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

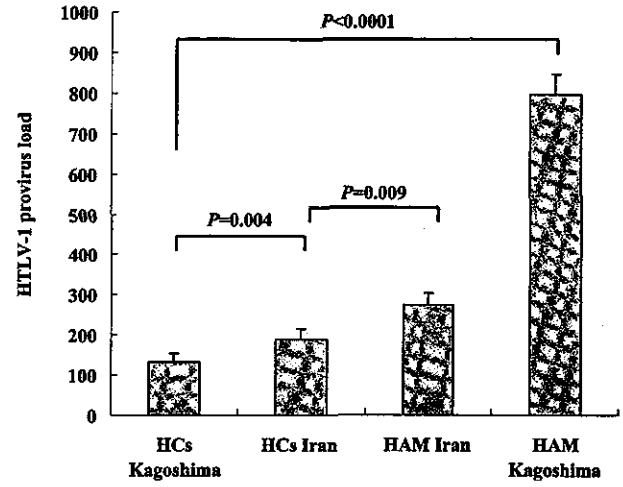

Fig. 1. HTLV-1 provirus load of Japanese and Iranian HTLV-1-infected individuals. Mean HTLV-1 copy numbers per 10^4 PBMCs for Japanese samples and per 10^4 nucleated cells for Iranian samples determined by quantitative PCR are shown. The HTLV-1 provirus load of Iranian HAM/TSP patients was significantly 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.

Iranian HCs was still significantly higher than that of Japanese HCs ($P=0{\cdot}004$, Mann–Whitney U test).

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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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

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To investigate non–human leukocyte antigen candidate genes that influence the outcome of human T cell lymphotropic virus (HTLV) type I infection, we analyzed 6 single-nucleotide polymorphisms in the interleukin (IL)–10 promoter region in 280 patients with HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP) and 255 HTLV-I–seropositive asymptomatic carriers from an area where HTLV-I is endemic. The IL-10 −592 A allele, which shows lower HTLV-I Tax–induced transcriptional activity than the C allele in the Jurkat T cell line, was associated with a >2-fold reduction in the odds of developing HAM/TSP ($P = .011$; odds ratio [OR], 0.50 [95% confidence interval, 0.30–0.86]) by reducing the provirus load in the whole cohort ($P = .009$, analysis of variance). Given the OR and the observed frequency of IL-10 −592 A, we demonstrate that this allele prevents ~44.7% (standard deviation, ±13.1%) of potential cases of HAM/TSP, which indicates that it defines one component of the genetic susceptibility to HAM/TSP in the cohort.

Human T-cell lymphotropic virus (HTLV) type I is the first characterized human retrovirus [1, 2] and is associated with adult T cell leukemia (ATL) [3, 4] and HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP) [5, 6]. Unlike HIV, HTLV-I causes no disease in a majority of infected subjects (healthy carriers [HCs]). However, ~2%–3% develop ATL, and another 2%–3% develop a disabling chronic inflammatory disease involving the central nervous system (HAM/TSP), eyes, lungs, or skeletal muscles [7]. The lifetime incidence for developing HAM/TSP is only 0.25% in Japan [8]. The factors that cause these different manifestations of HTLV-I infection are not fully understood. However, our previous population association study of >200 cases of HAM/TSP and >200 HTLV-I–seropositive HCs revealed several important risk factors for HAM/TSP. One of the major risk factors is the provirus load, as has been reported elsewhere [9]. The median provirus load was 16 times higher in patients with HAM/TSP than in HCs, and a high provirus load was also associated with an increased risk of progression to disease [10]. We next investigated HLA associations and found that the HLA-A*02 and -Cw*08 genes were associated with a lower HTLV-I provirus

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Table 1. Primers and restriction enzymes used for restriction fragment-length polymorphism analysis.

Polymorphism, primer direction	Primer sequence	Restriction enzyme	Reference (accession no.)[a]
−3575 (T/A)		TSP509I	25
Forward	5′-GTTTTTCCTTCATTTGCAGC-3′		
Reverse	5′-ACACTGTGAGCTTCTTGAGG−3′		
−2849 (G/A)		*Alw*I	AF295024
Forward	5′-CTGTAATCTCAGCACTCTGG-3′		
Reverse	5′-AGTTCAAGCCATTCTCCTGC-3′		
−2763 (C/A)		*Dde*I	25
Forward	5′-GAGGACTTGCACCAGGGAACT-3′		
Reverse	5′-TCCCGAGTAGCTGGGACTACA-3′		
−1082 (A/G)		*Mnl*I	26
Forward	5′-TCTGAAGAAGTCCTGATGTCACTG-3′		
Reverse	5′-ACTTTCATCTTACCTATCCCTACTTCC-3′		
−819 (T/C)		*Mae*III	27
Forward	5′-ATCCAAGACAACACTACTAA-3′		
Reverse	5′-TAAATATCCTCAAAGTTCC-3′		
−592 (A/C)		*Rsa*I	28
Forward	5′-CCTAGGTCACAGTGACGTGG -3′		
Reverse	5′-GGTGAGCACTACCTGACTAGC-3′		

[a] Accession numbers for GenBank/EMBL/DDBJ.

load and with protection from HAM/TSP, whereas HLA-DRB1*0101 and -B*5401 were associated with susceptibility to HAM/TSP; HLA-B*5401 was also associated with a higher provirus load in patients with HAM/TSP [11, 12]. We further examined the non-HLA host genetic factors that affect the risk of HAM/TSP and reported previously [13] that the tumor necrosis factor promoter −863 A allele predisposes toward HAM/TSP, whereas the stromal cell–derived factor–1 +801A 3′ untranslated region and interleukin (IL)–15 191 C alleles confer protection. In another study [14], we reported the association between variation in the HTLV-I *tax* gene and the risk of HAM/TSP. The *tax* subgroup A was more frequently observed in patients with HAM/TSP, and this effect was independent of HLA-A*02. These findings suggest that both host genetic factors and HTLV-I subgroup play a part in determining the risk of HAM/TSP.

