Table 1. List of 17 protein classifiers for categorization of normal donors, asymptomatic carriers, HAM/TSP, and ATL

Accession	Protein name	*P* value (Kruskal-Wallis test)	m/z	RT	Charge	Peptide score	Identity or homology threshold	Sequence
LPPL	Eosinophil lysophospholipase	2.3.E-03	409.722	47.4	2	36.3	27	MVQVWR
CH10	Heat shock 10-kDa protein, mitochondrial	2.5.E-03	430.721	40.6	2	26.2	21	GGIMLPEK
PRG2	Bone marrow proteoglycan	2.4.E-03	528.271	64.6	2	31.6	28	RLPFICSY
MOES	Moesin	8.1.E-04	532.253	26.8	2	46.2	29	EKEELMER
MNDA	Myeloid cell nuclear differentiation antigen	9.4.E-03	647.863	69.1	2	67.3	24	SLLAYDLGLTTK
GLYM	Serine hydroxymethyltransferase, mitochondrial	8.7.E-04	408.551	21.6	3	31.1	18	HADIVTTTTHK
PTMS	Parathymosin	9.7.E-04	453.875	17.8	3	41.2	25	AAEEEDEADPKR
TPIS	Triosephosphate isomerase	9.1.E-03	472.266	71.0	3	54.0	28	QSLGELIGTLNAAK
HSP71	Heat shock 70-kDa protein 1A/1B	9.7.E-03	563.307	65.5	3	93.8	21	IINEPTAAAIAYGLDR
CD6	T-cell differentiation antigen CD6	7.7.E-03	592.306	37.8	3	62.7	22	VLCQSLGCGTAVERPK
ANXA1	Annexin A1	4.4.E-04	612.347	61.5	3	57.0	17	RKGTDVNVFNTILTTR
ANXA6	Annexin A6	2.3.E-03	669.017	70.9	3	54.7	16	AMEGAGTDEKALIEILATR
SPTA2	Spectrin α chain, brain	5.4.E-03	409.718	28.8	2	42.7	30	EAGSVSLR
GLYM	Serine hydroxymethyltransferase, mitochondrial	1.1.E-03	428.240	57.0	2	42.8	27	SGLIFYR
DRB1s	HLA class II histocompatibility antigen, DRB1-1, 4, 10, 11, 13, 15, 16 β chain	1.0.E-02	478.216	25.8	2	55.9	25	AAVDTYCR
CAN2	Calpain-2 catalytic subunit	2.4.E-03	483.253	54.0	2	66.6	29	SDTFINLR
STAT1	Signal transducer and activator of transcription 1-α/β	7.3.E-03	486.290	21.7	2	39.1	29	KILENAQR
PRG2	Bone marrow proteoglycan	9.4.E-04	497.742	49.2	2	31.6	27	FQWVDGSR
CXCL7	Platelet basic protein	1.3.E-03	528.761	43.1	2	51.7	28	ICLDPDAPR

line SO-4 (Figure 5D), but not in the non-ATL leukemia cell line Jurkat (Figure 5E). On the other hand, an additional cell proliferation assay using siRNA against *SPTAN1* revealed that reduction of SPTA2 was not sufficient for the induction of cell death for ATL cells (supplemental Figures 3 and 4).

In addition, 3 proteins (PTMS, CH10, and GLYM) were also found to be upregulated in ATL cells. To address the roles of these proteins, a cell proliferation assay was conducted using 3 ATL cell lines treated with siRNAs against *PTMS*, *HSPE1* (gene symbol of CH10), or *SHMT2* (gene symbol of GLYM) (supplemental Figure 4). As a result, suppression of the *SHMT2* gene induced significant growth inhibition for all 3 ATL cell lines. Although si*HSPE1*-treated KOB cells showed a statistically significant decrease in cell growth rate, si*HSPE1* and si*PTMS* had only partial

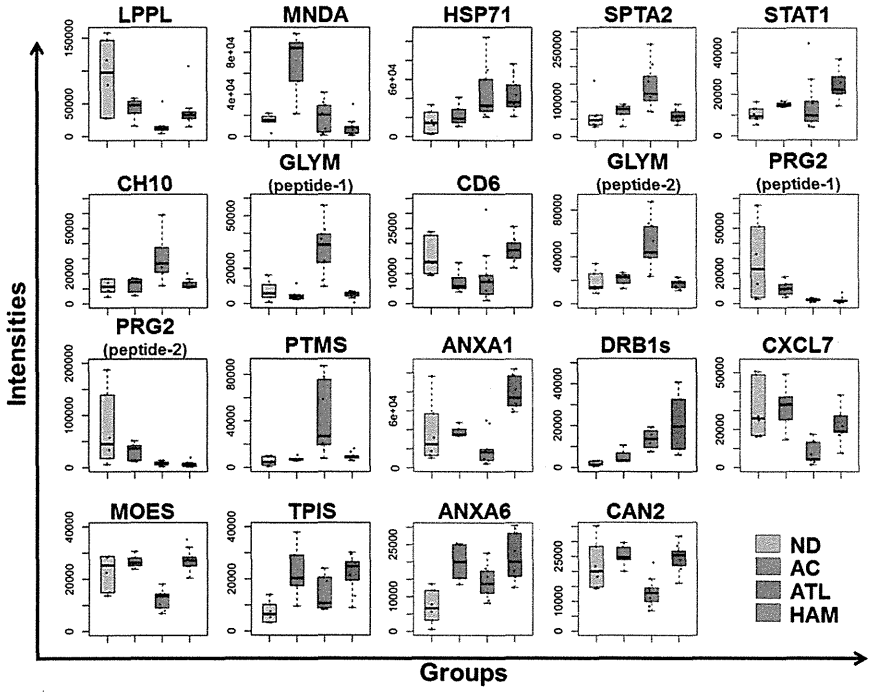

Figure 4. Summary of quantitative features for the 17 protein classifiers identified. The 19 box plots (see Table 1 for protein names) show the results of mass spectrometric quantification and protein identification. We finally identified 19 peptides out of 91 candidates in Figure 3, which were assigned to 17 proteins. Proteins identified from 2 distinct peptides were shown as GLYM (peptides 1 and 2) or PRG2 (peptides 1 and 2). The y-axis indicates normalized relative intensity of peptides in mass spectrometric data. ND, normal donors; AC, asymptomatic carriers.

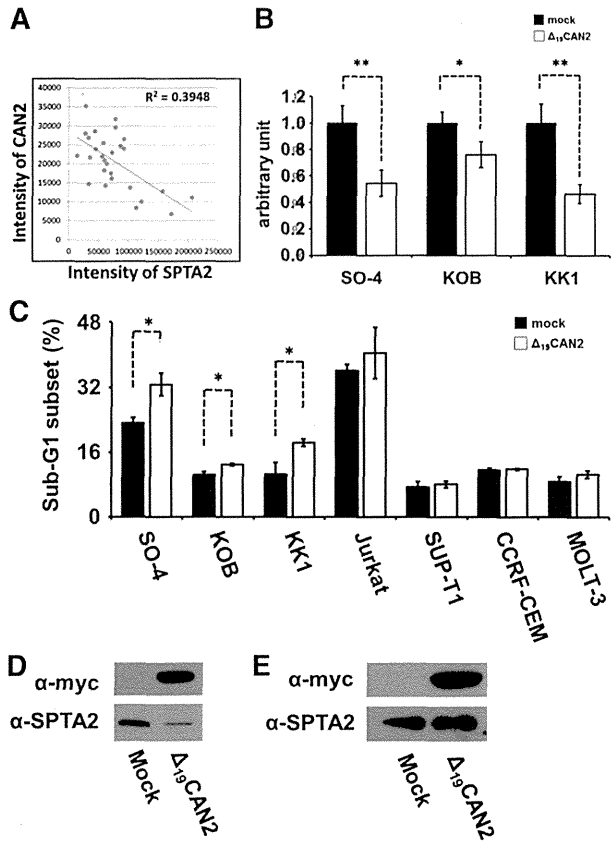

Figure 5. Rescue of CAN2 activity induced cell death in ATL cells. (A) Correlation between CAN2 and SPTA2 expression level in 27 cases. (B) Cell proliferation was measured by MTT assay on SO-4, KOB, and KK1 cells 36 hours after transfection of mock vector or Δ_{19}CAN2. *$P < .05$; **$P < .01$ by Student t test. (C) Overexpression of Δ_{19}CAN2 significantly accelerated cell death in 3 ATL (SO-4, KOB, and KK1) and 4 non-ATL (Jurkat, SUP-T1, CCRF-CEM, and MOLT-3) cell lines. **$P < .05$ by Student t test. The drastic attenuation of SPTA2 expression was observed after transfection of Δ_{19}CAN2 in SO-4 cells (D), but not in Jurkat cells (E). The immunoblot of anti-myc tag confirmed the expression of exogenous Δ_{19}CAN2.

or no effects on proliferation of ATL cell lines. To further confirm whether the overexpression of SPTA2, PTMS, CH10, or GLYM protein would be an ATL-specific molecular signature, the expression levels of these proteins in 8 clinical samples were evaluated by the mass spectrometric quantification technology MRM (supplemental Figures 5 and 6). Expression of SPTA2, GLYM, and CH10 in cells derived from ATL patients was significantly higher than that in cells derived from HAM/TSP patients. The level of PTMS also showed a clearly increasing tendency in the ATL patient group. Taken together, these results suggested that the deprivation of CAN2 activity and upregulation of GLYM in HTLV-1–infected T cells might have a key role at the onset or progression of ATL.

Discussion

In the past decade, proteomics technologies have developed dramatically for the purpose of obtaining more and more comprehensive and sensitive proteome maps in cells or clinical specimens. The performance of mass spectrometers in particular has exhibited remarkable progress; however, as for sensitivity and throughput, it has still been difficult to identify biomarkers from crude samples including body fluids or total cell lysate. A major reason could be that the range of protein concentration in the analyte is indeed much larger than the dynamic range of recent mass spectrometers.[12] The other essential factor to be improved for clinical proteomics is the capacity of the bioinformatics platform to allow analysis of a sufficient number of clinical samples in order to statistically overcome the significant individual variability.[13]

Concerning the first issue, we previously developed and applied various focused proteomic applications targeting molecular biochemical features including glycan structure biomarkers[14-16] and low-molecular-weight peptide biomarkers.[17] The preenrichment of subproteome fractions effectively reduces the complexity of crude samples and allowed us to identify potential serum cancer biomarkers successfully. Through our previous knowledge, we provide an approach for investigating infectious diseases by employing virus-infected cell-focused proteomics. In addition to HTLV-1, for instance, isolation of HIV-infected cells is highly desired because the frequency of these cells in AIDS patients' PBMCs is ~1 out of 10^4 to 10^5 cells.[18] Actually, we successfully demonstrated the effect of HTLV-1–infected cell isolation on the elimination of individual variability (Figure 2, supplemental Figure 2) and reliable identification of disease state–associated proteins (Figures 4 and 5). We further showed the potential of the next-generation bioinformatics platform Expressionist to remove the constraint on the capacity of data size acquired from high-end mass spectrometers. Expressionist covered whole discovery steps from processing of raw mass spectrometer data to statistical analyses (Figures 1 and 3, and supplemental Figure 1) and, importantly, could perform quantification analysis using a basically unlimited number of clinical samples. Hence, in parallel with the development of mass spectrometers, high-specification and inexpensive OMICS server systems are necessary for future diagnostic marker and therapeutic target discoveries using hundreds or thousands of clinical specimens.

