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# Expression of TSLC1 in patients with HAM/TSP

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## Abstract

Human T-lymphotropic virus type 1 (HTLV-1)–associated myelopathy/tropical spastic paraparesis (HAM/TSP) is chronic myelopathy characterized by slowly progressive spastic paraparesis and urinary dysfunction. A few biomarkers in the cerebrospinal fluid are known to be related to disease activity, but no biomarker has been reported in peripheral blood. This study aims to explore the expression level of the adhesion molecule during the expression level of the adhesion molecule among HAM/TSP disease activity. In lymphocyte function–associated antigen 1 and DNAX accessory molecule 1, no variation in expression levels specific to HTLV-1 infection was observed in CD4-positive T cells; however, TSLC1 expression was higher in HAM patients than in asymptomatic carriers and non-infected persons. TSLC1 tended to be higher in patients whose symptoms were worsening. On the contrary, the expression level of TSLC1 in CD8-positive T cells was lower in HAM patients than in asymptomatic carriers, and this tendency was stronger in patients whose symptoms had deteriorated. No significant correlation was found between TSLC1 and either of the transcription factors Tax or HBZ in any T cell group. Therefore, TSLC1 expression in CD4-positive T cells might be a useful biomarker of HAM/TSP disease activity.

**Keywords** Human T-lymphotropic virus type I (HTLV-I) · HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) · Tumor suppressor in lung cancer-1 (TSLC1) · Clinical symptoms

## Introduction

Human T cell leukemia virus type 1 (HTLV-1) is an oncogenic retrovirus that mainly infects human T cells (Seiki et al. 1983; Watanabe 1997). HTLV-1 infection is associated with adult T cell leukemia/lymphoma (ATLL) (Yamaguchi et al. 1983; Shimoyama et al. 1983) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al. 1985; Osame et al. 1986).

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HAM/TSP displays an inflammatory pathology in the central nervous system, and HTLV-1 preferentially infects T lymphocytes, mainly CD4-positive T cells (Richardson et al. 1990; Matsuoka et al. 1998; Yamano et al. 2005). HTLV-1-infected CD4-positive T cells break through the blood–brain barrier and invade the spinal cord. After that, HTLV-1-specific CD8-positive T cells invade the spinal cord to eliminate the infected cells. Neural cells are assumed to be involved in the inflammation caused by the severe immune response that occurs in the spinal cord and that injury to these neural cells leads to the onset of the disorder (Ijichi et al. 1993; Jacobson 2002; Osame 2002; Bangham and Osame 2005). Patients with HAM/TSP display symptoms such as gait disturbance, bladder disturbance, and rectal disturbance. Patients with a long history of illness eventually lose their ability to stand and walk because of the progressive dysfunction of the thoracic cord (Nakagawa et al. 1995). However, pathological studies have shown that severe inflammatory lesions of the spinal cord, which indicate high-level disease activity, can be detected in less than 10 years after the onset of HAM/TSP. Neopterin and CXCL10 in the cerebrospinal fluid are known as HAM/TSP biomarkers, but blood biomarkers are also required to evaluate disease activity and increase treatment efficacy.

CD4-positive T cell infection in the spinal cord is triggered when HAM/TSP develops or when the disease worsens (Matsuoka et al. 1998; Saito 2010). During the invasion of infected cells, infected cells first adhere to the vascular endothelium. Chronic persistence of spinal cord inflammation caused by HTLV-1 infected cells may further progress the conditions. Furthermore, the infiltration of infected cells worsens with time owing to the high expression of adhesion molecules. It has been reported that HTLV-1 infection increases the expression level of various molecules in infected cells.

This study evaluated lymphocyte function–associated antigen 1 (LFA-1; CD11a/CD18) (Fukudome et al. 1992; Kim et al. 2006), which is highly expressed in the HTLV-1 infected cell line, and DNAX accessory molecule 1 (DNAM-1), CD226 (Lofgren et al., 2010), which is a conjugate molecule of LFA-1 and is highly expressed in the blood cells of lupus erythematosus patients. In recent years, the tumor suppressor in lung cancer 1(TSLC1) (Sasaki et al. 2005; Nakahata et al. 2012) has indicated that the expression of ATLL-infected T cells is approximately 30-fold that of healthy individual T cells. TSLC1 may be a diagnostic marker for ATL progression (Makiyama et al. 2019). We examined whether the expression level of the adhesion molecule can be a new biomarker of HAM/TSP disease activity.

## Materials and methods

### Objects

Sixteen subjects with HAM/TSP, 6 asymptomatic HTLV-1 carriers, and 8 uninfected volunteers gave informed consent to be studied at Kansai Medical University Hospital. Peripheral blood was collected with an EDTA-added blood collection tube, and human peripheral blood mononuclear cells (PBMCs) were separated by the Ficoll method. The separated PBMCs were suspended in a cell preservation solution (Cell banker), dispensed, and then stored in liquid nitrogen. The stored PBMCs were thawed as necessary for use in experiments.

