研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ⅳ. 研究成果の刊行物・別冊

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Differences in viral and host genetic risk factors for development of human T-cell lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis between Iranian and Japanese HTLV-1-infected individuals

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Human T-cell lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a neurological disease observed only in 1–2% of infected individuals. HTLV-1 provirus load, certain HLA alleles and HTLV-1 *tax* subgroups are reported to be associated with different levels of risk for HAM/TSP in Kagoshima, Japan. Here, it was determined whether these risk factors were also valid for HTLV-1-infected individuals in Mashhad in northeastern Iran, another region of endemic HTLV-1 infection. In Iranian HTLV-1-infected individuals ($n=132$, 58 HAM/TSP patients and 74 seropositive asymptomatic carriers), although HLA-DRB1*0101 was associated with disease susceptibility in the absence of HLA-A*02 ($P=0{\cdot}038$; odds ratio = 2·71) as observed in Kagoshima, HLA-A*02 and HLA-Cw*08 had no effect on either the risk of developing HAM/TSP or HTLV-1 provirus load. All Iranian subjects possessed *tax* subgroup A sequences, and the protective effects of HLA-A*02 were observed only in Kagoshima subjects with *tax* subgroup B but not in those with *tax* subgroup A. Both the prevalence of HTLV-1 subgroups and the host genetic background may explain the different risks levels for HAM/TSP development in these two populations.

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INTRODUCTION

Human T-cell lymphotropic virus type 1 (HTLV-1) (Poiesz *et al.*, 1980; Yoshida *et al.*, 1982) is a causative agent of adult T-cell leukaemia (Hinuma *et al.*, 1981; Yoshida *et al.*, 1984) and the chronic neurodegenerative disorder HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain *et al.*, 1985; Osame *et al.*, 1986). Only a minority of HTLV-1-infected individuals develop HAM/TSP, and most infected individuals remain healthy throughout their lives. A previous seroepidemiological survey in Kyushu Island, in southwestern Japan, where Kagoshima prefecture is located, estimated the incidence of HAM/TSP among HTLV-1-infected persons at $3{\cdot}1 \times 10^{-5}$ cases per year; assuming a lifespan of 75 years, the lifetime incidence is therefore approximately 0·25% (Kaplan *et al.*, 1990). In HAM/TSP patients from Kagoshima, the median provirus load in peripheral blood mononuclear cells (PBMCs) is more than ten times higher than HTLV-1-seropositive asymptomatic carriers (HCs) and high provirus load is also associated with an increased risk of progression to disease (Nagai *et al.*, 1998). HTLV-1 provirus load has been correlated with progression of motor disability (Takenouchi *et al.*, 2003) and the risk of sexual transmission of HTLV-1 (Kaplan *et al.*, 1996). Thus, HTLV-1 provirus load is an important correlate of virus transmission as well as disease progression. A previous study

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).

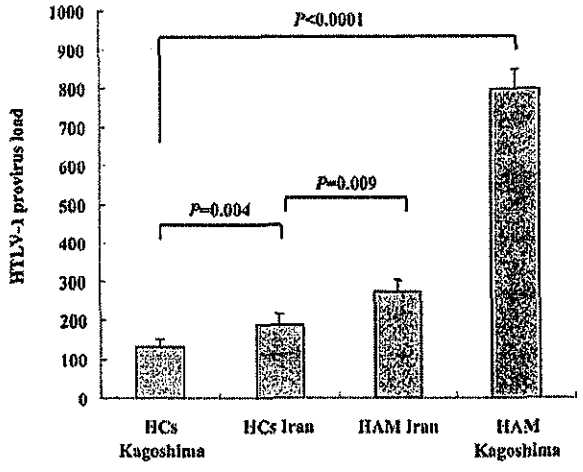

Fig. 1. HTLV-1 provirus load of Japanese and Iranian HTLV-1-infected individuals. Mean HTLV-1 copy numbers per 10^4 PBMCs for Japanese samples and per 10^4 nucleated cells for Iranian samples determined by quantitative PCR are shown. The HTLV-1 provirus load of Iranian HAM/TSP patients was significantly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

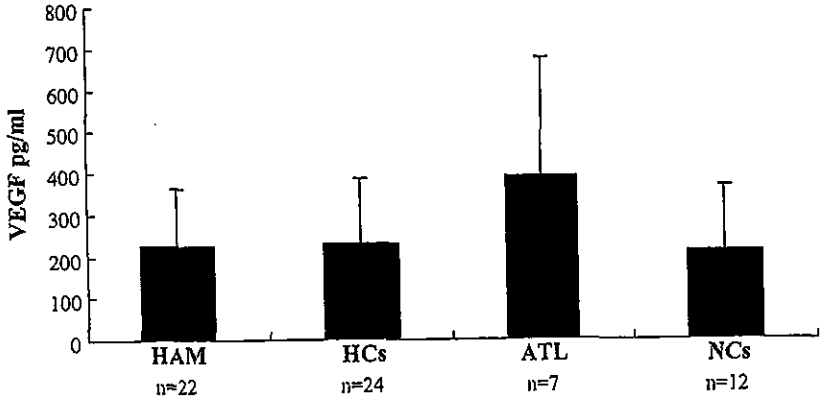

Fig. 1. Serum concentration of VEGF among HAM/TSP patients, ATL patients, asymptomatic HTLV-I carriers and seronegative controls. Serum VEGF levels from 22 HAM/TSP (224.62 ± 140.65), 7 ATL patients (390.54 ± 283.78), 24 asymptomatic HTLV-I carriers (HCs) (228.22 ± 156.99) and 12 seronegative healthy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