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Temporal Dynamics of Human T-Lymphotropic Virus Type I *tax* mRNA and Proviral DNA Load in Peripheral Blood Mononuclear Cells of Human T-Lymphotropic Virus Type I-Associated Myelopathy Patients

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Human T-cell lymphotropic virus type I (HTLV-I) is the etiologic agent of HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). High HTLV-I provirus load and *tax* mRNA level have been suggested as predictors of disease progression in patients with HAM/TSP, but little is known about the temporal variation in patients. To clarify the role of high proviral and *tax* mRNA loads and their fluctuations in the pathogenesis of HAM/TSP, we measured proviral load and *tax* mRNA in serially collected peripheral blood mononuclear cells (PBMCs) from nine patients with HAM/TSP during a long-term follow-up, by use of real-time polymerase chain reaction using tax primers. The real-time PCR quantitation revealed a wide range of variation of proviral loads (7.82–97.13 copies per 100 PBMCs) and tax mRNA (0.20–245.30 copies) among HAM/TSP patients. Patients showed three different patterns of HTLV-I tax mRNA loads during the course of the disease. Tax mRNA load showed a separate evolution with respect to the disease. The dynamic patterns of proviral load and mRNA Tax expression suggest that only the permanent presence of a basal level of tax mRNA, rather than the tax mRNA load, is related to the development of HAM/TSP. To our knowledge, this is the first longitudinal study to determine *tax* mRNA expression at different clinical stages. ***J. Med. Virol. 79:782–790, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: HTLV-I-associated myelopathy/tropical spastic paraparesis; HTLV-I *tax* mRNA load; HTLV-I proviral DNA load

INTRODUCTION

Human T-lymphotropic virus type I (HTLV-I) is the retrovirus causing adult T-cell leukemia (ATL) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [Uchiyama et al., 1977; Osame et al., 1986]. HAM/TSP is a neurological disease defined by a progressive and slow spastic paraparesis without remittance. The main feature of this progressive neurological disease is demyelinization and axonal loss of the pyramidal tract [Cartier et al., 1997; Sugisaki et al., 1998].

HAM/TSP patients generally have a large population of HTLV-I-infected T cells in peripheral blood mononuclear cells (PBMCs), an increased CTL response to Tax, and a higher immune response against HTLV-I antigens than asymptomatic carriers [Parker et al., 1992; Nagai et al., 1998]. High levels of HTLV-I proviral DNA, HTLV-I Tax mRNA, and anti-HTLV antibody have been associated with HAM/TSP and the progress of the disease [Nagai et al., 1998; Yamano et al., 2002]. The increment of HTLV-I provirus in PBMCs was explained by increased replication of HTLV-I-infected T cells in HAM/TSP patients by autocrine secretion of IL-2/IL-2R induced with the Tax function [Tendler et al., 1990]. However, the pathogenic mechanism is unresolved and may be due to either an autoimmune response directed at viral proteins homologous to neuronal proteins [Levin

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et al., 2002], inflammatory substances released by lymphocytes during the course of an immune response to HTLV-I-infected CD4+ T cells that have invaded the central nervous system [Jacobson, 2002], or a neurodegenerative process giving rise to indirect Tax action [Cartier and Ramirez, 2005].

Even though a high proviral DNA load is characteristic of patients with HAM/TSP, the expression of HTLV-I in PBMCs appears to be very low, which suggests that HTLV-I may be latent in the peripheral blood [Tochikura et al., 1985; Kinoshita et al., 1989; Gessain et al., 1990]. However, the presence of high frequencies of HTLV-I-specific CD4+ cells and activated CTLs in the peripheral blood supports the hypothesis that the virus is not latent [Daenke et al., 1996; Jeffery et al., 1999]. Moreover, a correlation between the HTLV-I proviral DNA load and the frequency of HTLV-I Tax-specific CD8+ T cells has been described in patients with HAM/TSP [Kubota et al., 2000; Nagai et al., 2001]. In addition, a correlation between the HTLV-I *tax* mRNA load and disease severity has been suggested in patients with HAM/TSP [Yamano et al., 2002]. Collectively, these data suggest that the Tax protein plays a central role in the development of HAM/TSP. Although Tax itself does not bind to DNA directly or function as an enzyme, its ability to regulate multiple cellular responses is conferred by its protein—protein interactions with various host cellular factors. Importantly, HTLV-I-mediated activation of the host T cell is induced primarily by the viral protein Tax, which influences transcriptional activation, signal transduction, cell cycle control, and apoptosis [Burton et al., 2000; Jeang, 2001]. Recently, it has been shown that the CD4+CD25+ T cell population (Treg cells) is the main reservoir for HTLV-I in HAM/TSP patients [Yamano et al., 2005]. In addition, a Tax–specific inhibition of foxp 3 mRNA expression has been demonstrated that may be associated with the suppression of CD4+CD25+ that in turn may be associated with the suppression of the Treg function [Yamano et al., 2005]. However, no longitudinal studies have characterized the HTLV-I *tax* messenger RNA (mRNA) expression in the relationship between the HTLV-I proviral DNA load and the progression of the disease in HAM/TSP patients.

