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 (*HCs*) and in normal control subjects

		Allele*						Genotype**					
		HAM/TSP		*HCs*		Control		*HAM/TSP*		*HCs*		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*, *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 $\chi^2=27.33(df=16)$, $p=0.038$.
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). 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 ($\chi^2=12.02$, $p=0.0005$, 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 *HCs* ($\chi^2=19.68$, $p=0.003$, $df=6$). 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 *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, $df=2$)

$\chi^2=27.33$ ($df=16$), $p=0.038$. 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).

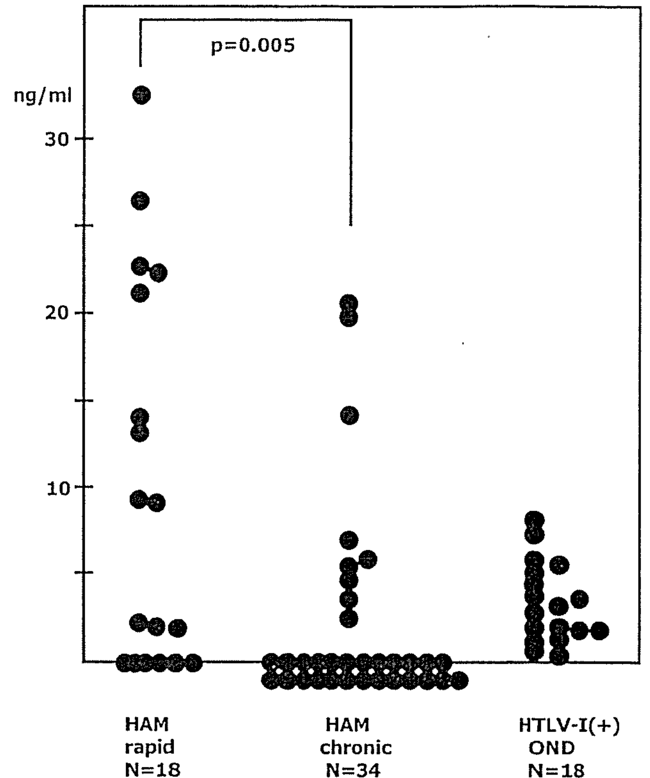

Fig. 2 The amount of aggrecan in *CSF* of patients with *HAM/TSP* showing rapid or slow progression, and other non-inflammatory disease (*OND*)

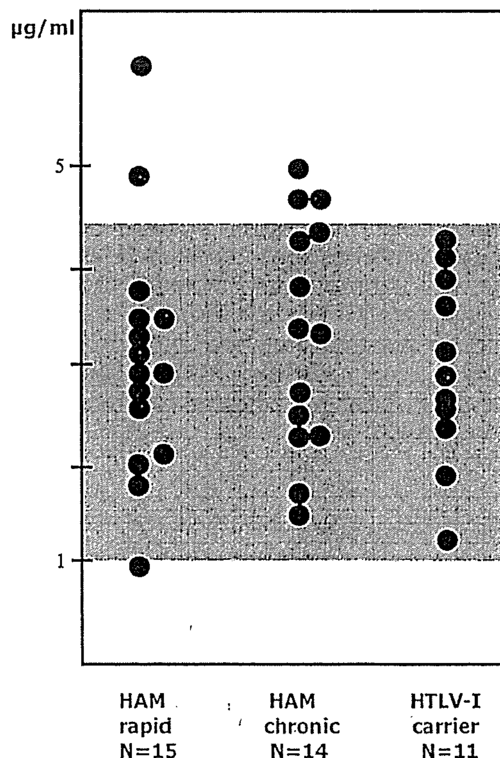

Fig. 1 The amount of aggrecan in serum (normal range 1–4.4 μg/ml, *shadowed area*) showed similar level among *HAM/TSP* patients with rapid progression, with slow progression and *HTLV-I* carriers (*HCs*)

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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Geographical distribution and disease associations of the CD45 exon 6 138G variant

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Abstract CD45 is crucial for normal lymphocyte signalling, and altered CD45 expression has major effects on immune function. Both mice and humans lacking CD45 expression are severely immunodeficient, and single-nucleotide polymorphisms in the CD45 gene that cause altered splicing have been associated with autoimmune and infectious diseases. Recently, we identified an exon 6 A138G polymorphism resulting in an increased proportion of activated CD45RO T cells and altered immune function. Here we report a significantly reduced frequency of the 138G allele in hepatitis C Japanese patients and a possibly reduced frequency in type I diabetes. The allele is widely distributed in the Far East and India, indicating that it may have a significant effect on disease burden in a large part of the human population.