In this study, we focused on the $CD4^+CD25^+CCR4^+$ T-cell subpopulation in which T helper 2, T helper 17, and regulatory T (Treg) cells were mainly involved.[10] The purpose for which we used this subset was to technically enrich the preferential viral reservoir cells and to strengthen reliability of screening results. However, investigating proteome behaviors of these subtypes in HTLV-1–associated diseases is also important physiologically because it has been frequently reported that deregulated Treg plays significant roles in pathogenesis of ATL and HAM/TSP. Indeed, aberrant proliferation of Treg cells is considered the main cause of immunodeficiency in ATL patients because of their innate immunosuppressive functions,[19] whereas abnormal production of interferon γ from infected Treg cells might induce chronic spinal inflammation in HAM/TSP patients.[20] Given the list of our 17 classifier proteins, activation of signal transducer and activator of transcription 1-α/β is the well-known key factor for HAM/TSP,[21] whereas upregulation of heat shock 70-kDa protein 1A/1B, CH10, and PTMS were reported in many other types of tumors.[22-24] The association of these 4 proteins with the etiology of HAM/TSP and ATL would be evident according to the previous work, supporting that our other candidates might similarly have a direct impact on the transformation of Treg cells after infection of HTLV-1. Particularly, the specific upregulation of GLYM in ATL cells represents the first evidence that excessive folate metabolism might be essential for the progression or survival of ATL cells because GLYM is a fundamental enzyme catalyzing the supply of glycine accompanying the conversion of tetrahydrofolate to 5,10-methylenetetrahydrofolate.[25] Indeed, the suppression of GLYM expression, which was confirmed to be upregulated in ATL patients, resulted in significant reduction of cell growth. This observation suggests that diminishing GLYM

expression or enzyme activity could be a promising strategy for molecular-targeting treatment of ATL. Together with the downregulation of CAN2 in the ATL cells shown in Figure 5, the proteins listed in Table 1 could provide the molecular basis for not only interpretation of physiological mechanisms in ATL or HAM/TSP but also development of novel therapeutic agents for HTLV-1–associated diseases.

CAN2 belongs to a Ca^{2+}-regulated cytosolic cysteine protease family, which includes 14 calpain isoforms.[26] The enzymatic activity of calpain is implicated in diverse physiological processes, such as cytoskeletal remodeling, cellular signaling, and apoptosis.[26] As an example of a spectrin-mediated apoptosis pathway, it was reported that CAN2 produced SPTA2 breakdown products following traumatic brain injury.[27] Because SPTA2 interacts with calmodulin and constructs the membrane cytoskeletons, its breakdown is considered a process of membrane structural changes during cell death.[28,29] This fact is concordant with our finding in ATL, suggesting that accumulation of SPTA2 in ATL cells can be attributed to the suppression of CAN2 expression and contribute to circumvent apoptosis. In the analysis of basal levels of CAN2 and SPTA2 in 7 cell lines (supplemental Figure 7), 3 ATL cell lines showed endogenous expression of CAN2 and moderate levels of SPTA2. On the other hand, 4 non-ATL leukemia cells demonstrated very high expression of SPTA2 and undetectable levels of CAN2. Although we found the downregulation of CAN2 and accumulation of SPTA2 in ATL cells, this tendency might be more distinctive in HTLV-1 (–) leukemia cells. Taken together, even though the expression level of CAN2 was indeed suppressed in ATL cells, the CAN2-SPTA2 apoptotic pathway itself might remain normal. In contrast, this pathway was considered to be impaired at multiple stages in HTLV-1 (–) leukemia cells because CAN2 expression was completely diminished (supplemental Figure 7) and overexpression of CAN2 could not reactivate the CAN2-SPTA2 apoptotic pathway (Figure 5B-E). In these cells, not only genetic downregulation of CAN2 but also inhibition of CAN2 enzymatic activity might be involved in the carcinogenesis.

In conclusion, comprehensive proteomic profiling of HTLV-1–infected T cells provided 17 disease-associated signature proteins, which have great potential for future clinical use as diagnostic biomarkers. As we described regarding the relationship between the CAN2-SPTA2 pathway and ATL phenotypes, further individual functional analyses will contribute to understanding the detailed molecular mechanisms involved in the onset or progression of HAM/TSP and ATL.

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Authorship

Contribution: M.I. and K.U. designed the study, performed experiments, analyzed results, and wrote the manuscript; A.T. and N.S. performed experiments; N.A., T.S., A.U., and Y.Y. collected the clinical samples and performed flow cytometric experiments; Y.N. and H.N. revised the manuscript; and all authors discussed the results and commented on the manuscript.

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PLOS | NEGLECTED TROPICAL DISEASES

CSF CXCL10, CXCL9, and Neopterin as Candidate Prognostic Biomarkers for HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis

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Abstract

Background: Human T-lymphotropic virus type 1 (HTLV-1) -associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a rare chronic neuroinflammatory disease. Since the disease course of HAM/TSP varies among patients, there is a dire need for biomarkers capable of predicting the rate of disease progression. However, there have been no studies to date that have compared the prognostic values of multiple potential biomarkers for HAM/TSP.

Methodology/Principal Findings: Peripheral blood and cerebrospinal fluid (CSF) samples from HAM/TSP patients and HTLV-1-infected control subjects were obtained and tested retrospectively for several potential biomarkers, including chemokines and other cytokines, and nine optimal candidates were selected based on receiver operating characteristic (ROC) analysis. Next, we evaluated the relationship between these candidates and the rate of disease progression in HAM/TSP patients, beginning with a first cohort of 30 patients (Training Set) and proceeding to a second cohort of 23 patients (Test Set). We defined "deteriorating HAM/TSP" as distinctly worsening function (≧3 grades on Osame's Motor Disability Score (OMDS)) over four years and "stable HAM/TSP" as unchanged or only slightly worsened function (1 grade on OMDS) over four years, and we compared the levels of the candidate biomarkers in patients divided into these two groups. The CSF levels of chemokine (C-X-C motif) ligand 10 (CXCL10), CXCL9, and neopterin were well-correlated with disease progression, better even than HTLV-1 proviral load in PBMCs. Importantly, these results were validated using the Test Set.

Conclusions/Significance: As the CSF levels of CXCL10, CXCL9, and neopterin were the most strongly correlated with rate of disease progression, they represent the most viable candidates for HAM/TSP prognostic biomarkers. The identification of effective prognostic biomarkers could lead to earlier detection of high-risk patients, more patient-specific treatment options, and more productive clinical trials.

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Introduction

Human T-lymphotropic virus type 1 (HTLV-1) is a human retrovirus associated with persistent infection of T-cells [1]. While the majority of HTLV-1-infected individuals remain asymptomatic, approximately 2.5–5% develop an aggressive T-cell malignancy, termed adult T-cell leukemia (ATL) [2,3] and 0.3–3.8% develop a serious chronic neuroinflammatory disease, termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [4–6]. Aside from Japan, endemic areas for this virus and the associated disorders are mostly located in developing countries in the Caribbean, South America, Africa, the Middle East, and Melanesia [7,8], which may explain why these conditions have remained ill-defined and virtually untreatable for so long [9].

HAM/TSP is characterized by unremitting myelopathic symptoms such as spastic paraparesis, lower limb sensory disturbance, and bladder/bowel dysfunction [10,11]. Although

Author Summary

HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a rare neurodegenerative disease caused by infection with human T-lymphotropic virus type 1 (HTLV-1). HTLV-1 infects 10–20 million people worldwide, and, depending on the region, 0.25–3.8% of infected individuals develop HAM/TSP. As the disease progresses, chronic inflammation damages the spinal cord and lower limb and bladder function gradually decline. In the worst cases, even middle-aged patients can become perpetually bedridden. Today, there are treatments that may alleviate the symptoms to a certain degree, but there is no cure that can halt disease progression, and there are no known biomarkers to indicate the level and speed of disease progression. In this study, we successfully identified three promising candidate biomarkers. We believe that the use of these biomarkers could lead to more accurate prognoses and more prudent, patient-specific treatment plans. We not only hope that these biomarkers are sensitive enough to use as selection criteria for clinical trials, but also that measurements of these biomarkers can be used to accurately evaluate drug effectiveness. In short, the biomarkers we identified have the potential to help more effectively treat current HAM/TSP patients and to pave the way for new drugs to potentially cure future HAM/TSP patients.

the symptoms of HAM/TSP have been well documented for quite some time, the rate at which these symptoms progress has only recently become a point of interest. The clinical course of HAM/TSP has classically been described very simply as insidious onset and continuous progression [12], but recent reports have hinted at a more complex, heterogeneous pool of patients with differing clinical needs. Recent studies have shown that although HAM/TSP usually progresses slowly and without remission as per the classical description, there is a subgroup of patients whose conditions decline unusually quickly and who may be unable to walk within two years of onset and another subgroup whose conditions decline unusually slowly and who may only display very mild symptoms [13–15]. It is only logical that these patients should receive treatments tailored to suit their individual needs rather than identically aggressive treatments. Unfortunately, clinicians are currently only able to distinguish between these different groups by observing the way a patient's disease progresses over time, usually years; clinicians often decide to treat the patients immediately and identically rather than wait and allow the disease to progress further. Therein lies the dire need for biomarkers with the power to forecast the rate and extent of disease progression and enable clinicians to make more accurate prognoses and prescribe the most appropriate and effective treatments in a timely manner.

Several candidate prognostic biomarkers with elevated levels in HAM/TSP patients have already been identified in the peripheral blood and cerebrospinal fluid (CSF). In the peripheral blood, such candidates include the HTLV-1 proviral load in peripheral blood mononuclear cells (PBMCs) and serum levels of the soluble IL-2 receptor (sIL-2R) [16,17]. The level of neopterin in the CSF has been reported to be a useful parameter for detecting cell-mediated immune responses in the spinal cord of HAM/TSP patients and the CSF anti-HTLV-1 antibody titer has been shown to be associated both with CSF neopterin levels and the severity of clinical symptoms [18–20]. In addition, several cytokines have been detected in the CSF and/or spinal cord of HAM/TSP patients, including interleukin (IL)-1β, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, and tumor necrosis factor (TNF)-α [21–24]. Some chemokines, such as chemokine (C-X-C motif) ligand (CXCL) 9, CXCL10, and chemokine (C-C motif) ligand (CCL) 5, have been shown to be substantially elevated in both the blood and the CSF with respect to asymptomatic carriers (ACs) or patients with other neurological diseases such as multiple sclerosis [25–28]. This is the first study to compare the adequacies of several of these candidate biomarkers for forecasting the rate of disease progression.