In this study, to determine the importance of HTLV-I *tax* mRNA expression in the progression of HAM/TSP, a quantitation of HTLV-I *tax* mRNA expression was developed by a real-time reverse transcription-polymerase chain reaction (RT-PCR) method. This paper provides a composite picture of proviral load and HTLV-I tax mRNA level during the follow-up of HAM/TSP patients.

MATERIALS AND METHODS

Patients and Samples

Nine patients with HAM/TSP and four asymptomatic HTLV-I carriers (Acs) were included in the study. All subjects resided in Santiago of Chile and had Spanish ethnic background. Infection of HTLV-I was confirmed by immunofluorescence assay and PCR [Ramirez et al., 2004]. The diagnosis of HAM/TSP was made according to the World Health Organization guidelines [Osame, 1990]. Other causes of progressive spastic paraparesis were excluded through clinical presentation according to clinical, neurophysiological, radiological (NMR), immunological, and CSF cytochemical studies [Cartier et al., 1989, 1992, 1995; Castillo et al., 1999]. Hematological tests were performed to detect leucemoid lymphocytes [Cabrera et al., 1999]. Disease severity was measured by using an expanded disability status scale (EDSS) [Kurtzke, 1983]. Blood samples (10 ml) were obtained during a follow-up of 1–9 years to study the *tax* mRNA load from nine HAM/TSP patients. Blood samples were obtained from four asymptomatic carriers. Three HAM/TSP patients (HAM/TSP-3, HAM/TSP-5 and HAM/TSP-7) died during the period of the study. The cause of death of HAM/TSP-3, HAM/TSP-5 and HAM/TSP-7 was pneumonia, acute lymphoma, and heart disease, respectively. HAM/TSP-1, HAM/TSP-2, HAM/TSP-3, and HAM/TSP-4 patients did not receive any pharmacological therapy during the period of the follow-up. HAM/TSP-5, HAM/TSP-6, HAM/TSP-7, HAM/TSP-8 and HAM/TSP-9 patients received therapy with corticosteroids during intermittent periods.

All cases were selected with an informed consent accepted previously. The study was carried out with the approval of the Ethics Committee of the El Salvador Hospital. The PBMCs were prepared by centrifugation over Ficoll-Hypaque gradients, and the cells were viably cryopreserved in liquid nitrogen until used.

Cell Lines

MT-2 cells are lines of CD4+ T cells infected with HTLV-I [Miyoshi et al., 1981]. K-562 cells are human T-cell lines not infected with HTLV-I. Both lines (5×10^5 cells) were cultivated for 10 days in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 100 U of penicillin/ml, 100 μg of streptomycin/ml, 4 nM glutamine, 1 mM sodium pyruvate and plating on 50-mm plastic flask at 37°C and 5% CO_2. The cellular suspension was centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. The cellular pellet was washed three times with PBS pH 7.2 and it was centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the pellet was used for PCR and RT-PCR assays.

Real-Time RT-PCR of Tax mRNA

RNA was extracted from PBMCs using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RT-PCR was performed using LyghtCycler–RNA Amplification Kit SYBR Green I (Roche Molecular Systems, Inc., Alameda, CA). Primers for the amplification of HTLV-I *tax* mRNA were previously described [Yamano et al., 2002]. The forward primer RPX-3 (5096–5115; 5′-ATCCCGTGGA-GACTCCTCAA-3′) and the reverse primer RPX-4+3 (7360–7338; 5′-CCAAACACGTAGACTGGGTATCC-3′)

were located upstream and downstream of the second splice junction site of HTLV-I *pX* (*tax/rex*) mRNA. The amplified DNA was 147 base pairs (bp). We used the human housekeeping gene hypoxanthine ribosyl transferase (*HPRT*) primers (BiosChile IGSA) for internal calibration. The amplified control DNA was 80 bp [Specht et al., 2001]. RT-PCR conditions were as follows: 100 ng RNA was added to 20 μl reaction mixture containing $1\times$LightCycler–RT-PCR reaction Mix SYBR Green I, $1\times$resolution solution, and $1\times$ LightCycler–RT-PCR enzyme Mix, 6.0 mM $MgCl_2$, 0.5mM each primer. The thermal cycler conditions were 15 min at 55°C to cDNA synthesis, and then 45 cycles of 10 sec at 95°C (denaturation) followed by 5 sec at 55°C (annealing) and 7 sec at 72°C (extension).

Standard curves for the value of HTLV-I *tax* mRNA and HPRT mRNA were generated using cDNA from MT-2 cells. MT-2 cDNA was serially diluted 10-fold with diethyl pyrocarbonate (DEPC) H_2O down to a 10^{-5} dilution, and sample cDNA from 100 ng RNA per reaction was applied and analyzed by this system. All standards and samples were assayed in triplicate. When a serial dilution of 10^0 to 10^{-5} of the MT-2 cell cDNA was used as the template for the real-time PCR, a specific signal of each increased in accordance with the increase of PCR cycles, but not in the negative control. The threshold cycle (C_t) values were used to plot a standard curve in which C_t decreased in linear proportion to the log of the template copy number.

