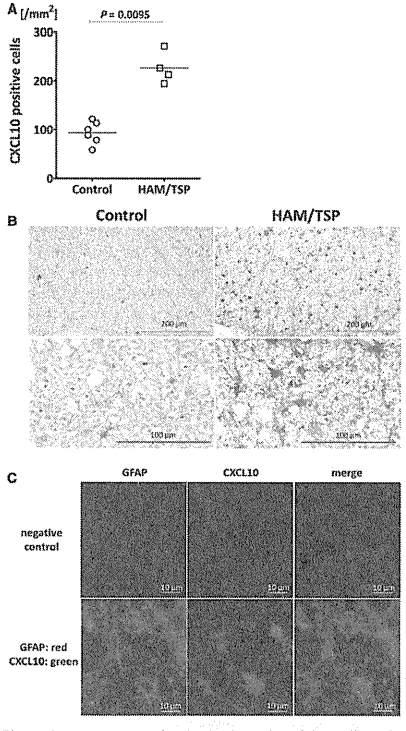


Figure 3 Astrocytes are the major CXCL10-producing cells in the spinal cords of patients with HAM/TSP. (**A**) Quantitative histological analysis (control: $n = 6$, HAM/TSP: $n = 4$). The numbers of CXCL10$^+$ cells in the spinal cord sections were counted under a microscope. The data represent the mean number of CXCL10$^+$ cells in three random fields of 1 mm$^2$ per sample. Horizontal bars represent the mean. Statistical analysis was performed using the Mann–Whitney U-test. (**B**) Representative immunohistochemistical images of CXCL10 in the thoracic spinal cord tissues from control individuals ($n = 6$) and patients with HAM/TSP ($n = 4$). CXCL10-positive cells are brown. *Upper* panel: low magnification; *Lower* panel: high magnification. (**C**) Representative immunofluorescent images of GFAP (red), a marker for astrocytes, and CXCL10 (green) in the thoracic spinal cord tissues from a control individual and a patient with HAM/TSP patient. Similar results were observed in images of spinal cord tissues obtained from two other patients with HAM/TSP.

HAM/TSP ($n = 4$) and control individuals ($n = 6$). CXCL10-positive staining was mainly observed in star-shaped cells with extensive and radiating cytoplasmic processes, indicating that CXCL10 is expressed in activated astrocytes in the thoracic spinal cord of patients with HAM/TSP (Fig. 3B). We also used immunofluorescence to confirm that CXCL10 is mainly expressed in astrocytes (GFAP$^+$ cells) (Fig. 3C).

## Co-culture with CD4$^+$ T cells from patients with HAM/TSP enhances CXCL10 production in U251 human astrocytoma cells

CXCL10, also known as an IFN-γ-inducible protein 10, is mainly produced in response to IFN-γ stimulation (Muller *et al.*, 2010). We used this fact to investigate the events leading to CXCL10 production by astrocytes in the spinal cords of patients with HAM/TSP. First, we compared the capacities of several purified cell populations within peripheral blood mononuclear cells to produce IFN-γ spontaneously, i.e. without any stimulation. We found that CD4$^+$ T cells exhibited the highest production of IFN-γ among peripheral blood mononuclear cells isolated from patients with HAM/TSP, and CD4$^+$ T cells from patients with HAM/TSP produced more IFN-γ than those from asymptomatic carriers (Fig. 4A and B left). No peripheral blood mononuclear cells isolated from healthy donors displayed any detectable level of IFN-γ production (data not shown). Interestingly, CD4$^+$ T cells from patients with HAM/TSP did not produce IL-17A, a proinflammatory cytokine known to play a key role in the pathogenic inflammatory response that characterizes multiple sclerosis (Fig. 4B, right) (Matusevicius *et al.*, 1999). Next, we used a co-culture system to confirm that CD4$^+$ T cells induce astrocytes to produce CXCL10 by releasing IFN-γ. CD4$^+$ T cells from patients with HAM/TSP induced CXCL10 production in U251 astrocytoma cells in a cell number-dependent manner (Fig. 4C), whereas CD4$^+$ T cells from healthy donors did not induce CXCL10 production (data not shown). Importantly, in the presence of anti-IFN-γ neutralizing antibodies, the supernatant from HAM/TSP patient CD4$^+$ T cell cultures stimulated significantly less CXCL10 production in U251 cells (Fig. 4D).

## Chemotaxis of peripheral blood mononuclear cells from patients with HAM/TSP due to CXCL10 and inhibition of chemotaxis by anti-CXCL10 neutralizing antibodies

To investigate the potential role of CXCL10 or CXCR3 as a therapeutic target for inhibiting the migration of proinflammatory cells into the CNS, we assessed whether neutralizing antibodies against CXCL10 or CXCR3 could inhibit the migration of peripheral blood mononuclear cells in patients with HAM/TSP through the use of an *in vitro* chemotaxis assay system. Human CXCL10 increased the chemotactic activity of peripheral blood mononuclear cells from patients with HAM/TSP by ~1.7-fold (Fig. 5A). Compared with isotype control monoclonal antibodies, the chemotactic activity due to CXCL10 was inhibited by anti-CXCL10 neutralizing antibodies (Fig. 5A; 65.9% inhibition, $P < 0.01$) but not by anti-CXCR3 antibodies (Fig. 5A; 9.2% inhibition, $P > 0.05$: not





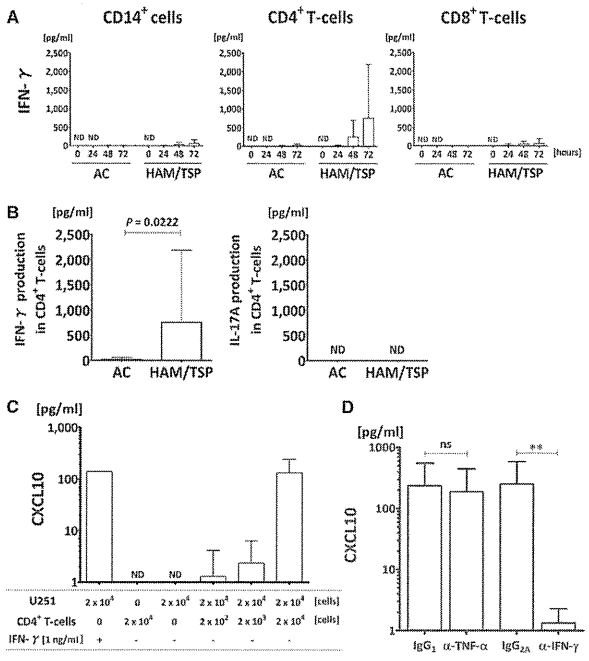


Figure 4 Co-culturing with CD4$^+$ T cells from patients with HAM/TSP increases CXCL10 production in a U251 human astrocytoma cell line. (**A**) Concentration of IFN-γ in supernatants of cultured CD4$^+$, CD8$^+$, or CD14$^+$ cells in peripheral blood mononuclear cells (PBMCs) from patients with HAM/TSP ($n = 6$) compared with that in peripheral blood mononuclear cells from asymptomatic carriers (AC, $n = 5$). These cells were cultured without any stimuli for 24, 48 and 72 h. ND = not detected. (**B**) Concentrations of IFN-γ (*left*) and IL-17A (*right*) in culture supernatants of cultured CD4$^+$ T cells for 72 h from patients with HAM/TSP compared with the concentrations in those from asymptomatic carriers. Patients with HAM/TSP: $n = 6$, asymptomatic carrier: $n = 5$. ND = not detected. Statistical analyses were performed using the Mann–Whitney U-test. (**C**) Concentration of CXCL10 produced by U251, a human astrocytoma cell line, co-cultured with CD4$^+$ T cells from patients with HAM/TSP ($n = 5$). ND = not detected. (**D**) Concentration of CXCL10 produced by U251 stimulated by the supernatant of cultured CD4$^+$ T cells of patients with HAM/TSP ($n = 5$) in the presence of neutralizing antibodies against IFN-γ and TNF-α, and isotype control antibodies for each. NS = not significant. $^{**}P < 0.01$. Error bars represent the mean ± SD.

significant). Next, we investigated whether or not this decreased migration would also be reflected in the absolute number of HTLV-1-infected cells among migrated cells. Chemotaxis assays revealed that the addition of human CXCL10 (0.25 μg/ml) increased the absolute number of HTLV-1-infected cells by ~2.1-fold (Fig. 5B) compared with isotype control monoclonal antibodies, and that this increase was largely inhibited by anti-CXCL10 neutralizing antibodies (Fig. 5B; 101.1% inhibition, $P < 0.01$) but only slightly by anti-CXCR3 antibodies (Fig. 5B; 65.7% inhibition, $P > 0.05$: not significant). Finally, we evaluated the degree to which the migrated cells were proliferating spontaneously, where spontaneous proliferation is defined as proliferation in the absence of exogenous antigens or stimulants (Itoyama *et al.*, 1988; Ijichi *et al.*, 1989). This is important because the level of spontaneous proliferation of peripheral blood mononuclear cells in patients with HAM/TSP is believed to reflect the cell proliferation that occurs in the CNS (Itoyama *et al.*, 1988; Ijichi *et al.*, 1989). Significantly less $^3$H-thymidine uptake, an assay for cell proliferation, was detected in the lower chemotaxis assay chamber following administration of anti-CXCL10 antibody than isotype control antibodies (Fig. 5C; 33.8% inhibition, $P < 0.05$).