To investigate further the non-HLA host genetic factors that influence the outcome of HTLV-I infection, we analyzed 6 single-nucleotide polymorphisms (SNPs) in the IL-10 promoter region and quantified the effect of each SNP on the risk of HAM/TSP, because recent studies have revealed a close association between IL-10 promoter polymorphisms and the outcome of certain viral infections, such as Epstein-Barr virus (EBV) [15], hepatitis B virus (HBV) [16], hepatitis C virus (HCV) [17], and HIV-1 [18], which suggests that particular polymorphisms in the IL-10 promoter contribute to the host immune reaction against viruses.

PATIENTS, MATERIALS, AND METHODS

Study population. Two hundred eighty patients with HAM/TSP were compared with 255 randomly selected HCs. All patients and control subjects were Japanese and resided in Kagoshima Prefecture, Japan. The diagnosis of HAM/TSP was made according to the World Health Organization diagnostic criteria [19]. All subjects provided written informed consent.

Detection of SNPs in the IL-10 promoter region. Polymerase chain reaction (PCR)–restriction fragment–length polymorphism analysis was performed for 6 SNPs. Primers and restriction enzymes used in the study are presented in table 1. A genomic PCR was performed with 50 ng of genomic DNA as template, 20 pmol of each primer, 5 mmol/L dNTP, reaction buffer provided by the manufacturer, and 1 U of Takara-Taq DNA polymerase (Takara) in a final volume of 50 μL. Fifteen microliters of the amplified PCR product was then digested for 12 h with the use of each restriction enzyme. Finally, digested PCR products were electrophoresed through a 2% agarose gel and visualized by ethidium bromide.

Provirus load measurement. To examine the HTLV-I provirus load, we performed a quantitative PCR method using an ABI Prism 7700 (PE-Applied Biosystems) with 100 ng of genomic DNA (~10^4 cells) from peripheral blood mononuclear cell (PBMC) samples, as reported elsewhere [10]. When β-actin was used as an internal control, the amount of HTLV-I provirus DNA was calculated by copy number of HTLV-I (pX) per 1 × 10^4 PBMCs = [(copy number of pX)/(copy number of β-

actin/2)] × 10^4. All samples were tested in triplicate. The lower limit of detection was 1 pX/10^4 PBMCs.

Cell line and plasmids. The human T-cell line Jurkat was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The expression vector pCG-Tax and the control vector pCG-BL were provided by Dr. J. Fujisawa (Kansai Medical University, Osaka, Japan). The pCG-Tax expression vector based on the human cytomegalovirus promoter for HTLV-I *tax* was constructed by inserting *tax* cDNA into the *Xba*I-*Bam*HI site of pCG-BL, as described elsewhere [20]. Human IL-10 promoter fragments (fragment −890 to +120; GenBank accession number X78437) were amplified by PCR from genomic DNA from 2 patients with HAM/TSP—one −592 AA homozygote and one CC homozygote—as described elsewhere [21]. The primers used to amplify the IL-10 region were IL-10 -890 (5′-AGC TCG AGA GTT GGC ACT GGT GTA CC-3′) and IL-10 AS (5′-ACT TCG AAG TTA GGC AGG TTG CCT G-3′). A promoter fragment that does not contain the −592 SNP, as well as the neighboring Sp-1 and Ets binding sites (fragment −571 to +120), was also amplified with the primers IL-10 −571 (5′-AAC CTC GAG GGA TAT TTA GCC CAC-3′) and IL-10 AS. The amplified products were subcloned into the pCR-Blunt II-TOPO vector (Invitrogen), and the sequences were confirmed. The correct insertions were subcloned into the *Xho*I polylinker site of the pGL2 Basic luciferase reporter vector (Promega), and sequences were confirmed again.

Transient transfection and luciferase assay. Five hundred thousand Jurkat cells were cotransfected with 2 μg of a reporter plasmid (IL-10 −592 A-Luc or IL-10 −592 C-Luc), together with 0.5 μg of either pCG-Tax or pCG-BL [20] and 300 ng of pRL-TK (Promega), to control transfection efficiency. The results of preliminary studies that measured luciferase activities from cell lysates at 24, 48, and 72 h after transfection indicated that the greatest luciferase activity was at 48 h after transfection. Therefore, after 48 h of cultivation at 37°C, cells were harvested, washed with PBS, and lysed in reporter lysis buffer (Promega). Luciferase assays were performed by use of the Dual Luciferase Assay System (Promega) and a TD-20/20 luminometer (Turner Designs). All assays were performed at least 3 times, each in duplicate.

Statistical and logistic-regression analysis. The χ^2 test was used to examine associations between HAM/TSP and the IL-10 promoter polymorphism. General linear model (GLM) analysis [22], which is a general form of multiple regression, was used to identify which factors were predictors of provirus load, in patients with HAM/TSP alone, in HCs alone, or in all subjects in the study. Logistic-regression analysis was used to identify which factors could be used to predict the odds of HAM/TSP and to fit an equation to estimate the risk in an individual of known genotype. The prevented fraction (Fp) of disease was calculated as described elsewhere [11].

