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Relapsing cervical cord lesions on MRI in patients with HTLV-I–associated myelopathy

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Human T lymphotropic virus type I (HTLV-I) is associated with adult T cell leukemia (ATL) and a chronic progressive disease of the CNS termed HTLV-I–associated myelopathy (HAM)/tropical spastic paraparesis (TSP).[1,2] The most common MRI findings of HAM/TSP are spinal cord atrophy predominantly involving the thoracic cord levels. In this article, we report two patients with HAM/TSP, who developed transient cervical cord lesions on MRI during the course of the illness.

Case reports. Patient 1 is 39-year-old woman who developed progressive gait and urinary disturbance when she was 22 years old. At age 35, she was diagnosed with HAM/TSP. MRI taken at age 37 showed spinal cord atrophy predominantly in the thoracic region (see figure E-1 on the *Neurology* Web site at www.neurology.org). Despite various treatments, her symptoms gradually worsened. At age 39, she noticed numbness and pain in the right upper limbs. On neurologic examination, muscle strength in the lower limbs was moderately decreased. Deep tendon reflexes were mildly exaggerated in the upper limbs and highly exaggerated in the lower limbs. Babinski signs were positive bilaterally. Superficial and deep sensations were disturbed below T10 level. She had urinary frequency and residual urine (170 mL). Anti-HTLV-I antibody was positive both in serum (×32,768) and in CSF (×512). HTLV-I provirus load was 55 copies/10^4 peripheral blood mononuclear cells. CSF showed increased protein content (53 mg/dL), IgG level of 12.5 mg/dL, and cell count of 7/mm^3. Myelin basic protein level was normal, and there were no oligoclonal bands detected in the CSF. Western blotting of CSF for anti-HTLV-I was positive for p19, p24, p28, p53, and env. Cervical MRI demonstrated swelling of the spinal cord at C5 to C6 levels with gadopentetate dimeglumine enhancement (figure). T2-weighted image showed high intensity signals at the same levels on sagittal section. Axial T2-weighted image at the C5 level showed high intensity signals in the lateral columns bilaterally. She was treated with IV high-dose methylpredonisolone followed by oral prednisolone treatment; cervical MRI gradually decreased with improvement of symptoms in the upper limb.

Patient 2 is a 72-year-old woman who developed progressive gait and pollakiuria at age 59 years and was subsequently diagnosed with HAM/TSP. Despite various treatments, her symptoms gradually worsened, and she became unable to walk. Since age 72, she noticed muscle weakness in the upper limbs. On neurologic examination, muscle strength in the lower limbs was moderately decreased. Deep tendon reflexes were mildly exaggerated in the upper limbs and highly exaggerated in the lower limbs. Babinski signs were positive bilaterally. She had difficulty in urination and residual urine (250 mL). Anti-HTLV-I antibody was positive both in serum (×131,072) and in CSF (×512). CSF showed increased protein content (53 mg/dL) and cell count (1/mm^3). Cervical MRI demonstrated swelling of the entire cervical spinal cord region (see figure E-2, A and B). T2-weighted image showed high intensity signals at the same level on sagittal section. Axial T2-weighted image at the C5 level showed high intensity signals in the lateral columns bilaterally. She was treated with IV high-dose methylprednisolone followed by oral prednisolone treatment; cervical MRI gradually decreased with improvement of muscle weakness in the upper limbs (figure E-2, C).

Discussion. The characteristic finding in these two patients is the appearance of abnormal lesions in the cervical cord on MRI during the long-standing course of the illness. Disappearance of high intensity lesions in the cervical cord after corticosteroid treatment suggests that inflammation or edematous changes may be the cause.

Recently, several cases of HAM/TSP with MRI abnormalities in the spinal cord have been reported.[3-5] These changes consisted of spinal cord swelling with high intensity signals and contrast enhancement from the cervical to the thoracic levels. Recently, we reported four patients who developed slowly progressive myelopathy with abnormal MRI lesions at the cervical cord levels; we proposed that these four cases may belong to a variant form of HAM/TSP, predominantly involving the cervical cord levels.[6] In the two patients presented here, MRI abnormalities at the cervical cord level were associated with worsening of symptoms in the upper limbs, suggesting that these MRI abnormalities also indicate active inflammatory changes. Neuropathologic analysis of HAM/TSP demonstrated diffuse parenchymal infiltration involving the entire spinal cord, including the cervical cord levels.[7] Therefore, abnormal MRI findings at the cervical cord also indicate active inflammatory changes in the current patients.

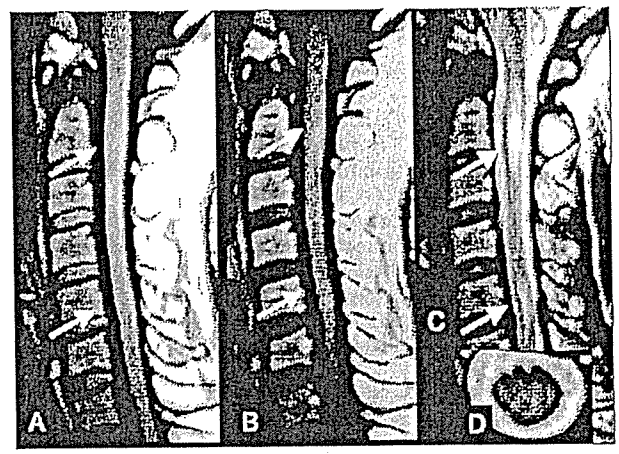

Figure. Cervical MRI showed focal swelling (A, arrows) with slight gadolinium enhancement (B, arrows) on T1-weighted imaging. T2-weighted image showed high intensity signals at the same level on sagittal section (C, arrows), which was located at the center of the spinal cord (D, C2 level).

Additional material related to this article can be found on the *Neurology* Web site. Go to www.neurology.org and scroll down the Table of Contents for the January 24 issue to find the title link for this article.

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HTLV-I viral escape and host genetic changes in the development of adult T Cell leukemia

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In the pathogenesis of adult T cell leukemia (ATL), an oncogenetic role of the human T cell lymphotropic virus type I (HTLV-I) Tax protein, viral escape from the host immune system, and host genetic changes have been proposed as contributory factors. We examined the premature stop codons in *tax* gene as one of the mutations that may lead to escape of HTLV-I from the cytotoxic T lymphocyte (CTL) response in HTLV-I carriers, to test whether a putative CTL escape mutant can emerge in the early stage of ATL development and whether HTLV-I infected cells with such a mutation can proliferate subsequently. We also examined deletion of cyclin-dependent kinase inhibitor 4 (*INK4*) genes and mutation of *p53* gene in combination with changes in the HTLV-I genome in acute type ATL to test whether host genetic changes promoted the malignant transformation of ATL cells that carry putative CTL escape mutations. The premature stop codon in *tax* gene existed in many non-ATL HTLV-I carriers as a minor population but not in the commonest HTLV-I sequence of the individual. This minor population with a premature stop codon did not expand subsequently in 3 asymptomatic carriers tested. There were cases who had a mutation or deletion in HTLV-I who also have either deletion of *INK4* genes or mutation in *p53* gene. Our findings suggest that CTL escape mutation can occur at an early stage of ATL development, and that certain host genetic changes favor the development of the aggressive form of ATL.
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Key words: viral escape; INK4; p53; ATL

Adult T cell leukemia (ATL) is a T cell malignancy with clonal proliferation of human T cell leukemia virus type I (HTLV-I) infected cells.[1,2] HTLV-I is also an etiologic agent for HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[3,4] ATL is subdivided into 4 types (smoldering, chronic, lymphoma and acute).[5] ATL has a long incubation period and smoldering and chronic type ATL sometimes transform into a more aggressive acute/lymphoma type of ATL, suggesting a multistep leukemogenesis model[6] for the development of ATL.

HTLV-I Tax protein is a key regulator for immortalization, transformation and oncogenesis of the HTLV-I infected lymphocytes through its interaction with many cellular proteins. For example, Tax binds to CBP/p300 and determines the accessibility of CBP/p300 to protein complexes on specific DNA elements,[7] resulting in Tax mediated trans-activation of viral genes[8] and growth factors,[9] or trans-repression of p18,[10] DNA polymerase β and bax genes.[11] Tax also modifies the cell cycle through binding $p16^{INK4A}$,[12] hDLG[13] and MAD1[14] and contributes to the development of ATL.

Tax also plays a role as an immunodominant target antigen for the cytotoxic T lymphocyte response (CTL)[15,16] to HTLV-I and Tax-expressing cells will be rejected by the host immune response. It is possible therefore that immortalized cells that elicit weaker CTL responses are selected during the development of ATL. We have reported previously several mutations and deletions in the *tax* gene in ATL that can escape from the host immune system.[17] A premature stop codon in the *tax* gene (substitution at nt. position 7464 from G–A) that was observed frequently in ATL, is one such escape mutation, because the resulting truncated Tax protein loses its transactivational activity[18] and expression of HTLV-I related proteins is diminished. This premature stop codon was also observed in the chronic type of ATL as a consensus sequence of the patient,[17] suggesting that Tax is dispensable at least in some chronic ATL cases.

These findings suggest that viral escape from CTL recognition is one of the important steps for the development of ATL; however host genetic changes such as cyclin-dependent kinase inhibitor genes and *p53* gene have also been observed in ATL.