### Cell separation

PBMCs were separated into CD4-positive T cells and CD8-positive T cells by using the following method. To isolate CD8-positive T cells, PBMCs were suspended in MACS buffer (2% FBS, PBS [pH 7.2]) and Biotin-Antibody Cocktail (Miltenyi Biotec, MACS CD8-positive T cell isolation kit). The mixture was incubated on ice. Thereafter, a 10-fold amount of MACS buffer was added, the mixture was centrifuged at room temperature at 300×*g*, and the supernatant was removed and suspended by adding MACS buffer. The suspension was applied to a column (Miltenyi Biotec, MACS® Separation Columns, 25 MS Columns) equipped with a magnet (Miltenyi Biotec, MiniMACS™ Separator), and the fractions that were not adsorbed on the column (other than CD8-positive T cells) were treated with CD4-positive T cells. The cells were collected in a tube, and the column was further washed 3 times with MACS buffer to collect additional cells. The collected cells were kept on ice until CD4-positive T cells were collected in the tube. The magnet was removed from the column, MACS buffer was added, the flow-through (CD8-positive T cell fraction) was collected in a tube, MACS buffer was passed through the column 3 more times. A tube for collecting CD4-positive T cells was then centrifuged at room temperature to remove the supernatant, which was then suspended in MACS buffer. Biotin-Antibody Cocktail (Miltenyi Biotec, MACS CD4-positive T cell isolation kit II) was added, and the mixture was incubated for on ice. Following incubation, 10 times the amount of MACS buffer was added, the mixture was centrifuged at room temperature at 300×*g*, the supernatant was removed, MACS buffer and Anti-Biotin MicroBeads (Miltenyi Biotec, MACS CD4-positive T cell isolation kit II) were added and suspended, and the mixture was incubated on ice. A 10-fold amount of MACS buffer was added, the mixture was centrifuged at room temperature at 300×*g*, and the supernatant was removed and suspended by adding MACS buffer. The suspension was applied to a column (Miltenyi Biotec, MACS® Separation Columns, 25 MS Columns), and fractions that were not adsorbed on the column (CD4-positive T cells) were collected in a tube. Three additional doses of MACS buffer were added to wash the column, and the cells were collected in a tube. Each cell fraction collected in a tube was centrifuged. The supernatant was discarded, transferred to a tube, and stored on ice.

### RNA extract

The cells (CD4-positive T cells and CD8-positive T cells) were immersed briefly in MACS buffer and suspended lightly by the vortex. Thereafter, Buffer RLT (Lysis buffer) was added, and the cell membrane was destroyed by vortexing. 70% EtOH was added, and the finished lysate was added to the column after vortexing and centrifuged at 8000×*g* at room temperature. After discarding the lysate that passed through the column, Buffer RW 1 (wash buffer) was added to the column and centrifuged at 8000×*g* at room temperature. After discarding the wash solution that passed through the column, Buffer RPE (wash buffer) was added to the column and centrifuged at 8000×*g* at room temperature. The wash solution that passed through the column was discarded, and the column was washed again with Buffer RPE. After discarding the wash solution that was passed through the column, the column was centrifuged at 8000×*g* at room

temperature without adding anything, and moisture was removed sufficiently from the column.

The column was replaced with a new collection tube, and RNase-free water were placed on the filter of the column. It was left to stand at room temperature and was centrifuged at 8000×*g* at room temperature. After centrifugation, the RNA extract collected in the collection tube was placed in a 1.5-ml tube of RNase-free water, and the purity and recovered the amount of RNA was measured with NanoDrop (Thermo Fisher Scientific NanoDrop 1000).

## cDNA synthesis and RT-PCR reaction

RNA 1 μg was used for cDNA synthesis. An RNA reaction solution (total volume: 20 μl) was prepared with 1 × RT Buffer (TOYOBO), 200 μM dNTP (TOYOBO), 3.125 μM random hexamer (TaKaRa), and 5 U/tube ReverTra-Ace (TOYOBO). The prepared solution was incubated in the following order to prepare cDNA: 30 °C, 10 min → 42 °C, 20 min → 99 °C, 5 min → 4 °C. and 5 min.

## DNA extract

DNA (CD4-positive T cell or CD8-positive T cell) fractionated by total PBMC and MACS sorting was recovered by the following method: PBS was added to the cell suspension and adjusted to 200 μl. Thereafter, RNase and Proteinase K were added and mixed, and the mixture was incubated at room temperature. A lysis buffer was added and was incubated at 55 °C for 10 min in a heat block after sufficient vortexing. 100% EtOH was added and suspended, transferred to a column, and centrifuged at 9100×*g* at room temperature. After centrifugation, the lysate that passed through the column was removed, wash buffer was added, and the mixture was centrifuged at 9100×*g* at room temperature. After centrifugation, the wash solution that passed through the column was removed, wash buffer was added, and the mixture was centrifuged at 9100×*g* at room temperature. After centrifugation, the wash solution that passed through the column was removed, and the column was further centrifuged at 13,000×*g* at room temperature to remove sufficient moisture from the column. The column was replaced with a new collection tube, elution buffer was added dropwise and placed on the filter, and the column was allowed to stand at room temperature. The centrifugation was performed at 13000×*g* at room temperature, and the DNA extract collected in the collection tube was collected in a 1.5-ml tube. The purity and yield of the recovered DNA extract were measured with NanoDrop.

## Quantitative PCR (for DNA)

HTLV-1 proviral loading was measured by quantitative PCR using 50 ng of DNA obtained from HTLV-1-infected PBMCs. The PCR reaction solution (total volume: 25 μl) was prepared by adding 1 × PCR buffer (Roche), 200 μM dNTP (Gene Amp®), 2.5 mM $MgCl_2$ (Roche), 200 nM F-primer, 200 nM R-primer, 200 nM Probe, and 1.25 U/tube Ampli Taq Gold (Roche AmpliTaq Gold®). Sample amplification (BIO-RAD My iQ™ - Optics Module) was performed with a standard known in advance by using a 96-well reaction plate (BIO-RAD). The thermal cycler condition was performed at 95 °C, 10 min → 95 °C, 15 s/60 °C, and 30 s (× 50 cycles). The proviral amount of HTLV-1 was calculated as follows: HTLV-1 pX/(human globin/2) × 100. Table 1 shows the primers and probe used.

