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Comparative molecular analysis of HTLV-I proviral DNA in HTLV-I infected members of a family with a discordant HTLV-I-associated myelopathy in monozygotic twins

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In order to elucidate the underlying mechanisms of a discordant case with HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) in monozygotic twins, we investigated HTLV-I *tax* sequences of 10–18 polymerase chain reaction-based clones each derived from peripheral blood mononuclear cells of the twins as well as their infected mother and an elder brother who also suffered from HAM/TSP. Sequence comparison revealed that three of the infected individuals including a twin with HAM/TSP shared the consensus *tax* sequence identical to the reference, ATK-1, but that of another healthy twin was different at five nucleotide positions including three nonsynonymous changes from ATK-1. This finding strongly suggested that different HTLV-I strains infected the monozygotic twins and the difference in infected proviral sequences determined the discordant clinical outcomes. Transfection and subsequent reporter assays failed to show a significant difference in transactivation activity on HTLV-I LTR and NF-κB elements between the products of the two sequences. Two HAM/TSP patients (a twin and elder brother) among three members infected with the ATK-1 type virus shared a paternal HLA allele which was absent in the healthy individual (mother). Genetic analysis of sequence variation in the *tax* sequences of the discordant twins showed that the *Dn/Ds* ratio was high in the healthy twin but low in the twin with HAM/TSP, implying the presence of more intense selection forces in the carrier. Our findings strongly suggested that a particular combination of HTLV-I strains with an HLA genotype would be a risk for HAM/TSP. *Journal of NeuroVirology* (2000) **6,** 275–283.

Keywords: HAM/TSP; tax sequence; HLA; proviral load; transactivation

Introduction

Human T-cell leukemia virus type-I (HTLV-I) is closely linked to HTLV-I associated myelopathy (HAM)/tropical spastic paraparesis (TSP) (Gessain *et al*, 1985; Osame *et al*, 1987). HTLV-I is a persistent virus, infecting 10–20 million people worldwide. Most infected individuals remain healthy, but 1–2% develop HAM/TSP. While the mechanisms by which HTLV-I causes HAM/TSP are unknown, a high HTLV-I provirus load and increased spontaneous proliferation of cultured peripheral T-cells are noted as characteristics of HAM/TSP (Yoshida *et al*, 1989; Kira *et al*, 1991).

We previously reported a high degree of homology in the nucleotide sequence between HTLV-I isolates from patients with HAM/TSP and ATL (Shirabe *et al*, 1990). However, extensive sequence analyses of HTLV-I proviruses in infected individuals have shown the presence of microvariation scattered throughout all regions of the proviral

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genome. Attempts to identify the disease-specific variants have been reported by many investigators. Comparisons of the HTLV-I proviral LTR-U3 and *env* sequences in peripheral blood mononuclear cells (PBMCs) of adult T-cell leukemia (ATL) and HAM/TSP patients failed to identify any disease-specific sequences (Daenke *et al*, 1990; Kinoshita *et al*, 1991). In contrast to these observations, Renjifo *et al* (1995) reported a *tax* mutation at position 7959 commonly found in PBMCs of patients with HAM/TSP, but the finding has not been reproduced by other research groups (Mahieux *et al*, 1995). Analysis of proviruses in the central nervous system (CNS) of HAM/TSP patients demonstrated frequent existence of defective proviruses that lack a part of the *tax* gene which encodes a viral transcriptional transactivator, Tax, suggesting a role for the *tax*-defective mutants in the development of HAM/TSP (Kira *et al*, 1994). The presence or absence of HTLV-I variants specific to HAM/TSP remains controversial. On the other hand, the ethnic background of HTLV-I infections and associated diseases was investigated in association with human leukocyte antigens (HLA) (alleles) and haplotypes (Usuku *et al*, 1988; Sonoda *et al*, 1996). Recent studies revealed that susceptibilities to certain infectious diseases are determined by the host genetic background (Hill, 1998). More recently, Jeffery *et al* (1999) demonstrated that specific HLA genotypes, such as HLA-A*02 and HLA-DRB1*0101, correlated with the high HTLV-I proviral load and were linked to the risk of HAM/TSP.

We previously reported a family including HTLV-I infected monozygotic twins, one of whom suffered from HAM/TSP (Motomura *et al*, 1990). In the present study, we investigated proviral *tax* sequences, transactivation activity of the protein product, proviral load, and immunologic parameters of four HTLV-I infected members of this family including the monozygotic twins, their mother and an elder brother also suffered from HAM/TSP.

Results

Family pedigree, proviral loads, and HLA serotypes of subjects

The pedigree of the family is shown in Figure 1. The family consisted of a mother and seven children including monozygotic twins. The mother, twins, and their eldest brother were infected with HTLV-I. The remaining four siblings were seronegative. Among the infected individuals, mother and a twin (twin B) remained as healthy carriers, but the elder brother and another twin (twin A) suffered from HAM/TSP for about 30 years. The father had died earlier (more than 20 years) following pontine hemorrhage, and the history suggestive of neurological symptoms was not available.

