JE, Dolin R(ed)："Principles and Practice of Infectious Diseases". Elsevier Churchill Livingstone, Philadelphia, 2005, pp. 2098-2118

3) Tajima K, The T- and B-cell Malignancy Study Group：The 4th nationwide study of adult T-cell leukemia/lymphoma(ATL in Japan)：Estimates of risk of ATL and its geographical and clinical features. Int J Cancer 45：237-243, 1990

4) 山口一成：本邦におけるHTLV-1感染及び関連疾患の実態調査と総合対策. 厚生労働科学研究費補助金研究事業研究班. 平成21年度総括研究報告書：1-25, 2010

5) Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H：Adult T-cell leukemia：clinical and hematologic features of 16 cases. Blood 50：481-492, 1977

6) Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, Matsumoto M, Tara M：HTLV-I associated myelopathy, a new clinical entity. Lancet 1：1031-1032, 1986

7) Mochizuki M, Watanabe T, Yamaguchi T, Takatsuki K, Yoshimura K, Shirao M, Nakashima S, Miyata N：HTLV-I uveitis：A distinct clinical entity caused by HTLV-I. Jpn J Cancer Res 83：236-239, 1992

8) Arisawa K, Soda M, Endo S, Kurokawa K, Katamine S, Shimokawa I, Koba T, Takahashi T, Saito H, Doi H, Shirahama S：Evaluation of adult T-cell leukemia/lymphoma incidence and its impact on non-Hodgkin lymphoma incidence in southwestern Japan. Int J Cancer 85：319-324, 2000

9) 宇都宮 與：ATLLの臨床. 臨床血液 47：1502-1513, 2006

10) 長崎県ATLウイルス母子感染防止研究協力事業連絡協議会：長崎県ATLウイルス母子感染防止研究事業（APP）報告書—20年のあゆみ—, 2008

11) Matsuoka M, Jeang KT：Human T-cell leukaemia virus type 1(HTLV-1)infectivity and cellular transformation. Nat Rev Cancer 7：270-280, 2007

12) Okochi K, Sato H, Hinuma Y：A retrospective study on transmission of adult T cell leukemia virus by blood transfusion：seroconversion in recipients. Vox Sang 46：245-253, 1984

13) Shioiri S, Stuver SO, Okayama A, Murai K, Shima T, Tachibana N, Tsubouchi H, Essex M, Mueller N：Intrafamilial transmission of HTLV-I and its association with anti-Tax antibody in an endemic population in Japan. Int J Cancer 75：15-18, 1998

14) Bartholomew C, Jack N, Edwards J, Charles W, Corbin D, Cleghorn FR, Blattner WA：HTLV-I serostatus of mothers of patients with adult T-cell leukemia and HTLV-I-associated myelopathy/tropical spastic paraparesis. J Hum Virol 1：302-305, 1998

15) Wilks R, Hanchard B, Morgan O, Williams E, Cranston B, Smith ML, Rodgers-Johnson P, Manns A：Patterns of HTLV-I infection among family members of patients with adult T-cell leukemia/lymphoma and HTLV-I associated myelopathy/tropical spastic paraparesis. Int J Cancer 65：272-273, 1996

16) Ueno S, Umeki K, Takajo I, Nagatomo Y, Kusumoto N, Umekita K, Morishita K, Okayama A：Proviral loads of human T-lymphotropic virus type 1 in asymptomatic carriers with different infection routes. Int J Cancer(In press).

17) Kinoshita. K, Hino S, Amagasaki T, Ikeda S, Yamada Y, Suzuyama J, Momita S, Toriya K, Kamihira S, Ichimaru M：Demonstration of adult T-cell leukemia virus antigen in milk from three sero-positive mothers. Jpn J Cancer Res(Gann) 75：103-105, 1984

18) Yamanouchi K, Kinoshita K, Moriuchi R, Katamine S, Amagasaki T, Ikeda S, Ichimaru M, Miyamoto T, Hino S：Oral transmission of humanT-cell leukemia virus type-Ⅰ into a common marmoset (Callithrix Jacchus)as an experimental model for milk-borne transmission. Jpn J Cancer Res(Gann) 76：481-487, 1985

19) Kinoshita K, Amagasaki T, Hino S, Doi H, Yamanouchi K, Ban N, Momita S, Ikeda S, Kamihira S, Ichimaru M, Katamine S, Miyamoto T, Tsuji Y, Ishimaru T, Yamabe T, Ito M, Kamura S, Tuuda T：Milk-borne transmission of HTLV-Ⅰ from carrier mothers to their children. Jpn J Cancer Res(Gann)78：674-680, 1987

20) 齋藤 滋：HTLV-1の母子感染予防に関する研

究. 平成21年度厚生労働科学特別研究報告書 (http://www.mhlw.go.jp/bunya/kodomo/boshi-hoken16/dl/02.pdf)

21) 泉 陽子 : HTLV-1母子感染予防対策について 行政の立場から. 日医雑誌 140 : 812-813, 2011

22) Kawase K, Katamine S, Morinouchi R, Miyamoto T, Kubota K, Igarashi H, Doi H, Tsuji Y, Yamabe T, Hino S : Maternal transmission of HTLV- I other than through breast milk : Discrepancy between the polymerase chain reaction positivity of cord blood samples for HTLV- I and the subsequent seropositivity of individuals. Jpn J Cancer Res 83 : 968-977, 1992

23) Hino S : Establishment of the milk-borne transmission as a key factor for the peculiar endemicity of human T-lymphotropic virus type 1 (HTLV-1) : the ATL Prevention Program Nagasaki. Proc Jpn Acad Ser B Phys Biol Sci 87 : 152-166, 2011

24) Okayama A, Stuver S, Matsuoka M, Ishizaki J, Tanaka G, Kubuki Y, Mueller N, Hsieh CC, Tachibana N, Tsubouchi H : Role of HTLV-1 proviral DNA load and clonality in the development of adult T-cell leukemia/lymphoma in asymptomatic carriers. Int J Cancer 110 : 621-625, 2004

25) Iwanaga M, Watanabe T, Utsunomiya A, Okayama A, Uchimaru K, Koh KR, Ogata M, Kikuchi H, Sagara Y, Uozumi K, Mochizuki M, Tsukasaki K, Saburi Y, Yamamura M, Tanaka J, Moriuchi Y, Hino S, Kamihira S, Yamaguchi K : Human T-cell leukemia virus type I (HTLV-1) proviral load and disease progression in asymptomatic HTLV-1 carriers : a nationwide prospective study in Japan. Blood 116 : 1211-1219, 2010

26) Iga M, Okayama A, Stuver S, Matsuoka M, Mueller N, Aoki M, Mitsuya H, Tachibana N, Tsubouchi H : Genetic evidence of transmission of human T cell lymphotropic virus type 1 between spouses. J Infect Dis 185 : 691-695, 2002

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ORIGINAL ARTICLE

# Clinical significance of CADM1/TSLC1/IgSF4 expression in adult T-cell leukemia/lymphoma

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Cell adhesion molecule 1 (CADM1/TSLC1) was recently identified as a novel cell surface marker for adult T-cell leukemia/lymphoma (ATLL). In this study, we developed various antibodies as diagnostic tools to identify CADM1-positive ATLL leukemia cells. In flow cytometric analysis, the percentages of $CD4^{+}CADM1^{+}$ double-positive cells correlated well with both the percentages of $CD4^{+}CD25^{+}$ cells and with abnormal lymphocytes in the peripheral blood of patients with various types of ATLL. Moreover, the degree of $CD4^{+}CADM1^{+}$ cells over 1% significantly correlated with the copy number of the human T-lymphotropic virus type 1 (HTLV-1) provirus in the peripheral blood of HTLV-1 carriers and ATLL patients. We also identified a soluble form of CADM1 in the peripheral blood of ATLL patients, and the expression levels of this form were correlated with the levels of soluble interleukin 2 receptor alpha. Moreover, lymphomas derived from ATLL were strongly and specifically stained with a CADM1 antibody. Thus, detection of $CD4^{+}CADM1^{+}$ cells in the peripheral blood, measurement of serum levels of soluble CADM1 and immunohistochemical detection of CADM1 in lymphomas would be a useful set of markers for disease progression in ATLL and may aid in both the early diagnosis and measurement of treatment efficacy for ATLL.

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**Keywords:** CADM1/IgSF4/TSLC1; ATLL

## INTRODUCTION

Adult T-cell leukemia/lymphoma (ATLL) results from infection with human T-lymphotropic virus type 1 (HTLV-1).[1,2] Following HTLV-1 infection, 2.1 to 6.6% of HTLV-1 carriers will develop ATLL, and most of the ATLL patients will die within a year.[3] An estimated 10-20 million people worldwide are infected with HTLV-1, and HTLV-1 is endemic in southwestern Japan, the island of Kyushu, Africa, the Caribbean Islands and South America.[4] ATLL cells are mainly derived from activated helper T cells with the $CD3^{+}$, $CD4^{+}$, $CD8^{-}$ and $CD25^{+}$ (also known as interleukin 2 receptor alpha (IL-2Rα)) cell surface markers.[2] A fraction of ATLL cases have been shown to also express forkhead box P3 (*FOXP3*), which is a master gene for regulatory T cells (T-reg), suggesting that some cases of ATLL may originate from HTLV-1-infected T-reg cells.[5,6] For diagnosis, identification of mono- or oligoclonal provirus integration events by Southern blot analysis is one of the definitive markers for ATLL. In addition to viral integration, ATLL cells with multi-lobulated nuclei (called 'flower cells') have been frequently seen in leukemia cells in the peripheral blood of ATLL patients. Hypercalcemia and high levels of either serum lactate dehydrogenase (LDH) or soluble IL-2Rα (sIL-2Rα) have been found to be unfavorable markers for ATLL; however, these markers are not specific for the diagnosis of ATLL.[7,8]

The developmental steps of ATLL after HTLV-1 infection have remained obscure for 30-40 years. HTLV-1 Tax is thought to be an important viral protein that functions in the maintenance of HTLV-1-infected lymphocytes;[9,10] however, expression of Tax protein was not detected in over 70% of ATLL cases because of genomic deletion and/or DNA methylation.[11-14] Recently, HTLV-1 basic leucine zipper (HBZ) was found to be constitutively expressed in ATLL cells and was shown to interact with JUN and CREB2 to regulate Tax expression.[15,16] HBZ also promotes $CD4^{+}$ T-cell proliferation in transgenic mice;[16] therefore, HBZ has important roles and functions not only in maintaining the virus life cycle but also in the maintenance of the HTLV-1-infected cells that contribute to disease pathogenesis. Although HBZ is expressed in the majority of ATLL cells, only 5% of HTLV-1 carriers develop ATLL, suggesting that additional factors besides viral infection are required for the development of ATLL.

To identify additional pathogenic factors or novel surface markers for ATLL, we collected gene expression profiles for acute-type ATLL. Using a comprehensive DNA microarray gene expression analysis, we recently demonstrated that cell adhesion molecule 1 (CADM1/TSLC1/IgSF4) is a novel cell surface maker for ATLL.[17] CADM1 was initially isolated as a tumor suppressor for lung cancers by genomic analysis. CADM1 expression is reduced in a variety of cancers by promoter methylation and is associated with poor prognosis and enhanced metastatic potential.[18] By contrast, we identified that high expression of CADM1 has an important role in enhanced cell-cell adhesion to the vascular endothelium, tumor growth and the organ infiltration of ATLL cells.[19]

In this study, we developed various antibodies for CADM1 to be used as diagnostic tools for identifying ATLL leukemia cells.

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We successfully identified ATLL cells in the peripheral blood and in lymphoma samples and detected the soluble form of CADM1 in the peripheral blood of ATLL patients using specific antibodies for CADM1. The CADM1 antibody may therefore represent a useful tool in the diagnosis of ATLL cells.

## MATERIALS AND METHODS

### Quantification of HTLV-1 proviral load

HTLV-1 proviral DNA load was determined by real-time PCR as previously described.[20] Briefly, genomic DNA from peripheral blood mononuclear cells (PBMCs) was extracted by proteinase K digestion and phenol/chloroform extraction and then subjected to a real-time TaqMan PCR assay using an ABI PRISM 7000 detection system (Perkin Elmer/Applied Biosystems, Forster City, CA, USA) with two sets of primers specific for the *pX* region of the HTLV-1 provirus and the human gene encoding the RNase P enzyme. The primers and the probe for the *RNase P* gene were purchased from Applied Biosystems; those for the *pX* region of the HTLV-1 provirus were described previously.[20] Genomic DNA of normal control PBMCs mixed with a plasmid DNA, which contained almost the whole genome of the HTLV-1 provirus (*SacI* site of 5′-LTR to *SacI* site of 3′-LTR), was used as a standard to quantify the proviral DNA copies. The copy number of the plasmid DNA was calculated based on the size and weight of the plasmid DNA, as measured by spectrophotometry. HTLV-1 proviral loads in some of the PBMC samples were measured by the Group of Joint Study on Predisposing Factors of ATL Development (JSPFAD, Japan) as described previously.[21] The amount of HTLV-1 proviral DNA was calculated as the copy number of HTLV-1 per 100 PBMC = ((copy number of *pX*)/(copy number of *RNase P*/2)) $\times$ 100.