## Quantitative RT-PCR (for cDNA)

Quantitative RT-PCR was performed using 100 ng of cDNA prepared from either CD4-positive T cell or CD8-positive T cells. RT-PCR samples (total volume: 20 μl) were prepared with PCR mixtures for 1 × SYBR Green, 200 nM F-primer, and 200 nM R-primer. Sample amplification (BIO-RAD My iQ™ - Optics Module) was performed using a 96-well reaction plate (BIO-RAD). The thermal cycler condition was performed at 95 °C, 10 min → 95 °C, 10 s/60 °C, 30 s (× 50 cycles) → 65 to 95 °C, and 10 s (melt curve). The expression level of each molecule was calculated as a value relative to the amount of *HPRT* expressed. Table 2 shows the primers used.

## FACS analysis

The expression analysis of cell surface molecules was performed using flow cytometry (BD FACS Calibur™ flow cytometer). PBMCs were washed with FACS buffer (2% FBS, PBS [pH 7.2]) and were centrifuged at room temperature and 300×*g*. The supernatant was removed and suspended in an appropriate amount of FACS buffer. After a cell count, it was dispensed into a $3 \times 10^5$ cells/1.5 ml tube. After dispensing, it was centrifuged 300×*g* at room temperature, the supernatant was removed and the pellet was suspended with FACS buffer. Then each antibody was added, the mixture was incubated in the dark for 30 min at room temperature, FACS buffer was added, and the mixture was washed and centrifuged at room temperature 300×*g*. The supernatant was removed, FACS buffer was added, and the mixture was washed and centrifuged at room temperature 300×*g*. The supernatant was removed, suspended in FACS buffer, and transferred to a mesh-equipped FACS tube. FACS analysis was then performed. The antibodies used are shown below.

FITC (Alexa 488): anti-TSLC1 (from Prof. Kazuhiro Morishita, Miyazaki University)
R-PE: anti-CD25 (antihuman mouse-IgG1: Bio Legend)
APC: anti-CD4 (antihuman mouse-IgG1: BD)
PerCP-Cy 5.5: anti-CD 8 (antihuman mouse IgG1: R&D)

**Table 1** Primer sets for this study (DNA). Primer & probe set used for proviral quantification by the TaqMan probe method

| Gene | Forward primer | Reverse primer | Probes |
|---|---|---|---|
| HTLV-1 pX | 5′-acaaagttaaccatgcttattatcagc-3′ | 5′-tctccaaacacgtagactgggt-3′ | 5′FAM-acaaagttaaccatgcttattatcagc-BHQ3′ |
| Human β-globin | 5′-tgaggagaagtctgccgttac-3′ | 5′-tggtctccttaaacctgtcttg-3′ | 5′FAM-tgaggagaagtctgccgttac-BHQ3′ |

### Osame's motor disability score

First, the disease activity of HAM/TSP patients was evaluated clinically, and the biomarkers of disease activity were identified. The extent of non-mobility was used as the index. Specifically, we evaluated the patients' degree of movement disorder over time and judged cases with the progressive deterioration of one grade or more on Osame's motor disability score within half a year from the time of pre-evaluation as "progressive" and judged disease activity as high. On the contrary, cases with no change in the grade were judged "stable," and disease activity was judged low. The test was carried out for the case where this unchanged state continued for the past 2 years. Quantitative PCR, quantitative RT-PCR, and FACS of each cell were performed at each time point, and the results were compared with the clinical symptoms.

### Stimulation by either IL-2/PHA or anti-CD3 antibody/anti-CD28 antibody

The CD8-positive T cells isolated by MACS from uninfected PBMCs were used for stimulation by either IL-2/PHA or anti-CD3 antibody/anti-CD28 antibody. A total of $3 \times 10^5$ cells were suspended in 1 ml culture medium (RPMI 1640 [SIGMA], 10% FBS, 0.1% penicillin streptomycin mixed solution [Nacalai Tesque]), dispensed into 24-well plates (Nunc) 50 U and PHA 10 μg/ml, and stimulated by plate-bound anti-CD3 antibody/anti-CD28 antibody. Plate bonding was performed by suspending each antibody in culture medium, dispensing the suspension into a 24-well plate, incubating it at 37 °C for 1 h, and then removing the supernatant. For the sample without stimulation, only IL-2 50 U was added to the culture medium. After incubation at 37 °C for 18–24 h, RNA extraction, cDNA preparation, and quantitative RT-PCR were performed for each sample according to the above procedure to measure the expression level of TSLC1.

## Results

### mRNA expression analysis in CD4-positive T cells (TSLC1, DNAM-1, LFA-1)

We analyzed adhesion mRNA expression in HTLV-1 infected CD4-positive T cells in vivo. No significant difference was observed in the expression levels of *DNAM-1* and *LFA-1* between HAM/TSP patients and asymptomatic carriers, but *TSLC1* was increased significantly in HAM/TSP patients compared with asymptomatic carriers (Fig. 1).

### Comparison of mRNA expression levels between groups (TSLC1, HBZ, Tax)

The total amount of *tslc1* in CD4-positive T cells was increased in HAM/TSP patients compared with asymptomatic carriers. Compared with the HAM/TSP patient group, which had stable symptoms, the total amount of *TSLC1* expressed was greater in the patient group with worse symptoms (Fig. 2a upper). Thus, we calculated the expression of *TSLC1* per infected cell by correcting the proviral loads. No significant difference was observed in each group (asymptomatic carriers, HAM/TSP stable symptoms and HAM/TSP progressive symptoms) (Fig. 2b upper). On the other hand, in CD8-positive T cells, no change was observed in *TSLC1* expression. However, there was a tendency for *TSLC1* expression to decrease between stable and worse symptoms in HAM/TSP patients (Fig. 2a lower). Furthermore, it was found that *TSLC1*