We hypothesized the existence of biomarkers capable of differentiating stable and deteriorating HAM/TSP patients. In this retrospective study, a preliminary experiment was first conducted to select the most promising candidate biomarkers by comparing blood and CSF levels in HAM/TSP patients and control subjects (Figure S1). Four candidate blood markers (sIL-2R, CXCL9, CXCL10, and proviral load) and five candidate CSF markers (CXCL9, CXCL10, neopterin, cell count, and anti-HTLV-1 antibody titer) were selected. To evaluate the relative effectiveness of these candidate biomarkers for predicting rate of disease progression, a classification system was created and HAM/TSP patients were designated as either deteriorating or relatively stable. The levels of candidate biomarkers were then compared between the two patient groups. In the current study, we identified three viable candidates for HAM/TSP prognostic biomarkers that could lead to more accurate prognoses and more prudent, patient-specific treatment plans.

Materials and Methods

Ethical considerations

The study was designed and conducted in accordance with the tenets of the Declaration of Helsinki. The protocol in this study was approved by the Ethics Review Committee of St. Marianna University School of Medicine (No. 1646). Prior to the collection of blood or CSF samples, all subjects gave written informed consent permitting the analysis of their samples for research purposes as part of their clinical care.

Subjects

Between April 2007 and February 2013, we enrolled 53 HAM/TSP patients according to the inclusion and exclusion criteria shown in Table 1, and divided them into two cohorts based on the chronological order of their doctor's visits: a 30-patient Training set and a 23-patient Test set. Demographics and clinical characteristics of the Training set and Test set are shown in Table 2 and Table 3, respectively. Between April 2007 and December 2009, we enrolled 22 HTLV-1-infected ACs as control subjects for blood analysis and eight HTLV-1-infected subjects (seven ACs, one patient with smoldering ATL) as control subjects for CSF analysis according to the inclusion and exclusion criteria shown in Table 1. These two groups were not mutually exclusive; some ACs donated both blood and CSF to this study. Demographics of control subjects as compared to the HAM/TSP patients are shown in Table S1.

Sample preparation

Blood and/or CSF samples were obtained within a one-hour window for each subject. Peripheral blood samples were collected in heparin-containing blood collection tubes and serum-separating tubes. Plasma and PBMCs were obtained from the former tubes and serum was obtained from the latter. PBMCs were isolated with standard procedures using Pancoll® density gradient centrifugation (density 1.077 g/mL; PAN-Biotech GmbH, Aidenbach, Germany). Plasma and serum samples were stored at −80°C until

Table 1. Inclusion and exclusion criteria for this study.

	HAM/TSP	Control for Blood	Control for CSF
Inclusion Criteria	Willing and able to give informed consent		
	HTLV-1 seropositive individuals conformed by CLEIA and Western blot		
	Diagnosed with HAM/TSP as defined by WHO criteria		Choose to provide CSF for the purposes of differential diagnosis
Exclusion Criteria	History of treatment with corticosteroids or other immunomodulating drugs (interferon, cyclosporin, methotrexate, etc.)		
	Diagnosed with an autoimmune disease or other chronic inflammatory disorder aside from HAM/TSP		
	Diagnosed with additional disease affecting gait disturbance (e.g. parkinsonism, rheumatoid arthritis, cervical spondylosis, brain infarction, etc.)		
	History of severe urinary infection, decubitus scars, pneumonia, deep venous thrombosis, or other condition potentially affecting disease course within the last four years	Diagnosed with HAM/TSP as defined by WHO criteria	
	Diagnosed with adult T-cell leukemia (ATL)		

CLEIA = chemiluminescent enzyme immunoassay.
doi:10.1371/journal.pntd.0002479.t001

use. CSF was collected in polypropylene tubes. A small amount of CSF was used for routine laboratory tests, which included total protein, cell count, and IgG level. The remaining CSF was aliquoted into cryotubes and stored at −80°C until undergoing further analysis. All tests in this study were performed on samples from these frozen stocks.

Measurement of blood candidate markers

The serum concentration of sIL-2R was determined using an ELISA (Cell Free N IL-2R; Kyowa Medex Ltd., Tokyo, Japan). HTLV-1 proviral load was measured using real-time PCR, following DNA extraction from PBMCs, as previously described [29–31]. Plasma levels of IL-1β, TNF-α, and IFN-γ were measured using a cytometric bead array (CBA) (BD Biosciences, Franklin Lakes, NJ USA), which was used according to the manufacturer's instructions. Plasma concentrations of CXCL9, CXCL10, CXCL11, and CCL5 were also measured using a CBA (BD Biosciences).

Measurement of CSF candidate markers

CSF cell count was determined using the Fuchs–Rosenthal chamber (Hausser Scientific Company, Horsham PA USA). Total protein and IgG levels in the CSF were measured using a pyrogallol red assay and a turbidimetric immunoassay, respectively. The anti-HTLV-1 antibody titer was determined using the gelatin particle agglutination test (Serodia-HTLV-1; Fujirebio, Tokyo, Japan). CSF concentration of sIL-2R was determined using an ELISA (Cell Free N IL-2R; Kyowa Medex). CSF neopterin level was measured using high-performance liquid chromatography. IFN-γ and six chemokines (CXCL9, CXCL10, CXCL11, CCL3, CCL4, and CCL5) were measured using a CBA (BD Biosciences). The CSF concentrations of three chemokines (CCL17, CCL20, and CCL22) and IL-17A were measured using commercially available ELISA kits (CCL17, CCL20, and CCL22: TECHNE/R&D Systems, Minneapolis, MN USA; IL-17A: Gen-Probe, San Diego, CA USA). All assays were conducted according to the respective manufacturers' instructions.

Table 2. Demographics and clinical characteristics of HAM/TSP patients (Training Set).

	Total	Stable HAM/TSP	Deteriorating HAM/TSP	
	n = 30	n = 14	n = 11	*p*-value*
Demographics				
Age, y**	58 [37–75]	54.5 [39–75]	62 [53–72]	0.0183†
Female sex	80.0%	64.3%	90.9%	0.1696‡
Clinical characteristics				
Age of onset, y**	48 [20–70]	33 [20–58]	57 [40–70]	0.0021†
Disease duration, y**	12.5 [1–33]	19 [7–33]	9 [1–13]	0.0021†
OMDS**	6 [2–11]	5 [2–9]	8 [5–11]	0.0065†

In the Training set, deteriorating patients were significantly older, experienced disease onset later in life, had been living with the disease for shorter periods, and were more severely disabled (OMDS).
*Stable HAM/TSP vs Deteriorating HAM/TSP.
**Data are expressed as median [range].
†By Mann-Whitney test.
‡By Fisher's exact test.
OMDS = Osame's Motor Disability Score.
doi:10.1371/journal.pntd.0002479.t002

Table 3. Demographics and clinical characteristics of HAM/TSP patients (Test Set).

	Total	Stable HAM/TSP	Deteriorating HAM/TSP	
	n = 23	n = 11	n = 9	*p*-value*
Demographics				
Age, y**	58 [22–75]	61 [22–75]	59 [48–68]	0.8491†
Female sex	78.3%	81.8%	77.8%	1.000‡
Clinical characteristics				
Age of onset, y**	43 [12–70]	40 [14–70]	51 [39–63]	0.0184†
Disease duration, y**	9 [2–41]	19 [5–41]	6 [2–14]	0.0148†
OMDS**	5 [2–8]	5 [4–8]	5 [4–8]	0.4526†

In the Test set, deteriorating patients experienced disease onset later in life and had been living with the disease for shorter periods, but there were no significant differences in current age or OMDS.
*Stable HAM/TSP vs Deteriorating HAM/TSP.
**Data are expressed as median [range].
†By Mann-Whitney test.
‡By Fisher's exact test.
OMDS = Osame's Motor Disability Score.
doi:10.1371/journal.pntd.0002479.t003

Classification system based on the natural history of HAM/TSP

The 53 total HAM/TSP patients without any history of HAM/TSP-targeting treatments were interviewed using a questionnaire (Figure S2) to determine the changes in Osame's Motor Disability Score (OMDS) over time (Figure S3). OMDS is a standardized neurological rating scale as a measure of disability [10] (Figure S1). Based on the changes in OMDS, "deteriorating cases" and "stable cases" were identified in both the Training set and Test set patient cohorts. Patients with deteriorating HAM/TSP were defined as those whose OMDS worsened ≥3 grades over four years and patients with stable HAM/TSP were defined as those whose OMDS remained unchanged or worsened 1 grade over four years. Patients whose OMDS worsened 2 grades over four years were excluded from the patient cohort in order to create a larger gap between the deteriorating and stable patient groups.

Statistical analysis

GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA USA) was used to plot graphs and perform statistical analyses. Differences between the two subject groups were tested using the Mann-Whitney U-test. Receiver operating characteristic (ROC) analysis was performed to examine the sensitivity and specificity of individual biomarkers. For the ROC analyses, an area under the ROC curve (AUC) of 1.0 was used to represent a perfect test with 100% sensitivity and 100% specificity, whereas an area of 0.5 was used to represent random discrimination. Spearman's rank correlation test was employed to investigate the correlation between the four CSF markers (CXCL10, CXCL9, neopterin, and cell count) and the proviral load in PBMCs. To compare the four CSF markers between three groups (HTLV-1-infected control, n = 8; stable HAM/TSP, n = 25; and deteriorating HAM/TSP, n = 20), we used the Kruskal–Wallis test followed by Dunn's post-hoc tests. P-values<0.05 were considered statistically significant.

