Correlation of human T-cell lymphotropic virus type 1 (HTLV-1) mRNA with proviral DNA load, virus-specific CD8+ T cells, and disease severity in HTLV-1–associated myelopathy (HAM/TSP)

Yoshihisa Yamano, Masahiro Nagai, Meghan Brennan, Carlos A. Mora, Samantha S Soldan, Utano Tomaru, Norihiro Takenouchi, Shuji Izumo, Mitsuhiro Osame, and Steven Jacobson

To investigate the role of viral expression in individuals infected with human T-cell lymphotropic virus type 1 (HTLV-1), a real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) of HTLV-1 tax messenger RNA (mRNA) using ABI Prism 7700 Sequence Detection System was developed. Using this system, the HTLV-1 tax mRNA load was compared with HTLV-1 proviral DNA load, HTLV-1 Tax protein expression, HTLV-1 Tax-specific CD8+ T-cell frequency, and disease severity of HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP). This approach was a sensitive and specific technique for the precise quantification of HTLV-1 tax mRNA. The total amount of HTLV-1 tax mRNA and mRNA expression level in HTLV-1–infected cells (mRNA/DNA ratio) were higher in HAM/TSP patients than in asymptomatic HTLV-1 carriers. The HTLV-1 tax mRNA load correlated with the HTLV-1 proviral DNA load ex vivo, the Tax protein expression in vitro, and the Tax-specific CD8+ T-cell frequency ex vivo. The HTLV-1 tax mRNA load also correlated with disease severity in HAM/TSP patients. These data suggest that increased HTLV-1 expression plays an important role in the pathogenesis of HAM/TSP, and the HTLV-1 tax mRNA level could be a useful predictor of disease progression in patients with HAM/TSP.

Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1) is a human retrovirus that infects approximately 10 million people worldwide. The majority of infected individuals remain healthy lifelong asymptomatic carriers (ACs), approximately 0.25% to 3% develop an inflammatory disease of the central nervous system termed HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP), and another 2% to 3% develop an aggressive mature T-cell malignancy termed adult T-cell leukemia (ATL). Patients with HAM/TSP generally harbor a much larger population of HTLV-1–infected T cells in peripheral blood mononuclear cells (PBMCs) and a higher immune response against HTLV-1 antigens than ACs. These findings indicate that an abundant viral load plays an important role in the pathogenesis of HAM/TSP, suggesting that high viral expression may be associated with the high immune response against the virus.

Even though a high proviral DNA load is characteristic of patients with HAM/TSP, the expression of HTLV-1 in PBMCs appears to be very low, which suggests that HTLV-1 may be latent in peripheral blood. However, this suggestion is difficult to reconcile with the observation of high activated immune responses in patients with HAM/TSP. Moreover, it has been reported that a significant proportion of PBMCs infected with HTLV-1 are capable of expressing HTLV-1 proteins after short-term cultivation in vitro, supporting the hypothesis that HTLV-1 is not inherently latent. Recently, a correlation between the HTLV-1 proviral DNA load and the frequency of HTLV-1 Tax-specific CD8+ T cells has been described in patients with HAM/TSP. Collectively, these data suggest HTLV-1 antigens continuously restimulate the cellular immune system in vivo. However, no reports have characterized the HTLV-1 tax mRNA expression in that relationship and in the pathogenesis of HAM/TSP; a rapid, sensitive, and precise quantification of HTLV-1 tax mRNA expression was developed by a real-time reverse transcription-polymerase chain reaction (RT-PCR) method using the ABI Prism 7700 Sequence Detection System. Because the level of human immunodeficiency virus type 1 (HIV-1) RNA has been found to be a valid predictor of the clinical progression of HIV-1–related disease and efficacy of anti-HIV therapy, it was also of interest to determine if the HTLV-1 tax mRNA load as quantified by this novel method might be a marker of disease activity and of relative prognostic value in HAM/TSP.

