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frequency of prednisolone (PSL) and methotrexate (MTX) use between HTLV-1 positive and negative patients. A nonparametric test (Wilcoxon sign rank test) was also used to compare change in disease activity markers before and after anti-TNF therapy. Log-rank test was used to compare the difference of the continuation periods of anti-TNF treatment between HTLV-1 positive and negative patients. P values of less than 0.05 were considered statistically significant. Data were analyzed by GraphPad Prism 5 for windows version 5.04 (GraphPad Software Inc., USA.).

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Results

Background characteristics of patients prior to anti-TNF therapy (Table)

The level of serum CRP was higher in HTLV-1 positive patients than in HTLV-1 negative patients (median [IQR] 4.1 [4.2] vs. 0.7 [1.3] mg/dl, p= 0.0003). TJC28, SJC28 and DAS28 did not differ between HTLV-1 positive and negative patients. There were no differences in disease activity, including low disease activity/remission rate according to EULAR improvement criteria between HTLV-1 positive and negative patients.

The efficacy of anti-TNF treatment

The efficacy of anti-TNF treatment was assessed at 3 months after the beginning of treatment (Figure 1). The rate of good response in HTLV-1 positive patients was lower than that in HTLV-1 negative patients (10 % vs. 50 %) and the rate of no response in HTLV-1 positive patients was higher than that in HTLV-1 negative patients (30 % vs. 5 %) (Figure 1A). The rate of low disease activity/ remission in HTLV-1 negative patients was higher than that in HTLV-1 positive patients (50 % vs. 10 %) (Figure 1B).

The levels of CRP, ESR and DAS28 at 3 months after anti-TNF therapy were significantly decreased in HTLV-1 negative patients (median [IQR] 0.7

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[illegible] vs. 0.1 [0.3] mg/dl, P= 0.0002, median [IQR] 65.0 [34.5] vs. 34.5 [25.7] mm/60min, P= 0.0004, and median [IQR] 5.2 [0.8] vs. 3.2 [0.8], P< 0.0001, respectively). On the other hand, in HTLV-1 positive patients, the levels of CRP and ESR at 3 months after anti-TNF therapy were lower than those before therapy (median [IQR] 4.1 [4.2] vs. 1.3 [3.4] mg/dl, P= 0.0645 and median [IQR] 74.5 [37.5] vs. 62.0 [44.5] mm/60min, P= 0.425, respectively); however, they did not reach statistical significance. DAS28 in HTLV-1 positive patients after the therapy was significantly lower than that before treatment (median [IQR] 5.8 [0.8] vs. 4.4 [1.1], P= 0.0137). The median levels of CRP, ESR, and DAS28 at 3 months after anti-TNF treatment in HTLV-1 positive patients were significantly higher than those in HTLV-1 negative patients (P= 0.003, P= 0.03 and P= 0.003, respectively) (Figure 1C).

During the 2-year observation period, anti -TNF therapy was discontinued in 6 HTLV-1 positive patients (2 cases due to adverse reaction and 4 cases due to lack of efficacy). On the other hand, anti -TNF therapy was discontinued only in 3 HTLV-1 negative patients (2 cases due to adverse reaction and one case due to lack of efficacy). Discontinuation rates due to any reason and due to insufficient effect were significantly higher in HTLV-1

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positive patients (60% and 40%) than in HTLV-1 negative patients (15% and 5%) (P= 0.0053 and P= 0.013, respectively) (Figure 2).

Signs, symptoms and laboratory data showed no indication of the development of ATL in HTLV-1 positive patients during the 2-year observation period.

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Discussion

The background levels of CRP in HTLV-1 positive patients with RA were higher than those in HTLV-1 negative patients with RA in the present study. HTLV-1 Tax protein has been reported to promote the production of IL-6 (8,9). Production of IL-6 from synovial cells was reported to be up-regulated in HTLV-1 positive patients with osteoarthritis (10). These data suggest that production of IL-6 could be up-regulated by HTLV-1 infection and may account for the high inflammation.