RESULTS

Association of the IL-10 −592 A allele with a lower risk of HAM/TSP. The median age of patients with HAM/TSP (60.0 years; range, 12–81 years; 69.0% female) was greater than that of HCs (41 years; range, 16–65 years; 57.6% female), and there were more females in the HAM/TSP group and an absence of subjects <16 or >65 years old from the HCs; however, these factors did not affect the frequency of individual HLA alleles (data not shown). In addition, because the prevalence of HAM/TSP in Kagoshima is <1% among individuals infected with HTLV-I, very few HCs in the present cohort would be expected to develop HAM/TSP. There were no significant differences in the distribution of all genotypes and allele frequencies between 102 patients with HAM/TSP and 102 HCs in 4 SNPs tested (table 2). The nucleotide at position −2849 was nonpolymorphic in 102 patients with HAM/TSP and 102 HCs. In contrast, the IL-10 −592 A/C SNP showed a significant difference in allele frequency. We therefore analyzed further a total of 280 patients with HAM/TSP and 255 HCs (table 2; $\chi^2 = 8.48$; 2 *df*; $P = .014$) and identified a significant association between possession of an A residue in the IL-10 promoter −592 A/C SNP and a reduced risk of HAM/TSP. Possession of the IL-10 −592 A allele was associated with a >2-fold reduction in the odds of developing HAM/TSP ($P = .011$; odds ratio [OR], 0.50 [95% confidence interval, 0.30–0.86]). Given this OR and the observed frequency of the IL-10 −592 A allele in Kagoshima, we can estimate the Fp [11]. Here, Fp = 44.7% (SD, ± 13.1%) when the prevalence rate of HAM/TSP is 0.01, which indicates that the IL-10 −592 A allele prevents ~44.7% (SD, ± 13.1%) of potential cases of HAM/TSP in the study population.

Association of the presence of the A allele with a lower provirus load in the whole Kagoshima cohort of HTLV-I–infected individuals. We next tested the hypothesis that, if a gene is associated with a protection from HAM/TSP, it is also associated with a reduction in provirus load in HCs, given that the risk of developing HAM/TSP is dependent on the provirus load [10]. Table 3 summarizes the HTLV-I provirus load in patients with HAM/TSP and HCs, subdivided according to their IL-10 −592 A/C genotype. Because histograms of provirus load exhibited right-skewed distributions, the standard statistical technique of logarithmic transformation [22] was also used to mitigate this feature, which resulted in the data being more amenable to statistical analysis by parametric methods. To confirm whether the IL-10 −592 A/C SNP is a significant predictor of provirus load in the entire cohort, we performed multiple-regression analysis (GLMs; see Patients, Materials, and Methods). The results showed that the IL-10 −592 A/C SNP is a

Table 2. Interleukin (IL)–10 polymorphisms among patients with human T cell lymphotropic virus (HTLV) type I–associated myelopathy/tropical spastic paraparesis (HAM/TSP) and healthy HTLV-I carriers (HCs).

Polymorphism	HAM/TSP	HCs	*P*
−3575(T/A)			1.00
TT	99 (97.1)	99 (97.1)	
AT	3 (2.9)	3 (2.9)	
AA	0 (0)	0 (0)	
−2849 (G/A)			NA
GG	102 (100)	102 (100)	
GA	0 (0)	0 (0)	
AA	0 (0)	0 (0)	
−2763 (C/A)			.24
CC	95 (93.1)	89 (87.3)	
AC	7 (6.9)	13 (12.7)	
AA	0 (0)	0 (0)	
−1082 (A/G)			.38
AA	93 (91.2)	88 (86.3)	
AG	9 (8.8)	14 (13.7)	
GG	0 (0)	0 (0)	
−819 (T/C)			1.00
CC	12 (11.8)	12 (11.8)	
TC	49 (48.0)	48 (47.1)	
TT	43 (42.2)	42 (41.2)	
−592 (A/C)			**.014**[a]
AA	117 (41.8)	101 (39.6)	
AC	117 (41.8)	131 (51.4)	
CC	46 (16.4)	23 (9.0)	

NOTE. Data are no. of samples (%). The IL-10 −592 A allele was associated with a >2-fold reduction in the odds of HAM/TSP ($P = .011$; odds ratio, 0.50 [95% confidence interval, 0.30–0.86]). The proportion of potential cases of HAM/TSP that are prevented by the presence of the IL-10 −592 A allele (the prevented fraction of disease) [11] was 44.7% (SD, ±13.1%) when prevalence rate of HAM/TSP was 0.01, indicating that IL-10 −592 A allele prevents ~44.7% (SD, ±13.1%) of potential cases of HAM/TSP in the study population. NA, not applicable.

[a] χ^2 for genotype, $\chi^2 = 8.48$.

significant predictor of provirus load in the entire cohort ($n = 535$; $P = .004$, Kruskal-Wallis test; $P < .01$, GLM on the log-transformed or -untransformed data). This SNP was also a significant predictor of provirus load in the HC group alone ($n = 255$; $P = .040$, Kruskal-Wallis test), but not in the HAM/TSP group ($n = 280$; $P = .243$, Kruskal-Wallis test). Also, presence or absence of the IL-10 −592 A allele was a significant predictor of the provirus load in the entire cohort ($n = 535$; $P = .001$, Mann-Whitney U test; $P < .005$, GLM), although this relationship was only marginally significant in the HC group ($n = 255$; $P = .103$; Mann-Whitney U test; $P < .13$, GLM). These analyses indicate that the IL-10 −592 A/C SNP was a significant predictor of the provirus load and that the presence of A allele was associated with a lower provirus load in the whole Kagoshima cohort of HTLV-I–infected individuals (table 3).