Keywords CD45 · A138G · Alternative splicing · Type I diabetes · Hepatitis C

CD45 (leucocyte common) antigen is a haemopoietic cell-specific tyrosine phosphatase which undergoes complex alternative splicing. In humans, naive T cells express CD45RA-containing isoforms, while most memory/effector T cells express the low molecular weight CD45RO isoform. The function of the isoforms remains obscure, although it is clear that CD45 is an important immunomodulator which regulates many signalling pathways in the immune response, from establishing a threshold for T cell antigen receptor (TCR) signalling to modulating

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cytokine production and responses, as well as regulating cell survival (Hermiston et al. 2003; Irie-Sasaki et al. 2003).

The importance of CD45 expression for normal lymphocyte development was first demonstrated in CD45-deficient mice (Kishihara et al. 1993; Byth et al. 1996), and animals with genetic polymorphisms influencing the balance of isoforms expressed show altered disease susceptibility and production of cytokines (Subra et al. 2001). It is likely therefore that genetic variants altering CD45 expression or the balance of different isoforms would affect disease susceptibility or progression in humans. This has been found to be the case. Individuals lacking CD45 expression exhibit severe combined immunodeficiency (Kung et al. 2000; Tchilian et al. 2001b), while the exon 4 C77G variant, which profoundly affects alternative splicing (Thude et al. 1995; Zilch et al. 1998), has been associated with altered susceptibility to various autoimmune and infectious diseases (Jacobsen et al. 2000; Tchilian et al. 2001a; Schwinzer et al. 2003; Vogel et al. 2003; Vyshkina et al. 2004). C77G carriers are relatively rare (allele frequency from 0 to 3.5% in Europe and North America), which has hampered more sophisticated studies of the influence of this variant on disease progression or immune function.

Table 1 Frequency of the CD45 exon 6 A138G polymorphism in control and disease groups from different regions

Disease group	Total number	AA (%)	AG (%)	GG (%)	% G allele frequency
Japanese Osaka controls	314	184	117	13	22.8
Hepatitis C cases	338	237	91	10	16.4**
Diabetes type I cases	190	132	52	6	16.8*
Cervical cancer cases	69	38	27	4	25.4
Japanese Kagoshima controls	79	54	25	0	15.8
HTLV HC	80	57	19	4	16.9
HAM/TSP	79	57	17	5	17.1
Japanese Wakayama	594	374	198	22	20.4
Vietnam malaria controls	225	164	55	6	14.9
Malaria cases	332	237	86	9	15.7
Vietnam dengue controls	226	158	66	2	15.5
Dengue cases	303	197	101	5	18.3
Dengue fever	108	74	32	2	16.7
Dengue haemorrhagic fever	60	43	17	0	14.2
India controls (lepromatous)	42	33	9	0	10.7
Leprosy cases (lepromatous)	62	51	11	0	8.9
Leprosy controls (tuberculoid)	59	52	7	0	5.9
Leprosy cases (tuberculoid)	62	49	13	0	10.5
India TB controls	180	155	22	3	7.8
India TB cases	263	231	32	0	6.1
Thai controls	146	95	43	6	18.8
Cambodian controls	20	12	8	0	20.0
Chinese controls	560	349	187	24	21
Peruvian controls	19	12	6	1	21.0

