Table 3
VEGF −634C/G SNP genotype and HTLV-I provirus load

	CC	CG	GG
HAM (n=202)	743.6 ± 110.9	704.0 ± 76.9	750.4 ± 93.4
HCs (n=202)	224.8 ± 59.0	200.0 ± 33.6	155.0 ± 27.6
All patients combined	441.6 ± 65.3	423.1 ± 42.9	411.0 ± 51.7

The values are shown as the mean tax value (tax copies/10^4 PBMCs) ± SE.

3.3. The VEGF −634 SNP is not a significant predictor of the HTLV-I proviral load in HAM/TSP patients and asymptomatic HTLV-I carriers

To test whether VEGF −634C/G SNP genotype is a significant predictor of the HTLV-I proviral load, we measured the proviral load of HTLV-I and compared it with VEGF −634C/G genotype in HAM/TSP patients and HCs. Our data indicated that there was no association between VEGF −634C/G genotype and HTLV-I proviral load (Table 3), CSF neopterin levels as well as serum HTLV-I antibody titers (data not shown) in our population. Also, the clinical course and disability of HAM/TSP were not specifically associated with the polymorphism and serum VEGF levels in HAM/TSP patients (data not shown).

4. Discussion

HTLV-I infection is of particular interest to the field of immunology as well as neurology because it persists at a remarkably high level despite a vigorous cellular and humoral immune response, and causes inflammatory demyelinating disease only in a minority of infected people. Although certain Tax subtypes were recently reported to carry different risks of HAM/TSP [7], viral factors alone are not sufficient to predict disease. Our recent observations as well as many reported findings strongly suggest that the outcome of HTLV-I infection mainly depends upon a host of genetic factors [17]. Especially, our recent case/control study in Kagoshima strongly supports this idea. In the Kagoshima population, possession of the HLA-class I genes, HLA-A*02 and Cw*08, each independently halve the odds of developing HAM/TSP, whereas possession of the HLA-class I gene, HLA-B*5401 and the HLA-class II gene, HLA-DRB1*0101, predispose a person to HAM/TSP [18,19]. Since HLA itself does not explain the entire disease onset of HAM/TSP, and a non-HLA candidate gene approach has already been shown to be successful in identifying markers in other infectious diseases [20,21], we are now focusing on non-HLA gene polymorphisms as candidate genes that are associated with development of HAM/TSP.

VEGF is a specific mitogen and survival factor for endothelial cells and a key promoter of angiogenesis in physiological and pathophysiological conditions, and promotes inflammatory processes by causing vascular leakage and mobilizing leukocytes [8]. Increased concentrations of free VEGF have been measured in a variety of autoimmune and infectious inflammatory diseases, including rheumatoid arthritis [22], POEMS syndrome [23,24], and Kawasaki disease [25,26]. More interestingly, VEGF expression was consistently upregulated in both acute and chronic multiple sclerosis plaques [27], suggesting that VEGF exacerbate the inflammatory response in autoimmune diseases of the central nervous system and migration of inflammatory cells into the lesions. Since HTLV-I-transformed cells secrete VEGF and bFGF proteins and induce angiogenesis in vitro via HTLV-I Tax-mediated transcriptional activation of VEGF promoter [10] and HAM/TSP is also associated with inflammatory cell infiltrations into central nervous system (CNS), we investigated the influence of VEGF gene polymorphism as well as serum concentration of VEGF in HTLV-I infection.

In the present study, there were no significant differences in any VEGF −634C/G genotypes between HAM/TSP patients and HCs. Also, there were no correlations between serum VEGF levels and CSF neopterin levels as well as serum anti-HTLV-I antibody titers. Furthermore, the clinical course and disability of HAM/TSP were not associated with the VEGF −634C/G polymorphism and serum VEGF levels in HAM/TSP patients, although two ATL patients with organ infiltration showed relatively higher concentration of VEGF in the serum. Taken together, our present results suggest that VEGF −634C/G genotype as well as serum concentrations of VEGF are not susceptibility factors for the development of HAM/TSP. It is still possible that VEGF might have an important role in the affected spinal cord lesion of HAM/TSP, as reported in both acute and chronic MS plaques [27], although further studies are needed to clarify this point.

In conclusion, our results indicate that VEGF in serum is not the suitable factor to evaluate the risk and disease activity of HAM/TSP.

Acknowledgements

The authors thank Dr. Hiroshi Takashima of Kagoshima University for providing genomic DNA samples of seronegative healthy controls. We also thank Ms. Tomoko Muramoto of Kagoshima University for her excellent technical assistance. This work was supported by the Japan Intractable Diseases Research Foundation (to M.S.), Japan Foundation for Aging and Health (to M.S.), Japanese Foundation for AIDS prevention, and the Grant in Aid for Research on Brain Science of the Ministry of Health, Labor and Welfare, Japan.

References

[1] Poiesz BJ, Ruscetti RW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and

cultured lymphocytes of a patient with cutaneous T cell lymphoma. Proc Natl Acad Sci U S A 1980;77:7415–9.
[2] Yoshida M, Miyoshi I, Hinuma Y. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. Proc Natl Acad Sci U S A 1982;79: 2031–5.
[3] Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A, et al. Antibodies to human T-lymphotropic virus type-1 in patients with tropical spastic paraparesis. Lancet 1985;2:407–10.
[4] Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, et al. HTLV-I associated myelopathy, a new clinical entity. Lancet 1986;1: 1031–2.
[5] Uchiyama T. Human T cell leukemia virus type I (HTLV-I) and human diseases. Annu Rev Immunol 1997;15:15–37.
[6] Kaplan JE, Osame M, Kubota H, Igata A, Nishitani H, Maeda Y, et al. The risk of development of HTLV-I associated myelopathy/tropical spastic paraparesis among persons infected with HTLV-I. J Acquir Immune Defic Syndr 1990;3:1096–101.
[7] Furukawa Y, Yamashita M, Usuku K, Izumo S, Nakagawa M, Osame M. Phylogenetic subgroups of human T cell lymphotropic virus (HTLV) type I in the tax gene and their association with different risks for HTLV-I-associated myelopathy/tropical spastic paraparesis. J Infect Dis 2000;182:1343–9.
[8] Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. Endocr Rev 1997;18:4–25.
[9] Awata T, Inoue K, Kurihara S, Ohkubo T, Watanabe M, Inukai K, et al. A common polymorphism in the 5′-untranslated region of the VEGF gene is associated with diabetic retinopathy in type 2 diabetes. Diabetes 2002;51:1635–9.
[10] El-Sabban ME, Merhi RA, Haidar HA, Arnulf B, Khoury H, Basbous J, et al. Human T-cell lymphotropic virus type 1-transformed cells induce angiogenesis and establish functional gap junctions with endothelial cells. Blood 2002;99:3383–9.
[11] Osame M. Review of WHO Kagoshima meeting and diagnostic guidelines for HAM/TSP. In: Blattner WA, editor. Human retrovirology. New York: Raven Press; 1990. p. 191–7.
[12] Nagai M, Usuku K, Matsumoto W, Kodama D, Takenouchi N, Moritoyo T, et al. Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: high proviral load strongly predisposes to HAM/TSP. J Neurovirol 1998;4:586–93.
[13] Nomoto M, Utatsu Y, Soejima Y, Osame M. Neopterin in cerebrospinal fluid: a useful marker for diagnosis of HTLV-I-associated myelopathy/tropical spastic paraparesis. Neurology 1991;41:457.
[14] Boiardi L, Casali B, Nicoli D, Farnetti E, Chen Q, Macchioni P, et al. Vascular endothelial growth factor gene polymorphisms in giant cell arteritis. J Rheumatol 2003;30:2160–4.
[15] Hayashibara T, Yamada Y, Onimaru Y, Tsutsumi C, Nakayama S, Mori N, et al. Matrix metalloproteinase-9 and vascular endothelial growth factor: a possible link in adult T-cell leukemia cell invasion. Br J Haematol 2002;116:94–102.
[16] Hayashibara T, Yamada Y, Miyanishi T, Mori H, Joh T, Maeda T, et al. Vascular endothelial growth factor and cellular chemotaxis: a possible autocrine pathway in adult T-cell leukemia cell invasion. Clin Cancer Res 2001;7:2719–26.
[17] Bangham CRM. The immune response to HTLV-I. Curr Opin Immunol 2000;12:397–402.
[18] Jeffery KJ, Usuku K, Hall SE, Matsumoto W, Taylor GP, Procter J, et al. HLA alleles determine human T-lymphotropic virus-1 (HTLV-I) proviral load and the risk of HTLV-I-associated myelopathy. Proc Natl Acad Sci U S A 1999;96:3848–53.
[19] Jeffery KJ, Siddiqui AA, Bunce M, Lloyd AL, Vine AM, Witkover AD, et al. The influence of HLA class I alleles and heterozygosity on the outcome of human T cell lymphotropic virus type I infection. J Immunol 2000;165:7278–84.
[20] McGuire W, Hill AVS, Allsopp CEM, Greenwood BM, Kwiatkowski D. Variation the TNF-alpha promoter region associated with susceptibility to cerebral malaria. Nature 1994;371:508–10.
[21] Seki N, Yamaguchi K, Yamada A, Kamizono S, Sugita S, Taguchi M, et al. Polymorphism of the 5′-flanking region of the tumor necrosis factor (TNF)-alpha gene and susceptibility to human T-cell lymphotropic virus type 1 (HTLV-I) uveitis. J Infect Dis 1999;180:880–3.
[22] Bottomley MJ, Webb NJ, Watson CJ, Holt L, Bukhari M, Denton J, et al. Placenta growth factor (PlGF) induces vascular endothelial growth factor (VEGF) secretion from mononuclear cells and is co-expressed with VEGF in synovial fluid. Clin Exp Immunol 2000; 119:182–8.
[23] Watanabe O, Maruyama I, Arimura K, Kitajima I, Arimura H, Hanatani M, et al. Overproduction of vascular endothelial growth factor/vascular permeability factor is causative in Crow-Fukase (POEMS) syndrome. Muscle Nerve 1998;21:1390–7.
[24] Soubrier M, Dubost JJ, Serre AF, Ristori JM, Sauvezie B, Cathebras P, et al. Growth factors in POEMS syndrome: evidence for a marked increase in circulating vascular endothelial growth factor. Arthritis Rheum 1997;40:786–7.
[25] Maeno N, Takei S, Masuda K, Akaike H, Matsuo K, Kitajima I, et al. Increased serum levels of vascular endothelial growth factor in Kawasaki disease. Pediatr Res 1998;44:596–9.
[26] Terai M, Yasukawa K, Narumoto S, Tateno S, Oana S, Kohno Y. Vascular endothelial growth factor in acute Kawasaki disease. Am J Cardiol 1999;83:337–9.
[27] Proescholdt MA, Jacobson S, Tresser N, Oldfield EH, Merrill MJ. Vascular endothelial growth factor is expressed in multiple sclerosis plaques and can induce inflammatory lesions in experimental allergic encephalomyelitis rats. J Neuropathol Exp Neurol 2002;61:914–25.

MAJOR ARTICLE

Polymorphism in the Interleukin-10 Promoter Affects Both Provirus Load and the Risk of Human T Lymphotropic Virus Type I–Associated Myelopathy/Tropical Spastic Paraparesis

Amir H. Sabouri,[1] Mineki Saito,[1,5] Alun L. Lloyd,[6] Alison M. Vine,[7] Aviva W. Witkover,[7] Yoshitaka Furukawa,[3] Shuji Izumo,[4] Kimiyoshi Arimura,[1] Sara E. F. Marshall,[8,a] Koichiro Usuku,[2] Charles R. M. Bangham,[7] and Mitsuhiro Osame[1]

Departments of [1]Neurology and Geriatrics and [2]Medical Information Science, Kagoshima University Graduate School of Medical and Dental Sciences, and [3]Division of Blood Transfusion Medicine, Kagoshima University Hospital, and [4]Department of Molecular Pathology, Center for Chronic Viral Diseases, Kagoshima University, Kagoshima, and [5]Japan Foundation for Aging and Health, Higashiura, Aichi, Japan; [6]Program in Theoretical Biology, Institute of Advanced Study, Princeton, New Jersey; [7]Department of Immunology, Imperial College, London, and [8]Oxford Transplantation Centre, Churchill Hospital, Oxford, United Kingdom

To investigate non–human leukocyte antigen candidate genes that influence the outcome of human T cell lymphotropic virus (HTLV) type I infection, we analyzed 6 single-nucleotide polymorphisms in the interleukin (IL)–10 promoter region in 280 patients with HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP) and 255 HTLV-I–seropositive asymptomatic carriers from an area where HTLV-I is endemic. The IL-10 −592 A allele, which shows lower HTLV-I Tax–induced transcriptional activity than the C allele in the Jurkat T cell line, was associated with a >2-fold reduction in the odds of developing HAM/TSP ($P = .011$; odds ratio [OR], 0.50 [95% confidence interval, 0.30–0.86]) by reducing the provirus load in the whole cohort ($P = .009$, analysis of variance). Given the OR and the observed frequency of IL-10 −592 A, we demonstrate that this allele prevents ~44.7% (standard deviation, ± 13.1%) of potential cases of HAM/TSP, which indicates that it defines one component of the genetic susceptibility to HAM/TSP in the cohort.

