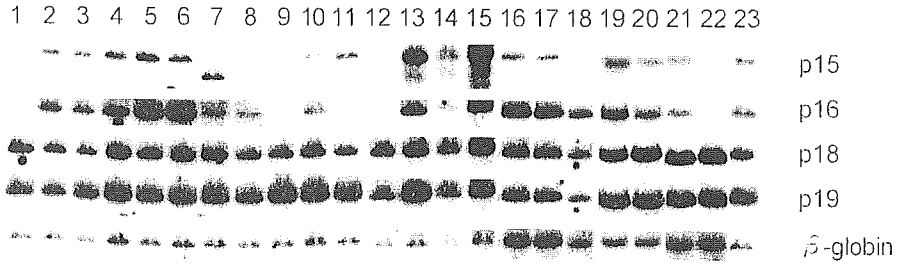

FIGURE 3 – Southern blot analysis of $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$ and β-globin in 23 acute ATL. Ten micrograms of genomic DNA was digested with Hind III and hybridized with each probe. Cases 1, 7–12, 14 and 22 has weak or decreased $p16^{INK4A}$ band when compared to the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

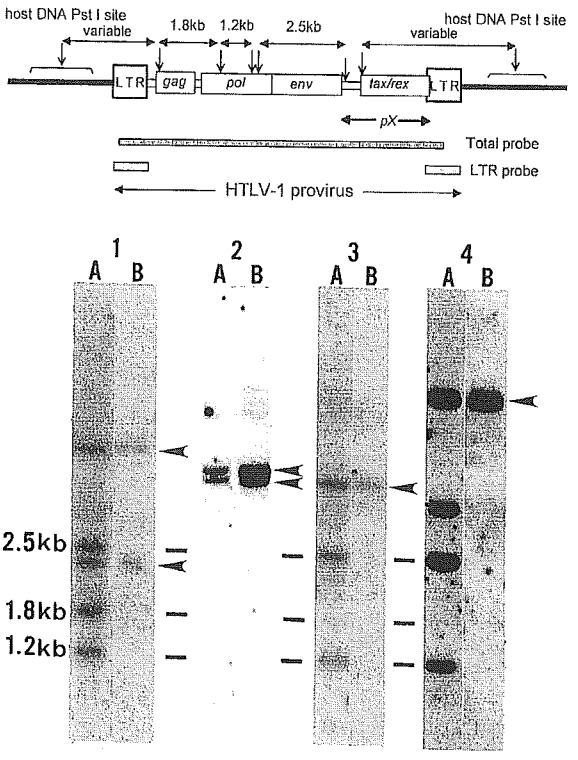

FIGURE 4 – Southern blot analysis of the HTLV-I provirus genome. Ten micrograms cellular DNA was digested with Pst I and subjected to standard Southern blot analysis. The filter was hybridized with a total HTLV-I probe (*a*) and then with a LTR probe (*b*). Arrowheads show the viral-cellular junction 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).

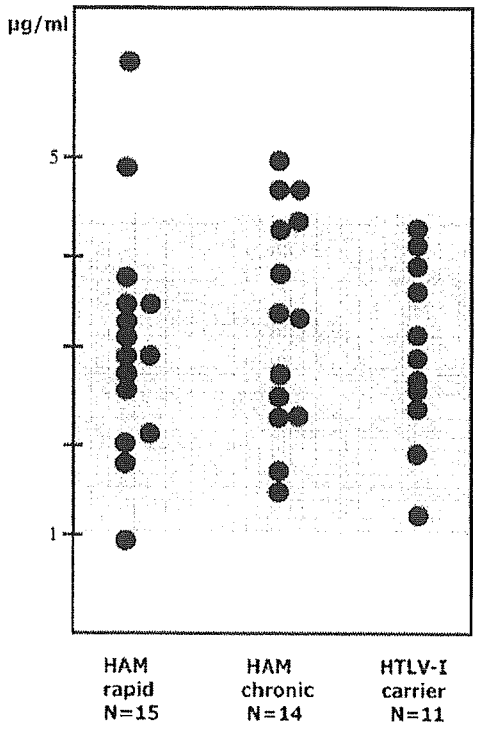

Fig. 1 The amount of aggrecan in serum (normal range 1–4.4 μg/ml, *shadowed area*) showed similar level among *HAM/TSP* patients with rapid progression, with slow progression and *HTLV-I* carriers (*HCs*)