# Discussion

Previous studies of HAM/TSP pathogenesis have revealed that chronic inflammation occurs in the spinal cords of patients with HAM/TSP (Saito and Bangham, 2012; Yamano and Sato, 2012);

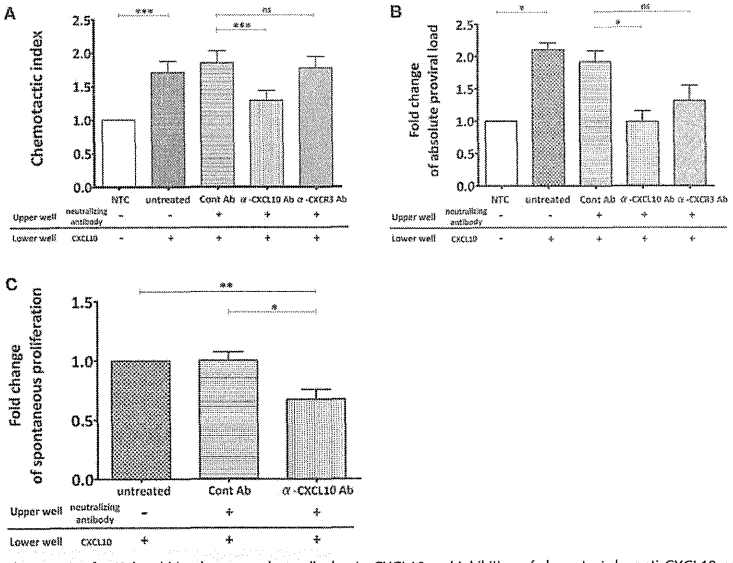


**Figure 5** Chemotaxis of peripheral blood mononuclear cells due to CXCL10 and inhibition of chemotaxis by anti-CXCL10 neutralizing antibody in cells from patients with HAM/TSP. **(A)** The migration-inducing effect of CXCL10 and the inhibitory effect of neutralizing antibody against CXCL10 versus its receptor, CXCR3. Peripheral blood mononuclear cells from patients with HAM/TSP ($n = 21$) migrated into the lower well in response to CXCL10, and treatment with anti-CXCL10 antibody significantly reduced the migration of peripheral blood mononuclear cells, as compared to anti-CXCR3 antibody and control antibody. **(B)** The inhibition of cell migration led to an overall decrease in migrated cells including HTLV-1-infected cells, effectively decreasing the absolute proviral load. Peripheral blood mononuclear cells used were collected from patients with HAM/TSP; $n = 4$. **(C)** The inhibition of cell migration led to an overall decrease in migrated cells which also means less spontaneous proliferation. Peripheral blood mononuclear cells used were collected from patients with HAM/TSP; $n = 7$. Error bars represent the mean ± SD. Statistical analyses were performed using the Friedman test followed by the Dunn test for multiple comparison. NS = not significant. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NTC = negative control; Cont Ab = isotype control monoclonal antibody; α-CXCL10 Ab = anti-CXCL10 monoclonal antibody; α-CXCR3 Ab = anti-CXCR3 monoclonal antibody.

however, the precise mechanisms by which these inflammatory lesions are formed and maintained remain unclear. We hypothesized that a positive feedback loop driven by chemokines may be responsible for the chronic inflammation associated with HAM/TSP. We identified CXCL10 as the principal chemokine responsible for inducing this chronic inflammation. We found for the first time astrocytes to be the main producers of CXCL10. Our data suggest that these astrocytes are stimulated to produce CXCL10 initially by IFN-γ released by infected T cells, where the infection appears to have produced changes in the cells that promote spontaneous IFN-γ production. In short, we inferred that spinal cord lesions found in patients with HAM/TSP arise when IFN-γ produced by HTLV-1-infected T cells induces astrocytes to secrete CXCL10, which attracts $CXCR3^+$ T cells, including more T cells infected with HTLV-1, thereby continuing the cycle. Furthermore, we demonstrated that an interruption of this pathway represents a promising strategy for treating HAM/TSP.

First, we identified the key chemokine involved in inducing the migration of cells to sites of inflammation. We compared the CSF and serum levels of several chemokines and demonstrated for the first time that CXCL10 is the only chemokine of those studied that is present at a significantly higher concentration in the CSF than in the sera of patients with HAM/TSP. Although previous reports indicate that CCL5 and CXCL9 levels are also elevated in the CSF of patients with HAM/TSP (Teixeira *et al.*, 2004; Tanaka *et al.*, 2008), we showed that these two chemokines exhibit a concentration gradient in the opposite direction (Fig. 1B). We previously measured the levels of other chemokines such as CCL3, CCL4, CXCL11, CCL17, CCL20 and CCL22 in the CSF and found that the levels of these chemokines are negligible in patients with HAM/TSP (Sato, in press). Importantly, we also previously demonstrated that CSF CXCL10 levels are correlated with the rate of disease progression (Sato, in press). These findings suggest that CXCL10 is crucial for the development of chronic inflammation in patients with HAM/TSP.





In the present study, we found a positive correlation between CSF CXCL10 levels and CSF cell counts (Fig. 1A), a high percentage of CXCR3-positive cells in the CSF (Fig. 2A and B; 92.4 ± 7.0%), and perivascular accumulation of $CXCR3^+$ cells in spinal cord lesions of patients with HAM/TSP (Fig. 2C). These results strongly indicate that a high concentration of CXCL10 in the spinal cord attracts $CXCR3^+$ cells that include proinflammatory cells (Qin *et al.*, 1998; Sallusto *et al.*, 1998; Thomas *et al.*, 2003). Intriguingly, the percentage of $CXCR3^+$ cells among peripheral blood mononuclear cells from patients with HAM/TSP was significantly lower than that observed in asymptomatic carriers and healthy donors, but not patients with adult T cell leukaemia (Supplementary Fig. 3). $CXCR3^+$ peripheral blood mononuclear cells are relatively few in patients with adult T cell leukaemia, perhaps because of an increase in $CCR4^+CXCR3^-$ tumour cells in the peripheral blood (Ishida *et al.*, 2003). Although the precise mechanism by which peripheral $CXCR3^+$ cells in patients with HAM/TSP become diminished remains unclear, we believe that many of these cells migrate into the CNS and contribute to the formation of spinal cord lesions. Other possible mechanisms include migration to lymphoid organs such as lymph nodes or the spleen. Because lymph nodes are important organs for CXCL10–CXCR3 interactions in patients suffering from various diseases (Groom *et al.*, 2012; Sung *et al.*, 2012), future studies analysing the lymph nodes of patients with HAM/TSP may provide a more complete understanding of HAM/TSP pathogenesis.

The discovery that CXCL10–CXCR3 interactions represent an important pathway for recruiting cells to the CNS in patients with HAM/TSP prompted us to search the spinal cord lesions of patients with HAM/TSP and identify the CXCL10-producing cells. Firstly, we confirmed that CXCL10-producing cells are more numerous in the spinal cords of patients with HAM/TSP than control individuals (Fig. 3A). We also compared high and low inflammatory regions within a single patient with HAM/TSP and found more CXCL10-producing cells in the more inflamed region (Supplementary Fig. 4), although the limitation of sampling from only a single individual prevents us from extrapolating too freely on the significance of this result. Although CXCL10 is secreted by several cell types such as monocytes, endothelial cells, fibroblasts and astrocytes in response to IFN-γ (Luster and Ravetch, 1987; Lee *et al.*, 2009), our study demonstrated that astrocytes are the major CXCL10-producing cells in thoracic spinal cord lesions in patients with HAM/TSP (Fig. 3). Notably, the astrocytes examined in this study were star-shaped with radiating cytoplasmic processes, indicating high cytological activity (Fig. 3B and C). In the CNS, CXCL10 is mainly produced by astrocytes; however, CXCL9 is primarily a product of microglial cells (Muller *et al.*, 2010). Therefore, the finding that CXCL10 production is substantially higher than CXCL9 production in the CSF (Fig. 1) suggests that astrocytes are very active in HAM/TSP. This finding supports a previous finding that gliosis is one of the main pathological features of HAM/TSP (Iwasaki, 1990; Izumo *et al.*, 1992).

Next, we investigated the mechanism by which astrocytes produce CXCL10 in patients with HAM/TSP. CXCL10 is generally not detectable in most non-lymphoid tissues under physiological conditions; however, its synthesis is easily induced by cytokines, particularly IFN-γ. Therefore, it was important to determine the source of IFN-γ that stimulates astrocytes to produce CXCL10 in patients with HAM/TSP. Interestingly, we have shown that $CD4^+$ T cells from patients with HAM/TSP spontaneously produce IFN-γ and induce CXCL10 production by U251 human astrocytoma cells via IFN-γ (Fig. 4), whereas $CD4^+$ T cells from healthy donors do not induce CXCL10 production (data not shown). These results support the hypothesis that there are interactions between HTLV-1-infected $CD4^+$ T cells and astrocytes in patients with HAM/TSP *in vivo* that may possibly initiate the first wave of CXCL10 production. Moreover, this CXCL10 production may further induce the trafficking of peripheral $CXCR3^+$ T cells. Importantly, we demonstrated that a number of peripheral $CXCR3^+$ T cells are infected with HTLV-1 (Fig. 2E), indicating that migration of peripheral $CXCR3^+$ T cells into the CNS can induce further secretion of IFN-γ that continues the vicious cycle. In fact, HTLV-1-infected $CD4^+$ T cells and IFN-γ-producing T cells have been detected in HAM/TSP spinal cord lesions (Umehara *et al.*, 1994; Moritoyo *et al.*, 1996; Matsuoka *et al.*, 1998). Notably, more than half of the $CXCR3^+$ T cells in the CSF of patients with HAM/TSP are $CD8^+$ T cells (Fig. 2D). It has been shown that $CD8^+$ cytotoxic T lymphocytes (CTLs), particularly HTLV-1-specific CTLs, have a high potential for secreting IFN-γ (Kubota *et al.*, 2000; Hanon *et al.*, 2001) and are abnormally elevated in the CSF and spinal cord lesions (Nagai *et al.*, 2001*a*, *b*; Matsuura *et al.*, 2010). Therefore, CXCL10 production by astrocytes may further boost the trafficking of $CXCR3^+$-infected $CD4^+$ T cells as well as $CXCR3^+CD8^+$ CTLs that secrete IFN-γ, leading to a positive feedback-driven chronic inflammatory loop.