**Table 2** Primer sets for this study (cDNA). Primer set used for mRNA quantification by the STBR green metod

| Gene | Forward primer | Reverse primer |
|---|---|---|
| human TSLC1 | 5′-atgatcgatatccagaaagacact-3′ | 5′-gtacttctagataccgctggg-3′ |
| human LFA-1 | 5′-tgagagcaggctatttgggttac-3′ | 5′-cggcccatgtgctggtat-3′ |
| human DNAM-1 | 5′-cgtgatgagattgactgtagccga-3′ | 5′-gggtgccttctgtgtatcccag-3′ |
| HTLV-1 pX | 5′-acaaagttaaccatgcttattatcagc-3′ | 5′-acacgtagactgggtatccgaa-3′ |
| HTLV-1 HBZ | 5′-agaacgcgactcaaccgg-3′ | 5′-tgacacaggcaagcatcg-3′ |
| human HPRT | 5′-ccttggtcaggcagtataatcca-3′ | 5′-ccaacaaagtctggcttatatccaa-3′ |

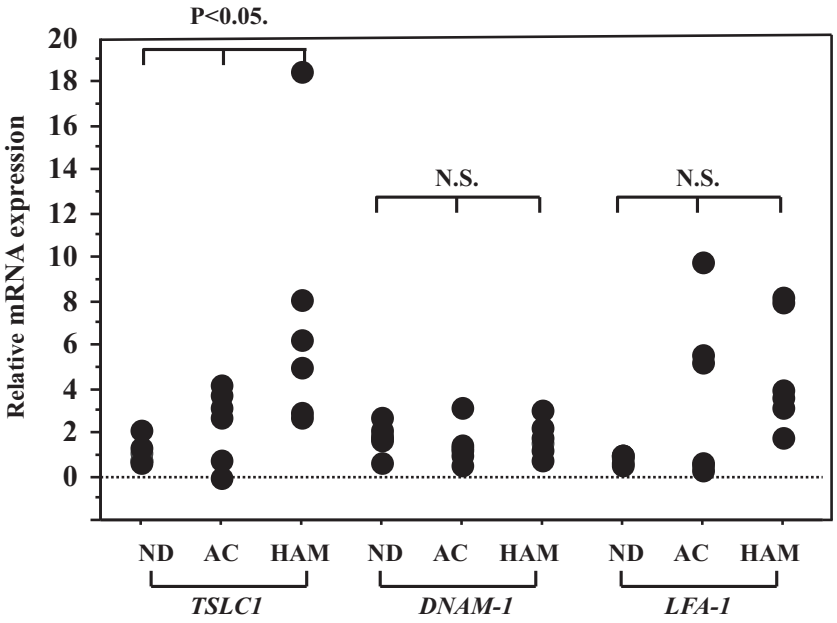


**Fig. 1** Comparison of the expression levels of TSLC1, DNAM-1, and LFA-1 in CD4-positive T cells. Normal donor (ND; $n = 6$), asymptomatic HTLV-1 infected carriers (AC; $n = 6$), HAM/TSP patients (HAM; $n = 6$), and mRNA expression levels of *TSLC1*, *DNAM-1*, and *LFA-1* in CD4-positive T cells

expression per cell was decreased by correcting with the provirus amount (Fig. 2b lower).

We compared the expression levels of the transcriptional activation factors *Tax* and *HBZ* in each group. The total amount of *tax* in both CD4-positive T cells and CD8-positive T cells was increased in HAM/TSP patients compared with asymptomatic carriers. In HAM/TSP patients, more *tax* was expressed in the progressive group. No significant difference was observed in the *HBZ* expression levels of CD4-positive T cells and CD8-positive T cells per cell (Fig. 2).

## Correlation analysis of TSLC1 expression level and Tax and HBZ expression levels in HTLV-1 infected persons

Considering that *TSLC1* expression may be regulated by *Tax* in comparing the expression level between groups, we verified whether there is a correlation between the expressions of *TSLC1* and *Tax*. However, there was no correlation between *TSLC1* and *Tax* expressions in both CD4-positive T cells (Fig. 3, upper left) and CD8-positive T cells (Fig. 3, upper right). Furthermore, we verified whether there is a correlation