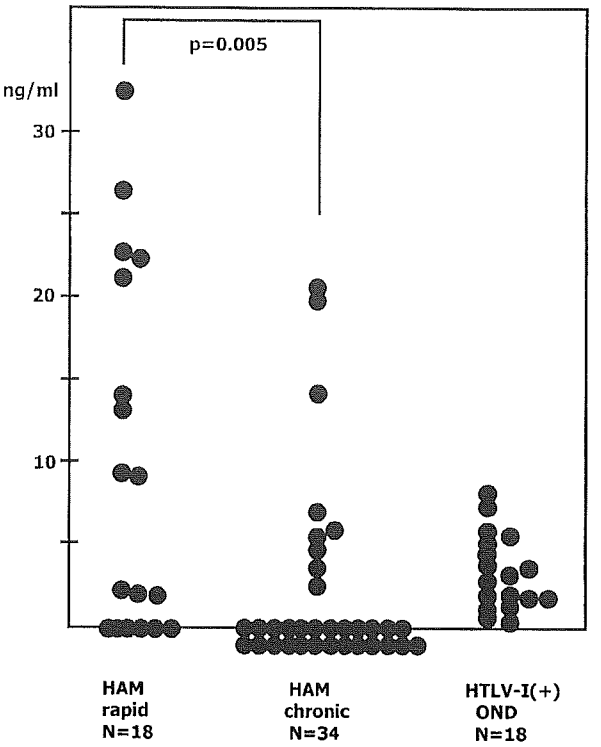

Fig. 2 The amount of aggrecan in *CSF* of patients with *HAM/TSP* showing rapid or slow progression, and other non-inflammatory disease (*OND*)

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 (*HC*s) group

		Allele*				Genotype**			
		HAM/TSP		*HC*s		*HAM/TSP*		*HC*s	
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 *HC*s 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 *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 ($\chi^2=11.03$, $p=0.0009$, 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 *HC*s ($\chi^2=9.28$, $df=2$, $p=0.009$)

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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話題

HAMの診断と治療の進歩

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〔KEYWORDS〕 HAM，診断，治療，HTLV-1プロウイルス量

1．はじめに

HTLV-1(human T lymphotropic virus type 1)は，主にCD4陽性Tリンパ球に感染するヒトレトロウイルスの一種である．HTLV-1-associated myelopathy(HAM)は，HTLV-1無症候性感染者(キャリアー)の一部から発症する慢性の膀胱直腸障害を伴う痙性脊髄麻痺であり，1986年に納，井形により新しい疾患単位として提唱された[1)]もので，後にカリブ海沿岸を中心に報告された熱帯性痙性脊髄麻痺(TSP)の一部と同一の疾患であることが確認された[2)]．HAM発見後すでに19年が経過し，この間に臨床像[3~5)]，発症病態[6~8)]，治療[9)]について精力的に研究が進められた．本稿では，特にHAMの臨床診断，検査所見，治療指針について，自験例をもとに最近の知見を加えて述べる．

2．HAMの現況

1993年のHAM全国調査では，HAM患者の実数は1,062名(そのうち九州700，関東33，北海道49)であった[10)]．その後，HAM患者の実数は1999年4月鹿児島で行われたHTLV国際学会のワークショップにおいて，世界で3,000人＋α，日本で1,422人と報告された[11)]．現在，鹿児島大学病院神経内科に登録されている患者数は495人である．HAM患者は，HTLV-1キャリアーの多い地域に広く分布しており，日本(特に九州，沖縄と四国，東北，北海道の一部)のほか，世界的にはカリブ諸島，アフリカ，イラン北東部，ヨーロッパの一部(ほとんどは感染地域からの移民)などに多くみられる．

