

The CD3 versus CD7 Plot in Multicolor Flow Cytometry Reflects Progression of Disease Stage in Patients Infected with HTLV-I

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Abstract

Purpose: In a recent study to purify adult T-cell leukemia-lymphoma (ATL) cells from acute-type patients by flow cytometry, three subpopulations were observed in a CD3 versus CD7 plot (H: CD3^{high}CD7^{high}; D: CD3^{dim}CD7^{dim}; L: CD3^{dim}CD7^{low}). The majority of leukemia cells were enriched in the L subpopulation and the same clone was included in the D and L subpopulations, suggesting clonal evolution. In this study, we analyzed patients with indolent-type ATL and human T-cell leukemia virus type I (HTLV-I) asymptomatic carriers (ACs) to see whether the CD3 versus CD7 profile reflected progression in the properties of HTLV-I-infected cells.

Experimental Design: Using peripheral blood mononuclear cells from patient samples, we performed multi-color flow cytometry. Cells that underwent fluorescence-activated cell sorting were subjected to molecular analyses, including inverse long PCR.

Results: In the D(%) versus L(%) plot, patient data could largely be categorized into three groups (Group 1: AC; Group 2: smoldering- and chronic-type ATL; and Group 3: acute-type ATL). Some exceptions, however, were noted (e.g., ACs in Group 2). In the follow-up of some patients, clinical disease progression correlated well with the CD3 versus CD7 profile. In clonality analysis, we clearly detected a major clone in the D and L subpopulations in ATL cases and, intriguingly, in some ACs in Group 2.

Conclusion: We propose that the CD3 versus CD7 plot reflects progression of disease stage in patients infected with HTLV-I. The CD3 versus CD7 profile will be a new indicator, along with high proviral load, for HTLV-I ACs in forecasting disease progression.

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Introduction

Human T-cell leukemia virus type I (HTLV-I) is the agent that causes HTLV-I-associated diseases, such as adult T-cell leukemia-lymphoma (ATL), HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and HTLV-I uveitis (HU) [1–3]. Approximately 10–20 million people are infected with the HTLV-I virus worldwide [4]. The lifetime risk of developing ATL is estimated to be approximately 2.5–5% [5,6]. ATL includes a spectrum of diseases that are referred to as smoldering-, chronic-, lymphoma-, and acute-type [7,8]. The chronic and smoldering types of ATL are considered indolent and are usually managed with watchful waiting until the disease progresses to aggressive

(lymphoma- or acute-type) ATL [9]. Because the prognosis of ATL is poor with current treatment strategies, factors to forecast progression to ATL from asymptomatic carriers (ACs) have been researched [10–13] in the hope that they will be useful for preventive therapy under development in the early malignant stage.

Various cellular dysfunctions induced by viral genes (e.g., tax and HBZ), genetic and epigenetic alterations, and the host immune system are considered to cooperatively contribute to leukemogenesis in ATL [14–16]. However, the complex mechanism may hinder determination of a clear mechanism of the pathology and make discovery of risk factors difficult. In a prospective nationwide study in Japan, high proviral load (VL,

Table 1. Clinical profile of patients infected with HTLV-I and normal controls.

Clinical subtype	Number of cases	Male	Female	Age (range)	WBC(μ l) (range)	Lymphocytes(%) (range)	Abnormal lymphocytes(%) (range)
HTLV-I AC	40	12	28	49.9 (28–70)	5525 (2680–10360)	35.9 (22.4–59.5)	0.9 (0.0–4.4)
Smoldering	7	4	3	55.3 (43–77)	5944 (3680–8710)	32.5 (13.4–47.5)	5.8 (0.7–16.5)
Chronic	7	4	3	52.7 (37–60)	9180 (4070–12790)	45.8 (35.0–61.5)	9.2 (3.4–12.7)
Acute	13	4	9	58.8 (42–74)	15328 (4450–41480)	16.3 (1.7–50.5)	40.3 (3.0–89.6)
Normal controls	10	6	4	47.4 (27–66)	ND	ND	ND

WBC: white blood cells (normal range, 3500–9100/ μ l).

AC: asymptomatic carrier.

ND: analysis were not performed.

Average of age, WBC, lymphocytes (%) and abnormal lymphocytes (%) are shown.

The proportion of abnormal lymphocytes in peripheral blood WBCs was evaluated by morphological examination.

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over 4.17 copies/100 peripheral blood mononuclear cells) was found to be a major risk factor for HTLV-I AC developing into ATL [13]. Although VL indicates the proportion of HTLV-I-infected cells, it does not indicate size or degree of malignant progression in each clone; *i.e.*, it does not directly indicate progression of disease stage in HTLV-I infection. Moreover, the majority of ACs with high VL remained intact during the study period, indicating that a more accurate indicator of progression is needed.

In our recent study to purify monoclonal ATL cells from acute-type patients by flow cytometry, three subpopulations were observed in a CD3 versus CD7 plot of CD4⁺ cells (H: CD3^{high}CD7^{high}, D: CD3^{dim}CD7^{dim}, L: CD3^{dim}CD7^{low}), and the majority of ATL cells were enriched in the L subpopulation [17]. Clonality analyses revealed that the D and L subpopulations contained the same clone, suggesting clonal evolution of HTLV-I-infected cells to ATL cells. From these findings, we speculated that the CD3 versus CD7 profile may reflect disease progression in HTLV-I infection. In this study, the CD3 versus CD7 profile by flow cytometry, combined with molecular (clonality and proviral load) characterizations, were analyzed in patients with various clinical subtypes (HTLV-I AC, and indolent and aggressive ATL). We found that the CD3 versus CD7 profile reflected disease progression of HTLV-I-infected cells to ATL cells. We also discuss the significance of this analysis as a novel risk indicator for HTLV-I ACs in forecasting progression to ATL.

Materials and Methods

Cell lines and patient samples

TL-Om1, an HTLV-I-infected cell line, established Dr. Hinuma's laboratory [18], was provided by Dr. Toshiki Watanabe (The University of Tokyo, Tokyo, Japan) and was cultured in RPMI-1640 medium containing 10% fetal bovine serum. Peripheral blood samples were collected from inpatients and outpatients at our hospital from August 2009 to November 2011. All patients with ATL were categorized according to Shimoyama's criteria [7,8]. Patients with various complications, such as autoimmune

disorder and systemic infections, were excluded. Lymphoma-type patients were excluded because ATL cells are not considered to exist in peripheral blood of this clinical subtype. In patients with ATL receiving chemotherapy, blood samples were collected before treatment or during the recovery phase between chemotherapy sessions. Samples collected from 10 healthy volunteers (mean age: 47.4 years; range: 27–66 years) were used as normal controls.

The present study was approved by the research ethics committee of the institute of medical science, the university of Tokyo. Subjects provided written informed consent.