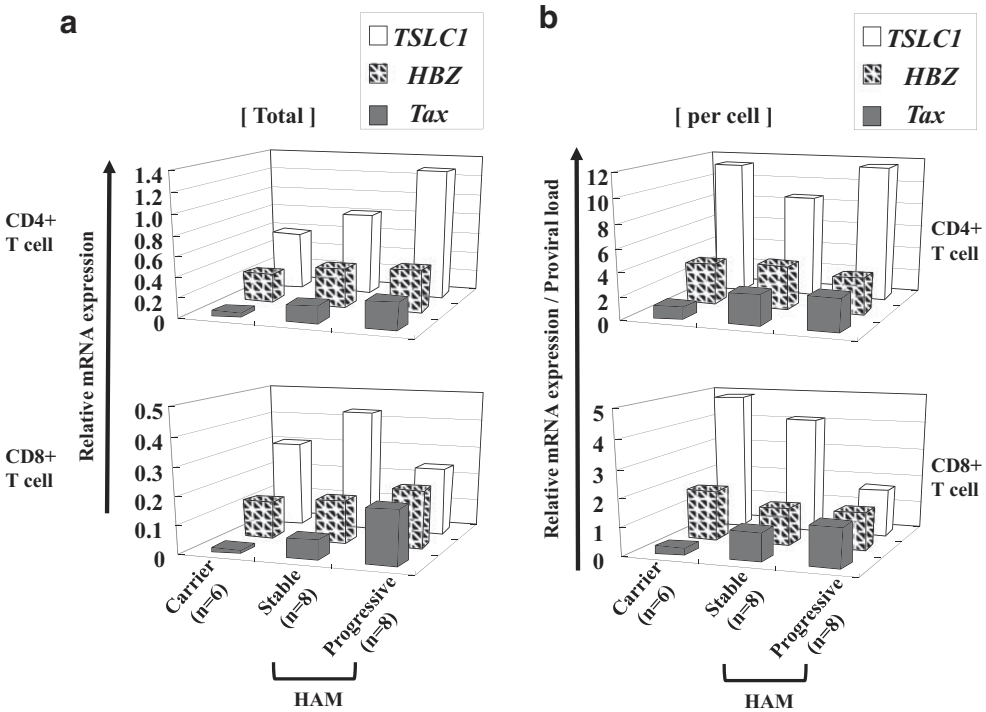


**Fig. 2** Comparison of the expression levels of TSLC1, HBZ, and Tax in groups of HTLV-1 infected individuals. TSLC1, HBZ, and Tax mRNA expression level in each group of HAM/TSP patients with different levels of disease activity (stable [$n = 8$] and progressive [$n = 8$]) and the asymptomatic HTLV-1 infected group (carrier; $n = 6$). **a** The expression level of each mRNA in CD4-positive T cells (upper) and CD8-positive T cells (bottom). **b** The amount of expression of each mRNA per infected cell corrected by the amount of provirus in CD4-positive T cells (upper) and CD8-positive T cells (bottom)

between the expression of *TSLC1* and *HBZ*. We confirmed that there is no correlation between the expressions of *TSLC1* and *HBZ* in both CD4-positive T cells (Fig. 3, lower left) and CD8-positive T cells (Fig. 3, lower right).

## Observation over time in the same individual

A comparative study of mRNA expression between the 3 patient groups (asymptomatic carriers, HAM/TSP stable symptoms, and HAM/TSP progressive symptoms) (Fig. 2) covers independent patients in each group. Therefore, the differences in the expression level of *TSLC1* in T cells obtained from comparative studies between the groups may be influenced by factors other than disease activity (e.g., genetic factors and living environment). Whether *TSLC1* expression measurement is useful as a disease activity biomarker should be verified not only in an intergroup test but also during both a period of high disease activity and a period when the symptoms are stable in the same individual.

The expression level of *TSLC1* in both the stable phase and progressive phase of symptoms was measured in HAM/TSP patients and was followed up over time. In CD4-positive T cells, the *TSLC1* expression level was high in 8 out of 10 patients when clinical symptoms were worsened (Fig. 4a left). In CD8-positive T cells, *TSLC1* expression tended to decrease in 6 out of 10 patients (Fig. 4a right) when symptoms worsened. Furthermore, the provirus loads which currently used as a biomarker was increased in 8 out of 10 patients (Fig. 4b.

Based on HTLV-1provirus loads and *TSLC1* expression level in CD4-positive T cells, the increase in *TSLC1* was expected to depend on the increase in the number of infected cells. There was no tendency to change the amount of *TSLC1* expression in each T cell when the clinical symptoms were stable(Fig. 4a lower). Also there was no clear change in proviral loads during the period when the clinical symptoms were stable (Fig. 4b lower).

## Differences in TSLC1 expression in clinical symptoms

Previously reported that TSLC1 is expressed on the cell surface and is involved in cell adhesion (Dewan et al. 2008) and immune response (Murakami 2005; Boles et al. 2005). Therefore, expression analysis by FACS was performed over time when the clinical symptoms in the same individuals of HAM/TSP patients were stable (disease stabilization period) and when the symptoms worsened (disease activity stage).

For CD4-positive T cells, although there were no changes in the expression level of TSLC1 in patients during the period of symptomatology, 4 out of 5 patients showed increased expression during the disease activity stage, and the results were the same as those obtained for mRNA expression (Fig. 5 a and b upper). In CD8-positive T cells, similar to CD4-positive T cells, there was no change in the expression level in patients during the symptomatology period, but TSLC1 expression was reduced in 2 out of 5 patients during the disease activity period (Fig. 5 a and b lower).

## Changes in the expression level of tslc1 by antigen stimulation

Persistent antigenic stimulation from HTLV-1 infected cells and excessive immune response to HTLV-1 infected cells due to the increased production of inflammatory cytokines have been observed in HAM/TSP patients (Usuku et al. 1988; Itoyama et al. 1988; Nakagawa et al. 1995). Therefore, the decrease in *TSLC1* expression in CD8-

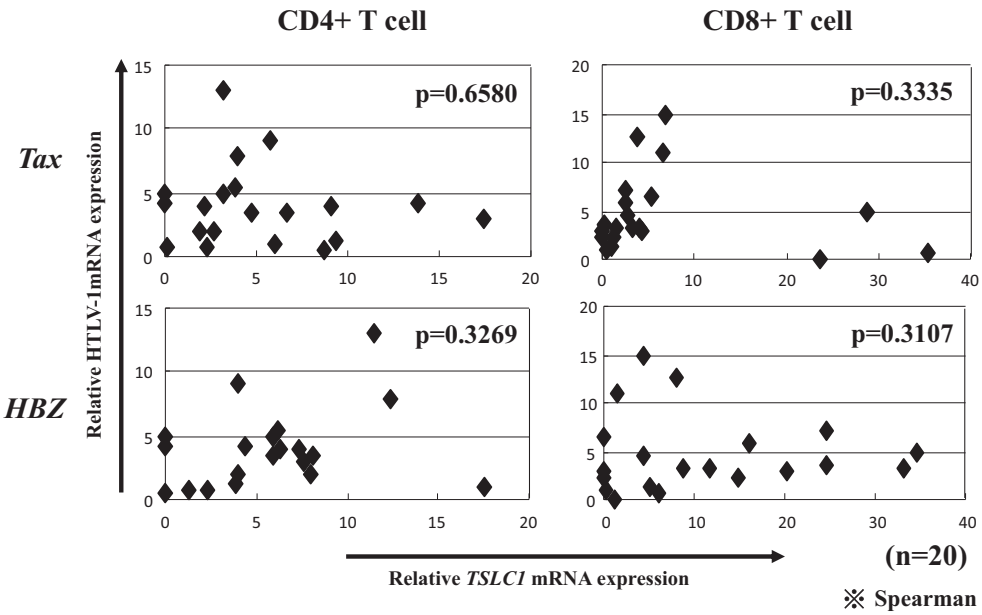


**Fig. 3** Correlation between the TSLC1 expression level and the Tax and HBZ expression levels in HTLV-1 infected persons. Correlation between *TSLC1* and *Tax* in CD4-positive T cells (upper left), the correlation between *TSLC1* and *HBZ* in CD4-positive T cells (lower left), the correlation between *TSLC1* and *Tax* in CD8-positive T cells (upper right), and the correlation between *TSLC1* and *HBZ* in CD8-positive T cells (lower right). All HTLV-1 infected persons ($n = 20$)