3．HAMの発症形式について

男女比は1：2.3と女性に多い．平均発症年齢は45.1±16.5歳である．主な感染経路は母乳による母子垂直感染で，そのほか輸血，夫婦間伝播(ほとんど男性から女性)などがある．自験例では，若年発症(発症年齢15歳以下)は21例(4.2%)，高齢発症(同65歳以上)は74例(14.9%)で，輸血後発症は39例(7.9%)であった．家族内発症は17家族においてみられた．年間生涯発症率は無症候性キャリアー全体の0.23%である[12)]．

4．HAMの診断

1）臨床症状

HAMの診断指針を表1に示す[13)]．初発症状として歩行障害，排尿障害，しびれ感，腰痛，便秘，感覚鈍麻，手指振戦の順に多くみられる．運動障害は足の筋肉のつっぱり(痙性)による歩行障害が主なもので(痙性対麻痺，痙性歩行)，内反足となる．しだいに痙性が強くなると，小走りができない，階段昇降困難，手すり歩行，車椅子移動と症状が進行していく．歩行障害の悪化と筋力低下に伴って下肢の筋萎縮を認めることもある．排尿障害は夜間頻尿に始まり，日中の頻尿(2時間以内の排尿)，残尿，尿失禁などがみられる．残尿のため，しだいに腹部の圧迫排尿，自己導尿となり，最終的に膀胱瘻の造設を余儀なくされる症例もある．尿意がなく尿閉で発症した症例も報告されている．感覚障害は異常知覚，感覚鈍麻が主

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表1　HAM/TSP 診断のための WHO 診断指針(1990)

Ⅰ．臨床診断
　慢性痙性対麻痺の多彩な臨床像が初診時からそろっているとは限らず，発症初期の HAM/TSP では単一の徴候または身体所見のみが認められることもある．
A．年齢ならびに性
　多くは孤発例で成人期発症，時に家系内発症や小児期発症，女性に多い．
B．発症様式
　通常緩徐な発症であるが，急激な発症のこともある．
C．主要な神経学的症候
　1．慢性痙性対麻痺，通常緩徐進行性．時に，初め進行した後に症状の停止する例あり．
　2．両下肢(特に近位部)の筋力低下．
　3．膀胱障害は通常は初期症状，便秘は通常後期症状，インポテンツや性欲減退も稀でない．
　4．刺痛，ジンジン感，灼熱感などのような感覚症状のほうが他覚的身体所見よりも優位．
　5．下肢に放散する下部腰痛が稀でない．
　6．振動覚はしばしば障害されるが，固有感覚はより保たれる．
　7．下肢反射亢進．しばしば足クローヌスや Babinski 徴候を伴う．
　8．上肢反射亢進．しばしば Hoffman 徴候や Tromner 徴候陽性．上肢脱力は認めないこともある．
　9．下顎反射の亢進例も存在．
D．より出現頻度の少ない神経学的所見
　小脳症状・視神経萎縮・難聴・眼振・その他の脳神経障害・手指振戦・アキレス腱反射の減弱または消失．(痙攣・認識力障害・痴呆・意識障害はほとんどみられることはない)
E．HAM/TSP に伴いうる他の神経学的症候
　筋萎縮・筋束性攣縮(稀)・多発筋炎・末梢神経障害・多発神経炎・脳神経炎・髄膜炎・脳症
F．HAM/TSP に伴いうる系統的症候
　肺胞炎・ブドウ膜炎・Sjögren 症候群・関節障害・血管炎・魚鱗癬・クリオグロブリン血症・単クローン性免疫グロブリン血症・成人 T 細胞白血病
Ⅱ．実験室的診断
　1．HTVL-Ⅰ抗体または抗原が血清ならびに髄液に存在すること．
　2．髄液に軽度のリンパ球性細胞増多をみることがある．
　3．血液あるいは髄液中に核の分葉したリンパ球を認めることがある．
　4．脳脊髄液中に軽度から中等度の蛋白増多を認めることがある．
　5．可能なら，血液あるいは脳脊髄液からの HTLV-Ⅰウイルスの分離．