## RESULTS

### Frequent expression of surface CADM1/TSLC1 among ATLL-derived cell lines

CADM1/TSLC1/IgSF4 was identified as a novel surface marker on ATLL cells by gene expression profiling using DNA microarray analysis and was found to be frequently expressed in leukemia cells from patients with acute-type ATLL.[17] We first analyzed the CADM1 protein levels in a panel of T-leukemia cell lines using a chicken anti-human CADM1 antibody (MBL, Nagoya, Japan). A 107 kDa band was clearly detected in whole-cell lysates from the KOB, KK1 and S1T cell lines (Figure 1a), which have been reported

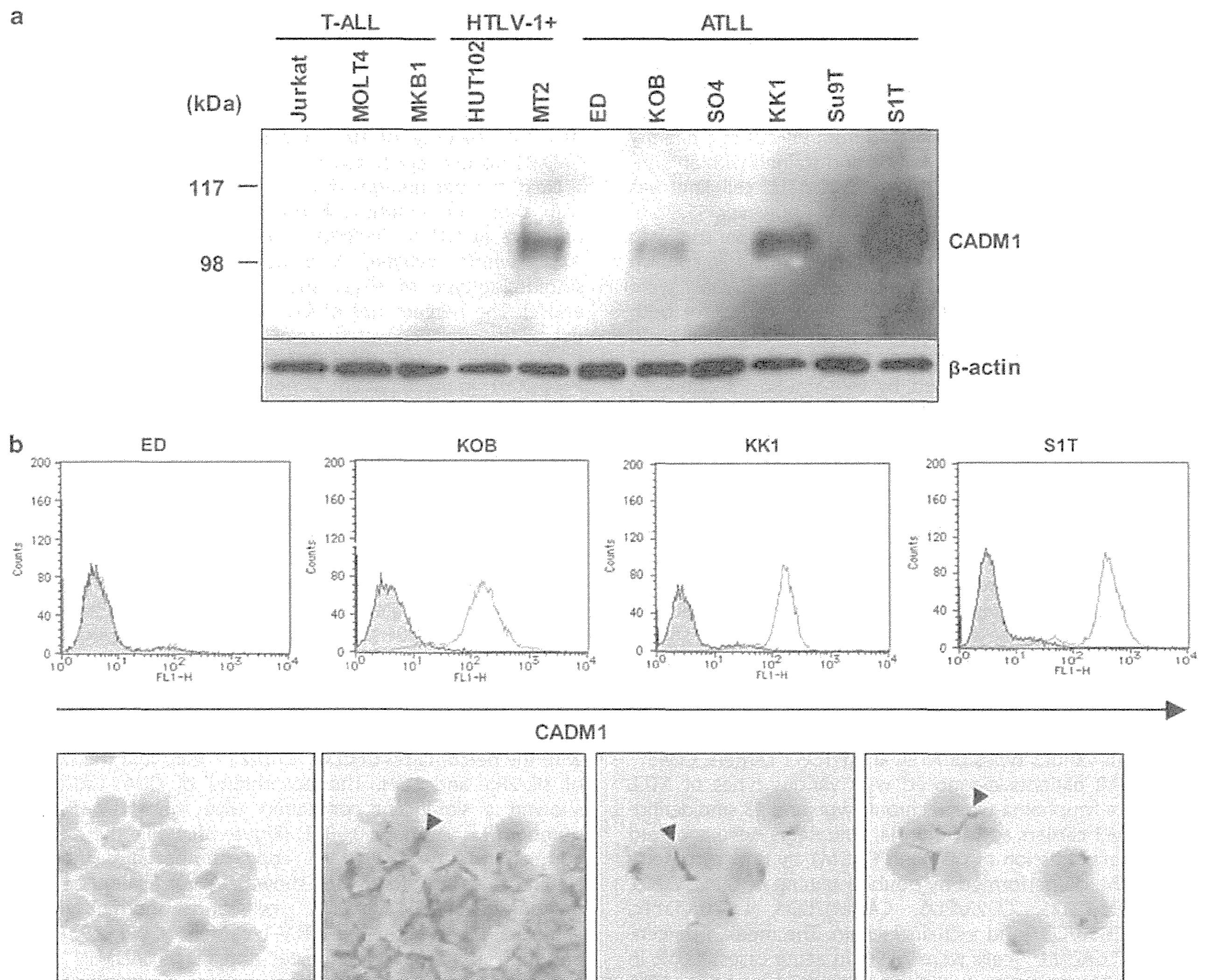


**Figure 1.** High CADM1 expression in ATLL analyzed by immunoblot, flow cytometry (FMC) and immunohistochemical staining (IHC). (**a**) Immunoblot analysis was performed on a series of T-lymphoid leukemia cell lines (three T-ALL, T-acute lymphoid leukemias; two HTLV-1 +, HTLV-1-infected cell lines; six ATLL, ATLL-derived cell lines) with a chicken anti-human CADM1 antibody. (**b**) A human anti-human CADM1 antibody (051-054), which was established by phage display, was used for FMC and IHC. The anti-CADM1 antibody was visualized by Alexa 488 in FMC and by horseradish peroxidase in IHC.



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to express CADM1 according to reverse transcriptase PCR and northern blot analysis.[17] To confirm CADM1 expression on the cell surface of ATLL cells, we examined CADM1 membrane expression by flow cytometry with an Alexa 488-labeled human anti-CADM1 antibody generated by phage-display technology.[22] Four ATLL cell lines were used for flow cytometry: CADM1-negative ED and CADM1-positive KOB, KK1 and S1T cell lines. In all three CADM1-positive cell lines, the fluorescence intensity of CADM1 expression was two logs greater than that of the isotype immunoglobulin G control (Figure 1b, upper panels), while only background levels of fluorescence could be seen in the CADM1-negative ED-ATLL cell line, which had high levels of DNA methylation in the CADM1 promoter region.[17] To evaluate the subcellular distribution of CADM1, immunohistochemical staining was performed on the same cell lines using the anti-CADM1 antibody (Figure 1b, bottom panels). CADM1 was highly concentrated at the cell–cell contact sites in the three CADM1-positive cell lines, and no staining of CADM1 was detected in the ED cell line. These data suggest that CADM1 expression in ATLL cells may promote cell-to-cell contact.

## Low levels of CADM1 expression in the T-reg fraction of peripheral lymphocytes

To examine the expression of CADM1 in peripheral blood T-lymphocytes of healthy volunteers, T-reg populations were analyzed for CADM1 expression because $CD4^+CD25^{high}$ T-reg cells are a potential source of ATLL cells.[5,6] Initially, the $CD4^+CD25^+$ cell fraction was separated from PBMCs of a healthy volunteer by the magnetic bead method and stained with an anti-CADM1 antibody. Almost 100% of the S1T-ATLL cell line was strongly stained with the anti-CADM1 antibody; however, 55.8% of the $CD4^+CD25^+$ cells were stained weakly in comparison with the high level of staining of S1T-ATLL cells (Figure 2a). To confirm whether the purified $CD4^+CD25^+$ cells expressing CADM1 were T-reg cells, the sorted $CD4^+CD25^+$ cells were stained for both FoxP3 (a master regulator in the development of T-reg cells) and CADM1. In all, 93% of the $CD4^+CD25^+$ double-positive cells in the peripheral blood were stained by the anti-FoxP3 antibody, while 37% of the cells were stained with both the anti-CADM1 and anti-FoxP3 antibodies (Figure 2b), suggesting that a fraction of the $CD4^+CD25^+FoxP3^+$ T-reg cells weakly expressed CADM1 on their cell surfaces.

We then determined the proportion of $CD4^+CADM1^+$ and $CD4^+CD25^+$ T cells in PBMCs from 10 healthy volunteers after selection with Cy5-labeled CD45 staining. On average, 7.3% of $CD45^+$ cells in PBMCs expressed CD4 and CD25, while only 0.6% of the cell population expressed CD4 and CADM1 (Figure 2c and representative fluorescence-activated cell sorting data are shown in Supplementary Figures 1a and b), indicating that the number of $CD4^+CADM1^+$ cells was significantly lower than the number of $CD4^+CD25^+$ cells in the PBMCs of healthy volunteers. To determine the percentage of $CD4^+CADM1^+$ cells in peripheral lymphocytes of various types of ATLL and HTLV-1 carriers, $CD45^+$ PBMCs from 40 patients diagnosed with various types of ATLL (7 acute-type, 4 lymphoma-type, 6 chronic-type and 23 smoldering-type), 51 HTLV-1 carriers and 10 normal volunteers were analyzed for the surface expression of CD4 and CADM1 by flow cytometry analysis, which was performed by double staining of CD12/CD19, CD3/CD8, CD4/CD25, CD23/CD5, CADM1/CD4, CD20/CD11c, CD16/CD56, CD30/CD7 and κ-chain/λ-chain. The median percentages of $CD4^+CADM1^+$ cells were 73.9% in acute cases, 72.4% in chronic cases (except for a patient with CD4-negative ATLL described below), 5.6% in lymphoma cases, 11.5% in smoldering cases, 4.4% in HTLV-1 carriers and 0.5% in normal volunteers (Figure 2d). In these subjects, the percentages of $CD4^+CD25^+$ cells were significantly correlated with those of $CD4^+CADM1^+$ cells ($R = 0.907$, $P < 0.0001$) (Figure 2e), suggesting that most of the ATLL cells were $CD4^+CD25^+CADM^+$. However, we also observed a cell surface profile of $CD3^+CD8^-$ (91.3%), $CD25^+CD4^-$ (81.5%) and $CD4^-CADM1^+$ (83.6%) in a case of chronic ATLL, suggesting that the surface markers of the ATLL cells represented $CD4^-CD8^-$ double-negative T lymphocytes that expressed CD25 and CADM1.

## CADM1 expression in leukemia cells from ATLL patients and HTLV-1-infected cells from HTLV-1 carriers

To confirm that most of the HTLV-1-infected ATLL cells were indeed in the $CD4^+CADM1^+$ cell fraction, PBMCs from an HTLV-1 carrier and two ATLL patients with chronic or smoldering ATLL were isolated and separated into CADM1-positive and CADM1-negative cell fractions by anti-CADM1 antibody-conjugated magnetic beads. The cell fractions were then analyzed for the expression of CD4 and CADM1 by fluorescence-activated cell sorting analysis (Supplementary Figure 2). In these three patients, 3.4 to 31.4% of PBMCs were positive for CD4 and CADM1. After separation by the magnetic CADM1 antibody, 73.5 to 96.5% of the cells were $CD4^+CADM1^+$. To assess whether these $CD4^+CADM1^+$ cells indeed represented the HTLV-1-infected cell population, the HTLV-1 status was determined by PCR of the proviral DNA with primers against the *HBZ* region of the HTLV-1 genome. As shown in Figure 3a, the HTLV-1 genomic sequence was detected in the three CADM1-positive cell fractions, while weak or no signal was detected in the CADM1-negative cell fractions, indicating that the majority of HTLV-1-positive cells are present in the CADM1-positive cell fractions.

Next, the percentages of $CD4^+CADM1^+$ cells were compared with those of abnormal lymphocytes or with the DNA copy numbers of HTLV-1 in PBMCs of patients with various types of ATLL, which included 6 acute-type, 8 chronic-type and 6 smoldering-type of ATLL, and 20 HTLV-1 carriers (Figures 3b and c). The percentages of $CD4^+CADM1^+$ cells showed a high degree of correlation with those of abnormal lymphocytes ($R = 0.791$, $P < 0.0001$) and with the HTLV-1 DNA copy numbers ($R = 0.677$, $P < 0.0001$) in these patient samples. Notably, in two samples from chronic- and smoldering-type ATLL patients, the number of $CD4^+CADM1^+$ cells was less than one-half of the number of HTLV-1 DNA copies (32.0% vs 107.97 copies and 30.0% vs 65.76 copies), which may be due to multiple copies of proviral DNA in the cells. In addition, the percentages of $CD4^+CADM1^+$ cells were correlated with the levels of sIL-2Rα ($R = 0.586$, $P < 0.0001$) and with the levels of LDH ($R = 0.486$, $P = 0.0015$) (Figures 3d and e). Consistent with earlier studies, both serum sIL-2Rα and LDH levels were correlated with the HTLV-1 DNA copy numbers ($R = 0.705$; $P < 0.0001$ and $R = 0.44$; $P = 0.0045$, respectively) in this study (data not shown).