Moderate or better responses have been reported in 70-80% of Japanese patients with RA who received treatment with IFX or ETN (11-13). In the present study, the response rate (moderate or better) to anti-TNF treatment in HTLV-1 negative RA patients was 95%, which is consistent with rates reported in the previous studies. In contrast, in HTLV-1 positive patients, the decrease in CRP and ESR at 3 months after anti-TNF therapy did not reach statistical significance. The levels of CRP, ESR and DAS28 in HTLV-1 positive patients were significantly higher than those in HTLV-1 negative patients. According to EULAR improvement criteria, the rate of low disease activity and remission in HTLV-1 positive patients was much

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lower than that in HTLV-1 negative patients (10 % vs. 50 %). Therefore, it is suggested that HTLV-1 positive patients with RA are resistant to anti-TNF therapy.

High serum levels of CRP have been reported as a factor in insufficient RA patient response to anti-TNF treatment (14). Therefore, it is still not clear whether the low response to anti-TNF in HTLV-1 positive patients is due to high inflammation or due to the HTLV-1 positivity itself. To clarify this question, a greater number of HTLV-1 patients with RA must be classified according to CRP level to examine the response to anti-TNF based on level of CRP. It is also not clear whether HTLV-1 positive patients with RA show insufficient response only to anti-TNF agents. Future study to clarify these questions is necessary.

During the 2-year observation period, there were no signs, symptoms, or laboratory data suggesting that HTLV-1 positive patients developed ATL. Viral markers such as HTLV-1 proviral loads or clonality of HTLV-1 infected cells were not measured in this study; however, a previous study of two HTLV-1 positive cases showed no change in these viral markers after receiving anti-TNF agents (7).

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This retrospective study has a number of limitations. The number of HTLV-1 positive and negative patients was only 10 and 20, respectively, and, therefore, too small to reach a conclusion about the difference in response to anti-TNF therapy. Because the incidence of ATL among HTLV-1 carriers has been reported as only one out of 1000 person-years, a prospective study including a greater number of HTLV-1 positive patients and with longer observation periods is necessary to clarify the risk of ATL. At the same time, proviral loads and clonality of HTLV-1 infected cells should be measured.

The results of this study raise the question of whether HTLV-1 infection should be measured when anti-TNF agents are administrated in patients with RA, especially in endemic areas. Further study including a greater number of patients with longer periods of observation is necessary to reach a definite conclusion.

Acknowledgement

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REFERENCES

1. Moot RJ, Naisbett-Groet B. The efficacy of biologic agents in patients with rheumatoid arthritis and an inadequate response to tumour necrosis factor inhibitors: a systematic review. Rheumatology 2012;51:2252-61
2. Satake M, Yamaguchi K, Tadokoro K. Current prevalence of HTLV-1 in Japan as determined by screening of blood donors. J Med Virol 2012;84:327-35
3. Watanabe T. HTLV-1 associated diseases. Int J Hematol 1997;66:257-78
4. Nishioka K, Sumida T, Hasunuma T. Human T lymphotropic virus type I in arthropathy and autoimmune disorders. Arthritis Rheum 1996;39:1410-8
5. Eguchi K, Origuchi T, Takashima H, Iwata K, Katamine S, Nagataki S. High seroprevalence of anti-HTLV-I antibody in rheumatoid arthritis. Arthritis Rheum 1996;39:463-6
6. Sato K, Maruyama I, Maruyama Y, Kitajima I, Nakajima Y, Higaki M, et al. Arthritis in patients infected with human T lymphotropic virus type I. Clinical and immunopathologic features. Arthritis Rheum 1991;34:714-21
7. Umekita K, Umeki K, Miyauchi S, Ueno S, Kusumoto N, Kubo K, et al. Use of anti-tumor necrosis factor biologics in the treatment of rheumatoid arthritis does not change human T-lymphotropic virus type 1 markers: a case series. Mod Rheumatol