Human T-cell lymphotropic virus (HTLV) type I is the first characterized human retrovirus [1, 2] and is associated with adult T cell leukemia (ATL) [3, 4] and HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP) [5, 6]. Unlike HIV, HTLV-I causes no disease in a majority of infected subjects (healthy carriers [HCs]). However, ~2%–3% develop ATL, and another 2%–3% develop a disabling chronic inflammatory disease involving the central nervous system (HAM/TSP), eyes, lungs, or skeletal muscles [7]. The lifetime incidence for developing HAM/TSP is only 0.25% in Japan [8]. The factors that cause these different manifestations of HTLV-I infection are not fully understood. However, our previous population association study of >200 cases of HAM/TSP and >200 HTLV-I–seropositive HCs revealed several important risk factors for HAM/TSP. One of the major risk factors is the provirus load, as has been reported elsewhere [9]. The median provirus load was 16 times higher in patients with HAM/TSP than in HCs, and a high provirus load was also associated with an increased risk of progression to disease [10]. We next investigated HLA associations and found that the HLA-A*02 and -Cw*08 genes were associated with a lower HTLV-I provirus

Received 9 February 2004; accepted 12 April 2004; electronically published 31 August 2004.

Financial support: Japan Intractable Diseases Research Foundation (to M.S.); Japanese Foundation for AIDS Prevention; Ministry of Health, Labor and Welfare, Japan (Grant-in-Aid for Research on Brain Science); Wellcome Trust (to C.R.M.B., A.M.V., and A.D.W.).

[a] Present affiliation: Department of Immunology, Imperial College, London, United Kingdom.

Reprints or correspondence: Dr. Mineki Saito, Dept. of Neurology and Geriatrics, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan (mineki@m3.kufm.kagoshima-u.ac.jp).

The Journal of Infectious Diseases 2004;190:1279–85

0022-1899/2004/19007-0011$15.00

Table 1. Primers and restriction enzymes used for restriction fragment-length polymorphism analysis.

Polymorphism, primer direction	Primer sequence	Restriction enzyme	Reference (accession no.)[a]
−3575 (T/A)		TSP509I	25
Forward	5′-GTTTTTCCTTCATTTGCAGC-3′		
Reverse	5′-ACACTGTGAGCTTCTTGAGG−3′		
−2849 (G/A)		*Alw*I	AF295024
Forward	5′-CTGTAATCTCAGCACTCTGG-3′		
Reverse	5′-AGTTCAAGCCATTCTCCTGC-3′		
−2763 (C/A)		*Dde*I	25
Forward	5′-GAGGACTTGCACCAGGGAACT-3′		
Reverse	5′-TCCCGAGTAGCTGGGACTACA-3′		
−1082 (A/G)		*Mnl*I	26
Forward	5′-TCTGAAGAAGTCCTGATGTCACTG-3′		
Reverse	5′-ACTTTCATCTTACCTATCCCTACTTCC-3′		
−819 (T/C)		*Mae*III	27
Forward	5′-ATCCAAGACAACACTACTAA-3′		
Reverse	5′-TAAATATCCTCAAAGTTCC-3′		
−592 (A/C)		*Rsa*I	28
Forward	5′-CCTAGGTCACAGTGACGTGG -3′		
Reverse	5′-GGTGAGCACTACCTGACTAGC-3′		

[a] Accession numbers for GenBank/EMBL/DDBJ.

load and with protection from HAM/TSP, whereas HLA-DRB1*0101 and -B*5401 were associated with susceptibility to HAM/TSP; HLA-B*5401 was also associated with a higher provirus load in patients with HAM/TSP [11, 12]. We further examined the non-HLA host genetic factors that affect the risk of HAM/TSP and reported previously [13] that the tumor necrosis factor promoter −863 A allele predisposes toward HAM/TSP, whereas the stromal cell–derived factor–1 +801A 3′ untranslated region and interleukin (IL)–15 191 C alleles confer protection. In another study [14], we reported the association between variation in the HTLV-I *tax* gene and the risk of HAM/TSP. The *tax* subgroup A was more frequently observed in patients with HAM/TSP, and this effect was independent of HLA-A*02. These findings suggest that both host genetic factors and HTLV-I subgroup play a part in determining the risk of HAM/TSP.

To investigate further the non-HLA host genetic factors that influence the outcome of HTLV-I infection, we analyzed 6 single-nucleotide polymorphisms (SNPs) in the IL-10 promoter region and quantified the effect of each SNP on the risk of HAM/TSP, because recent studies have revealed a close association between IL-10 promoter polymorphisms and the outcome of certain viral infections, such as Epstein-Barr virus (EBV) [15], hepatitis B virus (HBV) [16], hepatitis C virus (HCV) [17], and HIV-1 [18], which suggests that particular polymorphisms in the IL-10 promoter contribute to the host immune reaction against viruses.

PATIENTS, MATERIALS, AND METHODS

Study population. Two hundred eighty patients with HAM/TSP were compared with 255 randomly selected HCs. All patients and control subjects were Japanese and resided in Kagoshima Prefecture, Japan. The diagnosis of HAM/TSP was made according to the World Health Organization diagnostic criteria [19]. All subjects provided written informed consent.

Detection of SNPs in the IL-10 promoter region. Polymerase chain reaction (PCR)–restriction fragment–length polymorphism analysis was performed for 6 SNPs. Primers and restriction enzymes used in the study are presented in table 1. A genomic PCR was performed with 50 ng of genomic DNA as template, 20 pmol of each primer, 5 mmol/L dNTP, reaction buffer provided by the manufacturer, and 1 U of Takara-Taq DNA polymerase (Takara) in a final volume of 50 μL. Fifteen microliters of the amplified PCR product was then digested for 12 h with the use of each restriction enzyme. Finally, digested PCR products were electrophoresed through a 2% agarose gel and visualized by ethidium bromide.

Provirus load measurement. To examine the HTLV-I provirus load, we performed a quantitative PCR method using an ABI Prism 7700 (PE-Applied Biosystems) with 100 ng of genomic DNA (~10^4 cells) from peripheral blood mononuclear cell (PBMC) samples, as reported elsewhere [10]. When β-actin was used as an internal control, the amount of HTLV-I provirus DNA was calculated by copy number of HTLV-I (pX) per 1 × 10^4 PBMCs = [(copy number of pX)/(copy number of β-

actin/2)] × 10^4. All samples were tested in triplicate. The lower limit of detection was 1 pX/10^4 PBMCs.

Cell line and plasmids. The human T-cell line Jurkat was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The expression vector pCG-Tax and the control vector pCG-BL were provided by Dr. J. Fujisawa (Kansai Medical University, Osaka, Japan). The pCG-Tax expression vector based on the human cytomegalovirus promoter for HTLV-I *tax* was constructed by inserting *tax* cDNA into the *Xba*I-*Bam*HI site of pCG-BL, as described elsewhere [20]. Human IL-10 promoter fragments (fragment −890 to +120; GenBank accession number X78437) were amplified by PCR from genomic DNA from 2 patients with HAM/TSP—one −592 AA homozygote and one CC homozygote—as described elsewhere [21]. The primers used to amplify the IL-10 region were IL-10 -890 (5′-AGC TCG AGA GTT GGC ACT GGT GTA CC-3′) and IL-10 AS (5′-ACT TCG AAG TTA GGC AGG TTG CCT G-3′). A promoter fragment that does not contain the −592 SNP, as well as the neighboring Sp-1 and Ets binding sites (fragment −571 to +120), was also amplified with the primers IL-10 −571 (5′-AAC CTC GAG GGA TAT TTA GCC CAC-3′) and IL-10 AS. The amplified products were subcloned into the pCR-Blunt II-TOPO vector (Invitrogen), and the sequences were confirmed. The correct insertions were subcloned into the *Xho*I polylinker site of the pGL2 Basic luciferase reporter vector (Promega), and sequences were confirmed again.

Transient transfection and luciferase assay. Five hundred thousand Jurkat cells were cotransfected with 2 μg of a reporter plasmid (IL-10 −592 A-Luc or IL-10 −592 C-Luc), together with 0.5 μg of either pCG-Tax or pCG-BL [20] and 300 ng of pRL-TK (Promega), to control transfection efficiency. The results of preliminary studies that measured luciferase activities from cell lysates at 24, 48, and 72 h after transfection indicated that the greatest luciferase activity was at 48 h after transfection. Therefore, after 48 h of cultivation at 37°C, cells were harvested, washed with PBS, and lysed in reporter lysis buffer (Promega). Luciferase assays were performed by use of the Dual Luciferase Assay System (Promega) and a TD-20/20 luminometer (Turner Designs). All assays were performed at least 3 times, each in duplicate.

Statistical and logistic-regression analysis. The χ^2 test was used to examine associations between HAM/TSP and the IL-10 promoter polymorphism. General linear model (GLM) analysis [22], which is a general form of multiple regression, was used to identify which factors were predictors of provirus load, in patients with HAM/TSP alone, in HCs alone, or in all subjects in the study. Logistic-regression analysis was used to identify which factors could be used to predict the odds of HAM/TSP and to fit an equation to estimate the risk in an individual of known genotype. The prevented fraction (Fp) of disease was calculated as described elsewhere [11].

RESULTS

Association of the IL-10 −592 A allele with a lower risk of HAM/TSP. The median age of patients with HAM/TSP (60.0 years; range, 12–81 years; 69.0% female) was greater than that of HCs (41 years; range, 16–65 years; 57.6% female), and there were more females in the HAM/TSP group and an absence of subjects <16 or >65 years old from the HCs; however, these factors did not affect the frequency of individual HLA alleles (data not shown). In addition, because the prevalence of HAM/TSP in Kagoshima is <1% among individuals infected with HTLV-I, very few HCs in the present cohort would be expected to develop HAM/TSP. There were no significant differences in the distribution of all genotypes and allele frequencies between 102 patients with HAM/TSP and 102 HCs in 4 SNPs tested (table 2). The nucleotide at position −2849 was nonpolymorphic in 102 patients with HAM/TSP and 102 HCs. In contrast, the IL-10 −592 A/C SNP showed a significant difference in allele frequency. We therefore analyzed further a total of 280 patients with HAM/TSP and 255 HCs (table 2; $\chi^2 = 8.48$; 2 *df*; $P = .014$) and identified a significant association between possession of an A residue in the IL-10 promoter −592 A/C SNP and a reduced risk of HAM/TSP. Possession of the IL-10 −592 A allele was associated with a >2-fold reduction in the odds of developing HAM/TSP ($P = .011$; odds ratio [OR], 0.50 [95% confidence interval, 0.30–0.86]). Given this OR and the observed frequency of the IL-10 −592 A allele in Kagoshima, we can estimate the Fp [11]. Here, Fp = 44.7% (SD, ±13.1%) when the prevalence rate of HAM/TSP is 0.01, which indicates that the IL-10 −592 A allele prevents ~44.7% (SD, ±13.1%) of potential cases of HAM/TSP in the study population.

Association of the presence of the A allele with a lower provirus load in the whole Kagoshima cohort of HTLV-I–infected individuals. We next tested the hypothesis that, if a gene is associated with a protection from HAM/TSP, it is also associated with a reduction in provirus load in HCs, given that the risk of developing HAM/TSP is dependent on the provirus load [10]. Table 3 summarizes the HTLV-I provirus load in patients with HAM/TSP and HCs, subdivided according to their IL-10 −592 A/C genotype. Because histograms of provirus load exhibited right-skewed distributions, the standard statistical technique of logarithmic transformation [22] was also used to mitigate this feature, which resulted in the data being more amenable to statistical analysis by parametric methods. To confirm whether the IL-10 −592 A/C SNP is a significant predictor of provirus load in the entire cohort, we performed multiple-regression analysis (GLMs; see Patients, Materials, and Methods). The results showed that the IL-10 −592 A/C SNP is a

Table 2. Interleukin (IL)–10 polymorphisms among patients with human T cell lymphotropic virus (HTLV) type I–associated myelopathy/tropical spastic paraparesis (HAM/TSP) and healthy HTLV-I carriers (HCs).

Polymorphism	HAM/TSP	HCs	*P*
−3575(T/A)			1.00
TT	99 (97.1)	99 (97.1)	
AT	3 (2.9)	3 (2.9)	
AA	0 (0)	0 (0)	
−2849 (G/A)			NA
GG	102 (100)	102 (100)	
GA	0 (0)	0 (0)	
AA	0 (0)	0 (0)	
−2763 (C/A)			.24
CC	95 (93.1)	89 (87.3)	
AC	7 (6.9)	13 (12.7)	
AA	0 (0)	0 (0)	
−1082 (A/G)			.38
AA	93 (91.2)	88 (86.3)	
AG	9 (8.8)	14 (13.7)	
GG	0 (0)	0 (0)	
−819 (T/C)			1.00
CC	12 (11.8)	12 (11.8)	
TC	49 (48.0)	48 (47.1)	
TT	43 (42.2)	42 (41.2)	
−592 (A/C)			**.014**[a]
AA	117 (41.8)	101 (39.6)	
AC	117 (41.8)	131 (51.4)	
CC	46 (16.4)	23 (9.0)	

NOTE. Data are no. of samples (%). The IL-10 −592 A allele was associated with a >2-fold reduction in the odds of HAM/TSP ($P = .011$; odds ratio, 0.50 [95% confidence interval, 0.30–0.86]). The proportion of potential cases of HAM/TSP that are prevented by the presence of the IL-10 −592 A allele (the prevented fraction of disease) [11] was 44.7% (SD, ±13.1%) when prevalence rate of HAM/TSP was 0.01, indicating that IL-10 −592 A allele prevents 44.7% (SD, ±13.1%) of potential cases of HAM/TSP in the study population. NA, not applicable.

[a] χ^2 for genotype, $\chi^2 = 8.48$.

significant predictor of provirus load in the entire cohort ($n = 535$; $P = .004$, Kruskal-Wallis test; $P < .01$, GLM on the log-transformed or -untransformed data). This SNP was also a significant predictor of provirus load in the HC group alone ($n = 255$; $P = .040$, Kruskal-Wallis test), but not in the HAM/TSP group ($n = 280$; $P = .243$, Kruskal-Wallis test). Also, presence or absence of the IL-10 −592 A allele was a significant predictor of the provirus load in the entire cohort ($n = 535$; $P = .001$, Mann-Whitney *U* test; $P < .005$, GLM), although this relationship was only marginally significant in the HC group ($n = 255$; $P = .103$; Mann-Whitney *U* test; $P < .13$, GLM). These analyses indicate that the IL-10 −592 A/C SNP was a significant predictor of the provirus load and that the presence of A allele was associated with a lower provirus load in the whole Kagoshima cohort of HTLV-I–infected individuals (table 3).