The results of the present study and other studies show that the pathology of HAM/TSP is unique among immune disorders. Unlike other inflammatory disorders such as multiple sclerosis or rheumatoid arthritis that exhibit Th17 as well as Th1 involvement (Matusevicius *et al.*, 1999; Kirkham *et al.*, 2006), HAM/TSP pathogenesis appears to be dominated by the Th1 axis, particularly CXCL10–CXCR3 interactions. Our research indicates that the characteristics of HTLV-1-infected T cells may be responsible for the emphasis on the Th1 axis in HAM/TSP pathogenesis. We have reported that cultured $CD4^+$ T cells from patients with HAM/TSP clearly exhibit detectable production of IFN-γ (a Th1 cytokine) but not IL-17 (a Th17 cytokine) (Fig. 4B), and we previously demonstrated that HTLV-1-infected T cells in patients with HAM/TSP exhibit elevated IFN-γ and reduced IL-17 production (Yamano *et al.*, 2009). Furthermore, HAM/TSP peripheral blood contained more CXCL10 (Supplementary Fig. 1B) and fewer $CXCR3^+$ cells (Supplementary Fig. 3B) than asymptomatic carrier blood, suggesting that a greater number of $CXCR3^+$ cells had migrated out of the periphery due at least in part to chemotaxis induced by elevated CXCL10 production in the CNS. As the proviral loads of all the samples used in the above experiment were roughly identically high, it can be assumed that these characteristics are indeed features of HAM/TSP pathogenesis as opposed to simple consequences of having a high proviral load.

We suspect that a genetic predisposition for higher IFN-γ or CXCL10 production in response to HTLV-1 may exist. Recently, systems biology approaches were used to show that a subset of IFN-stimulated genes, including the gene encoding CXCL10, is overexpressed in peripheral blood mononuclear cells of patients with HAM/TSP compared with asymptomatic carriers

(Tattermusch *et al.*, 2012). It will be important to test for an association between genetic polymorphisms in interferon-associated genes and the presence of HAM/TSP in future studies. The existence of this genetic predisposition would strengthen the argument for Th1-dominance and explain why some infected individuals develop HAM/TSP, whereas others remain life-long asymptomatic carriers. Because it is well-known that interferons and products of interferon-stimulated genes mediate antiviral responses (Randall and Goodbourn, 2008), IFN-γ and CXCL10 production in HTLV-1-infected patients (Supplementary Fig. 1) may be considered a *normal immune response. However, once the production levels* surpass threshold and a CXCL10–CXCR3 amplification loop develops, it may begin to cause tissue damage. Possible reasons for CXCL10 overproduction in HAM/TSP include the presence of a high number of HTLV-1-infected T cells (Nagai *et al.*, 1998; Yamano *et al.*, 2002) and a genetic predisposition for higher IFN-γ and/or CXCL10 production in response to HTLV-1.

*The ideal therapeutic strategy for treating HAM/TSP would be* eradication of HTLV-1-infected cells, but this has yet to be proven possible. Another promising approach would be a receptor blockade using anti-CXCR3 neutralizing antibody, which has been reported to be effective at blocking CXCR3 activity (Van den Steen *et al.*, 2008). Although we were unable to validate this effect using our commercially available antibody, this certainly does not rule out a receptor blockade as a therapeutic candidate. Our relative success at disrupting inflammatory cell migration using anti-CXCL10 neutralizing antibodies (Fig. 5) suggests that targeting CXCL10 to interrupt *the positive feedback loop may be the more promising new strategy* for effectively treating HAM/TSP. A noteworthy potential advantage of anti-CXCL10 over anti-CXCR3 is that it may yield less severe side effects as only interactions with CXCL10 rather than all CXCR3 agonists would be blocked.

In conclusion, our data revealed novel insights into the pathogenic processes of HAM/TSP. Our results suggest that CXCL10 plays a pivotal role in the development of chronic inflammatory lesions where HTLV-1-infected T cells produce IFN-γ, which induces astrocytes to secrete CXCL10. This further boosts the trafficking of $CXCR3^+$-infected T cells that secrete IFN-γ, *leading to a virus-*induced CXCL10–CXCR3 inflammatory loop. Thus, HAM/TSP represents a pathological consequence of interactions that occur between the immune system and CNS. Understanding these complex interactions should provide new insights into the functional regulation of both systems and help uncover new therapeutic targets.



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## Supplementary material

Supplementary material is available at *Brain* online.

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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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## 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.

## 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 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, ètc.) | | |
| | 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.
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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-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: GenProbe, 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.
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**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.
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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.

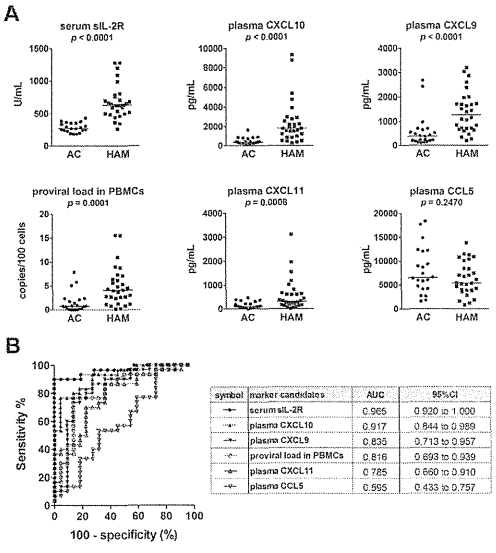
# Results

## 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-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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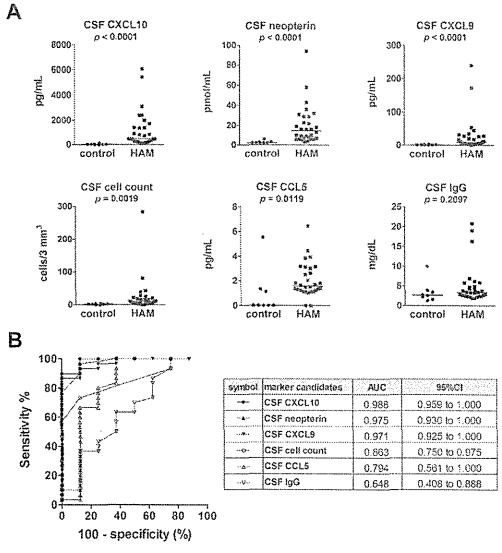


| symbol | marker candidates | AUC | 95%CI |
|---|---|---|---|
| | CSF CXCL10 | 0.988 | 0.959 to 1.000 |
| | CSF neopterin | 0.975 | 0.930 to 1.000 |
| | CSF CXCL9 | 0.971 | 0.925 to 1.000 |
| | CSF cell count | 0.863 | 0.750 to 0.975 |
| | CSF CCL5 | 0.794 | 0.581 to 1.000 |
| | CSF IgG | 0.648 | 0.408 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.
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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/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.

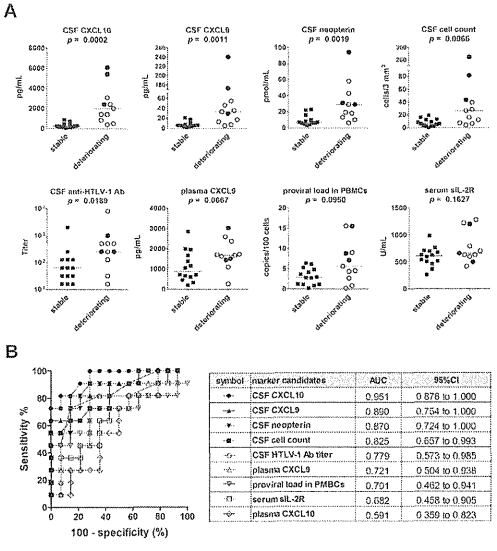
To investigate the potential influence of disease duration as a secondary variable, a new test group was created containing only those patients for whom the disease onset date was 7–13 years prior to the sample collection day. Patients fitting this criterion were selected from the 53 total available 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 | 0.860 | 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.
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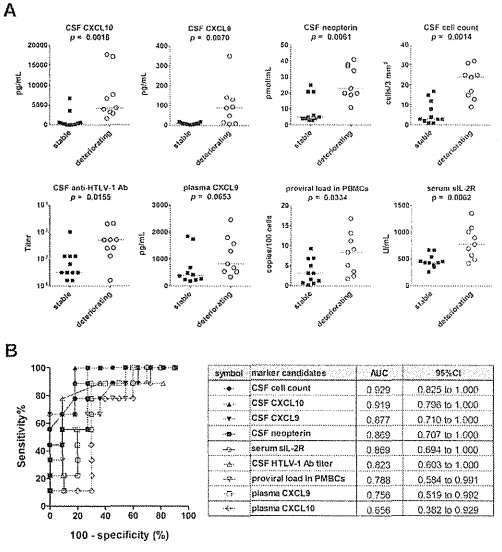


| symbol | marker candidates | AUC | 95%CI |
|---|---|---|---|
| | CSF cell count | 0.929 | 0.825 to 1.000 |
| | CSF CXCL10 | 0.919 | 0.798 to 1.000 |
| | CSF CXCL9 | 0.877 | 0.710 to 1.000 |
| | CSF neopterin | 0.869 | 0.707 to 1.000 |
| | serum sIL-2R | 0.869 | 0.694 to 1.000 |
| | CSF HTLV-1 Ab titer | 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.
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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].

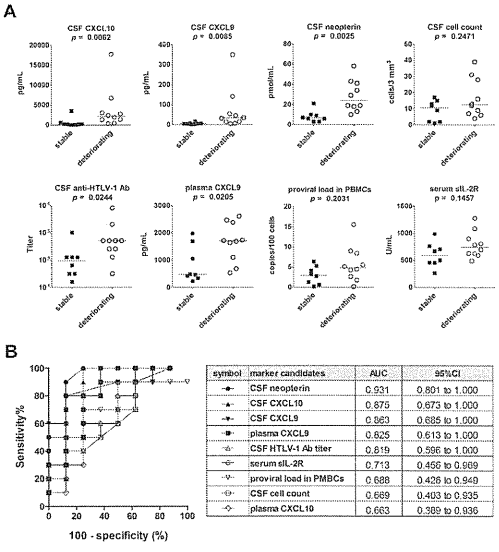
The HTLV-1 proviral load in the CSF as well as the ratio of the proviral load in the CSF to that in PBMCs have been reported to be effective for discriminating HAM/TSP patients from ACs or multiple sclerosis patients infected with HTLV-1 [38,39]. Some researchers have suggested that these values might 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 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 | 0.625 | 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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Regular Article

LYMPHOID NEOPLASIA

# Preapoptotic protease calpain-2 is frequently suppressed in adult T-cell leukemia

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**Key Points**

- Proteome-wide analysis of HTLV-1–infected T cells identified 17 biomarker proteins for the diagnosis of ATL or HAM/TSP patients.