To further evaluate the diagnostic efficacy of measuring CADM1-positive cells to detect HTLV-1-infected cells, the copy number of the HTLV-1 provirus in PBMCs of carriers was compared with the percentages of $CD4^+CADM1^+$ cells and the serum levels of sIL-2Rα and LDH. The percentage of $CD4^+CADM1^+$ cells showed a significant correlation with the HTLV-1 DNA copy number ($R = 0.921$, $P < 0.0001$) (Figure 3f), while there was a poor correlation between HTLV-1 copy number and the levels of sIL-2Rα and LDH (data not shown). A correlation between the percentage of $CD4^+CADM1^+$ cells and abnormal lymphocytes was also observed in the HTLV-1 carriers ($R = 0.819$, $P < 0.0001$), although abnormal lymphocytes and $CD4^+CADM1^+$ cells were very rare in these subjects (Supplementary Figure 3). On the basis of these data, in addition to the determination of copy numbers of HTLV-1 proviral DNA, quantification of $CD4^+CADM1^+$ cell number by flow cytometry may be useful for monitoring the number of HTLV-1-infected cells in the peripheral blood of ATLL patients and HTLV-1 carriers.

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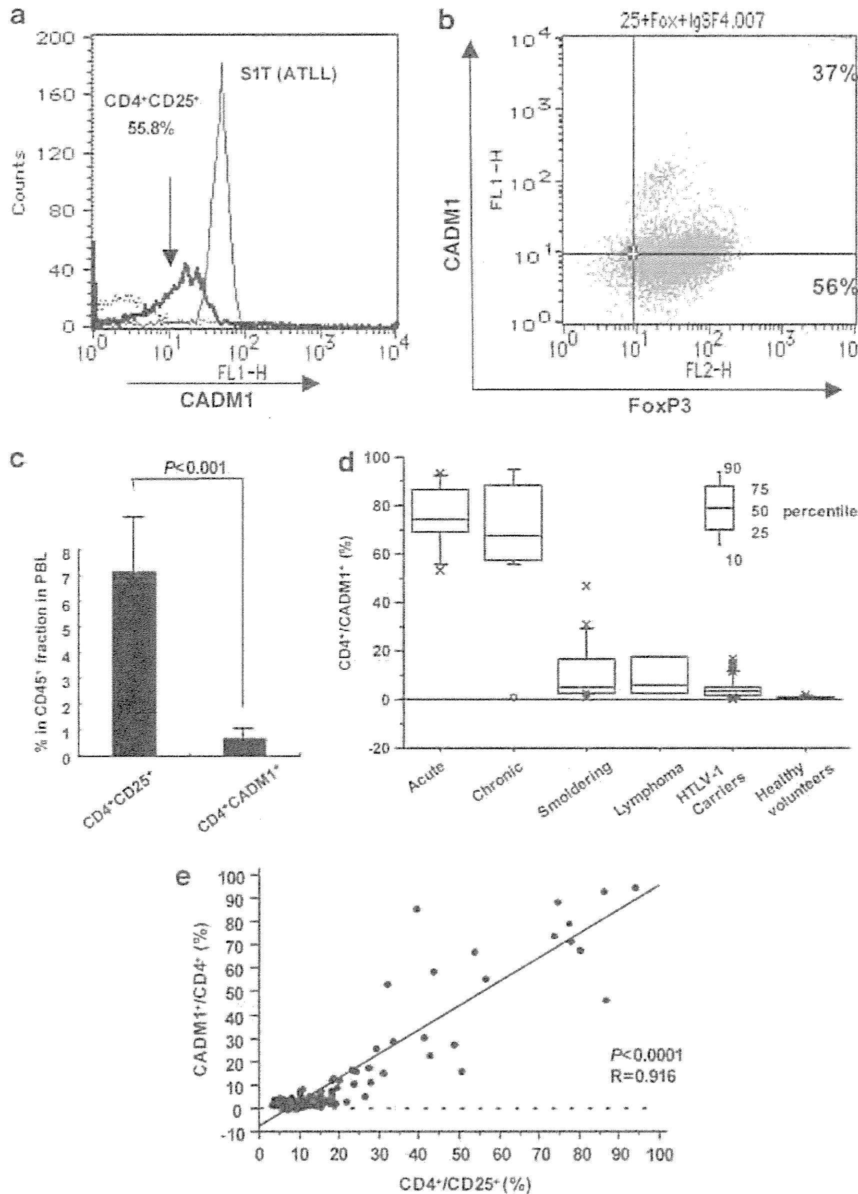


**Figure 2.** Flow cytometric analysis of CADM1 in T-reg lymphocytes, ATLL cells and HTLV-1-infected T cells. (**a**) Flow cytometric analysis of CADM1 expression in the CD4$^+$CD25$^+$ fraction from peripheral T lymphocytes. Each sample was stained with an Alexa 488-labeled anti-CADM1 antibody. The S1T-ATLL cell line with high CADM1 expression was used as a positive control. (**b**) The CD4$^+$CD25$^+$ fraction from peripheral lymphocytes was stained by the Alexa 488-labeled anti-CADM1 and PE-labeled anti-FoxP3 antibodies. (**c**) Comparison of percentages between the CD4$^+$CD25$^+$ and CD4$^+$CADM1$^+$ cell fractions in the CD45$^+$ fraction of peripheral blood lymphocytes. (**d**) Box plots are shown for the percentages of the CD4$^+$CADM1$^+$ cell fractions in CD45$^+$ peripheral blood lymphocytes from patients with various types of ATLL, HTLV-1 carriers and healthy volunteers. The data from a CD4-negative ATLL case are indicated by a white circle. (**e**) Comparison between CD4$^+$CADM1$^+$ and CD4$^+$CD25$^+$ cell fractions in CD45$^+$ peripheral blood lymphocytes from patients with various types of ATLL, HTLV-1 carriers and healthy volunteers. Spearman correlation coefficients were calculated to assess the association between CD4$^+$CADM1$^+$ and CD4$^+$CD25$^+$ cell fractions.

The soluble form of CADM1 is detected in the serum of ATLL patients

A soluble isoform of CADM1 consisting of the extracellular domain was recently isolated in murine mast cells.[23] We determined whether the soluble form of CADM1 was present in the serum of ATLL patients by western blot using a chicken anti-human CADM1 antibody. As a positive control, soluble CADM1 was produced by transfection of 293 cells with a construct encoding a soluble form of CADM1 (1 to 374 aa). The soluble CADM1 band (72 kDa) and the recombinant soluble form of CADM1 were clearly detected in the sera of five patients with acute-type ATLL but not in the sera of five healthy volunteers (Figure 4a). We screened the sera of 5 healthy controls and 25 ATLL patients (14 acute-type, 7 lymphoma-type, 2 smoldering-type and 2 HTLV-1 carrier) for the presence of soluble CADM1. We detected different levels of soluble CADM1 among these ATLL patients by western blot (data not shown). In addition, we compared the levels of soluble CADM1 in the serum and the percentages of CD4$^+$CADM1$^+$ cells in the peripheral blood (Supplementary Figure 4) and confirmed that high levels of soluble CADM1 are present in the serum of patients who had high numbers of CADM1$^+$ cells in the peripheral blood. As serum levels of soluble IL-2R$\alpha$ are correlated with the prognosis


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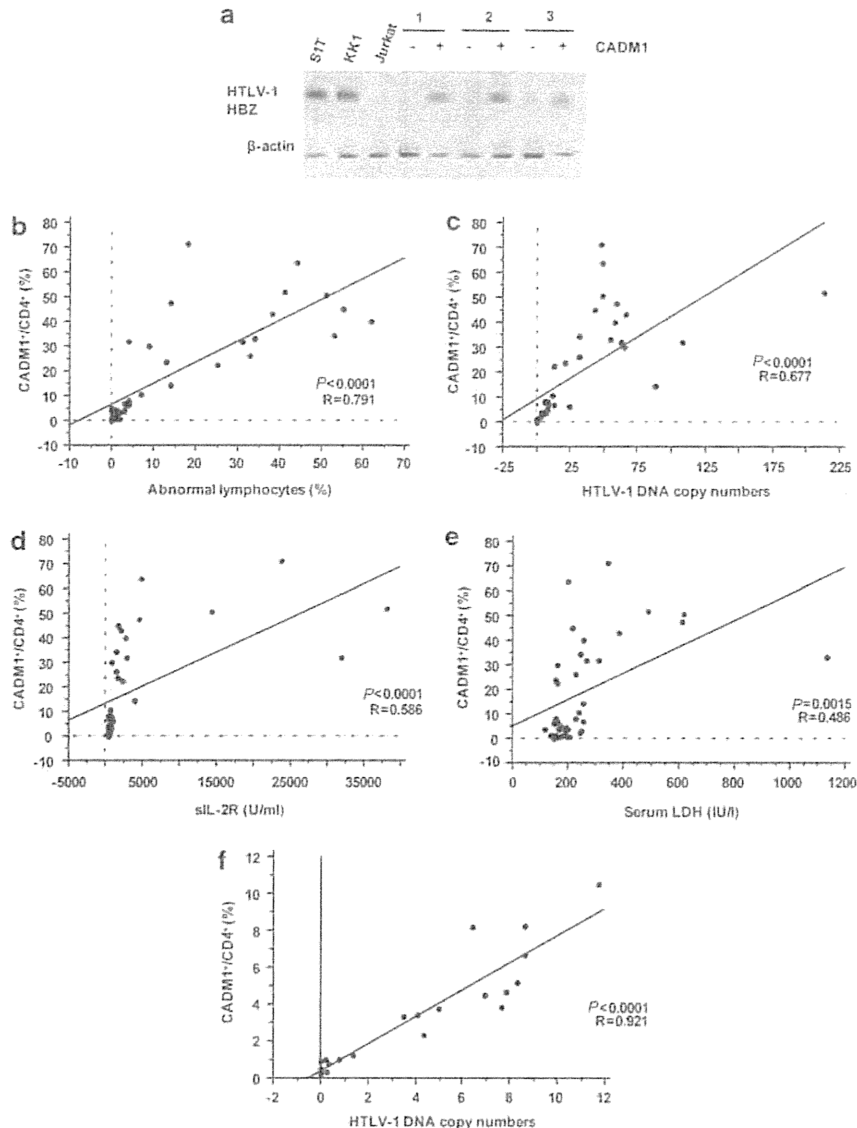


**Figure 3.** Correlation of the percentages of the CD4$^+$CADM1$^+$ fraction with the percentages of abnormal lymphocytes, HTLV-1 DNA copy number and the levels of soluble IL-2Rα and serum LDH in both various types of patients with ATLL and HTLV-1 carriers. (**a**) Identification of the HTLV-1 genome by PCR amplification after separation by CADM1-magnetic beads. After separation of the peripheral blood of three ATLL patients by magnetic beads, genomic DNA was extracted from both the CADM1 and non-CADM1 fractions and amplified by specific PCR primers for HTLV-1 *HBZ*. Two ATLL cell lines (S1T and KK1) were used as positive controls, and a T-ALL cell line (Jurkat) was used as a negative control for the HTLV-1 *HBZ*. Lane 1, smoldering ATLL; lane 2, chronic ATLL; lane 3, HTLV-1 carrier. (**b-e**) The percentage of the CD4$^+$CADM1$^+$ fraction was compared with the percentage of abnormal lymphocytes, the HTLV-1 DNA copy number and the levels of soluble IL-2Rα and serum LDH in both various types of patients with ATLL and HTLV-1 carriers. In (**d**), data from one acute-type patient were not included in the analysis because of the extremely high levels of soluble IL-2Ra (CD4$^+$CADM1$^+$, 32.9%; IL-2Ra, 96 900 U/ml). (**f**) The percentage of the CD4$^+$CADM1$^+$ fraction was compared with the HTLV-1 DNA copy number in HTLV-1 carriers.

of ATLL patients, we compared the serum levels of soluble CADM1 and soluble IL-2Rα in individual cases. As shown in Figure 4b, significantly higher levels of soluble CADM1 were detected in the serum of ATLL patients who had increased levels of soluble IL-2Rα; thus, serum CADM1 levels may be a diagnostic tool for the prediction of disease progression in ATLL.