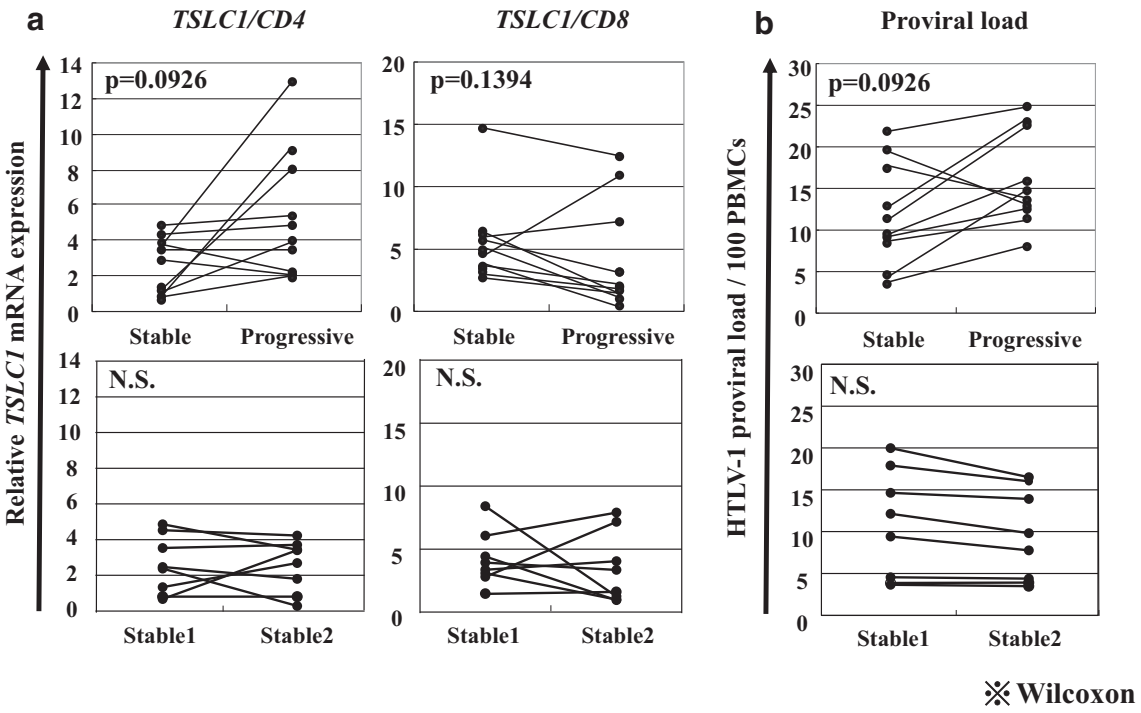


**Fig. 4** Comparison of the expression levels of TSLC1 in the same individuals at overtime. Changes the expression level of *TSLC1* (**a**) and in proviral loads (**b**) in either the symptoms worsened (upper) or stable periods (lower) in the same individual

positive T cells when clinical symptoms worsen might be attributed to the decrease in *TSLC1* expression level due to cell stimulation, such as antigen stimulation. Thus, we conducted cell stimulation experiments to clarify whether the decrease in the level of *TSLC1* expression is caused either indirectly by stimulation, such as antigen stimulation, or directly by HTLV-1 infection.

CD8-positive T cells isolated from the PBMCs of uninfected individuals were used in this experiment because the PBMCs of infected individuals may have already been stimulated by HTLV-1 infection either directly or indirectly. *TSLC1* expression was dramatically decreased following stimulation by either IL-2/PHA or anti-CD3 antibody/anti-CD28 antibody (Fig. 6).

## Discussion

Given that there is no curative treatment for HAM/TSP, interferon and steroids are administered to treat HAM/TSP symptoms (Nakagawa et al. 1996; Saito et al. 2004). Peripheral blood biomarkers, which are indicators of disease activity, are required to judge the appropriate timing of drug administration. We analyzed the adhesion molecules TSLC1, DNAM-1, and LFA-1 as new candidates for disease activity biomarkers. However, there was no significant difference mRNA expression levels between *DNAM-1* and *LFA-1* in both the asymptomatic carrier group and the HAM/TSP patient group. Therefore, mRNA quantification of these molecules is not useful for HAM/TSP disease activity (Fig. 1, right and middle). LFA-1 has been reported to express protein levels in various HTLV-1 infected cell lines (Fukudome et al. 1992; Kim et al. 2006), and from HTLV-1 infected cells of HAM/TSP patients. The protein levels of infected cells need to be analyzed in vivo in the future. DNAM-1 has not yet been reported for expression analysis in HTLV-1 infected cells, but is also a conjugated molecule of LFA-1 (Shirakawa et al., 2006). It is necessary to consider the analysis of DNAM-1.