**Adult T-cell leukemia (ATL) is one of the most aggressive hematologic malignancies caused by human T-lymphotropic virus type 1 (HTLV-1) infection. The prognosis of ATL is extremely poor; however, effective strategies for diagnosis and treatment have not been established. To identify novel therapeutic targets and diagnostic markers for ATL, we employed focused proteomic profiling of the $CD4^+CD25^+CCR4^+$ T-cell subpopulation in which HTLV-1–infected cells were enriched. Comprehensive quantification of 14 064 peptides and subsequent 2-step statistical analysis using 29 cases (6 uninfected controls, 5 asymptomatic carriers, 9 HTLV-1–associated myelopathy/tropical spastic paraparesis patients, 9 ATL patients) identified 91 peptide determinants that statistically classified 4 clinical groups with an accuracy rate of 92.2% by cross-validation test. Among the identified 17 classifier proteins, α-II spectrin was drastically accumulated in infected T cells derived from ATL patients, whereas its digestive protease calpain-2 (CAN2) was significantly downregulated. Further cell cycle analysis and cell growth assay revealed that rescue of CAN2 activity by overexpressing constitutively active CAN2 ($\Delta_{19}$CAN2) could induce remarkable cell death on ATL cells accompanied by reduction of α-II spectrin. These results support that proteomic profiling of HTLV-1–infected T cells could provide potential diagnostic biomarkers and an attractive resource of therapeutic targets for ATL. (*Blood.* 2013;121(21):4340-4347)**

## Introduction

Human T-lymphotropic virus type 1 (HTLV-1) is a human retrovirus that is the pathogenic agent of HTLV-1–associated diseases, such as adult T-cell leukemia (ATL) and HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP). Recent epidemiological studies revealed that HTLV-1 is endemic mainly in Japan, the Caribbean basin, Iran, Africa, South America, and the Melanesian islands.[1] Other estimates have shown that 20 million to 30 million people worldwide are infected with HTLV-1.[2] The infection is followed by a prolonged asymptomatic phase of 20 to 30 years, and 2% to 5% of the infected individuals develop ATL during their lifetime.[3] ATL is one of the most aggressive hematologic malignancies characterized by increased numbers of lymphocytes with multilobulated nuclei, so-called flower cells, in blood circulation. The prognosis is severe with the median overall survival period and 5-year survival rate of ATL patients of 7 months and 20%, respectively.[4] Recently, humanized anti-CCR4 (KW-0761) therapeutic antibody achieved a great improvement in ATL treatment in a phase 3 study. However, the disease control rate was restricted to 50%, and long-term prognosis has yet to be known.[5] For future improvements in the management of ATL, novel biomarkers for early diagnosis are urgently needed for early therapeutic intervention.

To date, comprehensive genomic or proteomic studies using $CD4^+$ T cells have been performed for this purpose,[6-9] but reproducibility and reliability of quantification results in the discovery phase were uncertain due to the diverse individual variety of HTLV-1–infected cell contents in $CD4^+$ T cells. To overcome the etiologic variety of samples, we focused on the $CD4^+CD25^+CCR4^+$ T-cell subpopulation since Yamano et al[10] recently revealed that HTLV-1 preferentially infected $CD4^+CD25^+CCR4^+$ T cells in both ATL and HAM/TSP patients. By targeting $CD4^+CD25^+CCR4^+$ T cells, we here provide the first quantitative proteome map illustrating molecular disorders in pathogenic human T cells directly associated with the onset or progression of ATL. The comprehensive and comparative interpretation of total proteome in infected cells, especially between asymptomatic HTLV-1 carriers and ATL patients, could immediately lead to specific candidates for biomarkers and drugs.

Another challenge to emphasize in this study is our recently established proteomic profiling technologies. It is indisputable that the greater the number of clinical samples analyzed, the more confidently statistical analysis can be undertaken in order to identify diagnostic markers and druggable targets. Despite this fact, previous proteomics reports could not provide high-throughput quantitative methodologies that were sufficient for dealing with even more than 10 clinical samples, excepting a study utilizing a surface enhanced laser desorption/ionization time of flight mass spectrometer. Although the surface enhanced laser desorption/ionization time of flight method drastically improved the performance in both quantification and throughput, allowing relative quantification

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analysis for 96 samples in several hours, at most only 250 unidentified protein peaks were detectable. In the present study, we integrated the proteomics server for the huge data set "Expressionist" (Genedata A.G., Basel, Switzerland) with high-end mass spectrometers to maximize the quality and quantity of protein catalogs transferred from mass spectrometers. We first describe the discovery phase providing a panel of novel diagnostic molecules from quantification of 14 064 peptides and identification of 4763 proteins. As the functional validation phase, we further examined the physiological potential of an identified diagnostic marker candidate, calpain-2 (CAN2), particularly concerning the association of its activity with survival or progression of ATL cells.

## Materials and methods

### PBMCs and cell lines

Peripheral blood mononuclear cells (PBMCs) from 6 normal donors, 5 asymptomatic carriers, and 9 HAM/TSP patients used in the screening analysis were collected in the St. Marianna University School of Medicine. Those from 9 ATL patients were collected in the Imamura Bun-in Hospital. PBMCs from 4 ATL patients used for the validation experiments were provided by the Joint Study on Predisposing Factors of ATL Development. The others from 4 HAM/TSP patients were collected in the St. Marianna University School of Medicine. The use of these human specimens in this study was approved by individual institutional ethical committees: the Ethical Committee of Yokohama Institute, RIKEN (approval code Yokohama H22-3); the Ethical Committee of St. Marianna University School of Medicine; the Institutional Review Board of Imamura Bun-in Hospital; and the Ethical Committee of the University of Tokyo (approval code 10-50). This study was conducted in accordance with the Declaration of Helsinki.

SO-4, KOB, and KK1 cells were kindly provided by Dr Yasuaki Yamada, cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Cell Culture Bioscience, Tokyo, Japan), 100 kU/L interleukin 2 (Cell Science & Technology Institute Inc., Tokyo, Japan), and 1 × antibiotic-antimycotic solution (Sigma-Aldrich, MO). Jurkat, SUP-T1, CCRF-CEM, and MOLT-3 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1 × antibiotic-antimycotic solution. All cell lines were grown at 37°C in 5% $CO_2$. $CD3^+CD4^+CD25^+CCR4^+$ T cells were isolated with anti-CD3-FITC (eBioscience, San Diego, CA), anti-CCR4-PE (Becton Dickinson, CA), anti-CD4-Cy7 (eBioscience), and anti-CD25-APC (eBioscience) on a Cell Sorter JSAN (Bay Bioscience, Hyogo, Japan).

### Sample preparation for mass spectrometric analysis

The $CD4^+CD25^+CCR4^+$ T cells were washed with phosphate-buffered saline 3 times and lysed in denaturation buffer (8 M urea in 50 mM ammonium bicarbonate). After sonication, reduction with 5 mM tris(2-carboxyethyl) phosphine (Sigma-Aldrich) at 37°C for 30 minutes, and alkylation with 25 mM iodacetamide (Sigma-Aldrich) at room temperature for 45 minutes, lysates were digested with Trypsin GOLD (Promega, WI) with protein/enzyme ratio of 25:1 at 37°C for 12 hours. The digested peptides were desalted with Oasis HLB μElution plate (Waters, MA). The collected samples were dried up with a Vacuum Spin Drier (TAITEC Co. Ltd., Saitama, Japan) and subjected to mass spectrometric analyses.

### Liquid chromatography tandem mass spectrometry (LC/MS/MS)

The digested peptides were separated on a 0.1 × 200 mm homemade $C_{18}$ column using a 2-step linear gradient, 2% to 35% acetonitrile for 95 minutes and 35% to 95% acetonitrile for 15 minutes in 0.1% formic acid with a flow rate of 200 nL/min. The eluting peptides were analyzed with a QSTAR-Elite mass spectrometer (AB Sciex, CA) in the smart information-dependent acquisition mode of Analyst QS software 2.0 (AB Sciex). The other parameters on QSTAR-Elite were shown as follows: DP = 60, FP = 265, DP2 = 15, CAD = 5, IRD = 6, IRW = 5, curtain gas = 20, and ion spray voltage = 2000 V.

### Two-dimensional (2D) LC/MS/MS

Tryptic digests of $CD4^+CD25^+CCR4^+$ T cells were dissolved in 10 mM ammonium formate in 25% acetonitrile and fractionated by a 0.2 × 250 mm monolith strong cation exchange column (GL Science, Tokyo, Japan). Peptides were eluted with an ammonium formate gradient from 10 mM to 1 M in curve = 3 mode for 70 minutes using a Prominence high-performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan). The eluate was fractionated into 20 fractions and analyzed individually by LTQ-Orbitrap-Velos mass spectrometer (Thermo Scientific, Bremen, Germany) accompanied with the Ultimate 3000 nano-HPLC system. The fractionated peptide samples were separated with the same gradient used in the QSTAR-Elite system described previously and analyzed by LTQ-Orbitrap-Velos acquiring a full MS scan on Fourier-transition mode with MS resolution = 60 000 and simultaneously MS/MS scans for the 20 most intense precursor ions in each MS spectrum on ion-trap mode with regular resolution. Other important parameters for LTQ-Orbitrap-Velos were as follows: capillary temp = 250, source voltage = 2 kV, MS scan range = mass-to-charge ratio (m/z) 400 to 1600, acquire data dependent CID MS/MS for top-20 intense precursors, and dynamic exclusion enabled during 30 seconds. For protein identification, all MS/MS spectra were searched against SwissProt database version 2012_06 (20 232 human protein sequences) using SEQUEST algorithm on ProteomeDiscoverer 1.3 software (Thermo Scientific) with the following parameters: MS tolerance = 3 ppm, MS/MS tolerance = 0.8 Da, maximum missed cleavages = 2, enzyme = trypsin, taxonomy = *Homo sapiens*, fixed modification = carbamidomethylation on cysteine, and variable modification = oxidation on methionine. We accepted the protein identification satisfying the false discovery rate <1% by Percolator false discovery rate estimation algorithm on ProteomeDiscoverer.