## High expression of CADM1 in ATLL-derived lymphomas

To examine the expression of CADM1 in tissue sections from lymphoma-type ATLL, formalin-fixed lymphoma samples from different types of malignant lymphomas were immunostained with the anti-CADM1 antibody. For these studies, we used a monoclonal antibody (1-10C) raised against the recombinant extracellular domain of the CADM1 protein. To confirm the reactivity of the anti-CADM1 antibody in formalin-fixed ATLL cells, cell pellets from various leukemia cell lines were fixed in 10% formalin, embedded in paraffin and stained for CADM1. The anti-CADM1 antibody specifically stained the surface of the CADM1-positive S1T-ATLL cell line but did not react with the CADM1-negative ED-ATLL and all non-ATLL cell lines (Figure 5a, panels 1 and 2, and Supplementary Figure 5a). Western blot analysis confirmed the lack of CADM1 expression in these cell lines (Figure 1a and Supplementary Figure 5b). We next performed immunostaining of lymph node biopsies from ATLL patients with malignant lymphoma using the anti-CADM1 antibody. As positive controls, we used erythrocytes and peripheral nerve tissue (Figure 5a, panels 3 and 4).[17,18] In addition, we examined CADM1

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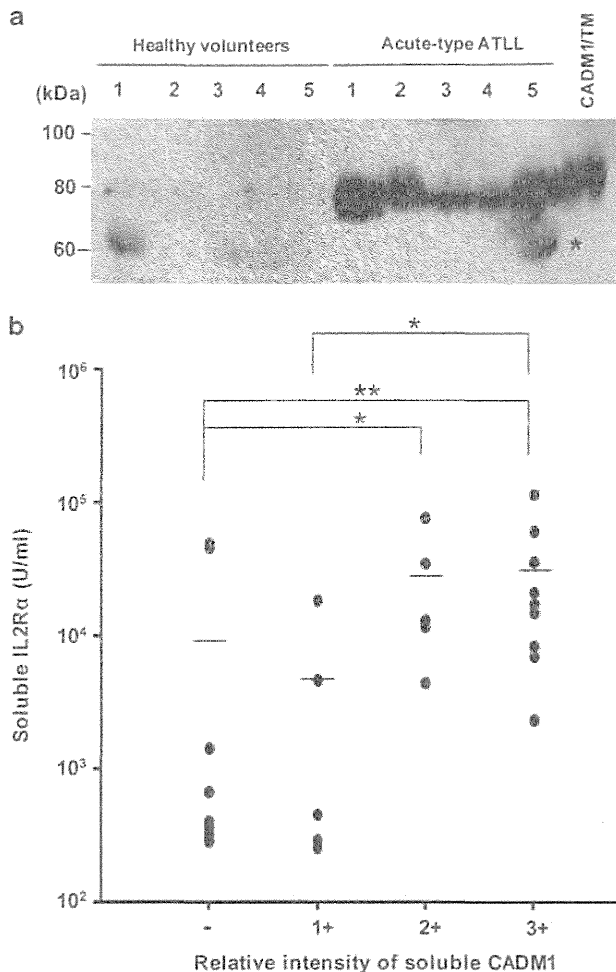


**Figure 4.** Identification of a soluble form of CADM1 in ATLL patients. (**a**) The soluble form of CADM1 in the peripheral blood from five healthy volunteers and five patients with acute-type ATLL was identified by immunoblot analysis using an anti-CADM1 antibody. The asterisk indicates an albumin band. Truncated CADM1 with an extracellular domain was purified from the culture supernatant of 293 cells after transfection of the CADM1 expression plasmid as a positive control. (**b**) The relative band intensity of CADM1 by immunoblot was compared with the level of sIL-2Rα in various serum samples from healthy volunteers, HTLV-1 carriers and ATLL patients. The band intensity was measured by the Image Gauge software (Fujifilm, Tokyo, Japan). The signal intensities were classified as either high (3+), medium (2+), low (1+) or undetectable (-). Asterisks indicate a significant difference between the band intensities of the groups (* $P<0.001$, ** $P<0.0001$).

expression in three cases of lymph nodes with reactive follicular and/or paracortical hyperplasia (reactive lymph nodes) and found that most of the lymphocytes in the reactive lymph nodes were negatively stained and <1% of the cells were positively stained (Figure 5a, panel 5). The staining pattern of the CADM1-positive cells in the reactive lymph nodes mainly shows a uniform cytoplasmic pattern rather than the specific membranous staining that was seen in ATLL cells (as shown below and in Figure 1b). The CADM1-positive cells in reactive lymph node possibly correspond to histiocytes, including dendritic cells because a subset of T-cell zone dendritic cells was reported to express CADM1 (Necl-2) within the lymph node.[24,25] We examined 90 tissue samples from patients with various types of lymphoma, including 36 patients with ATLL and 54 with non-ATLL lymphomas, using erythrocytes and nerve fascicles as positive controls. Of the non-ATLL samples, 29 cases were T- or NK-cell lymphomas, 37 cases were B-cell lymphomas and 2 cases were null-cell lymphomas. Using a four-grade scale to score CADM1 immunohistochemical staining (0 to 3+, Figure 5b), we found that 92% of ATLL lymphomas were positive for CADM1, and 50% of them were heavily stained and were scored 2+ or higher (Table 1). Of note, a few lymphoma cells showed diffuse cytoplasmic staining in addition to membrane staining with CADM1. Among the non-ATLL lymphomas, a few CADM1-positive cells were observed, the morphology of which was small to medium in size with normochromatic round to ovoid nuclei and lacking nuclear atypia (Figure 5c). Based on the morphology and the CADM1-staining patterns, the CADM1-positive cells in the non-ATLL lymphomas were not considered as lymphoma cells but may correspond to histiocytes, including dendritic cells, because these cells were similar to the CADM1-positive cells found in reactive lymph nodes (Figure 5a, panel 5 and Figure 5c). Based on these results, a high degree of cell membrane staining for CADM1 with a score of 2+ may provide high specificity in the diagnosis of ATLL, and combined staining with CADM1 and other T-cell-specific markers may be necessary for a more accurate diagnosis of lymphoma-type ATLL.

## DISCUSSION

In this study, we made a series of antibodies against CADM1 to be used as diagnostic tools for ATLL, such as for the identification and separation of ATLL and HTLV-1-infected cells, the detection of the soluble form of CADM1 in peripheral blood and the pathological identification of lymphoma-type ATLL after formalin fixation. Expression of CADM1 by flow cytometry was clearly detected on the surface of ATLL cells and HTLV-1-infected T lymphocytes, which was confirmed by detection of the HTLV-1 genome after separation by magnetic beads with a CADM1 antibody. The percentage of $CD4^+CADM1^+$ cells in the peripheral blood correlated highly with the DNA copy number of HTLV-1 in lymphocytes from HTLV-1 carriers and ATLL patients. In particular, we identified the soluble form of the CADM1 protein in the peripheral blood of HTLV-1 carriers and ATLL patients. The definitive diagnosis of ATLL is based on the confirmation of ATLL cells in the peripheral blood or in lymphoma tumors by detection of HTLV-1 genomic integration; therefore, measurement of serum levels of soluble CADM1 protein as well as detection of $CD4^+CADM1^+$ cells in the blood, when used in conjunction with other standard diagnostic methods, would be useful for identifying and monitoring disease progression in HTLV-1 carriers with increased accuracy and may aid in the early diagnosis and measurement of treatment effects for ATLL.

It has been proposed that HTLV-1 infects various types of cells, including T-reg cells and subsets of T helper cells (Th2 and Th17), in a cell-to-cell manner.[26-29] There is also evidence that ATLL cells act as T-reg cells that express CD4, CD25 and FoxP3 and are thought to contribute to the immune suppression of ATLL patients;[6] however, it was reported that CADM1 is expressed at low levels on resting naive T cells, and its expression is further downregulated 14 h following TCR activation.[30] Therefore, we determined the expression of CADM1 in the T-reg cell fraction of the peripheral blood of healthy volunteers. The results showed that a subset of the T-reg fraction weakly expressed CADM1, suggesting that CADM1 is not a major marker for the T-reg fraction and that CADM1 expression on ATL cells may reflect the fact that ATL cells originate from T-reg cells. As ATLL cells that constitutively express CD25 exhibited heterogeneous Foxp3 expression patterns,[5] a part of ATLL is likely derived from $FoxP3^+$ T-reg cells. In another report, a population of $FoxP3^+$ cells distinct from ATLL cells was shown to have a regulatory function and was found to impair the cell-mediated immune response to HTLV-1 in patients with ATLL.[31] Although we do not know whether the population of T-reg cells with weak expression of CADM1 in the


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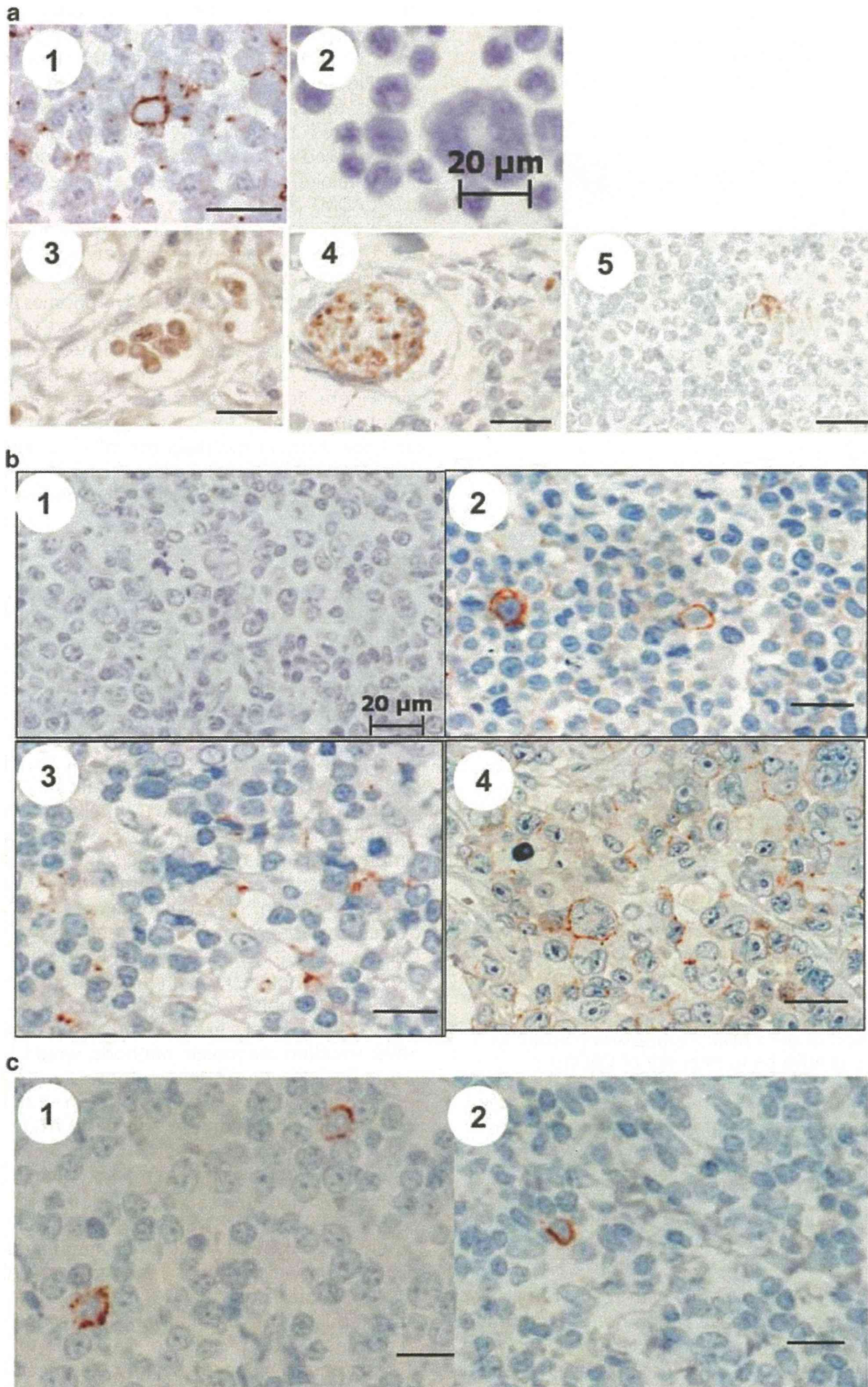


**Figure 5.** Expression of CADM1 in lymphoma-type ATLL. (**a**) Immunostaining of CADM1 in the S1T-ATLL cell line was used as a positive control (a1), and the ED-ATLL cell line was used as a negative control (a2) for CADM1 expression using an anti-CADM1 antibody (1-10C). Immunostaining of erythrocytes in the blood vessels (**c**), peripheral nerve cells (a3) and reactive lymph nodes (a4) using the same antibody. Scale bar, 20 μm. (**b**) The immunoreactivity for CADM1 was scored using a standardized four-tiered scoring scale as follows: staining in >30% of cells was scored as 3+ (b4); staining in >5% but <30% of cells was scored as 2+ (b3); staining in <5% of cells was scored as 1+ (b2); and a lack of staining was scored as 0 (b1). These images were taken from immunostained ATLL lymphoma sections. Scale bar, 20 μm. (**c**) Representative CADM1 immunostaining in B-cell (c1) and NK-cell (c2) lymphomas. Scale bar, 20 μm.