Flow cytometry and cell sorting

Peripheral blood mononuclear cells (PBMCs) were isolated from heparin-treated whole blood by density gradient centrifugation, as described previously [17]. Cells were stained using a combination of phycoerythrin (PE)-CD7, APC-Cy7-CD3, Pacific Blue-CD4, and Pacific Orange-CD14. Pacific Orange-CD14 was purchased from Caltag-Invitrogen (Carlsbad, CA). All other antibodies were obtained from BD BioSciences (San Jose, CA). Propidium iodide (PI; Sigma, St. Louis, MO) was added to the samples to stain dead cells immediately prior to flow cytometry. A BD FACS Aria instrument (BD Immunocytometry Systems, San Jose, CA) was used for all multicolor flow cytometry and cell sorting. Data were analyzed using the FlowJo software (Treestar, San Carlos, CA).

Quantification of HTLV-I proviral load by real-time quantitative polymerase chain reaction (PCR)

The HTLV-I proviral load in FACS-sorted PBMCs was quantified by real-time quantitative polymerase chain reaction (PCR; TaqMan method) using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) as described previously [13,17]. Briefly, 50 ng of genomic DNA was extracted from human PBMCs using a QIAamp DNA blood Micro kit (Qiagen, Hilden, Germany). Triplicate samples of the DNA were amplified. Each PCR mixture, containing an HTLV-I pX region-specific primer pair at 0.1 μ M (forward primer 5'-CGGATACCCAGTCTACGTGTT-3' and reverse primer 5'-

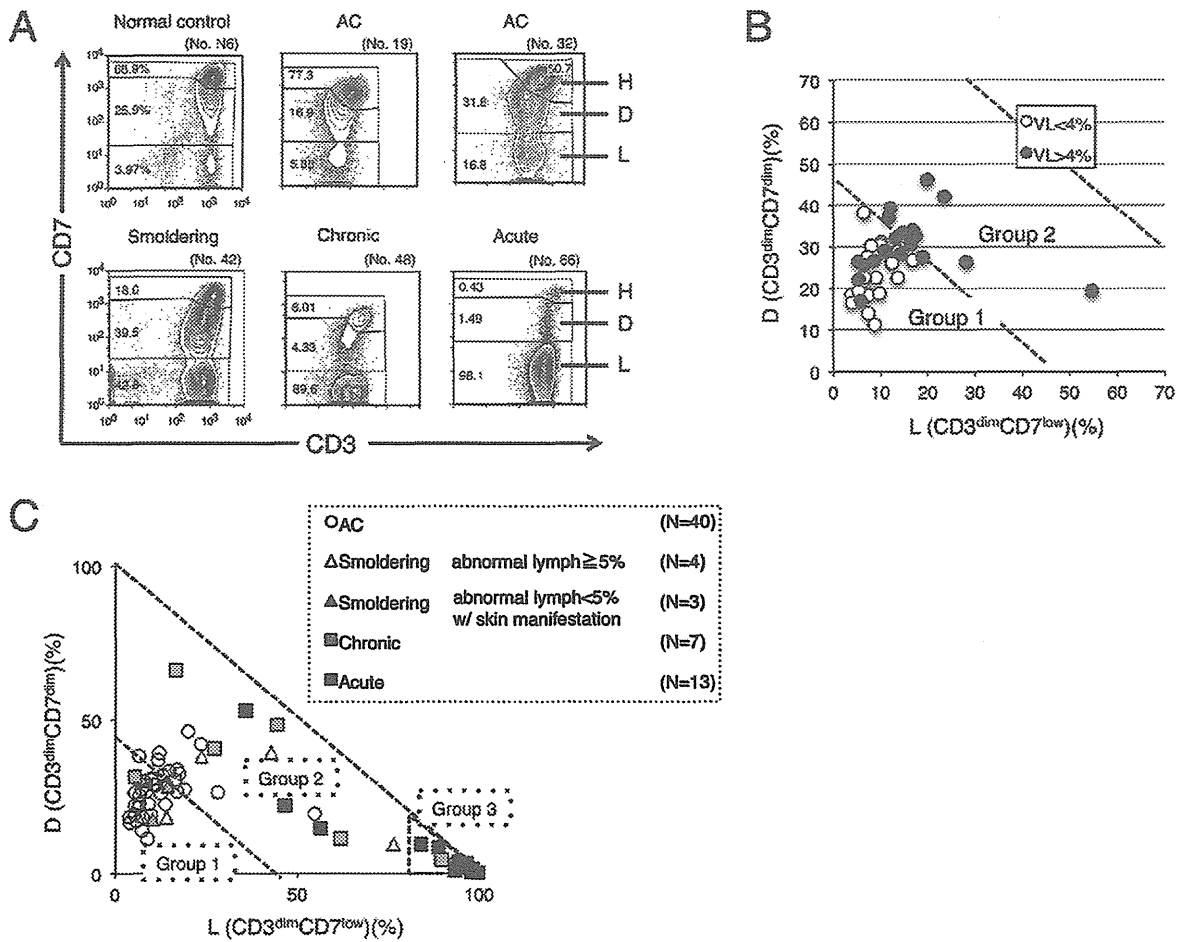


Figure 1. CD3 versus CD7 plots in flow cytometric analysis of patients who are asymptomatic HTLV-I carriers (ACs) and have various clinical subtypes of adult T-cell leukemia-lymphoma (ATL) suggest disease progression in HTLV-I infection. (A) Flow cytometric profile of an AC, various clinical subtypes of ATL (smoldering, chronic, and acute), and a normal control. Representative cases of CD3 versus CD7 plots in CD4⁺ cells are shown. (B) A two-dimensional plot of AC cases showing the percentage of the D and L subpopulations by flow cytometry. AC cases were divided into two groups according to HTLV-I VL (greater or less than 4%). The border line (45% of D+L subpopulations) between Group 1 and 2 was set based on proviral load (VL). All AC cases with less than 4% VL were included in Group 1. All AC cases included in Group 2 had greater than 4% VL. VL < 4%: n = 21; VL > 4%: n = 19. All VL data in this figure were provided from the database of the Joint Study on Predisposing Factors of ATL Development (JSPFAD). (C) A two-dimensional plot of all patients showing the percentage of the D and L subpopulations. The smoldering type was divided into two categories: smoldering type with greater than 5% abnormal lymphocytes and smoldering type with less than 5% abnormal lymphocytes with skin manifestation. The two diagonal dotted lines indicate 45% and 100% of D+L subpopulations (i.e., 55% and 0% of the H subpopulation). Data were categorized into three groups. doi:10.1371/journal.pone.0053728.g001

CAGTAGGGCGTGCAGATGTA-3'), FAM-labeled probe at 0.1 μM (5'- CTGTGTACAAGGCGACTGGTGCC-3'), and 1 × TaqMan Universal PCR master mix (Applied Biosystems), was subjected to 50 cycles of denaturation (95°C, 15 seconds) and annealing to extension (60°C, 1 minute), following an initial Taq polymerase activation step (95°C, 10 minutes). The RNase P control reagent (Applied Biosystems) was used as an internal control for calculating the input cell number (using VIC reporter dye). DNAs extracted from TL-Om1 and normal human PBMCs were used as positive and negative controls, respectively. The HTLV-I proviral load (%) was calculated as the copy number of the pX region per input cell number. To correct the deviation of

data acquired in each experiment, data from TL-Om1 (positive control) were adjusted to 100%, and the sample data were corrected accordingly by a proportional calculation.