### Label-free quantification analysis

The LC/MS/MS raw data were imported into the Expressionist RefinerMS module and subjected to the following data processing and relative quantification steps. The total work flow on the RefinerMS module is shown in supplemental Figure 1 (see the *Blood* Web site). The LC/MS/MS raw data set from 29 clinical samples was displayed in 2D planes (m/z vs retention time [RT]). The chromatogram grid was applied to all planes: scan counts = 10, polynom order = 3, and RT smoothing = 0. The planes were simplified by subtracting background noises using chromatogram chemical noise subtraction: RT window = 50 scans, quantile subtraction = 50%, and RT smoothing = 3 scans. After the noise subtraction, data points with intensity <10 were clipped to zero. The RT variety among 29 planes was adjusted by chromatogram RT alignment: RT transformation window = 0.2 minutes, RT search interval = 5 minutes, m/z window = 0.1 Da, and gap penalty = 1. Peaks were detected by chromatogram summed peak detection: summation window = 5 scans, overlap = 50, minimum peak size = 4 scans, maximum merge distance = 10 points, peak RT splitting = true, intensity profiling = max, gap/peak ratio = 1%, refinement threshold = 5, consistency threshold = 0.8, and signal/noise threshold = 1. The detected peaks were grouped into isotopic clusters derived from each molecule using 2-step chromatogram isotopic peak clustering. The first parameters were as follows: minimum charge = 1, maximum charge = 10, maximum missing peaks = 0, first allowed gap position = 3, RT window = 0.1 minute, m/z tolerance = 0.05 Da, isotope shape tolerance = 10, and minimum cluster size ration = 1.2. The second parameters were as follows: minimum charge = 1, maximum charge = 10, maximum missing peaks = 0, first allowed gap position = 3, RT window = 0.1 minute, m/z tolerance = 0.05 Da, and minimum cluster size ration = 0.6.

### Expression vectors and siRNA

For the $\Delta_{19}$CAN2 construct, the *CAPN2* fragment was amplified with primers 5′-CATGTCGACTCCCACGAGAGGGCCATCAAGT-3′ and 5′-CATTCTAGATCAAAGTACTGAGAAACAGAGCC-3′ from pBlueBacIII *CAPN2* and cloned into pEFBOS-Myc. Prior to the overexpression experiments, we confirmed that the sequence of the inserted *CAPN2* fragment was identical to the Mammalian Gene Collection sequence (accession number

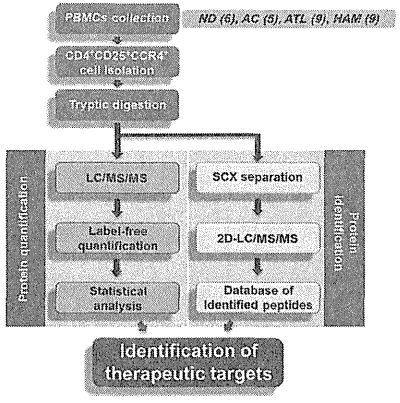


**Figure 1. Schematic overview of proteomic profiling for $CD4^+CD25^+CCR4^+$ cells.** PBMCs were collected from 6 normal donors, 5 asymptomatic carriers, 9 ATL patients, and 9 HAM/TSP patients, followed by isolation of the $CD4^+CD25^+CCR4^+$ subset using the cell-sorting system. The statistical candidate selection steps, including LC/MS/MS data processing, label-free quantification, and statistical analysis, were performed on the Expressionist proteome server. The protein identification database was separately established based on 2D LC/MS/MS analysis. ND, normal donors; AC, asymptomatic carriers.

BC021303). The 5-μg vector DNA was transfected to $1 \times 10^6$ cells. The small interfering RNAs (siRNAs) against *SPTAN1*, *PTMS*, *HSPE1*, and SHMT2 and siRNA universal negative control were purchased from Sigma-Aldrich. The 500-pmol siRNA oligo was transfected into $1 \times 10^6$ cells. The vectors and siRNAs were transfected into all cell lines except CCRF-CEM by Amaxa Nucleoportator transfection Kit V (Lonza, Cologne, Germany) and CCRF-CEM by Kit C (Lonza).

### Cell cycle analysis and proliferation assay

For the cell cycle analysis, $1 \times 10^5$ to $2 \times 10^5$ cells were washed and agitated in 0.1% Triton-X (Sigma-Aldrich) with 100 ng/mL of ribonuclease (Sigma-Aldrich). Following addition of 1 μg/mL propidium iodide, the flow cytometric analysis was performed on FACScalibur (Becton Dickinson). The data analysis was performed using FlowJo software (Tree Star Inc., OR). Doublet events were eliminated from analyses by proper gating on FL2-W/FL2-A primary plots before histogram analysis of DNA content. Cell proliferation was estimated by measuring cell metabolic activity using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) following the manufacturer's recommendation.

### Western blotting

Cells were lysed in lysis buffer [1% NP-40, 2 mM EGTA, 2 mM $MgCl_2$, 150 mM NaCl, 20 mM tris(hydroxymethyl)aminomethane–HCl (pH 7.5), 10% glycerol, containing the protease inhibitor cocktail Complete (Roche, IN)] and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto PVDF membranes. Following blocking with 4% Block Ace (Yukijirushi Nyugyo Inc., Tokyo, Japan), membranes were incubated with anti-myc (9E10; Sigma-Aldrich) or anti–α-II spectrin (Abcam, Cambridge, UK) antibodies. Membranes were then incubated with horseradish peroxidase–conjugated anti-mouse IgG (GE Healthcare, NJ) or anti-rabbit IgG (GE Healthcare), respectively, and visualized with Western Lightning kit (Perkin Elmer, MA).

### Multiple reaction monitoring (MRM)

$CD4^+$ T cells were isolated from PBMCs using flow cytometry. The tryptic digests of the isolated cells were analyzed by 4000 Q-TRAP mass spectrometer (AB Sciex) accompanied with Ultimate 3000 nano-HPLC system. The LC gradient was as follows: 2% to 30% acetonitrile for 10 minutes and 30% to 95% acetonitrile for 5 minutes in 0.1% formic acid with a flow rate of 300 nL/min. The MRM transitions monitored were m/z 409.7/375.2 for α-II spectrin (SPTA2); m/z 538.3/889.5 for parathymosin (PTMS); m/z 507.3/147.1 for heat shock 10-kDa protein, mitochondrial (CH10); m/z 490.3/147.1 for serine hydroxymethyltransferase, mitochondrial (GLYM); and m/z 581.3/919.5 for β-actin, respectively. Individual peak areas were normalized by the peak area of β-actin. Data acquisition was performed with ion spray voltage = 2300 V, curtain gas = 10 psi, nebulizer gas = 10 psi, and an interface heating temperature = 150°C. The parameters were set as follows: declustering potential = 60, entrance potential = 10, collision cell exit potential = 10, and dwell time for each transition = 10 seconds. Collision energy was optimized to achieve maximum intensity for each MRM transition as follows: 34.03 V for m/z 409.7/175.1, 24.68 eV for m/z 538.3/889.5, 23.32 eV for m/z 507.3/147.1, 37.57 eV for m/z 490.3/147.1, and 31.58 eV for m/z 581.3/919.5.

## Results

### Quantitative proteome profiling of $CD4^+CD25^+CCR4^+$ T cells

A schematic overview of the screening approach is shown in Figure 1. To identify diagnostic markers expressed in HTLV-1–infected T cells, a $CD4^+CD25^+CCR4^+$ subset of PBMCs from 6 uninfected volunteers, 5 asymptomatic carriers, 9 HAM/TSP patients, and 9 ATL patients was isolated by flow cytometry (Figure 2). The averaged proportion of $CD4^+CD25^+CCR4^+$ cells in $CD4^+$ T cells from 4 clinical groups was 6.48 ± 2.46%, 13.17 ± 13.06%, 20.55 ± 10.73%, and 55.83 ± 22.40%, respectively, indicating that the occupancy of viral reservoir cells varied drastically among both pathological groups and even individuals within a group. Enrichment of the infected cells was confirmed by viral load measurement of the used samples (supplemental Figure 2). As reported previously,[10] the viral load of $CD4^+CD25^+CCR4^+$ cells (37.91 copies/100 cells on average) was ~10 times higher than that of $CD4^+CD25^-CCR4^-$ cells (4.12 copies/100 cells on average), indicating that the former cells were evidently the HTLV-1–enriched fraction. This fact strongly supports the importance of enriching pathogenic cells for rigorous quantitative biomarker discovery.

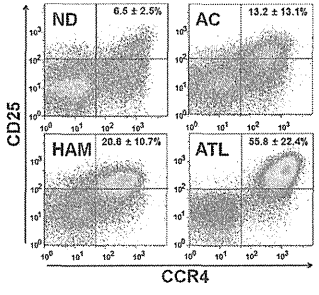


**Figure 2. Representative sorting results of $CD4^+CD25^+CCR4^+$ cells.** After labeling with anti-CD3-FITC, anti-CD4-Cy7, anti-CD25-APC, and anti-CCR4-PE, the $CD3^+CD4^+CD25^+CCR4^+$ fraction was isolated. The averaged content ± standard deviation (%) of $CD25^+CCR4^+$ cells out of $CD3^+CD4^+$ cells was calculated for each clinical group and is displayed in the upper right section of the panels.