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**Table 1.** Immunohistochemical staining of CADM1 in various types of lymphomas, including ATLL

| | *Case numbers* | *Staining scores* | | | | *Positive rates (%)* | |
|---|---|---|---|---|---|---|---|
| | | *Negative* | *1+* | *2+* | *3+* | *⩾1+* | *⩾2+* |
| ATLL | 36 | 3 | 15 | 14 | 4 | 33/36 (92) | 18/36 (50) |
| Non-ATLL | 54 | 37 | 16 | 1 | 0 | 17/54 (31) | 1/54 (1.8) |
| T/NK | 15 | 12 | 3 | 0 | 0 | 3/15 (20) | 0/15 (0) |
| B | 37 | 23 | 13 | 1 | 0 | 14/37 (38) | 1/37 (2.7) |
| Null | 2 | 2 | 0 | 0 | 0 | 0/2 (0) | 0/2 (0) |

Abbreviations: ATLL, adult T-cell leukemia/lymphoma; CADM1, cell adhesion molecule 1. The immunoreactivity for CADM1 was scored using a standardized four-tiered scoring scale as follows: staining in >30% of cells was scored as 3+; staining in >5% but <30% of cells was scored as 2+; staining in <5% of cells was scored as 1+; lack of staining was scored as 0.

PBMCs of healthy volunteers is the cellular origin for ATLL cells, CADM1 is thought to be one of the major markers for the various types of ATLL cells. In fact, we observed strong expression of CADM1 in rare cases of ATLL characterized by the $CD4^+CD8^+$, $CD4^-CD8^+$ or $CD4^-CD8^-$ phenotypes (data not shown); therefore, the $CADM1^{high}$ population of T-lymphocytes in peripheral blood can be considered ATLL cells.

The question of why CADM1 is strongly expressed on the surface of various types of ATLL remains unclear. Previously, we investigated whether the expression of CADM1 was induced by HTLV-1/Tax expression and found that Tax protein expression did not activate the expression of CADM1 in JPX-9 cells (data not shown). We also introduced a Tax expression vector into MOLT4 and 293T cells and determined the expression level of CADM1. We found that Tax could not induce CADM1 expression in these cells, suggesting that Tax expression is not related to the high expression of CADM1. As HBZ is known to be constitutively expressed in both HTLV-1-infected cells and ATLL cells and can modulate transcription of cellular genes,[16] it is possible that HBZ activates CADM1 expression. We also speculate that $CADM1^{high}$ expression in ATLL cells may be associated with transcriptional abnormalities in ATLL cells through the accumulation of genomic or epigenomic alterations. In this study, we found a good correlation between HTLV-1 copy numbers and the percentages of $CD4^+CADM1^+$ cells in the peripheral blood of HTLV-1 carriers, suggesting that HTLV-1 carriers with high percentages of $CD4^+CADM1^+$ cells could be associated with progressive genetic alterations and might be at high risk for developing ATLL.

Recent studies have shown that a few markers, such as CCR4 and CD70, are unique ATLL surface markers.[32,33] Although the proportion of $CD4^+CCR4^+$ cells and $CD4^+CD70^+$ cells in the PBMCs from healthy individuals were found to be approximately 5%,[27,33] the proportion of $CD4^+CADM1^+$ cells was <1% (Figure 2); therefore, measurement of $CADM1^+$ T cells is particularly efficient in the diagnosis of HTLV-1 infection in individuals who carry a small number of HTLV-1-infected cells. We have demonstrated previously that CADM1 has important functions in increasing cell adhesion and mediating progression to organ invasion.[19] In this study, we succeeded in isolating a low percentage of both HTLV-1-infected cells from the PBMCs of HTLV-1 carriers with high HTLV-1 copy numbers and ATLL cells from patients with ATLL. The sorted HTLV-1-infected cells and ATLL cells could become useful tools for transcriptional and/or genomic analysis that may be used to compare their results with those of PBMCs from either healthy volunteers or peripheral leukemia cells from patients with ATLL. The results may provide important information on the expression patterns and/or genomic abnormalities that occur at the early stages of HTLV-1 infection and/or ATLL development.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

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## REFERENCES

1 Yoshida M, Miyoshi I, Hinuma Y. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc Natl Acad Sci USA* 1982; **79**: 2031-2035.
2 Takatsuki K. Discovery of adult T-cell leukemia. *Retrovirology* 2005; **2**: 16.
3 Arisawa K, Soda M, Endo S, Kurokawa K, Katamine S, Shimokawa I *et al.* Evaluation of adult T-cell leukemia/lymphoma incidence and its impact on non-Hodgkin lymphoma incidence in southwestern Japan. *Int J Cancer* 2000; **85**: 319-324.
4 Proietti FA, Carneiro-Proietti AB, Catalan-Soares BC, Murphy EL. Global epidemiology of HTLV-I infection and associated diseases. *Oncogene* 2005; **24**: 6058-6068.
5 Karube K, Ohshima K, Tsuchiya T, Yamaguchi T, Kawano R, Suzumiya J *et al.* Expression of FoxP3, a key molecule in CD4CD25 regulatory T cells, in adult T-cell leukaemia/lymphoma cells. *Br J Haematol* 2004; **126**: 81-84.
6 Chen S, Ishii N, Ine S, Ikeda S, Fujimura T, Ndhlovu LC *et al.* Regulatory T cell-like activity of Foxp3+ adult T cell leukemia cells. *Int Immunol* 2006; **18**: 269-277.
7 Yasuda N, Lai PK, Ip SH, Kung PC, Hinuma Y, Matsuoka M *et al.* Soluble interleukin 2 receptors in sera of Japanese patients with adult T cell leukemia mark activity of disease. *Blood* 1988; **71**: 1021-1026.
8 Kamihira S, Atogami S, Sohda H, Momita S, Yamada Y, Tomonaga M. Significance of soluble interleukin-2 receptor levels for evaluation of the progression of adult T-cell leukemia. *Cancer* 1994; **73**: 2753-2758.
9 Akagi T, Ono H, Shimotohno K. Characterization of T cells immortalized by Tax1 of human T-cell leukemia virus type 1. *Blood* 1995; **86**: 4243-4249.
10 Furukawa Y, Kubota R, Tara M, Izumo S, Osame M. Existence of escape mutant in HTLV-I tax during the development of adult T-cell leukemia. *Blood* 2001; **97**: 987-993.
11 Tamiya S, Matsuoka M, Etoh K, Watanabe T, Kamihira S, Yamaguchi K *et al.* Two types of defective human T-lymphotropic virus type I provirus in adult T-cell leukemia. *Blood* 1996; **88**: 3065-3073.
12 Koiwa T, Hamano-Usami A, Ishida T, Okayama A, Yamaguchi K, Kamihira S *et al.* 5′-long terminal repeat-selective CpG methylation of latent human T-cell leukemia virus type 1 provirus *in vitro* and *in vivo*. *J Virol* 2002; **76**: 9389-9397.
13 Takeda S, Maeda M, Morikawa S, Taniguchi Y, Yasunaga J, Nosaka K *et al.* Genetic and epigenetic inactivation of tax gene in adult T-cell leukemia cells. *Int J Cancer* 2004; **109**: 559-567.
14 Taniguchi Y, Nosaka K, Yasunaga J, Maeda M, Mueller N, Okayama A *et al.* Silencing of human T-cell leukemia virus type I gene transcription by epigenetic mechanisms. *Retrovirology* 2005; **2**: 64.
15 Basbous J, Arpin C, Gaudray G, Piechaczyk M, Devaux C, Mesnard JM. The HBZ factor of human T-cell leukemia virus type I dimerizes with transcription factors JunB and c-Jun and modulates their transcriptional activity. *J Biol Chem* 2003; **278**: 43620-43627.
16 Satou Y, Yasunaga J, Yoshida M, Matsuoka M. HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells. *Proc Natl Acad Sci USA* 2006; **103**: 720-725.
17 Sasaki H, Nishikata I, Shiraga T, Akamatsu E, Fukami T, Hidaka T *et al.* Overexpression of a cell adhesion molecule, TSLC1, as a possible molecular marker for acute-type adult T-cell leukemia. *Blood* 2005; **105**: 1204-1213.
18 Murakami Y. Involvement of a cell adhesion molecule, TSLC1/IGSF4, in human oncogenesis. *Cancer Sci* 2005; **96**: 543-552.
19 Dewan MZ, Takamatsu N, Hidaka T, Hatakeyama K, Nakahata S, Fujisawa J *et al.* Critical role for TSLC1 expression in the growth and organ infiltration of adult T-cell leukemia cells *in vivo*. *J Virol* 2008; **82**: 11958-11963.
20 Tanaka G, Okayama A, Watanabe T, Aizawa S, Stuver S, Mueller N *et al.* The clonal expansion of human T lymphotropic virus type 1-infected T cells: a comparison between seroconverters and long-term carriers. *J Infect Dis* 2005; **191**: 1140-1147.
21 Shimizu Y, Takamori A, Utsunomiya A, Kurimura M, Yamano Y, Hishizawa M *et al.* Impaired Tax-specific T-cell responses with insufficient control of HTLV-1 in a subgroup of individuals at asymptomatic and smoldering stages. *Cancer Sci* 2009; **100**: 481-489.
22 Kurosawa G, Akahori Y, Morita M, Sumitomo M, Sato N, Muramatsu C *et al.* Comprehensive screening for antigens overexpressed on carcinomas via


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isolation of human mAbs that may be therapeutic. *Proc Natl Acad Sci USA* 2008; **105**: 7287-7292.
23 Koma Y, Ito A, Wakayama T, Watabe K, Okada M, Tsubota N *et al.* Cloning of a soluble isoform of the SgIGSF adhesion molecule that binds the extracellular domain of the membrane-bound isoform. *Oncogene* 2004; **23**: 5687-5692.
24 Galibert L, Diemer GS, Liu Z, Johnson RS, Smith JL, Walzer T *et al.* Nectin-like protein 2 defines a subset of T-cell zone dendritic cells and is a ligand for class-I-restricted T-cell-associated molecule. *J Biol Chem* 2005; **280**: 21955-21964.
25 Takeuchi A, Itoh Y, Takumi A, Ishihara C, Arase N, Yokosuka T *et al.* CRTAM confers late-stage activation of CD8+ T cells to regulate retention within lymph node. *J Immunol* 2009; **183**: 4220-4228.
26 Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J *et al.* Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int Immunol* 1999; **11**: 81-88.
27 Baatar D, Olkhanud P, Sumitomo K, Taub D, Gress R, Biragyn A. Human peripheral blood T regulatory cells (Tregs), functionally primed CCR4+ Tregs and unprimed CCR4- Tregs, regulate effector T cells using FasL. *J Immunol* 2007; **178**: 4891-4900.
28 Lim HW, Lee J, Hillsamer P, Kim CH. Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+ regulatory T cells. *J Immunol* 2008; **180**: 122-129.
29 Hieshima K, Nagakubo D, Nakayama T, Shirakawa AK, Jin Z, Yoshie O. Tax-inducible production of CC chemokine ligand 22 by human T cell leukemia virus type 1 (HTLV-1)-infected T cells promotes preferential transmission of HTLV-1 to CCR4-expressing CD4+ T cells. *J Immunol* 2008; **180**: 931-939.
30 Yeh JH, Sidhu SS, Chan AC. Regulation of a late phase of T cell polarity and effector functions by Crtam. *Cell* 2008; **132**: 846-859.
31 Toulza F, Nosaka K, Takiguchi M, Pagliuca T, Mitsuya H, Tanaka Y *et al.* FoxP3+ regulatory T cells are distinct from leukemia cells in HTLV-1-associated adult T-cell leukemia. *Int J Cancer* 2009; **125**: 2375-2382.
32 Yoshie O, Fujisawa R, Nakayama T, Harasawa H, Tago H, Izawa D *et al.* Frequent expression of CCR4 in adult T-cell leukemia and human T-cell leukemia virus type 1-transformed T cells. *Blood* 2002; **99**: 1505-15011.
33 Baba M, Okamoto M, Hamasaki T, Horai S, Wang X, Ito Y *et al.* Highly enhanced expression of CD70 on human T-lymphotropic virus type 1-carrying T-cell lines and adult T-cell leukemia cells. *J Virol* 2008; **82**: 3843-3852.

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ORIGINAL ARTICLE

# HTLV-I virological and histopathological analysis in two cases of anti-centromere-antibody-seropositive Sjögren's syndrome

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**Abstract**
*Introduction* The aim of this study was to show the clinical and pathological characteristics of anti-centromere-antibody (ACA)-seropositive Sjögren's syndrome (SS) in two anti-human T-cell leukemia virus type I (HTLV-I)-seropositive patients.
*Methods* One patient was an HTLV-I carrier whereas the other was diagnosed with HTLV-I-associated myelopathy (HAM). Background data including serum HTLV-I titers, viral loads, and cytokine profiles were recorded. Azocarmine with aniline blue (Azan)–Mallory staining and immunohistochemistry of the labial salivary glands (LSGs) and a muscle biopsy specimen from the HAM patient were performed.
*Results* Serum transforming growth factor beta (TGF-$\beta$), tumor necrosis factor alpha (TNF-$\alpha$), and HTLV-I viral load were high in the HAM-SS patient compared with the HTLV-I carrier. Fibrous change in LSG was prominent in the HAM-SS patient. Although TGF-$\beta$ expression was similar in the two patients, expression of HTLV-I-related proteins including p12, p28, group-specific antigen (GAG), and nuclear factor kappa-B (NF-$\kappa$B) in the LSG were dominantly detected in the HAM-SS patient. Frequency of TGF-$\beta$ staining in HTLV-I-seropositive SS patients without ACA, HTLV-I-seronegative SS patients with ACA, and HTLV-I-seronegative SS patients without ACA was lower than that of the previous two patients.
*Conclusion* A high HTLV-I viral load in situ is supposed to promote the production of cytokines, especially TGF-$\beta$, resulting in the fibrous change of LSG in ACA-seropositive SS patients.