Inverse long PCR

For clonality analysis, inverse long PCR was performed [17]. First, 1 μg of genomic DNA extracted from the FACS-sorted cells was digested with *EcoRI* and *PstI* at 37°C overnight. Purification of DNA fragments was performed using a QIAEX2 gel extraction kit (Qiagen). The purified DNA was self-ligated with T4 DNA ligase (Takara Bio, Otsu, Japan) at 16°C overnight. The circular DNA obtained from the *EcoRI* digestion fragment was then digested

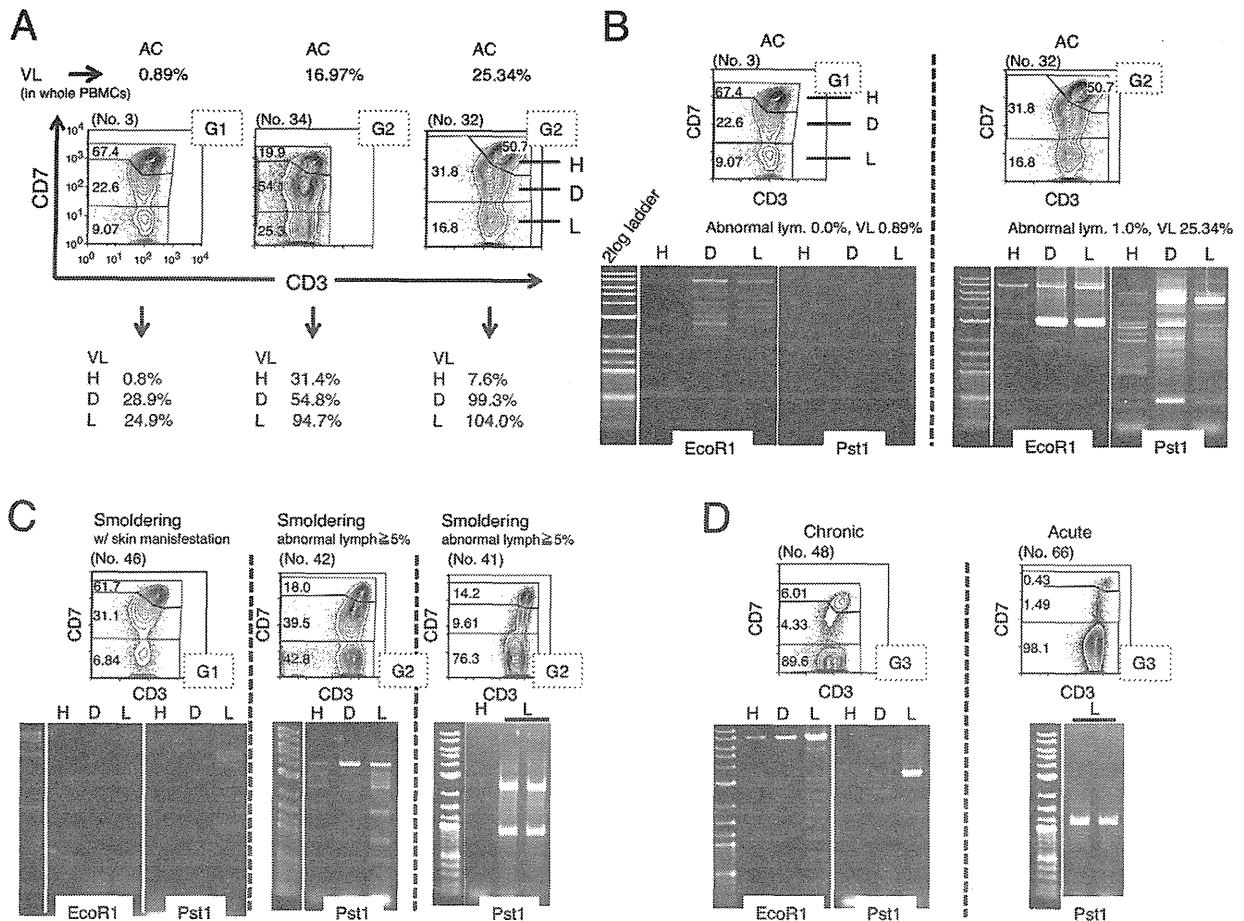


Figure 2. HTLV-I proviral load (VL) and clonality in each subpopulation, based on the CD3 versus CD7 plot. (A) The three subpopulations (H, D, L) based on the CD3 versus CD7 plot were subjected to fluorescence-activated cell sorting (FACS) and VL analysis. Three representative cases are shown. G1 or G2 in the dotted box indicates Group 1 or Group 2, categorized by the percentage of the D and L subpopulations, respectively. (B)–(D) Analysis of clonality in the three subpopulations based on the CD3 versus CD7 plot. Genomic DNA was extracted from FACS-sorted cells of each subpopulation and subjected to inverse long polymerase chain reaction (PCR). Representative data of two cases of AC (B), three cases of smoldering type, including one with skin manifestations (C), and cases of a chronic type and an acute type (D) are shown. PCR was performed in duplicate (black bars) in cases when a sufficient amount of DNA was obtained. doi:10.1371/journal.pone.0053728.g002

with *Mlu*I, which cuts the pX region of the HTLV-I genome and prevents amplification of the viral genome. Inverse long PCR was performed using Takara LA *Taq* polymerase (Takara Bio). For the *Eco*RI-treated template, the forward primer was 5'-TGCCTGACCCTGCTTGCTCAACTCTACGTCCTTG-3' and the reverse primer was 5'-AGTCTGGGCCCT-GACCTTTTCAGACTTCTGTTTC-3'. For the *Pst*I-treated group, the forward primer was 5'-CAGCCCATTCTATAGCACTCTCCAGGAGAG-3' and the reverse primer was 5'-CAGTCTCCAACACGTAGACTGGGTATCCG-3. Each 50- μ L reaction mixture contained 0.4 mM of each dNTP, 25 mM MgCl₂, 10 \times LA PCR buffer II containing 20 mM Tris-HCl and 100 mM KCl, 0.5 mM of each primer, 2.5 U LA *Taq* polymerase, and 50 ng of the processed genomic DNA. The reaction mixture was subjected to 35 cycles of denaturation (94°C, 30 seconds) and annealing to extension (68°C, 8 minutes). Following PCR, the products were subjected to electrophoresis on 0.8% agarose gels. In samples from which a sufficient amount of DNA was extracted, PCRs were performed in duplicate.