The signaling pathway governed by G1 cyclins, cyclin-dependent kinases (CDK), pRb and E2F plays a major regulatory role during G1 to S transition in the cell cycle.[19,20] The complex formed by CDK4 and D-type cyclins controls the passage of cells through G1 phase, and the function of CDK4/CDK6 complexes is inhibited by a number of inhibitor of CDK4 (INK4), *i.e.*, $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$.[21–25] The human $p16^{INK4A}$ and $p15^{INK4B}$ genes are situated within 30kb on chromosome 9p21.[26] $p18^{INK4C}$ and $p19^{INK4D}$ proteins also inhibit the activities of D-type CDK.[23–25] Among these INK4, however, $p16^{INK4A}$ is most impaired frequently in tumor cells.[27] There is another tumor suppressor gene named $p14^{ARF}$,[28] encoded in an alternative reading frame (ARF) of the $p16^{INK4A}$ gene. The *p53* gene is another tumor suppressor gene.[29] Mutations of the *p53* gene have been found in several malignancies including ATL.[30]

We wondered when a premature stop codon in the tax gene, a putative CTL escape mutant, emerged during the development of ATL, and whether HTLV-I with this stop codon would subsequently proliferate. To this end, we examined the occurrence of a premature stop codon in the tax gene in 219 asymptomatic carriers and 143 HAM/TSP patients. We also examined the proportion of HTLV-I infected cells with this stop codon in 3 asymptomatic carriers at different time points to test whether such HTLV-I infected cells continuously proliferate in asymptomatic HTLV-I carriers without ATL. We also examined the deletion of cyclin-dependent kinase (CDK) 4 inhibitor genes ($p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$) in 23 acute ATL patients and mutations of *p53* gene in 22 ATL patients to investigate whether additional host genetic changes favor the development of the aggressive form of ATL.

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Informed consent was obtained from all HTLV-I carriers and patients. This research was approved by the institutional review boards of the author's institutions, and human experimentation guidelines of the US Development of Health and Human Services and those of the author's institutions were followed in the conduct of clinical research.

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Material and methods

Study population

Two hundred and nineteen HTLV-I seropositive asymptomatic blood donors (AC) and 143 cases of HAM/TSP whose *tax* gene had not been sequenced in our previous study[17] were examined for the stop codon in the *tax* gene. Twenty-three cases of acute type ATL whose tax genes were sequenced in the previous study[17] were tested for the deletion of cyclin-dependent kinase inhibitor 4 genes and 22 of these cases were tested for a mutation in the *p53* gene. All cases were of Japanese ethnic origin and resided in Kagoshima prefecture (Japan). The diagnosis and clinical subtyping of ATL were made according to Shimoyama's criteria.[5] The diagnosis of HAM/TSP was made according to WHO diagnostic criteria.[31]

Proviral load measurement

The HTLV-I provirus load in peripheral blood mononuclear cells (PBMC) was measured in HAM/TSP patients and HC as described.[32] A quantitative PCR reaction was carried out using the ABI PRISM 7700 sequence detector (Perkin-Elmer Applied Biosystems, Tokyo, Japan). The amount of HTLV-I proviral DNA was calculated as follows: copy number of HTLV-I (*tax*) per 10^4 PBMC = [copy number of *tax*/(copy number of β-actin/2)] × 10^4. The lower limit of detection was 1 copy/10^4 PBMC.

Statistical analysis

The Mann-Whitney *U*-test was used for statistical analysis of HTLV-I provirus load and the variables were treated as continuous.

Restriction fragment length polymorphism analysis of the HTLV-I tax gene

Substitution at nt. position 7464 from G–A on the *tax* gene created a premature stop codon[17] and also created a Bln I restriction site (CCTGGG–CCTAGG). This stop codon was observed frequently in ATL in the commonest sequence of the individual ATL patients. Restriction fragment length polymorphism (RFLP) analysis using Bln I was done on 219 AC and in 143 HAM/TSP cases. Nested polymerase chain reaction (PCR) was carried out on the extracted DNA to amplify proviral DNA and the amplified product was digested with Bln I. One hundred nanograms of DNA were amplified by 35 cycles of PCR using expand high fidelity PCR system (Boehringer Mannheim, Tokyo, Japan) and 1 μM primers (PX01+: 5′-TCGAAACAGCCCTGCAGATA-3′ [7257–7276] and PX02−: 5′-TGAGCTTATGATTTGTCTTCA-3′ [8447–8467]). After the first PCR reaction, 1 μl aliquots of the amplified products were subjected to further 20 cycles of the second PCR using internal primers (PX11+: 5′-ATACAAAGTTAACCATGCTT-3′ [7274–7293] and PX11−: 5′-GGGTTCCATGTATCCATTTC-3′ [7644–7663]). Each PCR cycle consisted of denaturation at 94°C for 60 sec, annealing at 58°C for 75 sec, extension at 72°C for 90 sec and extension of the final cycle at 72°C for 10 min. Two μl of the nested PCR product was digested with 5 U of Bln I (Takara, Tokyo, Japan) in 10 μl volume at 37°C for 18 hr and was then electrophoresed on 1% agarose gel.

Proportion of HTLV-I infected cells with stop codon in the tax gene in asymptomatic carriers at different time points

RFLP analysis showed that there are AC and HAM/TSP patients that have a stop codon in the *tax* gene as a minor subpopulation of HTLV-I infected cells. To test whether such HTLV-I infected cells with a premature stop codon in the *tax* gene that can escape from the host immune response to HTLV-I can expand subsequently as a major population, we carried out RFLP analysis at different time points in 3 asymptomatic carriers. Case 1 was examined with samples taken on June 25 1999 and June 26 2000. Case 2 was examined with samples taken on Jan 14 2000 and Jan 26 2001. Case 3 was examined with samples taken on May 26 2000, Jan 25 2002 and Oct. 22 2004. RFLP analysis suggested that in Case1, the proportion of HTLV-I infected cells with the premature stop codon decreased after 1 year. In this case, to quantify the ratio of HTLV-I infected cells with the premature stop codon to HTLV-I infected cells without this premature stop codon, the nested PCR product was cloned into pCR-Blunt II-TOPO vector (Zero Blunt TOPO PCR cloning kit: Invitrogen), transformed into competent *E. coli* cells and spread on LB plates containing 50 μg/ml kanamycin. Colonies from the plate were cultured overnight in LB medium containing 50 μg/ml kanamycin, and plasmids containing subcloned tax genes were extracted. Purified plasmids containing the subcloned tax gene were digested with Eco RI and Bln I and then electrophoresed on 1 % agarose gel. When the subcloned *tax* gene was cleaved by Bln I, the subclone was judged as having the stop codon, and if uncleaved, the subclone was judged as not having the stop codon. The proportion of HTLV-I cells that carry the stop codon was then calculated.

Southern blot analysis of $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ *and* $p19^{INK4D}$ *and HTLV-I*

Southern blot analysis of $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$ was carried out in 23 cases with acute type ATL. Southern blot analysis of HTLV-I was also carried out. High molecular weight DNA was extracted by a standard method using phenol extraction. In Southern blot analysis for cyclin-dependent kinase inhibitor genes ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, $p19^{INK4D}$), 10μg of genomic DNA was digested with Hind III, separated on a 1% agarose gel, and transferred to a nylon membrane. Probes used in hybridization were a EcoRI–XhoI fragment of $p16^{INK4A}$ cDNA, EcoRI–XhoI fragment of $p15^{INK4B}$ cDNA, BamIII–BamIII fragment of $p18^{INK4C}$ cDNA and EcoRI–EcoRI fragment of $p19^{INK4D}$ cDNA. All of these probes were provided from Dr. Hirai (Banyu Tsukuba Research Institute). The same filters were rehybridized successively with the respective probes. Nylon membranes were also hybridized with β-globin probe. Probe DNA fragments were labeled with α-^{32}P-dCTP by random priming. Blots were hybridized at 65°C for 12 hr in a mixture containing 4× SSC (1× SSC; 0.15 M NaCl, 0.015 M sodium citrate) and 50μg of sonicated and denatured salmon sperm DNA and then washed in 0.1% sodium dodecylsulfate (SDS) and 1× SSC at 65°C for 30 min, and autoradiographed, then exposed to a imaging plate and analyzed by a laser image analyzer (MAC-BAS-1000). Southern blot analysis of HTLV-I was also done in our previous study[17] with 10μg of genomic DNA digested with Pst I and hybridized with total sequence of HTLV-I as a probe. The same filters were rehybridized with a ^{32}P-labeled HTLV-1 long terminal repeat (LTR) probe.

Sequence of p53 *gene*

The sequence of *p53* was examined in 22 ATL cases. Three *p53* fragments were amplified using nested PCR: (*i*) 371 bp encompassing the entire exon 4; (*ii*) 499 bp encompassing the entire exons 5 and 6; and (*iii*) 692 bp encompassing the entire exons 7 and 8. The primers used for PCR encompassing exon 4 were sense 5′-AACGTTCTGGTAAGGACAAGGG-3′ (p53_41) and antisense 5′-AAGGGTGAAGAGGAATCCCAAA-3′ (p53_42) for the first PCR and sense 5′-AGGACCTGGTCCTCTGACTG-3′ (p53_43) and antisense 5′-ATACGGCCAGGCATTGAAGT-3′ (p53_44) for the second PCR. The primers used for PCR encompassing exons 5 and 6 were sense 5′-TAGTGGGTTGCAGGAGGTGCTT-3′ (p53_51) and antisense 5′-GCAGGAGAAAGCCCCCCTACTG-3′ (p53_62) for the first PCR and sense 5′-TATCTGTTCACTTGTGCCCT-3′ (p53_53) and antisense 5′-GGCCACTGACAACCACCCTT-3′ (p53_64) for the second PCR. The primers used for PCR encompassing exons 7 and 8 were sense 5′-GACAGAGCGAGATTCCATCTCA-3′ (p53_71) and antisense 5′-GCTGGTGTTGTTGGGCAGTGCT-3′ (p53_82) for the first PCR and sense 5′-AGGTCTCCCCAA- GGCGCACTGG-3′ (p53_73) and antisense 5′-GGCATAACTGCACCCTTGGTCT-3′ (p53_84) for the second PCR. One hundred nanogram DNA was amplified by 35 cycles for the first PCR using the Expand