**Keywords** HTLV-I infection · Anti-centromere antibody · Sjögren's syndrome · Cytokine

**Abbreviations**

| | |
|---|---|
| ACA | Anti-centromere antibody |
| ANA | Anti-nuclear antibody |
| CSF | Cerebrospinal fluid |
| HAM | HTLV-I-associated myelopathy |
| HTLV-I | Human T-cell leukemia virus type I |
| IFN-$\gamma$ | Interferon gamma |
| MNC | Mononuclear cell |
| LSG | Labial salivary gland |
| SS | Sjögren's syndrome |
| TGF-$\beta$ | Transforming growth factor beta |
| TNF-$\alpha$ | Tumor necrosis factor alpha |

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## Introduction

Human T-cell leukemia virus type I (HTLV-I) is known to be one of the causative agents of Sjögren's syndrome (SS) [1, 2]. Our previous epidemiologic studies show a close association between HTLV-I and SS [3, 4]. In addition, we found a significantly high prevalence of SS in patients with HTLV-I-associated myelopathy (HAM) [3, 5]. On the other hand, anti-centromere antibody (ACA) is known as a second class of autoantibodies in SS patients [6, 7]. Our previous report revealed that ACA is detected in only 4 % of HTLV-I-seropositive SS cases, demonstrating that HTLV-I might not be involved in the pathogenesis in ACA-seropositive SS patients [8]. However, if HTLV-I infection coincidently occurs in ACA-seropositive SS patients, the influence of ACA on HTLV-I-associated SS might become obvious. In this study, we report two cases of ACA-seropositive SS patients who were also seropositive for anti-HTLV-I antibody. One patient was complicated with HAM, whereas the other was an HTLV-I carrier. The variation in HTLV-I viral road in these patients appears to explain the differences in labial salivary gland (LSG) histopathology and cytokine profile.

## Patients and methods

### Patients

#### *Case 1*

This was a 61-year-old female patient who complained of sicca symptoms. Both ACA and anti-HTLV-I antibody measured by chemiluminescent enzyme immunoassay (CLEIA) were highly positive, as shown in Table 1. As no other symptoms or signs, including in the neuromuscular systems, were found in this patient, she was classified as an HTLV-I carrier.

#### *Case 2*

A 57-year-old female patient who complained of sicca symptoms and myalgia was diagnosed with HAM based on the diagnostic guidance for HAM determined by the Ministry of Health, Labour and Welfare. She had slowly progressive and symmetrical pyramidal tract damage with positive anti-HTLV-I antibody in both serum and cerebrospinal fluid (CSF). Antibodies against gp46, p53, p24, and p19 of HTLV-I in CSF were all positive. Serum ACA was also positive at a high titer (Table 1). She also suffered from inflammatory myopathy as evidenced by the elevation of muscle enzymes and by magnetic resonance imaging and muscle biopsy findings.

Both patients were diagnosed with SS according to the revised criteria [9], as proposed by the American–European Consensus Group. In both cases, HTLV-I viral roads in sera and serum cytokines including tumor necrosis factor alpha (TNF-$\alpha$), interferon gamma (IFN-$\gamma$), and transforming growth factor beta (TGF-$\beta$) were measured. For comparison, we studied the three groups of patients: (1) HTLV-I-seropositive SS patients without ACA, (2) HTLV-I-seronegative SS patients with ACA, and (3) HTLV-I-seronegative SS patients without ACA with respect to TGF-$\beta$ immunostaining of LSG (four patients each in three groups).

### LSG biopsy

LSG biopsy from the lower lip was performed under local anesthesia in SS patients. Informed consent to use biopsy samples was obtained from all participating patients at the commencement of the study. The study was conducted with the approval of the human ethical committee of our institution. The classifications of Chisholm and Mason [10] were used to determine the severity of mononuclear cell (MNC) infiltration.

### Azan–Mallory staining and immunohistochemistry of labial salivary glands

Formalin-fixed, paraffin-embedded sections (3-μm thick) from the LSGs of these ACA-seropositive SS patients were used for azocarmine with aniline blue (Azan)–Mallory staining and immunohistochemistry. The sections were then stained using the Histofine Simple Stain Kit (Nichirei Co., Tokyo, Japan) with mouse anti-human CD4, CD8, CD20, and CD68 antibodies (DakoCytomation, Glostrup, Denmark), mouse anti-HTLV-I [p19, p28, and group-specific antigen (GAG)] antibody (Chemicon International Inc., Temecula, CA, USA), mouse anti-nuclear factor kappa B (NF-$\kappa$B) p65 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and mouse anti-TGF-$\beta$ antibody (LifeSpan BioSciences, Inc., Seattle, WA, USA). Briefly, endogenous peroxidase was inactivated in a 3 % hydrogen peroxide ($H_2O_2$) solution after microwave epitope retrieval. These sections were then blocked with 5 % normal horse serum, followed by incubation with monoclonal and polyclonal antibodies in a humid chamber for 60 min at room temperature. After incubation, all sections, including the negative control sections, were treated with peroxidase-conjugated secondary antibodies for 30 min. The color was developed by soaking the sections in 3.3′-diaminobenzidine (DAB) and $H_2O_2$ for 10 min, followed by counterstaining by soaking the sections in hematoxylin solution. Negative

**Table 1** Background information and serum data of the human T-cell leukemia virus type I (HTLV-I)-associated anti-centromere antibody (ACA)-seropositive patients

| | Case 1<br>HTLV-I carrier with ACA-seropositive SS | Case 2<br>HAM with ACA-seropositive SS |
|---|---|---|
| Age and gender | 61 years old, female | 57 years old, female |
| Xerostomia | Positive | Positive |
| Xerophthalmia | Positive | Negative |
| Schirmer test (right/left mm; <5 mm: positive) | 5/4 | 11/11 |
| Saxon test (g/2 min; <2 g: positive) | 1.47 | 2.7 |
| ANA: pattern | 160×, centromere | 640×, centromere |
| Anti-SS-A antibody: normal 10–30 U/ml | 0.7 | 0.9 |
| Anti-SS-B antibody: normal 15–25 U/ml | 0.9 | 0.5 |
| ACA: normal <16 index | 172.8 | 165.0 |
| IgG: normal 870–1,700 mg/dl | 1,712 | 1,623 |
| Rheumatoid factor: normal <15 IU/ml | 11.4 | 17.0 |
| Sialography[a] (Rubin and Holt) | Stage 1 | Stage 2 |
| Lip biopsy grade[b] (Chisholm and Mason) | 3 | 3 |
| LST (cpm) PHA(+)/no stimulation | 105,936/617 | 184,859/19,319 |
| LST (cpm) ConA(+)/no stimulation | 160,934/617 | 102,299/19,319 |
| Serum anti-HTLV-I antibody: normal <1.0 COI | >45 | >45 |
| Serum viral load (copies/$10^4$ cells) | <53 | 373 |
| Serum TNF-α: normal 0.6–2.8 pg/ml | 1.0 | 2.9 |
| Serum IFN-γ: normal <0.1 IU/ml | <0.1 | <0.1 |
| Serum TGF-β: normal 1.56–3.24 ng/ml | 2.76 | 12.6 |

Anti-SS-A Ab and anti-SS-B Ab (Mesacup SS-A/Ro test and SS-B/La test; Medical and Biological Laboratories, Nagoya, Japan) and ACA (Mesacup-2 test CENP-B; Medical and Biological Laboratories, Nagoya, Japan) were measured using an enzyme-linked immunosorbent assay (ELISA) kit. Serum anti-HTLV-I antibody was measured by chemiluminescent enzyme immunoassay, and HTLV-I viral load was measured by the FastStart DNA Master Hybridization probe method. Serum TNF-α and TGF-β were measured by ELISA. Serum IFN-γ was measured by enzyme immunoassay. Data shown represent the period before treatments with agents such as glucocorticoids or immunosuppressive agents

*SS* Sjögren's syndrome, *ANA* anti-nuclear antibody, *COI* cutoff index, *ConA* concanavalin A, *cpm* count per minute, *HAM* HTLV-I-associated myelopathy, *Ig-G* immunoglobulin G, *LST* lymphocyte stimulation test, *PHA* phytohemagglutinin, *TNF* tumor necrosis factor, *IFN* interferon *TGF* transforming growth factor

[a] Sialography grading was determined by Rubin and Holt. Stages 1 and 2 represent punctate and globular patterns, respectively

[b] Grading defined by Chisholm and Mason: the presence of at least one focus of mononuclear cells per 4 $mm^2$ section = grade 3

control sections were treated with mouse immunoglobulin (Ig)G1.

## Results

### Clinical and serological data with cytokine profile

As shown in Table 1, a high ACA titer was detected in both patients. Serum IgG was almost normal, which is characteristic in ACA-seropositive SS patients [6]. As patient 2 was diagnosed with HAM, spontaneous proliferation of MNCs was significantly higher than in patient 1. Serum HTLV-I viral load was 373 copies/$10^4$ cells in patient 2, which is obviously higher than in patient 1 (<53 copies/$10^4$ cells). Serum TNF-α and TGF-β levels in patient 2 were increased compared with those in patient 1, although serum IFN-γ in both patients was within normal limits.

### Azan–Mallory staining and immunohistochemical analysis

MNC infiltration was similar in both patients; however, Azan–Mallory staining showed a stronger fibrosis in patient 2 than in patient 1 (Fig. 1). In patient 2, TGF-β was highly stained in infiltrating MNCs and vessels, except in ductal and acinar cells. TGF-β staining, although weaker than MSG, was also performed in the muscle in patient 2. Accordingly, infiltration of CD4+ lymphocytes, which were dominant compared with CD20 and CD68, was shown in the LSGs of both patients (Fig. 2). Although CD8+ lymphocytes were also scattered in LSGs, CD4+

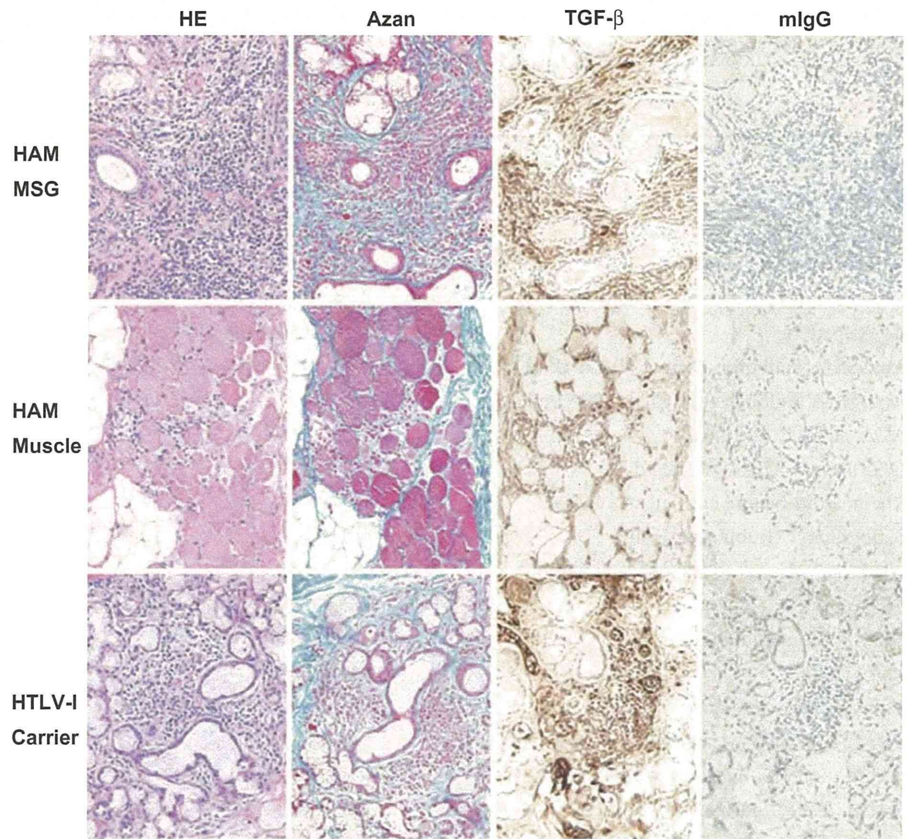


**Fig. 1** Azocarmine with aniline blue (Azan)–Mallory staining and transforming growth factor beta (TGF-$\beta$) immunostaining in the labial salivary gland (LSG). Azan–Mallory staining and immunohistochemistry after epitope retrieval were performed for formalin-fixed, paraffin-embedded sections (3-μm thick) from the LSG using the Histofine Simple Stain Kit (Nichirei Co., Tokyo, Japan). The primary antibodies used for immunohistochemistry were TGF-$\beta$ and mouse immunoglobulin (Ig)G1 (×200). Hematoxylin was used as a counterstain