The overall genotype distribution and allele frequencies are shown. Statistically significant differences (assessed by continuity corrected chi-square) in the frequency of G allele carriers between cases and respective controls are indicated with asterisks. There were no truly significant departures from Hardy–Weinberg equilibrium in any of the populations tested. Ethical approval was obtained for all cohorts genotyped, from the relevant authorities. The presence of the A138G allele was detected using alkaline-mediated differential interaction (AMDI), which is based on alkaline-mediated detection of ARMS-PCR products using double-stranded DNA specific dyes (Bartlett et al. 2001; Stanton et al. 2003) in all Japanese, Thai, Cambodian and Peruvian samples. Two separate reaction mixes containing the forward (GCAGAAGTGCTTGAAGATT) and one of the two reverse primers GCATAGTCAGACCTGAGCT (for the A allele) and GCATAGTCAGACCTGAGCC (for the G allele) were used. Annealing temperatures were 52°C and 55°C for the A and G alleles, respectively. All of the Indian and Vietnamese samples were genotyped using the Sequenom Mass-Array MALDI-TOF primer extension assay (Jurinke et al. 2002) (http://www.sequenom.de/) under standard conditions. Primers for A138G were P1: ACGTTGGATGACCTCCAACACCACCATCAC, P2: ACGTTGGATGGAAG ACACTACTAGAGCAGC and extension P: ACACCACCATCACAGCGAAC. Where different samples from the same population had been typed by the Sequenom and AMDI techniques, there was no more than a 1% difference in frequencies between the two samples typed using the different techniques

HTLV HC Human T lymphotropic virus in healthy controls, *HAM/TSP* HTLV-1-associated myelopathy/tropical spastic paraparesis patients, *TB* tuberculosis

*p=0.019; **p=0.0028

We recently described another CD45 polymorphism, exon 6 A138G, which is common at least in Japan (Stanton et al. 2003). This variant showed a relatively strong protective effect in Graves' disease and hepatitis B, and a suggestive, but not significant, reduction in the frequency of the variant in hepatitis C patients (Boxall et al. 2004). Preliminary data indicate that 138G individuals have more activated CD45RO T cells and synthesize more interferon gamma (IFN-γ) (Boxall et al. 2004). The high frequency of 138G-carrying individuals in Japan suggests that the G allele might also affect susceptibility and pathogenesis in other autoimmune or infectious diseases and might have a wider geographical distribution.

We therefore extended our earlier studies by analysing an additional 138 Japanese controls and 165 further hepatitis C patients all from the Osaka region. We also examined the frequency of the A138G single-nucleotide polymorphism (SNP) in patients with type I diabetes and cervical cancer, a malignant disease with viral aetiology. As shown in Table 1, we have now analysed a total of 314 controls and 338 hepatitis C cases.

Our previous data have been combined with those added in this study, as there was no significant heterogeneity between the two sets of samples. The overall frequency of the presence of the G vs A allele (namely, AA vs AG + GG) is significantly different in the hepatitis C patients compared to the controls, p=0.0028 (odds ratio=0.6, 95% confidence interval=0.43–0.84), using Yates continuity corrected chi-square. Even when using the Bonferroni correction to allow for multiple comparisons (12 in this case), the p value remains significant at p=0.0336. In this case, however, the correction is conservative because there is an a priori suggestion of an association between hepatitis C and another CD45 variant C77G (our unpublished results), and our previous data suggested an effect of the A138G variant, and also because of the effect of the A138G variant on immune phenotype and function (Boxall et al. 2004).

There is a suggestion of a decreased frequency of the G allele in type I diabetes p=0.019, which, however, would not remain significant when the number of comparisons is taken into account. Nevertheless, it is intriguing that, given our previous data on Graves' disease, the A138G may have effects on two autoimmune disease. Interestingly, both Graves' disease and diabetes are influenced by another polymorphism, namely, in the CTLA-4 gene, suggesting that their pathogenesis may share immunological mechanisms and that A138G should be added to the list of common alleles with low penetrance, in addition to the major histocompatibility genes, which have already been shown to be factors in the development of autoimmune diseases (Rioux and Abbas 2005). There was no significant difference between the cervical cancer patients and controls.

