ロウイルスの場合，多種の血清型があり同定不能の例が多い．

- 種々のウイルス抗体の検査法として，従来の補体結合抗体法や中和抗体法よりも非常に感度の高い ELISA 法が開発され，発病初期の診断に有用である．
- 髄液ウイルス抗体価の ELISA 法による測定は病原診断に大いに有用である．本来，髄腔内抗体産生を反映するものであるが，血液脳関門の破綻による血清抗体の髄腔内への leakage の可能性を考えて，抗体価比（血清抗体価/髄液抗体価）あるいは抗体価指数（髄液抗体価/血清抗体価÷髄液アルブミン濃度/血清アルブミン濃度）を算出しなければならない．これらの値はすべてのウイルスで一定ではないが，ちなみに単純ヘルペス脳炎では前者が 20 以下，後者が 1.91 以上とされている．
- SSPE，PML の髄液では特徴的所見はなく正常のことが多い．しかし，SSPE の場合，IgG が著明に高く麻疹ウイルス抗体価も高値であるとともに，オリゴクローナルバンドが出現することがある．いずれの疾患でも麻疹ウイルス，JC ウイルスの検出のための PCR 法が開発されている．
- プリオン病の髄液も特徴的所見はなく，まず異常がないと考えてよい．しかし，14-3-3 蛋白やニューロン特異的エノラーゼ（NSE）あるいは最近の報告では総 tau 蛋白が発病初期より増加することがあり，早期診断の参考になる．
- 遺伝性プリオン病が疑われる場合，末梢血白血球のプリオン蛋白遺伝子解析によって点変異などの遺伝子異常の有無を検索する．
- マイコプラズマによる髄膜炎・脳炎が疑われる場合はマイコプラズマ血球凝集抗体価および寒冷凝集素価の上昇が参考になる．

Ⅳ 診断がつかない場合に考えること

- 髄膜炎・脳炎の場合，治療開始の遅れは死亡率，後遺症など予後に大きく影響する．したがって，病原診断に至らなくても早期に可能性の考えられるものに対する治療を開始することが望ましい．特に，ウイルス性脳炎が疑われる場合，散発性脳炎の中では単純ヘルペス脳炎の頻度が最も高いこと，また本脳炎に対する治療薬（アシクロビル，ビラダビン）の安全性の高さなどより考えて早期に治療を開始することが望ましい．

- 後で病原診断ができる可能性があるため，血清，髄液は必ず来院時と回復期のペアで保存しておくことが重要である．
- 感染病原体以外で惹起される髄膜炎・脳炎様の病態があり，急性散在性脳脊髄炎，神経ベーチェット病，各種膠原病あるいはサルコイドーシスによる脳障害，癌性髄膜炎，ライ症候群なども考慮する必要がある．

参考 HTLV-I関連脊髄症について

ヒトレトロウイルスの一つであるHTLV-I（human T-lymphotropic virus type-I）感染によってひき起こされる慢性脊髄炎である．臨床的には排尿障害を伴った緩徐進行性の痙性対麻痺を示す．本疾患は1986年Osameらによって発見された．西南日本を中心に多発しているが，原因不明の痙性対麻痺の症例では抗HTLV-I抗体を測定してみる必要がある．

（中村龍文）

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Serum concentration and genetic polymorphism in the 5′-untraslated region of VEGF is not associated with susceptibility to HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) in HTLV-I infected individuals

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Abstract

HTLV-I- associated myelopathy/tropical spastic paraparesis (HAM/TSP) is one outcome of human T-cell lymphotropic virus type I (HTLV-I) infection. It remains unknown why the majority of infected people remain healthy whereas only approximately 2–3% of infected individuals develop the disease. Recently, it has been reported that increased plasma concentrations of VEGF were significantly related to high ATL cell infiltration, and the viral transactivator Tax activates the VEGF promoter, linking the induction of angiogenesis to viral gene expression. To investigate whether VEGF promoter −634C/G single nucleotide polymorphism (SNP) and serum concentration of VEGF are associated with the development of HAM/TSP, we studied a group of 202 HAM/TSP patients, 202 asymptomatic HTLV-I seropositive carriers (HCs) and 108 seronegative healthy controls (NCs) in Kagoshima, Japan by using PCR-RFLP analysis. The serum concentration of VEGF was also compared among patients with HAM/TSP, ATL, HCs as well as with NCs. Our results indicate that both VEGF gene polymorphism and serum VEGF levels are not specifically associated with the risk of HAM/TSP in our cohort.