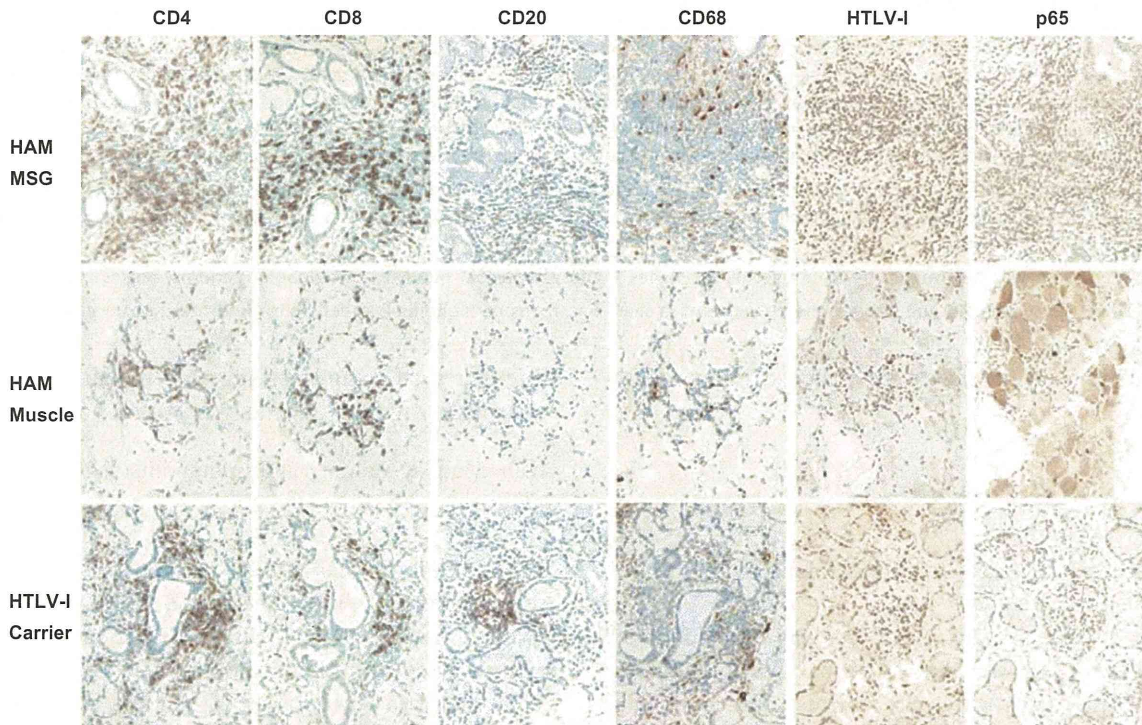


**Fig. 2** Immunohistochemistry in the labial salivary gland (LSG). Immunohistochemistry after epitope retrieval was performed for formalin-fixed, paraffin-embedded sections (3-μm thick) from the LSG using the Histofine Simple Stain Kit (Nichirei Co., Tokyo, Japan). The primary antibodies used for immunohistochemistry were CD4, CD8, CD20, nuclear factor kappa B (NF-$\kappa$B) (p65), and human T-cell leukemia virus type I (HTLV-I) [p19, p28, group-specific antigen (GAG)]. Lymph node from a patient with adult T-cell leukemia was used as a positive control for staining HTLV-I-related proteins (data not shown) (×200). Hematoxylin was used as a counterstain

and CD8+ lymphocytes were found in a muscle specimen from patient 2. It is interesting to note that HTLV-I-related proteins including p19, p28, and GAG were detected in the nuclei of a large percentage of infiltrating MNCs in LSGs and in the muscle specimen in patient 2, which was in accordance with the distribution of NF-$\kappa$B p65.

### TGF-$\beta$ immunostaining in SS in the presence or absence of anti-HTLV-I antibody or ACA

We finally showed TGF-$\beta$ immunostaining according to the presence of anti-HTLV-I antibody or ACA (Fig. 3). We performed these experiments in four patients each in three groups and show representative results (Fig. 3). In the HTLV-I-seropositive SS patients without ACA, TGF-$\beta$ was dominantly found in vascular endothelial cells or fibrous tissues in LSG; however, the frequency of TGF-$\beta$+ cells (patients A–D in Fig. 3) appeared to be lower than the patients in cases 1 and 2 in Fig. 1. In the HTLV-I-seronegative SS patients with ACA, TGF-$\beta$ was seen in infiltrating MNCs, vascular endothelial cells, and fibrous tissues in LSG. Then, in the HTLV-I-seropositive SS patients without ACA, TGF-$\beta$ expression was similar to HTLV-I-seronegative SS patients with ACA (patients E–H in Fig. 3). In contrast, TGF-$\beta$ expression was less in HTLV-I-seronegative patients without ACA (patients I, K, L) compared with other groups. In a HTLV-I-seronegative SS patient without ACA (as in patient J), TGF-$\beta$ was not found in fibrous cells but in MNCs.

## Discussion

Both HTLV-I and ACA are known to contribute to SS [1–8]; however, this coincidence of HTLV-I and ACA is supposed to occur with low frequency [8]. Our two cases presented here are rare but may illustrate the in vivo role of HTLV-I in patients with ACA-seropositive SS. Although both patients showed grade 3 MNC infiltration in LSGs, results from exocrine function tests, including Schirmer test and Saxon test in patient 1, were worse than those in patient 2. Except for the degree of MNC infiltration in LSGs, other factors such as aquaporin-5 distribution or type 3 muscarinic receptors [11, 12] might affect lacrimal and salivary secretion. With respect to MNC infiltration into the LSG, both cases showed similar findings. However, there were significant differences in fibrosis determined by Azan–Mallory staining and cytokine profiles.

As patient 2 was diagnosed with HAM, the HTLV-I viral load was high in comparison with patient 1, a finding that is consistent with previous reports [13]. Striking differences were observed in the Azan–Mallory staining findings; however, both patients showed high TGF-$\beta$ expression in LSGs. TGF-$\beta$ is a key cytokine for promoting the fibrotic process; thus, the prominent fibrosis of LSG is believed to be driven by TGF-$\beta$. Fibrosis was found in the LSG of both patients, which might be explained to some extent by the presence of ACA, as we previously reported [6]. However, a recent report found that HTLV-I basic-leucine zipper (bZIP) factor enhances TGF-$\beta$ signaling through the p300 coactivator [14]. As strong expression of HTLV-I-related proteins was found in the LSG of patient 2, the TGF-$\beta$ signaling pathways were suggested to be promoted in situ by HTLV-I, resulting in marked fibrosis. A similar phenomenon might occur in the muscle of patient 2, resulting in inflammatory myopathy. We previously reported that myopathy or uveitis was one characteristic of HTLV-I-seropositive SS patients [15]. With respect to a low level of IFN-$\gamma$, Santos et al. [16] demonstrated that administration of exogenous TGF-$\beta$ induced a decrease of IFN-$\gamma$ in cells from HTLV-I carriers, suggesting the possibility of the modulation of IFN-$\gamma$ by TGF-$\beta$ in HTLV-I-seropositive individuals. The high TNF-$\alpha$ level in patient 2 may also be driven by HTLV-I, as indicated for TGF-$\beta$.

To show the involvement of HTLV-I and ACA toward TGF-$\beta$ expression, we examined TGF-$\beta$ immunostaining for HTLV-I-seropositive patients without ACA, HTLV-I-seronegative patients with ACA, and HTLV-I-seronegative without ACA (Fig. 3). Although the precise quantitative analysis was not performed in this study, it may demonstrate that TGF-$\beta$ expression in vascular endothelial cells and fibrous tissues of LSGs is more prominent in SS patients positive for both anti-HTLV-I antibody and ACA (two cases in Fig. 1) compared with SS patients positive for either one alone [two groups (patients A–H in Fig. 3)]. Accordingly, TGF-$\beta$ expression in the above-mentioned sites was less in SS patients who were not positive for either anti-HTLV-I antibody or ACA (patients I–L in Fig. 3) in comparison with other groups. Therefore, we speculate that the synergistic effect of HTLV-I infection with ACA-carrying status induces the expression of TGF-$\beta$ in LSGs, especially in vascular endothelial cells and fibrous tissue of SS patients (Fig. 4). However, we also found intense expression of TGF-$\beta$ in MNCs even in HTLV-I-seronegative patients without ACA. As fibrous change determined by Azan–Mallory staining was not so significant in these patients, TGF-$\beta$ in MNCs of LSGs may not be directly associated with the fibrotic process. In fact, TGF-$\beta$ is known to be produced by CD4+ T lymphocytes [17] and influenced by other cytokines, such as IFN-$\gamma$ [18]. Therefore, the two phenomena—Azan–Mallory-stain-proven fibrosis and TGF-$\beta$ expression—should be carefully determined in patients with SS. Further studies with a larger number of participants and more definitive qualification approaches are necessary to prove our hypothesis.

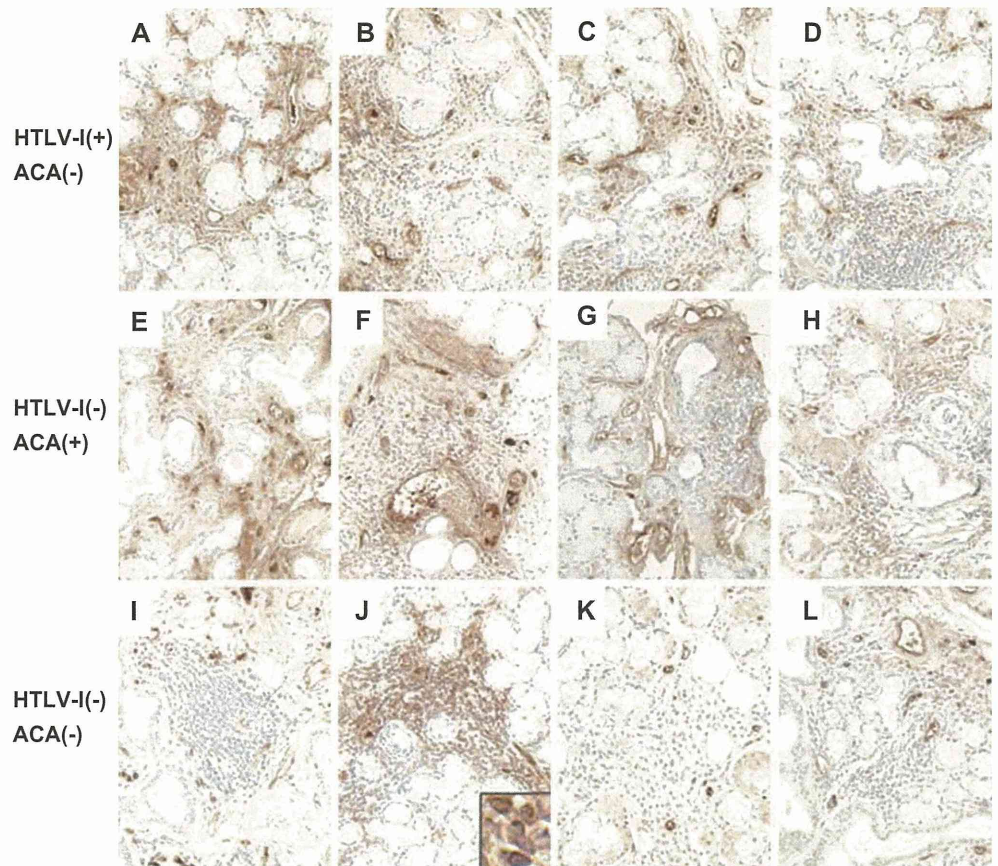


**Fig. 3** Expression of transforming growth factor beta (TGF-$\beta$) in human T-cell leukemia virus type I (HTLV-I)-seropositive Sjögren's syndrome (SS) patients without anti-centromere-antibody (ACA), HTLV-I-seronegative SS patients with ACA, and HTLV-I-seronegative SS patients without ACA. Immunohistochemistry for TGF-$\beta$ after epitope retrieval was performed for formalin-fixed, paraffin-embedded sections (3-µm thick) from the labial salivary gland (LSGs) using the Histofine Simple Stain Kit (Nichirei Co., Tokyo, Japan). Staining was performed for four HTLV-I-seropositive SS patients without ACA (patients A–D), four HTLV-I-seronegative SS patients with ACA (patients E–H), and four HTLV-I-seronegative SS patients without ACA (patients I–J). For patient J, TGF-$\beta$-positive MNCs are shown in the *inset* (×200). Hematoxylin was used as a counterstain