*IL-10 −592 A/C SNP—significant predictor of HAM/TSP even after accounting for provirus load or HLA-A*02.* As was already mentioned, there was a significant association between the odds of developing HAM/TSP and the IL-10 −592 A/C SNP genotype according to the results of single-factor χ^2 analysis at both the allele and the genotype level. To confirm whether the IL-10 −592 A/C SNP genotype remains a significant predictor of HAM/TSP even after taking into account the other significant predictors identified by our previous analyses, such as provirus load and HLA-A*02, we performed logistic-regression analysis. As a result, in logistic-regression analysis that included HTLV-I provirus load and IL-10 −592 A/C SNP genotype treated as a 3-level factor (i.e., AA vs. AC vs. CC), the IL-10 −592 A/C SNP remained significant as a predictor of HAM/TSP ($P = .043$). We can calculate the risk for HAM/TSP by ln(odds of HAM/TSP) = −4.1212–0.5668 (if AC)–0.0235 (if CC) + 2.0764 × $\log_{10}$ (pX/10^4 PBMCs). When we treated the IL-10 −592 A/C SNP genotype as a 2-level factor, inclusion of the absence or presence of the A allele was not significant when $\log_{10}$(pX/10^4 PBMCs) was included ($P = .399$). However, the inclusion of the absence or presence of C was significant when $\log_{10}$(pX/10^4 PBMCs) was included ($P = .047$). Therefore, we conclude that the IL-10 −592 A/C SNP genotype has predictive power for HAM/TSP even after we accounted for the HTLV-I provirus load. Next, to test whether the IL-10 −592 A/C SNP genotype remains a predictor of HAM/TSP even after we accounted for HLA-A*02, we further performed the logistic-regression analysis using samples that are available on both IL-10 −592 A/C SNP and HLA-A*02 ($n = 402$). In logistic-regression analysis that included the HLA-A*02 and IL-10 −592 A/C SNP genotype, both HLA-A*02 ($P = .001$) and IL-10 −592 A/C SNP ($P = .014$) remained significant as predictors of HAM/TSP. In this case, we can calculate the risk for HAM/TSP by the equation ln(odds of HAM/TSP) = 0.4321–0.8876 (if A*02-positive)–0.2242 (if AC) + 0.7488 (if CC). In conclusion, the IL-10 −592 A/C SNP remains as a significant predictor of HAM/TSP even after taking into account the effects of the 2 known significant predictors of the risk of HAM/TSP—provirus load and HLA-A*02.

Effect of IL-10 −592 A/C SNP on HTLV-I Tax–mediated IL-10 promoter activity. To examine the functional significance of the −592 A/C SNP in HTLV-I infection, a 1010-bp promoter of the IL-10 gene (−890 to +120) carrying either the C or the A allele was inserted upstream of the luciferase gene in the pGL2-Basic plasmid vector, and luciferase assays were done. Because many polymorphisms in the IL-10 gene have been identified, numerous combinations of these polymorphisms may exist. Although our Kagoshima cohort of patients with HAM/TSP is the world's largest, <300 patients are available for analysis, so it would be meaningless to analyze all combinations of the IL-10 SNPs. The only sequence difference between the 2 reporter vectors was

Table 3. Interleukin (IL)–10 −592 A/C single-nucleotide polymorphism (SNP) genotype and human T cell lymphotropic virus (HTLV) type I provirus load.

Group	AA	AC	CC
HAM/TSP (280)	679.0 ± 58.2 (117)	785.8 ± 63.8 (117)	959.3 ± 139.6 (46)
HC (255)	77.2 ± 13.7 (101)	129.6 ± 15.7 (131)	194.6 ± 50.1 (23)
All patients combined (535)	400.2 ± 37.8 (218)	439.2 ± 37.5 (248)	704.4 ± 103.8 (69)

NOTE. Values are the average *tax* value (no. of *tax* copies/10^4 PBMCs) ± SE. The IL-10 −592 A/C SNP was a significant predictor of provirus load in the entire cohort ($n = 535$; $P = .004$, Kruskal-Wallis test; $P < .01$, general linear model analysis on log-transformed or -untransformed data) and of provirus load in the HTLV-I–seropositive asymptomatic carriers alone ($n = 255$; $P = .040$, Kruskal-Wallis test) but not in the HAM/TSP group ($n = 280$; $P = .243$, Kruskal-Wallis test). Values in parentheses are nos. of individuals tested. HAM/TSP, associated myelopathy/tropical spastic paraparesis; HC, healthy carrier.

the residue at position −592, which allowed us to estimate the functional differences associated with the −592 A or C residues alone. The results of the experiments showed that the functional differences were associated with the −592 A or C residues alone on HTLV-I Tax–mediated IL-10 promoter activity. These results showed that the ectopic expression of the Tax protein in Jurkat T cells increased IL-10 promoter activity by ~3 times with the A construct and 6 times with the C construct, compared with HCs ($P < .01$, Mann-Whitney *U* test) (figure 1). In contrast, the promoter fragment (fragment −571 to +120), which does not contain −592 SNP, as well as the neighboring Sp-1 and Ets binding site, was not transactivated by Tax. The basal luciferase activity without the transfecting Tax-expression vector (i.e., with transfecting empty vector, pCG-BL) did not differ between the A and C constructs. These results indicated that Tax directly transactivates the IL-10 promoter and that the C allele is more effective for Tax-mediated transcription than the A allele.

DISCUSSION

IL-10 is an important immunoregulatory cytokine that is involved in inflammatory responses, autoimmune diseases, and the response to infectious agents [23]. Although IL-10 has been reported to suppress the synthesis of proinflammatory cytokines from T cells and monocytes/macrophages, animal models have suggested that the overexpression of IL-10 in vivo can cause organ-specific autoimmune diseases, such as Sjögren syndrome [24] and type 1 diabetes [25]. Therefore, IL-10 is not regarded simply as an immunoinhibitory cytokine but also as a powerful immunostimulatory cytokine. Because transgenic mice containing the HTLV-I *tax* gene under the control of the viral long-terminal repeat (LTR) have previously been shown to develop an exocrinopathy involving the salivary and lachrymal glands that resembles Sjögren syndrome [26], which is frequently observed in patients with HAM/TSP [27], and be-

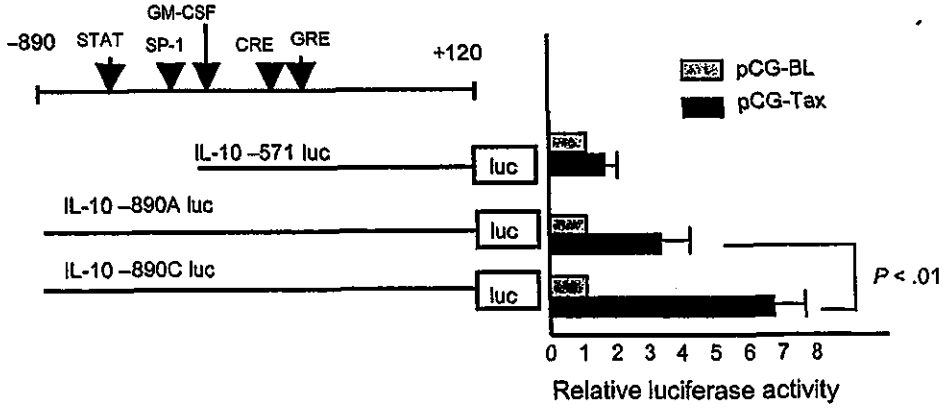

Figure 1. Interleukin (IL)–10 −592 A/C polymorphism and the Tax-mediated transcription of the IL-10 promoter. Jurkat cells were transfected with human T cell lymphotropic virus (HTLV) type I Tax expressing (pCG-Tax) or control (pCG-BL) vector and luciferase (luc) reporter constructs containing the full-length IL-10 promoter with −592 AA (−890 A-luc) or CC (−890 C-luc) or luc reporter plasmid without the specificity protein (Sp)–1 or −592 A/C SNP (−572 luc) sites. *Gray bars*, Luc activity of each reporter plasmid with control vector pCG-BL. *Black bars*, Luc activity of each reporter plasmid with Tax-expressing vector pCG-Tax. The activities are given relative to the activity of each reporter plasmid with control vector pCG-BL, which was defined as 1. The mean ± SD from 3 independent experiments is shown. The basal luciferase activity with pCG-BL was not different between −890 A-luc and −890 C-luc. The difference of luciferase activity with pCG-Tax between −890 A-luc and −890 C-luc was statistically significant ($P < .01$, Mann-Whitney *U* test). CRE, cyclic AMP response element; GM-CSF, granulocyte macrophage colony-stimulating factor; GRE, glucocorticoid response element; STAT, signal transducer and activator of transcription.

cause IL-10 mRNA expression was induced by HTLV-I Tax in both transiently and stably transfected Jurkat cells [28], it is likely that Tax directly transactivates the IL-10 promoter. The resulting overexpression of Tax in vivo may cause a Sjögren-like syndrome via an IL-10–mediated mechanism.

The implication of a heritable genetic basis for IL-10 production is supported by the concordance of IL-10 production in monozygotic twins, which suggests that genetic polymorphism could account for up to 75% of the observed variation in IL-10 production [29]. As was already mentioned, several studies have shown an association between particular polymorphisms in the human IL-10 promoter region and the outcome of certain viral infections, such as EBV [15], HBV [16], HCV [17], and HIV-1 [18]. In view of the immunomodulatory and anti-inflammatory effects of IL-10, we initially hypothesized that genetically determined lower production of IL-10 (associated with the allele −592 A) might influence disease susceptibility to HAM/TSP. This is the case for HIV-1 infection, because individuals with the IL-10 −592 AA genotype have been reported to be at higher risk of HIV-1 infection and rapid progression to AIDS [18]. In contrast, the present data show that, in HTLV-I infection, possession of the IL-10 −592 A allele prevented ~44.7% (SD, ± 13.1%) of potential cases of HAM/TSP and was also a significant predictor for a lower provirus load in the entire cohort.

The −592 A/C SNP is located between the Sp1 and Ets binding site within the region between −652 and −571 nt that is necessary for IL-10 transcription [21]. It is of interest that previous reports have indicated that Tax transactivates the parathyroid hormone–related protein promoter by forming a ternary complex between Tax, Ets, and Sp-1, which acts on the promoter Sp-1 and Ets binding sites [30]. Another report showed that the HTLV-I LTR also contains a motif related to the Ets-binding sequence, named TRE-2S [31]. More important, 1 copy of the cyclic AMP response element (CRE)–like 21-bp sequence and TRE-2S in the HTLV-I LTR, contributes to the transactivation of viral gene via a ternary complex formed between Tax, Gli2 (TRE-S binding Gli oncogene family protein), and CRE-binding protein [32]. These findings suggest that a common mechanism of the HTLV-I Tax–mediated transactivation of the promoter of target genes ternary complexes formed with 2 different transcription factors. Furthermore, the results also suggest that the IL-10 promoter −592 A/C SNP, which lies between the Sp-1 and Ets binding sites, affects Tax-mediated transcription. Indeed, our cotransfection study using a Tax-expressing vector and Jurkat cells demonstrated that a IL-10 −592 luciferase vector carrying the high producer allele (C) showed higher Tax-mediated transcription than that of low producer allele (A), whereas a promoter fragment (fragment −571 to +120) that does not contain −592 SNP, as well as the neighboring Sp-1 and Ets binding site, was not transactivated by Tax. These findings suggested that HTLV-I Tax directly transactivates the IL-10 promoter and that the −592 A/C SNP affects Tax-induced transcription—that is, that the C allele is more effective than the A allele in mediating the Tax-induced transcription of IL-10. In future studies, it may be interesting to test whether Tax, Ets, and Sp-1 form a ternary complex on the IL-10 promoter and whether the −592 SNP affects this complex formation.

Among >90 non-HLA candidate gene loci that we have so far examined, the IL-10 −592 A/C SNP is the only non-HLA candidate gene locus associated with a significant reduction in both the provirus load and the risk of HAM/TSP. This observation is exactly analogous to the argument that we previously reported for HLA-A*02 and -Cw*08, where, in each case, possession of the allele was associated with both a significant reduction in provirus load in the HCs and a significant reduction in the risk of HAM/TSP [11, 12]. Thus, one possible mechanism for the observed IL-10 promoter effect is that increased the production of IL-10 reduces the efficiency of immune surveillance of HTLV-I infection—for example, by reducing the number or the activity of HTLV-I–specific cytotoxic T lymphocytes. However, the IL-10 promoter genotype remained a significant predictor of the risk of HAM/TSP even after taking the provirus load into account. This observation suggests that IL-10 increases the risk of HAM/TSP by another mechanism in addition to an apparent effect on provirus load.

In conclusion, we report that the IL-10 −592 A allele, which is associated with lower HTLV-I Tax–mediated transcriptional activity, influences both the provirus load in HTLV-I–infected individuals and the susceptibility to HAM/TSP in the Kagoshima cohort. This effect remains significant even after taking into account the other 2 known major predictors of HAM/TSP risk in this cohort—provirus load and HLA-A*02 genotype—which suggests a powerful argument in favor of a real physiological effect of this polymorphism. Further functional studies to clarify the role of IL-10 in HTLV-I infection may reveal immunotherapeutic strategies that would retard the development of HAM/TSP.