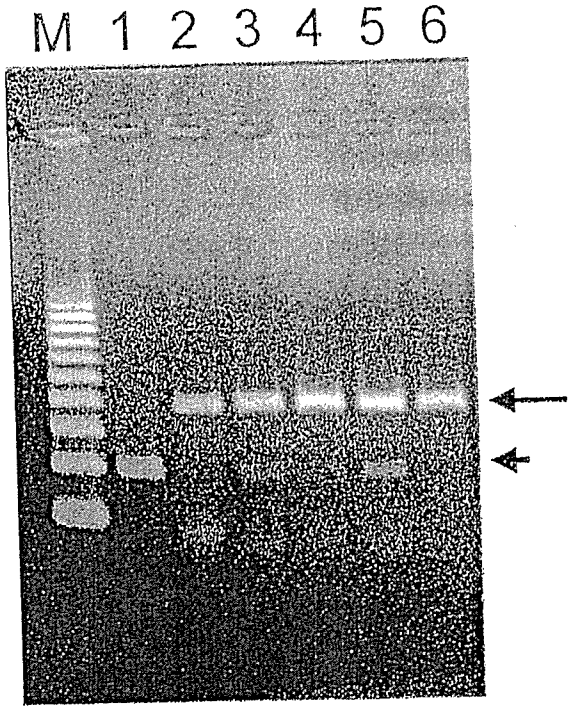

FIGURE 1 – RFLP analysis of *tax* gene. Nested PCR products of *tax* was digested by restriction enzyme Bln I. PCR product in ATL case with a premature stop codon at nt. 7464 in HTLV-I genome was completely cut by Bln I (*lane 1*), whereas in ATL case without the stop codon at nt. 7464 was not cut by Bln I (*lane 2*). PCR products in HAM case (*lane 3*) and in AC case (*lane 5*) was partially cut by Bln I, suggesting existence of a minor population of HTLV-I infected cells with the premature stop codon at nt. 7464 in HTLV-I *tax* genome, whereas in other HAM case (*lane 4*) and in AC case (*lane 6*), PCR products were not cut by Bln I. Long arrow indicates the nested PCR product and short arrow indicates the band cut by Bln I. M, 100 base marker.

high-fidelity PCR system (Boehringer Mannheim, Tokyo, Japan) and 1 μM of each primers. After the first PCR , 1 μl of aliquots of the amplified products were subjected to an additional 20 cycles of the second PCR using internal primers. Each PCR cycle consisted of denaturation at 95°C for 60 sec, annealing at 60°C for 75 sec, extension at 72°C for 120 sec and extension of the final cycle at 72°C for 10 min. Amplified DNA products were purified using QIA quick purification kit (Qiagen, Tokyo, Japan) and 0.1μg of PCR products were sequenced using dye terminator DNA sequencing kit (Applied Biosystems, Tokyo, Japan) with 3.2 pmol of each primers (p53_43 and p53_44 for exon 4, p53_53 and p53_64 for exons 5 and 6, p53_73 and p53_84 for exons 7 and 8) in an automatic sequencer (377 DNA Sequencer, Applied Biosystems).

Results

Premature stop codon in the tax *gene in AC and in HAM/TSP patients*

In 219 asymptomatic carriers and in 143 HAM/TSP patients, there was no case that had a premature stop codon in the *tax* gene in the commonest sequence of the individual. There are cases, however, who had HTLV-I infected cells with this premature stop codon in the *tax* gene as a minor population of the HTLV-I infected cells. Figure 1 shows representative results. In the ATL case with a premature stop codon in the HTLV-I *tax* gene, all of the nested PCR product was cut by Bln I (Fig. 1, *lane 1*). Under the same experimental conditions, there were no AC or HAM/TSP patients whose nested PCR products were completely cut by Bln I, but there was partial cleavage in some cases (Fig. 1., *lanes 3,5*).

TABLE I – HTLV-I PROVIRUS LOAD IN AC AND IN HAM/TSP IN ASSOCIATION WITH OR WITHOUT THE PREMATURE STOP CODON IN TAX GENE AS MINOR POPULATION OF HTLV-I INFECTED CELLS[1]

	AC (n = 219)		HAM (n = 143)	
	+	–	+	–
n	79	140	78	65
Median	166*	34*	523	420

[1]HTLV-I copy number per 10^{-4} PBMC was represented. n = number of subjects. + Subjects with the premature stop codon in tax gene as minor population of HTLV-I infected cells detected by RFLP analysis – Subjects without premature stop codon in tax gene; HAM, patients with HAM; AC, asymptomatic carriers. *p-value < 0.001 by Mann-Whitney U-test.

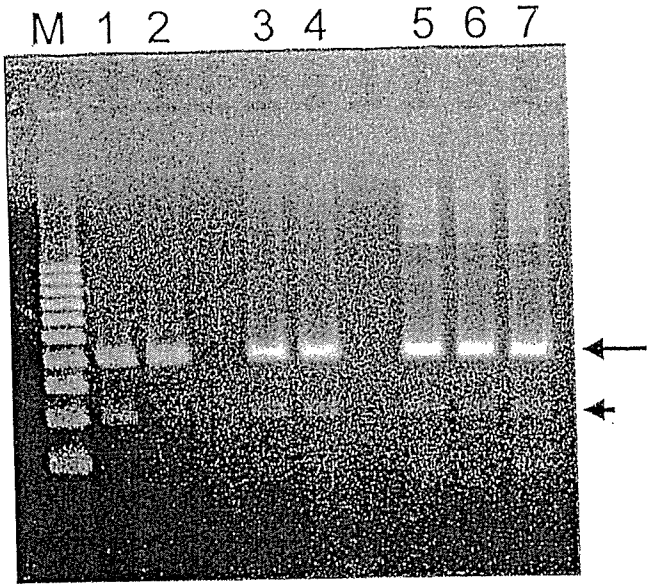

FIGURE 2 – RFLP analysis of *tax* gene at different time points. RFLP analysis of nested PCR product of *tax* gene digested by Bln I was done in 3 asymptomatic carriers at different time points. Case 1 (*lane 1* at June 25 1999 and *lane 2* at June 26 2000). Case 2 (*lane 3* at Jan 14 2000 and *lane 4* at Jan 26 2001). Case 3 (*lane 5* at May 26 2000, *lane 6* at Jan 25 2002 and *lane 7* at Oct. 22 2004). Long arrow indicates the nested PCR product and short arrow indicates the band cut by Bln I. M, 100 base marker.

There were 79 cases of 219 AC (36.1%) and 78 cases of 143 HAM/TSP patients (54.5%) that had HTLV-I infected cells with the premature stop codon in the *tax* gene as a minor population of the individuals (Table I).

The median provirus load in AC who had the premature stop codon in the *tax* gene as a minor population of HTLV-I infected cells was 166 and the median provirus load in AC who did not have this premature stop codon in the HTLV-I infected cells was 34.5, and this difference was significant ($p < 0.001$). The median provirus load in HAM patients who had the premature stop codon in *tax* gene as a minor population of HTLV-I infected cells was 523 and the median provirus load in HAM patients who did not have this premature stop codon in HTLV-I infected cells was 420, and this difference was not significant ($p = 0.305$) (Table I).

Proportion of HTLV-I infected cells with a stop codon in the tax gene as a minor population in asymptomatic carriers at different time points

In 3 asymptomatic carriers having the premature stop codon in the *tax* gene as a minor population of HTLV-I infected cells, we examined whether this minor population expanded subsequently.

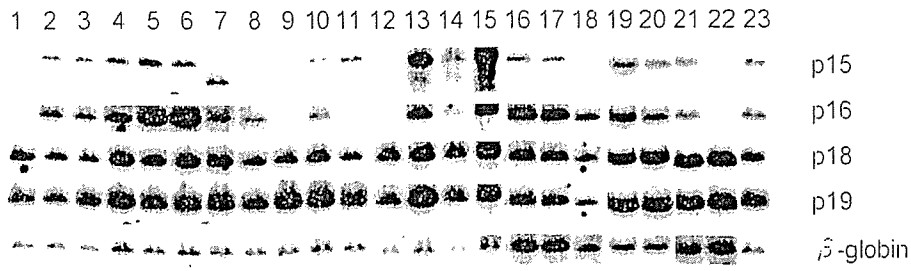

FIGURE 3 – Southern blot analysis of $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$ and β-globin in 23 acute ATL. Ten micrograms of genomic DNA was digested with Hind III and hybridized with each probe. Cases 1, 7–12, 14 and 22 has weak or decreased $p16^{INK4A}$ band when compared to the density of β-globin band. Cases 1, 8–10, 12, 14, 18 and 22 has weak or decreased $p15^{INK4B}$ band and Case 7 has a shorter $p15^{INK4B}$ band. Abbreviations: p15, $p15^{INK4B}$; p16, $p16^{INK4A}$; p18, $p18^{INK4C}$; p19, $p19^{INK4D}$.

Figure 2 shows that this minor population with the premature stop codon in the *tax* gene in these 3 asymptomatic carriers did not expand subsequently (*Case 1, lane 1,2*; *Case 2, lane 3,4*; *Case 3, lanes 5–7*) . In Case 1, the proportion of the population with premature stop codon decreased subsequently when analyzed by RFLP (Fig. 2, *lanes 1,2*). To quantify this, we subcloned the PCR product and counted the number of clones that had a stop codon at different time points. The number of subclones that had a premature stop codon in the *tax* gene were 9 of 59 (15.3%) on June 25 1999, and was 2 of 63 (3.2%) on June 26 2000.

Deletion of cyclin-dependent kinase 4 inhibitor genes and mutations in HTLV-I provirus in acute type ATL patients

Judged from the density of the $p16^{INK4A}$ gene band in Southern blot hybridization compared to the density of the band of β-globin using an image analyzer, 9 of 23 (39.1%) acute-type ATL patients deleted the $p16^{INK4A}$ gene in leukemic cells (Fig. 3). Similarly, an absent or weak hybridization band indicating deletion of the $p15^{INK4B}$ gene was observed in 8 of 23 acute type ATL patients and a shorter size of the $p15^{INK4B}$ gene band indicating partial deletion or rearrangement of this gene was observed in one patient. No genetic alteration was detected by Southern blot analysis in $p18^{INK4C}$ and in $p19^{INK4D}$.

Case 9 had the stop codon in the *tax* gene but did not have deletion in the HTLV-I provirus genome by Southern blotting (Fig. 4, *lane 1A,1B*), and $p15^{INK4B}$ and $p16^{INK4A}$ were deleted (Table II). Case 11 had only one LTR band by Southern blotting (data not shown) and $p16^{INK4A}$ was deleted. Case 22 had a large deletion in HTLV-I genome suggested by the Southern blotting (Fig. 4, *lanes 2A,2B*), and $p15^{INK4B}$ and $p16^{INK4A}$ were deleted.