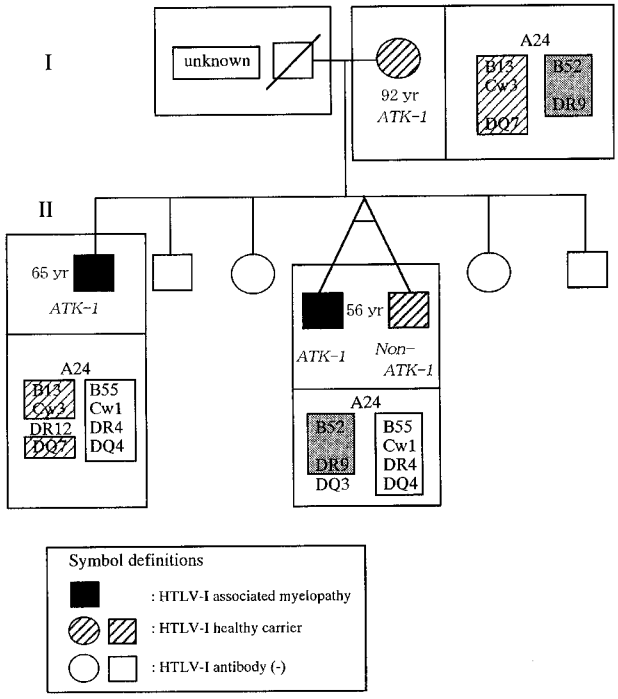

Figure 1 Family pedigree showing HLA serotypes and HTLV-I genotypes. HTLV-I genotypes are italicized (*ATK-1* or *non ATK-1*). Shaded HLA serotypes: derived from mother to twins. Hatched HLA serotypes: derived from mother to eldest brother. A24 was observed in all members studied. DR12 and DQ3 are considered to be derived from mother, but not detected by serotyping. Open HLA serotypes: derived from deceased father who could not be examined. Identical twins shared completely identical HLA types.

Serotyping of HLA revealed that different maternal HLA alleles were transmitted to the twins and the elder brother. Twins shared HLA B52 and DR9 with their mother, but the elder brother had B13, Cw3, and DQ7. On the other hand, twins and the brother shared HLA B55, Cw1, DR4, and DQ4, which were absent in the mother and thus considered to be of paternal origin.

Proviral loads in PBMCs of the four infected individuals were quantified by the competitive PCR method (Furuya *et al*, 1997). HTLV-I proviral loads were quantified three times in each PBMCs. These experiments resulted in almost same results. Twin A and elder brother, both suffered from HAM/TSP, revealed high proviral loads, 31.0 and 23.5 copies/1000 cells, respectively, in comparison to those of healthy carriers, while the proviral load in twin B and mother was 4.0 and 0.4 copies/1000 cells, respectively. Similarly high titers of anti-HTLV-I antibody were detected in sera of twin A and twin B.

Nucleotide sequence analysis of HTLV-I tax region in infected family members

The whole open reading frame (ORF) encoding Tax was amplified by PCR from PBMC genomic