All assays were carried out in triplicate and the average value was used for calculations. The relative HTLV-I *tax* mRNA load was calculated by the following formula: HTLV-I *tax* mRNA load = (value of *tax*)/(value of HPRT) $\times$ 10000.

Real-Time PCR of Proviral DNA

The HTLV-I proviral DNA load was measured using LightCycler DNA Master SYBR Green I Kit (Roche Molecular Systems, Inc., Alameda, CA). DNA was extracted from 1×10^6 PBMCs according to a method described previously [Ehrlich et al., 1990]. An extraction control used identical procedures without cells. Protective clothing, separate equipment, newly prepared reagents, ultraviolet irradiation and others measures to prevent contamination were routinely used. We amplified the following genomic regions: 158 bp of HTLV-I *tax* with primers SK43/SK44, and 268 bp of β-globin gene with primers PC04 and GH20 for internal calibration [Ehrlich et al., 1990]. RT-PCR conditions were as follows: 100 ng DNA was added to 20 μl reaction mixture containing $1\times$ LightCycler DNA Master SYBR Green I, 4.0 mM $MgCl_2$, 0.5mM each primer. The thermal cycler conditions were 4 min at 95°C (hot start), and then 45 cycles of 10 sec at 95°C (denaturation) followed by 5 sec at 55°C (annealing) and 7 sec at 72°C (extension). All assays were carried out in triplicate and the average value was used for calculations. The HTLV-I proviral DNA load was calculated by the following formula: copy number of HTLV-I (*pX*) per 100 cells = (copy number of *pX*)/(copy number of β-globin/2) $\times$ 100.

Statistical Analysis

The Mann–Whitney *U* test was used to compare the data between patients with HAM/TSP and Acs. Linear regression analysis was used to test the relation between HTLV-I proviral DNA load, HTLV-I *tax* mRNA load, and mRNA/DNA ratio. The Spearman rank correlation was used to test the correlation of EDSS with HTLV-I *tax* mRNA, proviral DNA and mRNA/DNA ratio. Statistical analyses were carried out with the statistical packages Statgraphics 5.0 (Mann–Whitney *U* test) and Stata 6.0 (Spearman rank correlation).

RESULTS

Precision, Sensitivity and Specificity of Real-Time RT-PCR

To determine the interassay coefficient of variance of this real-time HTLV-I *tax* RT-PCR system, cDNA from HTLV-I-infected MT-2 cells was diluted serially with cDNA from HTLV-I-non-infected H9 cells, and the HTLV-I *tax* mRNA load of each dilution was measured three times (Fig. 1a). To estimate the accuracy of this method, we calculated the coefficient of variance (CV%) for each amount. The mean interassay CV% was 16.2%, which is consistent with the CV% measuring HTLV-I proviral DNA using real-time PCR methods [Yamano et al., 2002].

To test the intra-assay coefficient of variance of this real-time HTLV-I *tax* RT-PCR system, PBMCs from three patients with HAM/TSP were separated into three samples per individual. RNA was extracted from each sample, cDNA was synthesized, and the HTLV-I *tax* mRNA load for each sample was determined (Fig. 1b). The CV% was calculated for each patient. The mean value of intra-assay CV% was 11.2%, which is consistent with the CV% measuring HIV-1 RNA [Sun et al., 1998]. These data demonstrate that this real-time HTLV-I *tax* RT-PCR assay is accurate and reliable for quantitation of HTLV-I *tax* mRNA.

To determine the sensitivity of this real-time RT-PCR assay, the HTLV-I *tax* mRNA load was determined from MT-2 cells diluted serially with PBMCs from a healthy donor (HD) not infected with HTLV-I. As shown in Figure 1c, the HTLV-I *tax* mRNA signal was detected in a dose-dependent manner with a sensitivity limit as low as two MT-2 cells in 1×10^6 PBMCs.

To test the specificity of this assay, cDNA from HTLV-I-non-infected H9, HIV-1-infected H9 cells and PBMCs from HTLV-I-non-infected HDs (n = 3) were analyzed. No signal was observed from any of these HTLV-I-non-infected cells (results not shown).