Results

CD3 versus CD7 profile in flow cytometry in various clinical subtypes of patients infected with HTLV-I

The clinical profiles of the 77 cases analyzed in this study are shown in Table 1. According to the gating procedure, as shown in Figure S1 [17], we constructed a CD3 versus CD7 plot of CD4⁺ cells in PBMCs of various clinical subtypes from patients infected with HTLV-I and normal controls. The three subpopulations (CD3^{high}CD7^{high}, CD3^{dim}CD7^{dim}, and CD3^{dim}CD7^{low}) observed are referred to as the H, D, and L subpopulations, respectively. Representative results for each clinical subtype of HTLV-I infection are shown in Figure 1A. Regarding the data for an acute-type patient (no. 66), the dominant population was the L subpopulation, in which we previously demonstrated that monoclonal ATL cells are enriched [17]. Regarding the AC (no. 19), the CD3 versus CD7 profile was close to that of the normal control, although in some AC cases, such as no. 32, the profile differed from that of the normal control, because in contrast to case no. 19,

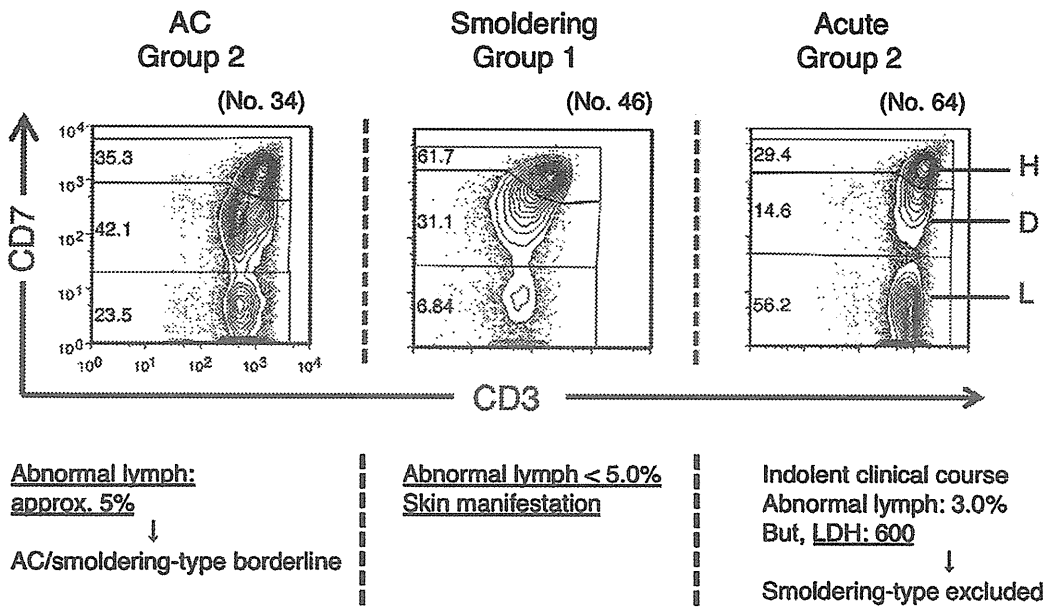


Figure 3. Study of exceptional cases categorized by proportion of the CD3^{dim}CD7^{dim} (D) and CD3^{dim}CD7^{low} (L) subpopulations. Left: An HTLV-I AC patient who was categorized in Group 2 in the D(%) versus L(%) plot. Middle: A patient with smoldering-type ATL who was categorized in Group 1. Right: A patient with acute-type ATL who was categorized in Group 2. doi:10.1371/journal.pone.0053728.g003

these cases had increased D and L subpopulations. Regarding the data for indolent-type disease (smoldering and chronic), increases in the D and L subpopulations were intermediate between ACs and patients with acute-type disease. These representative flow cytometric data suggest that continuity in the CD3 versus CD7 profile seemed to exist among the various clinical subtypes of patients infected with HTLV-I.

The proportions of D and L subpopulations in all AC cases analyzed are shown in Figure 1B. Because the high HTLV-I proviral load (VL) in whole PBMCs, a VL of >4%, was reported to be a major risk indicator for progression to ATL [13], a borderline was set based on VL. Group 1, the area under the diagonal line (D+L = 45%), included all AC cases with VLs of <4%. ACs with VLs of >4% were distributed between Groups 1 and 2. The proportions of D and L subpopulations in normal controls are shown in Figure S2. In this plot, all data for normal controls were distributed in Group 1. Data for all clinical subtypes are shown in Figure 1C. Most data for acute-type patients were located in the area beyond 80% of the L subpopulation and we designated this area as Group 3. Group 2, which is located between Group 1 and Group 3, included the majority of indolent-type (smoldering and chronic) cases. From these results, the three groups in the D(%) versus L(%) plot seemed to represent disease stage in each case.

Proviral load and clonality in each subpopulation in the CD3 versus CD7 plot

To further characterize each subpopulation (H, D, and L) in the CD3 versus CD7 plot, cells in each subpopulation were FACS-sorted and subjected to analysis of VL to determine the percentage of HTLV-I-infected cells in each subpopulation. Results for representative cases are shown in Figure 2A. The VL in whole PBMCs of an AC (no. 3) was low (0.89%). As expected, the VL in H, the major subpopulation, was low (0.8%). However, VLs in the D and L subpopulations were considerably higher (28.9% and

24.9%, respectively), indicating that HTLV-I-infected cells are relatively concentrated in these subpopulations. In the cases with high VLs in whole PBMCs (no. 32 with 25.34%; no. 34 with 16.97%), the VLs were also higher in the D and L subpopulations, and almost all cells in the L subpopulation were HTLV-I-infected.

In HTLV-I infection, progression to ATL requires several pathological steps, including clonal expansion [15]. To further characterize the three subpopulations based on the CD3 versus CD7 plot, we analyzed clonality in each subpopulation in patients with various clinical subtypes using the inverse long PCR method. Figure 2B shows two cases of AC. In the left case (no. 3), included in Group 1 in the D(%) and L(%) plot, multiple bands suggestive of multiple small clones were detected in the three subpopulations. However, no major band suggestive of a dominant clone was observed. In the right case (no. 32), included in Group 2, inverse long PCR of the FACS-sorted subpopulations suggested that the D and L subpopulations contained a major clone (Figure 2B). The D subpopulation had bands of the same size as those of the L subpopulation, indicating that the two distinct subpopulations contained a common major clone. Eleven cases of AC were included in Group 2. All three cases analyzed by Southern blotting (whole blood samples) were positive for clonal bands (Figure S3). In Figure 2C, data for three smoldering cases are shown. In case no. 46 (left), whose only manifestation was a skin eruption with few abnormal lymphocytes (less than 5% of white blood cells) in the peripheral blood, only faint minor bands suggestive of small clones were observed. In contrast, in the other two cases (nos. 42 and 41), intense bands suggestive of major clones were observed in both the D and L subpopulations. In no. 41 (right), weak bands were not visible, which suggested the selection of dominant clones. In Figure 2D, data for a chronic-type case and an acute-type case are shown. In both cases, intense bands in the L subpopulation suggest the existence of a major clone. The series of clonality analyses indicated that a major clone became more evident and the clinical