Mutations in p53 *gene*

Sequencing of p53 was carried out from codons 33–307 (exons 4–8) in 22 acute ATL cases. Mutations were found in 3 cases. All of them were homozygous missense mutations. Case 13 had a mutation at codon 285 (Glu to Lys) in exon 8. This case had only one LTR band by Southern blot analysis of HTLV-I (Fig. 4, *lanes 3A,3B*). In this case (Case 13), 1.8 kb band was absent when hybridized with a total HTLV-I probe, suggesting a large deletion encompassing the 5′ LTR through the first Pst I restriction site. Case 17 had a mutation at codon 266 (Gly to Arg) in exon 8 and had a deletion in HTLV-I genome by Southern blot analysis of HTLV-I. Case 19 had a mutation at codon 193 (His to Leu) in exon 6. There were also ATL cases that had deletion in HTLV-I provirus genome but did not have deletion in INK4 genes and did not have mutation in *p53* gene. Case 15 represent one such ATL case. Case 15 had only one LTR band when hybridized with a LTR probe (Fig. 4, *lane 4B*) and there was a larger size of band instead of 1.8 kb band when hybridized with a total HLTV-I probe (Fig. 4, *lane 4A*), suggesting a deletion encompassing the 5′ LTR through the first Pst I restriction site. The deletions and mutations observed in the HTLV-I provirus genome, INK genes and *p53* gene are summarized in Table II. There were 9 cases of acute ATL who had a putative CTL escape mutation in the HTLV-I provirus genome and 4 of these 9 patients had either a deletion in *INK4A* or *INK4B* gene or *p53* gene.

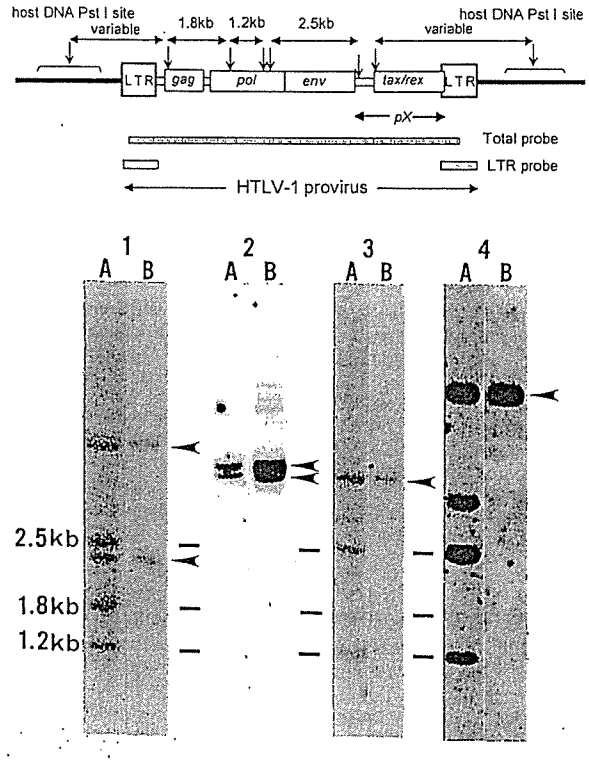

FIGURE 4 – Southern blot analysis of the HTLV-I provirus genome. Ten micrograms cellular DNA was digested with Pst I and subjected to standard Southern blot analysis. The filter was hybridized with a total HTLV-I probe (*a*) and then with a LTR probe (*b*). Arrowheads show the viral-cellular junction bands with LTR probe, in addition to 3 internal bands (2.5kb, 1.8kb, 1.2kb). Schematic illustration of the HTLV-I genome, restriction map, and probes are shown on the upper column. (↓) Pst I site. *Lane 1* (Case 9 in Table II): Typical patient with ATL showing 3 major internal bands (2.5kb, 1.8kb, 1.2kb) with additional 2 viral-cellular junction bands (arrow heads). *Lane 2* (Case 22 in Table II): Three major internal bands are absent (*a*), but 2 LTR bands are observed, suggesting a large deletion in HTLV-I proviral genome. *Lane 3* (Case 13 in Table II): 1.8kb band is absent when hybridized with a total HTLV-I probe (*a*) and there is only one LTR band hybridized with a LTR probe (*b*), suggesting a large deletion encompassing the 5′ LTR through the first Pst I restriction site. *Lane 4* (Case 15 in Table II): Another example of ATL who have only one LTR band hybridized with a LTR probe (*b*) and 1.8kb band is absent hybridized with a total HTLV-I probe (*a*).

TABLE II – MUTATION/DELETION IN HTLV-I GENOME, p15, p16 AND p53 GENES IN ACUTE ATL

Case no.	Southern blot/mutation analysis	Southern blot analysis		Mutation analysis		
	HTLV-I	p15	p16	codon	p53 nucleotide substitution	amino acid
1		Del	Del			
2						
3						
4						
5	G7464A[1]					
6						
7		Del	Del			
8		Del	Del			
9	G7464A[1]	Del	Del			
10		Del	Del			
11	1 LTR[3]		Del			
12		Del	Del			
13	1 LTR[3]			285	GAG-AAG	Glu-Lys
14		Del	Del			
15	1 LTR[3]					
16						
17	PD			266	GGA-AGA	Gly-Arg
18		Del				
19				193	CAT-CTT	His-Leu
20						
21	A7337G[2]			not examined		
22	PD	Del	Del			
23	PD					

[1]G7464A: substitution at nt. position 7464 from G–A that creates a premature stop codon in tax.–[2]A7337G: substitution at nt. position 7337 from A–G that causes an amino acid change from Gly – Arg that is an putative escape mutation.[17]–[3]1LTR Cases that show only one LTR band by Southern blot analysis using HTLV-I LTR as a probe. PD, partial deletion; Del, deletion.

Discussion

The purpose of our present study was to examine when the premature stop codon in the *tax* gene, a putative escape mutation from the anti-HTLV-I CTL response can emerge in the proviral population, and to examine whether such HTLV-I infected cells with escape mutation will subsequently proliferate even in an asymptomatic carrier. We found that the premature stop codon in the *tax* gene exist frequently in HTLV-I carriers as a minor population of the individual carriers: however, such a minor population did not expand subsequently, and deletions in certain host genes still favored the development of ATL.

In our study, we focused on the mutation in *tax* gene especially the premature stop codon of *tax*. Although we could not detect this premature stop codon in the *tax* gene in the majority of ATL patients, there were 4 cases among 55 ATL patients as we reported previously[17] and 4 cases among 47 ATL patients were reported in another study.[33] In addition to this premature stop codon, deletions in the *tax* gene,[17] deletion of 5′-LTR[33] that is a promoter of viral genes, and silencing of the *tax* gene[34] have been reported previously in ATL, suggesting that ATL cells that do not express HTLV-I-encoded proteins are selected by the cytotoxic T cell (CTL) response to HTLV-I, during the development of ATL. A premature stop codon in the *tax* gene is one such escape mutation that is observed in some ATL patient as the commonest sequence in the individual.[17] It was clear that this premature stop codon in the ATL cell emerged after the viral transmission, because the HTLV-I *tax* sequence in the family member was identical with other nucleotide alterations specific for this family except this premature stop codon.[17] It was not clear, however, if this premature stop codon emerged in the early stage of ATL development, or whether the mutation emerged after ATL had developed. To infer at which stage the premature stop codon could have emerged, and to investigate whether there is any HTLV-I carrier with this premature stop codon in the *tax* gene in the commonest proviral sequence of the individual, we examined this premature stop codon in the tax gene in 219 AC and in 143 HAM/TSP patients. Although the premature stop codon in the *tax* gene did not exist as the commonest sequence in any AC or HAM/TSP patients, a stop codon in the *tax* gene was observed as a minor population of the HTLV-I infected cells in many AC and in HAM/TSP patients. This observation was consistent with our previous study by direct sequencing in which we found no AC or HAM/TSP patients that had this premature stop codon as the commonest sequence of the individual.[17] The observation is also consistent with our previous study of a small number of AC and HAM/TSP patients that the premature stop codon in the *tax* gene is present in a minor population in some of the HTLV-I carriers.[35] In our present study, we found that many AC and HAM/TSP patients carried the premature stop codon in the *tax* gene in a minor population of the infected cells. We also reported previously a chronic type of ATL with this premature stop codon as the commonest sequence of the individual.[17] These findings suggest that this premature stop codon emerged in the early stages of ATL development rather than as a consequence of genetic instability after the progression to an aggressive form of ATL. The median HTLV-I provirus load was significantly higher in AC who had a minor population of HTLV-I infected cells with this premature stop codon in the *tax* gene compared to AC who did not have the premature stop codon in the *tax* gene as a minor population, but was not different in HAM patients regardless of the presence of premature stop codon in the *tax* gene in HTLV-I infected cells. We do not know why this difference happens, but it is possible to speculate that the proportion of the role of viral transcription *vs.* cell division in maintaining the provirus load is different among AC and HAM because, the HTLV-I sequence mutation are caused frequently by the reverse transcriptase, but caused rarely by the host DNA polymerase.

Although the RFLP analysis of the premature stop codon in the *tax* gene was not quantitative, there were HTLV-I carriers with a significant proportion of HTLV-I infected cells with this premature stop codon, as judged from the density of the band cut by the Bln I restriction enzyme. To investigate whether such HTLV-I infected cells were already in the process of developing ATL, we followed 3 asymptomatic carriers who had this premature stop codon in the tax gene as minor population (Fig. 2). In these 3 carriers, the population with the premature stop codon in the *tax* gene

did not expand, although the observation time was 1 year in each of 2 cases and 4 years in one case. Interestingly, in one case, in which we quantified the proportion of HTLV-I infected cells with the premature stop codon in the *tax* gene, the proportion of HTLV-I infected cells with this premature stop codon decreased after one year. The provirus load was 64 when the proportion of HTLV-I infected cells had the premature stop codon in tax gene was 15.3%, but the provirus load was 5 after a year when the proportion of HTLV-I infected cells with the premature stop codon in tax gene decreased to 3.2%. These findings suggest that, although HTLV-I infected cells with escape mutation can accumulate in non-ATL HTLV-I carriers, HTLV-I infected cells with a mutation that abolishes the function of Tax lose their proliferative advantage and progressively disappear. It is likely that an escape mutation that abolishes the function of Tax should appear after the immortalization of HTLV-I infected cells for the subsequent development of ATL.