HTLV-I *tax* mRNA and Proviral Load in Samples From HAM/TSP Patients and Acs

The first analysis was to study separately each sample of the HAM/TSP patients and Acs. HTLV-I tax mRNA

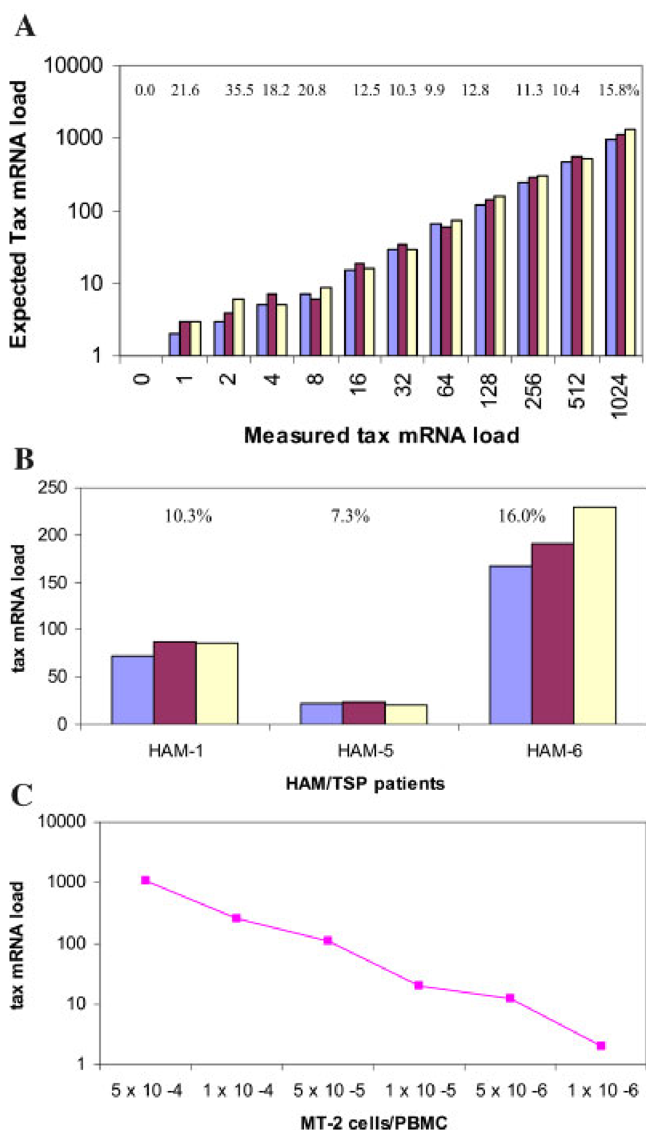

Fig. 1. Interassay and intra-assay precision, and sensitivity of real-time RT-PCR. Interassay CV% (**A**), intra-assay CV% (**B**), and sensitivity (**C**). The coefficients of variation (CV%) are [SD of HTLV-I tax mRNA load/average of HTLV-I tax mRNA load] × 100.

and the provirus was determined in 32 samples of PBMCs from nine HAM/TSP patients and four Acs.

Table I and Figure 2 show that the HTLV-I *tax* mRNA load ranged from 0.20 to 245.30 (average = 104.64) in 26 samples from HAM/TSP patients and from 0.00 to 0.89 (average = 0.25) in 8 samples from Acs. The HTLV-I *tax* mRNA load in HAM/TSP patients was significantly greater than in Acs ($P = 0.00004$, Mann–Whitney *U* test). HTLV-I *tax* mRNA from HAM/TSP patients was 418.56 times greater than Acs. 96.15% of the samples of HAM/TSP patients had values of mRNA over 10, and 46.15% were over 100. By contrast, 100% of the samples from Acs had mRNA values less than one. A greater dispersion in the values of *tax* mRNA was observed among HAM/TSP patients (standard deviation = 65.32) than Acs (standard deviation = 0.34). The absolute amounts of HTLV-I tax mRNA before adjusting by the HPRT value in HTLV-I-infected individuals were 10^3 to 10^5 times lower than that in MT-2 cells.

The HTLV-I provirus load ranged from 7.82 to 97.13 (average = 50.55) in 26 samples from HAM/TSP patients and from 0.46 to 3.58 (average = 1.63) in 8 samples from Acs (Table I and Fig. 2). In addition, the HTLV-I proviral DNA load in HAM/TSP patients was significantly higher than in Acs ($P = 0.00002$, Mann–Whitney *U* test). The HTLV-I provirus load from HAM/TSP patients was 31.01 times higher than from Acs individuals. All of the samples of HAM/TSP patients had values of HTLV-I provirus over 5; 96.15% were over 10, and 38.46% were above 50. In contrast, all samples from Acs had mRNA lower than 4. A greater variation in provirus load was observed among HAM/TSP patients (standard deviation = 24.33) compared to Acs (standard deviation = 0.94). The absolute amount of proviral HTLV-I before adjusting by the β-globin value in HTLV-I-infected individuals was 10^3 to 10^6 times less than that in MT-2 cells.

Comparison of HTLV-I *tax* mRNA Load With HTLV-I Proviral DNA Load Ex Vivo

To determine during the follow-up of patients if there was a correlation between the HTLV-I proviral DNA load and the HTLV-I *tax* mRNA load in HTLV-I-infected individuals, a real-time quantitative HTLV-I DNA PCR assay was used to measure the HTLV-I proviral DNA load in PBMCs from HAM/TSP patients and Acs. Table I shows that the HTLV-I *tax* mRNA load ranged from 0.2 to 245.3 in HAM/TSP patients and from 0 to 0.89 in Acs. The *tax* mRNA load was significantly greater in HAM/TSP patients than in Acs ($P = 0.00004$). Furthermore, the HTLV-I proviral DNA load was related significantly to the HTLV-I *tax* mRNA load in HAM/TSP patients ($P = 0.00005$, $r^2 = 0.924$, linear regression analysis, Fig. 3).