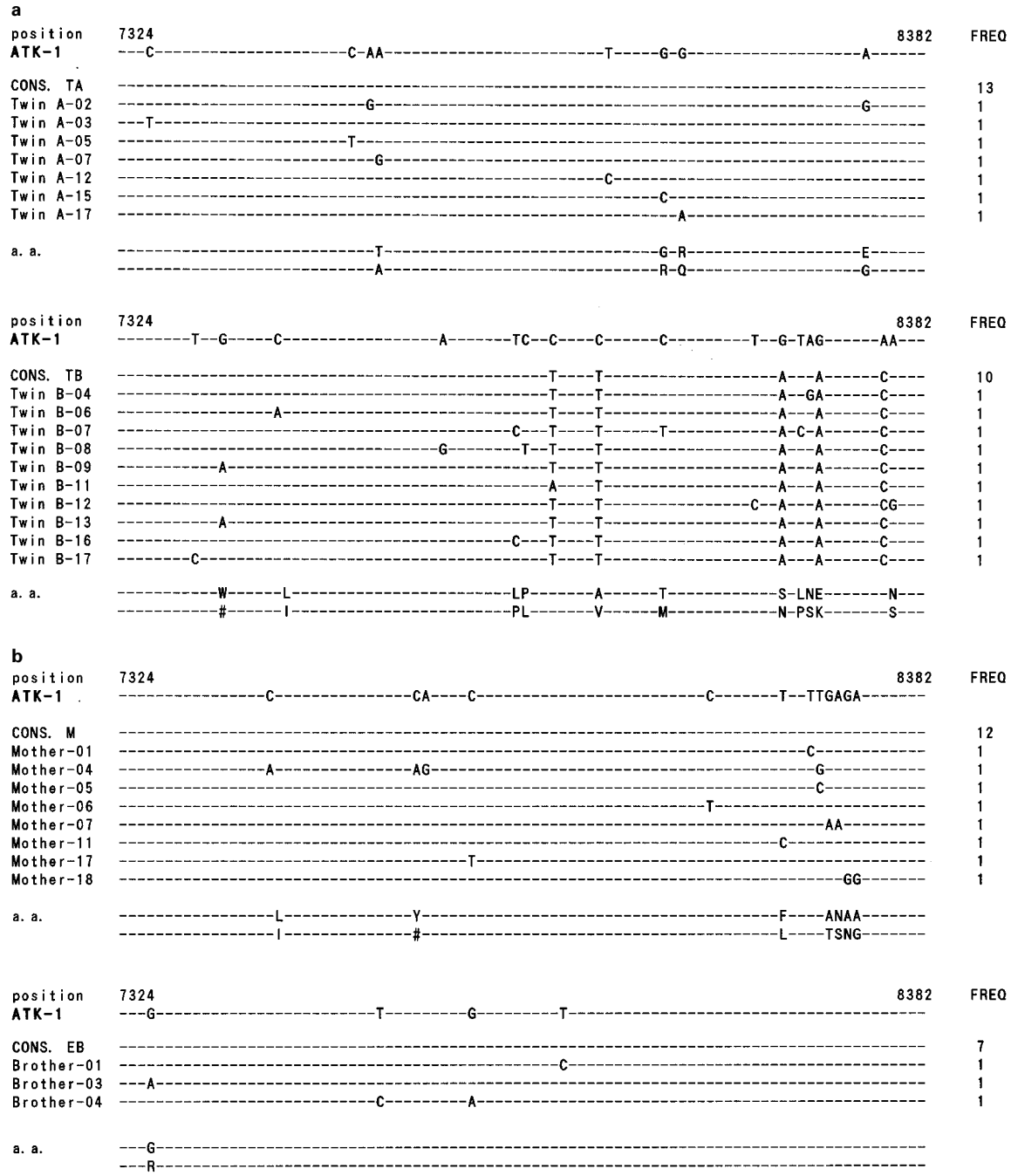

Figure 2 Nucleotide sequence variation among *in vivo*-derived HTLV-I *tax* clones. The whole *tax*-ORF amplified from sample DNA was ligated into pCG, and randomly selected plasmid clones were sequenced. Numbers at the top of the figure represent the position of the nucleotide in reference to the ATK-1 sequence (Seiki *et al*, 1983). Nucleotide sequences of each 18 *tax* clones derived from both twins A and twin B (**a**), and their mother (**b**). The nucleotide sequence of the *tax* gene of twin B reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession number AB038239. Nucleotide sequences of 10 *tax* clones derived from elder brother (**b**). Nucleotide sequences are aligned in comparison with the reference ATK1. Dots indicate sequences identical to the reference. At the lower part in each figure, amino acid changes (single letter abbreviations) or stop codon (#) by corresponding mutations are presented. The dominant sequences in the individual are indicated as consensus sequence (CONS). The number of clones is identical to a given nucleotide sequence (FREQ).

DNA of the four infected individuals and the products were ligated into the expression plasmid, pCG. Ten or 18 clones were randomly isolated from each individual and their nucleotide sequences were determined. A consensus sequence was found among the clones of each

individual. Those of twin A, mother, and elder brother were identical to the reference, ATK-1 (Figure 2a,b). On the other hand, the consensus sequence of twin B was different at five nucleotide positions from ATK-1, three of which resulted in differences in amino acids of the protein product (Figure 2a). One substitution at nucleotide position 7959 was identical to that previously reported to be closely associated with HAM/TSP (Renjifo *et al*, 1995). The proportions of the consensus sequences among the clones were 13/18, 10/18, 12/18, and 7/10 in twin A, twin B, mother, and elder brother, respectively.

The remaining clones included 1–4 nucleotide substitutions from each consensus sequence. These substitutions were randomly distributed throughout the whole region of the *tax* ORF. Frequencies of nucleotide substitution in two healthy carriers (twin B and mother), 0.78 and 0.72/clone, respectively, were higher than those of two HAM/TSP patients (twin A and elder brother), 0.44 and 0.40, respectively (Table 1). Rates of nonsynonymous substitution were also high in the healthy carriers, 0.71 and 0.54, respectively, compared to HAM/TSP patients, 0.50 and 0.25, respectively (Table 1). We further analyzed the substitutions in twin A and B by the algorithm developed by Nei and Gojobori (1986). The rates of nonsynonymous substitutions per nonsynonymous site (*Dn*) of twin A and B were 0.00051 and 0.00152, respectively, and those of synonymous substitutions per synonymous site (*Ds*) were 0.00223 and 0.00075, respectively. The *Dn/Ds* ratio of twin A, 0.23, was much lower than that of twin B, 2.04 (Table 2), suggesting the presence of more intense selection forces in the latter.

Table 2 Comparison of the *Dn/Ds* ratios for the *tax* gene

Case	Dn	Ds	Dn/Ds
Twin A (HAM/TSP)	0.00051	0.00223	0.23
Twin B (healthy carrier)	0.00152	0.00075	2.04

The rate of synonymous changes per synonymous site and the rate of nonsynonymous changes per nonsynonymous site and the *Dn/Ds* ratio was calculated according to the method of Nei and Gojobori (1986) for the full-length tax gene.

Table 1 Analysis of the sequence variation for the *tax* gene

Subjects	*Number of clones*	*Number of substitutions*	*Substitutions/ clone*	*Nonsynonymous substitutions/substitutions*
Twin A[a]	18	8	0.44	0.50
Twin B[b]	18	14	0.78	0.71
Mother[b]	18	13	0.72	0.54
Elder brother[a]	10	4	0.40	0.25

[a]HAM/TSP patient; [b]healthy carrier. Clone sequences were compared with the subject's own consensus sequence.