Acknowledgments

We thank Jun-ichi Fujisawa, Kansai Medical University, Osaka, Japan, for the gift of pCG-BL and pCG-Tax plasmids; and Y. Nishino and T. Muramoto, Kagoshima University, for their excellent technical assistance.

References

1. Poiesz BJ, Ruscetti RW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T cell lymphoma. Proc Natl Acad Sci USA 1980;77:7415–9.
2. Yoshida M, Miyoshi I, Hinuma Y. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. Proc Natl Acad Sci USA 1982;79:2031–5.

3. Hinuma Y, Nagata K, Misaka M, et al. Adult T cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. Proc Natl Acad Sci USA 1981; 78:6476–80.
4. Yoshida M, Seiki M, Yamaguchi K, Takatsuki K. Monoclonal integration of human T-cell leukemia provirus in all primary tumors of adult T-cell leukemia suggests causative role of human T-cell leukemia virus in disease. Proc Natl Acad Sci USA 1984; 81:2534–7.
5. Gessain A, Barin F, Vernant JC, et al. Antibodies to human T-lymphotropic virus type–1 in patients with tropical spastic paraparesis. Lancet 1985; 2: 407–10.
6. Osame M, Usuku K, Izumo S, et al. HTLV-I associated myelopathy, a new clinical entity [letter]. Lancet 1986; 1:1031–2.
7. Uchiyama T. Human T cell leukemia virus type I (HTLV-I) and human diseases. Annu Rev Immunol 1997; 15:15–37.
8. Kaplan JE, Osame M, Kubota H, et al. The risk of development of HTLV-I associated myelopathy/tropical spastic paraparesis among persons infected with HTLV-I. J Acquir Immune Defic Syndr 1990; 3:1096–101.
9. Yoshida M, Osame M, Kawai H, et al. Increased replication of HTLV-I in HTLV-I–associated myelopathy. Ann Neurol 1989; 26:331–5.
10. Nagai M, Usuku K, Matsumoto W, et al. Analysis of HTLV-I provirus load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: high provirus load strongly predisposes to HAM/TSP. J Neurovirol 1998; 4:586–93.
11. Jeffery KJM, Usuku K, Hall SE, et al. HLA alleles determine human T-lymphotropic virus–I (HTLV-I) provirus load and the risk of HTLV-I–associated myelopathy. Proc Natl Acad Sci USA 1999; 96:3848–53.
12. Jeffery KJM, Siddiqui AA, Bunce M, et al. The influence of HLA class I alleles and heterozygosity on the outcome of human T cell lymphotropic virus type I infection. J Immunol 2000; 165:7278–84.
13. Vine AM, Witkover AD, Lloyd AL, et al. Polygenic control of human T lymphotropic virus type I (HTLV-I) provirus load and the risk of HTLV-I–associated myelopathy/tropical spastic paraparesis. J Infect Dis 2002; 186:932–9.
14. Furukawa Y, Yamashita M, Usuku K, Izumo S, Nakagawa M, Osame M. Phylogenetic subgroups of human T cell lymphotropic virus (HTLV) type I in the *tax* gene and their association with different risks for HTLV-I–associated myelopathy/tropical spastic paraparesis. J Infect Dis 2000; 182:1343–9.
15. Helminen ME, Kilpinen S, Virta M, Hurme M. Susceptibility to primary Epstein-Barr virus infection is associated with interleukin-10 gene promoter polymorphism. J Infect Dis 2001; 184:777–80.
16. Miyazoe S, Hamasaki K, Nakata K, et al. Influence of interleukin-10 gene promoter polymorphisms on disease progression in patients chronically infected with hepatitis B virus. Am J Gastroenterol 2002; 97:2086–92.
17. Yee LJ, Tang J, Gibson AW, Kimberly R, Van Leeuwen DJ, Kaslow RA. Interleukin 10 polymorphisms as predictors of sustained response in antiviral therapy for chronic hepatitis C infection. Hepatology 2001; 33:708–12.
18. Shin HD, Winkler C, Stephens JC, et al. Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10. Proc Natl Acad Sci USA 2000; 97:14467–72.
19. Osame M. Review of WHO Kagoshima meeting and diagnostic guidelines for HAM/TSP. In: Blattner WA, ed. Human retrovirology: HTLV. New York: Raven Press 1990:191–7.
20. Fujisawa J, Toita M, Yoshimura T, Yoshida M. The indirect association of human T-cell leukemia virus tax protein with DNA results in transcriptional activation. J Virol 1991; 65:4525–8.
21. Ma W, Lim W, Gee K, et al. The p38 mitogen-activated kinase pathway regulates the human interleukin-10 promoter via the activation of Sp1 transcription factor in lipopolysaccharide-stimulated human macrophages. J Biol Chem 2001; 276:13664–74.
22. Grafen A, Hails R. Modern statistics for the life sciences. Oxford: Oxford University Press, 2002.
23. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001; 19:683–765.
24. Saito I, Haruta K, Shimura M, et al. Fas ligand-mediated exocrinopathy resembling Sjögren's syndrome in mice transgenic for IL-10. J Immunol 1999; 162:2488–94.
25. Wogensen L, Lee MS, Sarvetnick N. Production of interleukin 10 by islet cells accelerates immune-mediated destruction of β cells in nonobese diabetic mice. J Exp Med 1994; 179:1379–84.
26. Green JE, Hinrichs SH, Vogel J, Jay G. Exocrinopathy resembling Sjögren's syndrome in HTLV-I tax transgenic mice. Nature 1989; 341:72–4.
27. Nakagawa M, Izumo S, Ijichi S, et al. HTLV-I-associated myelopathy: analysis of 213 patients based on clinical features and laboratory findings. J Neurovirol 1995; 1:50–61.
28. Mori N, Gill PS, Mougdil T, Murakami S, Eto S, Prager D. Interleukin-10 gene expression in adult T-cell leukemia. Blood 1996; 88:1035–45.
29. Westendorp RG, Langermans JA, Huizinga TW, et al. Genetic influence on cytokine production and fatal meningococcal disease. Lancet 1997; 349:170–3.
30. Dittmer J, Pise-Masison CA, Clemens KE, Choi KS, Brady JN. Interaction of human T-cell lymphotropic virus type I Tax, Ets1, and Sp1 in transactivation of the PTHrP P2 promoter. J Biol Chem 1997; 272: 4953–8.
31. Tanimura A, Teshima H, Fujisawa J, Yoshida M. A new regulatory element that augments the Tax-dependent enhancer of human T-cell leukemia virus type 1 and cloning of cDNAs encoding its binding proteins. J Virol 1993; 67:5375–82.
32. Dan S, Tanimura A, Yoshida M. Interaction of Gli2 with CREB protein on DNA elements in the long terminal repeat of human T-cell leukemia virus type 1 is responsible for transcriptional activation by tax protein. J Virol 1999; 73:3258–63.

ELSEVIER

Journal of Neuroimmunology 156 (2004) 188–194

Journal of
Neuroimmunology

www.elsevier.com/locate/jneuroim

Longer dinucleotide repeat polymorphism in matrix metalloproteinase-9 (MMP-9) gene promoter which correlates with higher HTLV-I Tax mediated transcriptional activity influences the risk of HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP)

Daisuke Kodama[a], Mineki Saito[a,b,*], Wataru Matsumoto[a], Amir H. Sabouri[a], Shuji Izumo[c], Kimiyoshi Arimura[a], Koichiro Usuku[d], Charles R.M. Bangham[e], Mitsuhiro Osame[a]

[a]*Department of Neurology and Geriatrics, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan*

[b]*Japan Foundation for Aging and Health, Higashiura, Aichi 470-2101, Japan*

[c]*Department of Molecular Pathology, Center for Chronic Viral Deseases, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan*

[d]*Department of Medical Information Science, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan*

[e]*Department of Immunology, Imperial College, Norfolk Place, W2 1PG, London, UK*

Received 11 March 2004; received in revised form 7 July 2004; accepted 16 July 2004

Abstract

Matrix metalloproteinase-9 (MMP-9) has been reported to be expressed in various inflammatory disorders including human T cell lymphotropic virus type I (HTLV-I) associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-I-infected T-cells expressed high levels of MMP-9 via viral transactivator Tax mediated activation of the MMP-9 promoter. To investigate whether the d(CA) repeat polymorphism in MMP-9 promoter affects the risk of developing HAM/TSP, we compared the allele frequencies between 200 HAM/TSP patients and 200 HTLV-I seropositive asymptomatic carriers (HCs). The longer d(CA) repeat alleles of MMP-9 promoter, which was associated with higher Tax-mediated transcriptional activity, was more frequently observed in HAM/TSP patients than HCs ($p<0.01$ by Mann-Whitney *U*-test). The length alteration of this d(CA) repeat in the MMP-9 promoter may cause phenotypic differences among HTLV-I infected infiltrating cells and may thereby be in part responsible for the development of HAM/TSP.

Keywords: MMP-9; Dinucleotide repeat polymorphism; Promoter; HTLV-I; HAM/TSP

1. Introduction

Human T-cell lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Osame et al., 1986) is a chronic inflammatory progressive disease of the central nervous system (CNS). Although a majority of HTLV-I infected people remain healthy throughout life, only 1-2% of infected people develop HAM/TSP (Kaplan et al., 1990). Our population association study has revealed that one of the major risk factors for developing HAM/TSP is the provirus load (Nagai et al., 1998). The major histocompatibility genes HLA-A*02 and Cw*08 were associated with a lower HTLV-I provirus load and with protection from HAM/TSP whereas HLA-DRB1*0101 and B*5401 were

* Corresponding author. Department of Neurology and Geriatrics, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan. Tel.: +81 99 275 5332; fax: +81 99 265 7164.

E-mail address: mineki@m3.kufm.kagoshima-u.ac.jp (M. Saito).

0165-5728/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneuroim.2004.07.007

associated with susceptibility to HAM/TSP, and B*5401 was also associated with a higher provirus load in HAM/TSP patients (Jeffery et al., 1999, 2000). Since an association of HLA-DRB1*0101 with disease susceptibility was only evident in the absence of the protective effect of HLA-A*02, and an immunodominant epitope of the HTLV-I transactivator protein Tax (Tax11–19) is restricted by HLA-A*02, these results are consistent with the hypothesis that a strong class-I restricted CTL limits HTLV-I replication and reduces the risk of HAM/TSP (Bangham, 2000). Another study indicated an association between HTLV-I *tax* gene variation and the risk of HAM/TSP (Furukawa et al., 2000). The *tax* subgroup A was more frequently observed in HAM/TSP patients and this effect was independent of HLA-A*02. These findings suggest that both host genetic factors and HTLV-I subgroup play a part in determining the risk of HAM/TSP. Some non-HLA host genetic polymorphisms also affect the risk of HAM/TSP. The TNF alpha promoter-863 A allele predisposed to HAM/TSP, whereas SDF-1+801A 3′ UTR, and IL-15 191 C alleles conferred protection against disease (Vine et al., 2002).

Matrix metalloproteinase-9 (MMP-9, 92-kDa type IV collagenase, gelatinase B, CLG4B) is known as a proteolytic enzyme whose main substrate is collagen IV, a major component of extracellular matrix (ECM) of the blood–brain barrier (BBB). MMP-9 participates in in vivo migration of leukocytes, BBB damage, and regulation of inflammatory response by chemokines and cytokines (Alexander and Werb, 1989; Opdenakker and Van Damme, 1994). The main immunohistochemical characteristic of active-chronic inflammatory lesions of the spinal cords of HAM/TSP is the infiltration of T lymphocytes/macrophages in patients (Umehara et al., 1993), and both MMP-2 and MMP-9 were expressed in these infiltrating cells (Umehara et al., 1998; Giraudon et al., 2000). Since MMP-9 levels in both serum and cerebrospinal fluid (CSF) were found to be higher than in normal controls (Umehara et al., 1998), MMP-9 on mononuclear cells may be a key molecule in causing the BBB damage in observed HAM/TSP. Recently, it has been reported that HTLV-I-infected T-cell lines expressed high levels of MMP-9 compared with uninfected T-cell lines, and the viral transactivator protein Tax of HTLV-I activates the MMP-9 promoter and induces MMP-9 expression in T cells (Mori et al., 2002). Another report suggested that the length of the d(CA) repeat located in MMP-9 promoter was related to the transcriptional activity and that heterogeneity of the length of the d(CA) repeat exists in the Japanese population (Shimajiri et al., 1999). These previous findings prompted us to investigate whether there is any correlation between the risk of developing HAM/TSP and the length of the d(CA) repeat in the promoter. In this study we compared the allele frequencies of the d(CA) repeat polymorphism in the MMP-9 promoter region of between 200 HAM/TSP patients and 200 HCs. We further examined the effect of the d(CA) repeat length on the Tax-mediated transcriptional activity of the MMP-9 promoter in a human T cell line as well as MMP-9 levels in cerebrospinal fluid (CSF) of HAM/TSP patients.

2. Materials and methods

2.1. Patients

Two hundred cases each of HAM/TSP and HCs were analyzed. All cases and controls were of Japanese and resided in Kagoshima Prefecture, where HTLV-I is endemic, in southern Japan. The diagnosis of HAM/TSP was made according to the World Health Organization diagnostic criteria (Osame, 1990). All samples were taken under written informed consent.