Keywords: VEGF; Single nucleotide polymorphism; HAM/TSP; HTLV-I; Disease susceptibility; Proviral load

1. Introduction

Human T-cell lymphotropic virus type I (HTLV-I) [1,2] infection is closely associated with a slowly progressive neurological disease called HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [3,4]. Infection with HTLV-I is estimated to affect 10 million to 20 million people worldwide [5]. However, only a minority of infected individuals develops HAM/TSP, by mechanisms incompletely understood [6]. Since it has been reported that the subtype of the viral transactivator Tax is associated with the risk of developing HAM/TSP [7], many other reported findings suggest that host factors are most important to determine the risk of HAM/TSP.

Vascular endothelial growth factor (VEGF) is a major mediator of vascular permeability and angiogenesis. Dysregulated VEGF expression has been implicated as a major contributor to the development of a number of common disease pathologies [8]. A recent report indicated that among seven common polymorphisms in the promoter region, genotype distribution of the −634C/G single nucleotide polymorphism (SNP) differed significantly ($P=0.011$) between patients with and without diabetic retinopathy, and that C allele was significantly increased in patients with retinopathy compared with those without retinopathy ($P=0.0037$) [9]. On the other hand, it has recently been reported that HTLV-I-transformed cells secrete VEGF and basic fibroblast growth factor (bFGF) proteins and induce

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angiogenesis in vitro, via HTLV-I Tax-induced transcriptional activation of the VEGF promoter [10]. Therefore, it may be possible that altered vessel permeability and activated endothelial cells are involved in the pathogenesis of HAM/TSP.

To test this possibility, we examined the serum concentration of VEGF as well as promoter gene polymorphism to assess its possible role in HAM/TSP.

2. Patients and methods

2.1. Study population

The study population consisted of 202 patients with HAM/TSP, 202 asymptomatic HTLV-I seropositive carriers (HCs) and 108 seronegative healthy controls (NCs), all residing in HTLV-I endemic Kagoshima Prefecture in Southern Japan. The diagnosis of HAM/TSP was done in accordance with World Health Organization criteria [11]. Clinical characteristics of the patients are shown in Table 1. All samples were taken with the consent of the patients.

2.2. Isolation and cryopreservation of PBMC and DNA extraction

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 (PBS) containing 1% fetal calf serum (FCS). 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.

2.3. Genomic PCR analysis

In order to amplify a 469 base pair fragment containing the −634C/G SNP in VEGF promoter, 50 ng of genomic DNA was PCR-amplified with the primers (forward: 5′-TTG CTT GCC ATT CCC CAC TTG A-3′ and reverse: 5′-CCG AAG CGA GAA CAG CCC AGA A-3′) by 1 unit of Takara-Taq DNA polymerase® (Takara, Tokyo, Japan) in a final volume of 50 μl. PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 54 °C for 60 s, and elongation at 72 °C for 60 s with a final extension at 72 °C for 10 min. The 15 μl of PCR product was then digested for 12 h using 5 units of *Bsm-FI* (New England Biolabs, MA) restriction enzyme, resulting in fragments of 338 and 131 bp in length if −634G is used or in fragments of 469 bp in length if −634C is used [9]. Finally, digested PCR products were electrophoresed through a 2% agarose gel and visualized with ethidium bromide.

2.4. Quantification of VEGF in serum

The serum VEGF concentration was measured in duplicate using a commercial ELISA kit (R&D Systems, Minneapolis, Minnesota). All samples were quickly frozen and stored at −80 °C until the time of the assay. The assay system used is sensitive to typically less than 9.0 pg/ml. Optical density at 450 nm was measured on the Immuno-Mini NJ-2300 (Nippon Inter Med, Tokyo, Japan) and VEGF concentration was determined by linear regression from a standard curve using the VEGF supplied with the kit as standard. The intra-assay coefficient of variation (CV) of the VEGF ELISA was 6.7%, and the inter-assay CV was 8.8%.

2.5. Quantification of HTLV-I provirus load, CSF neopterin 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 PBMC samples as reported previously [12]. 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 that is measured at each cycle. Based on the standard curve created by four known concentrations of template, the concentrations of unknown samples were determined. Using β-actin as an internal control, the amount of HTLV-1 proviral DNA was calculated by the following formula: copy number of HTLV-1 (pX) per 1×10^4 PBMC=[(copy number of pX)/(copy number of β-actin/2)] $\times 10^4$. All samples were performed in triplicate. Neopterin levels were evaluated by HPLC with fluorometric detection methods [13]. Serum and CSF antibody titers to

Table 1
Clinical characteristics of HAM/TSP patients and asymptomatic HTLV-I carriers (HCs)

	Age	Male/Female	Anti-HTLV-I antibodies[a]	HTLV-I proviral load[b]	Neopterin in CSF[c]
HAM/TSP (n=202)	57.3 ± 11.9[d]	60/142	26,364 ± 41,347	725.2 ± 656.5	111.9 ± 112.4
HCs (n=202)	39.5 ± 12.9	96/106	1514 ± 1467	191.2 ± 312.9	N/A

N/A: not applicable.