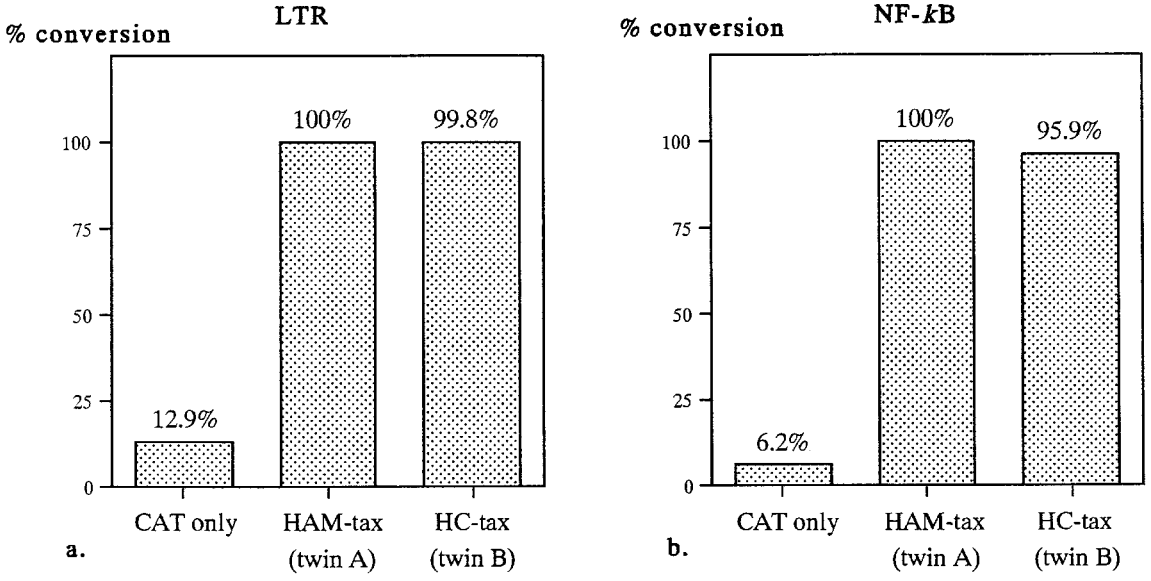

Figure 3 Transfection and CAT-ELISA in 293T cells were performed as described in Materials and methods. There was no significant difference in transactivation between twin A (HAM/TSP: the consensus sequence of *tax* region was identical to wild-type *tax*) and twin B (healthy HTLV-I carrier).

Transactivation potentials of Tax proteins encoded by two consensus tax *sequences derived from monozygotic twins are equivalent*

In order to elucidate the biological consequence of the difference in primary structure between the consensus *tax* clones of twin A and B, transcriptional transactivation potentials of their protein products were compared by chloramphenicol acetyltransferase (CAT) ELISA in 293T cells cotransfected with a reporter plasmid, pCHL4 or pκB3-CAT. In the two reporters, CAT gene expression was under the control of HTLV-I LTR and κB enhancer/promoter, respectively. As shown in Figure 3, non-ATK-1 type Tax from twin B activated CAT in both reporters as efficiently as ATK-1 type Tax from twin A.

Discussion

The exact microvariation of HTLV-I or host genetic background involved in the pathogenesis of HAM/TSP remains unclear at present. We followed a family with monozygotic twins infected with HTLV-I. Interestingly, one of the twins was a HAM/TSP patient while the other remains healthy. Since the genetic backgrounds of monozygotic twins should be identical, it is unlikely that HLA genotypes were involved in the discordant clinical outcomes of HTLV-I infection in the twins. One of the testable possibilities was that microvariations between the viruses in the twins might play a role in the discordant development of HAM/TSP. This prompted us to investigate the genetic variation in the *tax* region of proviruses in PBMCs from monozygotic twins and two other infected relatives including another patient with HAM/TSP.

Sequence analysis revealed that each infected individual harbored a single consensus *tax* sequence. Mother, elder brother and twin A shared the consensus sequence identical to ATK-1, but that of twin B was different at five nucleotide positions including three nonsynonymous substitutions from ATK-1. This clearly indicated that different HTLV-I strains were the cause of infection in the monozygotic twins. The reason for existence of different consensus *tax* sequences of twin B and mother are unknown. Infected members had never received blood transfusion and there is a possibility that consensus *tax* sequence of twin B exists as a minor clone in the HTLV-I clones of mother. It is conceivable that ATK-1 type HTLV-I infection was a risk for HAM/TSP in their genetic background, and some of amino acid changes between the two consensus sequences played a key role in the discordant HAM/TSP in the monozygotic twins.

Renjifo *et al* (1995) previously proposed a *tax* (C-T) mutation at position 7959 as a risk of HAM/TSP. However, in the present study we found this mutation in the virus of a healthy twin B, but not in that of twin A with HAM/TSP. This strongly argues against their proposal.