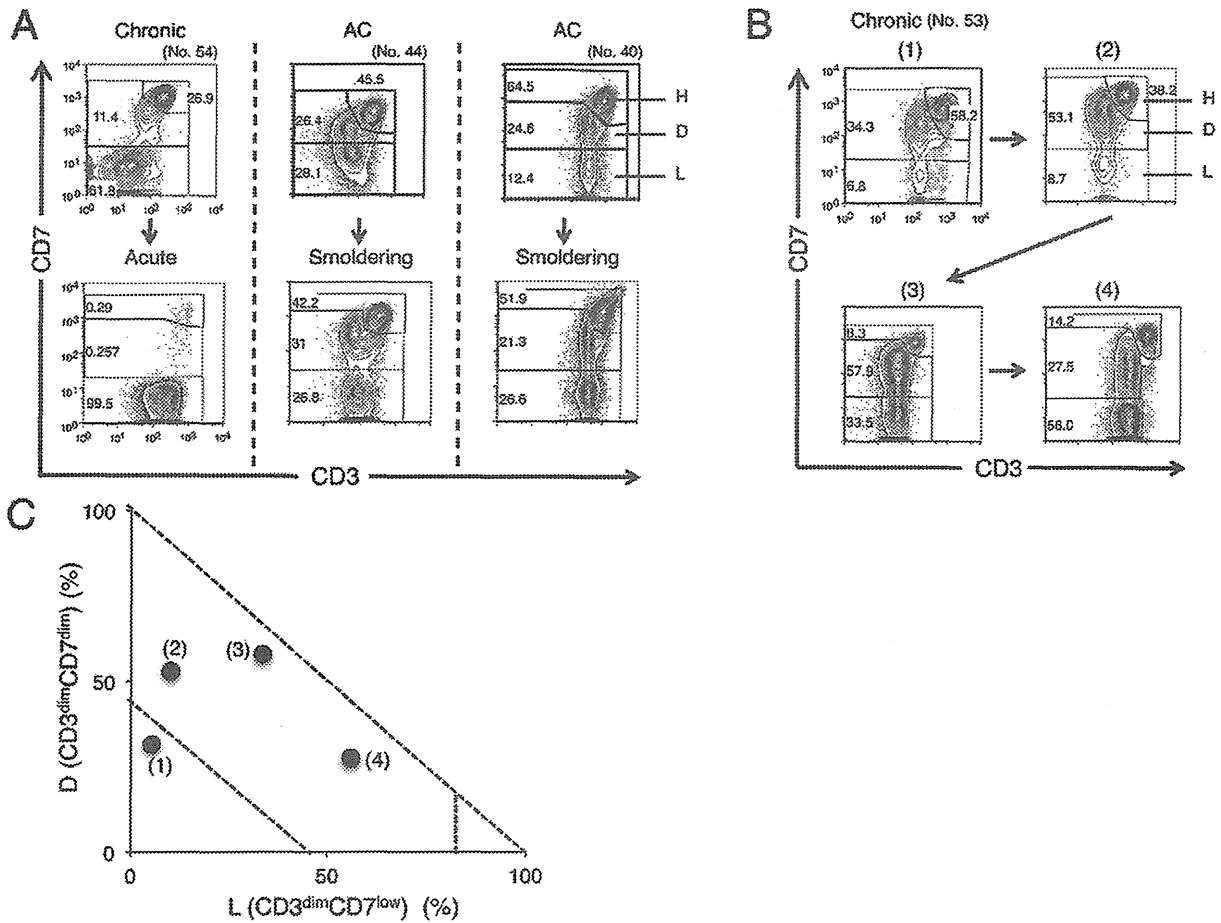


Figure 4. Alteration in the CD3 versus CD7 profile by flow cytometry in accordance with disease progression. (A) Change in the CD3 versus CD7 profile in representative cases. In all three cases shown, change in clinical data (e.g., abnormal lymphocyte, LDH) resulted in progression of the clinical subtype. (B) Change in the CD3 versus CD7 profile in a time course in the case of chronic-type ATL. Clinical data are shown in Table S1. (C) Flow cytometric data in (B) are summarized in the D(%) versus L(%) plot. doi:10.1371/journal.pone.0053728.g004

stage became more advanced as the D and L subpopulations increased.

Clinical evaluation of exceptional cases categorized by proportions of the CD3^{dim}CD7^{dim} (D) and CD3^{dim}CD7^{low} (L) subpopulations

As noted above, the D(%) versus L(%) plot generally represented disease stage in HTLV-I infection. However, we observed one case of chronic-type disease and three cases of smoldering-type disease in Group 1 and three cases of acute-type disease in Group 2. Furthermore, some ACs with VLs of >4% were observed in Group 2. Representative data from these apparently exceptional cases are shown in Figure 3. On the left, a case of AC (no. 34) observed in Group 2 is shown. 4.7% of lymphocytes in this blood sample were abnormal and clonality analysis by Southern blotting showed oligoclonal bands suggestive of clones of substantial size (Figure S3). These clinical data suggest that the disease stage would be around the AC/smoldering borderline. In the middle, a case of a smoldering type (no. 46) observed in Group 1 is shown. In this case, the percentage of abnormal lymphocytes in the peripheral blood was only 1%, but she had a histologically proven ATL lesion

in the skin and was diagnosed with smoldering-type ATL. The other two smoldering cases categorized in Group 1 were the same as this case. These results indicate that ATL cells in these three smoldering cases infiltrated the skin, but not the peripheral blood. On the right, a case of acute-type disease categorized as Group 2 (no. 64) is shown. The clinical course of this patient was relatively indolent compared with typical acute-type disease. He had skin infiltration of ATL cells, but no lymph node swelling. However, LDH exceeded 1.5 times the upper limit of the normal range, which excludes a diagnosis of smoldering-type disease. Other acute-type cases categorized in Group 2 were diagnosed as such according to Shimoyama's criteria, but also had the same indolent clinical course as case no. 64. These cases should have been regarded as indolent ATL.