2.2. Determination of number of d(CA) repeats

Fresh PBMCs were obtained by Histopaque-1077 (Sigma) density gradient centrifugation and isolated samples were stored in liquid nitrogen until use. Genomic DNA was extracted from PBMCs using a QIAamp blood kit (Qiagen) according to the manufacturer's instructions. To determine the length of the d(CA) repeat in the MMP-9 promoter region, genomic DNA was subjected to PCR amplification. Two oligonucleotide primers (20 pmol each) 5′-TTG CCT GAC TTG GCA GTG GAG ACT GC-3′ (forward: −210 to −185 nt) and 5′-TGT TGT GGG GGC TTT AAG GAG-3′ (reverse: −33 to −13 nt), based on the human MMP-9 gene sequences, were used for PCR with 50 ng of genomic DNA as template, 5 mM dNTP, reaction buffer provided by the manufacturer, and 1 unit of Takara-Taq DNA polymerase® (Takara, Tokyo, Japan) in a final volume of 50 μl. After initial denaturing at 94 °C for 5 min, PCR was performed for 35 cycles of denaturing at 94 °C for 1 min, annealing at 60 °C for 1 min and polymerase extension at 72 °C for 1 min followed by final 10 min extension at 72°C. In this PCR reaction, 3 pmol out of 20 pmol of forward primer had been end-labeled with 6-FAM (PE-Applied Biosystems, Tokyo, Japan). An aliquot of each PCR product was subjected to electrophoresis on a 5% polyacrylamide sequencing gel after heat denature and quickly chilled on ice, and the resulting bands were compared with DNA size markers to determine the length of the d(CA) repeats using Genescan software (PE-Applied Biosystems).

2.3. Quantification of HTLV-I provirus load and anti-HTLV-I antibody titers

To examine the HTLV-I provirus load, we carried out a quantitative PCR method using ABI Prism 7700™ (PE-Applied Biosystems) with 100 ng of genomic DNA (roughly equivalent to 10^4 cells) from PBMCs samples as reported previously (Nagai et al., 1998). In this method, the 5′

nuclease activity of Taq polymerase cleaves a nonextendible hybridization probe during the extension phase of PCR. This cleavage generates a specific fluorescent signal which is measured at each cycle. Based on the standard curve created by four known concentrations of template, the concentration of unknown samples was determined. Using β-actin as an internal control, the amount of HTLV-I provirus DNA was calculated by the following formula: copy number of HTLV-I (pX) per 1×10^4 PBMC=[(copy number of pX)/(copy number of β-actin/2)]$\times10^4$. All samples were performed in triplicate. Serum and CSF antibody titers to HTLV-I were determined by a particle agglutination method (Serodia-HTLV-I®, Fujirebio).

2.4. Construction of reporter genes for luciferase assay

To test the possibility whether the length of d(CA) repeats in MMP-9 promoter affects the Tax mediated transcription, a human promoter of MMP-9 gene (−664 to +20) was inserted upstream of the luciferase gene in the pGL2-Basic plasmid vector. Human MMP-9 promoter fragments were amplified by PCR from genomic DNA of the patients that contained 23, 21, and 18 d(CA) repeats. Two primers containing restriction enzyme recognition sites (*Xho*I for MMP-9 29B and *Hin*dIII for MMP-9R, which described in bold-faced letters as follows) were used to amplify the MMP-9 promoter (MMP-9 29B: 5′-GCC **CTC GAG** GGC TGC TAC TGT CCC CTT TA-3′ MMP-9R: 5′-GCC CAA **GCT** TGC CAC CTG GTG AGG GCA GAG GTG T-3′). The amplified products were subcloned into the pCR®-Blunt II-TOPO® vector (Invitrogen, Carlsbad, CA), and the sequences were confirmed. The correct insertions were excised from the TOPO vector by *Xho*I and *Hin*dIII, inserted into the *Xho*I and *Hin*dIII site of pGL2 Basic luciferase reporter vector (Promega, Madison, WI), and sequences were confirmed again.

2.5. Luciferase assay

Human T-cell line Jurkat was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100μg/ml streptomycin. Expression vector pCG-Tax and control vector pCG-BL were kindly provided by Dr. J. Fujisawa at Kansai Medical University, Osaka, Japan. The pCG-Tax expression vector based on the human cytomegalovirus promoter for HTLV-I *tax* was constructed by inserting *tax* cDNA into the *Xba*I–*Bam*HI site of pCG-BL as described previously (Fujisawa et al., 1991). 5×10^5 Jurkat cells were cotransfected with 2 μg of a reporter plasmid (MMP-9 dCA23-Luc, MMP-9 dCA21-Luc or MMP-9 dCA18-Luc), 0.5 μg of either pCG-Tax or pCG-BL (Fujisawa et al., 1989) and 300 ng of pRL-TK (Promega) to control transfection efficiency. Preliminary studies with measurement of luciferase activities from cell lysates at 12, 24, and 48 h after transfection indicated that the greatest luciferase activity was at 48 h following transfection. Therefore, after 48 h cultivation at 37 °C, cells were harvested, washed with phosphate buffered saline (PBS), and lysed in reporter lysis buffer (Promega). Luciferase assay were performed using the Dual Luciferase Assay System (Promega) and TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Luciferase activity was normalized for transfection efficiency. All assays were performed at least three times, each in duplicate.

2.6. Quantification of MMP-9 and neopterin in Cerebrospinal fluid (CSF)

MMP-9 concentration in CSF was measured in duplicate using a commercial ELISA kit (Amersham Pharmacia Biotech, USA). The assay system used is sensitive to typically less than 4.0 ng/ml. Optical density at 450 nm was measured on the ImmunoMini NJ-2300 (Nippon Inter Med, Tokyo, Japan) and MMP-9 concentration was determined by linear regression from a standard curve using the MMP-9 supplied with the kit as standard. The intra-assay coefficient of variation (CV) of this assay was 4.9%, and the inter-assay CV was 8.6%. Neopterin levels were evaluated by high-performance liquid chromatography with fluorimetric detection methods (Nomoto et al., 1991).

2.7. Statistical analysis

Mann–Whitney *U*-test was used for comparing the length of MMP-9 promoter d(CA) repeats between HAM/TSP patients and HCs. Comparison of whole allele distribution between patients with HAM/TSP and HCs was also performed using a chi-square test for 2×11 contingency table with a significance level $p<0.01$. The distribution of each allele and genotype of the d(CA) repeat polymorphism of the MMP-9 promoter gene in HAM/TSP patients was compared with those in HCs using a chi-square test for 2×2 (for allele) or 2×3 (for genotype) contingency table. A Bonferroni multiple adjustment (Motulsky, 1995) was made to the level of significance because of the multiple comparisons for d(CA) repeat allele frequencies. This level was set at $p<0.0051$ [$p=1-0.95^{(1/10)}$].

3. Results

3.1. Clinical characteristics of HAM/TSP patients and asymptomatic HTLV-I carriers

Clinical characteristics of HAM/TSP patients and HTLV-I seropositive asymptomatic carriers (HCs) participating in this study are summarized in Table 1. The median age of HAM/TSP patients (57.5 years) was greater than that of the HCs (42.2 years). The sex ratio of males/females in the HAM/TSP group was 1:2.7, whereas the ratio was 1:1.3 in the HCs. However, there was no correlation between the HTLV-I provirus load and age at blood sampling or duration of disease

Table 1
Clinical characteristics of HAM/TSP patients and HTLV-I seropositive asymptomatic carriers (HCs) participated in this study

	HAM/TSP (n=200)	HCs (n=200)	p value
Age	57.5±11.4	42.2±13.2	<0.01
Sex			
Male	54	87	
Female	146	113	
Serum anti-HTLV-I antibody titer (median)[a]	×8192	×1024	<0.01
CSF anti-HTLV-I antibody titer (median)[a]	×64		
HTLV-I provirus load in PBMCs[b]	686.4±47.1	185.6±31.2	<0.01
Neopterin in CSF (mean±SE, pmol/ml)[c]	79.2±10.1		

[a] Serum and CSF antibody titers to HTLV-1 were determined by a particle agglutination method.
[b] The values of HTLV-I provirus load are shown as the average *tax* value (*tax* copies/10^4 PBMCs)±SE.
[c] Neopterin levels were evaluated by HPLC with fluorimetric detection methods. Normal: <30 pmol/ml.

in the Kagoshima population (Jeffery et al., 1999; Nagai et al., 1998). Because the prevalence of HAM/TSP in Kagoshima is low (≤1%) among HTLV-I seropositives, very few HCs would be expected to develop HAM/TSP. Both serum anti-HTLV-I antibody titer and HTLV-I provirus load were significantly higher in HAM/TSP than HCs. The CSF neopterin level was increased in HAM/TSP patients (normal: <30 pmol/ml). These laboratory features were consistent with our previous observations (Nakagawa et al., 1995).

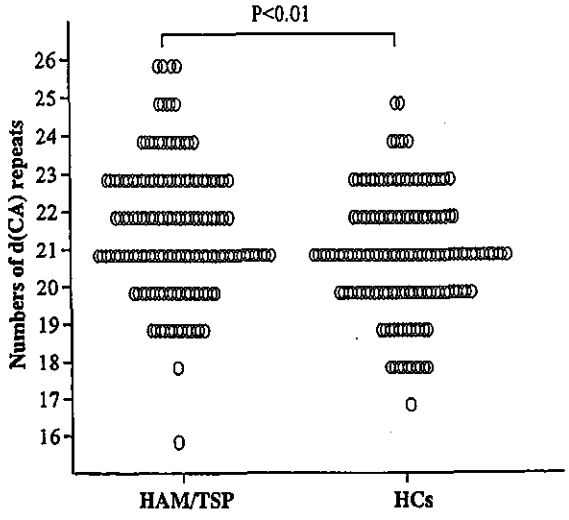

Fig. 1. Distribution of d(CA)n repeats in HAM/TSP patients and HTLV-I seropositive asymptomatic carriers (HCs). The longer d(CA) repeat alleles of MMP-9 promoter was more frequently observed in HAM/TSP patients than HCs (p<0.01 by Mann-Whitney U-test).

3.2. Length of d(CA) repeats in MMP-9 promoter was significantly longer in HAM/TSP patients than HTLV-I seropositive asymptomatic carriers

The number of d(CA) repeats was compared between patients with 200 each of HAM/TSP and HCs by fractionating PCR-amplified DNA fragments on denaturing polyacrylamide sequencing gels. As previously reported (Shimajiri et al., 1999), most of the samples tested had two MMP-9 alleles that contained 20 or more d(CA) repeats

Table 2
Distribution of dinucleotide repeat polymorphisms in the MMP-9 gene promoter

	Allele*				Genotype**			
	HAM/TSP		HCs		HAM/TSP		HCs	
d(CA)	Obs	Freq (%)	Obs	Freq (%)	homo-zygote	hetero-zygote	home-zygote	hetero-zygote
26	4	1.00	0	0	0	4	0	0
25	6	1.50	2	0.50	1	4	0	2
24[a]	18	4.50	6	1.50	3	12	0	6
23[b]	76	19.00	44	11.00	5	66	2	40
22	52	13.00	45	11.25	3	46	2	41
21	189	47.25	198	49.50	44	101	49	100
20[c]	38	9.50	84	21.00	1	36	3	78
19	15	3.75	12	3.00	1	13	2	8
18	1	0.25	8	2.00	0	1	0	8
17	0	0	1	0.25	0	0	0	1
16	1	0.25	0	0	0	1	0	0
Total	400	100	400	100	58	284	58	284

[a] p=0.023 (χ^2=5.20, Odds Ratio 3.09, 95%C.I. 1.22–7.88). Susceptive for HAM/TSP.
[b] p=0.002 (χ^2=9.42, Odds Ratio 1.90, 95%C.I. 1.27–2.83). Susceptive for HAM/TSP.
[c] p<0.001 (χ^2=19.58, Odds Ratio 0.39, 95%C.I. 0.26–0.60). Protective for HAM/TSP.
* Comparison of whole allele distribution between patients with HAM/TSP and HTLV-I seropositive asymptomatic carriers (HCs) was performed using a chi-square test for 2×11 contingency table with a significance level p<0.01. This analysis revealed χ^2=46.37 (df=10), p<0.001. The distribution of each allele of the d(CA) repeat polymorphism of the MMP-9 promoter gene in patients with HAM/TSP patients was also compared with those in HCs using a chi-square test for a 2×2 contingency table.
** The p value of genotype was calculated by χ^2 test with a 2×3 contingency table. This analysis revealed that the genotype of 23 repeat was frequently observed in HAM/TSP than HCs (χ^2=10.59, df=2, p=0.005), whereas the genotype of 20 repeat was frequently observed in HCs than HAM/TSP (χ^2=23.34, df=2, p<0.0001).

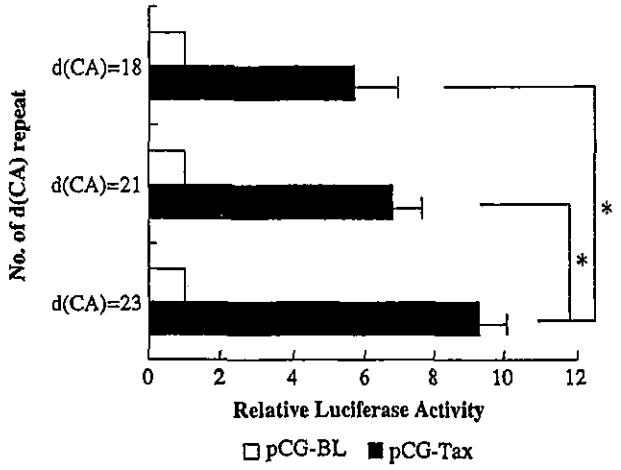

Fig. 2. HTLV-I Tax mediated trans-activation of MMP-9 promoter correlates with the length of the d(CA) repeat. Jurkat cells were transfected with HTLV-I Tax expressing (pCG-Tax) or control (pCG-BL) vector and luciferase reporter constructs containing the d(CA)18 or d(CA)21 or d(CA)23 repeat. Open bars represent luciferase activity of each reporter plasmid with control vector pCG-BL. Solid bars represent luciferase activity of each reporter plasmid with Tax expressing vector pCG-Tax. The activities are given relative to the activity of each reporter plasmid with control vector pCG-BL, which was defined as 1. The mean values±SD from three independent experiments are shown. Maximal luciferase activity was observed when reporter plasmid containing d(CA)23 was used (*$p<0.05$ by Mann-Whitney U-test).