To adjust the HTLV-I *tax* mRNA expression level in HTLV-I–infected PBMCs, we calculated the mRNA/DNA ratio by dividing the HTLV-I *tax* mRNA load by the HTLV-I proviral DNA load. The mean value of the mRNA/DNA ratio was 187.88 in HAM/TSP patients and 11.99 in Acs. The mRNA/DNA ratio of HAM/TSP patients was statistically greater than that of Acs ($P = 0.00005$, Mann–Whitney *U* test, Table I).

Comparison of Disease Severity With HTLV-I Proviral DNA Load, HTLV-I *tax* mRNA Load, and mRNA/DNA Ratio in HAM/TSP Patients

Clinical features of HAM/TSP patients included in this study are shown in Table I. The HAM/TSP patients with the longest duration of illness were usually more severely affected. This series of patients had 4–22 years of paraparesis. It was observed that the clinical marker was the main factor associated with the progress of the illness according to the EDSS indicator (Table I). A direct correlation between dysfunctionality and the *tax* mRNA load was not detected (Fig. 4a). For example,

TABLE I. Clinical Features, HTLV-I tax mRNA Load, HLTV-I Proviral Load, and mRNA/DNA Ratio of nine Chilean HAM/TSP Patients and four Acs

Patient	Age	Sex	Years after admission	EDSS	Duration of illness (year)	Motor involvement	Sensitive	Associated pathology	Other pathologies	Tax mRNA load	Proviral DNA load	mRNA/DNA
HAM-1	49	F	0	3	15	1	–	1, 2	1, 2	193.28	91.1	212.16
			1	4	16	1	+			12.15	18.17	66.87
			5	5	20	2	+			73.25	38.45	190.51
			7	6	21	2	+			81.07	47.06	172.27
HAM-2	51	F	0	5	9	2	+	1	1, 2	178.01	85.27	208.76
			2	6	11	2	+			0.20	7.82	2.56
			3	6	12	3	+			93.74	44.45	210.89
			9	7	18	3	+			116.35	63.21	184.07
HAM-3	52	M	0	7	17	2	+	1, 3, 4	1	203.07	75.49	269.00
			1[a]	8	18	4	+			134.96	48.52	278.15
HAM-4	37	M	0	6	4	2	+	1	1	113.68	43.62	260.61
			1	7	5	3	+			245.3	97.13	252.55
			7	8	11	4	+			167.46	84.03	199.29
HAM-5	56	M	0	4	6	1	–	1, 3	–	118.74	55.29	214.76
			2[b]	4	8	1	–			22.31	14.91	149.63
HAM-6	60	M	0	2	9	1	–	1, 3	1, 3	87.03	33.54	259.48
			1	4	10	1	–			167.94	74.26	226.15
			6	6	15	2	–			196.01	81.22	241.33
HAM-7	52	F	0	2	5	1	–	–	–	63.27	37.01	170.95
			1[c]	4	6	1	–			94.66	44.14	214.45
HAM-8	46	F	0	4	6	1	–	–	1	45.39	36.11	125.70
			1	5	7	2	+			66.21	47.03	140.78
			7	6	13	2	+			112.07	53.45	209.67
HAM-9	48	F	0	5	8	2	–	1, 2	1	29.58	25.92	114.12
			2	6	10	2	+			43.12	29.16	147.87
			6	7	14	3	+			61.73	38.03	162.32
Mean	50.1				13.7					104.64	50.55	187.88
median										94.2*	45.74**	204.02***
AC-1	53	M	0	–	–					0.89	2.09	44.50
			2	–	–					0.65	3.58	21.67
AC-2	61	F	0	–	–					0.00	1.36	0.00
			2	–	–					0.00	1.74	0.00
AC-3	37	M	0	–	–					0.00	0.46	0.00
			4	–	–					0.32	1.78	17.98
AC-4	48	F	0	–	–					0.00	0.87	0.00
			1							0.14	1.19	11.76
Mean	49.8									0.25	1.63	11.99
median										0.07*	1.55**	5.88***

HTLV-I tax mRNA, HTLV-I proviral DNA load, and mRNA/DNA ratio were higher in HAMTSP patients than in Acs with statistical significance by the Mann–Whitney U test: $^{*}P = 0.00004$, $^{**}P = 0.00002$, $^{***}P = .00005$. The EDSS score was not correlated with HTLV-I tax mRNA load, HTLV-I proviral DNA load, or mRNA/DNA ratio ($P = 0.2936$, 0.3267 and 0.1862, respectively) by Spearman rank correlation analysis.
Motor involvement: spastic gait without support (1), spastic gait with support (2), wheelchair (3), and bedridden (4).
Associated pathology: dacryosialadenitis (1), cognitive impairment (2), cutaneous lymphoma (3), and Hepatic cirrhosis (4).
[a]HAM-3 died 3 years after last evaluation.
[b]HAM-5 died 1 year after last evaluation.
[c]HAM-7 died 4 years after last evaluation.