Next, we examined whether host genetic changes promote the malignant transformation of HTLV-I infected cells even in ATL cells that can escape from the host immune response to HTLV-I. To this end, we examined 4 known cyclin dependent kinase inhibitors (INK) and *p53* gene, in combination with the alterations in the HTLV-I genome. In the case of $p16^{INK4A}$ and $p15^{INK4B}$ genes, there is a report that these genes are deleted in many ATL patients,[36] whereas 18^{INK4C} and $p19^{INK4D}$ are not deleted.[37,38] This is consistent with our finding that 9 of 23 (39.1%) acute-phase ATL samples had a deletion in $p16^{INK4A}$ gene and 8 of these patients (36%) who deleted $p16^{INK4A}$ also had deletion or rearrangement of $p15^{INK4B}$, whereas $p18^{INK4C}$ and $p19^{INK4D}$ were not deleted. In our ATL cases that deleted $p16^{INK4A}$ or $p15^{INK4B}$, there were 3 cases that could potentially escape from the host CTL response to HTLV-I. One had a premature stop codon in the *tax* gene, one other case had only one LTR region, and the other had a large deletion in the HTLV-I genome. Each of these genomic alterations could lead to escape from the host immune response to HTLV-I. In the case of *p53* gene, we sequenced the entire exons 4–8, where are highly conserved regions of the *p53* gene,[40] and are also identified as hot spots for mutations in several malignancies including ATL.[30] The missense mutations of *p53* gene observed in our study were at codons 193, 266 and 285 where mutation had been reported in other malignancies (anonymous ftp to ftp.ebi.ac.uk, in the directory/pub/databases/p53) and 2 of them had alterations in the HTLV-I provirus genome. These deletions in the HTLV-I genome could also lead to escape from the host immune response to HTLV-I. Regarding the host genetic changes in the development of ATL, Yamada *et al.*[39] reported that 3 chronic ATL cases who progressed to acute type lost the $p16^{INK4A}$ gene alone or the $p15^{INK4B}$ and $p16^{INK4A}$ genes at their exacerbation phase.[39] Hatta *et al.* reported an ATL patient with a homozygously deleted $p16^{INK4A}$ gene in the chronic phase who rapidly progressed to acute ATL and died within 6 months of the initial diagnosis.[36] There was also a case reported in which the *p53* gene was intact at chronic stage but was mutated when the disease progressed to acute type ATL.[30] These reports suggest that deletion/mutation of tumor suppressor genes such as $p16^{INK4A}$ gene and *p53* in ATL are not a result of genetic instability after the exacerbation of ATL, but are factors that predict poor prognosis. We speculate that in the early stage of HTLV-I infection, Tax gives advantage for proliferation of HTLV-I infected cells, but those cells that continuously express HTLV-I viral proteins are likely to be rejected by host immune system. If an escape mutation emerges after the immortalization of the HTLV-I infected cells, cells that carry such mutations are selected by the host immune system and when additional host genetic changes have accumulated, such as in the $p16^{INK4A}$ or p53 gene, HTLV-I infected cells will progress further to an aggressive form of ATL.

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ORIGINAL PAPER

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Genetic variability in the extracellular matrix protein as a determinant of risk for developing HTLV-I-associated neurological disease

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Abstract Aggrecan, which is a well-known proteoglycan in joint cartilage, also exists in the spinal cord and plays an important role in maintaining water content in the extracellular matrix structure. In this study, we first examined the variable number of tandem repeat (VNTR) polymorphism of the *aggrecan* gene in 227 HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients, in 217 HTLV-I-infected healthy carriers (HCs), and in 85 normal controls. The VNTR allele 28 (1,630 bp) was more frequently observed in HAM/TSP patients than in HCs (χ^2=12.02, p=0.0005, odds ratio 1.79, 95% C.I. 1.29–2.50) and in controls (χ^2=13.43, p=0.0002, odds ratio 2.54, 95% C.I. 1.52–4.25), although this allele was not related to disease progression or to HTLV-I provirus load. We also found that the aggrecan concentration in cerebrospinal fluid (CSF) from rapidly progressive HAM/TSP patients was significantly higher than in slowly progressive patients (corrected p=0.0145) but not in infected non-inflammatory neurological other disease controls (OND) (corrected p=0.078). We then analyzed this aggrecan VNTR polymorphism in the different set of patients with HAM/TSP (n=58) and healthy carriers (n=70). This analysis, again, revealed that allele 28 was detected more frequently in HAM/TSP group than in HCs (χ^2=11.03, p=0.0009, odd ratio 3.04, 95% C.I. 1.55–5.97). The reproducibility of our study was regarded as a second- or third-class association by comparing combined p values and the Better Associations for Disease and GEnes (BADGE) system. Our results suggest that aggrecan polymorphism can be a novel genetic risk factor for developing HAM/TSP.

Keywords Aggrecan · Extracellular matrix · HTLV-I · VNTR · HAM/TSP

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Introduction

HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic progressive inflammatory disease of the spinal cord, which occurs in only 1–2% of HTLV-I-infected individuals (Gessain et al. 1985; Osame et al. 1986). As we have previously reported, that the main HTLV-I-harboring cells in the spinal cord of HAM/TSP patients are not neuronal cells but $CD4^+$ T cells (Moritoyo et al. 1996), a T-cell-mediated immunologic process initiated by HTLV-I infection can be a possible pathological process of HAM/TSP. Although the factors that cause different manifestations of HTLV-I infection are not fully understood, our recent population association studies of more than 200 cases each of HAM/TSP and HTLV-I-infected healthy carriers (HCs) in Kagoshima, an endemic area of HTLV-I infection in Japan, have revealed several important risk factors (Jeffery et al. 1999, 2000; Nagai et al. 1998; Vine et al. 2002). One of the major risk factors for developing HAM/TSP is the provirus load. The median provirus load was approximately 16 times higher in HAM/TSP patients than in HCs, and a high provirus load is also associated with an increased risk for

progression to HAM/TSP (Nagai et al. 1998). We have also reported that *HLA-A*02* and *Cw*08* genes were associated with a lower HTLV-I provirus load and protection from HAM/TSP, whereas *HLA-DRB1*0101* and *B*5401* were associated with susceptibility to HAM/TSP (Jeffery et al. 1999, 2000). Moreover, we have revealed non-HLA genetic risk factors such as TNF−α, SDF-1, and IL-15 (Vine et al. 2002), as well as the association between *HTLV-I Tax* gene variation and the risk for HAM/TSP (Furukawa et al. 2000). From these observations, we now can identify approximately 88% of cases of HAM/TSP in the Kagoshima cohort.

Our detailed clinical analysis of 213 patients with HAM/TSP has revealed that 17% showed arthropathy (Nishioka et al. 1989) characterized by erythema, swelling, and severe pain on moving which mainly occur in large joints (Nakagawa et al. 1995). As the recent study by Levin et al. identified an autoantibody against heterogeneous nuclear ribonuclear protein-A1 (hnRNP-A1) which cross-reacts with HTLV-I Tax protein in IgG isolated from HAM/TSP patients (Levin et al. 2002), it is possible that host recognition of 'self' molecules that mimic HTLV-I contributes to the tissue damage seen in HAM/TSP and its accompanying arthropathy. If this is the case, an immune reaction against a protein that exists in both the spinal cord and joint may be a good candidate for autoantigen.

Human aggrecan is a major extracellular matrix protein expressed in both joint cartilage and the spinal cord, and consists of a core protein and attached glycosaminoglycan (GAG) side chains (Asher et al. 1995; Doege et al. 1991; Milev et al. 1998; Moon et al. 2003; Takahashi-Iwanaga et al. 1998; Watanabe et al. 1998). The reported functions of aggrecan are, first, to maintain the high water content in the extracellular matrix, and second, to act as a barrier against cell migration and a guide for axonal growth in the central nervous system (CNS) along with other chondroitin sulfate (CS) proteoglycans such as phosphocan, neurocan, and versican (Adams et al. 1993; Ang et al. 1999; Asher et al. 2000; Grumet et al. 1993; Moon et al. 2003; Oohira et al. 2000; Perris and Perissinotto 2000). Some reports provide evidence that aggrecan is produced by astrocytes in the perineurial region of the CNS (Matthews et al. 2002; Takahashi-Iwanaga et al. 1998).

Interest in aggrecan function has been increasing as a result of recent research on autoimmune and inflammatory arthritis (Glant et al. 1998; Poole 1998; Zhang et al. 1998b). There are reports showing that aggrecan may act as an immunogenic epitope of T and B cells both in vivo and in vitro. Once the G1 domain has been removed from the core protein of aggrecan by the enzyme aggrecanase (Feng et al. 1998; Zhang et al. 1998a), the molecule discloses a T-cell epitope. It has also been reported that a decrease of CS content elicits a T-cell immune response, whereas a decrease of keratan sulfate (KS) content elicits a B-cell response (Glant et al. 1998).

Based on these findings, we wished to consider the possibility that genetically determined characteristics of extracellular matrix proteins and their degradation are related to the pathogenesis of HAM/TSP. To test this possibility, we analyzed the variable number of tandem repeat (VNTR) polymorphism that was recently identified in the second exon of the *aggrecan* gene, and which encodes a CS attachment site (Doege et al. 1997), in 227 HAM/TSP patients, 217 HCs, and 85 normal controls, and in 58 HAM/TSP patients and 70 HCs. We also examined the protein level of *aggrecan* in both serum and CSF from HTLV-I-infected individuals.