(Table 2). The analysis revealed that the length of d(CA) repeats in HAM/TSP patients was significantly longer in whole HAM/TSP patients than HCs ($p<0.01$, Mann–Whitney U-test) (Fig. 1). The comparison of whole allele distribution between two groups by using a chi-square test for 2×11 contingency table also showed the same result with a significance level $p<0.01$ ($\chi^2=46.37$ [$df=10$], $p<0.001$) (Table 2). We also compared the distribution of each allele of the d(CA) repeat polymorphism in HAM/TSP patients and HCs using a chi-square test for a 2×2 contingency table. The d(CA)23 and d(CA)24 repeat alleles was frequently observed in HAM/TSP than HCs ($p=0.002$ and $p=0.023$, respectively), whereas d(CA)20 repeat allele was frequently observed in HCs than HAM/TSP patients ($p<0.001$) (Table 2). The p value of genotype calculated by chi-square test with a 2×3 contingency table revealed that the genotype of 23 repeat was frequently observed in HAM/TSP than HCs ($\chi^2=10.59$, $df=2$, $p=0.005$), whereas the genotype of 20 repeat was frequently observed in HCs than HAM/TSP ($\chi^2=23.34$, $df=2$, $p<0.0001$). These results indicated that the longer d(CA) repeat alleles was more frequently observed in HAM/TSP patients than HCs. The observed frequency of alleles in HCs was very similar to the frequency previously reported in a Japanese population (Shimajiri et al., 1999).

3.3. Effect of the length of d(CA) repeats on HTLV-I Tax mediated trans-activation of MMP-9 promoter

To investigate whether the length of the d(CA) repeat in MMP-9 promoter affects the HTLV-I Tax-mediated transcription of MMP-9 promoter, a MMP-9 promoter carrying either d(CA)18, d(CA)21 or d(CA)23 was inserted upstream of the luciferase gene in the pGL2-Basic plasmid vector and luciferase assays were carried out. Since the only sequence difference among the reporter vectors used for luciferase assay was the length of the d(CA) repeats, we can quantify the functional differences associated with the d(CA) repeat length alone. We showed that ectopic expression of Tax protein in Jurkat T cells increased MMP-9 promoter activity by approximately 9.7 (mean±SD=9.69±0.52) times in dCA23-Luc, 7.3 (7.25±0.51) times in dCA21-Luc, and 5.8 (5.79±0.80) times in dCA18-Luc reporter, compared with control, suggesting that Tax trans-activates MMP-9 promoter more effectively in longer d(CA) repeats containing promoter than shorter one ($p<0.05$ by Mann–Whitney's U-test) (Fig. 2).

3.4. The CSF levels of MMP-9 was not correlated with the d(CA) repeat length in HAM/TSP patients

We quantified the CSF MMP-9 levels in forty HAM/TSP patients to examine the relationship between MMP-9 and the d(CA) repeat length. MMP-9 in the CSF was detectable only in the two patients with severe clinical symptoms (those who became unable to walk within three years after onset of the disease) out of 40 samples tested (2/40: 5.0%) (Table 3). There was no correlation between the d(CA) repeat length in the MMP-9 promoter and the CSF levels of MMP-9.

4. Discussion

In this study, we demonstrated that the mean length of the d(CA) repeat polymorphism in MMP-9 promoter was

Table 3
Clinical and laboratory findings of HAM/TSP patients who was positive for MMP-9 in CSF

Patients	Age/ sex	Duration (years)	MMP-9 genotype[a]	Provirus load[b]	Anti-HTLV-I antibody titer		CSF neopterin[c] (pmol/ml)	CSF MMP-9 (ng/ml)
					Serum	CSF		
HAM1	60/M	2	23/19	700	×65336	×4096	108	5.97
HAM2	39/M	3	21/21	1417	×131072	×32768	281	4.68

[a] Length of d(CA) repeats in each allele.
[b] HTLV-1 (pX) copy number per 1×10^4 PBMCs by quantitative PCR.
[c] Normal<30 pmol/ml.

significantly greater in HAM/TSP patients than HCs ($p<0.01$ by Mann–Whitney U-test and a chi-square test for 2×11 contingency table). Our results also confirmed an earlier report (Shimajiri et al., 1999) that longer d(CA) MMP-9 promoter alleles were associated with higher transcriptional activity. The d(CA) repeat have been found in many eukaryotic and prokaryotic genes and has been confer regulatory effects on gene transcription by conformational transition from B-DNA to Z-DNA (Nordheim and Rich, 1983; Tripathi and Brahmachari, 1991). The existence of d(CA) repeat in promoter region was reported to have effect of up-regulation in some genes and down-regulation in other genes. For example, in case of acetyl-CoA carboxylase gene, d(CA) repeat in promoter region suppress promoter activity (Tae et al., 1994). The d(CA) repeat polymorphism in the promoter of the MMP-9 gene is present approximately 90 bp upstream from transcriptional initiation site and there are several important transcription factor-binding sites around this microsatellite. Therefore we hypothesized that the polymorphism of this microsatellite is linked to the transcriptional activity of the MMP-9 gene, since previous study indicated that longer d(CA) repeats correlates with the binding affinity of the nuclear protein(s) (Peters et al., 1999) and higher transcriptional activity (Shimajiri et al., 1999).

MMP-9 is a member of the gelatinase subgroup of the MMP gene family, and digests type IV collagens and gelatins (Sellebjerg and Sorensen, 2003). Since type IV collagen is a major constituent of the basal lamina along with laminin, heparin sulfate and proteoglycan, it is possible that once MMP-9 is released from HTLV-I infected cells and activated in spinal cord, the enzyme could attack the extracellular matrix components in the basal lamina around CNS blood vessels, then opening the blood brain barrier (BBB). Indeed, previous reports indicated that transient contact between astrocytes and T lymphocytes activated by HTLV-I infection led to increased production of MMP-3 and MMP-9 in astrocytes via T cell-produced inflammatory cytokines and integrins (Giraudon et al., 2000), and MMP-9 was expressed in spinal cord infiltrating mononuclear cells of HAM/TSP patients (Umehara et al., 1998). We have detected MMP-9 in CSF only in 5.0% (2 out of 40 cases) cases of HAM/TSP patients, consistent with the previous report by Umehara et al. which also showed that MMP-9 in the CSF was detectable only in a part of HAM/TSP patients (18.9%: 8 out of 46 cases) (Umehara et al., 1998). Thus, majority of the HAM/TSP patients did not show the increased MMP-9 levels in CSF. Since MMP-9 expression in the spinal cord lesion of HAM/TSP was restricted only in the infiltrating mononuclear cells (Umehara et al., 1998), MMP-9 concentrations in CSF may not be able to reflect tissue expression of MMP-9 exactly.

It is well known that HTLV-I Tax protein can also transactivate many inflammatory cytokines that are associated with cell growth and differentiation. One of these cytokines, IL-15, which dose-dependently induces MMP-9 and TIMP-1 secretion in PBMCs and T cells (Constantinescu et al., 2001), was expressed at higher levels in PBMCs from HAM/TSP patients than in those from normal controls (Azimi et al., 1999). Interestingly, a report by Yu et al. indicated that the cell surface hyaluronan receptor CD44 can localize proteolytically active MMP-9 to the surface of carcinoma cell lines and promotes MMP-9 proteolytic activity that correlates with tumor growth and invasiveness (Yu and Stamenkovic, 1999). Since we previously reported that a CD44 splice variant (v6) was highly expressed in PBMCs and spinal cord infiltrating CD4 positive cells of HAM/TSP patients (Matsuoka et al., 2000), it is possible that these CD44v6 positive cells more efficiently localize MMP-9 on their cell surface, therefore promote the inflammatory cell infiltration of the spinal cord as observed in HAM/TSP patients. If this is a case, MMP-9 may be a good candidate target molecule for treatment of HAM/TSP. Recent study by Ikegami et al. showed that selective MMP inhibitor BPHA (*N*-biphenyl sulfonyl-phenylalanine hydroxamic acid) could inhibit migration activity of $CD4^+$ T cells derived from HAM/TSP patients in vitro (Ikegami et al., 2002).

In conclusion, our present study revealed that the longer d(CA) repeat alleles of MMP-9 promoter, which correlated with higher Tax-mediated transcriptional activity, were more frequently observed in HAM/TSP patients than HCs. This observation is further evidence of an important role of MMP-9 in HAM/TSP pathology.

Acknowledgments

The authors thank Prof. Jun-ichi Fujisawa of Kansai Medical University, Osaka, Japan, for the gift of pCG-BL and pCG-Tax plasmids. The authors also thank Ms Y. Nishino and T. Muramoto of Kagoshima University for their excellent technical assistance. This work was supported by the Japan Intractable Diseases Research Foundation (to M.S.), Japanese Foundation for AIDS prevention, the Grant in Aid for Research on Brain Science of the Ministry of Health, Labor and Welfare, Japan.

References

Alexander, C.M., Werb, Z., 1989. Proteinases and extracellular matrix remodeling. Curr. Opin. Cell Biol. 1, 974–982.

Azimi, N., Jacobson, S., Leist, T., Waldmann, T.A., 1999. Involvement of IL-15 in the pathogenesis of human T lymphotropic virus type I-associated myelopathy/tropical spastic paraparesis: implications for therapy with a monoclonal antibody directed to the IL-2/15R beta receptor. J. Immunol. 163, 4064–4072.

Bangham, C.R.M., 2000. The immune response to HTLV-I. Curr. Opin. Immunol. 12, 397–402.

Constantinescu, C.S., Grygar, C., Kappos, L., Leppert, D., 2001. Interleukin 15 stimulates production of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by human peripheral blood mononuclear cells. Cytokine 13, 244–247.

Fujisawa, J., Toita, M., Yoshida, M., 1989. A unique enhancer element for the trans activator (p40 tax) of human T-cell leukemia virus type I that is distinct from cyclic AMP-and 12-*O*-tetradecanoylphorbol-13-acetate-responsive elements. J. Virol. 63, 3234–3239.

Fujisawa, J., Toita, M., Yoshimura, T., Yoshida, M., 1991. The indirect association of human T-cell leukemia virus tax protein with DNA results in transcriptional activation. J. Virol. 65, 4525–4528.

Furukawa, Y., *Yamashita, M., Usuku, K.*, Izumo, S., Nakagawa, M., Osame, M., 2000. Phylogenetic subgroups of human T cell lymphotropic virus (HTLV) type I in the tax gene and their association with different risks for HTLV-I-associated myelopathy/tropical spastic paraparesis. J. Infect. Dis. 182, 1343–1349.

Gessain, A., Barin, F., Vernant, J.C., Gout, O., Maurs, L., Calende, A., de The, G., 1985. Antibodies to human T-lymphotropic virus type-I in patients with tropical *spastic paraparesis. Lancet* 2, 407–410.

Giraudon, P., Szymocha, R., Buart, S., Bernard, A., Cartier, L., Belin, M.F., Akaoka, H., 2000. T lymphocytes activated by persistent viral infection differentially modify the expression of metalloproteinases and their endogenous inhibitors, TIMPs, in human astrocytes: relevance to HTLV-I-induced neurological disease. J. Immunol. 164, 2718–2727.

Ikegami, M., Umehara, F., Ikegami, N., Maekawa, R., Osame, M., 2002. Selective matrix *metalloproteinase inhibitor*, N-biphenyl sulfonyl phenylalanine hydroxamic acid, inhibits the migration of CD4+T lymphocytes in patients with HTLV-I-associated myelopathy. J. Neuroimmunol. 127, 134–138.

Jeffery, K.J.M., Usuku, K., Hall, S.E., Matsumoto, W., Taylor, G.P., Procter, J., Bunce, M., Ogg, G.S., Welsh, K.I., Weber, J.N., Lloyd, A.L., Nowak, M.A., Nagai, M., Kodama, D., Izumo, S., Osame, M., Bangham, C.R.M., 1999. HLA alleles determine human T-lymphotropic virus-I (HTLV-I) proviral load and the risk of HTLV-I-associated myelopathy. Proc. Natl. Acad. Sci. U. S. A. 96, 3848–3853.

Jeffery, K.J.M., Siddiqui, A.A., Bunce, M., Lloyd, A.L., Vine, A.M., Witkover, A.D., Izumo, S., Usuku, K., Welsh, K.I., Osame, M., Bangham, C.R.M., 2000. The influence of HLA class I alleles and heterozygosity on the outcome of human T cell lymphotropic virus type I infection. J. Immunol. 165, 7278–7284.

Kaplan, J.E., Osame, M., Kubota, H., Igata, A., Nishitani, H., Maeda, Y., Khabbaz, R.F., Janssen, R.S., 1990. The risk of development of HTLV-I associated myelopathy/ tropical spastic paraparesis among persons infected with HTLV-I. J. Acquir. Immune Defic. Syndr. 3, 1096–1101.

Matsuoka, E., Usuku, K., Jonosono, M., Takenouchi, N., Izumo, S., Osame, M., 2000. CD44 splice variant involvement in the chronic inflammatory disease of the spinal cord: HAM/TSP. J. Neuroimmunol. 102, 1–7.

Mori, N., Sato, H., Hayashibara, T., Senba, M., Hayashi, T., Yamada, Y., Kamihira, S., Ikeda, S., Yamasaki, Y., Morikawa, S., Tomonaga, M., Geleziunas, R., Yamamoto, N., 2002. Human T-cell leukemia virus type I Tax transactivates the matrix metalloproteinase-9 gene: potential role in mediating adult T-cell leukemia invasiveness. Blood 99, 1341–1349.

Motulsky, H., 1995. Multiple Comparisons in "Intitutive Biostatistics". Oxford University Press, New York.