We also measured the amount of HTLV-I proviral DNA in PBMC of twin A and B by competitive PCR method and demonstrated that the proviral load of twin A was almost eight times higher than that of twin B. The proviral loads of twin A and elder brother were higher than those of twin B and mother, indicating that proviral load is closely related to the development of HAM/TSP. The viral transcriptional transactivator, Tax, activates its own viral promoter in the LTR sequence and is thought to be indispensable for viral replication. In addition, Tax has the potential to upregulate the expression of various cellular genes involving cell proliferation, such as interleukin-2 (IL-2), IL-2 receptor, IL-6, GM-CSF, and c-*fos* (Inoue *et al*, 1986; Cross *et al*, 1987; Fujii *et al*, 1988; 1991; Miyatake *et al*, 1988). It is conceivable that Tax plays a key role in the high virus load in HAM/TSP and disease progression. A possible biological consequence of variation in the two *tax* consensus sequences was examined, but there was no significant difference in transactivation activity on HTLV-I LTR and κB between the products of the two sequences. The high proviral load in twin A could thus not be explained by a higher transactivation activity of ATK-1 type Tax.

Discordant virus sequences found in twin A and B may cause different immunological responses due to the altered interaction of substituted viral epitopes with some HLA molecules. Two of the three members infected with the ATK-1 type virus, twin A and the elder brother, developed HAM/TSP. Because the mother remains healthy, combination of maternal HLA types and ATK-1 type virus is unlikely to be a risk for HAM/TSP. According to HLA serotypes in these family members, a distinct maternal HLA allele was transmitted to each of twin A and the elder brother. On the other hand, the two HAM/TSP patients shared HLA B55, Cw1, DR4, and DQ4 which were absent in mother and thus presumed to have originated from a paternal allele. It is conceivable that some of these HLA molecules might interact with peptides of ATK-1 type Tax, but not with non-ATK-1 type, and cause HAM/TSP. Identification of the immunodominant epitopes in HTLV-I *tax* protein restricted by the HLA allele of the monozygotic twins is underway in our laboratory.

Niewiesk and Bangham (1996) analyzed the viral HTLV-I *tax* quasispecies of HAM/TSP patients and healthy carriers using the algorithm developed by Nei and Gojobori (1986). They showed that the ratio of nonsynonymous to synonymous changes (*Dn/Ds* ratios) in HTLV-I proviral *tax* sequences from healthy seropositive subjects was higher than that in HAM/TSP patients. In contrast, Saito *et al* (1995) reported highly frequent variations of the pX sequence within both of HAM/TSP patients and asymptomatic carriers. In the present study, non-

synonymous substitutions were markedly high in *tax* from twin B rather than twin A, supporting the findings reported by Niewiesk *et al* (1996). The high *Dn/Ds* ratios theoretically imply the presence of intense selection forces. On the basis of the low *Dn/Ds* ratio and an extremely high virus load in HAM/TSP patients, Nowak and Bangham (1996) postulated that the outcome of HTLV-I infection was primarily determined by the CTL response of the individual. A high CTL response results in a low proviral load and selects escape variants with a higher rate of amino acid changes (Niewiesk *et al*, 1994; Bangham *et al*, 1999). In contrast, low CTL responders develop a high proviral load with less sequence diversity, and the resulting chronic T cell activation causes tissue damages such as those seen in HAM/TSP patients. However, the role of the CTL response in generating HTLV-I quasispecies remains controversial. In the present study, either of maternal HLA class 1 alleles, which would determine the CTL response, was shared by her two HAM/TSP children. Involvement of the selection force other than CTL, such as antibody, in the high *Dn/Ds* ratio of healthy carriers should also be considered.

In summary, the present study showed that different HTLV-I strains infected the monozygotic twins, suggesting that the difference in infected proviral sequences may play a role in their discordant clinical outcomes. Transfection and subsequent reporter assays failed to show a significant difference between the products of the two sequences in transactivation activity on HTLV-1 LTR and NF-κB elements. Moreover, two HAM/TSP patients among three family members infected with an identical virus shared a paternal HLA allele, which was absent in the remaining healthy individual. These results strongly suggest that a particular combination of HTLV-I sequences with an HLA genotype would be a risk for HAM/TSP.

Materials and methods

Materials

We followed a familial case of HAM/TSP, who was one of monozygotic twins (twin A); the other twin remained an asymptomatic HTLV-I carrier (twin B). The probability of twins being monozygotic was 99.999% (Motomura *et al*, 1990). The sera and CSF samples from four infected individuals were tested for anti-HTLV-I antibodies by the particle agglutination (PA) method. The proband was a 56-year-old man who suffered from spastic paraplegia with neurogenic bladder for 36 years. Twin A met the criteria for HAM/TSP proposed by Osame *et al* (1987). Twin B was seropositive for anti-HTLV-I antibody, but had no myelopathy. Twins had never received blood transfusion. All samples were taken under informed consent.

HLA serotyping

Serologic typing for HLA class I and II antigens was performed by the immunomagnetic HLA typing technique (Vartdal *et al*, 1986).

DNA extraction

PBMCs were obtained from heparinized venous blood by Ficoll-Conray gradient centrifugation (Daiichi Pharmaceutical, Tokyo, Japan), as described previously (Mizokami *et al*, 1996). Approximately 10^7 cells were lysed in 0.5 ml of lysing buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 0.5% SDS) and treated with proteinase K (100 μg/ml) for 2 h at 50°C. After phenol/chloroform extraction and ethanol precipitation, high molecular weight DNA was resuspended in 10 mM Tris (pH 8.0) and 1 mM EDTA.