HAM：HTLV-Ⅰ関連脊髄症，TSP：熱帯性痙性脊髄対麻痺　〔文献 13)より引用〕

で，レベルを伴わない感覚障害(75.6%)が多い．初期より振動覚低下がみられる．自律神経障害として，頑固な便秘，皮膚乾燥，脊髄レベル以下の発汗低下がしばしば認められ，皮膚乾燥が強いと魚鱗癬様の皮膚症状を呈することもある．バビンスキー反射，チャドック反射など病的反射の陽性，腹壁反射の消失(99%)を認める[4]．HAM の治療効果の判定にも用いられる機能評価スケールを**表2**に示した[14,15]．脊髄症状以外の症状では，小脳症状(3.2%)，パーキンソニズム(5.5%)，筋萎縮性側索硬化症様症状(1%)[16,17]，末梢神経障害[18]が認められる．頸髄病変を有する HAM の報告もある[19]．慢性に経過する HAM 患者のなかで，2年間のうちに3段階以上運動障害度が悪化する一群(急速進行群)が自験例で17例(3.4%)にみられた．急速進行群は緩徐進行群と比較すると運動障害度7(伝い歩き不能)以上の重症例が多く，下肢の痙性が強い．検査所見で，急速進行群は髄液抗 HTLV-1 抗体価(PA 法)および髄液ネオプテリン値が高いという特徴がある[4]．

2）検査所見

血清抗 HTLV-1 抗体価高値，血清 IgE 低値，各種自己抗体陽性(リウマチ因子 18.3%，抗核抗体 22%，抗 SS-A 抗体 23.4%，抗 SS-B 抗体 9.7%，platelet associated IgG：PA IgG 30%など)，NK 活性低下，末梢血 HTLV-1 プロウイルス量高値[20]，髄液抗 HTLV-1 抗体陽性を認めるほか，髄液ネオプテリン値高値，髄液内 IgG 産生亢進などもみられる．ATL 細胞様の異型リンパ球を末梢血，髄液中に認めることもある．電気生理検査では，傍脊柱筋針筋電図で脱神経所見を，下肢 SEP で中枢神経伝導速度遅延を認めるほか，下肢 SSR の消失も認められる．頭部 MRI では，大脳深部白質病変を高頻度(55%)に認め

表2　HAMの機能障害の評価

運動機能障害重症度	
Grade	Disability
0	歩行・走行ともに異常を認めない
1	走るスピードが遅い
2	歩行異常(つまづき，膝のこわばり)
3	かけ足不能
4	階段昇降に手すり必要
5	片手によるつたい歩き
6	片手によるつたい歩き不能：両手なら10 m以上可
7	両手によるつたい歩き5 m以上，10 m以内可
8	両手によるつたい歩き5 m以内可
9	両手によるつたい歩き不能，四つんばい移動可
10	四つんばい移動不能，いざり等移動可
11	自力では移動不能，寝返り可
12	寝返り不能
13	足の指も動かせない

排尿障害の重症度	
頻尿	0：正常
残尿	1：わずかに存在
尿失禁	2：明らかに存在
	3：著明に存在
(残尿2：圧迫排尿，残尿3：自己導尿)	
3つの症状の合計点数で表す．	

〔文献15)より改変して引用〕

表3　HAM発症にかかわるリスク要因

- ウイルス側
  - Taxサブタイプ A は発症させやすい
- 宿主側
  1) HLA遺伝子
     発症抑制：HLA-A*02，Cw*08
     発症促進：HLA-DRB 1*0101，B*5401
  2) Non-HLA遺伝子
     発症抑制：IL-10 promoter-592 A/C SNP：A allele
     Vitamin D receptor exon 9 ApaI 多型
     SDF-1-801A3' UTR
     発症促進：TNF-α-893 A/C SNP：A allele
     アグリカン VNTR 1630 bp allele
     MMP-9 promoter d(CA)n repeat の延長

SDF：stromal cell-derived factor 1，MMP：マトリックスメタロプロテアーゼ，TNF：腫瘍壊死因子，SNP：single nucleotide polymorphism