In this study, the HTLV-1 tax mRNA load in PBMCs from patients with HAM/TSP and ACs was measured and compared with HTLV-1 proviral DNA load, HTLV-1 Tax protein expression, HTLV-1 Tax-specific CD8+ T-cell frequency, and disease severity in patients with HAM/TSP. The data presented demonstrate that increased HTLV-1 expression plays an important role in the pathogenesis of HAM/TSP, and the HTLV-1 tax mRNA level could be a useful predictor of disease progression in HAM/TSP patients.
Patients, materials, and methods

Subjects and cells

The study population consisted of 16 patients with nontreated HAM/TSP (HAM-1 to HAM-16), 8 asymptomatic HTLV-1 carriers (AC-1 to AC-8), and 5 seronegative healthy donors (HDs). Infection of HTLV-1 was confirmed by enzyme-linked immunosorbent assay (Abbot Laboratories, North Chicago, IL) and Western blot analysis (Gene Labs, Singapore). The diagnosis of HAM/TSP was assessed according to the World Health Organization guidelines.24 The HAM-2 to -5, -11, -13, and -16 and AC-4, -6, and -7 individuals were positive for HLA-A*0201. All samples were taken under informed consent. The clinical features of the patients included in this study are shown in Table 1. Disease severity was assessed by using an expanded disability status scale (EDSS).25 MT-2 cells and Hut 102 cells are lines of human CD4+ T cells infected with HTLV-1.26,27 Jurkat cells are human T-cell lines not infected with HTLV-1.

Real-time RT-PCR of complementary DNA

RNA was extracted from PBMCs using RNasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized using TaqMan Gold RT-PCR Kit (Applied Biosystems, Foster City, CA). For cDNA synthesis from extracted mRNA, 3 μg mRNA, 10 μL 10 × TaqMan RT buffer, 22 μL MgCl2 (25 mM), 20 μL dNTPs mixture (at a final concentration of 500 μM each), 5 μL random hexamers (50 μM), 2 μL RNase inhibitor (20 U/μL), and 2.5 μL (50 U/μL) Moloney murine leukemia virus reverse transcriptase were added to a total volume of 100 μL. Samples were incubated at 25°C for 10 minutes and 48°C for 30 minutes. Reactions were stopped by heating to 95°C for 5 minutes.

Primers used for the amplification of HTLV-1 tax cDNA were slightly modified from the sequence previously reported28 to be suitable for this system. The forward primer RPX-3 (5096-5115; 5′-ATCCCCTTGGGAGACTCTCAAA-3′) and the reverse primer RPX-4 (7360-7338; 5′-CCAAACACGTAGCTGGTGATTCC-3′) were located upstream and downstream of the second splice junction site of HTLV-1 pX (tax/rex) mRNA. The amplified DNA was 147 base pairs (bp). The probe (5172-5183, 7302-7312; 5′-TCCAAACACGTG-GCCCCACTACC-3′) surrounded the second splice junction site of HTLV-1 pX (tax/rex) mRNA. We used the human housekeeping gene hypoxanthine ribosyl transferase (HPRT) primers and probe set (Applied Biosystems) for internal calibration. This internal control appeared to be most stable within PBMCs from HDs, ACs, HAM/TSP patients, and ATL patients by using the TaqMan Human Endogenous Control Plate (Applied Biosystems). PCR conditions were as follows: 10 μL cDNA solution synthesized from 300 ng RNA was added to 40 μL reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM EDTA, 60 mM MgCl2, 0.3 μM each primer, 0.2 μM TaqMan probe, 200 μM each of dATP, dGTP, and dCTP, 400 μM dUTP, 0.5 U AmpErase uracil-N-glycosylase (UNG), and 0.25 U AmpliTaq Gold DNA polymerase (AmpliTaq Gold; Applied Biosystems). The thermal cycler conditions were: 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C.