Finally, we have employed a special criterion proposed as the Better Association for Disease and GEnes (BADGE) system (Manly 2005) to assure the reproducibility of our genetic association study. This is because some genetic association studies have problems on reproducibility. In fact, several studies have shown poor reproducibility (Becker et al. 2004; Cardon and Bell 2001; Colhoun et al. 2003; Hirschhorn et al. 2002; Ioannidis et al. 2001; Redden and Allison 2003). This novel system is simple to use and is useful when one needs to estimate reproducibility in the absence of direct experimental replication.

Materials and methods

Study population

The genomic DNA sequences of the *aggrecan* gene were compared among 227 HAM/TSP patients, randomly selected 217 HCs, and 85 normal controls. All cases, HCs, and controls were Japanese and resided in Kagoshima Prefecture, which is an endemic area of HTLV-I infection in Japan. All HCs and normal controls were blood donors and were not related to the patients. The diagnosis of HAM/TSP was made according to the World Health Organization diagnostic criteria (Osame 1990). Sex and ages of subjects were as follows: HAM/TSP group, 69 males and 158 females, 23–76 (mean 57) years old; HC group, 101 males and 116 females, 20–74 (mean 50) years old; control group, 35 males and 50 females, 35–55 (mean 48) years old. The second set of DNA samples were derived from 58 patients with HAM/TSP and 70 HCs from our area. Sex and ages of subjects of this second group were as follows: HAM/TSP group, 20 males and 38 females, 40–65 (mean 50) years old; HC group, 30 males and 40 females, 35–50 (mean 42) years old.

To measure the level of aggrecan in serum and CSF, we used serum samples from 33 HAM/TSP patients and from 11 HCs and CSF samples from 52 HAM/TSP patients, CSF samples from 18 HTLV-I-infected non-inflammatory other disease controls (OND) (five motor neuron disease, four spinocereberal degenaration, two Parkinson's disease, two quadriceps myopathy, one thyroid dysfunction, one essential tremor, one hemifacial spasm, one arrhythmia, and one leg fracture). There was no paired sample of serum and CSF.

We defined rapidly progressive HAM/TSP patients as those who became unable to walk within 3 years after onset of the disease. Sex and ages of rapidly progressive HAM/TSP patients were seven males and 11 females, 48–65 (mean 55) years old, and those of chronic HAM/TSP patients were 11 males and 23 females, 40–64 (mean 54) years old. All samples were taken under written informed consent. The

Ethical Committee of Kagoshima University Faculty of Medicine approved this study.

Serum, CSF, and genomic DNA preparation

Fresh peripheral blood mononuclear cells (PBMCs) were obtained by Histopaque-1077 (Sigma, Tokyo, Japan) density gradient centrifugation, and washed three times with phosphate buffered saline (PBS) with 1% fetal calf serum (FCS). Isolated PBMCs were cryopreserved in liquid nitrogen until use. Genomic DNA was extracted from PBMCs using a QIAamp blood kit (Qiagen Ltd, Tokyo, Japan) according to the manufacturer's instructions. The CSF and serum samples were also collected, and stored at −70°C until use.

Determination of polymorphism and provirus load measurement

The *aggrecan* gene contains a large exon (exon 12) of 3.5 kb, which encodes the entire glycosaminoglycan (GAG) attachment regions of its core protein (Doege et al. 1991). This region consists of numerous repeated sequences, including a particularly highly conserved set of repeats in the CS attachment site. The VNTR polymorphism of the *aggrecan* gene in exon 10 has already been reported (Doege et al. 1997) This VNTR can be detected by PCR as different lengths of PCR products. A genomic PCR was performed with 20 ng of genomic DNA as template, 50 pmol of each primer (forward: 5′-TAG AGG GCT CTG CCT CTG GAG TTG-3′ and reverse: 5′-AGG TCC CCT ACC GCA GAG GTA GAA-3′), 20-mM deoxynucleotide triphosphates (dNTPs), 15-mM $MgCl_2$, reaction buffer provided by the manufacturer, and one unit of Takara-Taq DNA polymerase (Takara, Tokyo, Japan) in a final volume of 10 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 58°C for 60 s, and elongation at 72°C for 60 s with a final extension at 72°C for 10 min. PCR products were separated on 1% agarose gels, visualized by ethidium bromide staining, after which the products were determined. Several of the alleles differ by only one repeat in size (59 bp), and care was taken to identify these alleles using appropriate gels and size markers. Two independent readers scored the alleles.

Provirus load of the samples was measured by a quantitative PCR method using an ABI Prism 7700 (PE-Applied Biosystems) (Nagai et al. 1998).

Quantification of aggrecan in serum and CSF

Serum, as well as CSF aggrecan concentration, was measured in duplicate using a commercial ELISA kit (BiSource Europe S.A., Nivelles, Belgium). According to the manufacturer's instruction, the kit detects aggrecan and aggrecan fragments, and the assay system used is sensitive to detect 0.9 ng/ml of aggrecan in samples. Serum aggrecan levels in normal adults ranged between 1 and 4.4 µg/ml, whereas no information was available regarding CSF levels. When we needed to separate CSF aggrecan amounts into two groups, we selected 0.9 ng/ml as the cut-off level, as this value was the lowest value of the cut-off range and there was no previous report measuring CSF aggrecan concentration. Optical density at 450 nm was measured on the ImmunoMini NJ-2300 (Nippon Inter Med, Tokyo, Japan) and aggrecan concentration was determined by linear regression from a standard curve using the aggrecan supplied with the kit as standard.

Statistical analysis

Statistical analysis was performed using the SPSS for Windows release 7.0, run on an IBM-compatible computer (Analytical software, Version 7, Tallahassee, FL, USA). Comparison of whole-allele distribution between patients with HAM/TSP and HCs was performed using a chi-square test for 2×9 contingency table with a significance level $p<0.05$. The distribution of each allele and genotype of the VNTR polymorphism of the *aggrecan* gene in patients with HAM/TSP patients was compared with those in HCs using a chi-square test for a 2×92 and 2×3 contingency table. Bonferroni multiple adjustments (Motulsky 1995) were made to the level of significance because of the multiple comparisons for VNTR allele frequencies. This level was set at $p<0.0057$ ($p=1-0.95^{(1/9)}$).

To assure reproducibility of our study, we have combined p values from the analysis on two sets of populations and have compared the combined p values to the BADGE (Manly 2005).

Serum and CSF aggrecan levels in patients and controls in three different groups were compared using either ANOVA or Kruskal–Wallis test. A p value less than 0.05 was considered statistically significant. When aggrecan levels in three groups were different, multiple comparisons were done by Scheffe's test. We also performed multiple-hypotheses testing when it was needed, and the level was set at $p<0.017$ ($p=1-0.95^{(1/3)}$).

Results

Frequency of aggrecan VNTR allele 28 was significantly higher in HAM/TSP than HCs and normal control

We applied two-step analysis on our cohort. We first typed 100 samples from each group observing nine aggrecan VNTR alleles, and found the difference between the groups [$\chi^2=18.18$ ($df=8$), $p=0.019$]. We then proceeded to analyze whole samples in this study (227 HAM/TSP patients and 217 HCs) (Table 1).

Comparison of whole allele distribution among patients with HAM/TSP, in HCs and normal controls was performed using a chi-square test for 3×9 contingency table with a significance level $p<0.05$. This analysis has revealed

Table 1 Distribution of aggrecan *VNTR* polymorphism in *HAM/TSP* patients, in healthy carriers (*HC*s) and in normal control subjects

		Allele*						Genotype**					
		HAM/TSP		*HC*s		Control		*HAM/TSP*		*HC*s		Control	
Allele	Length	Obs	Freq (%)	Obs	Freq (%)	Obs	Freq (5)	Homozygote	Heterozygote	Homozygote	Heterozygote	Homozygote	Heterozygote
32	1858	2	0.4	2	0.4	1	0.6	0	2	0	2	0	1
29	1687	18	3.9	12	2.7	6	3.5	2	14	3	6	0	6
28	1630	115	25.3	69	15.9	20	11.8	23	69	12	45	5	10
27	1573	141	31	155	35.7	57	33.5	32	77	37	81	13	31
26	1516	90	19.8	102	23.5	44	25.9	15	60	22	58	8	28
25	1459	62	13.6	53	12.2	25	14.7	4	54	9	35	1	23
22	1288	23	5	37	8.5	15	8.8	6	11	11	15	3	9
21	1231	2	0.4	3	0.6	1	0.6	1	0	1	1	0	1
18	1060	1	0.2	1	0.2	1	0.6	0	1	0	1	0	1
		454		434		170							

* Comparison of whole allele distribution among patients with *HAM/TSP*, *HC*s and normal controls was performed using a chi-square test for 3×9 contingency table with a significance level $p<0.05$. This analysis has revealed $\chi^2=27.33$(*df*=16), $p=0.038$.
Comparisons of whole allele distribution between each two groups were performed using a chi-square test for 2×9 contingency table with a significance level $p<0.05$. This analysis has revealed $\chi^2=17.84$(*df*=8), $p=0.02$ (*HAM/TSP* vs *HC*s), $\chi^2=16.53$(*df*=8), $p=0.035$ (HAM/TSP vs normal controls), and $\chi^2=3.24$(*df*=8), $p=0.918$ (*HC*s vs normal controls). The distribution of each allele of the *VNTR* polymorphism of the *aggrecan* gene in patients with *HAM/TSP* patients was compared with those in *HC*s using a chi-square test for a 2×2 contingency table. Allele 28 has been detected more frequently in patients group than HCs ($\chi^2=12.02$, $p=0.0005$, odds ratio 1.79, 95% C.I. 1.29–2.50)
**: The p value of genotype among three groups was calculated by χ^2 test with a 3×3 contingency table. This analysis revealed that genotype of the 28 repeat was frequently observed in *HAM/TSP* than *HC*s ($\chi^2=19.68$, $p=0.003$, *df*=6). Then p values of genotype in each two groups were calculated by χ^2 test with a 2×3 contingency table. This analysis revealed that genotype of the 28 repeat was frequently observed in *HAM/TSP* than in *HC*s ($\chi^2=10.41$, $p=0.005$, *df*=2) and in *HAM/TSP* than in normal controls ($\chi^2=14.65$, $p=0.0007$, *df*=2), but not in *HC*s and in normal controls ($\chi^2=3.31$, p=0.19, *df*=2)

$\chi^2=27.33$ (*df*=16), $p=0.038$. Comparisons of whole allele distribution between each two groups were performed using a chi-square test for 2×9 contingency table with a significance level $p<0.05$. This analysis has revealed $\chi^2=17.84$ (*df*=8), $p=0.02$ (HAM/TSP vs HCs), $\chi^2=16.53$ (*df*=8), $p=0.035$ (HAM/TSP vs normal controls), and $\chi^2=3.24$ (*df*=8), $p=0.918$ (HCs vs normal controls).