る．通常，脊髄MRIの異常を認めないが，慢性期では胸髄萎縮を，急速進行群では脊髄腫脹を認めることがある．髄液抗HTLV-1抗体価(PA法)が2倍から8倍以下の群(低力価群)が存在し，1,024倍以上の高力価群と比べて発症年齢が遅い，やや運動障害が軽い，バビンスキー反射および排尿障害の出現率が低い，血清IgGが低い，髄液ネオプテリン値が低いという特徴がある[4]．また，高力価群には急速進行群に属する症例が多く含まれている(自験例17例中10例)．最近，HAM患者の血清中に中枢神経のBetz神経細胞に反応する自己抗体である，抗heterogenous nuclear ribonuclear protein-A 1(hnRNP-A 1)抗体が高頻度に検出されるという報告があり[7]，HAMの病態への関与が示唆されている．

5. HAM発症にかかわる危険因子からみた診断

HAM発症要因として，ウイルス側および宿主側の様々な因子が解析されつつある(表3)．HTLV-1 taxのサブタイプにはサブタイプA，サブタイプBがあり，サブタイプAのウイルスに感染している個体ではHAMを発症しやすいことが報告されている[21]．一方，宿主側因子として，HLA遺伝子のうちHLA-A*02，Cw*08が発症抑制，HLA-DRB 1*0101，B*5401が発症促進に関与することが報告されている(表3)．われわれは，これらHAM発症に関与する複数のHLA，非HLA宿主遺伝子多型とHTLV-1ウイルスサブタイプを用いた多変量解析から，HAM

表4　HAM発症リスク計算式
(Best-fit logistic regression equation for the risk of HAM/TSP in the Kagoshima HTLV-1 infected cohort：n=402)

Factor, condition	ln(odds of HAM/TSP)	Odds ratio(P)
Constant	−1.716	
Age	−(0.145×age)+(0.003×age^2)	
Provirus load	+(0.460×load)+(0.487×load^2)	
TNF-α−863 A$^+$	+3.057−(4.616×load)+(1.476×load^2)	
SDF-1+801 GA	−0.808	0.45(0.042)
SDF-1+801 AA	−1.689	0.18(0.003)
HLA-A*02$^+$	−0.638	0.53(0.043)
HLA-Cw*08$^+$	−0.894	0.41(0.046)
HTLV-1 subgroup B	−1.587	0.20(0.017)

例) 60歳，$\log_{10}$(No. of tax copies/10^4 PBMCs)=2.5，TNF-α−863 A$^-$，SDF-1+801 AA，HLA-A*02$^-$，HLA-Cw*08$^+$，HTLV-1 subgroup Bの感染者の場合：ln(odds of HAM/TSP)=−1.716−(0.145×60)+(0.003×60^2)+(0.46×2.5)+(0.487×2.5^2)+3.057−(4.616×2.5)+(1.476×2.5^2)−1.689−0.894−1.587=1.14975　よってこの症例のodds of HAM/TSP=exp(1.14975)=3.157403

発症リスクを計算する式を示した(表4)[22]．この式により求めたHAM発症リスク(オッズ比)を用いて，非典型的なHAMを鑑別できる可能性がある[23]．実際に，最近，頸髄病変を認めた非典型的HAM症例のなかに，オッズ比が高値であった症例が存在したことが報告されている[19]．

6．合併症

Tリンパ球性肺胞症(70%)，シェーグレン症候群(25%)，関節症(20%)，ぶどう膜炎(15%)，多発筋炎(5%)，魚鱗癬様皮疹，ATL　8例(1.6%：くすぶり型を含むと11例：2.2%)などがある．

7．HAMの病理

神経病理所見では，胸髄中下部の外側皮質脊髄路を中心にした脱髄と神経鞘および軸索の変性脱落が認められ，血管周囲から脊髄実質に広がる炎症性単核細胞の浸潤を伴っている．胸髄以外にも，数は少ないものの大脳においても同様に単核細胞の浸潤が認められることが報告されている[24]．