Table 1. HTLV-1 tax mRNA load, HTLV-1 proviral DNA load, mRNA/DNA ratio, HTLV-1 Tax-specific CD8+ T-cell frequency, and clinical profiles of HAM/TSP patients and ACs

<table>
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<tr>
<th>Patients</th>
<th>Age, y</th>
<th>Sex</th>
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<th>tax mRNA load</th>
<th>Provil DNA load</th>
<th>mRNA/DNA ratio</th>
<th>Tax-specific CD8+ T-cell frequency, %</th>
<th>EDSS</th>
<th>Duration of illness, y</th>
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<td>22.44†</td>
<td>88.67‡</td>
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* indicates subjects who are not HLA-A*0201. HTLV-1 tax mRNA load, HTLV-1 proviral DNA load, and mRNA/DNA ratio were higher in HAM/TSP patients than in ACs with statistical significance by the Mann-Whitney U test: *P = .0014; †P = .0003; ‡P = .0433. The EDSS score significantly correlated with both HTLV-1 tax mRNA load and mRNA/DNA ratio (P = .0109 and .0089, respectively), but not with HTLV-1 proviral DNA load (P = .5238) by Spearman rank correlation analysis.
were 2 minutes at 50°C to activate AmpErase UNG enzyme, 10 minutes at 95°C to inactivate UNG and activate AmpliTaq Gold DNA polymerase, and then 45 cycles of 15 seconds at 95°C (denaturation) followed by 1 minute at 60°C (annealing and extension).

Standard curves for the value of HTLV-1 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) H2O down to a 10^{-4} dilution, and sample cDNA from 300 ng RNA per well was applied and analyzed by this system. The amplification of standard cDNA and sample cDNA was carried out in a 96-well reaction plate. All standards and samples were assayed in triplicate. Each plate always contained the same standard. When a serial dilution of 10^{0} to 10^{-4} 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 (Ct) values were used to plot a standard curve in which Ct decreased in linear proportion to the log of the template copy number. The correlation values of standard curves were always more than 99%.

The relative HTLV-1 tax mRNA load was calculated by the following formula: HTLV-1 tax mRNA load = (value of tax)/(value of HPRT) × 10,000.

Real-time PCR of DNA
The HTLV-1 proviral DNA load was measured using this same TaqMan system as previously described.10,21 DNA was extracted from 1 × 10^{6} cells using Puregene DNA Isolation Kit (Genta, Minneapolis, MN) and 100 ng of the sample DNA solution per well was analyzed by this system. The HTLV-1 proviral DNA load was calculated by the following formula: copy number of HTLV-1 tax (pX) per 100 cells = (copy number of pX)/ (copy number of β-actin/2) × 100.

HTLV-1 Tax protein expression in PBMCs
The PBMCs (5 × 10^{5}) were placed on a culture well (round bottom 96-well plate) in 200 μL RPMI 1640 supplemented with t-glutamine, penicillin, streptomycin, and 5% human AB serum. Harvested cells were washed with phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS) and 0.1% NaCl, and incubated with antihuman CD4-PE (Caltag Laboratories, Burlingame, CA) and antihuman CD8-Tricolor monoclonal antibodies (mAbs; Caltag Laboratories) for 20 minutes at 4°C. Following washing with PBS containing 1% FCS and 0.1% NaCl, cells were fixed and permeabilized with 4% formaldehyde and 0.1% saponin (CytoFix/Cytoperm kits, Pharmingen, SanDiego, CA) for 20 minutes at 4°C. After washing with 0.1% saponin buffer (Perm/Wash solution, Pharmingen), cells were incubated with anti-HTLV-1 Tax mAb (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HTLV-1 Tax hybridoma 168A51-42 from Dr Beatrice Langton) for 20 minutes at 4°C. Following washing unbound antibody with 0.1% saponin buffer, fluorescein isothiocyanate (FITC)-conjugated goat F(ab')2 anti-mouse IgG2a was used as second antibody for labeling anti-HTLV-1 Tax mAb. After a 20-minute incubation, cells underwent a final wash. Flow cytometric analyses were performed using a FACS Calibur (Becton Dickinson, Mountain View, CA).

