTLV-1, which has four open reading frames and encodes the unique regulatory and accessory proteins, for quantitative PCR because pX gene is retained by defective proviruses that are sometimes present in the infected individuals. Using β-actin as an internal control, the amount of HTLV-1 proviral DNA was calculated through the following formula: copy number of HTLV-1 (pX) per 1×10^4 PBMC $= [(\text{copy number of pX})/(\text{copy number of β-actin}/2)] \times 10^4$. All samples were performed in triplicate. Serum and CSF antibody titers to HTLV-1 were determined by a particle agglutination method (Serodia-HTLV-1®, Fujirebio).

2.9. Statistical analysis

We made a paired comparison of changes in T cell phenotypes, HTLV-1 provirus load, NK cell activity, OMDS, and UDS scores between LcS treatments (before and 4 weeks after LcS treatment) by using a Wilcoxon signed rank test. The results represent the mean ± standard deviation (S.D.) where applicable. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Clinical improvement of HAM/TSP with LcS treatment

We studied 10 HAM/TSP patients who received a LcS preparation, which contains approximately 4×10^{10} viable LcS, orally once a day for 4 weeks. All 10 patients showed the rate of excellent to fair response by overall evaluation of efficacy (see Patients and methods). Motor dysfunction (OMDS) was improved after treatment in 5 out of 10 patients ($p = 0.157$, Wilcoxon signed rank test). One patient (HAM 4) who had needed a walking cane before treatment became able to walk without assistance after treatment. Urinary symptoms (UDS) were improved in all 10 patients ($p = 0.0085$, Wilcoxon signed rank test; Table 1). Nine out of 9 patients showed decreased spasticity on neurological examination ($p = 0.015$, Wilcoxon signed rank test; Table 1). No adverse effect and laboratory findings were observed. The concomitant administration of prednisolone was not associated with the specific clinical outcome against LcS treatment (Table 1).

3.2. Laboratory findings

Simultaneous staining with CD45RA and CD27 mAbs has been demonstrated to identify subpopulations of human $CD8^{high+}$ T cells that were distinct for both phenotypic and functional properties [37]. From this staining, unprimed naïve T cells were defined as $CD45RA^+CD27^+$, antigen-experienced effector T cells that parallel with CTLs were defined as $CD45RA^+CD27^-$, and memory T cells were defined as $CD45RA^-CD27^+$. The $CD45RA^-CD27^-$ population was found to contain both effector and memory type T cells. In accordance with this phenotype, $CD45RA^+CD27^-$–but not $CD45RA^-CD27^+$–T cells have cytolytic activity without previous in vitro stimulation [37]. Our data have shown that the frequency as well as the absolute number of all the T cell phenotypes examined were not significantly changed in patients who received 4 weeks of daily oral LcS administration (Table 3). Interestingly, although the frequency as well as the absolute number of $CD16^+CD56^+/CD3^-$ NK cells and inhibitory NK receptor $NKG2A^+$ cells, which was a selectively decreased on T cells in HAM/TSP patients [34], showed no significant change after 4 weeks of LcS treatment, NK cell activity, which was reported to be decreased in HAM/TSP patients [32,33], was significantly increased after LcS treatment (Table 3). However, the concomitant administration of prednisolone was not associated with specific changes of NK cell activity against LcS administration (Fig. 1).

3.3. Provirus load of HTLV-1

The change in the HTLV-1 provirus load was measured by competitive PCR before and 4 weeks after LcS administration. The copy number of HTLV-1 per 1×10^4 PBMCs was not significantly changed after 4 weeks of LcS

Table 3
Absolute number, frequency of cell subsets and HTLV-1 provirus copy number in peripheral blood mononuclear cells (PBMCs) from patients with human T lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) who received *Lactobacillus casei* strain Shirota daily for 4 weeks

Cell type	Before treatment		After treatment		P^a	
	Absolute count, cells × 10^2/mm^3	Frequency, %	Absolute count, cells × 10^2/mm^3	Frequency, %	Absolute count	Frequency
$CD4^+$	5.79±4.54	26.38±12.71	5.96±3.16	27.13±11.09	0.674	0.575
$CD8^{high+}$	3.89±1.61	20.47±6.93	4.72±2.02	22.60±9.01	0.208	0.327
Naive in $CD8^{high+}$	1.13±1.71	5.24±7.11	0.93±1.06	4.25±4.08	1.000	0.674
Memory in $CD8^{high+}$	4.84±2.60	24.05±6.44	5.25±2.13	24.47±7.37	0.401	0.889
Effector in $CD8^{high+}$	7.56±4.46	37.35±10.81	7.57±2.56	36.10±9.93	1.000	0.674
Effector/memory in $CD8^{high+}$	5.99±2.36	33.36±14.54	7.21±3.47	34.07±13.78	0.263	0.889
$CXCR3^+$	4.38±2.38	21.07±4.28	3.93±1.16	18.78±6.57	0.735	0.398
$CXCR3^+$ in $CD4^+$	2.03±2.13	8.26±5.06	1.60±0.75	7.46±3.28	0.866	0.612
$\delta\gamma T^+$	0.40±0.25	2.28±1.46	0.47±0.34	2.35±1.83	0.208	0.674
$NKG2A^+$	0.72±0.43	3.68±1.84	0.74±0.37	3.84±2.39	0.674	0.889
$CD16^+CD56^+/CD3^-$	3.14±1.32	18.63±11.33	3.38±1.73	16.13±8.81	0.575	0.635
NK cell activity (%)[b]	26.54±16.13		39.43±15.48		**0.015**	
HTLV-1 provirus load[c]	867.38±874.62		641.75±343.12		0.401	