Nagai, M., Usuku, K., Matsumoto, W., Kodama, *D., Takenouchi, N.*, Moritoyo, T., Hashiguchi, S., Ichinose, M., Bangham, C.R.M., Izumo, S., Osame, M., 1998. Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: high proviral load strongly predisposes to *HAM/TSP. J. Neurovirology* 4, 586–593.

Nakagawa, M., Izumo, S., Ijichi, S., Kubota, R., Arimura, K., Kawabata, M., Osame, M., 1995. HTLV-I-associated myelopathy: analysis of 213 patients based on clinical and laboratory findings. J. Neurovirology 1, 50–61.

Nomoto, M., Utatsu, Y., Soejima, Y., Osame, M., 1991. Neopterin in cerebrospinal fluid: a useful marker for diagnosis of HTLV-I-associated myelopathy/tropical spastic paraparesis. Neurology *41, 457.*

Nordheim, A., Rich, A., 1983. The sequence (dC-dA)n·(dG-dT)n forms left-handed Z-DNA in negatively supercoiled plasmids. Proc. Natl. Acad. Sci. U. S. A. 80, 1821–1825.

Opdenakker, G., Van Damme, J., 1994. Cytokine-regulated proteases in autoimmne diseases. Immunol. Today 15, 103–107.

Osame, M., 1990. Review of WHO Kagoshima meeting and diagnostic guidelines for HAM/TSP. In: Blattner, W.A. (Ed.), Human Retrovirology: HTLV. Raven Press, New York, pp. 191–197.

Osame, M., Usuku, K., Izumo, S., Ijichi, N., Amitani, H., Igata, A., Matsumoto, M., Tara, M., 1986. HTLV-I associated myelopathy, a new clinical entity. Lancet 1, 1031–1032.

Peters, D.G., Kassam, A., St. Jean, P.L., Yonas, H., Ferrell, R.E., 1999. Functional polymorphism in the matrix metalloproteinase-9 promoter as a potential risk factor for intracranial aneurysm. *Stroke 30, 2612–2616.*

Sellebjerg, F., Sorensen, T.L., 2003. Chemokines and matrix metalloproteinase-9 in leukocyte recruitment to the central nervous system. Brain Res. Bull. 61, 347–355.

Shimajiri, S., Arima, N., Tanimoto, A., Murata, Y., Hamada, T., Wang, K.Y., Sasaguri, Y., 1999. Shortened microsatellite d(CA)21 sequence down-regulates promoter activity of matrix metalloproteinase 9 gene. FEBS Lett. 455, 70–74.

Tae, H.J., Luo, X., Kim, K.H., 1994. Role of CCAAT/Enhancer-binding protein and its binding site on repression and derepression of acetyl-CoA carboxylase gene. J. Biol. Chem. 269, 10475–10484.

Tripathi, J., Brahmachari, S.K., 1991. Distribution of simple repetitive (TG/CA)n and (CT/AG)n sequences in human and rodent genomes. J. Biomol. Struct. Dyn. 9, 387–397.

Umehara, F., Izumo, S., Nakagawa, M., Ronquillo, A.T., Takahashi, K., Sato, E., Osame, M., 1993. Immunocytochemical analysis of the cellular infiltrate in the spinal cord lesions in HTLV-I-associated myelopathy. J. Neuropathol. Exp. Neurol. 52, 424–430.

Umehara, F., Okada, Y., Fujimoto, N., Abe, M., Izumo, S., Osame, M., 1998. Expression of matrix metalloproteinase and tissue inhibition of metalloproteinase in HTLV-I-associated myelopathy. J. Neuropathol. Exp. Neurol. 57, 839–849.

Vine, A.M., Witkover, A.D., Lloyd, A.L., Jeffery, K.J., Siddiqui, A., Marshall, S.E., Bunce, M., Eiraku, N., Izumo, S., Usuku, K., Osame, M., Bangham, C.R.M., 2002. Polygenic control of human T lymphotropic virus type I (HTLV-I) provirus load and the risk of HTLV-I-associated myelopathy/tropical spastic paraparesis. J. Infect. Dis. 186, 932–939.

Yu, Q., Stamenkovic, I., 1999. Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. Genes Dev. 13, 35–48.

Chronic progressive cervical myelopathy with HTLV-I infection

Variant form of HAM/TSP?

F. Umehara, MD; S. Nagatomo, MD; K. Yoshishige, MD; M. Saito, MD; Y. Furukawa, MD; K. Usuku, MD; and M. Osame, MD

Abstract—*Objective:* To investigate the role of human T-lymphotrophic virus type I (HTLV-I) infection in four patients who developed slowly progressive myelopathy with abnormal MRI lesions in the cervical cord levels. *Methods:* Clinical and neuroradiologic examinations were performed, and the odds that an HTLV-I-infected individual of specified genotype, age, and provirus load had HTLV-I-associated myelopathy (HAM)/tropical spastic paraparesis (TSP) were calculated. *Results:* Anti-HTLV-I antibodies were positive in both the serum and the CSF in all of the patients. Biopsied sample from spinal cord lesions showed inflammatory changes in Patient 1. Patient 2 had a demyelinating type of sensorimotor polyneuropathy. Two of the three patients examined showed high risk of developing HAM/TSP in virologic and immunologic aspects. *Conclusion:* These four cases may belong to a variant form of HAM/TSP, predominantly involving the cervical cord levels.

NEUROLOGY 2004;63:1276–1280

Human T-lymphotropic virus type I (HTLV-I) is associated with adult T-cell leukemia and a chronic progressive disease of the CNS called HTLV-I-associated myelopathy (HAM)/tropical spastic paraparesis (TSP).[1,2] The pathology of the disease involves the spinal cord, predominantly the thoracic level with atrophy of the lateral columns.[3] These lesions are associated with perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, proliferation of astrocytes, and fibrillary gliosis.[4]

In this article, we report four patients with slowly progressive cervical myelopathy. The calculated risk of HAM/TSP in two patients showed a high value, comparable with those of HAM/TSP and higher than those of a healthy HTLV-I carrier. Because the clinical and laboratory findings of these four cases show similarities to those of HAM/TSP, we propose that these four cases may be a variant form of HAM/TSP.

Case reports. *Patient 1.* A 56-year-old man had a year-long history of progressive gait disturbance and numbness in the upper and lower limbs. He visited a neurosurgeon, who discovered abnormal lesions in the cervical cord levels. To exclude the possibility of intramedullary spinal cord tumor, laminectomy and biopsy from the enhanced lesions were performed. Pathology revealed perivascular lymphocytic infiltration with degenerative changes of the spinal cord (figure 1). Immunohistochemical analysis revealed predominant infiltration of lymphocytes and macrophages. The patient was then referred to the Department of Neurology at the Kagoshima University Hospital.

General examinations were unremarkable. On neurologic examinations, he had normal consciousness and mentality. Cranial nerves were intact. Muscle strength in the upper and lower limbs was moderately decreased, and there was moderate muscle atrophy. Deep tendon reflexes were mildly exaggerated in the upper limbs and highly exaggerated in the lower limbs. Chaddock signs were positive bilaterally. Superficial and deep sensations were disturbed in the lower limbs. He had urinary disturbance. Anti-HTLV-I antibody was positive both in serum (×1,028) and in CSF (×64). CSF showed increased protein content (137 mg/dL), IgG level of 12.5 mg/dL, normal cell count (3/mm^3), and normal neopterin level (27 pmol/mL; normal <30 pmol/L). Myelin basic protein level was likewise normal, and there were no oligoclonal bands detected in the CSF. Western blotting of CSF for anti-HTLV-I was positive for p19, p24, p28, p53, and env. Nerve conduction studies were normal. Somatosensory evoked potentials, by stimulating the left tibial nerve, showed marked delay of central conduction time (N20: 23 milliseconds; P40: 48 milliseconds). Needle electromyography revealed fibrillation potentials in the lower limbs. Cervical MRI demonstrated swelling of the spinal cord at C5 to C6 levels with gadolinium-diethylenetriaminepentaacetate (Gd-DTPA) enhancement (figure 2). T2-weighted imaging showed high-intensity signals at the same level on sagittal section. Axial T2-weighted imaging at the C5 level showed high-intensity signals in the lateral columns bilaterally. The patient was treated with IV high-dose methylprednisolone

Additional material related to this article can be found on the *Neurology* Web site. Go to www.neurology.org and scroll down the Table of Contents for the October 12 issue to find the title link for this article.

From the Departments of Neurology and Geriatrics (Drs. Umehara, Nagatomo, Yoshishige, Saito, and Osame), Division of Blood Transfusion Medicine (Dr. Furukawa), and Department of Medical Information Science (Dr. Usuku), Graduate School of Kagoshima University, Japan.

Received February 13, 2004. Accepted in final form June 1, 2004.

Address correspondence and reprint requests to Dr. F. Umehara, Department of Neurology, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima 8900075, Japan; e-mail: umehara@m2.kufm.kagoshima-u.ac.jp

Figure 1. Spinal cord pathology of Patient 1. There was massive perivascular infiltration of mononuclear cells. Hematoxylin–eosin; bar = 100 μm.

followed by oral prednisolone, but his symptoms did not improve. In the course of the following 5 years, his symptoms gradually worsened. Repeat MRI revealed atrophy of the cervical cord with high-intensity lesions at the C5 level, which may have been residual lesions of the spinal cord biopsy (figure 3). Gd-DTPA enhancement was negative.

Patient 2. A 73-year-old man noted difficulty with fine hand movements in July 2002. This gradually worsened to involve the lower extremity so that by September 2002, he could not climb a ladder. Numbness in the upper and lower limbs then developed. He also had constipation and urinary disturbance. He consulted us in November 2002. Past history was unremarkable except for blood transfusion when he was 35 years old.

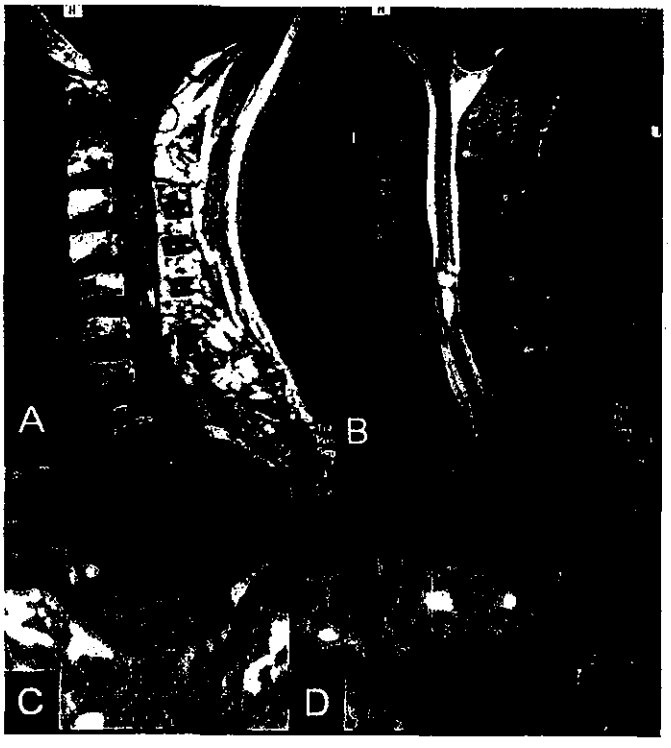

Figure 2. MRI of Patient 1, before treatment (November 1998): gadolinium-enhanced T1-weighted imaging (sagittal: A, axial: C) and T2-weighted imaging (sagittal: B, axial: D). Note spinal cord (C5 to C6) swelling with high intensity on T2-weighted imaging.

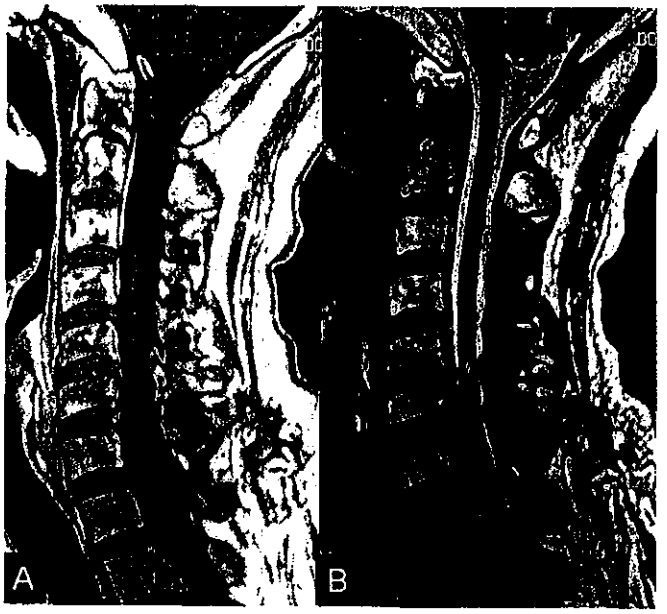

Figure 3. MRI of Patient 1: sagittal T1-weighted imaging (A) and T2-weighted imaging (B) after treatment (2003). Swelling of spinal cord with high intensity on T2-weighted imaging was markedly reduced.