Identification of HTLV-1 Tax-specific CD8+ T cells
The HTLV-1 Tax 11-19 peptide is a dominant epitope recognized by HLA-A2-restricted CD8+ T cells in HAM/TSP patients.13,28 HTLV-1 Tax11-19/HLA-A*0201-specific CD8+ T cells were analyzed using a phycoerythrin (PE)-conjugated HLA-A*0201 tetramer (provided by NIAID MHC Tetramer Core Facility, Atlanta, GA, and NIH AIDS Research and Reference Reagent Program) for HLA-A*0201 subjects. The tetramer was loaded with HTLV-1 Tax 11-19 peptide (LLFGYPVYV, single-letter amino acid codes), which was synthesized and 95% purified by high-performance liquid chromatography (HPLC; New England Peptide, Fitchburg, MA). Tetramer loaded with HIV Gag 77-85 peptide (SLYNVTAVL) was used as a negative control. PBMCs from HLA-A*0201 subjects were washed and incubated with PE-conjugated HLA-A*0201 tetramer, antihuman CD4-FITC (Caltag Laboratories), and antihuman CD8-Tricolor mAbs (Caltag Laboratories) for 30 minutes at 4°C, and then analyzed by flow cytometry. The frequencies of HTLV-1 Tax-specific CD8+ T cells were expressed as a percentage of total CD8+ T cells.

Statistical analysis
The Mann-Whitney U test was used to compare the data between patients with HAM/TSP and ACs. Simple regression analysis was used to test the correlation between HTLV-1 proviral DNA load, HTLV-1 tax mRNA load, mRNA/DNA ratio, and HTLV-1 Tax-specific CD8+ T-cell frequency. To test the correlation of EDSS with HTLV-1 proviral DNA load, HTLV-1 tax mRNA load and mRNA/DNA ratio, the Spearman rank correlation was used.

Results
Accuracy of real-time RT-PCR
To determine the interassay coefficient of variance of this real-time HTLV-1 tax RT-PCR system, cDNA from the HTLV-1-infected MT-2 cell was diluted serially with cDNA from the HTLV-1–noninfected Jurkat cell, and HTLV-1 tax mRNA load of each dilution was measured 5 times (Figure 1A). In this study, the HTLV-1 tax mRNA load was calculated as the value of HTLV-1 tax mRNA divided by the value of HPRT as described in “Patients, materials, and methods.” To estimate the accuracy of this system, we calculated the intercoefficient of variance (CV%) in each amount. The mean interassay CV% was 19.3%, which is consistent with the CV% measuring HTLV-1 proviral DNA using real-time PCR methods.10,29

To test the intra-assay coefficient of variance of this real-time
HTLV-1 tax RT-PCR system, PBMCs from 3 patients with HAM/TSP were separated into 5 samples per individual. RNA was extracted from each sample, cDNA was synthesized, and HTLV-1 tax mRNA load for each sample was determined (Figure 1B). The CV% was calculated for each patient. The mean value of intra-assay CV% was 19.2%, which is consistent with the CV% measuring HIV-1 RNA.30 These data demonstrate that this real-time HTLV-1 tax RT-PCR assay is accurate and reliable for quantitation of HTLV-1 tax mRNA.

Sensitivity and specificity of real-time RT-PCR

To determine the sensitivity of this real-time RT-PCR assay, the HTLV-1 tax mRNA load was determined from MT-2 cells diluted serially with PBMCs from a healthy donor not infected with HTLV-1. As shown in Figure 2A, the HTLV-1 tax mRNA signal could be detected in a dose-dependent manner with a sensitivity limit as low as one MT-2 cell in 106 PBMCs.

To test the specificity of this assay, cDNA from HTLV-1–noninfected Jurkat cells and PBMCs from HTLV-1–noninfected HDs (n = 5) were analyzed. No signal was observed from any of these HTLV-1– cells (Figure 2B). MT-2 cDNA without RT also showed no signal. Two HTLV-1–infected cell lines were tested for expression of HTLV-1 tax mRNA by this system (Figure 2B). The HTLV-1 tax mRNA was detected in these 2 cell lines, with Hut 102 cells expressing 20 times more HTLV-1 tax mRNA than MT-2 cells. This difference is consistent with previous reports demonstrating that Hut 102 cells have 10 to 17 HTLV-1 proviral copies per cell.29,31-33