IL-10 −592 A/C SNP—significant predictor of HAM/TSP even after accounting for provirus load or HLA-A*02. As was already mentioned, there was a significant association between the odds of developing HAM/TSP and the IL-10 −592 A/C SNP genotype according to the results of single-factor χ^2 analysis at both the allele and the genotype level. To confirm whether the IL-10 −592 A/C SNP genotype remains a significant predictor of HAM/TSP even after taking into account the other significant predictors identified by our previous analyses, such as provirus load and HLA-A*02, we performed logistic-regression analysis. As a result, in logistic-regression analysis that included HTLV-I provirus load and IL-10 −592 A/C SNP genotype treated as a 3-level factor (i.e., AA vs. AC vs. CC), the IL-10 −592 A/C SNP remained significant as a predictor of HAM/TSP ($P = .043$). We can calculate the risk for HAM/TSP by ln(odds of HAM/TSP) = −4.1212–0.5668 (if AC)–0.0235 (if CC) + 2.0764 × $\log_{10}$ (pX/10^4 PBMCs). When we treated the IL-10 −592 A/C SNP genotype as a 2-level factor, inclusion of the absence or presence of the A allele was not significant when $\log_{10}$(pX/10^4 PBMCs) was included ($P = .399$). However, the inclusion of the absence or presence of C was significant when $\log_{10}$(pX/10^4 PBMCs) was included ($P = .047$). Therefore, we conclude that the IL-10 −592 A/C SNP genotype has predictive power for HAM/TSP even after we accounted for the HTLV-I provirus load. Next, to test whether the IL-10 −592 A/C SNP genotype remains a predictor of HAM/TSP even after we accounted for HLA-A*02, we further performed the logistic-regression analysis using samples that are available on both IL-10 −592 A/C SNP and HLA-A*02 ($n = 402$). In logistic-regression analysis that included the HLA-A*02 and IL-10 −592 A/C SNP genotype, both HLA-A*02 ($P = .001$) and IL-10 −592 A/C SNP ($P = .014$) remained significant as predictors of HAM/TSP. In this case, we can calculate the risk for HAM/TSP by the equation ln(odds of HAM/TSP) = 0.4321–0.8876 (if A*02-positive)–0.2242 (if AC) + 0.7488 (if CC). In conclusion, the IL-10 −592 A/C SNP remains as a significant predictor of HAM/TSP even after taking into account the effects of the 2 known significant predictors of the risk of HAM/TSP—provirus load and HLA-A*02.

Effect of IL-10 −592 A/C SNP on HTLV-I Tax–mediated IL-10 promoter activity. To examine the functional significance of the −592 A/C SNP in HTLV-I infection, a 1010-bp promoter of the IL-10 gene (−890 to +120) carrying either the C or the A allele was inserted upstream of the luciferase gene in the pGL2-Basic plasmid vector, and luciferase assays were done. Because many polymorphisms in the IL-10 gene have been identified, numerous combinations of these polymorphisms may exist. Although our Kagoshima cohort of patients with HAM/TSP is the world's largest, <300 patients are available for analysis, so it would be meaningless to analyze all combinations of the IL-10 SNPs. The only sequence difference between the 2 reporter vectors was

Table 3. Interleukin (IL)–10 −592 A/C single-nucleotide polymorphism (SNP) genotype and human T cell lymphotropic virus (HTLV) type I provirus load.

Group	AA	AC	CC
HAM/TSP (280)	679.0 ± 58.2 (117)	785.8 ± 63.8 (117)	959.3 ± 139.6 (46)
HC (255)	77.2 ± 13.7 (101)	129.6 ± 15.7 (131)	194.6 ± 50.1 (23)
All patients combined (535)	400.2 ± 37.8 (218)	439.2 ± 37.5 (248)	704.4 ± 103.8 (69)

NOTE. Values are the average *tax* value (no. of *tax* copies/10^4 PBMCs) ± SE. The IL-10 −592 A/C SNP was a significant predictor of provirus load in the entire cohort ($n = 535$; $P = .004$, Kruskal-Wallis test; $P < .01$, general linear model analysis on log-transformed or -untransformed data) and of provirus load in the HTLV-I–seropositive asymptomatic carriers alone ($n = 255$; $P = .040$, Kruskal-Wallis test) but not in the HAM/TSP group ($n = 280$; $P = .243$, Kruskal-Wallis test). Values in parentheses are nos. of individuals tested. HAM/TSP, associated myelopathy/tropical spastic paraparesis; HC, healthy carrier.

the residue at position −592, which allowed us to estimate the functional differences associated with the −592 A or C residues alone. The results of the experiments showed that the functional differences were associated with the −592 A or C residues alone on HTLV-I Tax–mediated IL-10 promoter activity. These results showed that the ectopic expression of the Tax protein in Jurkat T cells increased IL-10 promoter activity by ~3 times with the A construct and 6 times with the C construct, compared with HCs ($P < .01$, Mann-Whitney U test) (figure 1). In contrast, the promoter fragment (fragment −571 to +120), which does not contain −592 SNP, as well as the neighboring Sp-1 and Ets binding site, was not transactivated by Tax. The basal luciferase activity without the transfecting Tax-expression vector (i.e., with transfecting empty vector, pCG-BL) did not differ between the A and C constructs. These results indicated that Tax directly transactivates the IL-10 promoter and that the C allele is more effective for Tax-mediated transcription than the A allele.