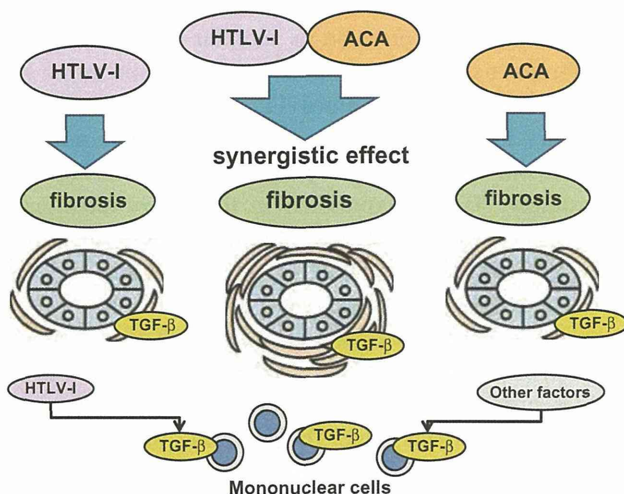


**Fig. 4** Hypothesis for fibrotic alternation of salivary glands in Sjögren's syndrome (SS) patients through human T-cell leukemia virus type I (HTLV-I) infection and anti-centromere-antibody (ACA)-carrying status. From the results of the this study, HTLV-I- and ACA-carrying status induce fibrosis in labial salivary glands (LSGs). Furthermore, synergistic effects of HTLV-I infection with ACA-carrying status are assumed from the results of azocarmine with aniline blue (Azan)–Mallory staining. However, transforming growth factor beta (TGF-$\beta$), especially in mononuclear cells (MNCs), is also induced in HTLV-I infection and ACA-carrying status

In summary, we report two cases of ACA-seropositive SS found in HTLV-I-seropositive individuals and compared these patients with HTLV-I-seropositive SS patients without ACA, HTLV-I-seronegative SS patients with ACA, and HTLV-I-seronegative SS patients without ACA. The predominant characteristics were found in a patient with HAM, which was believed to have been caused by elevated HTLV-I viral load and subsequent cytokine production. Elements other than TGF-$\beta$ are also suggestive of influencing fibrotic alternation of LSGs in patients with SS.

**Conflict of interest** None.

## References

1. Terada K, Katamine S, Eguchi K, Moriuchi R, Kita M, Shimada H, et al. Prevalence of serum and salivary antibodies to HTLV-1 in Sjögren's syndrome. Lancet. 1994;344(8930):1116–9.
2. Nakamura H, Kawakami A, Eguchi K. Mechanisms of autoantibody production and the relationship between autoantibodies and the clinical manifestations in Sjögren's syndrome. Transl Res. 2006;148:281–8.
3. Nakamura H, Eguchi K, Nakamura T, Mizokami A, Shirabe S, Kawakami A, et al. High prevalence of Sjögren's syndrome in patients with HTLV-I associated myelopathy. Ann Rheum Dis. 1997;56:167–72.

4. Hida A, Imaizumi M, Sera N, Akahoshi M, Soda M, Maeda R, et al. Association of human T lymphotropic virus type I with Sjogren syndrome. Ann Rheum Dis. 2010;69:2056–7.
5. Nakamura H, Kawakami A, Tominaga M, Hida A, Yamasaki S, Migita K, et al. Relationship between Sjögren's syndrome and human T-lymphotropic virus type I infection: follow-up study of 83 patients. J Lab Clin Med. 2000;135:139–44.
6. Nakamura H, Kawakami A, Hayashi T, Iwamoto N, Okada A, Tamai M, et al. Anti-centromere antibody-seropositive Sjögren's syndrome differs from conventional subgroup in clinical and pathological study. BMC Musculoskelet Disord. 2010;11:140.
7. Katano K, Kawano M, Koni I, Sugai S, Muro Y. Clinical and laboratory features of anti-centromere antibody positive primary Sjögren's syndrome. J Rheumatol. 2001;28:2238–44.
8. Hida A, Kawabe Y, Kawakami A, Migita K, Tominaga M, Nakamura H, et al. HTLV-I associated Sjögren's syndrome is aetiologically distinct from anti-centromere antibodies positive Sjögren's syndrome. Ann Rheum Dis. 1999;58:320–2.
9. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis. 2002;61:554–8.
10. Chisholm DM, Mason DK. Labial salivary gland biopsy in Sjögren's disease. J Clin Pathol. 1968;21:656–60.
11. Beroukas D, Hiscock J, Jonsson R, Waterman SA, Gordon TP. Subcellular distribution of aquaporin 5 in salivary glands in primary Sjögren's syndrome. Lancet. 2001;358:1875–6.
12. Tsuboi H, Matsumoto I, Wakamatsu E, Nakamura Y, Iizuka M, Hayashi T, et al. New epitopes and function of anti-M3 muscarinic acetylcholine receptor antibodies in patients with Sjögren's syndrome. Clin Exp Immunol. 2010;162:53–61.
13. Nagai M, Usuku K, Matsumoto W, Kodama D, Takenouchi N, Moritoyo T, et al. Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: high proviral load strongly predisposes to HAM/TSP. J Neurovirol. 1998;4:586–93.
14. Zhao T, Satou Y, Sugata K, Miyazato P, Green PL, Imamura T, et al. HTLV-1 bZIP factor enhances TGF-$\beta$ signaling through p300 coactivator. Blood. 2011;118:1865–76.
15. Eguchi K, Matsuoka N, Ida H, Nakashima M, Sakai M, Sakito S, et al. Primary Sjögren's syndrome with antibodies to HTLV-I: clinical and laboratory features. Ann Rheum Dis. 1992;51:769–76.
16. Santos SB, Porto AF, Muniz AL, Luna T, Nascimento MC, Guerreiro JB, et al. Modulation of T cell responses in HTLV-1 carriers and in patients with myelopathy associated with HTLV-1. Neuroimmunomodulation. 2006;13:145–51.
17. Hansen G, McIntire JJ, Yeung VP, Berry G, Thorbecke GJ, Chen L, et al. CD4(+) T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation. J Clin Invest. 2000;105:61–70.
18. McCartney-Francis NL, Wahl SM. Dysregulation of IFN-gamma signaling pathways in the absence of TGF-beta 1. J Immunol. 2002;169:5941–7.

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ORIGINAL ARTICLE

# Musculoskeletal ultrasonography assists the diagnostic performance of the 2010 classification criteria for rheumatoid arthritis

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**Abstract**
*Objective* We investigated whether musculoskeletal ultrasonography (MSKUS) assists the diagnostic performance of the 2010 rheumatoid arthritis (RA) classification criteria.
*Methods* Sixty-nine early arthritis patients were consecutively enrolled. None of the patients had been treated. In MSKUS of bilateral wrist and finger joints from 22 sites, the findings obtained by gray-scale and power Doppler (PD) assessment were graded on a semiquantitative scale from 0 to 3. Plain magnetic resonance imaging (MRI) of both wrist and finger joints was also examined. Diagnosis of RA was defined by the initiation of disease-modifying antirheumatic drugs within the first 3 months. The diagnostic performance of the patients was evaluated at entry using 2010 RA classification criteria in conjunction with MSKUS.
*Results* The indispensable MSKUS finding for differentiating RA was the presence of a PD grade 2 or 3 that was superior to 2010 RA classification criteria or MRI-proven bone edema. We propose that the decision tree algorithm of 2010 RA classification criteria with PD grade 2 or 3 reveals the best discriminative ability.
*Conclusion* MSKUS, especially with a strong PD signal, is very useful to assist the diagnostic performance of the 2010 RA classification criteria in the early recognition of RA.

**Keywords** Rheumatoid arthritis · 2010 RA classification criteria · Ultrasonography · Power Doppler · MRI

**Abbreviations**

| | |
|---|---|
| ACPA | Anticyclic citrullinated peptide antibody |
| ACR | American College of Rheumatology |
| CRP | C-reactive protein |
| DMARDs | Disease-modifying antirheumatic drugs |
| ESR | Erythrocyte sedimentation rate |
| EULAR | European League Against Rheumatism |
| Gd-DTPA | Gadolinium-diethylenetriamine pentaacetic acid |
| GS | Gray-scale |
| IP | Interphalangeal |
| MCP | Metacarpophalangeal |

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|---|---|
| MMP | Matrix metalloproteinase |
| MRI | Magnetic resonance imaging |
| MSKUS | Musculoskeletal ultrasonography |
| NPV | Negative predictive value |
| PD | Power Doppler |
| PIP | Proximal interphalangeal |
| PPV | Positive predictive value |
| RA | Rheumatoid arthritis |
| RF | Rheumatoid factor |
| SJC | Swollen joint counts |
| T2T | Treat to target |
| TJC | Tender joint counts |

## Introduction

Early diagnosis and the treat to target (T2T) strategy are now indispensable for managing rheumatoid arthritis (RA) [1]. Application of the T2T strategy using the tight control approach in patients with RA, especially those with early-stage RA, has been shown to improve RA outcomes [1, 2]. Thus, the early recognition of RA is a great benefit in managing patients with RA. The 1987 American College of Rheumatology (ACR) classification criteria for RA [3] are not designed for early classification of RA. Consequently, to identify patients with erosive arthritis early, a task force of experts from both the ACR and the European League Against Rheumatism (EULAR) derived new classification criteria [4]. These new criteria, the 2010 RA classification criteria, have been verified to classify patients early as having RA more efficiently than the 1987 criteria; however, a substantial population is not still classified as having RA, even by the 2010 RA classification criteria [4].

Although physical examination is still the gold standard by which to identify the presence of arthritis [4], it has come to be apparent that modern imaging techniques such as musculoskeletal ultrasonography (MSKUS) and magnetic resonance imaging (MRI) are more sensitive than physical examination for detecting joint injury in patients with RA, especially early-stage RA [5–9]. MSKUS is well tolerated and can image a large number of joints at multiple time points over a relatively short period of time [10, 11]. Varying kinds of joint injury, including synovitis, tenosynovitis, and bone erosion, can be recorded by gray-scale (GS) and power Doppler (PD) [5–8, 10–13]. We recently reported the utility of PD to reflect clinical disease activity as well as serum biomarkers in patients with RA [14].

We speculated that the detection sensitivity for synovitis would be increased if MSKUS was routinely incorporated into clinical practice for patients with early arthritis. The objective of the study reported here was to evaluate whether the findings of MSKUS, in comparison with MRI, assist the diagnostic performance of the 2010 RA classification criteria.

## Materials and methods

### Patients

Sixty-nine early arthritis patients suspected of having RA were consecutively recruited. Patients who could be classified as non-RA at first visit were excluded. In addition, we excluded patients who had experience with disease-modifying antirheumatic drugs (DMARDs), including biologics and glucocorticoids. All patients were recruited from the Unit of Translational Medicine, Department of Immunology and Rheumatology, Graduate School of Biomedical Sciences, Nagasaki University, and the Department of Internal Medicine, Nagasaki Municipal Hospital, from May 2010 through February 2011. The duration from the appearance of symptoms to entry into the study in these 69 patients was <1 year. Patients gave their informed consent to be subjected to the protocol that was approved by the Institutional Review Board of Nagasaki University. This study was a prospective single-center observational study. Follow-up periods were at least 6 months.

### Clinical and laboratory assessment

A clinical diagnosis of RA was comprehensively made by Japan College of Rheumatology (JCR)-certified rheumatologists (AK, HN, SY, and KE) using clinical histories, physical findings, blood tests including rheumatoid factor (RF) (Dade Behring, Marburg, Germany; cutoff value, 14 IU/ml), anticyclic citrullinated peptide antibodies (ACPA) (DIASTAT Anti-CCP, Axis-Shield, Dundee, UK; cutoff value, 4.5 U/ml), C-reactive protein (CRP) (Eiken Chemical Co., Ltd., Tokyo, Japan), erythrocyte sedimentation rate (ESR), matrix metalloproteinase 3 (MMP-3) (Daiichi Pure Chemicals, Fukuoka, Japan), 2010 RA classification criteria, plain radiography, ultrasound (US) findings, and MRI findings. All patients underwent the examinations except for MRI every 1–3 months. If JCR-certified rheumatologists introduced DMARDs within the first 3 months according to the above information, patients are diagnosed as having RA. Therefore, not only 2010 RA classification criteria but other information, such as MSKUS, MRI, and clinical course, are actually involved in these processes.

### US examination

Each patient underwent a US assessment on the same day as the clinical evaluation by a JCR-certified rheumatologist