Semi-quantitative assay of HTLV-I proviral DNA

The following oligonucleotide primers were used to amplify a 246 bp fragment of the HTLV-IpX gene: primer (+) ACTGTAGAGCTGAGCCGATAACG positioned at 7283–7306 and primer (−) ACTGTAGAGCTGAGCCGA positioned at 7506–7529 (Sawaday Technology Co., Japan). PCR MIMIC construction kit (Clontec Laboratories Inc., Fabian Way, CA, USA) was used for competitive PCR (Furuya *et al*, 1997). A cDNA fragment (633 bp) the v-*erb*B gene was used as the competitive internal standard in PCR amplification for quantitation of the target gene. Two rounds of PCR amplification were performed. In the first round, two composite primers were used. Each composite primer has the target gene primer sequence attached to a short sequence designed to hybridize to opposite standards of heterologous DNA fragments [primer (+) CAAGTTTCGTGAGCTGA positioned at 128–148, primer (−) TTGAGTCCATGGGGAGCTTT positioned at 544–564]. Then, aliquots of the first PCR were amplified by using only the gene-specific primers. This insured that all mimic molecules have a complete gene-specific primer sequences. The size of the mimic molecules was 436 bp by selecting appropriate sequences of the v-*erb*B fragment for composite primers. Following the second PCR amplification, the mimic molecules were purified by passage through CHROMA SPIN+TE 100 columns. The yield of mimic molecules was calculated and diluted to 100 attomole/μl. A twofold dilution series was used to measure HTLV-I proviral DNA (from 2 M1=6000 copies/μl to 2 M10=12 copies/μl). In the next step, 2 μl of serially diluted aliquots was added to the PCR. DNA yield was approximately 50 ng from 3000 cells. Then, 2 μl of DNA (3–25 ng/μl of DNA) and 2 μl of mimic molecules were added to 46 μl of a reaction mixture consisting of 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM dNTP, 20 pmol HTLV-IpX primers, and 2.5 units Taq polymerase (Takara Biomedicals, Japan). PCR mixtures were

subjected to 35 cycles in a DNA thermal cycler (Perkin-Elmer Corporation, Norwalk, CT, USA). Each cycle consisted of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and polymerization at 72°C for 90 s. The amplified products were size-fractionated by electrophoresis on 1% agarose gel containing 0.5 mg/ml ethidium bromide. PCR products were visualized by ultraviolet transillumination, and the intensity of each band was quantified by densitometry. Provirus load was expressed as the number of copies per one thousand PBMCs.

Assays for anti-HTLV-I antibody
Sera and CSF samples of twins were tested for the presence of anti-HTLV-I antibodies, using a commercial particle agglutination (PA) kit.

Plasmids and cells
The expression vector pCG has been described and characterized previously (Tanaka and Herr, 1990). The pCHL4 contained the CAT gene driven by a *Sma*I-*Bg*II 1630 bp fragment of HTLV-I LTR (Ohtani *et al*, 1987). The κB3 CAT contained the CAT gene driven by a *Bam*HI-*Xba*I from BL CAT-3 (Jantzen *et al*, 1987). We used the 293T cell line, kidney embryonal carcinoma cell line. The cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

PCR
The whole *tax* ORF of the HTLV-I provirus was amplified by nested PCR using 0.5 μg of DNA in a 50 μl cocktail containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.25 mM dNTPs, 1 μM each primers, and 2.6 units of High Fidelity Taq Polymerase (Boehringer Mannheim, Mannheim, Germany) (Roy *et al*, 1999). The primers used were: *tax* 11 (5′-GATAGCAAACCGTCAAGCACAG-3′; positions 7158–7179) and *tax* 6 (5′-TCCTGAACTGTCTC-CACGCTTT-3′; positions 8650–8629) for the first amplification, and *tax* 15-XB (5′-TACTCTAGAAC-CATGGCCCACTTCCCAGG-3′; positions 7324–7337) and *tax* 30-Bam (5′-CTGAGGATCAGAG-CCTTAGTCT-3′; positions 8409–8397) for the second amplification. *Xba*I and *Bam*HI restriction sites (underlined) were included in *tax* 15-XB and *tax* 30-Bam primers, respectively, to allow subsequent subcloning into pCG. PCR was performed in a thermal cycler (Biometra) for 30 cycles: denaturation at 94°C for 30 s (2 min on the first cycle), annealing at 60°C for 30 s, and extenuation at 72°C for 70 s (7 min on the last cycle). For the nested PCR, 1/10 volume of the first-step PCR product purified by Superec II column (Takara Co., Japan) was further amplified for 27 cycles using the inner primer pair. The thermal cycle program was almost the same as that used for the first PCR except the annealing temperature was 58°C for the first six cycles and 63°C for the following 21 cycles. The nested PCR products were size-fractionated by electrophoresis at 100 V for 30 min on 0.7% agarose gel (Seakem GTG) containing 0.5 mg/ml ethidium bromide.