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(In press)

8. Mori N, Shirakawa F, Shimizu H, Murakami S, Oda S, Yamamoto K, et al. Transcriptional regulation of the human interleukin-6 gene promoter in human T-cell leukemia virus type I-infected T-cell lines: evidence for the involvement of NF-kappa B. Blood 1994; 84: 2904-11

9. Mori N, Shirakawa F, Abe M, Kamo Y, Koyama Y, Murakami S, et al. Human T-cell leukemia virus type I tax transactivates the interleukin-6 gene in human rheumatoid synovial cells. J Rheumatol 1995;22:2049-54

10. Yoshihara Y, Tsukazaki T, Osaki M, Nakashima M, Hasui K, Shindo H. Altered expression of inflammatory cytokines in primary osteoarthritis by human T lymphotropic virus type I retrovirus infection: a cross-sectional study. Arthritis Res Thr 2004;6(4):R347-54

11. Tanaka Y, Takeuchi T, Inoue E, Saito K, Sekiguchi N, Sato E, et al. Retrospective clinical study on the notable efficacy and related factors of infliximab therapy in a rheumatoid arthritis management group in Japan: one-year clinical outcomes (RECONFIRM-2). Mod Rheumatol 2008;18:146-152

12. Kameda H, Ueki Y, Saito K, Nagaoka S, Hidaka T, Atsumi T, et al. Etanercept (ETN) with methotrexate (MTX) is better than ETN monotherapy in patients with

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active rheumatoid arthritis despite MTX therapy: a randomized trial. Mod Rheumatol 2010;20:531-38

13. Kameda H, Kanbe K, Sato E, Ueki Y, Saito K, Nagaoka S, et al. Continuation of methotrexate resulted in better clinical and radiographic outcomes than discontinuation upon starting etanercept in patients with rheumatoid arthritis: 52-week results from the JESMR study. J Rheumatol 2011;38:1585-92

14. Yamanaka H, Tanaka Y, Sekiguchi N, Inoue E, Saito K, Kameda H, et al. Retrospective clinical study on the notable efficacy and related factors of infliximab therapy in a rheumatoid arthritis management group in Japan (RECONFIRM). Mod Rheumatol 2007;17:28-32

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Figure Legend

Figure 1. Efficacy of anti-tumor necrosis factor 3 months after the beginning of treatment.

A, Response rate of HTLV-1 positive (n= 10) and negative patients (n= 20) with rheumatoid arthritis (RA) according to European League Against Rheumatism (EULAR) improvement criteria.

B, Disease activity of HTLV-1 positive (n= 10) and negative (n= 20) patients with RA according to EULAR improvement criteria.

C, Changes in the levels of C-reactive protein, erythrocyte sedimentation rate and disease activity score in 28 joints at 3 months (3M) after anti-TNF therapy.

HDA: high disease activity, MDA: moderate disease activity, LDA: low disease activity, REM: remission. HTLV-1: human T- lymphotropic virus type 1.

Figure 2. Continuation rate of anti-tumor necrosis factor therapy in human T-lymphotropic virus type 1 positive (n= 10) and negative (n= 20) patients with rheumatoid arthritis.

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HTLV-1: human T-cell lymphotropic virus type 1.