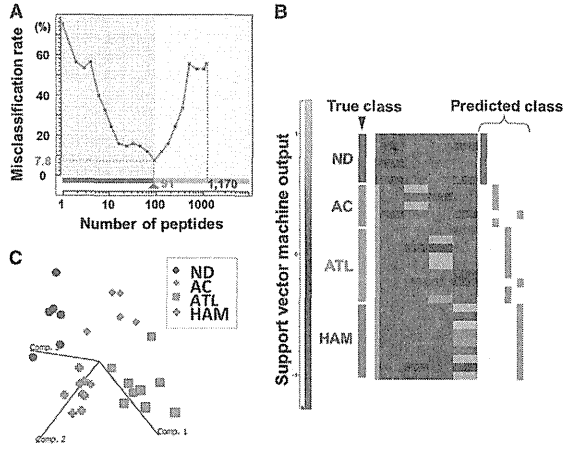


**Figure 3. Statistical extraction of candidate therapeutic targets.** The 14 064 nonredundant peptides detected were subjected to a 4-group Kruskal-Wallis test (ND, AC, ATL, and HAM), resulting in identification of 1170 first candidates ($P < .01$). ND, normal donors; AC, asymptomatic carriers. (A) Next, the Expressionist ranking method further narrowed down the candidates to 91 peptides based on SVM-REF so that the misclassification rate in the cross-validation test became minimum, 7.8%. (B) The predicted classification result by leave-one-out cross-validation test. The 27 out of 29 cases were successfully classified into the true classes. (C) The three-dimensional plot shows the additional assessment for the classification power of 91 classifiers by principal component analysis. Comp. 1 to 3 indicate principal components 1 to 3.

An accurately adjusted number of $CD4^+CD25^+CCR4^+$ cells from 29 cases were digested with trypsin and subjected to LC/MS/MS analysis individually. Because recent mass spectrometers often deal with data on the order of hundreds of megabytes per sample, it has been considered almost impossible to calculate a data set larger than a gigabyte from large-scale clinical samples on desktop computers. Hence, we constructed a proteomics server equipped with a 12-core central processing unit, 36 SAS hard disks, and 192-GB physical memories driving the Expressionist, which was designed to combine the database module, the data processing module, and the statistical analysis module into a single integrative platform for genomics, proteomics, and metabolomics. The detailed work flow for data processing and quantification for 29 LC/MS/MS raw data was described in the "Materials and methods" and is illustrated in supplemental Figure 1. Finally, 68 454 nonredundant peaks were detected and grouped into 37 143 isotopic clusters, or molecules. As tryptic peptides should appear as multivalent ions in electrospray ionization mass spectra, 23 079 singly charged ions were removed, resulting in utilization of 14 064 peptide signals for further statistical selection of diagnostic markers.

### Statistical identification of candidate diagnostic markers for ATL

A stepwise statistical extraction was employed for the effective identification of proteins, which demonstrated specific up- or downregulation in the ATL group. In the first stage, a 4-group Kruskal-Wallis test was performed to roughly extract the candidates showing a significantly distinct expression level among 4 clinical groups. Here we set the cutoff line at $P < .01$ and obtained 1170 first candidate peptides simply because the isolated peptide set using this criterion showed the best performance in the following prediction model.

Next, we selected the final candidates by the support vector machine–recursive feature elimination algorithm in the Expressionist Analyst module. Support vector machine–recursive feature elimination is a candidate elimination method based on SVM, which enabled us to improve the classification outputs by selecting the best-performing peptide set among initially provided candidates.[11] As a result, a combination of 91 peptides showed the lowest misclassification rate (7.78%) in a leave-one-out cross-validation test (Figure 3A-B). To evaluate the classification efficiency of 91 selected candidates, the principal component analysis was performed. Figure 3C shows the three-dimensional plot of 29 clinical samples based on the 3 best-explainable components, which illustrated statistically clear segregation among the 4 clinical groups. These assessments indicated that the 91 peptides should be a sufficient set of classifiers that closely associated with the pathological characteristics of the 4 clinical groups.

Based on an independently constructed 6279-protein identification database for $CD4^+CD25^+CCR4^+$ cells using 2D LC/MS/MS (see details in "Materials and methods"), 19 peptides among the 91 candidate peptides were successfully assigned to 17 proteins listed in Table 1. The mass spectrometric quantification profiles for the 19 peptides are also shown in Figure 4 (box plots).

### Recovering CAN2 activity induced cell death in ATL cells

Our diagnostic marker discovery for ATL identified an enzyme-substrate pair, CAN2 and SPTA2, which demonstrated significantly aberrant expression level in ATL patients (Figure 4). Interestingly, the intensities of the 2 proteins in 27 screening cases (without 2 statistical outliers in Figure 4) showed a clearly inverse correlation ($R^2 = 0.395$, Figure 5A). To examine whether CAN2 downregulation and/or SPTA2 upregulation might be essential for the growth of ATL cells, the enzymatic activity of CAN2 was rescued by overexpressing the constitutively active form of CAN2 ($\Delta_{19}$CAN2) in 3 ATL cell lines, SO-4, KOB, and KK1. After 36 hours of transfection, significant inhibition of cell proliferation (Figure 5B) and induction of sub-G1 transition was observed by activation of CAN2 in 3 ATL cells, but not in 4 non-ATL leukemia cell lines (Figure 5C). Furthermore, overexpression of $\Delta_{19}$CAN2 drastically attenuated the expression level of SPTA2 in the ATL cell

**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 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.

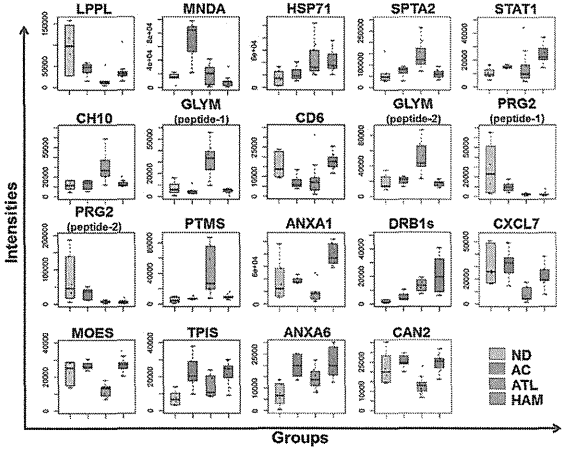


**Figure 4. Summary of quantitative features for the 17 protein classifiers identified.** The 19 box plots (see Table 1 for protein names) show the results of mass spectrometric quantification and protein identification. We finally identified 19 peptides out of 91 candidates in Figure 3, which were assigned 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.



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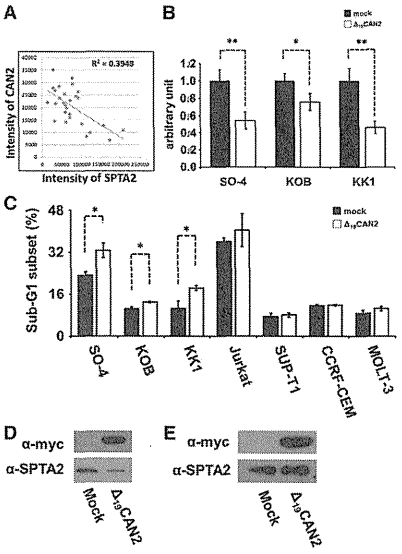


**Figure 5. Rescue of CAN2 activity induced cell death in ATL cells.** (A) Correlation between CAN2 and SPTA2 expression level in 27 cases. (B) Cell proliferation was measured by MTT assay on SO-4, KOB, and KK1 cells 36 hours after transfection of mock vector or $\Delta_{19}$CAN2. *$P < .05$; **$P <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

## 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 down-regulation 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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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 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.

**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® 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.

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## Competing interests

None declared.

## Ethical approval

Not required.

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原　著

# Human T-lymphotropic virus type I 感染者における腎移植の影響

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**要旨**：Human T-lymphotropic virus type I（HTLV-1）感染者における腎移植の安全性評価を目的とし，文献収集により成人T細胞白血病（ATL）およびHTLV-1関連脊髄症（HAM）の発症について解析した。さらに，免疫抑制療法によるATL，HAM発症リスク増加への影響を検討するため，ステロイド治療によるHTLV-1プロウイルス量の変動を解析した。その結果，腎移植後にATL 11例，HAM 6例の症例報告を認め，そのうちATL 2例およびHAM 6例では移植が感染源であった。また，ATL 11例のうち9例は若年発症であり移植との関連性が示唆された。またステロイド治療前後のプロウイルス量に有意な変化はなかった。HTLV-1陽性ドナーからの腎移植はレシピエントに感染および関連疾患発症の危険がある。またHTLV-1陽性レシピエントの関連疾患発症率については症例数が少ないため明確なエビデンスは得られず，腎移植の適応については慎重な判断が求められる。

**キーワード**　HTLV-1，ATL，HAM，腎移植



## 緒　言

ドナーからレシピエントへの病原微生物の伝播および免疫抑制療法による感染症の悪化は，臓器移植における重要な問題である。臓器移植におけるCytomegalovirus，BK virusなど比較的頻度が高い感染症に対する知見は集積してきているが，human T-lymphotropic virus type I（HTLV-1）感染に関する報告は乏しく，対応も定まっていない[1)]。

HTLV-1は，感染者が全国に100万人以上存在し，2～5%に極めて予後不良の成人T細胞白血病(adult T cell leukemia：ATL)，0.25～3.8%に神経難病であるHTLV-1関連脊髄症（HTLV-1-associated myelopathy：HAM）を引き起こす。HTLV-1は血漿中にはほとんど検出できないため，抗HTLV-1抗体の検出により感染を診断する。また，末梢血単核球のゲノムにウイルス遺伝子が組み込まれたプロウイルス（proviral load：PVL）をウイルス量の指標として用いており，PVL高値はATLおよびHAM発症のリスク因子である[2, 3)]。HTLV-1の感染経路には母乳を介する垂直感染と，性交渉や輸血による水平感染がある。ATLは通常垂直感染者に50～60年の潜伏期間を経て発症する一方で，HAMはすべての感染経路により数年以上の潜伏期間で発症する。HAMは感染T細胞に起因する脊髄の慢性炎症により，進行性の痙性対麻痺や膀胱直腸障害などを呈する疾患で，その治療には炎症抑制のためステロイドなどが用いられるが，明確な治療法は確立されていない[2)]。