Cloning and sequencing
Bands of the expected size were digested with *Xba*I and *Bam*HI, and purified by the QIA quick Gel Extraction kit (QIAGEN, Japan). The isolated, amplified DNA was subcloned into the expression plasmid pCG. After transformation of *Escherichia coli* (DH5α), the recombinant clones were randomly picked up and the plasmid DNA was prepared by the QIAprep Spin Miniprep Kit (QIAGEN). The whole *tax* ORF (1059 bp) in the plasmid DNA was sequenced by the dideoxy chain termination method (Thermo Sequenase core sequencing kit, Amersham Life Science, Buckinghamshire, UK) using the two types of 5′ Texas Red-labeled primers corresponding to the flanking vector sequences (HSV-tK sequence; 5′-GCCCAGCGCCTTGTAGAA-3′, and rabbit-β-globin sequence; 5′-TAGC-GAAAAAGAAAGAAC-3′), and analyzed on an automatic sequencer (SQ5500; Hitachi Co., Tokyo, Japan). A sequence comparison was carried out using the DDBJ database (J02029).

Calculation of Dn/Ds
We analyzed the HTLV-I tax quasispecies with an algorithm from Nei and Gojobori (1986). In this method, the rate of nonsynonymous changes per nonsynonymous site (*Dn*) and the rate of synonymous changes per synonymous (*Ds*) can be calculated by pairwise comparison of related sequences.

Transfection
Transient transfection was performed by the lipofection method. Plasmid DNA mixed with 8 μl of Lipofectamine (GIBCO/BRL) was added to subconfluent cells cultured in 2.5 ml of serum-free DMEM in a 6 cm-diameter dish. After incubation for 8 h at 37°C, 2.5 ml of DMEM containing 20% FBS was added, and at 24 h, the medium was replaced with DMEM containing 10% FBS. Cells were used for the following assays 48 h after transfection.

CAT-ELISA
293T cells co-transfected with pCG-tax (0.5 μg) and reporter plasmids were harvested and lysed 48 h after transfection. These constructs were used to test the transactivation activity on HTLV-1 LTR CAT and κB-containing enhancer in 293T cells. Efficiency of each transfection was monitored by co-transfecting RSV-β gal. The transactivation activity was quantified with the CAT-ELISA kit (5 Prime→3 Prime, Inc., USA) according to the instructions provided by the manufacturer. The resultant color

development is proportional to the concentration of CAT protein in each microwell. Absorbences were measured with an optical densitometer.

Acknowledgments

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References

Bangham CRM, Hall SE, Jeffery KJ, Vine AM, Witkover A, Nowak MA, Wodarz D, Usuku K, Osame M (1999). Genetic control and dynamics of the cellular immune response to the human T-cell leukaemia. *Phil Trans R Soc Lond* **354:** 691–700.

Cross SL, Feinberg MB, Wolf JB, Holbrook NJ, Wong-Staal F, Leonard WJ (1987). Regulation of the human interleukin 2 receptor a chain promoter: activation of a non-functional promoter by the transactivator gene of HTLV-I. *Cell* **49:** 47–56.

Daenke S, Nightingale S, Cruickshank JK, Bangham CRM (1990). Sequence variants of human T-cell lymphotropic virus type I from patients with tropical spastic paraparesis and adult T-cell leukemia do not distinguish neurological from leukemic isolates. *J Virol* **64:** 1278–1282.

Fujii M, Sassone-Corsi P, Verma IM (1988). c-fos promoter trans-activation by the tax 1 protein of human T-cell leukemia virus type I. *Proc Natl Acad Sci USA* **85:** 8526–8530.

Fujii M, Niki T, Matsuda M, Nomura N, Seiki M (1991). HTLV-I tax induces expression of various immediate early serum responsive genes. *Oncogene* **6:** 1023–1029.

Furuya T, Nakamura T, Shirabe S, Nishiura Y, Tsujino A, Goto H, Eguchi K, Nakamura H, Nagataki S (1997). Heightened transmigrating activity of CD-4 positive T cells through reconstituted basement membranes in patients with human T-cell lymphotropic virus type I-associated myelopathy. *Proc Assoc Am Physicians* **109:** 228–236.

Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A, de Thé G (1985). Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet* **ii:** 1247–1248.

Hill AVS (1998). The immunogenetics of human infectious diseases. *Annu Rev Immunol* **16:** 593–617.

Inoue J, Seiki M, Taniguchi T, Tsuru S, Yoshida M (1986). Induction of interleukin 2 receptor gene expression by p40x encoded by human T-cell leukemia virus type I. *EMBO J* **5:** 2883–2888.

Jantzen HM, Strähle U, Gloss B, Stewart F, Schmid W, Boshart M, Miksicek R, Schütz G (1987). Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. *Cell* **49:** 29–38.

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

Kinoshita T, Tsujimoto A, Shimotohono K (1991). Sequence variations in LTR and env regions of HTLV-I do not discriminate between the virus from patients with HTLV-I associated myelopathy and adult T-cell leukemia. *Int J Cancer* **47:** 491–495.

Kira J, Koyanagi Y, Yamada T, Itoyama Y, Goto I, Yamamoto N, Sasaki H, Sakaki Y (1991). Increased HTLV-I proviral DNA in HTLV-I associated myelopathy: A quantitative polymerase chain reaction study. *Ann Neurol* **29:** 194–201.