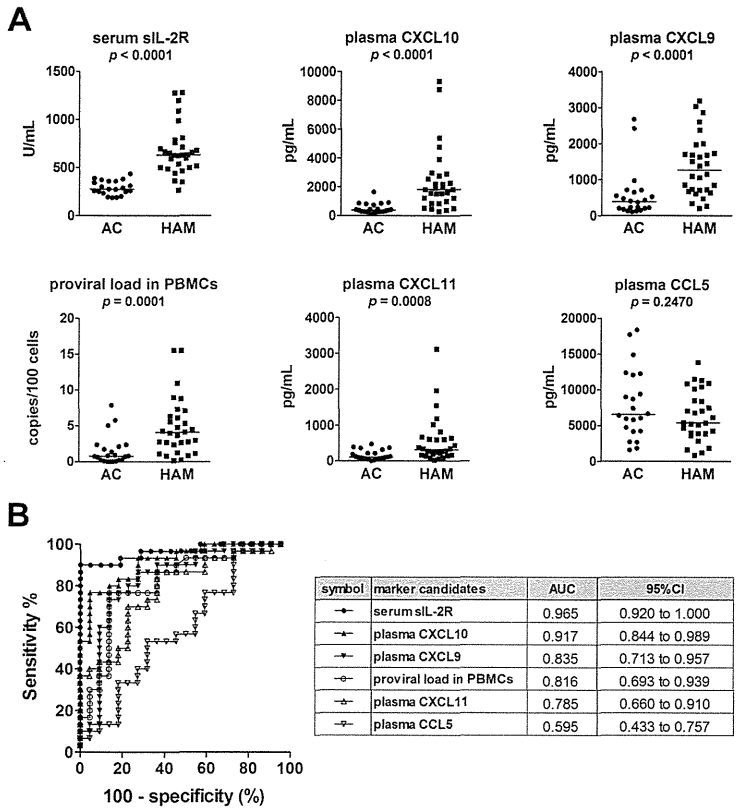
Results

Identification of biomarkers elevated in the blood of HAM/TSP patients

In order to identify candidate blood markers for HAM/TSP, the concentrations of IL-1β, TNF-α, and IFN-γ were measured in plasma samples from four ACs and four HAM/TSP patients. Plasma levels of IL-1β and TNFα were below the detection limits (<2.3 pg/mL and <1.2 pg/mL, respectively) except in one patient with HAM/TSP. Plasma IFN-γ levels showed no significant differences between ACs and HAM/TSP patients (median 10.4 pg/mL and 13.9 pg/mL, respectively). Therefore, these quantities were not measured in additional samples (Figure S1). The proviral DNA load in PBMCs, serum sIL-2R, and plasma levels of the chemokines CXCL9, CXCL10, CXCL11, and CCL5 were also measured in 22 ACs and 30 HAM/TSP patients without any history of immunomodulating treatments, including corticosteroids, IFN-α, and immunosuppressive drugs. The results revealed that serum levels of sIL-2R, plasma levels of CXCL10 and CXCL9, and proviral DNA load in PBMCs were markedly higher in HAM/TSP patients compared to ACs ($p \leq 0.0001$, Figure 1A). These quantities were then compared using ROC analysis to determine which parameters were superior markers for HAM/TSP. From the results of the ROC analysis, we determined that serum sIL-2R and plasma CXCL10 had the highest potential for distinguishing HAM/TSP patients from ACs with high sensitivity and specificity (area under the ROC curve [AUC]>0.9), followed by plasma CXCL9 and HTLV-1 proviral load in PBMCs (0.8<AUC<0.9) (Figure 1B). Thus, four candidate blood biomarkers were selected for further investigation: serum sIL-2R, plasma CXCL10, plasma CXCL9, and HTLV-1 proviral load in PBMCs.

Identification of biomarkers elevated in the CSF of HAM/TSP patients

In order to identify candidate CSF markers for HAM/TSP, elevated levels of various potential markers were screened for in CSF samples from HAM/TSP patients. CSF IL-17A was detectable (>3.0 pg/mL) in only one of eight HAM/TSP patients screened (including six deteriorating-type patients), and the level in this one patient (deteriorating-type) was negligible (4.0 pg/mL). CSF IFN-γ was detectable (>1.8 pg/mL) in only 3 of 10 HAM/TSP patients screened (six deteriorating patients), and the levels in all three were negligible (range 3.3–4.2 pg/mL). Therefore, these cytokines were not measured in additional patients. Total protein, cell count, IgG, neopterin, sIL-2R, and nine chemokines (CXCR3 ligands: CXCL9, CXCL10, and CXCL11; CCR5 ligands: CCL3, CCL4, and CCL5; CCR4 ligands: CCL17 and CCL22; CCR6



symbol	marker candidates	AUC	95%CI
	serum sIL-2R	0.965	0.920 to 1.000
	plasma CXCL10	0.917	0.844 to 0.989
	plasma CXCL9	0.835	0.713 to 0.957
	proviral load in PBMCs	0.816	0.693 to 0.939
	plasma CXCL11	0.785	0.660 to 0.910
	plasma CCL5	0.595	0.433 to 0.757

Figure 1. Selection of candidate biomarkers in the blood by comparing HAM/TSP patients and asymptomatic carriers. (**A**) Serum levels of soluble IL-2 receptor (sIL-2R), proviral loads in peripheral blood mononuclear cells (PBMCs), and plasma levels of four chemokines (chemokine (C-X-C motif) ligand (CXCL) 9, CXCL10, CXC11, and chemokine (C-C motif) ligand (CCL) 5) were compared between HAM/TSP patients (HAM; n = 30) and asymptomatic carriers (AC; n = 22). Horizontal bars indicate the median values. The Mann-Whitney *U*-test was used for statistical analysis. (**B**) Receiver operating characteristic (ROC) analysis was employed to assess the sensitivities and specificities of the six markers exhibited in part (A) for discriminating HAM/TSP patients from ACs: greater proximity of the ROC curve to the upper left corner indicates higher sensitivity and specificity of the marker. AUC = area under the ROC curve; 95% CI = 95% confidence interval.
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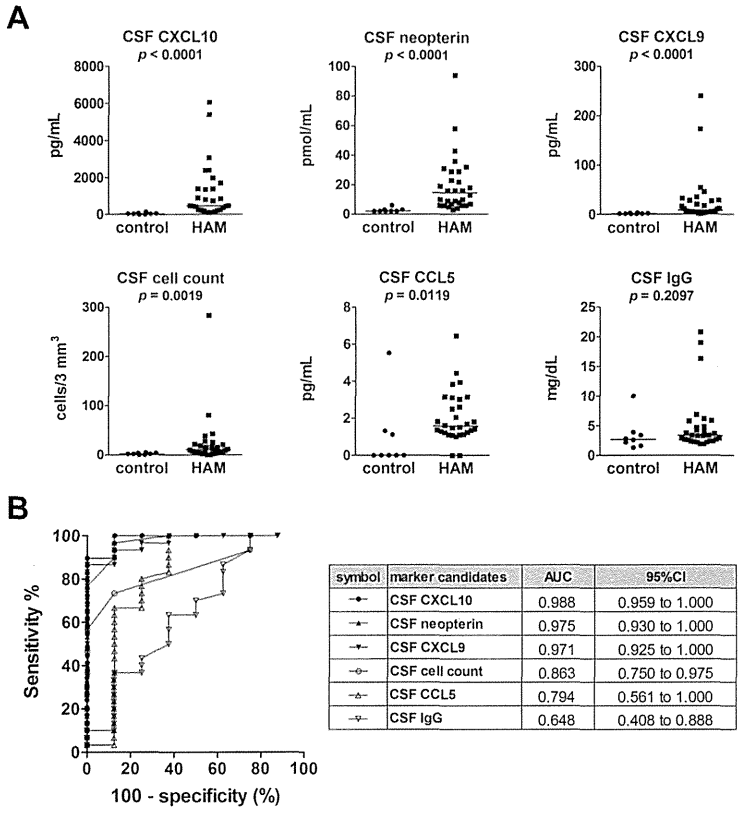
ligand: CCL20) were also measured in the CSF of 30 untreated HAM/TSP patients and in eight HTLV-1-infected control subjects (seven ACs and one patient with smoldering ATL). The results indicated that CSF levels of CXCL10, neopterin, and CXCL9 were remarkably higher in HAM/TSP patients compared to control subjects ($p<0.0001$ overall, Figures 2A and S4) and that CSF levels of cell count and CCL5 were less so but still significantly higher ($p = 0.0019$ and $p = 0.0119$, respectively; Figure 2A). By contrast, there were no differences in the CSF levels of IgG and total protein between HAM/TSP patients and control subjects, and CSF sIL-2R levels were only detectable in a single HAM/TSP patient (data not shown). ROC analysis showed that the CSF levels of CXCL10, neopterin, CXCL9, and CSF cell count could be used to relatively accurately distinguish HAM/TSP patients from control subjects (AUC>0.8) (Figure 2B). Therefore, these four CSF markers were selected as candidates for further investigation. It should be noted that the sensitivity of CSF cell count was very low (36.7%) when compared to the other three: CXCL10 (83.3%), CXCL9 (86.7%), and neopterin (76.7%) (Figure S5).

Identification of biomarkers correlated with rate of HAM/TSP disease progression

In short, we selected nine markers: eight markers chosen based on the analyses described above and CSF anti-HTLV-1 antibody titer, which is a known diagnostic marker for HAM/TSP. To determine which biomarkers were associated with HAM/TSP disease progression, the levels of these nine markers were compared between the deteriorating and stable HAM/TSP patient groups (see Methods for definitions of deteriorating and stable). The results revealed that all five CSF markers were significantly higher in the deteriorating group compared to the stable group (Figure 3A), but that none of the four blood markers, including proviral load, were significantly different between the two groups. The deteriorating group included three patients with particularly rapidly progressive HAM/TSP, defined as those who had been confined to wheelchairs (OMDS: ≥ grade 6) within two years after the onset of symptoms [13,14] (black circles in Figures 3A and S3B). These rapid progressors exhibited high levels of the CSF markers and high proviral loads. ROC analysis revealed that the levels of the CSF markers (CXCL10, CXCL9, neopterin, and cell count), but not anti-HTLV-1 antibody titer, distinguished clearly between patients with deteriorating HAM/TSP and stable HAM/TSP (AUC>0.8, Figure 3B).

Validation of nine candidate biomarkers using the Test Set

To validate the results obtained using the Training Set, the same nine markers were compared between deteriorating and stable patients using the Test Set (a second cohort of 23 HAM/



symbol	marker candidates	AUC	95%CI
	CSF CXCL10	0.988	0.959 to 1.000
	CSF neopterin	0.975	0.930 to 1.000
	CSF CXCL9	0.971	0.925 to 1.000
	CSF cell count	0.863	0.750 to 0.975
	CSF CCL5	0.794	0.561 to 1.000
	CSF IgG	0.648	0.408 to 0.888

Figure 2. Selection of candidate biomarkers in the cerebrospinal fluid (CSF) by comparing HAM/TSP patients and control subjects. (**A**) CSF levels of total protein, cell count, IgG, neopterin, sIL-2R, and nine chemokines (CCL3, CCL4, CCL5, CXCL9, CXCL10, CXC11, CCL17, CCL20, and CCL22) were measured and compared between HAM/TSP patients (HAM; n = 30) and HTLV-1-infected control subjects (control; n = eight: seven ACs and one ATL patient). Data is shown for the top six CSF markers ranked according to the significance of the difference between the HAM/TSP patients and the control subjects. Horizontal bars indicate the median values. The Mann-Whitney *U*-test was used for statistical analysis. (**B**) ROC analysis was employed to assess the sensitivities and specificities of the six markers exhibited in part (A) for discriminating HAM/TSP patients from controls. AUC = area under the ROC curve; 95% CI = 95% confidence interval.
doi:10.1371/journal.pntd.0002479.g002

TSP patients that had not undergone HAM/TSP-targeting treatment). As shown in Figure 4A, the results indicated that the levels of five CSF markers, proviral load in PBMCs, and serum sIL-2R were significantly higher in deteriorating cases than in stable cases. Among them, CSF levels of CXCL10, CXCL9, neopterin, and CSF cell count exhibited particularly high sensitivities and specificities for detecting the deteriorating HAM/TSP cases in the Test set as well as Training set (AUC>0.8, Figures 4B and S1).