We also analysed the frequency of this polymorphism in another Japanese population from the Kagoshima region of southern Japan, where human T lymphotropic virus type I (HTLV-1) infection is endemic (Jeffery et al. 2000). No significant difference in the frequency was found between 79 controls, 80 asymptomatic healthy carriers (HTLV HC) and 79 HTLV-1-associated myelopathy/tropical spastic paraparesis patients (HAM/TSP). The relatively large difference in the frequency of the A138G allele between the Kagoshima and Osaka controls is puzzling and needs more investigation, especially as 594 samples from the southern county of Wakayama prefecture (Shirakawa et al. 1997) showed a frequency of 20.4% for the A138G polymorphism which is very similar to that found in Osaka. It may be interesting in the future to see if this difference between the Kagoshima and other Japanese regions can be confirmed and, if so, whether it is specific only to the A138G CD45 variant or is reflected in the frequency of other polymorphisms.

Table 2 CD45 A138G gene frequency in different geographical locations

Location	Total number	% G allele frequency
Osaka (Japan)	314	22.8
Kagoshima (Japan)	238	16.6
Wakayama (Japan)	594	20.4
Vietnam	1254	16.2
Thailand	146	18.8
China	560	21
Cambodia	20	20
India	668	7.5
Peru	19	21

Data from disease and control groups were combined when no significant difference between them was found

We further investigated the frequency of the A138G SNP in other Far Eastern populations, examining cohorts of Vietnamese malaria (Tran et al. 1996) and dengue cases and controls (Loke et al. 2001; Cao et al. 2002; Phuong et al. 2004). The variant allele is present in Vietnam at a frequency of 15%, but there was no significant difference between the 332 malaria cases and 225 controls. Nor was there any significant difference between 303 dengue cases and 226 respective controls nor between 108 Dengue fever and 60 Dengue haemoraghic fever cases in a second well characterised cohort (Cao et al. 2002; Phuong et al. 2004). The G allele was detected in populations of 146 Thais and 20 Cambodians with frequencies of 18.8% and 20% respectively. In a large cohort of Chinese samples (Peisong et al. 2004) we also found a variant frequency of 21%. These data indicate that many Far Eastern Oriental populations have a relatively high frequency of the 138G allele.

We also tested samples from India and found an overall frequency for the A138G SNP of about 7.5%. No significant differences in the genotype distributions between lepromatous or tuberculoid leprosy patients and their respective matched controls from Calcutta were found (Roy et al. 1999). Similarly no significant difference in the overall frequency amongst the 263 Indian tuberculosis (TB) patients and 180 controls from Madurai was found. Interestingly, no G138G homozygotes were detected amongst the TB patients. However, this is not statistically

significant and so more samples clearly need to be analysed to establish whether there is a possible recessive effect in TB.

Since the A138G polymorphism was not found in cohorts of Africans and at a frequency of less than 1% in Western Europeans (Stanton et al. 2003), it was of interest, given the opportunity, to test samples from South American Peruvian Quechua Indians where we found the G allele at a high frequency in a small cohort of 21 individuals (21%) (Delgado et al. 2004). A summary of our overall data on the geographical distribution of the A138G allele is shown in Table 2. In cases where there are no differences in frequency between disease and control groups (Kagoshima, Vietnam and India), we have combined the data to calculate an overall frequency.

In summary, our data show a clearly significant protective effect of the 138G allele in hepatitis C. There may also be a similar effect in diabetes in Japanese from Osaka. The effects of the CD45 variants on disease are most likely subtle and not easy to establish in multifactorial diseases because of the likely complex interactions with other genetic and environmental factors. In both autoimmune and infectious diseases, large numbers of genes controlling immune responses have been implicated in contributing to the control of incidence and severity. These include genes that affect the threshold for triggering immune responses and genes that affect the quality of responses through cytokine production (Rioux and Abbas 2005). Both the threshold of response and cytokine signalling can be influenced by CD45 variants (Hermiston et al. 2003; Boxall et al. 2004; Do et al. 2005).

In the present study, we show that the A138G CD45 polymorphism is present at a high frequency in the Far East and at a lower frequency in India. Given its wide geographical distribution and the evidence of relevant functional effects, the variant 138G allele may well have a considerable impact on human disease.

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