HTLV-1感染者の臓器移植には2つの問題がある。1つはHTLV-1陽性ドナー（D+）からHTLV-1陰性レシピエント（R−）にHTLV-1が感染し，ATLやHAMを発症する危険性である。本邦ではD+死体腎移植のみ禁忌で生体腎移植の施行は各施設の判断によるが，明確な判断根拠はない。もう1つはHTLV-1陽性レシピエント（R+）におけるATLおよびHAMの発症率が免疫抑制療法などによって増加する危険性である。

今回われわれは，HTLV-1感染者における腎移植の安全性を評価するため，腎移植後のATLおよびHAMの発症に関する文献を収集しレビューした。さらに，免疫抑制療法のHTLV-1感染症への影響

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**表 1** 腎移植後に発症した HTLV-1 関連疾患の報告

| 著者（年） | 抗 HTLV-1 抗体 D/R | 発症時年齢（歳）/ 性 | 移植から発症までの期間 | 免疫抑制薬 |
|---|---|---|---|---|
| ATL | | | | |
| Zanke, et al.（1989）[12] | ?/? | 43/ 男 | 2 年 | CyA/PSL |
| Tsurumi, et al.（1992）[13] | −/+ | 32/ 男 | 4 年 | CyA/MZ/PSL |
| Williams, et al.（1994）[14] | ?/? | 42/ 男 | 13 年 | AZA/PSL |
| Jenks, et al.（1995）[15] | −/+ | 61/ 男 | 9 ヵ月 | CyA/AZA/PSL |
| 毛利，他（2000）[16] | ?/? | 49/ 女 | 3 年 | TAC/AZA/PSL |
| Ichikawa, et al.（2000）[17] | ?/− | 42/ 男 | 10 年 | CyA/AZA/PSL |
| | ?/? | 32/ 男 | ? | ? |
| | ?/? | 32/ 男 | ? | ? |
| Hoshida, et al.（2001）[18] | ?/? | 43/ 男 | ? | ? |
| | ?/? | 56/ 男 | ? | ? |
| | ?/− | 47/ 女 | ? | ? |
| HAM | | | | |
| 土岐，他（2000）[19] | ?/− | 48/ 男 | 4 年 | CyA/MZ/PSL |
| Nakatsuji, et al.（2000）[20] | +/− | 49/ 男 | 4 年 | CyA/MMF/PSL |
| 新谷，他（2002）[21] | +/− | 56/ 男 | 7 年 | CyA/MZ/PSL |
| Toro, et al.（2003）[5] | +/− | 55/ 男 | <2 年 | CyA |
| | +/− | 53/ 女 | <2 年 | CyA |
| Inose, et al.（2010）[22] | ?/− | 40/ 男 | 10 ヵ月 | CyA/MP |

抗 HTLV-1 抗体 D/R：donor/recipient の移植前の抗 HTLV-1 抗体の有無，?：不明または記載なし
AZA：azathiopurine，CyA：cyclosporine A，PSL：prednisolone，MMF：mycophenolate mofetil，MP：methylprednisolone，MZ：mizoribine，TAC：tacrolimus

を検討するため，ステロイド治療の PVL への影響を解析した。

## 対象・方法

### 1. 文献検索方法

腎移植後の ATL および HAM の発症に関する文献は，Pubmed，SciVerse Scopus，医学中央雑誌を用いて 2012 年 9 月までの文献を検索した。検索語は，“HTLV-1”，“adult T cell leukemia”，“HTLV-1-associated myelopathy”，および “kidney transplantation” を用いた。

### 2. HAM 患者の PVL の測定および解析

2007 年 4 月から 2011 年 9 月の間に聖マリアンナ医科大学の HAM 専門外来を定期受診した HAM 患者のうち，受診後に初めて prednisolone 内服治療（PSL）または methylprednisolone pulse 療法（MP pulse）を行い，12 ヵ月以上 PSL を継続した患者を対象とした。HAM の治療歴がある患者，ステロイド以外の免疫抑制薬を使用中の患者は除外した。ステロイド投与量は，主治医が病状・病勢をもとに決定した。

ステロイド治療開始前，治療開始 3 ヵ月後および 12 ヵ月後の末梢血単核球の HTLV-1 PVL（copies/100 cells）を定量的 PCR により測定した[4]。PVL の比較は Friedman 検定の後，治療前値を対照として Dunn 検定を行い，危険率 5% 未満を統計学的有意とした。統計処理は Graphpad Prism 5 and Prism statistics（GraphPad Software 社，米国）を用いた。

本研究は聖マリアンナ医科大学倫理委員会の承認を得，ヘルシンキ宣言に基づく倫理的原則を遵守して実施した。参加者には研究内容を十分に説明し文書による同意を得た。

## 結　果

### 1. 腎移植と HTLV-1 関連疾患に関する報告

1）症例報告

腎移植後の ATL 発症の報告は 7 報 11 例で，9 例が 50 歳未満であった（**表 1**）。術前の感染状態は 7 例で不明であったが，D−/R+ および D 不明 /R− が各 2 例存在した。HAM 発症の報告は 5 報 6 例で，全例 R− であった（**表 1**）。Toro らの報告では，1 名の HTLV-1 感染者からの死体移植により腎移植 2 名と肝移植 1 名が 2 年以内に HAM を発症した[5]。

2）HTLV-1 感染者に施行した腎移植の症例集積報告

単施設の 9 ～ 16 例からなる報告が 6 報あった（**表 2**）。合計 69 症例だが，新垣ら[6] と 1998 年および 2005 年の中村ら[7, 8] は同一グループであり，症例の重複





**表 2** HTLV-1 感染者に施行した腎移植の症例集積報告

| 著者（年） | 患者数（人数） | 抗 HTLV-1 抗体 D/R（人数） | 年齢（歳） | 観察期間（年） | 免疫抑制薬（人数） | ATL，HAM の発症 |
|---|---|---|---|---|---|---|
| 新垣，他（1995）[6] | 9 | +/−3<br>−/+ 2<br>+/+ 4 | 31.1（8.6） | 3.3（2.3） | CyA/AZA/PSL 3<br>CyA/MZ/PSL 3<br>CyA/AZA/PSL/ALG 1<br>CyA/MZ/PSL/ALG 2 | なし |
| Nakamura, et al.（1998）[7] | 15 | +/− 6<br>−/+ 2<br>+/+ 5<br>+/? 1<br>?/+ 1 | 39.5（6.4） | 2.6（2.8） | CyA/AZA/PSL 4<br>CyA/MZ/PSL 3<br>CyA/AZA/PSL/ALG 2<br>CyA/MZ/PSL/ALG 3<br>CyA/AZA/MZ/PSL/ALG 3 | なし |
| Tanabe, et al.（1998）[23] | 16 | +/− 0<br>−/+ 16<br>+/+ 0 | 34.3* | 8* | CyA/AZA/MP 16 | なし |
| Nakamura, et al.（2005）[8] | 10 | +/− 4<br>−/+ 2<br>+/+ 4 | 32.2（8.5） | 12.9（4.6） | CyA/AZA/PSL 3<br>CyA/MZ/PSL 3<br>CyA/AZA/PSL/ALG 1<br>CyA/MZ/PSL/ALG 2<br>TAC/MMF/PSL/BXM 1 | なし |
| Naghibi, et al.（2011）[24] | 10 | +/− 0<br>−/+ 8<br>+/+ 2 | 42.0（13.6） | 4.3（1.2） | CyA/PSL 1<br>CyA/MMF/PSL 7<br>CyA/MMF/PSL/ALG 1<br>CyA/MMF/MP/DZM 1 | なし |
| Shirai, et al.（2012）[25] | 9 | +/− 0<br>−/+ 5<br>+/+ 3<br>?/+ 1 | 54.3（8.1） | 2.8（3.1） | CyA/MMF/MP 1<br>CyA/MMF/MP/BXM 4<br>TAC/MMF/MP/BXM 2<br>TAC/MMF/MP/BXM/RXM 2 | なし |
| 計 | 69 | +/− 13<br>−/+ 35<br>+/+ 18<br>+/? 1<br>?/+ 2 | 34.4（6.9）<br>39.6*<br>37.4（12.1）<br>48.0<br>49.5（2.1） | 9.0（5.0）<br>6.2*<br>4.9（3.9）<br>4.0<br>6.8（3.9） | CyA 64/TAC 5<br>AZA 33/MZ 19/MMF 19<br>PSL 43/MP 26<br>ALG 15/BXM 9/DZM 1/RXM 2 | なし |

抗 HTLV-1 抗体 D/R：donor/recipient の移植前の抗 HTLV-1 抗体の有無，?：不明
AZA：azathiopurine，ALG：anti-lymphocyte globulin，BXM：basiliximab，CyA：cyclosporine A，DZM：daclizumab，PSL：prednisolone，MMF：mycophenolate mofetil，MP：methylprednisolone，MZ：mizoribine，RXM：rituximab，TAC：tacrolimus
年齢および観察期間は平均値（標準偏差），*：平均値

により実際は 60 例程度と考えられた。平均年齢 37.9 歳と若年者が多く，ATL および HAM の発症は全く認められなかった。しかし，新垣ら[6] および中村ら（1998）[7] は，D+/R− 移植を施行した 3 名および 6 名のレシピエントにおいて移植後に抗 HTLV-1 抗体が陽転化したと報告している。D+/R− 移植件数の記載はなく，腎移植による感染率は不明であった。

### 2. ステロイドによる HTLV-1 PVL の変化

1）患者背景

対象は，HAM 専門外来を定期受診した患者 21 名（男性 5 名，女性 16 名，年齢中央値 63 歳（範囲 40 ～ 72））。治療内容は PSL 内服 16 名，MP pulse 5 名であった（**表 3**）。ステロイドの 1 日投与量は PSL 群 2.5 mg（2.5 ～ 5），MP pulse 群は全例 MP 500mg を 3 日間点滴静注し，後療法の PSL は 5.0mg（4 ～ 10）であった。