Demographic and clinical characteristics of the subjects

The demographics of the HAM/TSP patients versus the control subjects for both the blood tests and CSF analyses were compared and evaluated for statistical significance (Table S1). There were no significant differences in age or gender distribution between the HAM/TSP patients and either control subject group.

Similarly, the demographic and clinical characteristics of stable versus deteriorating HAM/TSP subjects in both the Training and Test sets are shown in Tables 2 and 3, respectively. There were no significant differences in age or gender distribution among either set, but deteriorating patients in both sets were significantly older at disease onset and had been living with the disease for shorter periods of time. Deteriorating patients in the Training set scored higher OMDS values than their stable counterparts ($p<0.01$), but there was no such significant difference in the Test set.

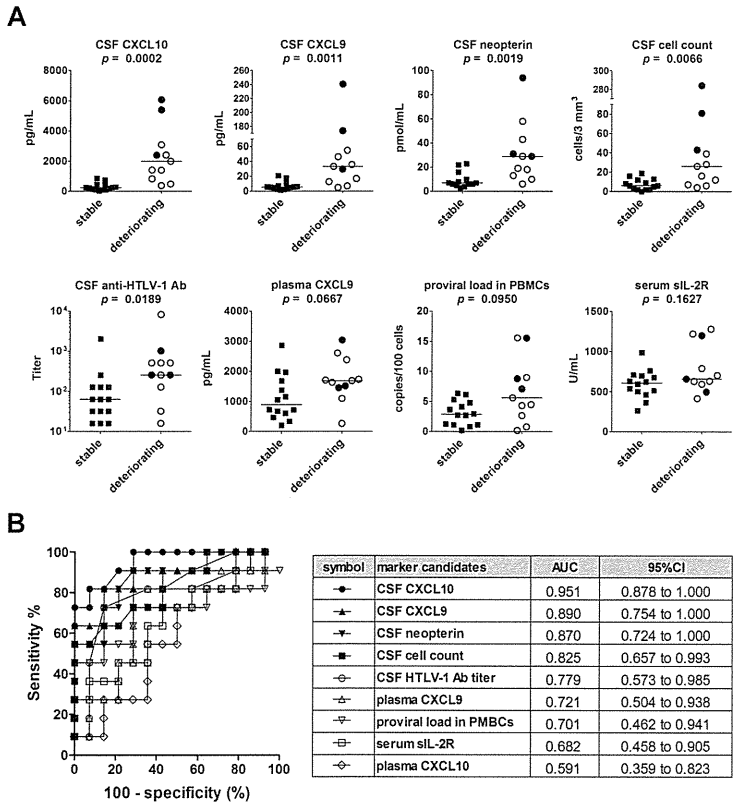
To investigate the potential influence of disease duration as a secondary variable, a new test group was created containing only those patients for whom the disease onset date was 7–13 years prior to the sample collection day. Patients fitting this criterion were selected from the 53 total available from both the Training and Test sets: eight stable patients and ten deteriorating patients; we confirmed that there was no significant difference in disease duration between these two groups. The results remained consistent with our previous findings: CSF CXCL10, CXCL9, and neopterin were all elevated in deteriorating patients with respect to stable patients ($p<0.01$, Figure 5).

Follow-up mini-study on biomarker levels over time

Four stable HAM/TSP patients were left completely untreated and followed for a period of three to five years. Within this time, one patient rose one grade on the OMDS scale, and the other three experienced no change in OMDS grade at all. The levels of CSF CXCL10 and neopterin remained consistently low over time (Figure S6).

Discussion

To date, there have been few well-designed studies that have evaluated the relationship between biomarkers and HAM/TSP disease progression. In a previous retrospective study with 100 untreated HAM/TSP patients, a significant association was



symbol	marker candidates	AUC	95%CI
●	CSF CXCL10	0.951	0.878 to 1.000
▲	CSF CXCL9	0.890	0.754 to 1.000
▼	CSF neopterin	0.870	0.724 to 1.000
■	CSF cell count	0.825	0.657 to 0.993
○	CSF HTLV-1 Ab titer	0.779	0.573 to 0.985
△	plasma CXCL9	0.721	0.504 to 0.938
▽	proviral load in PMBCs	0.701	0.462 to 0.941
□	serum sIL-2R	0.682	0.458 to 0.905
◇	plasma CXCL10	0.591	0.359 to 0.823

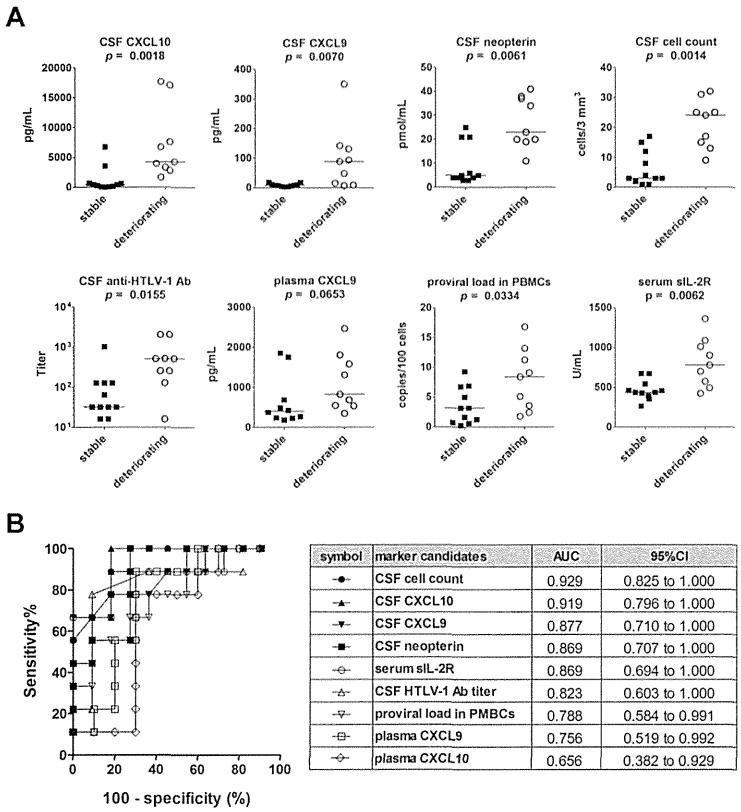
Figure 3. Identification of biomarkers associated with clinical progression of HAM/TSP. (**A**) Five CSF marker candidates (CXCL10, CXCL9, neopterin, cell count, and anti-HTLV-1 antibody titer) and four blood marker candidates (proviral load in PBMCs, serum sIL-2R, plasma CXCL9, and plasma CXCL10) were compared among a cohort of patients called the Training Set (deteriorating HAM/TSP, n = 11; stable HAM/TSP, n = 14). Data is shown for the top eight CSF markers ranked according to the significance of the difference between the deteriorating and stable subjects. Black circles indicate patients with particularly rapidly progressive HAM/TSP. Horizontal bars indicate the median values. The Mann-Whitney *U*-test was used for statistical analysis. (**B**) ROC analysis was employed to assess the sensitivities and specificities of the nine markers listed above for discriminating deteriorating HAM/TSP patients from stable patients. AUC = area under the ROC curve; 95% CI = 95% confidence interval.
doi:10.1371/journal.pntd.0002479.g003

demonstrated to exist between higher HTLV-1 proviral load in PBMCs and poor long-term prognosis; however, the predictive value of high proviral load appeared to be too low to qualify it as a marker for disease progression in clinical practice [32]. Here we conducted a retrospective study to compare for the first time the relationships of PBMC proviral load and several inflammatory biomarker candidates to disease progression in untreated HAM/TSP patients.

In this study, elevated CSF cell count, neopterin concentration, and CSF levels of CXCL9 and CXCL10 were well-correlated with disease progression over the four year period under study, better even than HTLV-1 proviral load in PBMCs (Figures 3 and 4). As CSF pleocytosis, CSF CXCL10, CSF CXCL9, and CSF neopterin are known indicators of inflammation in the central nervous system [33,34], our findings indicate that the rate of HAM/TSP progression is more closely reflected by the amount of inflammatory activity in the spinal cord than by the PBMC proviral load. However, we also found a significant correlation between PBMC proviral load and the levels of the CSF markers identified in this study (Figure S7), indicating that a higher PBMC proviral load does indeed suggest more inflammation in the spinal cord and therefore a poorer long-term prognosis. These findings are consistent with the theory that HAM/TSP is the result of an excess of inflammatory mediators caused by the presence of HTLV-1-infected T-cells [35–37].

The HTLV-1 proviral load in the CSF as well as the ratio of the proviral load in the CSF to that in PBMCs have been reported to be effective for discriminating HAM/TSP patients from ACs or multiple sclerosis patients infected with HTLV-1 [38,39]. Some researchers have suggested that these values might be associated with the rate of disease progression, but there has been only one small cohort study and one case report investigating this point, and so the significance of this experimental evidence is still questionable [40,41]. In addition to statistical validation with multiple, larger cohorts, it would also be beneficial to use precise definitions for progressive versus stable patients, as we have done in this study. Although the volume of CSF available per sample was too limited to measure CSF proviral load in the present study, we plan to incorporate CSF proviral load in a future prospective study and compare its usefulness to that of other biomarker candidates.

From our results, we concluded that of the potential biomarkers under study, CXCL10, CXCL9, and neopterin are the most fit for determining the level of spinal cord inflammation, and thus the most fit for predicting disease progression in HAM/TSP patients. Although the CSF cell count is an easily measurable inflammatory marker, it is not sensitive enough to reliably detect the level of spinal cord inflammation. Numerous patients with CSF cell counts within the normal range exhibited high levels of other inflammatory markers, such as neopterin and CXCL10 (Figure S5). In fact, it has been reported that CSF pleocytosis is present in only approximately 30% of HAM/TSP patients [42]. Furthermore, in



symbol	marker candidates	AUC	95%CI
	CSF cell count	0.929	0.825 to 1.000
	CSF CXCL10	0.919	0.796 to 1.000
	CSF CXCL9	0.877	0.710 to 1.000
	CSF neopterin	0.869	0.707 to 1.000
	serum sIL-2R	0.869	0.694 to 1.000
	CSF HTLV-1 Ab titer	0.823	0.603 to 1.000
	proviral load in PMBCs	0.788	0.584 to 0.991
	plasma CXCL9	0.756	0.519 to 0.992
	plasma CXCL10	0.656	0.382 to 0.929