HTLV-1 tax mRNA load in HAM/TSP patients and ACs

Using this novel method, HTLV-1 tax mRNA load was analyzed in PBMCs from HAM/TSP patients and ACs. As demonstrated in Table 1 and Figure 3, the HTLV-1 tax mRNA load ranged from 0.06 to 187.90 in HAM/TSP patients and 0.00 to 5.58 in ACs. The HTLV-1 tax mRNA load in HAM/TSP patients was significantly higher than that in ACs (P = .0014, Mann Whitney U test) as reported previously.34 The absolute amount of HTLV-1 tax mRNA before adjusting by the HPRT value in HTLV-1–infected individuals was 103 to 106 times less than that in MT-2 cells, which is consistent with previous reports.18,34

Comparison of HTLV-1 tax mRNA load with HTLV-1 proviral DNA load ex vivo

To determine if there was a correlation between the HTLV-1 proviral DNA load and the HTLV-1 tax mRNA load in HTLV-1–infected individuals, a real-time quantitative HTLV-1 DNA PCR assay21 was used to measure the HTLV-1 proviral DNA load in PBMCs from HAM/TSP patients and ACs. As previously reported,10,21 the HTLV-1 proviral DNA load, which represented a population of infected cells in PBMCs, was greater in HAM/TSP patients than ACs (P = .0003, Mann Whitney U test, Table 1). Furthermore, the HTLV-1 proviral DNA load was correlated significantly with the HTLV-1 tax mRNA load in HAM/TSP patients (P < .0001, r2 = 0.812, simple regression analysis, Figure 4).

To adjust the HTLV-1 tax mRNA expression level in HTLV-1–infected PBMCs, we calculated the mRNA/DNA ratio by dividing the HTLV-1 tax mRNA load by HTLV-1 proviral DNA load. The mean value of the mRNA/DNA ratio was 109.82 for HAM/TSP patients and 45.93 for ACs. The mRNA/DNA ratio of HAM/TSP patients was statistically higher than that of AC (P = .0433, Mann Whitney U test, Table 1).

Comparison of HTLV-1 tax mRNA load with HTLV-1 Tax protein expression in vitro

Because increased levels of mRNA may be associated with increased protein production, it was important to determine if there was a correlation between the HTLV-1 tax mRNA load and the amount of HTLV-1 Tax protein expression. PBMCs from 4
HAM/TSP patients (HAM-1, -11, -14, -15) were cultured for 48 hours in culture medium without mitogenic stimuli. PBMC samples were taken after 0, 12, 24, and 48 hours, respectively, and the HTLV-1 tax mRNA load and HTLV-1 Tax protein expression level as measured by FACS analysis using monoclonal anti-Tax antibodies were determined. As shown in Figure 5, a close correlation was found between the HTLV-1 tax mRNA load and the percentage of cells expressing HTLV-1 Tax protein. The detection of HTLV-1 tax mRNA and the expression of HTLV-1 Tax protein were coincident and peaked at approximately 12 to 24 hours of in vitro culture (Figure 5).

Comparison of HTLV-1 tax mRNA load with HTLV-1 Tax-specific CD8+ T-cell frequency ex vivo

The high levels of HTLV-1 proviral DNA load detected in PBMCs from HAM/TSP patients have been shown previously to correlate with high frequencies of virus-specific CD8+ T cells.20,21 It was therefore of interest to determine if HTLV-1 tax mRNA load also correlated with the frequency of HTLV-1 Tax-specific CD8+ T cells. The frequency of HTLV-1 Tax 11-19/HLA-A*0201-specific CD8+ T cells was analyzed using a PE-conjugated HLA-A*0201 tetramer for HLA-A*0201 subjects (HAM-2, -5, -11, -13, -16 and AC-4, -6, -7). The percentages of HTLV-1 Tax 11-19/HLA-A*0201-specific CD8+ T cells in the total amount of CD8+ T cells are summarized in Table 1. As reported previously,20,21 the HTLV-1 proviral DNA load significantly correlated with the frequency of HTLV-1 Tax-specific CD8+ T cells in HAM/TSP patients (P = .0191, r2 = 0.699, simple regression analysis, Table 1). Furthermore, the HTLV-1 tax mRNA load was also significantly correlated with the frequency of HTLV-1 Tax-specific CD8+ T cells in HAM/TSP patients (P = .0013, r2 = 0.893, simple regression analysis; Table 1 and Figure 6). HTLV-1 Tax 11-19/HLA-A*0201-specific CD8+ T cells have also been reported in PBMCs of ACs35 and although in this study the sample size of HLA-A*0201+ ACs was too small to compare statistically, increased mRNA load in this group was associated with a higher frequency of HTLV-1 Tax-specific CD8+ T cells (Table 1).