Changes in the CD3 versus CD7 profile in flow cytometry with disease progression

In several cases, we could obtain time-sequential samples (Figure 4). The patient (no. 54) shown on the left in Figure 4A progressed from chronic-type to acute-type disease. In flow cytometric analysis, decreases in the H and D subpopulations

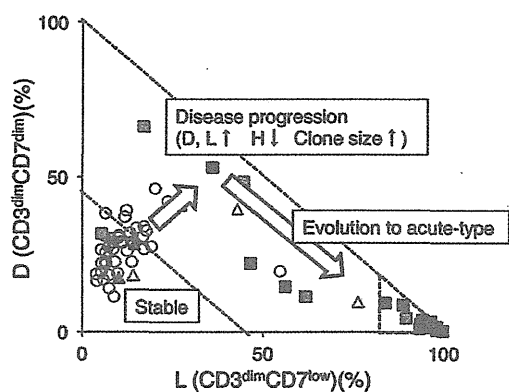


Figure 5. Summary of the study: the CD3 versus CD7 profile reflects progression of disease stage in patients infected with HTLV-I. In the percentage of D (CD3^{dim}CD7^{dim}) versus L (CD3^{dim}CD7^{low}) plot, Group 1 includes the majority of AC cases. As disease stage progresses, the CD3 versus CD7 profile then changes. With downregulation of CD3 and CD7, the D and L subpopulations increase gradually (Group 2). During this step, clones in the D and L subpopulations increase in size. Further accumulation of genetic alterations will result in rapid expansion of ATL clones—i.e., evolution to acute-type ATL. In this step, the CD3 versus CD7 profile will progress from Group 2 to 3.

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and an increase in the L subpopulation were observed, indicating that disease progression correlated well with the change in the CD3 versus CD7 profile. The patients in the middle (no. 44) and on the right (no. 40) were included in Group 2 at the AC stage and later progressed to smoldering-type ATL. Although variation in the change of the flow cytometric profile was seen between these patients, the results suggest that ACs in Group 2 are at high risk of developing ATL.

The patient in Figure 4B (no. 53) was initially diagnosed with AC and later progressed to chronic-type ATL. Although the initial clinical course was stable, an increase in abnormal lymphocyte numbers was later observed, and low-dose VP-16 therapy (50 mg/day) was initiated because of hypoxemia due to lung infiltration of ATL cells. Table S1 and Figure 4C show summaries of the clinical data and the flow cytometric analyses, respectively. The flow cytometric data correlated well with disease progression.

Discussion

Findings in our previous analysis of acute-type ATL samples prompted our analysis of various clinical subtypes of patients infected with HTLV-I to examine whether the CD3 versus CD7 profile reflects the progression of oncogenesis in HTLV-I-infected cells [17]. Representative flow cytometric data shown in Figure 1A suggested that the CD3 versus CD7 profile changed during disease progression. As the disease stage progressed, the D and L subpopulations increased with concomitant decreases in the CD3^{high}CD7^{high}(H) subpopulation. Figure 1C, a summary of the flow cytometric data of all cases analyzed, reveals that the two-dimensional plot of the proportions of the D versus L subpopulations could divide all cases into three groups. Group 1, the area under the diagonal line, equivalent to 55% of the H subpopulation in which all normal controls were included (Figure S2), contained the majority of HTLV-I ACs. Group 2, the area between the diagonal line and the 80% L subpopulation line, contained the majority of acute-type cases were included in this group. Group 3, the area beyond 80% of the L subpopulation, and the majority of acute-type cases were included in this group. Group 2, located between Groups 1 and 3 (i.e., less than 55% of the H subpopulation and 80% of the L

subpopulation), included indolent-type (smoldering and chronic) cases and some AC cases. These results suggest that the CD3 versus CD7 expression profile reflects disease stage. Initially, both the D and L subpopulations gradually and simultaneously increased. However, at the clinically advanced stage, the increase in the L subpopulation was prominent. The change is considered to reflect the biological difference between the D and L subpopulations, which needs to be clarified.

In HTLV-I infection, the small clones of infected cells are considered to coexist from the AC stage [19,20]. A selected clone from the multiple small clones then grows and progresses to the malignant state, and the emergence of a dominant clone indicates disease progression in ATL [19,20]. As shown in Figure 2B–D, major bands suggesting dominant clones were evident in patients with progressed clinical subtypes or those in the advanced group in the CD3 versus CD7 profile, and major bands existed exclusively in the D and L subpopulations. These data also support the idea that increases in the D and L subpopulations correlate with the progression of disease stage. AC cases in Group 2 had high HTLV-I proviral loads (>4%; Figure 1B) and clear major bands were observed by inverse long PCR in these cases (Figure 2B, right). Sasaki *et al.* reported that two cases of HTLV-I AC with oligoclonal bands on Southern blots and high VLs (20%) had progressed to ATL by 4 and 3.5 years later [21]. The two cases may correspond to HTLV-I AC in Group 2 proposed in our study. In fact, two cases of ACs in our series that were included in Group 2 progressed to smoldering ATL (Figure 4A). AC cases in Group 2 could be regarded as advanced carriers. Our flow cytometric analysis could apparently discriminate high-risk AC cases from stable ones. Follow-up analysis of these cases is warranted to determine whether AC cases included in Group 2 progress to ATL. Flow cytometric data for these AC cases included in Group 2 (Figure 1A and 1C) were similar to those for indolent ATL cases in Group 2. These ACs in Group 2 can be considered essentially the same as smoldering ATL cases. Some of the ACs categorized according to Shimoyama's criteria should in fact be separated and regarded as a subtype together with at least some of the smoldering ATL cases.

Iwanaga *et al.* reported that high HTLV-I proviral load (>4%) in whole PBMCs was a risk factor for progression to ATL [13]. In Figure 1B, the ACs with VLs>4% were distributed between Groups 1 and 2. These findings suggest that not all ACs with high VLs are currently in an advanced stage, although they may have the potential to develop ATL in the future.

In general, the categorization by flow cytometric profile correlated well with the current classification of clinical subtypes, with some exceptional cases of acute-type and smoldering-type disease (Figure 3). The only manifestation of three smoldering cases categorized in Group 1 was skin lesions; they fell into Group 1 because they showed minimal abnormalities in peripheral blood [22]. Three acute-type ATL cases categorized in Group 2 had indolent clinical courses. A diagnosis of acute-type disease is made when the indolent-type and lymphoma-type are excluded, according to Shimoyama's criteria. The CD3 versus CD7 plot may discriminate the cases that will follow an indolent clinical course from the aggressive acute-type ATL.

The VL in each subpopulation indicated that HTLV-I-infected cells were relatively concentrated in the D and L subpopulations (representative data are shown in Figure 2A). These data are consistent with downregulation of CD3 and CD7 being relevant to HTLV-I infection, although cells without HTLV-I infection may also contribute to this change to some extent, as a substantial subpopulation of T cells has been reported to be CD7-deficient under physiological [23,24] and certain pathological conditions,

including autoimmune disorders and viral infection [25–29]. To more precisely analyze phenotypic changes in HTLV-I-infected cells, markers that indicate HTLV-I infection should be incorporated in future studies.