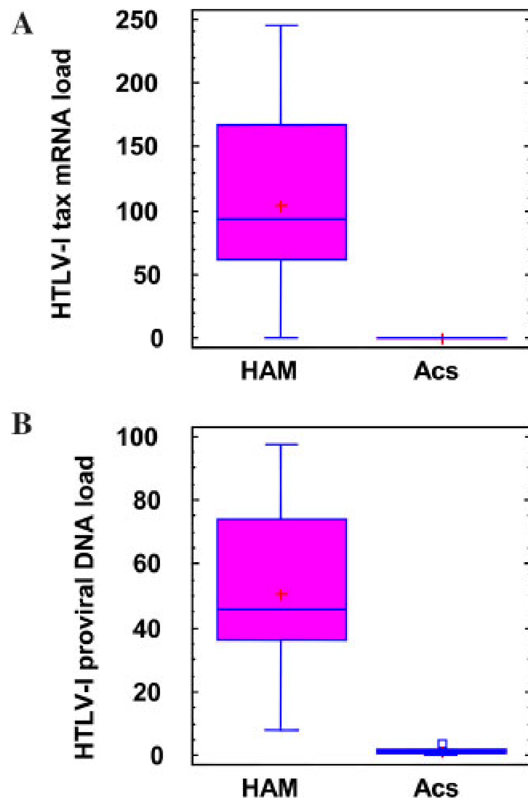

Fig. 2. HTLV-I tax mRNA and proviral DNA load in HAM/TSP patients and Acs. HTLV-I tax mRNA (**A**) and proviral DNA (**B**) load in PBMCs from nine HAM/TSP patients and four asymptomatic HTLV-I carriers (Acs). HTLV-I *tax* mRNA and proviral DNA load were significantly higher ($P = 0.00004$ and $P = 0.00002$, respectively) in HAM/TSP patients compared to Acs.

HAM/TSP-1 patient had 15 years of paraparesis with a functional status of three and a *tax* mRNA load of 193.28. This patient progressed to functional status six 7 years later, however the *tax* mRNA load was 81.07. Furthermore, patients with high HTLV-I *tax* mRNA load and a long evolution of the disease (HAM/TSP-2, HAM/TSP-3, HAM/TSP-4) had more severe dysfunction, with EDSS scores above 7 (patients essentially restricted to a wheelchair). In addition, we found a patient with low HTLV-I *tax* mRNA load (HAM/TSP-9) who had an EDSS of 7.

A direct relation was not found between the *tax* mRNA load and the clinical stage or progress of disease in most studied cases ($P = 0.2936$, Spearman rank correlation, Fig. 4a). Several patients showed progress of disease but had a decrease of *tax* mRNA load. The levels of *tax* mRNA load were associated with three different patterns during the follow-up of patients. Some patients showed an initial decrease of *tax* mRNA load, followed by an increase to a steady state, for example patients HAM/TSP-1 and HAM/TSP-2 (Fig. 4b). Patient HAM/TSP-4 showed an initial increment and a subsequent decrease of *tax* mRNA level and another two patients (HAM/TSP-3, and HAM/TSP-5) showed only a decrease, although these patients had previously 17 and 6 years of paraparesis, respectively (Fig. 4c). Patients HAM/TSP-6, HAM/TSP-7, HAM/TSP-8, and HAM/TSP-9, who had 5–9 previous years of evolution of the illness, showed a constant increment of the levels of mRNA (Fig. 4d).

DISCUSSION

A direct correlation has been described between the level of provirus, the tax mRNA load and the progress of HAM/TSP [Nagai et al., 1998; Manns et al., 1999; Yamano et al., 2002]. Higher DNA proviral and tax mRNA loads were found in patients with greater neurological commitment compared to infected healthy persons. The tax mRNA load has been suggested as a predictor of the progress of HAM/TSP [Yamano et al., 2002]. Nevertheless, those studies used cross sampling of different patients, which does not allow validation of the results. If those studies are analyzed, both the proviral and the tax mRNA loads have extensive ranges