DISCUSSION

IL-10 is an important immunoregulatory cytokine that is involved in inflammatory responses, autoimmune diseases, and the response to infectious agents [23]. Although IL-10 has been reported to suppress the synthesis of proinflammatory cytokines from T cells and monocytes/macrophages, animal models have suggested that the overexpression of IL-10 in vivo can cause organ-specific autoimmune diseases, such as Sjögren syndrome [24] and type 1 diabetes [25]. Therefore, IL-10 is not regarded simply as an immunoinhibitory cytokine but also as a powerful immunostimulatory cytokine. Because transgenic mice containing the HTLV-I *tax* gene under the control of the viral long-terminal repeat (LTR) have previously been shown to develop an exocrinopathy involving the salivary and lachrymal glands that resembles Sjögren syndrome [26], which is frequently observed in patients with HAM/TSP [27], and be-

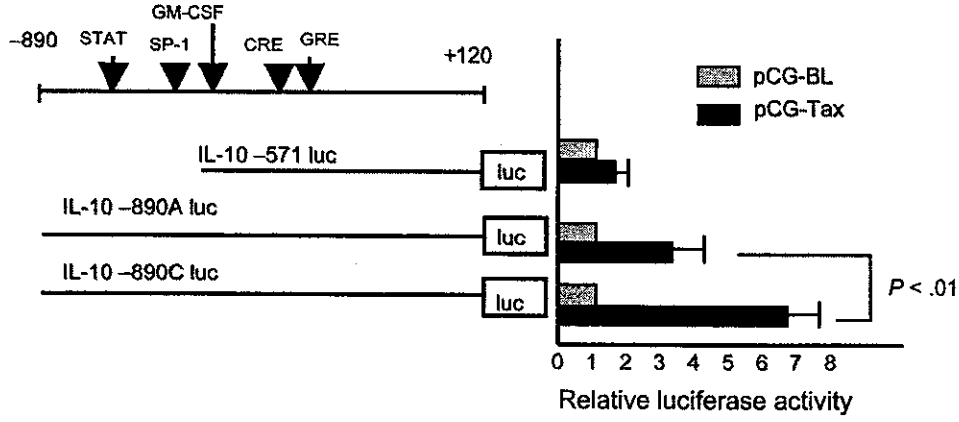

Figure 1. Interleukin (IL)–10 −592 A/C polymorphism and the Tax-mediated transcription of the IL-10 promoter. Jurkat cells were transfected with human T cell lymphotropic virus (HTLV) type I Tax expressing (pCG-Tax) or control (pCG-BL) vector and luciferase (luc) reporter constructs containing the full-length IL-10 promoter with −592 AA (−890 A-luc) or CC (−890 C-luc) or luc reporter plasmid without the specificity protein (Sp)–1 or −592 A/C SNP (−572 luc) sites. *Gray bars,* Luc activity of each reporter plasmid with control vector pCG-BL. *Black bars,* Luc activity of each reporter plasmid with Tax-expressing vector pCG-Tax. The activities are given relative to the activity of each reporter plasmid with control vector pCG-BL, which was defined as 1. The mean ± SD from 3 independent experiments is shown. The basal luciferase activity with pCG-BL was not different between −890 A-luc and −890 C-luc. The difference of luciferase activity with pCG-Tax between −890 A-luc and −890 C-luc was statistically significant ($P < .01$, Mann-Whitney U test). CRE, cyclic AMP response element; GM-CSF, granulocyte macrophage colony-stimulating factor; GRE, glucocorticoid response element; STAT, signal transducer and activator of transcription.

cause IL-10 mRNA expression was induced by HTLV-I Tax in both transiently and stably transfected Jurkat cells [28], it is likely that Tax directly transactivates the IL-10 promoter. The resulting overexpression of Tax in vivo may cause a Sjögren-like syndrome via an IL-10–mediated mechanism.

The implication of a heritable genetic basis for IL-10 production is supported by the concordance of IL-10 production in monozygotic twins, which suggests that genetic polymorphism could account for up to 75% of the observed variation in IL-10 production [29]. As was already mentioned, several studies have shown an association between particular polymorphisms in the human IL-10 promoter region and the outcome of certain viral infections, such as EBV [15], HBV [16], HCV [17], and HIV-1 [18]. In view of the immunomodulatory and anti-inflammatory effects of IL-10, we initially hypothesized that genetically determined lower production of IL-10 (associated with the allele −592 A) might influence disease susceptibility to HAM/TSP. This is the case for HIV-1 infection, because individuals with the IL-10 −592 AA genotype have been reported to be at higher risk of HIV-1 infection and rapid progression to AIDS [18]. In contrast, the present data show that, in HTLV-I infection, possession of the IL-10 −592 A allele prevented ~44.7% (SD, ±13.1%) of potential cases of HAM/TSP and was also a significant predictor for a lower provirus load in the entire cohort.

The −592 A/C SNP is located between the Sp1 and Ets binding site within the region between −652 and −571 nt that is necessary for IL-10 transcription [21]. It is of interest that previous reports have indicated that Tax transactivates the parathyroid hormone–related protein promoter by forming a ternary complex between Tax, Ets, and Sp-1, which acts on the promoter Sp-1 and Ets binding sites [30]. Another report showed that the HTLV-I LTR also contains a motif related to the Ets-binding sequence, named TRE-2S [31]. More important, 1 copy of the cyclic AMP response element (CRE)–like 21-bp sequence and TRE-2S in the HTLV-I LTR, contributes to the transactivation of viral gene via a ternary complex formed between Tax, Gli2 (TRE-S binding Gli oncogene family protein), and CRE-binding protein [32]. These findings suggest that a common mechanism of the HTLV-I Tax–mediated transactivation of the promoter of target genes ternary complexes formed with 2 different transcription factors. Furthermore, the results also suggest that the IL-10 promoter −592 A/C SNP, which lies between the Sp-1 and Ets binding sites, affects Tax-mediated transcription. Indeed, our cotransfection study using a Tax-expressing vector and Jurkat cells demonstrated that a IL-10 −592 luciferase vector carrying the high producer allele (C) showed higher Tax-mediated transcription than that of low producer allele (A), whereas a promoter fragment (fragment −571 to +120) that does not contain −592 SNP, as well as the neighboring Sp-1 and Ets binding site, was not transactivated by Tax. These findings suggested that HTLV-I Tax directly transactivates the IL-10 promoter and that the −592 A/C SNP affects Tax-induced transcription—that is, that the C allele is more effective than the A allele in mediating the Tax-induced transcription of IL-10. In future studies, it may be interesting to test whether Tax, Ets, and Sp-1 form a ternary complex on the IL-10 promoter and whether the −592 SNP affects this complex formation.