2）PVL の変化

PSL 群の PVL は治療開始 3 ヵ月後有意に低下したが（中央値は開始前 13.3，3 ヵ月後 8.0，$p<0.01$），12 ヵ月後は有意な変化を認めなかった（14.3，$p>0.05$）（**図 1**）。MP pulse 群の PVL は，3 ヵ月，12 ヵ月ともに有意な変化を認めなかった（それぞれ 23.3，15.0，20.5，$p>0.05$）（**図 1**）。PSL 群，MP pulse 群の PVL 高値各 1 例に PVL の著明な低下を認めたが，著明に上昇した症例はなかった。また，ATL 発症例はなかった。

表3 患者背景

| | PSL | MP pulse |
|---|---|---|
| 患者数（人数） | 16 | 5 |
| 男／女（人数） | 4/12 | 1/4 |
| 年齢（歳） | 57.5（40～68） | 70.0（62～72） |
| 罹病期間（年） | 10.5（4～28） | 3.0（2～41） |
| PVL（copies/100cells） | 13.3（0.3～98.0） | 24.3（2.5～69.9） |
| 納の運動障害重症度 | 6.5（2～8） | 8.0（3～12） |
| ステロイド投与量（mg/day） | 2.5（2.5～5） | 500mg，3days<br>後療法 5.0（4～10） |

PSL：prednisolone 内服群，MP pulse：methylprednisolone pulse 群，PVL：HTLV-1 proviral load
数値は中央値（範囲）

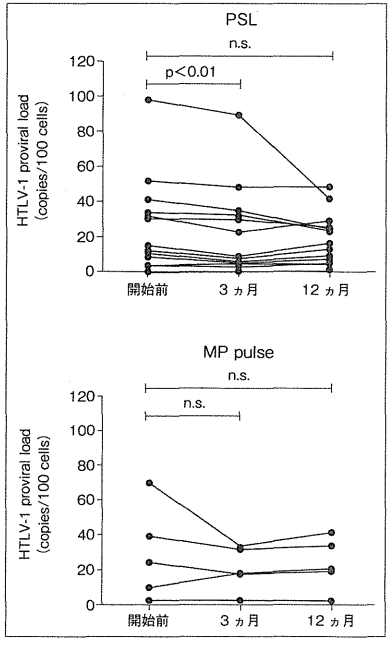


図1 ステロイド治療開始前後の HTLV-1 proviral load の推移
統計解析は Friedman 検定後，Dunn 検定を行った。
PSL：prednisolone 内服群，MP pulse：methylprednisolone pulse 群

## 考 察

今回，症例集積報告のまとめ（表2）では，腎移植を施行した69例に ATL および HAM の発症は認められなかったが，症例報告のまとめ（表1）では，腎移植後に ATL 11例および HAM 6例の発症を認めた。これらの報告では D/R における腎移植前の感染不明例が多く，D+/R− 腎移植での HTLV-1 感染率および R+ 腎移植の ATL および HAM の発症率に関する結論は得られなかった。また，ステロイド治療により HTLV-1 PVL は上昇しなかった（図1）。

まず D+/R− 腎移植についてであるが，HTLV-1 感染および関連疾患発症の危険が存在することが示された。しかし，情報不足のため感染率や発症率の算出はできず，そのリスク評価は困難であるのが現状である。また，米国から D+ 死体肝移植 81 例で ATL および HAM の発症はなかったとの報告があるが[9]，HTLV-1 と HTLV-2 を区別しておらず，また確認検査未施行で多くの偽陽性を含む可能性が高い，平均観察期間 1.21 年と短いなどの問題があり，安全性の評価はやはり困難である。ATL は致死的で HAM も機能予後不良の難治性疾患であることから，そのリスクと腎移植のベネフィットバランスを検討するためには，今後，全国的かつ継続的な情報の集積が必要と考えられる。その際，PVL によりリスクが異なる可能性も考えられるので，PVL 情報も蓄積することによりリスクの層別化が可能となるかもしれない。

次に R+ 腎移植において免疫抑制療法が ATL 発症を促進するか否かについてであるが，腎移植後 ATL の症例報告の9例が 30～40 歳代と通常の ATL よりも若年発症であること，さらに R− からの発症を認め，腎移植による感染後比較的短期間での ATL 発症を示していることから，移植に伴う免疫抑制療法が ATL 発症を促進する可能性が示唆される。HTLV-1 感染細胞の制御には免疫系による監視が重要で，その重度な機能低下は感染細胞の増加に繋がる。造血幹細胞移植後に再発した ATL が Cyclosporine A（CyA）中止により消褪したという報告は，免疫抑制薬の危険性を示唆している[10]。一方，69例の症例集積報告（表2）では ATL や HAM の発症がないことから，発症率は低いことが示唆され，腎移植による生命予後改善効果の方が大きい可能性もあり，リスクベネフィットについて慎重に判断することが重要と考える。今回の検討では，HAM 患者の検体ではあるがステロイドにより PVL は上昇しなかった。さらに最近，HAM 患者7名に対する 48 週間の CyA 療法（trough 濃度中央値 87ng/mL）でも PVL は上昇しなかったことが報告された[11]。以上の結果からは，維持用量の CyA，PSL および MP pulse では PVL は上昇しないことが示唆されるが，現在の腎移植後の標準的免疫抑制療法（calcineurin 阻害薬，代謝拮抗薬およびステロイド併用療法）の影響および腎移植のリスクとベネフィットを明らかにするには，感染経路や移植前からの PVL の推移を含む長期的なデータの蓄積が必要であろう。

欧米は HTLV-1 感染率が低いため術前検査が必須ではないが[1]，浸淫地域である日本では，明確な指標が必要であると考えられる。PVL の測定は現在のところ一部の研究機関でのみ可能で，HTLV-1 研究組織と協力し確実なデータの蓄積が望まれる。

## 結 論

HTLV-1 感染者における腎移植の影響について検討した。HTLV-1 陽性ドナーからの腎移植はレシピエントに感染および関連疾患発症のリスクを伴う。HTLV-1 陽性レシピエントの関連疾患発症率が腎移植により上昇するというエビデンスは得られなかったが，報告の症例数や観察期間は十分ではなく，その適応は慎重に判断する必要がある。したがって HTLV-1 感染者における腎移植の安全性評価の精度を高めるために，今後の情報集積が必要である。

利益相反自己申告：申告すべきものなし

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## Kidney Transplantation and Human T-Lymphotropic Virus Type I-Infected Patients

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To assess the safety of kidney transplantation to and from patients infected with human T-lymphotropic virus type I (HTLV-1), past cases were reviewed using literature (12 case reports and 6 case series) available on PubMed, SciVerse Scopus and Ichushi-Web (for Japanese literature). Data points analyzed include : age, seropositivity of HTLV-1 in donor and recipient, immunosuppressive agents, follow-up period, the incidence rate of adult T cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Among the case reports there were 11 patients who developed ATL and 6 patients who developed HAM/TSP following kidney transplantation ; of these, 2 ATL and all 6 HAM/TSP patients were seronegative before transplantation. Among all 6 case series, which included 69 transplants in which either the donor or recipient or both were HTLV-1-positive, no patients developed ATL or HAM/TSP post-transplant. In addition, at our hospital, we used blood samples from HAM/TSP patients treated with oral prednisolone or methylprednisolone pulse therapy to assess the influence of corticosteroids on HTLV-1 proviral load. Proviral loads were not significantly different from before to 12 months after the corticosteroid therapies. In conclusion, HTLV-1 can be transmitted via transplantation and cause ATL or HAM/TSP in some recipients. The influence of the immunosuppressive drugs used with kidney transplants remains to be elucidated.

**key words** HTLV-1, ATL, HAM/TSP, kidney transplantation

白血病
非定型白血病および特殊型

# HTLV-1関連脊髄症(HAM)

HTLV-1-associated myelopathy(HAM)

Key words：HAM，疫学，診断，治療，予後

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## 1. 概　　念

HTLV-1関連脊髄症(HTLV-1-associated myelopathy: HAM)は，成人T細胞性白血病リンパ腫(ATL)の原因ウイルスであるhuman T-lymphotropic virus type 1(HTLV-1)の感染者(キャリア)の一部に発症する，進行性の脊髄障害を特徴とする神経難病である．1986年に納らにより一つの疾患単位として提唱され[1]，2009年度からは国の難治性疾患克服研究事業の対象疾患(いわゆる難病)に認定されている．

## 2. 疫　　学

日本では，HTLV-1キャリアの生涯において約0.3％の確率で発症すると推定されている[2]．患者の分布は西日本を中心に全国に広がっており，特に九州・四国・沖縄に多く，ATLの分布と一致していた．最近の全国疫学調査では，全国の患者数は約3,000人と推定され，関東などの大都市圏で患者数が増加していることが明らかとなりつつある．

HTLV-1の感染経路として，主として母乳を介する母子感染と，輸血，性交渉による水平感染が知られているが，そのいずれでも発症することが知られている．輸血後数週間で発症した例もあり，感染成立後長期のキャリア状態を経て発症するATLとは異なっている．輸血後発症するHAMの存在の指摘により，1986年11月より献血時の抗HTLV-1抗体のスクリーニングが開始され，以後，輸血後発症はなくなった．発症は中年以降の成人が多いが(平均発症年齢は40歳代)，10歳代，あるいはそれ以前の発症と考えられる例も存在する．男女比は1：2と女性に多く，男性に多いATLと対照的である．

表1　HAMの初期症状

- 何となく歩きにくい，両下肢のつっぱり感，足がもつれる，つまずく，走ると転びやすい，などの歩行障害に関する症状
- 排尿障害や便秘も，早期から自覚されることが多く，尿閉や頻尿，繰り返す膀胱炎で泌尿器科を受診しHAMと診断されることもある
- まれに，持続する両下肢のしびれ感，痛みなどを早期から認めることがある

## 3. 診断と鑑別診断

HAMは，早期の診断と治療介入が重要で，病気を見逃さない注意が必要である．**表1**のような症状の患者を診たら，HAMという疾患を思い浮かべることが重要である．HAMを疑ったらすぐに神経内科医への紹介を考慮してほしい．症状や診察所見の組み合わせは特徴的であるので，神経内科医であれば診断は比較的容易であることが多い．

痙性対麻痺を呈しHAMの可能性が考えられる場合，血清中の抗HTLV-1抗体の有無をEIA法またはPA法でスクリーニングし，陽性の場

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