[a] Anti-HTLV-I antibodies were titrated by the particle agglutination method.

[b] Tax copy number per 1×10^4 PBMCs.

[c] Neopterin levels were evaluated by HPLC with fluorometric detection methods.

[d] The values are shown as the mean ± SD.

Table 2
Summary of VEGF -634C/G SNP data

Allele	HAM/TSP	HCs	NCs	p value[a]	Genotype	HAM/TSP	HCs	NCs	p value[b]
C	181 (44.8)[c]	183 (45.3)	93 (43.1)	0.89 (HAM-HCs)	CC	41 (20.3)	40 (19.8)	20 (18.5)	0.92 (HAM-HCs)
G	223 (55.2)	221 (54.7)	123 (56.9)	0.68 (HAM-Normal)	CG	99 (49.0)	103 (51.0)	53 (49.1)	0.91 (HAM-Normal)
				0.59 (HCs-Normal)	GG	62 (30.7)	59 (29.2)	35 (32.4)	0.84 (HCs-Normal)
Total	404	404	216			202	202	108	

HCs: asymptomatic HTLV-I carriers.
NCs: seronegative healthy controls.
[a] p values are calculated by χ^2-test with 2×2 contingency table.
[b] p values are calculated by χ^2-test with 2×3 contingency table.
[c] Numbers in parentheses are percentage.

HTLV-I were determined by a particle agglutination method (Serodia-HTLV-I®, Fujirebio).

2.6. Statistical analysis

Comparisons of genotype frequency among HAM/TSP patients, HCs and NCs were calculated by the chi-squared test. For multiple comparisons, we used Sheffe's F to analyze statistical difference. Mann–Whitney U-test was used to compare serum VEGF levels between the various clinical groups. Significance was considered at $p < 0.05$.

3. Results

3.1. VEGF promoter gene polymorphism in HAM/TSP patients, asymptomatic HTLV-I carriers and seronegative healthy controls

The functional promoter polymorphism at position −634C/G SNP in the VEGF promoter had been previously reported from Saitama, Japan to be associated with diabetic retinopathy with a significantly increased frequency of the CC genotype [9]. However, in the present study, no significant differences were observed among HAM/TSP patients, HCs and NCs genotype or gene frequencies (Table 2). In all groups (HAM/TSP patients, HCs and NCs) the genotype frequencies were distributed according to Hardy–Weinberg equilibrium. Interestingly, the allele and genotype frequencies of VEGF −634C/G SNP in Kagoshima population was very similar to previously reported type 2 diabetic patients with retinopathy, but not without retinopathy [9]. Recently reported allele and genotype frequencies of VEGF −634C/G SNP from Italian control population also showed similar results with our present study [14]. Thus −634C/G SNP in the VEGF promoter was not associated with the risk for HAM/TSP in Kagoshima population.

3.2. Serum concentration of VEGF among HAM/TSP, ATL patients, asymptomatic HTLV-I carriers and seronegative controls

There was no significant difference in serum VEGF levels among 22 HAM/TSP (224.62 ± 140.65), 7 ATL patients (390.54 ± 283.78), 24 asymptomatic HTLV-I carriers (228.22 ± 156.99) and 12 NCs (209.89 ± 159.02) (Fig. 1). Two ATL patients with organ infiltration of ATL cell showed relatively high serum VEGF levels (ATL1: 652.0 pg/ml; ATL 2: 857.5 pg/ml) than other patients, consisting with previous reports [15,16].

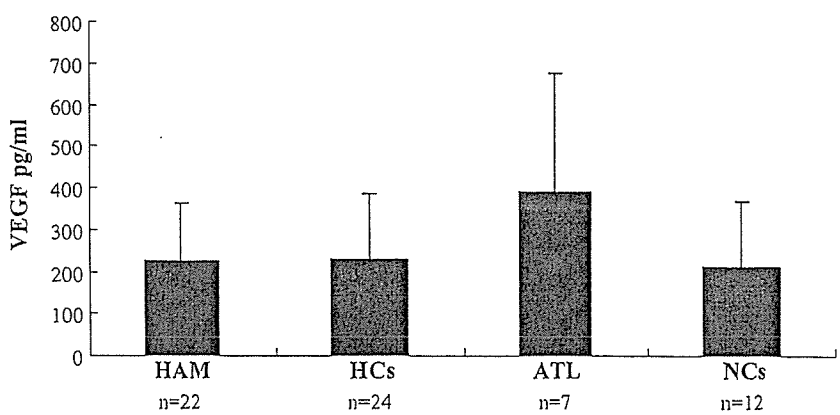

Fig. 1. Serum concentration of VEGF among HAM/TSP patients, ATL patients, asymptomatic HTLV-I carriers and seronegative controls. Serum VEGF levels from 22 HAM/TSP (224.62 ± 140.65), 7 ATL patients (390.54 ± 283.78), 24 asymptomatic HTLV-I carriers (HCs) (228.22 ± 156.99) and 12 seronegative healthy controls (NCs) (209.89 ± 159.02) were determined using ELISA. Bars show the mean ± standard deviation in each group.