8．治療

これまでに施行された治療を表5にまとめた．治療にあたり，まずHAMに伴う合併症の有無を精査することが重要である．初期治療として，特に急速進行群に対しては，メチルプレドニゾロンによるステロイドパルス療法を施行後，経口ステロイド剤による維持療法を行う．ステロイド療法には，抗炎症作用のみならず，末梢血単核球中のHTLV-1プロウイルス量を減らす効果も認められる．また，初期治療として肺合併症，網膜症がない場合には，天然型インターフェロン-α製剤(スミフェロン®)が使われる(これは，HAMに対する唯一の健康保険適用のある薬剤である)．インターフェロン-α 300万単位を30日間連日筋注し，以後週1回の投与とする．減量後に，治療開始後いったん減少したHTLV-1プロウイルス量が再び上昇することがあるので，そのような症例に対しては後療法として少量の経口ステロイド剤(5〜10 mg/日)を併用する．インターフェロン-αにはHTLV-1プロウイルス量を減らすとともに，活性化T細胞を減少させる効果がある[14]．慢性期の症例に対しては，経口ステロイド剤の少量持続療法[25,26]やビタミンC大量療法(1.5〜3 g/日)が有効である．経口ステロイド療法の際には，骨粗鬆症とステロイド糖尿に留意することが必要である．肺合併症や尿路感染を繰り返すケースでは，エリスロマイシンを使用する．関節炎を伴う症例では，サラゾスルファピリジンが有効な場合がある．痙性麻痺が強い場合は，筋弛緩剤を併用し，リハビリテーションを施行する．排尿障害に対しては，神経因性膀胱に対する治療を行う．腰・下肢痛については，原因となるものを精査したうえで，対症的にカルバマゼピン，抗うつ薬の投与や，半導体レーザーによる星状神経節近傍照射，鍼治療などの物理療法も積極的に取り入れる．将来的には，炎症細胞の中枢神

表5　現在までのHAMの治療

治療法	投与量	投与期間	施行例	有効率(A)	有効率(B)
1　副腎皮質ホルモン					
1）経口投与	10～80 mg/日	連日，隔日/1～3か月	247	78.1%	63.0%
2）大量点滴投与	500～1,000 mg/日	1～3日	14	78.6	50
3）髄注	50～70 mg/日	1～5回	5	80	40
2　血液浄化療法					
1）リンパ球除去術	約1×10⁹個/回	3～6回	9	77.8	44.4
2）プラズマフェレーシス	1回1.5～2.0 *l* の血液を免疫吸着カラムで処理	4～6回	7	42.9	42.9
3　インターフェロン-α					
1）筋注	300万単位/日	30日	32	62.5	21.9
2）吸入	100万単位/日	30日	11	81.8	27.3
4　アザチオプリン	50～100 mg/日	1～3か月	9	55.6	22.2
5　ビタミンC	1,500～3,000 mg/日	4日か5日連続投与後2日休薬/4週間	89	58.4	13.4
6　ペントキシフィリン	300 mg/日	2～4週	5	60	20
7　エリスロマイシン	600 mg/日	1～3か月	25	48	16
8　サラゾスルファピリジン	1,000～1,500 mg/日	1～3か月	24	50	12.5
9　ミゾリビン	100～150 mg/日	1～3か月	17	47.1	11.8
10　フォスホマイシン	静注4 g/日	2週間			
	その後経口2 g	2週間	14	78.6	7.1
11　TRH	静注2 mg/日	5日間	16	56.3	6.3
12　グリセオール	400～600 mg/日	3～5日間	3	66.7	0
13　ヒト免疫グロブリン	2.5～5 g/日	1～3日間	3	33.3	0
14　ダナゾール	200～300 mg/日	4～6週間	2	0	0
15　エペリゾン塩酸塩	100～150 mg/日	4～6週間	6	100	50
16　AZT+3TC	3TC 300 mg，AZT 400 mg	4～12週間	12	58.3	25

有効率(A)：やや有効以上の効果の割合，有効率(B)：有効以上の割合，TRH：甲状腺刺激ホルモン放出ホルモン
〔文献9)より引用して改変〕

経への移行を阻害するMMP(マトリックスメタロプロテアーゼ)阻害剤，TNF-αによる神経障害を抑制するための抗TNF薬，ウイルスの増殖を抑制する逆転写酵素阻害剤や，HTLV-1特異的プロテアーゼ阻害剤などの治療薬が現在検討中または開発中であり，臨床的に使用可能になるものと考えられる．