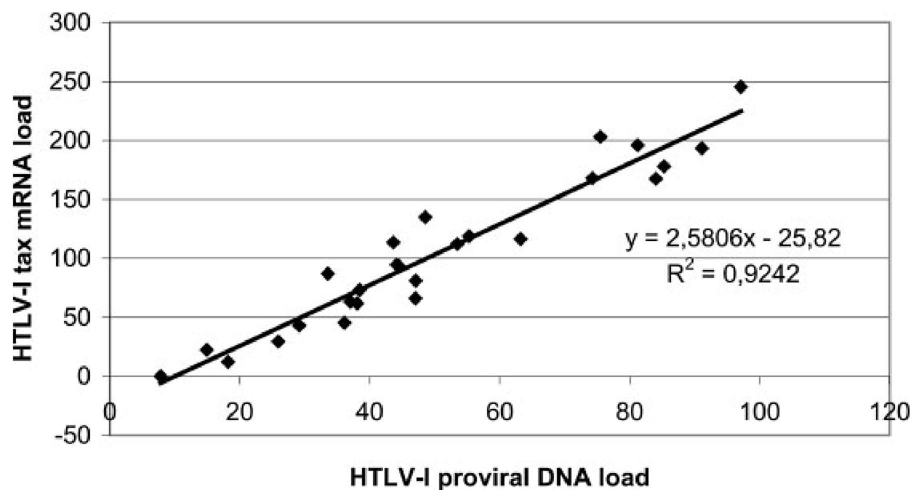

Fig. 3. Correlation between HTLV-I proviral DNA load and HTLV-I *tax* mRNA load in HAM/TSP patients. A statistically significant correlation ($P = 0.00005$, $r^2 = 0.924$) between the HTLV-I proviral DNA load and HTLV-I *tax* mRNA load was observed in PBMCs from HAM/TSP patients.

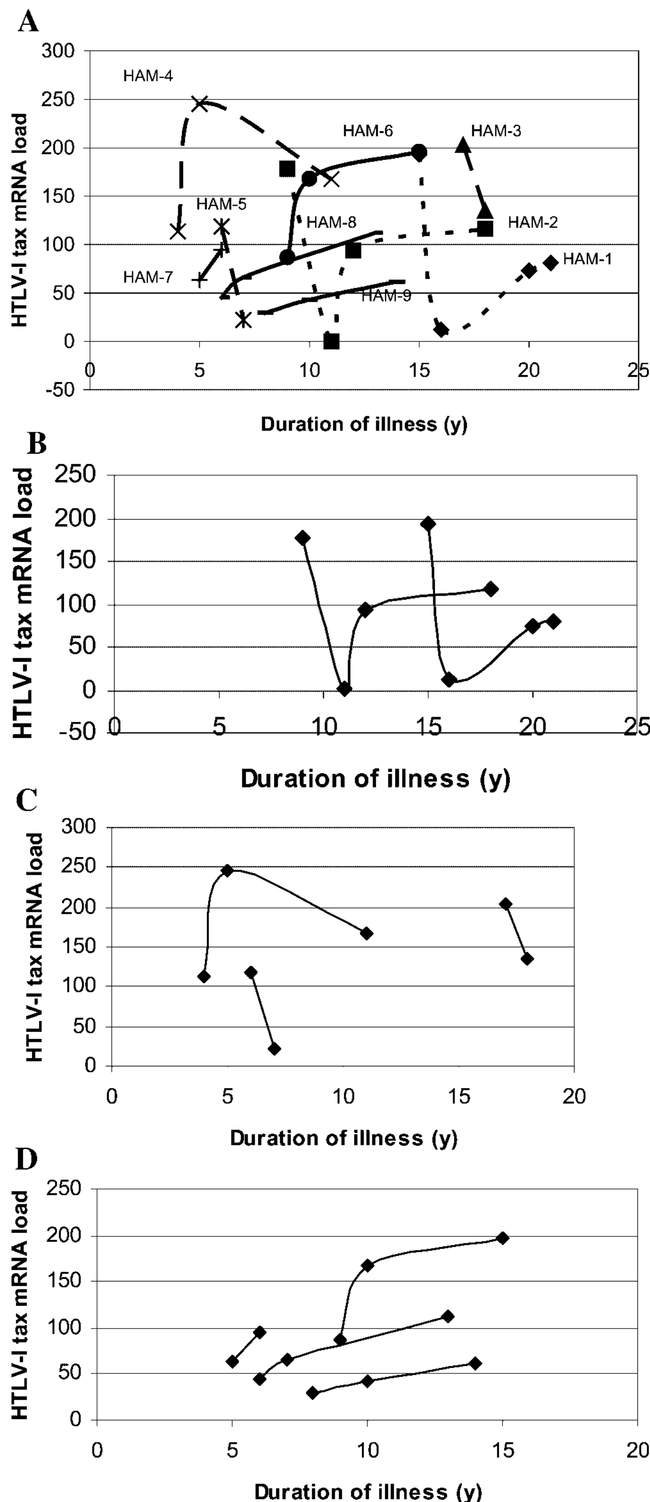

Fig. 4. Variation of HTLV-I tax mRNA load in nine HAM/TSP patients during the elapsed time of illness. All patients (**A**). The EDSS score was not correlated with HTLV-I tax mRNA load ($P = 0.2936$, Spearman rank correlation analysis). Patients HAM-1 and HAM-2 showed pattern 1, decrease, increase and stabilization (**B**). Patients HAM-3, HAM-4 and HAM-5 had pattern 2, decrease (**C**). Patients HAM-6, HAM-7, HAM-8 and HAM-9 presented pattern 3, increase (**D**).

of variation which include different subjects with similar levels of motor dysfunction [Yamano et al., 2002]. In addition, Kubota et al. (1993) detected fluctuations in the DNA proviral load in six patients with HAM/TSP. They showed that the amount of HTLV-I proviral DNA in six HAM/TSP patients was 3–50 times greater than that of eight Ac individuals. In addition, they followed up HAM/TSP patients for 1–3 years, and they found that the amount of HTLV-I proviral DNA fluctuated from four to 10-fold. These data demonstrated that the amount of HTLV-I proviral DNA fluctuates in the clinical course of the disease.