Comparison analysis between groups showed that *TSLC1* was elevated in CD4-positive T cells with high disease activity (Fig. 2a). However, given that the comparison of expression among the groups covered independent patients, we considered the possibility that differences in *TSLC1* expression levels in CD4-positive T cells were influenced by factors other than disease activity (e.g., genetic factors and living environment). Therefore, to examine whether the measurement of *TSLC1* expression is useful of disease activity, we conducted a comparative analysis between the high disease activity period in the same individual and the period during which symptoms are stable. As a result, the expression level of *TSLC1* in CD4-positive T cells in the same individual was 8 in 10 people and tended to increase when clinical symptoms worsened (Fig. 4a). On the contrary, the level of *TSLC1*

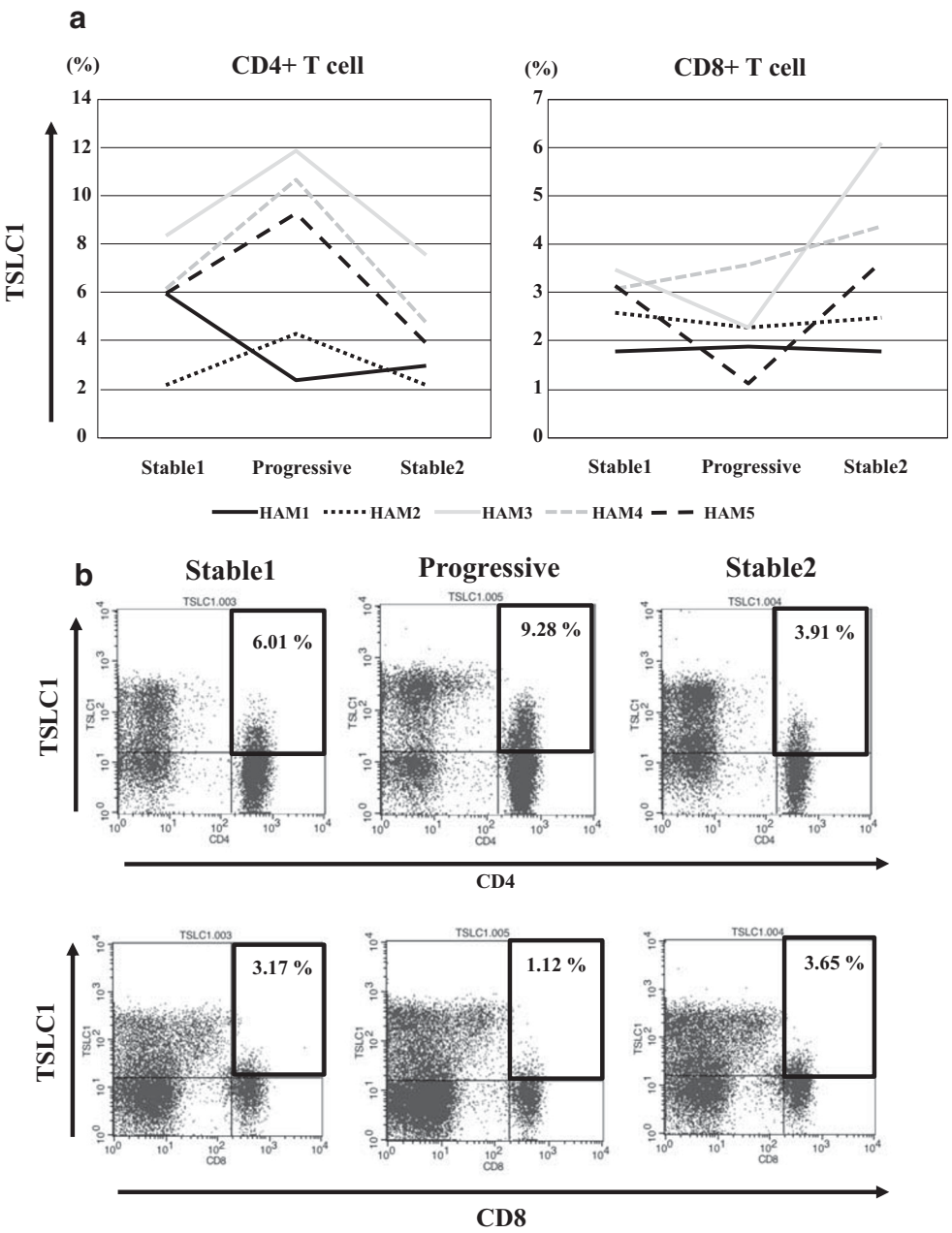


**Fig. 5** Comparison of the expression levels of TSLC1 in clinical symptoms. Results of cell surface TSLC1 expression analysis of HAM/TSP patients by the FACS method in 5 patients individuals (**a**) and representative FACS analysis data (**b**). Stable1 (stable periods) → Progressive (symptoms worsened) → Stable2 (stable periods)

expression was not significantly different in each group when corrected for each infected cell by using proviral loads (Fig. 2b). This finding suggested that the enhancement of *TSLC1* expression in CD4-positive T cells is dependent on the number of infected cells. Proviral loads that are currently being used as a biomarker for disease activity were also measured, and was found to increase in 8 out of 10 people when symptoms worsened (Fig. 4b). Based on these findings, the increase in *TSLC1* was predicted to depend on the increase infected cells number. However, there were several cases in which no change in *TSLC1* expression was observed despite increased HTLV-1 proviral loads. Conversely, there was also a case, which there were no change proviral loads despite increased *TSLC1* expression levels. The amount of *TSLC1* expressed did not necessarily depend on the number of infected cells. An analysis of the TSLC1 molecule by FACS also showed increased expression in CD4-positive T cells during the disease activity stage (Fig. 5a, b). There was a TSLC1-specific upregulation of HAM/TSP during the disease activity period. Considering that the pathogenesis of HAM/TSP is abnormal infiltration of HTLV-1 infected cells into the central nervous system (Umehara et al. 1994; Kubota et al. 1994; Matsuoka et al. 1998), the enhancement of expression of the adhesion molecule TSLC1 in the disease activity stage can reasonably