9．おわりに

HAMは，生命予後が比較的良好な疾患ではあるが，難治性で合併症も多く，患者の負担が大きいため，根治療法の開発が切望されている．現在，HAM患者会(アトムの会)が結成されており，ホームページ(http://www.minc.ne.jp/~hamtomo/)上で患者同志の情報交換が可能である．HAMに対しては，医療関係者を含めた多くの人々による治療，心のケア，福祉サービスなどの多方面にわたるサポートが必要であり，今後ともますますその充実が望まれる．

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Design and synthesis of a new polymer-supported Evans-type oxazolidinone: an efficient chiral auxiliary in the solid-phase asymmetric alkylation reactions

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Abstract—Wang resin-supported Evans' chiral auxiliary (**23**) was designed based on a novel polymer-anchoring strategy, which utilizes the 5-position of the oxazolidinone ring, and its new synthetic route applicable to multi-gram preparation in just a day was developed. Solid-phase Evans' asymmetric alkylation on **23**-derived *N*-acylimide resin and following lithium hydroperoxide-mediated chemoselective hydrolysis afforded the corresponding α-branched carboxylic acids in desired high stereoselectivities (up to 97% ee) and moderate to good overall yield (up to 70%, for 3 steps), which were comparable to those of the conventional solution-phase methods. Furthermore, recovery and recycling of the polymer-supported chiral auxiliary were successfully achieved without decreasing the stereoselectivity of the product. Therefore, this is the first successful example that the solid-phase Evans' asymmetric enolate-alkylation was efficiently performed on the solid-support, and it is concluded that the connection to the solid-support via the 5-position of the oxazolidinone ring is an ideal strategy in the solid-phase Evans' chiral auxiliary.

1. Introduction

Evans' chiral oxazolidinone is one of the efficient auxiliaries for preparing chiral building blocks necessary to synthesize molecules possessing the accurate spatial configuration of specific functional groups.[1,2] Its generality and reliability with high optical purity have already been established in a variety of efficient asymmetric syntheses of low molecular weight chiral compounds and complicated natural products.[3–5] Moreover, its potential is expanding in the study of novel asymmetric reactions.[6]

Solid-phase organic synthesis has been developed as a rapid and diversified method in organic chemistry.[7] As compared to solution-phase, the solid-phase technology provides a simple procedure 'filtration' for rapidly achieving the isolation of desired compounds or recovering expensive reagents or catalysts attached onto the solid-support for recycling. Hence, many useful reagents or catalysts, especially those used in chiral synthesis, in solution-phase methods have been intensively and successfully applied to the solid-phase methods so far.[8] However, some solid-supported chiral auxiliaries are problematic in achieving high quality of stereoselective reactions.[9] One of such well-known examples is pseudoephedrine[10] grafted onto the Merrifield resin. This solid-supported auxiliary showed lower stereoselectivity in asymmetric alkylation (approx. 85% ee) in comparison to the corresponding solution-phase experiments.

Evans' oxazolidinone has also been applied to the solid-phase stereoselective reactions such as enolate-alkylation reaction,[11] aldol reaction,[12] Diels–Alder cycloaddition[13] and 1,3-dipolar cycloaddition.[14] However, undesired results similar to those observed in the solid-supported pseudoephedrine case were reported, especially in the fundamental solid-phase asymmetric enolate-alkylation reaction which prepares optically active α-branched carboxylic acid derivatives.[11b] Indeed, maximum stereoselectivity was 90% ee in asymmetric benzylation using the auxiliary resin **1** (Fig. 1A).[15] Moreover, a side reaction was reported in the preparation of solid-supported L-serine derived chiral oxazolidinone **2**.[16] Therefore, to improve the efficiency in stereoselective reactions, we previously reported a

Keywords: Evans' oxazolidinone; Polymer-supported chiral auxiliary; Asymmetric synthesis; Solid-phase organic synthesis; Solid-phase asymmetric alkylation; Recovery and recycling.
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