Our experimental design offers advantages because the proviral and *tax* mRNA loads were studied in nine patients with HAM/TSP during a follow-up that fluctuated between 1 and 9 years. A greater level of provirus and mRNA were found compared to the controls, corroborating values observed by other investigators [Kubota et al., 2000; Nagai et al., 2001]. Nevertheless, a large temporal variation was found in the provirus and *tax* and in the level of the mRNA loads in patients independent of the functional damage.

The progress of the motor commitment increased in parallel with the *tax* mRNA load in four patients (HAM/TSP-6, HAM/TSP-7, HAM/TSP-8 and HAM/TSP-9). These four patients had less than 15 years of evolution. The five remaining patients showed fluctuations and even important decreases of the tax mRNA load that itself did not correlate with the motor damage. These findings are interesting because they indicate that the *tax* mRNA load does not always enlarge and may not be correlated with the progress of the illness.

This result coincides with diverse investigators who have observed that there is a high HTLV-I proviral load in PBMCs from patients with HAM/TSP, and the proviral load has been reported to fluctuate in individual patients during the course of the disease [Nagai et al., 2001; Takenouchi et al., 2003]. The proviral load of taken serially PBMCs as well as of cerebrospinal fluid (CSF) cells from patients with HAM/TSP were measured in a long-term follow-up and compared these with their clinical manifestations [Takenouchi et al., 2003]. They detected a wide distribution of proviral load, however, the proviral load in individual patients was relatively stable during this study. The proviral loads in CSF cells were higher than proviral loads in PBMCs in individual patients. The ratio of proviral loads in CSF/PBMC cells, but not the absolute load, in either compartment, could be significantly associated with the clinical progression of the disease and especially with recent onset of HAM/TSP.

Our results show that the quantity of provirus and *tax* mRNA are not associated directly with the functional damage of the subjects. The progression of the illness was only associated with the individual increase of these molecular parameters in four patients. In the other five patients, the indicators diminished or they varied independently of the progression of the illness, which is constant and without remissions. Thus, the progression of the disease may depend of the vulnerability of the

patient. Also, this lack of correlation between the *tax* mRNA load and the progression of HAM/TSP explains the progressive damage in the seronegative patients; although they still maintained very low levels of *tax* provirus and *tax* mRNA, the disease progressed to an indistinguishable form of those seropositive HAM/TSP patients that have high levels of Tax [Cartier and Ramirez, 2005].

If a correlation exists between the proviral or tax mRNA levels and the magnitude of the damage in the cortico-spinal tracts due to a constant increase of the proviral or tax mRNA loads, theoretically this should accelerate the progression of the lesions. Nevertheless, this does not occur in the HAM/TSP patients, suggesting that the increase of the synthesis of tax mRNA may be product of the temporal dynamics of the virus during the first decade of the paraparesis. Later, only the presence of a low quantity of Tax would be necessary to initiate or to maintain the degeneration of the axons in the cortico-spinal tract that are lost during the progression of HAM/TSP.

Likewise, the HAM/TSP neuropathology shows a symmetrical degeneration of the cortico-spinal tract expressed especially in the dorsal and lumbar segments and an involve of Goll tracts at the cervical level [Cartier et al., 1997]. The degenerative damage of the longest axons could be explained by the constant presence of Tax in the extracellular space of the CNS and the capacity of Tax to bind with kinase or phosphatases enzymes [Haller et al., 2002; Fu et al., 2003] that could have some influence in the axonal transport [Cartier and Ramirez, 2005]. Electron microscopy studies of HAM/TSP have shown an abnormal accumulation of neurofilaments and Hirano bodies in some axons [Liberski et al., 1999], and the immunohistochemical results show APP accumulation in axons [Umehara et al., 2000]. Both suggest that axonal transport showing a degenerative process arises from an indirect action of Tax protein.

Our findings show that the absolute levels of tax mRNA and the ratio mRNA/DNA by themselves are not necessarily predictors of the velocity in the progress of the HAM/TSP. We have shown that there are temporary fluctuations in *tax* mRNA and proviral loads in different samples from the same patient. In addition, these fluctuations in most cases do not correlate with the advance of the illness. Without doubt, it is mandatory to carry out additional longitudinal studies in HAM/TSP patients in which the CD4+ and CD8+ counts, and tax mRNA loads in PBMCs and in cells of the spinal fluid be determined simultaneously. These longitudinal studies are necessary to advance understanding of the pathogenesis of HAM/TSP.

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