Allele 28 was observed in 25.3% of HAM/TSP patients, whereas, only 15.9% of HCs and 11.8% of normal controls carried this allele. We, therefore, compared the distribution of allele 28 in patients with HAM/TSP and in HCs, and that in normal controls using a chi-square test for a 2×2 contingency table. As nine alleles appeared in our analysis, we set $p<0.0057$ ($p=1-0.95^{(1/9)}$) using the Bonferroni adjustment for multiple comparisons. Allele 28 has been detected more frequently in patients group than in HCs ($\chi^2=12.02$, $p=0.0005$, odd ratio 1.79, 95% C.I. 1.29–2.50) and than in normal controls ($\chi^2=13.43$, $p=0.0002$, odd ratio 2.54, 95% C.I. 1.52–4.25).

The p value of genotype in three groups was calculated first by χ^2 test with a 3×3 contingency table. This analysis revealed that genotype of the 28 repeat was frequently observed in HAM/TSP than in HCs in and normal controls ($\chi^2=19.68$, $p=0.003$, *df*=6). We, then, calculated the p values of genotype between two groups by χ^2 test with a 2×3 contingency table. This analysis revealed that genotype of the 28 repeat was frequently observed in HAM/TSP than in HCs ($\chi^2=10.41$, $p=0.005$, *df*=2) and in HAM/TSP than in normal controls ($\chi^2=14.65$, $p=0.0007$, *df*=2), but not in HCs and in normal controls ($\chi^2=3.31$, $p=0.19$, *df*=2). The observed frequency of alleles other than allele 28 was very similar to the frequency reported in a European population (Doege et al. 1997).

Table 2 Distribution of aggrecan 1630-bp allele in HAM/TSP patients at different provirus load

Provirus load	Total number of Patients	Patients with allele 1630	Frequency (%)
<100	28	10	35.7
<300	33	12	36.3
<600	48	18	37.5
<1000	34	14	41.2
<2000	46	18	39.1
>2000	16	8	50.0

Provirus load is presented as number of the cells in 10^4 PBMC
Mann–Whitney's U test has revealed that the distribution of allele 1630 positive patients is not different at different provirus load ($p=0.402$)

We assessed the reproducibility of our study by comparing combined p values and the BADGE system (Manly 2005). We first multiplied the p value for the 3×9 χ^2-square test from the first population (0.038) and that of the 2×9 χ^2-square test from the second population (0.0001). This yielded the combined p value of 0.0000038. This estimate suggested that the association of our study should be regarded as a second-class association in the BADGE system. We also tried to assess the reproducibility on the test applied to allele 28. We multiplied the p value from the first population (0.0005) with that from the second population (0.0009) and applied Bonferroni correction by multiplying 9 on each p value. This produced the combined p value of 0.00004 as a third-class association.

The possession of allele 28 was not related to disease progression or HTLV-I provirus load

Of 52 HAM patients with CSF aggrecan analyzed, eight patients with allele 1630 showed rapid progression while ten were without this allele. A chi-square test for 2×2 contingency table revealed that disease progression was not correlated with allele possession (χ^2=0.188, p=0.66, odds ratio 1.29, 95% C.I. 0.41–4.12).

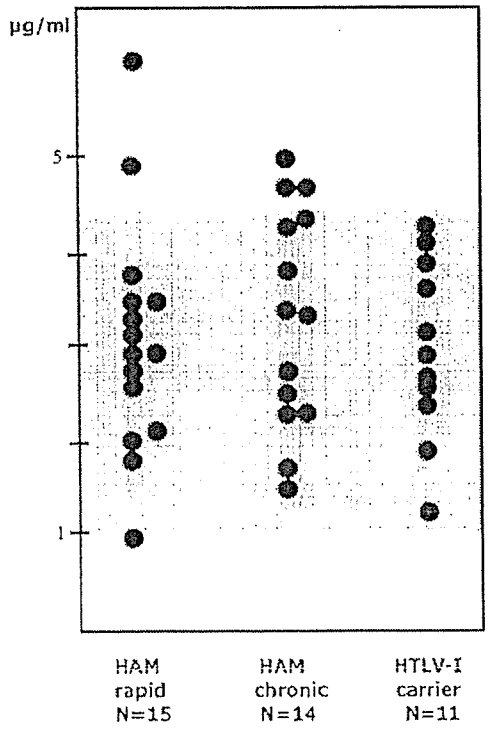

Fig. 1 The amount of aggrecan in serum (normal range 1–4.4 μg/ml, *shadowed area*) showed similar level among *HAM/TSP* patients with rapid progression, with slow progression and *HTLV-I* carriers (*HCs*)

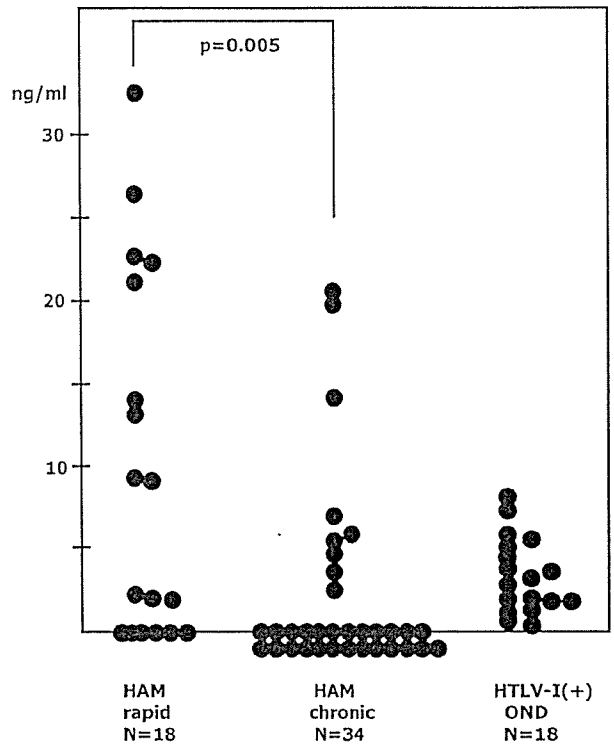

Fig. 2 The amount of aggrecan in *CSF* of patients with *HAM/TSP* showing rapid or slow progression, and other non-inflammatory disease (*OND*)

The distribution of allele 1630 positive patients in the entire sample of 205 HAM patients

In these samples, the provirus load was measured and was not correlated with the provirus load (Table 2) (Mann–Whitney's U test, p=0.402). We measured the provirus load of samples when we had an approval to measure the provirus load.

The CSF level of aggrecan was significantly higher in rapidly progressive HAM/TSP than in chronic HAM/TSP

We, next, compared the serum aggrecan level in rapidly progressive and chronically progressive HAM/TSP patients and HCs (Fig. 1). However, there was no significant difference among these three groups (F=0.78, p=0.47). We then compared CSF aggrecan levels among rapidly and chronically progressive HAM/TSP patients as well as OND (Fig. 2). The results showed that aggrecan levels in CSF in the three different groups were different by Kruskal–Wallis test (H=13.45, df=2, p=0.0006, corrected p=0.0018) and the level in the rapidly progressive HAM/TSP patients was significantly higher than that in the chronically progressive HAM/TSP (p=0.0049, corrected p=0.0145) but not in that of OND (p=0.026, corrected p=0.078) (Scheffe's test).

Table 3 Distribution of aggrecan *VNTR* polymorphism in the second group of *HAM/TSP* patients compared with the second healthy carrier (*HCs*) group

		Allele*				Genotype**			
		HAM/TSP		*HCs*		*HAM/TSP*		*HCs*	
Allele	Length	Obs	Freq (%)	Obs	Freq (%)	Homozygote	Heterozygote	Homozygote	Heterozygote
32	1858	0	0	2	1.5	0	0	0	2
29	1687	3	2.6	7	5	1	1	2	3
28	1630	31	26.8	15	10.8	7	17	3	9
27	1573	42	36.3	49	35	5	32	8	33
26	1516	17	14.7	34	24.3	0	17	3	28
25	1459	13	11.3	26	18.6	0	13	1	24
22	1288	10	8.4	1	0.8	0	10	0	1
18	1060	0	0	2	1.5	0	0	0	2

* Comparison of whole allele distribution among patients with *HAM/TSP* and *HCs* in the second group was performed using a chi-square test for 2×9 contingency table with a significance level $p<0.05$. This analysis has revealed $\chi^2=31.09$($df=8$), $p=0.0001$. The distribution of each allele of the *VNTR* polymorphism of the *aggrecan* gene in patients with *HAM/TSP* patients was compared with those in *HCs* using a chi-square test for a 2×2 contingency table. Allele 28 has been detected more frequently in patients group than HCs ($\chi^2=11.03$, $p=0.0009$, odds ratio 3.04, 95% C.I. 1.55–5.97)

** The p value of genotype was calculated by χ^2 test with a 2×3 contingency table. This analysis revealed that the genotype of 28 repeat was frequently observed in *HAM/TSP* than *HCs* ($\chi^2=9.28$, $df=2$, $p=0.009$)

Aggrecan VNTR analysis in the different set of patients with HAM/TSP and HCs

We, then, analyzed this aggrecan VNTR polymorphism in the different set of patients with HAM/TSP ($n=58$) and healthy carriers ($n=70$) (Table 3). We performed this second analysis to ensure our first observation. Comparisons of whole allele distribution between two groups were performed using a chi-square test for 2×9 contingency table with a significance level $p<0.05$. This analysis has revealed $\chi^2=31.09$($df=8$), $p=0.0001$. Allele 28 was observed in 26.8% of HAM/TSP patients and 10.8% of HCs in this second analysis. We compared the distribution of allele 28 in patients with HAM/TSP and that in HCs using a chi-square test for a 2×2 contingency table. The allele 28 was detected more frequently in HAM/TSP group than in HCs ($\chi^2=11.03$, $p=0.0009$, odds ratio 3.04, 95% C.I. 1.55–5.97). The p value of genotype was calculated by χ^2 test with a 2×3 contingency table. This analysis revealed that the genotype of 28 repeat was frequently observed in HAM/TSP than HCs ($\chi^2=9.28$, $df=2$, $p=0.009$).