A summary of this study is shown in Figure 5. In the CD3 versus CD7 profile, most AC cases were included in Group 1, in which the D and L subpopulations were relatively small. Consistent with disease progression to smoldering- and chronic-type ATL, a decrease in the H subpopulation and increases in the D and L subpopulations occur (Group 2). In this step, increases in the sizes of clones in the D and L subpopulations are observed. Further expansion of the leukemic clone results in progression to acute-type ATL in which the L subpopulation has expanded (Group 3). According to a study by Yamaguchi *et al.*, the natural course of ATL is to progress from the HTLV-I carrier state through the intermediate state, smoldering ATL, and chronic ATL, and finally to the acute ATL, indicating a process of multistage leukemogenesis [19]. We consider this study to successfully link the progressive clinical status and phenotypic changes in HTLV-I-infected cells. However, the way in which this profile reflects multistep oncogenesis in HTLV-I infection at the molecular level remains unclear. Further molecular analyses of the three subpopulations will help in understanding the mechanism(s).

Supporting Information

Figure S1 Representative flow cytometric analysis of an HTLV-I asymptomatic carrier (patient no. 32). The CD3 versus CD7 plot of CD4⁺ cells was constructed according to the gating procedure shown in this figure. In the plot, we designated three subpopulations: H (CD3^{high}CD7^{high}), D (CD3^{dim}CD7^{dim}), and L (CD3^{dim}CD7^{low}). (PPTX)

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Figure S2 A two-dimensional plot of 10 normal controls showing the percentage of the D and L subpopulations. (PPTX)

Figure S3 Southern blot analysis of clonal integration of the HTLV-I provirus. Representative data (AC, No. 34) are shown. In *EcoRI* or *PstI* digestion, a band indicated by a red arrow represents the monoclonal integration of the provirus. The band pattern indicates that two major clones coexist. This analysis was performed by a commercial laboratory (SRL, Tokyo, Japan). (PPTX)

Table S1 Clinical data in a case of chronic-type ATL (No. 53). Proportion of abnormal lymphocytes in the peripheral blood WBC were evaluated by morphological examination. LDH: Lactate dehydrogenase (normal range, 120–240 U/L) sIL-2R: soluble interleukin-2 receptor (normal range, 122–496 U/ml). (XLSX)

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Author Contributions

Conceived and designed the experiments: KT AT KU. Performed the experiments: SK YT. Analyzed the data: EW NW TI NO. Contributed reagents/materials/analysis tools: MI MT KU NO. Wrote the paper: SK KU.

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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)-1 β , 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 adult T-cell leukemia (ATL)	Diagnosed with HAM/TSP as defined by WHO criteria	

CLEIA = chemiluminescent enzyme immunoassay.
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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-1 β , 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 n = 30	Stable HAM/TSP n = 14	Deteriorating HAM/TSP 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.

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

	Total n = 23	Stable HAM/TSP n = 11	Deteriorating HAM/TSP 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.

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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-1 β , TNF- α , and IFN- γ were measured in

plasma samples from four ACs and four HAM/TSP patients. Plasma levels of IL-1 β and TNF- α 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

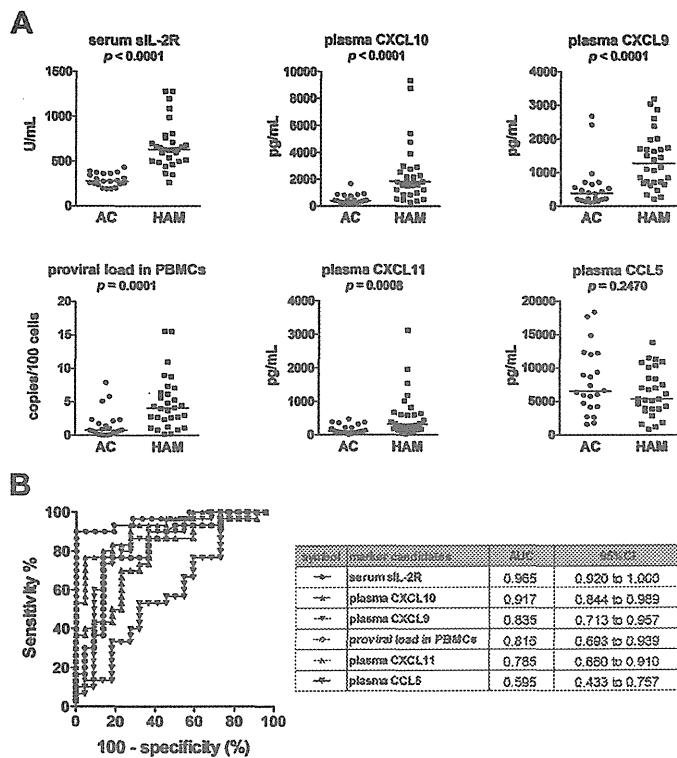


Figure 1. Selection of candidate biomarkers in the blood by comparing HAM/TSP patients and asymptomatic carriers. (A) Serum levels of soluble IL-2 receptor (sIL-2R), proviral loads in peripheral blood mononuclear cells (PBMCs), and plasma levels of four chemokines (chemokine (C-X-C motif) ligand (CXCL) 9, CXCL10, CXCL11, 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. doi:10.1371/journal.pntd.0002479.g001

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: \geq 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/

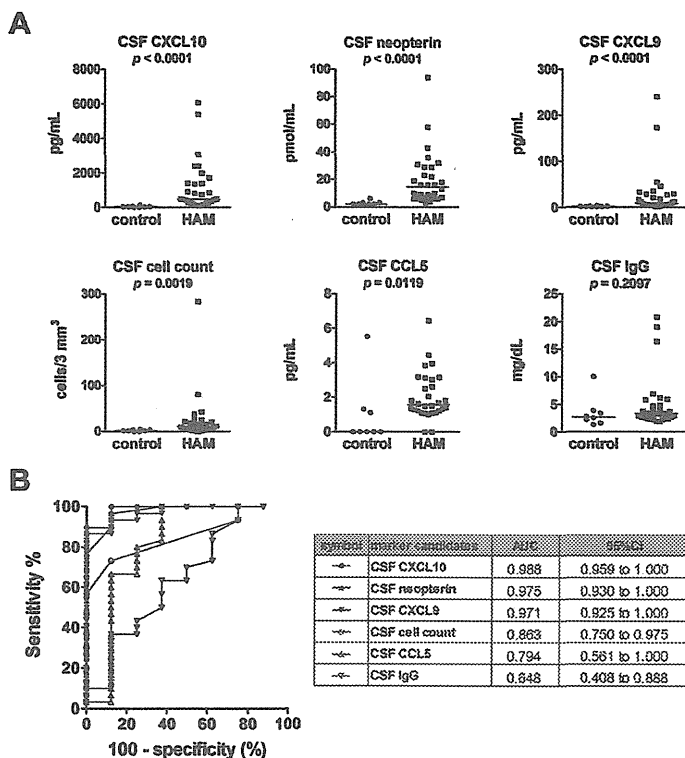


Figure 2. Selection of candidate biomarkers in the cerebrospinal fluid (CSF) by comparing HAM/TSP patients and control subjects. (A) CSF levels of total protein, cell count, IgG, neopterin, sIL-2R, and nine chemokines (CCL3, CCL4, CCL5, CXCL9, CXCL10, CXCL11, CCL17, CCL20, and CCL22) were measured and 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