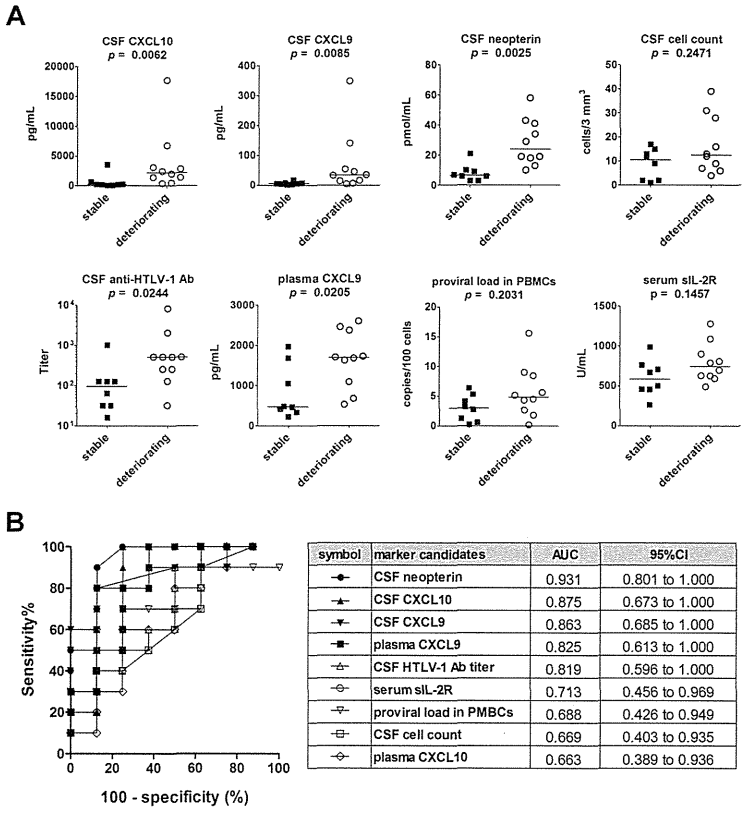
Figure 4. Validation of potential markers using the Test Set. (**A**) Five CSF marker candidates (CXCL10, CXCL9, neopterin, cell count, and anti-HTLV-1 antibody titer) and four blood marker candidates (proviral load in PBMCs, serum sIL-2R, plasma CXCL9, and plasma CXCL10) were compared among a second cohort of patients called the Test Set (deteriorating HAM/TSP, n = 9; stable HAM/TSP, n = 11). Data is shown for the top eight CSF markers ranked according to the significance of the difference between the deteriorating and stable subjects. Horizontal bars indicate the median values. The Mann-Whitney *U*-test was used for statistical analysis. (**B**) ROC analysis was employed to assess the sensitivities and specificities of the nine markers listed above for discriminating deteriorating HAM/TSP patients from stable patients. AUC = area under the ROC curve; 95% CI = 95% confidence interval.
doi:10.1371/journal.pntd.0002479.g004

our study, there was no significant difference in CSF cell count between the control subjects and the stable HAM/TSP patients (Figure S8).

We also explored the possibility of combining multiple biomarkers via multiple logistic regression to form a combination more sensitive and specific than individual markers, but the results indicated that there is not much to be gained from combinations (data not shown).

While there were no significant demographic differences between subject groups, the clinical characteristics of stable versus deteriorating HAM/TSP patients of course differed widely (Tables 2, 3, and S2). We confirmed the already well-reported statistic that deteriorating patients experience HAM/TSP onset relatively late in life [12,14,20] ; our data also reflected the short disease duration expected of deteriorating patients, who by definition progress through the disease more rapidly than their stable counterparts. As patients in all groups were of similar age at sample collection, the significant difference in age of onset should not have any impact on our findings. However, it was necessary to consider the possibility that those patients in a later stage of the disease (i.e. those listed with longer disease durations) might possess elevated or diminished biomarker levels regardless of rate of disease progression. We confirmed that this difference in disease duration was not a confounding factor in our selection of candidate biomarkers by comparing stable and deteriorating HAM/TSP patients with similar disease durations (7–13 years), and we were able to obtain results consistent with our earlier findings (Figure 5). Finally, the OMDS values for the stable and deteriorating patient groups in the Test set were perfectly identical, eliminating the need to consider the possibility that the biomarkers could have been elevated according to disease severity regardless of rate of progression.

The main limitation of our retrospective study is that our samples were collected from patients at the end of the four year period during which the extent of progression was analyzed as opposed to the beginning of the four year period, which would have been optimal for directly measuring their prognostic powers. Of course, the patients with severe HAM/TSP symptoms began undergoing treatment soon after sample collection, rendering any observations on disease course after sample collection un-useable for analysis in this study. While this situation is non-ideal, we hypothesize that biomarker levels in a given patient do not substantially change over a few years' time. We were actually able to monitor the biomarker levels of four untreated HAM/TSP patients over 3–5 years, and the levels remained relatively stable in all four subjects over time (Figure S6), supporting our hypothesis. However, these were all stable HAM/TSP patients (hence the lack of treatment), and so we cannot rule out the possibility that biomarker levels in untreated deteriorating patients may dramatically rise, fall, or fluctuate. The results of the analysis of patients with similar disease durations (Figure 5) also support our hypothesis that disease duration is not an important determinant



symbol	marker candidates	AUC	95%CI
●	CSF neopterin	0.931	0.801 to 1.000
▲	CSF CXCL10	0.875	0.673 to 1.000
▼	CSF CXCL9	0.863	0.685 to 1.000
■	plasma CXCL9	0.825	0.613 to 1.000
△	CSF HTLV-1 Ab titer	0.819	0.596 to 1.000
○	serum sIL-2R	0.713	0.456 to 0.969
▽	proviral load in PMBCs	0.688	0.426 to 0.949
□	CSF cell count	0.669	0.403 to 0.935
◇	plasma CXCL10	0.663	0.389 to 0.936

Figure 5. Comparison of potential markers in stable and deteriorating HAM/TSP patients with similar disease durations. (**A**) Five CSF marker candidates (CXCL10, CXCL9, neopterin, cell count, and anti-HTLV-1 antibody titer) and four blood marker candidates (proviral load in PBMCs, serum sIL-2R, plasma CXCL9, and plasma CXCL10) were compared among all patients from both the Training and Test Sets pooled together with similar disease durations (range: 7–13 years; no significant difference in duration between stable (n = 8) and deteriorating (n = 10) groups). Data is shown for the top eight CSF markers ranked according to the significance of the difference between the deteriorating and stable subjects. Horizontal bars indicate the median values. The Mann-Whitney *U*-test was used for statistical analysis. (**B**) ROC analysis was employed to assess the sensitivities and specificities of the nine markers listed above for discriminating deteriorating HAM/TSP patients from stable patients while controlling for disease duration. AUC = area under the ROC curve; 95% CI = 95% confidence interval.
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of biomarker levels, but it is of course not conclusive. We expect that a prospective study in the future will reveal the answer to this question.

The results of this study indicate that CXCL9 and/or CXCL10 may play a key role in the pathogenesis of HAM/TSP by recruiting more inflammatory cells to the spinal cord lesions. In this study, we measured the levels of the chemokines in the CSF that might play a part in inducing the migration of T-helper (Th) cells. $CD4^+$ Th cells differentiate from naïve T-cells to members of the Th subset (e.g., Th1, Th2, Th17, or Treg cells), and each one expresses its own characteristic chemokine receptors [43]. Usually, Th1 cell express CCR5/CXCR3 receptors, Th2 and Treg cells express CCR4, and Th17 express CCR6. Interestingly, CCR4 ligands (CCL17 and, CCL22) and the CCR6 ligand (CCL20) were not detected in the CSF of HAM/TSP patients. Moreover, of the CCR5 ligands, only CCL5 was elevated, but only slightly, and there was no association with rate of disease progression. Of the CXCR3 ligands, only CXCL9 and CXCL10 were correlated with the rate of disease progression. These results show that the pathology of HAM/TSP is unique among immune disorders in that, unlike other inflammatory disorders such as multiple sclerosis or rheumatoid arthritis that exhibit Th17 as well as Th1 involvement, the chemokine involvement in HAM/TSP is Th1-dominant. In a previous study, cytokines produced by HTLV-1-infected T-cells in HAM/TSP patients were analyzed, and the results showed that IFN-γ was elevated and IL-17 reduced [43,44]. Taken together, the results of these studies indicate that the characteristics of HTLV-1-infected T-cells themselves may be responsible for the Th1-dominant chemokine production observed in HAM/TSP. Also, these results suggest that the CXCR3-ligand (CXCL9 and CXCL10) interactions play an important role in the pathophysiology of HAM/TSP. Recently it was established that these CXCR3-ligand interactions are extremely important for the pathogenesis of several neurological disorders [33]. Therefore, future research on the significance of these interactions in the pathogenic process of HAM/TSP will be important for clarifying the suitability of CXCL9 and CXCL10 as biomarkers or therapeutic targets.

In conclusion, in this retrospective study, we have demonstrated that CSF levels of CXCL10, CXCL9, and neopterin are promising candidate prognostic biomarkers for HAM/TSP. These biomarkers may provide a means for the early identification of patients at increased risk of debilitating disease progression, those that may need anti-inflammatory therapies to limit or prevent this, and for evaluating the efficacy of such therapies. This initial identification of prognostic biomarkers for HAM/TSP should be followed by a future multicenter prospective clinical study.

Supporting Information

Figure S1 Diagram illustrating the biomarker selection process. A total of 26 biomarker candidates including 9 in the blood and 17 in the CSF underwent the following selection processes: 1) pre-screening of the cytokines for presence in HAM/TSP patients, 2) selection for markers elevated in HAM/TSP patients with respect to controls (AUC>0.8), 3) selection for markers elevated in deteriorating HAM/TSP patients with respect to stable patients (AUC>0.8) in a cohort termed the Training Set, 4) validation of the selected markers by evaluating again (AUC>0.8) in a second cohort termed the Test Set. The darkening of an arrow's color represents that marker's failure to meet the selection criteria, and the termination of an arrow indicates that no further testing was conducted for that marker. CYT = cytokine, HTLV-1 PVL = HTLV-1 proviral load, Ab Titer = anti-HTLV-1 antibody titer, AUC = area under the ROC curve.
(TIF)

Figure S2 Questionnaire on the development of motor disability over time as measured using Osame's Motor Disability Score (OMDS). The first and second columns indicate the OMDS numerical value and description, respectively. Doctors interviewed the patients and filled in the table according to the following instructions: in the bottom row, write the ages at which symptoms listed to the left first appeared, and above the age check the box in the row corresponding to the symptom.
(TIF)

Figure S3 Rate of disease progression in HAM/TSP patients without any history of HAM/TSP-targeting treatment. Each line illustrates the change in OMDS over time for an individual patient after disease onset for (**A**) all patients in the Training Set (n = 30) and (**B, left**) only deteriorating patients (n = 11) including three particularly rapidly progressive patients (shown as solid black circles) and (**B, right**) only stable patients (n = 14).
(TIF)

Figure S4 Comparison of CSF levels of nine chemokines in control subjects and HAM/TSP patients. The CSF levels of nine chemokines (CCR5 ligands: CCL3, CCL4, and CCL5; CXCR3 ligands: CXCL9, CXCL10, and CXCL11; CCR4 ligands: CCL17 and CCL22; CCR6 ligand: CCL20) were compared between control subjects (control; n = 8) and HAM/TSP patients (HAM; n = 30). Horizontal bars indicate median values. The Mann-Whitney *U*-test was used for statistical analysis.
(TIF)