Data are mean±S.D. of 10 HAM/TSP patients (except for NK cell activity, $n=9$). Significant P value is in bold.
[a] Calculated using the Wilcoxon signed rank test.
[b] NK cell activity measured against K562 target cells at E/T ratio of 50/1 by the Cr release assay ($n=9$).
[c] HTLV-1 (pX) copy number per 1×10^4 PBMC by quantitative PCR.

treatment (Table 3). HTLV-1 antibody titers in sera and CSF also showed no significant change after 4 weeks of LcS treatment (data not shown).

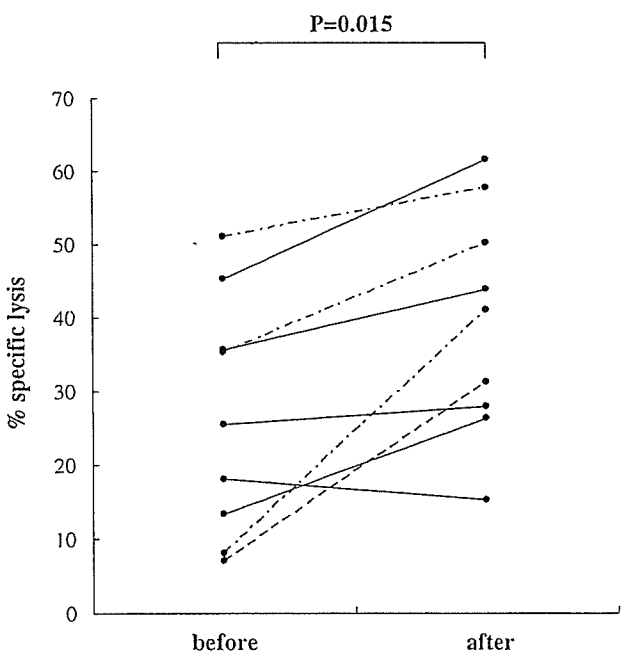

Fig. 1. Increased NK cell activity in PBMCs of HAM/TSP patients after oral administration of fermented milk drink containing *Lactobacillus casei* strain Shirota (LcS). The NK cell activity was significantly increased after LcS treatment ($n=9$, $p=0.015$, Wilcoxon's signed rank test). Each patient was given LcS preparation, which contains approximately 4×10^{10} viable LcS, orally twice a day for 4 weeks. NK cell activity was measured against K562 target cells at E/T ratio of 50/1 by the Cr release assay. Four patients out of ten received low dose oral prednisolone throughout the study period. None of the patients except for these four had received any immunomodulators. ------ Patients who received 5 mg/day oral prednisolone. ----: Patient who received 10 mg/day oral prednisolone.

4. Discussion

In this study, we demonstrated that clinical improvement was observed in all the HAM/TSP patients following 4 weeks of daily oral administration of LcS. Especially, improvement of urinary symptoms (UDS) was observed in all the 10 patients examined ($p=0.0085$, Wilcoxon signed rank test). LcS was also effective for motor dysfunction (OMDS) and spasticity (MAS) on lower extremity. Nine out of 10 patients showed decreased spasticity on neurological examination whereas OMDS score was improved only in 5 out of 10 patients. Thus, differences in OMDS score did not reach statistical significance ($p=0.157$, Wilcoxon signed rank test) whereas MAS scores did ($p=0.015$, Wilcoxon signed rank test). Although transient and mild diarrhea was previously reported in minor population (3 out of 65, 4.6%) after LcS administration [38], no adverse effect was observed in our patients.

Laboratory findings revealed that the NK cell activity in HAM/TSP patients was significantly increased after LcS treatment, but that HTLV-1 provirus load as well as the frequencies and absolute numbers of all the cell phenotypes examined were not significantly changed after treatment. In contrast, we have previously reported that the frequency of memory cells ($CD45RA^-CD27^+$) within $CD8^{high+}$, $CXCR3^+$ cells and HTLV-1 provirus load were significantly decreased after IFN-α treatment [22]. These findings suggest the possibility that NK cell activity is involved in the mechanism of clinical efficacy in LcS treatment, whereas Th1 type T cells and decreased HTLV-1 proviral load is associated with IFN-α effectiveness. Our present observation is consistent with the previous observation by Nagao et al. [39], who reported that in a healthy human subject, the frequency and number of NK cells, $CD4^+$ T