General findings were likewise unremarkable. Neurologic examinations showed normal consciousness and mentality. Cranial nerves were intact. There was muscle weakness in the upper and lower limbs. Coordination in the upper limbs was poor. Deep tendon reflexes were mildly exaggerated in the upper limbs and highly exaggerated in the lower limbs. Babinski signs were positive bilaterally. Superficial sensations were decreased below the C5 level, and deep sensation was disturbed in the lower limbs. There was a tendency to fall when standing, and he was unable to walk alone. Laboratory tests were as follows: Anti-HTLV-I antibody was positive in both serum (×2,048) and CSF (×256). Western blotting of CSF for HTLV-I was positive for p19, p24, p28, p53, and env. For CSF, cell count was 10/mm^3, protein level 64 mg/dL, IgG level 9.8 mg/dL, IgG index 0.66, and neopterin level 52 pmol/mL. Nerve conduction study showed diffuse slowing of both motor and sensory nerve conduction velocity in the upper and lower limbs with prolonged F-wave latencies. Sural nerve biopsy revealed mildly decreased densities of large and small myelinated fibers. Many fibers had thinner myelin sheaths compared with their axon diameter. On teased fiber analysis, fibers with de- and remyelination (paranodal demyelination: 7%, segmental demyelination: 5%, segmental remyelination: 20%) and axonal degeneration (5%) increased. Cervical MRI revealed swelling of the spinal cord without Gd-DTPA enhancement (see figure E-1 on the *Neurology* Web site at www.neurology.org). T2-weighted imaging showed high-intensity lesions from the C3 to C7 levels, which were located mainly in the posterior and lateral columns bilaterally. High-dose methylprednisolone (1,000 mg/day for 3 days) followed by oral prednisolone treatment gradually improved his muscle weakness in the upper and lower limbs with disappear-

ance of both high-intensity lesions and swelling of the cervical cord levels. One and one-half years after the treatment, MRI revealed spinal cord atrophy mainly at the middle thoracic levels without abnormal intensity.

Patient 3. In January 2003, a 51-year-old woman noticed difficulty in walking, which gradually worsened in the following months. She visited us in April 2003. She had been experiencing pemphigus vulgaris since age 17. General examinations revealed whole-body skin eruption. Neurologic examinations showed normal consciousness and mentality. Cranial nerves were intact. There was resting and postural tremor of both hands. Muscle strength in the upper limbs was normal but was mildly reduced in the lower limbs. Deep tendon reflexes were mildly exaggerated in the upper limbs and highly exaggerated in the lower limbs. Babinski signs were positive bilaterally. Superficial sensations were decreased in the distal parts of the lower limbs, and deep sensation was markedly disturbed in the lower limb. She was unable to stand. Laboratory tests as follows: Anti-HTLV-I antibody was positive in both serum (×8,192) and CSF (×512). Serum deoxytimidine kinase activity was 73.4 U/L (<5.0). For CSF, cell count was 26/mm^3, protein level 96 mg/dL, glucose level 45 mg/dL, and IgG index 0.99. Oligoclonal bands were positive. Nerve conduction studies were unremarkable. Cervical MRI revealed swelling of the spinal cord without Gd-DTPA enhancement (see figure E-2 on the *Neurology* Web site). T2-weighted imaging showed high-intensity lesions from the C3 to T3 levels, which were located mainly in the posterior columns bilaterally. High-dose methylprednisolone (1,000 mg/day for 3 days) followed by oral prednisolone treatment gradually improved her lower limb weakness. Both high-intensity lesions and swelling of the cervical cord levels decreased. During the following 6 months, her symptoms gradually improved.

Patient 4. In November 1998, a 68-year-old man noticed difficulty in walking, which gradually worsened. In February 1999, he was diagnosed as having lumbar canal stenosis and underwent laminectomy of the fourth lumbar vertebrae. Numbness and pain below the level of the navel developed 2 weeks after the operation. He also noticed constipation and urinary disturbance. He visited us in June 1999. He had cerebral infarction and had had angina pectoris since 1993. General findings were unremarkable. Neurologic examinations showed normal consciousness and mentality. Cranial nerves were intact. Muscle strength was mildly reduced in the upper limbs and markedly reduced in the lower limbs. Deep tendon reflexes were mildly exaggerated in the upper limbs and highly exaggerated in the lower limbs. Babinski signs were positive bilaterally. Superficial sensations were decreased below the T3 level, and deep sensation was disturbed in the lower limbs. He was unable to stand. Laboratory tests are as follows: Anti-HTLV-I antibody was positive in both serum (×4,096) and CSF (×32). For CSF, cell count was 2/mm^3, protein level 167 mg/dL, IgG level 29 mg/dL, IgG index 0.66, and neopterin level 52 pmol/mL. Nerve conduction studies were unremarkable. Cervical MRI revealed swelling of the spinal cord without Gd-DTPA enhancement (see figure E-3 on the *Neurology* Web site). T2-weighted imaging showed high-intensity lesions from the C2 to T5 levels, which were located mainly in the posterior and lateral columns bilaterally. High-dose methylprednisolone (1,000 mg/day for 3 days) followed by oral prednisolone and cyclophosphamide pulse therapy (1,000 mg/day for 1 day) gradually improved his muscle weakness in all four limbs. There was also decrease of both high-intensity lesions and swelling of the cervical cord levels. During the following 4 years, his symptoms remained stable.

Materials and methods. *Evaluation of risk of HAM/TSP.* We calculated the odds that an HTLV-I-infected individual living in Kagoshima prefecture, of specified genotype, age, and provirus load, had HAM/TSP by an equation specifically developed for this population.[5] As all four patients in this report live in Kagoshima prefecture, blood samples from Patients 1 to 3 were analyzed upon obtaining informed consent. For the control subjects, we used the study cohort, which consisted of 52 patients with HAM/TSP attending the Department of Neurology and Geriatrics, Kagoshima University (Kagoshima, Japan), and 47 healthy carriers (HCs) of HTLV-I randomly selected from the same geographic location, as described elsewhere.[6,7] All individuals screened were of Japanese descent and resided within Kagoshima prefecture (Kyushu, Japan). The diagnosis of HAM/TSP was made in accordance with World Health Organization criteria.[8] Peripheral blood was obtained from all individuals upon obtaining of informed consent. Fresh peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation using a Histopaque-1077 instrument (Sigma, Tokyo, Japan) and washed three times with phosphate-buffered saline containing 1% fetal calf serum. Isolated PBMCs were cryopreserved in liquid nitrogen until use. Genomic DNA was extracted from PMBCs using a QIAamp blood kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

Genotyping methods for non–human leukocyte antigen candidate genes. For each candidate gene, we went on to do genotyping either by DNA sequencing or by PCR with allele-specific primers as previously described.[6]

Human leukocyte antigen typing. The results of the molecular genotyping of class I and class II human leukocyte antigen (HLA) loci in this cohort have been reported elsewhere.[6,7]

HTLV-I genotyping. Two subgroups (A and B) of the cosmopolitan genotype of HTLV-I are present in Kagoshima, Japan. Molecular typing of the HTLV-I *tax* gene was done as described elsewhere to identify the HTLV-I subgroup present in each infected subject.[9]

Provirus load measurement. The provirus load in PBMCs was measured using real-time PCR with an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). With use of β-actin as an internal control, the amount of HTLV-I proviral DNA was calculated by the following formula: copy number of HTLV-I (pX) per 1×10^4 PBMCs = ([copy number of pX]/[copy number of β-actin/2]) $\times 10^4$. All samples were performed in triplicate. All samples were amplified and analyzed in triplicate, as described elsewhere.[10]

Odds of developing HAM/TSP. We calculated the odds that an HTLV-I-infected individual of specified genotype, age, and provirus load had HAM/TSP by using the equation based on the logistic regression analysis in the Kagoshima cohort as previously described.[6] A worked example is as follows: HTLV-I-infected individual in Kagoshima, 60 years old, with a $\log_{10}$ (provirus load) of 2.5 with the genotype *TNF -863A*$^+$, *SDF-1 + 801AA*, *HLA-A*02*$^-$, *HLA-Cw*08*$^+$, HTLV-I subgroup B has a predicted n odds of HAM/TSP of $-1.716 - (0.145 \times 60) + (0.003 \times 60^2) + (0.46 \times 2.5) + (0.487 \times 2.5^2) + 3.057 - (4.616 \times 2.5) + (1.476 \times 2.5^2) - 1.689 - 0.894 - 1.587 = 1.864$. That is, this HTLV-I-infected individual's odds of developing HAM/TSP is equal to $\exp(-1.864) = 0.155$.

Results. The results are summarized in the table and in figure 4. Among the patients with HAM/TSP and HC, 39 cases showed odds above 3.0. In these 39 cases, 37 cases (95%) had HAM/TSP, and only 2 cases (5%) were HCs. Except for Patient 3, the odds for HAM/TSP in Patients 1 and 2 were comparable with those of HAM/TSP, higher than those of an HC.

Discussion. The four patients had several features in common: 1) progressive cervical myelopathy with

Table Analysis of risk factors for developing HAM/TSP

Patient no.	Sex	ln odds of HAM	Odds of HAM	Age, y	*tax**	Log_{10} *tax*	*HLA*-DR1*	*HLA*-A02*	*HLA*-Cw08*	Genotype *TNF-α*	Genotype *SDF-1*	Genotype *IL-10*	HTLV-I subgroup
1	M	2.30	9.9	56	344	2.54	+	−	−	CC	GG	AC	B
2	M	3.40	29.9	73	198	2.30	−	−	+	AC	AA	AC	B
3	F	−1.20	0.3	51	128	2.11	−	+	−	CC	GA	AC	B

* Amount of human T-lymphotropic virus type I (HTLV-I) proviral DNA/10^4 peripheral blood mononuclear cells.
HAM/TSP = HTLV-I-associated myelopathy/tropical spastic paraparesis.

a duration of several months to years, 2) abnormal lesions in the cervical to upper thoracic cord levels with or without Gd-DTPA enhancement, 3) anti-HTLV-I antibodies positive in both serum and CSF, and 4) high levels of HTLV-I proviral load in PBMCs. We screened for other causes of myelopathy including neurosarcoidosis, parasitic myelitis, multiple sclerosis, atopic myelitis, and Sjögren syndrome, but these diseases were unlikely in any of the four cases. We then suspected that these cases might be associated with HTLV-I infection.

The most striking difference from the typical HAM/TSP is the presence of abnormal MRI lesions in the cervical cord. There was swelling of the spinal cords with high-intensity lesions, which were located mainly in bilateral posterior columns, posterior horns, or lateral columns. In Case 1, biopsy samples revealed massive lymphocytic perivascular infiltration in the parenchyma. In Cases 2, 3, and 4, abnormal MRI findings in the cervical cord diminished after corticosteroid treatment or long-term follow-up. These findings suggest that the abnormal cervical MRI lesions may be inflammatory in nature. This is in contrast to the ordinary type of HAM/TSP, the hallmark finding being because spinal cord atrophy predominantly involves the thoracic cord levels without Gd-DTPA enhancement. Recently, atypical MRI findings have been reported in patients with HAM/TSP. In two of the cases,

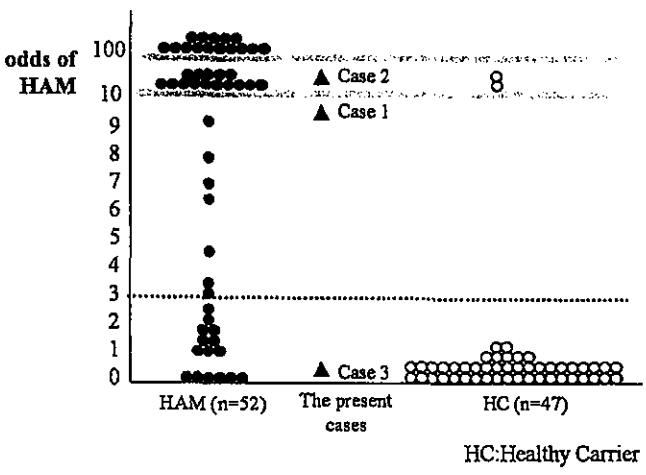

Figure 4. Odds of developing human T-lymphotropic virus type I–associated myelopathy (HAM)/tropical spastic paresis (TSP). Note Patients 1 and 2 showed high odds of developing HAM/TSP.

T2-weighted imaging showed high signal intensity lesions from the cervical to the thoracic cord levels with Gd-DTPA enhancement.[11] The other two cases of HAM patients revealed swelling of the spinal cord with Gd-DTPA enhancement and faint high T2 signal intensities.[12,13] This is not at all surprising because there is usually intense perivascular inflammation with destruction of the blood–brain barrier in actively inflamed lesions of the spinal cord.[3,4] Neuropathology of spinal cord biopsy specimens from Gd-enhanced spinal cord lesions in a patient with HAM/TSP demonstrated infiltration of the leptomeninges and adjacent spinal cord parenchyma by numerous mononuclear cells.[14] Thus, abnormal MRI lesions in the current four cases are not incompatible with HAM/TSP.

In Case 2, a demyelinating polyneuropathy complicated his condition. Complications of peripheral neuropathy have been reported in patients with HAM/TSP,[15] and sural nerve pathology of patients with peripheral neuropathy and HAM/TSP showed nonspecific changes including both chronic demyelinating changes and axonal loss. Thus, the polyneuropathy in Case 2 may be associated with HTLV-I infection.

To further confirm the role of HTLV-I infection in these cases, we calculated the risk of developing HAM/TSP by the best-fit logistic regression equation for the risk of HAM/TSP in the Kagoshima HTLV-I-infected cohort.[6] This equation allowed for the correct identification of 88% of cases of HAM/TSP. The algorithm, however, can be justifiably used to calculate the odds of HAM/TSP only in a Kagoshima cohort and may not be applicable to another population. Our results confirmed that individuals having odds of >3.0 have a high risk of developing HAM/TSP. Taking this into consideration, Patients 1 and 2 had comparably high risks of developing HAM/TSP. Therefore, HTLV-I infection may not only be coincidental but could be closely associated with the neurologic disorders in these patients.

Acknowledgment

The authors thank Dr. Arlene R. Ng for critical reading of the manuscript.

References

1. Osame M, Matsumoto M, Usuku K, et al. Chronic progressive myelopathy associated with elevated antibodies to human T-lymphotropic type I and adult T cell leukemia-like cells. Ann Neurol 1987;21:117–122.
2. Gessain A, Barin F, Vernant JC, et al. Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. Lancet 1985;2:407–410.