Discussion

In this study, we report three findings. First, allele 28 (1630 bp) of the *aggrecan* gene was more frequently observed in HAM/TSP patients than in HCs and in normal controls. This frequent distribution of allele 28 was observed also in the different set of HAM/TSP patients and HCs. The reproducibility of our study was assessed by comparing combined p values and the BADGE system (Manly 2005) and was regarded as a second- or third-class association. Second, possession of allele 28 was not related to the disease progression or HTLV-I provirus load. Finally, the rapidly progressive HAM/TSP patients showed a higher aggrecan concentration in the CSF than the chronically progressive HAM/TSP patients.

Recent genetic analysis of the *aggrecan* gene has shown that it has 18 exons and that there is a polymorphic region in the 12th exon, which is the CS attachment site (Doege et al. 1991). This site has a VNTR of 57 bp. Using this VNTR, several reports have analyzed whether there is a correlation between osteoarthritis (OA) of the hand and a particular allele of the *aggrecan* gene (Horton et al. 1998). Another study of *aggrecan* gene VNTR polymorphism has shown that individuals with shorter VNTR tend to develop multilevel disc degeneration at an earlier age (Kawaguchi et al. 1999). Even though no disease association of aggrecan VNTR has been shown in chronic inflammatory or immunological disease of the nervous system, the reported nature and function of aggrecan and these association studies prompted us to investigate its relation to HTLV-I-related neurological diseases. Ours is the second report of aggrecan VNTR allele distribution in the Asian population, but the first study to examine the association between aggrecan polymorphism and a neurological disease. Regarding allele 28, Kawaguchi et al. (Kawaguchi et al. 1999) reported that allele frequency was 9.4% in their studied population, whereas we have observed 25.3% in patients with HAM/TSP, 15.9% in HCs and 11.8% in normal controls from our area (Table 1). We have, again, shown that the allele frequency of this allele 28 was 26.8% in HAM/TSP patients and 10.8% in HCs (Table 3). This has shown that the allele 28 is indeed increased in our patient population and there was no possibility for a population stratification artifact. To estimate the reproducibility of our study, we have employed the BADGE system to describe genetic association (Manly 2005). As shown in the results, the association of whole allele distribution of *aggrecan* gene to HAM/TSP has reached second-class and the association of allele 28 has reached a third-class association. We have, therefore,

assumed that our study suggests reproducibility under conservative assumptions for traits previously mapped to a chromosome or a small region.

Aggrecan was recently reported to be produced by astrocytes and to exist in the perineurial region of the CNS (Matthews et al. 2002). In general, aggrecan degenerates with age and is cleaved between the G1 domain and the KS binding domain by proteolysis with the enzyme aggrecanase (Lark et al. 1997). Fragments of aggrecan are produced by aging, mechanical processes and/or activation of cleaving enzymes. Once these fragments activate T cells, these T cells can infiltrate into the CNS through the blood–brain barrier and initiate inflammatory CNS diseases (Buzas et al. 1995; Lemons et al. 1999; Mikecz et al. 1988; Zhang et al. 1998b). The reported lower concentration of CS and lack of KS in brain aggrecan (Buzas et al. 1995;Glant et al. 1998; Koppe et al. 1997) may be related to this elicited immune response in the CNS, as decrease of CS or KS content are reported to generate T- or B-cell immune response (Glant et al. 1998). Previous studies on the pathological mechanism of HAM/TSP have revealed that the main disease process is T-cell-mediated inflammation of the thoracic spinal cord (Izumo et al. 2000; Umehara et al. 1993). Taking these findings together, it is of interest to know that the length of the CS attachment site determined by VNTR may have a correlation with HAM/TSP.

Next, we were not able to show the correlation between possession of allele 28 and disease progression or HTLV-I provirus load. This may be because the genetic background we have found in this study can be one independent factor in causing HAM/TSP. Our previous studies have revealed that higher provirus load correlates with strong inflammation of the spinal cord and that the load is related to the deterioration of motor disability in 64 HAM/TSP patients followed up for 10 years (Matsuzaki et al. 2001). We also reported that there were HAM/TSP patients with lower provirus load (Nakagawa et al. 1995). From these observations, we speculated that tissue damage during immune inflammation might not only be controlled only by the strength of the inflammation itself but by the strength of the tissue structure as well. Weak inflammation is sufficient when inflammation occurs in a genetically determined weak tissue. In this regard, our present study may open a novel approach in finding the cause of HTLV-I-related neurological diseases.

To investigate whether aggrecan leakage correlates with disease progression, we measured aggrecan concentration in sera of HAM/TSP patients and HCs, and in CSF of HAM/TSP patients and OND. We found higher CSF aggrecan concentration in rapidly progressive HAM/TSP patients than in chronically progressive patients. As our previous clinical analysis of HAM/TSP patients showed that the patients with later disease onset and knee-joint arthritis showed faster progression of the disease (Nakagawa et al. 1995), we speculated that aggrecan that leaked into the CSF was caused by the degradation of spinal cord tissue secondary to inflammation induced by HTLV-I infection. We also showed that the degree of aggrecan degradation was higher in rapidly progressive patients in this study, and would, therefore, like to propose that the concentration of aggrecan in CSF may be a marker for denaturing in the spinal cord. Although HAM/TSP is reported to occur more frequently in female and we have observed slightly more male cases in rapid progressive group than expected by the reported ratio, age of onset is the only factor, so far, that has been shown to correlate with the disease progression rate (Nakagawa et al. 1995). To find a correlation between sex and disease progression, we may need to measure aggrecan concentration in more cases. To our knowledge, this is the first study to show the presence of aggrecan in CSF. Analysis of CSF aggrecan in other neurological diseases may clarify the significance of this molecule.

As aggrecan and other proteoglycan family molecules play a role in neuronal regeneration and tissue repair after CNS injury (Davies et al. 1997; Gates et al. 1996; Koppe et al. 1997; Lemons et al. 1999), our present observation suggests the possibility that the genetically determined nature of aggrecan determines the efficiency of tissue damage of the spinal cord. This may explain the axonal damage of the spinal cord observed in HAM/TSP patients (Umehara et al. 2000). Profound spinal tissue damage after acute inflammation caused by HTLV-I infected T cells may lead to an acute course of the disease, and insufficient or excessive repair of spinal tissue due to the genetic background may accumulate in a chronic course of the disease. Further studies are necessary to clarify these points.

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'*O*-Acyl isopeptide method' for the efficient synthesis of difficult sequence-containing peptides: use of '*O*-acyl isodipeptide unit'

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Abstract—A novel '*O*-acyl isodipeptide unit', Boc-Thr(Fmoc-Val)-OH **5** has been successfully used for the efficient synthesis of a difficult sequence-containing pentapeptide based on the '*O*-acyl isopeptide method', in which racemization-inducible esterification could be omitted, suggesting that the use of *O*-acyl isodipeptide units allows the application of this method to fully automated protocols for the synthesis of long peptides or proteins.

The synthesis of 'difficult sequence'-containing peptides is one of the most problematic areas in peptide chemistry. These peptides are often obtained with low yield and purity in solid-phase peptide synthesis (SPPS).[1] These difficult sequences are generally hydrophobic and promote aggregation in solvents during synthesis and purification. This aggregation is attributed to intermolecular hydrophobic interaction and hydrogen bond network among resin-bound peptide chains, resulting in the formation of extended secondary structures such as β-sheets.[1]

In regard to the synthesis of difficult sequence-containing peptides, we have recently disclosed an '*O*-acyl isopeptide method',[2] in which a native amide bond at a hydroxyamino acid residue, for example, Ser was isomerized to the ester bond, followed by an *O–N* intramolecular acyl migration reaction (Fig. 1A). The method has been successfully applied to the efficient synthesis of difficult sequence-containing peptides such as Ac-Val-Val-Ser-Val-Val-NH_2 **1** (Fig. 1B)[2b,d,e] and Alzheimer's disease-related amyloid β peptide (Aβ) 1–42.[2c–g]

Our studies indicated that the isomerization of the peptide backbone at only one position of the whole peptide sequence, that is, formation of the ester, significantly changed the unfavorable secondary structure of the difficult sequence-containing peptides, leading to improved coupling and deprotection efficacy during SPPS. Mutter et al.[3a,c] and Carpino et al.[3b] have also confirmed the efficacy of the '*O*-acyl isopeptide method'. Herein, a novel '*O*-acyl isodipeptide unit', Boc-Thr(Fmoc-Val)-OH **5** was successfully used to efficiently synthesize a difficult sequence-containing pentapeptide (Ac-Val-Val-Thr-Val-Val-NH_2 **3**).

In the synthesis of peptide **3** by standard SPPS using Fmoc-amino acids,[4] an undesired peptide, Fmoc-Val-Val-Thr-Val-Val-NH_2 was obtained at a similar rate to peptide **3** after the final deprotection (Fig. 2A). This indicated that the Fmoc group of the pentapeptide-resin was not deprotected during SPPS, similar to what we have previously reported for the synthesis of **1**.[2b,d,e] This suggests that the highly hydrophobic nature of Fmoc-peptide-resin prevented the base from accessing the Fmoc group, thus forming insoluble micro-aggregates on the resin. Further purification of **3** in preparative scale HPLC was laborious due to the extremely low solubility of the product (the solubility of **3** in H_2O, MeOH, and DMSO being 0.008 ± 0.003, 0.059 ± 0.004 and 1.89 ± 0.14 mg mL^{-1}, respectively). When the

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