Among >90 non-HLA candidate gene loci that we have so far examined, the IL-10 −592 A/C SNP is the only non-HLA candidate gene locus associated with a significant reduction in both the provirus load and the risk of HAM/TSP. This observation is exactly analogous to the argument that we previously reported for HLA-A*02 and -Cw*08, where, in each case, possession of the allele was associated with both a significant reduction in provirus load in the HCs and a significant reduction in the risk of HAM/TSP [11, 12]. Thus, one possible mechanism for the observed IL-10 promoter effect is that increased the production of IL-10 reduces the efficiency of immune surveillance of HTLV-I infection—for example, by reducing the number or the activity of HTLV-I–specific cytotoxic T lymphocytes. However, the IL-10 promoter genotype remained a significant predictor of the risk of HAM/TSP even after taking the provirus load into account. This observation suggests that IL-10 increases the risk of HAM/TSP by another mechanism in addition to an apparent effect on provirus load.

In conclusion, we report that the IL-10 −592 A allele, which is associated with lower HTLV-I Tax–mediated transcriptional activity, influences both the provirus load in HTLV-I–infected individuals and the susceptibility to HAM/TSP in the Kagoshima cohort. This effect remains significant even after taking into account the other 2 known major predictors of HAM/TSP risk in this cohort—provirus load and HLA-A*02 genotype—which suggests a powerful argument in favor of a real physiological effect of this polymorphism. Further functional studies to clarify the role of IL-10 in HTLV-I infection may reveal immunotherapeutic strategies that would retard the development of HAM/TSP.

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Serum concentration and genetic polymorphism in the 5′-untraslated region of VEGF is not associated with susceptibility to HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) in HTLV-I infected individuals

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Abstract

HTLV-I- associated myelopathy/tropical spastic paraparesis (HAM/TSP) is one outcome of human T-cell lymphotropic virus type I (HTLV-I) infection. It remains unknown why the majority of infected people remain healthy whereas only approximately 2–3% of infected individuals develop the disease. Recently, it has been reported that increased plasma concentrations of VEGF were significantly related to high ATL cell infiltration, and the viral transactivator Tax activates the VEGF promoter, linking the induction of angiogenesis to viral gene expression. To investigate whether VEGF promoter −634C/G single nucleotide polymorphism (SNP) and serum concentration of VEGF are associated with the development of HAM/TSP, we studied a group of 202 HAM/TSP patients, 202 asymptomatic HTLV-I seropositive carriers (HCs) and 108 seronegative healthy controls (NCs) in Kagoshima, Japan by using PCR-RFLP analysis. The serum concentration of VEGF was also compared among patients with HAM/TSP, ATL, HCs as well as with NCs. Our results indicate that both VEGF gene polymorphism and serum VEGF levels are not specifically associated with the risk of HAM/TSP in our cohort.

Keywords: VEGF; Single nucleotide polymorphism; HAM/TSP; HTLV-I; Disease susceptibility; Proviral load

1. Introduction

Human T-cell lymphotropic virus type I (HTLV-I) [1,2] infection is closely associated with a slowly progressive neurological disease called HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [3,4]. Infection with HTLV-I is estimated to affect 10 million to 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.

Vascular endothelial growth factor (VEGF) is a major mediator of vascular permeability and angiogenesis. Dysregulated VEGF expression has been implicated as a major contributor to the development of a number of common disease pathologies [8]. A recent report indicated that among seven common polymorphisms in the promoter region, genotype distribution of the −634C/G single nucleotide polymorphism (SNP) differed significantly (P= 0.011) between patients with and without diabetic retinopathy, and that C allele was significantly increased in patients with retinopathy compared with those without retinopathy (P=0.0037) [9]. On the other hand, it has recently been reported that HTLV-I-transformed cells secrete VEGF and basic fibroblast growth factor (bFGF) proteins and induce

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angiogenesis in vitro, via HTLV-I Tax-induced transcriptional activation of the VEGF promoter [10]. Therefore, it may be possible that altered vessel permeability and activated endothelial cells are involved in the pathogenesis of HAM/TSP.

To test this possibility, we examined the serum concentration of VEGF as well as promoter gene polymorphism to assess its possible role in HAM/TSP.

2. Patients and methods

2.1. Study population

The study population consisted of 202 patients with HAM/TSP, 202 asymptomatic HTLV-I seropositive carriers (HCs) and 108 seronegative healthy controls (NCs), all residing in HTLV-I endemic Kagoshima Prefecture in Southern Japan. The diagnosis of HAM/TSP was done in accordance with World Health Organization criteria [11]. 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 PBMC 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. Genomic PCR analysis

In order to amplify a 469 base pair fragment containing the −634C/G SNP in VEGF promoter, 50 ng of genomic DNA was PCR-amplified with the primers (forward: 5′-TTG CTT GCC ATT CCC CAC TTG A-3′ and reverse: 5′-CCG AAG CGA GAA CAG CCC AGA A-3′) by 1 unit of Takara-Taq DNA polymerase® (Takara, Tokyo, Japan) in a final volume of 50 μl. PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 54 °C for 60 s, and elongation at 72 °C for 60 s with a final extension at 72 °C for 10 min. The 15 μl of PCR product was then digested for 12 h using 5 units of *Bsm-FI* (New England Biolabs, MA) restriction enzyme, resulting in fragments of 338 and 131 bp in length if −634G is used or in fragments of 469 bp in length if −634C is used [9]. Finally, digested PCR products were electrophoresed through a 2% agarose gel and visualized with ethidium bromide.