Comparison of disease severity with HTLV-1 proviral DNA load, HTLV-1 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 1. Because HAM/TSP is a chronic progressive disease, patients with a long duration of illness are usually severely affected.36 However, there were 3 patients (HAM-3, -4, -5) who showed relatively mild progression and had a long duration of illness as reflected by relatively low scores on the EDSS (Table 1). The EDSS index is a neurologic measure of disability that is weighted toward ambulation.25 Of interest is the observation that the HTLV-1 tax mRNA loads of all 3 of these patients were low, even though 2 of the 3 patients (HAM-3, -4) harbored average levels of HTLV-1 proviral DNA load. Furthermore, patients with high HTLV-1 tax mRNA load and a long duration of illness (HAM-11, -13, -15, -16) had more severe disease with EDSS scores above 7 (patients essentially restricted to a wheelchair).

To examine the possibility that quantitative real-time PCR for HTLV-1 DNA and RNA determinations might have prognostic value in HAM/TSP disease progression, the EDSS values of these patients were statistically compared with HTLV-1 proviral DNA load, HTLV-1 tax mRNA load, and mRNA/DNA ratio by Spearman rank correlation analysis (Table 1). The EDSS score significantly correlated with both HTLV-1 tax mRNA load and mRNA/DNA ratio (P = .0109 and .0089, respectively), but not with HTLV-1 proviral DNA load (P = .5236). Thus, HTLV-1 tax mRNA load and mRNA/DNA ratio may have relative prognostic value for the assessment of disease progression in HAM/TSP.

Discussion

Using a newly established real-time RT-PCR assay to quantitate HTLV-1 tax mRNA load, this study demonstrated a significant difference in the HTLV-1 tax mRNA load between patients with HAM/TSP and ACs. Furthermore, HTLV-1 tax mRNA load significantly correlated with the HTLV-1 proviral DNA load, the HTLV-1 Tax protein expression, and the frequency of HTLV-1 Tax 11-19/HLA-A*0201-specific CD8+ T cells. Collectively, these results support previous studies demonstrating a relationship between the HTLV-1 proviral DNA load and the frequency of HTLV-1 Tax-specific CD8+ T cells in HAM/TSP patients,20 the existence of chronically activated HTLV-1 Tax-specific CD8+ T cells,23 and the existence of high anti-HTLV-1 IgM antibodies suggesting continuous immune stimulation in vivo.37 These results indicate that HAM/TSP patients have more HTLV-1 proviral DNA load than ACs, which may be associated with higher mRNA expression resulting in heightened virus-specific immune responses. This profile
These observations support and extend previous reports describing infected cells express HTLV-1 indicate that in HAM/TSP patients a higher proportion of HTLV-1 DNA ratio) was higher in HAM/TSP patients than in ACs. These results between these 2 groups. This study demonstrated that adjusting the tors 16,43 and CD8+ T cells, which have reported to be associated with the development of HTLV-I associated myelopathy/associated myelopathy/ tropical spastic paraparesis among persons infected with HTLV-I. J Acquir Immune Defic Syndr. 1990;3:1096-1101.


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Correlation of human T-cell lymphotropic virus type 1 (HTLV-1) mRNA with proviral DNA load, virus-specific CD8+ T cells, and disease severity in HTLV-1-associated myelopathy (HAM/TSP)

Yoshihisa Yamano, Masahiro Nagai, Meghan Brennan, Carlos A. Mora, Samantha S Soldan, Utano Tomaru, Norihiro Takenouchi, Shuji Izumo, Mitsuhiro Osame and Steven Jacobson