Figure S5 Low sensitivity of CSF cell count for detection of HAM/TSP. (**A**) Sensitivities of four potential CSF markers for detection of HAM/TSP. For CSF CXCL10, CXCL9, and neopterin, dotted lines indicate reference values, defined as mean for control subjects +3 standard deviations. For CSF cell count, the dotted line represents the pre-established reference value of 15/3 mm^3. The sensitivity of CSF cell count was much lower than those of the other CSF markers. (**B**) Direct comparison of the sensitivities of CSF cell count and the other three CSF markers. The horizontal dotted lines all represent the reference value for CSF cell count (≤15/3 mm^3), and each vertical dotted line indicates the reference value for each of the other CSF markers. With these lines drawn, one can see in the shaded area the numerous patients with CSF cell counts within the normal range but abnormally high levels of each of the other inflammatory markers, thus directly illustrating the comparatively low sensitivity of CSF cell count.
(TIF)

Figure S6 Changes in levels of CSF markers and OMDS over time in four untreated HAM/TSP patients. The three graphs illustrate the changes over time in CSF CXCL10 (top), neopterin (middle), and OMDS (bottom) for four untreated stable HAM/TSP patients. The patients were observed for 60 months (No. 1), 56 months (No. 2), 49 months (No. 3), and 39 months (No. 4).
(TIF)

Figure S7 Significant positive correlation between the proviral load in PBMCs and four CSF markers. HTLV-1 proviral load in PBMCs was compared with the levels of each of four CSF markers (CXCL10, CXCL9, neopterin, and cell count) in HAM/TSP patients (n = 53). Data analysis was performed using the Spearman's rank correlation test.
(TIF)

Figure S8 Significant higher CSF levels of CXCL10, CXCL9, and neopterin even in stable HAM/TSP compared to controls. The levels of four CSF markers (CXCL10, CXCL9, neopterin, and cell count) were compared among three groups (HTLV-1-infected controls, n = 8; stable HAM/TSP patients, n = 25; and deteriorating HAM/TSP patients, n = 20) assembling patients from both Training and Test Sets combined. The horizontal bar indicates the median value for each group. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's post-hoc tests. ns: not significant, * $P<0.05$, *** $P<0.001$.
(TIF)

Table S1 Demographics of HAM/TSP patients and control subjects. There were no significant differences in the demographics of HAM/TSP patients versus control subjects.
(DOCX)

Table S2 Demographics and clinical characteristics of HAM/TSP patients (Training set + Test Set). Among the HAM/TSP patients from the Training and Test Sets pooled together, deteriorating patients experienced disease onset significantly later in life and had lived with the disease for shorter periods.
(DOCX)

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Author Contributions

Conceived and designed the experiments: YY TS SJ SI. Performed the experiments: TS HA NA JY. Analyzed the data: TS AU NA NY HA JY EI TU YH KN TN. Contributed reagents/materials/analysis tools: YY AU YH. Wrote the paper: YY TS ACR.

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Commentary and point of view

Utility of HTLV proviral load quantification in diagnosis of HTLV-1-associated myelopathy requires international standardization

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The geographic distribution of Human T-cell Lymphotropic Virus Type 1 (HTLV-1) infection makes one thing clear: except Japan, most of the estimated 20 million infected individuals are clustered within communities with limited health care access [1].

Given that the majority of infected persons remain disease-free, one of the challenges of the clinical management of HTLV-1-infected patients with myelopathic symptoms is to establish the definite diagnosis of HTLV-Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP). Asymptomatic carriers may complain of a range of symptoms that cannot be excluded from an association with HTLV-1, such as dry eyes, urinary incontinence and constipation. Current HAM/TSP diagnostic procedures are based on criteria established by the World Health Organization (WHO), which consists of a list of neurological signs and symptoms in HTLV-1 seropositive subjects. In addition, imaging of the central nervous system is essential to exclude other neurological diseases with similar clinical features, whilst isolation of HTLV-1 proviral in the cerebrospinal fluid (CSF) [2] is a positive finding, especially when the viral load in CSF lymphocytes is greater than in PBMCs [3]. However, these complementary tests are not readily available in many communities. In our experience, patients rarely present with all the essential features of HAM/TSP to meet the diagnostic requirements of the complete syndrome. To complement WHO criteria, a new classification strategy, the Belem Criteria, based on three diagnostic ascertainment levels was proposed [4]. HTLV-1-infected patients with neurological defects are categorized as: (i) *Definite HAM/TSP:* patients who meet the established WHO criteria with a complete clinical presentation; (ii) *Probable HAM/TSP:* patients with a myelopathic mono-symptomatic presentation, in which other diseases resembling HAM/TSP have been excluded; (iii) *Possible HAM/TSP:* patients who present with a complete or incomplete clinical picture; however, other disorders resembling HAM/TSP cannot be excluded.

In recent years, several studies have demonstrated a clear association between HAM/TSP and HTLV-1 proviral load [5–11]. Moreover, compelling evidence indicates that patients with other

Abbreviations: HTLV, human T-cell lymphotropic virus; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; PBMC, peripheral blood mononuclear cells; PVL, proviral load.

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Table 1
Methodological characteristics of HTLV-1 proviral load measurements using PCR method from asymptomatic carriers and HAM/TSP patients in endemic countries for HTLV-1-infection.

Author, year	Country	PCR method	Region amplified	% infected cells asymptomatic	Number of asymptomatic carriers	% infected cells HAM/TSP	Number of HAM/TSP patients
Nagai et al., 1998 [8]	Japan	TaqMan	pX	0.3[a]/3.2[a]	200	5.4	202
Manns et al., 1999 [7]	Jamaica	TaqMan	pX	1.1	50	4.9	27
Olindo et al., 2005 [7]	Martinique	TaqMan	pol	0.8	34	8.1	100
Montanheiro et al., 2005 [10]	Brazil	TaqMan	pol	2.7	45	6.8	44
Best et al., 2006 [5]	Peru	SYBR green	pX	5.6	33	18	35
Silva et al., 2007 [11]	Brazil	TaqMan	pX	1.0	93	6.3	197
Grassi et al., 2011 [14]	Brazil	TaqMan	pol	0.7	189	11.6	47
Furtado et al., 2012 [15]	Brazil	SYBR[a] Green	pol	0.5	75	3.4	78
Demontis et al., 2012 [3]	United Kingdom	SYBR Green	Tax	1.8	211	14.7	85

#-Number of evaluated patients, % infected cells: data represents median.
[a] DNA sample from whole blood. All other DNA samples were from peripheral blood mononuclear cells.

HTLV-1-associated inflammatory conditions, such as infective dermatitis [12] or keratoconjunctivitis sicca [13], display significantly higher levels of proviral load compared to asymptomatic carriers.

In a published study conducted in Bahia, Brazil, we found that a proviral load cut-off value of 50,000 copies/10^6 PBMCs (5%), differentiated asymptomatic carriers from HAM/TSP patients with 87% sensitivity and 81% specificity [14]. Using the Belem criteria, only 22% of probable and 17% of definite HAM/TSP patients' HTLV-1 proviral loads fell below this cut-off value. If this threshold had been included as an additional criterion to diagnose HAM/TSP, 73% of patients from the probable group would be reclassified as definite.

Furtado et al. [15] also attempted to establish a proviral load cut-off value to distinguish asymptomatic carriers from HAM/TSP patients in Minas Gerais (Brazil). Using whole blood, a cut-off of 114 HTLV-1 copies/10^4 white blood cells (1.14%), offered 78.2% sensitivity and 28% specificity in patients with HAM/TSP. This value is considerably lower than what was observed in Bahia. As members of the HAM/TSP Clinical Trial Study Group (HAM/TSP-CTSG) we performed a literature review of proviral loads reported in asymptomatic carriers and HAM/TSP patients (Table 1). The percentage of infected cells was approximately 6× higher in HAM/TSP patients (median 6.8%, 5.4–18%) than in asymptomatic carriers (median 1.1%, 0.3–5.6%). Despite overlapping ranges, the trends are consistent across studies. However, study groups used diverse methods to measure proviral load, including different regions of the targeted HTLV-1 genome, the DNA sample source, e.g. whole blood cells or isolated PBMCs and cells quantified from patient samples. In personal consultation with HAM/TSP CTSG members we established that 7.04% and 10% of patients with definite HAM/TSP, living in Bahia, Brazil and Japan respectively, have a proviral load of <1% in PBMCs. These patients have only mild disease. In contrast, all definite HAM/TSP patients from UK or USA had a proviral load of >1% [3]. Based on the reviewed data and our own observations across four centres (Bahia/Japan/UK/USA) a single cut-off for asymptomatic versus HAM/TSP or for definite versus probable HAM/TSP cannot be recommended.

We therefore propose an international pilot study in an attempt to achieve consistent results in order to evaluate HTLV-1 proviral load in PBMCs of asymptomatic carriers as well as patients with probable, possible and definite HAM/TSP living in HTLV-1 endemic and non-endemic areas. By testing these patients at several chosen established laboratories blinded to samples' origins, we hope to take initial steps towards the standardization of proviral load quantification technique, with the ultimate goal of determining a relevant proviral load cutoff value to distinguish asymptomatic carriers from HAM/TSP patients. At the same time, we recognize that, due to variation from assay to assay, there are limitations with respect to viral load quantification [16,17]. In the future, the HTLV research community might propose the incorporation of a WHO international standard to aid in clinical applications by soliciting assistance from such groups as Standardization of Genome Amplification Techniques or National Institute for Biological Standards and Control.

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Competing interests

None declared.

Ethical approval

Not required.

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Potential Contribution of a Novel Tax Epitope–Specific CD4$^+$ T Cells to Graft-versus-Tax Effect in Adult T Cell Leukemia Patients after Allogeneic Hematopoietic Stem Cell Transplantation

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective treatment for adult T cell leukemia/lymphoma (ATL) caused by human T cell leukemia virus type 1 (HTLV-1). We previously reported that Tax-specific CD8$^+$ cytotoxic T lymphocyte (CTL) contributed to graft-versus-ATL effects in ATL patients after allo-HSCT. However, the role of HTLV-1–specific CD4$^+$ T cells in the effects remains unclear. In this study, we showed that Tax-specific CD4$^+$ as well as CD8$^+$ T cell responses were induced in some ATL patients following allo-HSCT. To further analyze HTLV-1–specific CD4$^+$ T cell responses, we identified a novel HLA-DRB1*0101–restricted epitope, Tax155–167, recognized by HTLV-1–specific CD4$^+$ Th1-like cells, a major population of HTLV-1–specific CD4$^+$ T cell line, which was established from an ATL patient at 180 d after allo-HSCT from an unrelated seronegative donor by in vitro stimulation with HTLV-1–infected cells from the same patient. Costimulation of PBMCs with both the identified epitope (Tax155–167) and known CTL epitope peptides markedly enhanced the expansion of Tax-specific CD8$^+$ T cells in PBMCs compared with stimulation with CTL epitope peptide alone in all three HLA-DRB1*0101$^+$ patients post–allo-HSCT tested. In addition, direct detection using newly generated HLA-DRB1*0101/Tax155–167 tetramers revealed that Tax155–167-specific CD4$^+$ T cells were present in all HTLV-1–infected individuals tested, regardless of HSCT. These results suggest that Tax155–167 may be the dominant epitope recognized by HTLV-1–specific CD4$^+$ T cells in HLA-DRB1*0101$^+$–infected individuals and that Tax-specific CD4$^+$ T cells may augment the graft-versus-Tax effects via efficient induction of Tax-specific CD8$^+$ T cell responses. *The Journal of Immunology*, **2013, 190: 4382–4392.**

Human T cell leukemia virus type 1 (HTLV-1) is the causative agent of a highly aggressive CD4$^+$ T cell malignancy, adult T cell leukemia/lymphoma (ATL) (1, 2). This virus has infected 10–20 million people worldwide, especially in southern Japan, the Caribbean basin, South America, Melanesia, and equatorial Africa (3). Approximately 5% of HTLV-1–seropositive individuals develop ATL, and another 2–3% develop a slow progressive neurologic disorder known as HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) or various chronic inflammatory diseases (4). The majority of HTLV-1–infected individuals remain asymptomatic throughout their lives.