2.4. Quantification of VEGF in serum

The serum VEGF concentration was measured in duplicate using a commercial ELISA kit (R&D Systems, Minneapolis, Minnesota). All samples were quickly frozen and stored at −80 °C until the time of the assay. The assay system used is sensitive to typically less than 9.0 pg/ml. Optical density at 450 nm was measured on the Immuno-Mini NJ-2300 (Nippon Inter Med, Tokyo, Japan) and VEGF concentration was determined by linear regression from a standard curve using the VEGF supplied with the kit as standard. The intra-assay coefficient of variation (CV) of the VEGF ELISA was 6.7%, and the inter-assay CV was 8.8%.

2.5. Quantification of HTLV-I provirus load, CSF neopterin and anti-HTLV-I antibody titers

To examine the HTLV-I provirus load, we carried out a quantitative PCR method using ABI Prism 7700™ (PE-Applied Biosystems) with 100 ng of genomic DNA (roughly equivalent to 10^4 cells) from PBMC samples as reported previously [12]. In this method, the 5′ nuclease activity of Taq polymerase cleaves a nonextendible hybridization probe during the extension phase of PCR. This cleavage generates a specific fluorescent signal that is measured at each cycle. Based on the standard curve created by four known concentrations of template, the concentrations of unknown samples were determined. Using β-actin as an internal control, the amount of HTLV-1 proviral 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)] $\times 10^4$. All samples were performed in triplicate. Neopterin levels were evaluated by HPLC with fluorometric detection methods [13]. Serum and CSF antibody titers to

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

	Age	Male/Female	Anti-HTLV-I antibodies[a]	HTLV-I proviral load[b]	Neopterin in CSF[c]
HAM/TSP (n=202)	57.3 ± 11.9[d]	60/142	26,364 ± 41,347	725.2 ± 656.5	111.9 ± 112.4
HCs (n=202)	39.5 ± 12.9	96/106	1514 ± 1467	191.2 ± 312.9	N/A

N/A: not applicable.

[a] Anti-HTLV-1 antibodies were titrated by the particle agglutination method.

[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 ± SD.

Table 2
Summary of VEGF -634C/G SNP data

Allele	HAM/TSP	HCs	NCs	p value[a]	Genotype	HAM/TSP	HCs	NCs	p value[b]
C	181 (44.8)[c]	183 (45.3)	93 (43.1)	0.89 (HAM-HCs)	CC	41 (20.3)	40 (19.8)	20 (18.5)	0.92 (HAM-HCs)
G	223 (55.2)	221 (54.7)	123 (56.9)	0.68 (HAM-Normal)	CG	99 (49.0)	103 (51.0)	53 (49.1)	0.91 (HAM-Normal)
				0.59 (HCs-Normal)	GG	62 (30.7)	59 (29.2)	35 (32.4)	0.84 (HCs-Normal)
Total	404	404	216			202	202	108	

HCs: asymptomatic HTLV-I carriers.
NCs: seronegative healthy controls.
[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.

HTLV-I were determined by a particle agglutination method (Serodia-HTLV-I®, Fujirebio).

2.6. Statistical analysis

Comparisons of genotype frequency among HAM/TSP patients, HCs and NCs were calculated by the chi-squared test. For multiple comparisons, we used Sheffe's F to analyze statistical difference. Mann–Whitney U-test was used to compare serum VEGF levels between the various clinical groups. Significance was considered at $p < 0.05$.

3. Results

3.1. VEGF promoter gene polymorphism in HAM/TSP patients, asymptomatic HTLV-I carriers and seronegative healthy controls

The functional promoter polymorphism at position −634C/G SNP in the VEGF promoter had been previously reported from Saitama, Japan to be associated with diabetic retinopathy with a significantly increased frequency of the CC genotype [9]. However, in the present study, no significant differences were observed among HAM/TSP patients, HCs and NCs genotype or gene frequencies (Table 2). In all groups (HAM/TSP patients, HCs and NCs) the genotype frequencies were distributed according to Hardy–Weinberg equilibrium. Interestingly, the allele and genotype frequencies of VEGF −634C/G SNP in Kagoshima population was very similar to previously reported type 2 diabetic patients with retinopathy, but not without retinopathy [9]. Recently reported allele and genotype frequencies of VEGF −634C/G SNP from Italian control population also showed similar results with our present study [14]. Thus −634C/G SNP in the VEGF promoter was not associated with the risk for HAM/TSP in Kagoshima population.

3.2. Serum concentration of VEGF among HAM/TSP, ATL patients, asymptomatic HTLV-I carriers and seronegative controls

There was no significant difference in serum VEGF levels among 22 HAM/TSP (224.62 ± 140.65), 7 ATL patients (390.54 ± 283.78), 24 asymptomatic HTLV-I carriers (228.22 ± 156.99) and 12 NCs (209.89 ± 159.02) (Fig. 1). Two ATL patients with organ infiltration of ATL cell showed relatively high serum VEGF levels (ATL1: 652.0 pg/ml; ATL 2: 857.5 pg/ml) than other patients, consisting with previous reports [15,16].