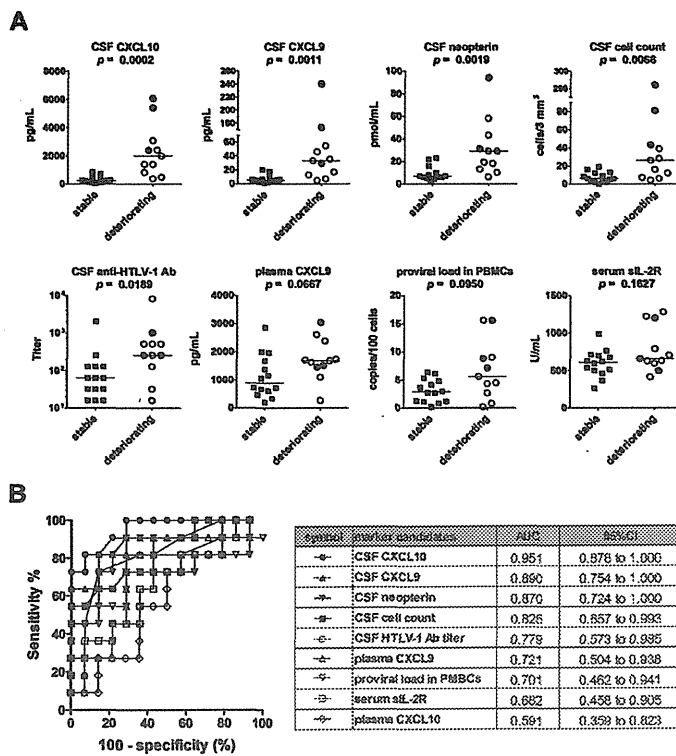


Figure 3. Identification of biomarkers associated with clinical progression of HAM/TSP. (A) Five CSF marker candidates (CXCL10, CXCL9, neopterin, cell count, and anti-HTLV-1 antibody titer) and four blood marker candidates (proviral load in PBMCs, serum sIL-2R, plasma CXCL9, and plasma CXCL10) were compared 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

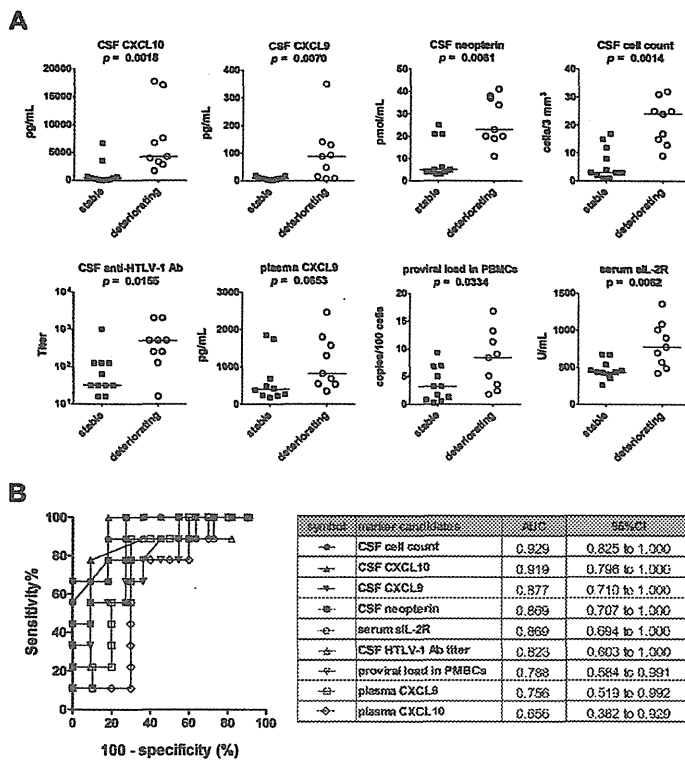


Figure 4. Validation of potential markers using the Test Set. (A) Five CSF marker candidates (CXCL10, CXCL9, neopterin, cell count, and anti-HTLV-1 antibody titer) and four blood marker candidates (proviral load in PBMCs, serum sIL-2R, plasma CXCL9, and plasma CXCL10) were compared among a second cohort 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

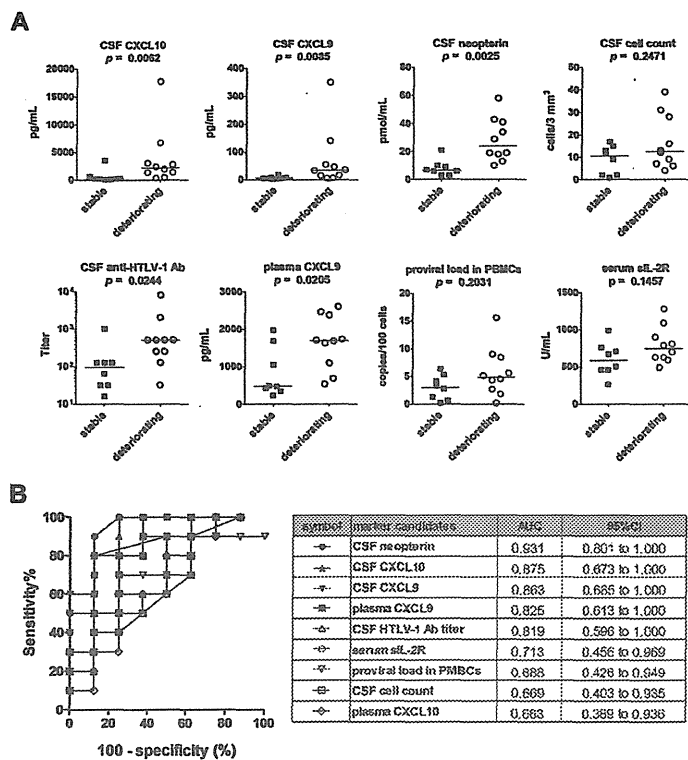


Figure 5. Comparison of potential markers in stable and deteriorating HAM/TSP patients with similar disease durations. (A) Five CSF marker candidates (CXCL10, CXCL9, neopterin, cell count, and anti-HTLV-1 antibody titer) and four blood marker candidates (proviral load in PMBCs, serum sIL-2R, plasma CXCL9, 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. doi:10.1371/journal.pntd.0002479.g005

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 $15/3 \text{ mm}^3$. 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 ($\leq 15/3 \text{ mm}^3$), 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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