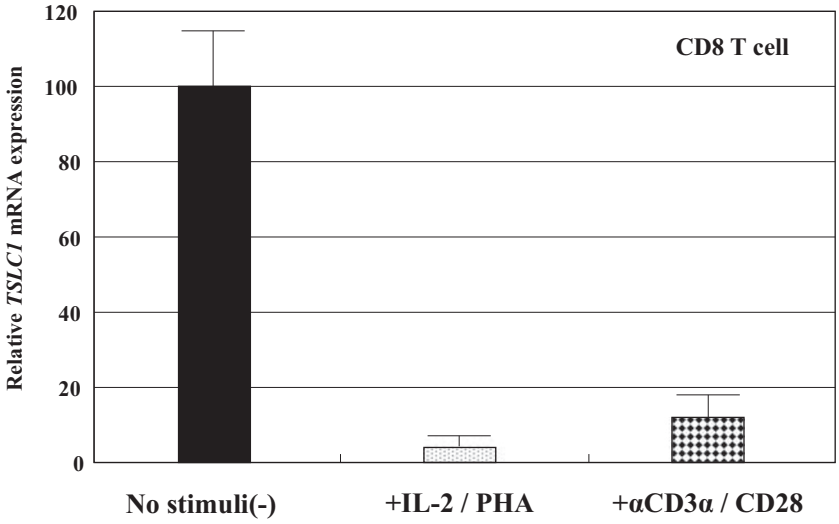


**Fig. 6** Changes in the expression level of TSLC1 by antigen stimulation. Changes in the expression level of *TSLC1* when immune stimulation (either PHA or anti-CD3 antibody/anti-CD28 antibody) was applied to CD8-positive T cells separated by MACS from non-infected HTLV-1. PHA: PHA 10 μg/ml and IL-2 50 U/ml; αCD3αCD28: plate-bound anti-CD3 antibody/anti-CD28 antibody and IL-2 50 U/ml. The mean and standard deviation is shown for three independent experiments

explain the bystander hypothesis. Henceforth, there may be a need to analyze the relationship between the expression level of TSLC1 and its capacity for infiltration in infected cells.

*Tax* and *HBZ* are known as transcriptional activators (Seiki et al. 1983; Sodroski et al. 1984; Fujisawa et al. 1985; Yasunaga and Matsuoka 2007), the increased expression of TSLC1 may be caused by these molecules. *Tax* expression in HAM/TSP patients was higher in CD4-positive T cells than in CD8-positive T cells compared with asymptomatic carriers and that HAM/TSP patients exhibited hyperactivity when the symptoms worsened. *Tax* expression may also reflect the disease activity of HAM/TSP. Therefore, a correlation analysis was performed to examine the direct effect of *Tax* on *TSLC1* expression. No significant correlation was observed, thus indicating that *TSLC1* expression is independent of *Tax* expression and *HBZ* (Fig. 3). Based on these results, TSLC1 was considered a biomarker of disease activity that was independent of Tax and HBZ. The results of this study were not inconsistent with a report indicating that Tax is expressed in trace amounts in vivo and is correlated with the proviral loads and disease activity (Yamano et al. 2002). However, transient hyperactivation occurs ex vivo (Kinoshita et al. 1989; Hanon et al. 2000). The clinical sample we used was stored in liquid nitrogen, and we consider the possibility that the freezing and thawing of the samples enhanced *Tax* expression. *Tax* expression freshly frozen and thawed samples differ significantly (data not shown). Therefore, it is necessary to carefully examine *Tax* expression as a biomarker of disease activity, and it seems that fresh blood needs to be used to perform sufficient analysis.

The *TSLC1* expression levels in CD8-positive T cells between the asymptomatic carrier group and the HAM/TSP patient group did not show a definite trends. However, the HAM/TSP patient group tended to decrease *TSLC1* expression levels when symptoms worsened. In the correction per cell using the proviral loads, the tendency of decreasing *TSLC1* expression level was more pronounced (Fig. 2). HTLV-1 has an infectivity rate of up to 10% in CD8-positive T cells (Hanon et al. 2000), and TSLC1 is expressed in trace amounts even in uninfected cells (Sasaki et al. 2005). This suggests that TSLC1 expression is decreased in HTLV-1 uninfected CD8-positive T cells in HAM/TSP patients. In HAM/TSP patients, persistent antigenic stimulation from HTLV-1 infected cells and the hyperproliferation of inflammatory cytokines (Nagasato et al. 1991; Kitze et al. 1996) cause excessive immune responses (Usuku et al. 1988; Itoyama et al. 1988; Nakagawa et al. 1995). Thus, we observed the effect of immunostimulatory *TSLC1* expression levels due to the potential reduction of *TSLC1* expression by this excess immune response (Fig. 6). Stimulation decreased the expression of *TSLC1* in CD8-positive T cells, thus indicating that this decreased expression was not specific to HTLV-1. This finding suggests that similar results may be obtained in inflammatory diseases such as other infectious diseases and collagen diseases. The significance of TSLC1 in CD8-positive T cells would seem to require further verification in the future. It has been reported that the number of infected cells is high in HAM/TSP patients despite the very high occurrence of HTLV-1-specific cytotoxic T lymphocytes (CTLs); this finding suggests a decrease in CTL function (Nagai et al. 2001). Considering that TSLC1 is an adhesion molecule that acts in immune response (Murakami 2005; Boles et al. 2005; Dewan et al. 2008), the functional deterioration of CTL observed in HAM/TSP might be due to the decreased expression of TSLC1. Therefore, further research is necessary into the correlation between the expression level of TSLC1 and CTL function to suppress the expression of TSLC1 in CTL, promote expression, and analyze the change in CTL function.

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## Compliance with ethical standards

The institutional review boards of the authors' institutions approved this research and written informed consent was obtained from all study participants.

**Conflict of interest** The authors declare that they have no competing interests.

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