Kira J, Koyanagi Y, Yamada T, Itoyama Y, Tateishi J, Akizuki S, Kishikawa M, Baba E, Nakamura M, Suzuki J, Nakamura T, Nakamura N, Yamamoto N, Goto I (1994). Sequence heterogeneity of HTLV-I proviral DNA in the central nervous system of patients with HTLV-I associated myelopathy. *Ann Neurol* **36:** 149–156.

Mahieux R, De Thé G, Gessain A (1995). The tax mutation at nucleotide 7959 of human T-cell leukemia virus type 1 (HTLV-1) is not associated with tropical spastic paraparesis/HTLV-1-associated myelopathy but is linked to the cosmopolitan molecular genotype. *J Virol* **69:** 5925–5927.

Miyatake S, Seiki M, Malefijt RD, Heike T, Fujisawa J, Takebe Y, Nishida J, Shilomai J, Yokota T, Yoshida M, Arai K, Arai N (1988). Activation of T-cell derived lymphokine genes in T cells and fibroblasts: effects of human T-cell leukemia virus type I p40x protein and bovine papilloma virus encoded E2 protein. *Nucleic Acid Res* **16:** 6547–6566.

Mizokami A, Eguchi K, Kawakami A, Ida H, Kawabe Y, Tsukada T, Aoyagi T, Maeda K, Morimoto C, Nagataki S (1996). Increased population of high fluorescence 1F7 (CD26) antigen on T cells in synovial fluid of patients with rheumatoid arthritis. *J Virol* **16:** 6547–6566.

Motomura M, Nakamura T, Nagasato K, Shibayama K, Kubo S, Nakasono I, Tsujihata M, Sonoda S, Yashiki S, Nagataki S (1990). HTLV-I associated myelopathy in an identical twin. *Lancet* **336:** 55.

Nei M, Gojobori T (1986). Simple method for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol Biol Evol* **3:** 418–426.

Niewiesk S, Daenke S, Parker CE, Taylor G, Weber J, Nightingale S, Bangham CRM (1994). The transactivator gene of human T-cell leukemia virus type-I is more variable within and between healthy carriers than patients with tropical spastic paraparesis. *J Virol* **68:** 6778–6781.

Niewiesk S, Bangham CRM (1996). Evolution in a chronic RNA virus infection: selection on HTLV-I tax protein differs between healthy carriers and patients with tropical spastic paraparesis. *J Mol Evol* **42:** 452–458.

Nowak MA, Bangham CRM (1996). Population dynamics of immune responses to persistent viruses. *Science* **272:** 74–79.

Ohtani K, Nakamura M, Saito S, Noda T, Ito H, Sugamura K, Hinuma Y (1987). Identification of two distinct elements in the long terminal repeat of HTLV-I responsible for maximum gene expression. *EMBO J* **21:** 117–122.

Osame M, Matsumoto M, Usuku K, Izumo S, Ijichi S, Ijichi N, Amitani H, Tara M, Igata A (1987). Chronic progressive myelopathy associated elevated antibodies to HTLV-I and adult T-cell leukemia cells. *Ann Neurol* **21:** 117–122.

Renjifo B, Borrero I, Essex M (1995). Tax mutation associated with tropical spastic/human T-cell leukemia virus type I-associated myelopathy. *J Virol* **69:** 2611–2616.

Roy BC, Mizokami A, Futsuki YI, Moriuchi R, Katamine S (1999). Functional loss of the HTLV-U Tax protein encoded by variant proviruses in infected individuals. *Acta Med Nagasaki* **43:** 40–47.

Saito M, Furukawa Y, Kubota R, Usuku K, Sonoda S, Izumo S, Osame M, Yoshida M (1995). Frequent mutation in pX region of HTLV-I is observed in HAM/TSP patients, but is not specifically associated with the central nervous system lesions. *J Neurovirol* **1:** 286–294.

Seiki M, Hattori S, Hirayama Y, Yoshida M (1983). Human adult T-cell leukemia virus: Complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. *Proc Natl Acad Sci USA* **80:** 3618–3622.

Shirabe S, Nakamura T, Tsujihata M, Nagataki S, Seiki M, Yoshida M (1990). Retrovirus from human T-cell leukemia virus type I-associated myelopathy is the same strain as a prototype human T-cell leukemia virus type I. *Arch Neurol* **47:** 1258–1260.

Sonoda S, Fujiyoshi T, Yashiki S (1996). Immunogenetics of HTLV-I/II and associated diseases. *J AIDS Hum Retrovirol* **13** (Suppl 1): 119–123.

Tanaka M, Herr W (1990). Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. *Cell* **60:** 375–386.

Usuku K, Sonoda S, Osame M, Yashiki S, Takahashi K, Matsumoto M, Sawada T, Tsuji K, Tara M, Igata A (1988). HLA haplotype-linked high immune responsiveness against HTLV-I in HTLV-I-associated myelopathy: comparison with adult T-cell leukemia/lymphoma. *Ann Neurol* **23** (Suppl 1): 143–150.

Vartdal F, Gaudernack G, Funderud S (1986). HLA class I and II typing cells positively selected from blood by immunomagnetic isolation – a fast and reliable technique. *Tissue Antigen* **28:** 301–312.

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