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.

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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

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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

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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-

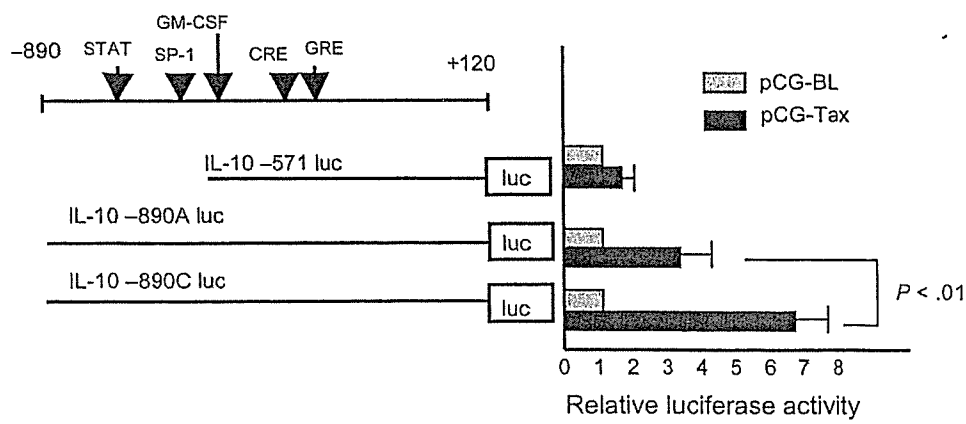

Figure 1. Interleukin (IL)–10 −592 A/C polymorphism and the Tax-mediated transcription of the IL-10 promoter. Jurkat cells were transfected with human T cell lymphotropic virus (HTLV) type I Tax expressing (pCG-Tax) or control (pCG-BL) vector and luciferase (luc) reporter constructs containing the full-length 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.

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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)

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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

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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-I 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).

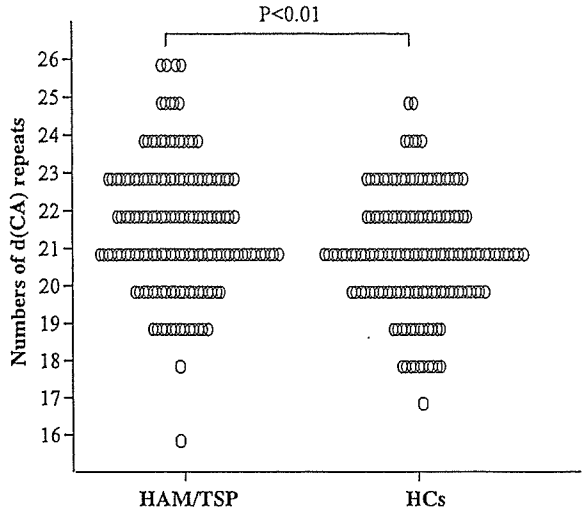

Fig. 1. Distribution of d(CA)n repeats in HAM/TSP patients and HTLV-I seropositive asymptomatic carriers (HCs). The longer d(CA) repeat alleles of MMP-9 promoter was more frequently observed in HAM/TSP patients than HCs (p<0.01 by Mann-Whitney U-test).

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).

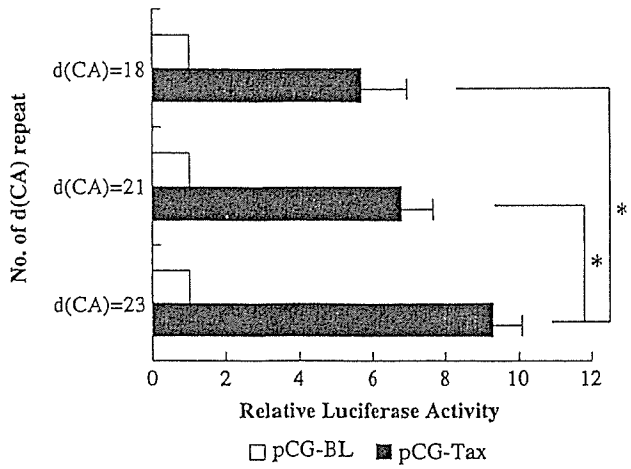

Fig. 2. HTLV-I Tax mediated trans-activation of MMP-9 promoter correlates with the length of the d(CA) repeat. Jurkat cells were transfected with HTLV-I Tax expressing (pCG-Tax) or control (pCG-BL) vector and luciferase reporter constructs containing the d(CA)18 or d(CA)21 or d(CA)23 repeat. Open bars 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-I (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.

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