ATL is characterized by extremely poor prognosis, mainly because of intrinsic drug resistance to cytotoxic agents. It has been reported that allogeneic hematopoietic stem cell transplantation (allo-HSCT), but not autologous HSCT, improved the outcome of ATL (5, 6). In previous clinical studies carried out by the ATL allo-HSCT Study Group, the overall survival rate within 3 y after allo-HSCT with reduced intensity conditioning (RIC) was 36% (7). HTLV-1 proviral load became and remained undetectable in some ATL patients with complete remission after allo-HSCT, suggesting that it is an effective treatment for ATL (7–9). In these studies, we reported that donor-derived HTLV-1 Tax-specific CD8$^+$ CTLs were induced in some ATL patients who achieved complete remission after allo-HSCT (10). These CTLs were able to lyse recipient–derived HTLV-1–infected T cells in vitro, suggesting potential contributions to graft-versus-leukemia effects. CD8$^+$ T cells, especially CTLs, generally play an important role in controlling viral replication in various infections, such as those

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Abbreviations used in this article: AC, asymptomatic carrier; allo-HSCT, allogeneic stem cell transplantation; ATL, adult T cell leukemia/lymphoma; HAM/TSP, HTLV-1–associated myelopathy/tropical spastic paraparesis; HTLV-1, human T cell leukemia virus type 1; ILT, IL-2–dependent T cell line; LCL, lymphoblastoid B cell line; rhIL-2, recombinant human IL-2; RIC, reduced intensity conditioning; Treg, regulatory T.

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involving HIV, hepatitis B virus, and hepatitis C virus. In HTLV-1 infection, HTLV-1–specific $CD8^+$ T cells predominantly recognize the Tax Ag and are believed to contribute to controlling infected cells (11, 12). A high frequency of functional Tax-specific $CD8^+$ T cells can be detected in HAM/TSP patients and some asymptomatic carriers (ACs), whereas most ATL patients and a small population of ACs show severely reduced Tax-specific $CD8^+$ T cell responses (13, 14). The mechanism underlying the suppression of HTLV-1–specific $CD8^+$ T cell responses in these patients has not yet been fully elucidated.

For induction and maintenance of virus-specific CTLs, virus-specific $CD4^+$ Th cell responses are required in many virus infections (15–19). However, there are only a few reports of HTLV-1–specific Th cell responses (20–23), presumably because of their susceptibility to HTLV-1 infection in vivo and in vitro (24). Preferential HTLV-1 infection in HTLV-1–specific $CD4^+$ T cells could be one of the reasons for immune suppression in ATL patients. In addition, it has been reported that a higher frequency of $CD4^+FOXP3^+$ regulatory T (Treg) cells is observed in infected individuals compared with uninfected healthy donors. The frequency of Tax^- Treg cells, which are a major population of Treg cells in infected individuals, is negatively correlated with HTLV-1–specific CTL responses (25). HTLV-1 basic leucine zipper factor might also be involved in immune suppression, because HTLV-1 basic leucine zipper was constitutively expressed in infected cells (26) and inhibited the activity of IFN-γ promoters by suppressing NFAT and AP-1 signaling pathways, resulting in the impaired secretion of Th1 cytokines from $CD4^+$ Th cells in a transgenic mouse model (27) These reports suggest that both the dysfunction of HTLV-1–specific $CD4^+$ Th cells and the increased number of uninfected Treg cells might be implicated in the immunosuppression observed in ATL patients. Conversely, in HAM/TSP patients, $CD4^+$ T cells are predominantly found in early active inflammatory spinal cord lesions (28, 29) with spontaneous production of proinflammatory, neurotoxic cytokines, such as IFN-γ and TNF-α (30), suggesting their contributions to the pathogenesis of HAM/TSP. However, the precise roles of HTLV-1–specific $CD4^+$ T cells in HTLV-1 infection remain unclear.

In some ATL patients who achieved complete remission after allo-HSCT, it has been suggested that donor-derived HTLV-1 Tax-specific CTLs may contribute to elimination of ATL cells (graft-versus-Tax effects) (10). We believe that $CD4^+$ T cells also play a critical role in the graft-versus-ATL effects because $CD4^+$ T cells are required for induction and maintenance of optimal CTL responses (15–19). It therefore is important to clarify the role of HTLV-1–specific $CD4^+$ T cells in the effects for understanding HTLV-1–specific T cell immunity in ATL patients after allo-HSCT and for developing new vaccine strategies to prevent recurrence of ATL.

Several studies have reported some HTLV-1–specific $CD4^+$ T cell epitopes restricted by different HLA haplotypes (20–23). The helper functions of these epitopes in HTLV-1–specific CTL responses in HTLV-1–infected individuals have not been well understood. However, Jacobson et al. (20) showed that $CD4^+$ T cells specific for Env gp46 196–209, an epitope restricted by HLA-DQ5 or -DRw16, exhibited a cytotoxic function by directly recognizing HTLV-1–infected cells. This observation raises the possibility that some HTLV-1–specific $CD4^+$ T cells may contribute to the graft-versus-ATL effects through their cytotoxic function in ATL patients after allo-HSCT.

In the current study, we demonstrated that both $CD4^+$ and $CD8^+$ Tax-specific T cell responses were induced in patients after allo-HSCT with RIC for ATL. To further analyze HTLV-1–specific $CD4^+$ T cell responses in ATL patients after allo-HSCT, we determined a novel HLA-DRB1*0101–resricted epitope, Tax155–167, recognized by HTLV-1–specific $CD4^+$ Th1-like cells, a major population of HTLV-1–specific $CD4^+$ T (T4) cell line, which was established from a patient in complete remission following allo-HSCT with RIC. Costimulation with oligopeptides corresponding to the Th1 epitope, Tax155–167, together with a known CTL epitope led to robust expansion of Tax-specific $CD8^+$ T cells in PBMCs from three HLA-DRB1*0101^+ patients after allo-HSCT tested. Furthermore, Tax155–167-specific $CD4^+$ T cells were found to be maintained in all HTLV-1–infected HLA-DRB1*0101^+ individuals tested, regardless of HSCT, by direct detection with newly generated HLA-DRB1*0101/Tax155–167 tetramers. Our results suggest that Tax155–167 may be a dominant epitope recognized by HTLV-1–specific $CD4^+$ T cells in HTLV-1–infected individuals carrying HLA-DRB1*0101 and that Tax-specific $CD4^+$ T cells may strengthen the graft-versus-ATL effects through efficient induction of Tax-specific CTL responses.

Materials and Methods

Subjects

A total of 18 ATL patients who underwent allo-HSCT with RIC regimen, and one HTLV-1–seronegative (#365) and two seropositive donors (one AC #310 and one HAM/TSP patient #294) carrying HLA-DRB1*0101 donated peripheral blood samples after providing written informed consent. Approximately one-half of these patients received allogeneic peripheral blood stem cell transplantation from HLA-A-, B-, and -DR–identical sibling donors. The other half received allogeneic bone marrow cells from HLA-A–, B–, and DR–identical seronegative unrelated donors (Table I). These patients were the participants of clinical studies organized by the ATL allo-HSCT Study Group, supported by the Ministry of Health, Welfare, and Labor of Japan. This study was also reviewed and approved by the Institutional Ethical Committee Review Board of the Tokyo Medical and Dental University.

Generation of cell lines derived from patients and donors

PBMCs were isolated using Ficoll-Paque PLUS (GE Healthcare, Buckinghamshire, U.K.) density gradient centrifugation and stored in liquid nitrogen in Bambanker stock solution (NIPPON Genetics, Tokyo, Japan) until required. These were used in part to obtain HTLV-1–infected IL-2–dependent T cell lines (ILT) and EBV-transformed lymphoblastoid B cell lines (LCL). ILT-#350 was spontaneously immortalized during long-term culture of PBMCs from patient #350 before allo-HSCT and maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 20% FCS (Sigma Aldrich, St. Louis, MO) and 30 U/ml recombinant human IL-2 (rhIL-2; Shionogi, Osaka, Japan). LCL-#307, -#341, and -#350 were established by maintaining PBMCs from ATL patients #307, #341, and #350, respectively, after allo-HSCT. These PBMCs were maintained in RPMI 1640 medium containing 20% FCS, following infection with the EBV-containing culture supernatant of the B95-8 cell line, LCL-Kan, derived from a healthy individual was also used.

Synthetic peptides

A total of 18 overlapping peptides, 12- to 25-mer in length, spanning the central region of Tax (residues 103–246) were purchased and used for epitope mapping (Scrum Tokyo, Japan) (Table II). HLA-A*2402–restricted CTL epitopes (Tax301–309, SFHSLHLLF) (10) were used for in vitro stimulation of Tax-specific CTLs (Hokudo, Sapporo, Japan).

GST–Tax fusion protein–based immunoassay

HTLV-1 Tax-specific T cell responses were evaluated using GST–fusion proteins of the N-terminal (residues 1–127), central (residues 113–237), and C-terminal (residues 224–353) regions of HTLV-1 Tax (GST–Tax-A, -B, and -C, respectively) as described previously (13, 31). PBMCs (1×10^6 cells/ml) were incubated with or without a mixture of GST–Tax-A, -B, and -C proteins (GST–TaxABC) in 200 μl RPMI 1640 medium supplemented with 10% FCS. After 4 d, the supernatant was collected, and the concentration of IFN-γ in the supernatant was determined using an OptiEIA Human IFN-γ ELISA Kit (BD Biosciences, San Jose, CA). The minimum detectable dose for this assay was determined to be 23.5 pg/ml IFN-γ. $CD8^+$ cells were depleted from PBMCs by negative selection using Dynabeads M-450 CD8 (Invitrogen, Carlsbad, CA), according to the