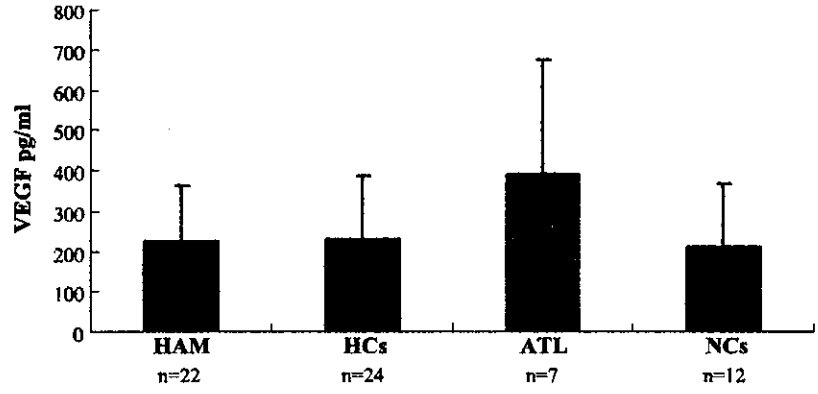

Fig. 1. Serum concentration of VEGF among HAM/TSP patients, ATL patients, asymptomatic HTLV-I carriers and seronegative controls. Serum VEGF levels from 22 HAM/TSP (224.62 ± 140.65), 7 ATL patients (390.54 ± 283.78), 24 asymptomatic HTLV-I carriers (HCs) (228.22 ± 156.99) and 12 seronegative healthy controls (NCs) (209.89 ± 159.02) were determined using ELISA. Bars show the mean ± standard deviation in each group.

Table 3
VEGF −634C/G SNP genotype and HTLV-I provirus load

	CC	CG	GG
HAM (n=202)	743.6 ± 110.9	704.0 ± 76.9	750.4 ± 93.4
HCs (n=202)	224.8 ± 59.0	200.0 ± 33.6	155.0 ± 27.6
All patients combined	441.6 ± 65.3	423.1 ± 42.9	411.0 ± 51.7

The values are shown as the mean tax value (tax copies/10^4 PBMCs) ± SE.

3.3. The VEGF −634 SNP is not a significant predictor of the HTLV-I proviral load in HAM/TSP patients and asymptomatic HTLV-I carriers

To test whether VEGF −634C/G SNP genotype is a significant predictor of the HTLV-I proviral load, we measured the proviral load of HTLV-I and compared it with VEGF −634C/G genotype in HAM/TSP patients and HCs. Our data indicated that there was no association between VEGF −634C/G genotype and HTLV-I proviral load (Table 3), CSF neopterin levels as well as serum HTLV-I antibody 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-I 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. Although certain Tax subtypes were recently reported to carry different risks of HAM/TSP [7], viral factors alone are not sufficient to predict disease. Our recent observations as well as many reported findings strongly suggest that the outcome of HTLV-I infection mainly depends upon a host of genetic factors [17]. Especially, our recent case/control study in Kagoshima strongly supports this idea. In the Kagoshima population, possession of the HLA-class I genes, HLA-A*02 and Cw*08, each independently halve the odds of developing HAM/TSP, whereas possession of the HLA-class I gene, HLA-B*5401 and the HLA-class II gene, HLA-DRB1*0101, predispose a person to HAM/TSP [18,19]. Since HLA itself does not explain the entire disease onset of HAM/TSP, and a non-HLA candidate gene approach has already been shown to be successful in identifying markers in other infectious diseases [20,21], we are now focusing on non-HLA gene polymorphisms as candidate genes that are associated with development of HAM/TSP.

VEGF is a specific mitogen and survival factor for endothelial cells and a key promoter of angiogenesis in physiological and pathophysiological conditions, and promotes inflammatory processes by causing vascular leakage and mobilizing leukocytes [8]. Increased concentrations of free VEGF have been measured in a variety of autoimmune and infectious inflammatory diseases, including rheumatoid arthritis [22], POEMS syndrome [23,24], and Kawasaki disease [25,26]. More interestingly, VEGF expression was consistently upregulated in both acute and chronic multiple sclerosis plaques [27], suggesting that VEGF exacerbate the inflammatory response in autoimmune diseases of the central nervous system and migration of inflammatory cells into the lesions. Since HTLV-I-transformed cells secrete VEGF and bFGF proteins and induce angiogenesis in vitro via HTLV-I Tax-mediated transcriptional activation of VEGF promoter [10] and HAM/TSP is also associated with inflammatory cell infiltrations into central nervous system (CNS), we investigated the influence of VEGF gene polymorphism as well as serum concentration of VEGF in HTLV-I infection.

In the present study, there were no significant differences in any VEGF −634C/G genotypes between HAM/TSP patients and HCs. Also, there were no correlations between serum VEGF levels and CSF neopterin levels as well as serum anti-HTLV-I antibody titers. Furthermore, the clinical course and disability of HAM/TSP were not associated with the VEGF −634C/G polymorphism and serum VEGF levels in HAM/TSP patients, although two ATL patients with organ infiltration showed relatively higher concentration of VEGF in the serum. Taken together, our present results suggest that VEGF −634C/G genotype as well as serum concentrations of VEGF are not susceptibility factors for the development of HAM/TSP. It is still possible that VEGF might have an important role in the affected spinal cord lesion of HAM/TSP, as reported in both acute and chronic MS plaques [27], although further studies are needed to clarify this point.

In conclusion, our results indicate that VEGF in serum is not the suitable factor to evaluate the risk and disease activity of HAM/TSP.

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