	HTLV-1 positive (N= 10)	HTLV-1 negative (N=20)	P-value †
No.	10	20	-
Age , median (IQR) years	70.0 (8.5)	68.5 (11.7)	0.98
Duration, median (IQR) years	5.0 (5.0)	9.0 (19.5)	0.21
Disease activity markers			
CRP, median (IQR) mg/dl	4.1 (4.2)	0.7 (1.3)	0.0003
ESR, median (IQR) mm/60min	74.5 (37.5)	65.0 (34.5)	0.15
TJC28, median (IQR)	4.5 (4.3)	4.5 (4.0)	0.72
SJC28, median (IQR)	4.5 (3.8)	2.0 (5.0)	0.35
DAS28, median (IQR)	5.8 (0.8)	5.2 (0.8)	0.18
Disease activity according to EULAR criteria			
High disease activity (DAS28: >5.1) [%]	80	70	-
Moderate disease activity (DAS28: 3.2 - 5.1) [%]	20	25	-
Low disease activity (DAS28: <3.2) [%]	0	5	-
Serological markers			
RF positive [%]	80	80	>0.99
ACPA positive [%]	90	100	0.33
Treatment			
DMARDs (%)	33	45	>0.99
Methotrexate median mg/week (IQR) [%]	10 (2.0) [50]	8.0 (2.5) [75]	0.23
Predonisolon median mg/day (IQR) [%]	5.5 (2.0) [90]	3.5 (3.0) [70]	0.37

Baseline characteristics of patients.
HTLV-1 = human T-lymphotrophic virus type 1; IQR = interquartile range; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; TJC28 = tender joint counts in 28 joints; SJC28 = swollen joint counts in 28 joints; DAS28 = disease activity score in 28 joints; RF= rheumatoid factor; ACPA = anti-citrullinated protein antibody; DMARDs = disease-modifying antirheumatic drugs
† By Mann-Whiteny U test, Fisher's exact test

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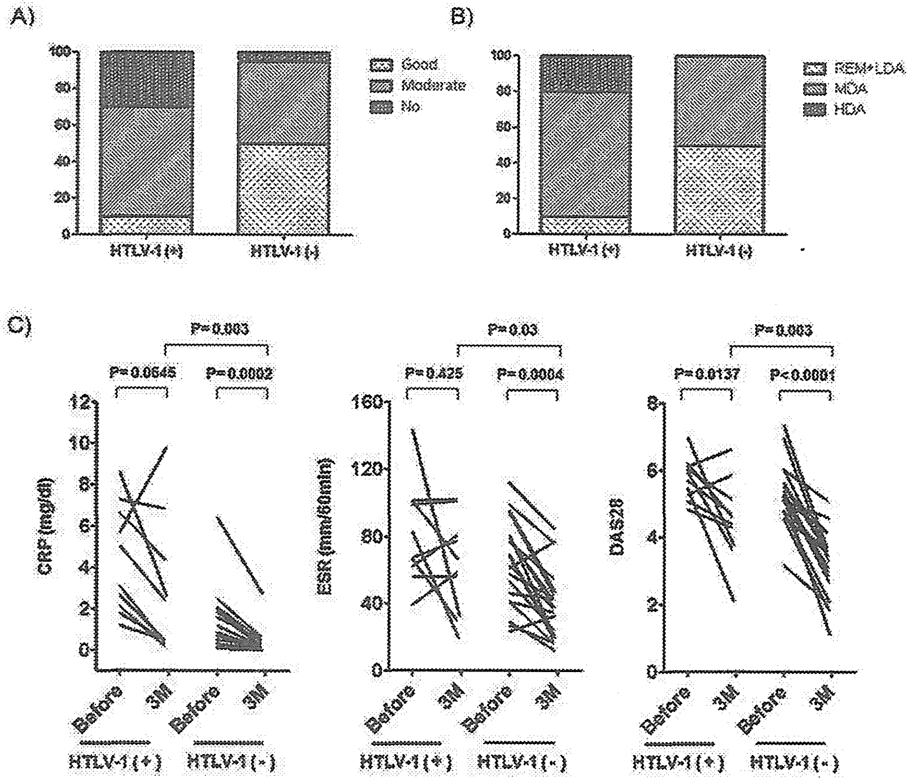

Efficacy of anti-tumor necrosis factor 3 months after the beginning of treatment.
A, Response rate of HTLV-1 positive (n= 10) and negative patients (n= 20) with rheumatoid arthritis (RA) according to European League Against Rheumatism (EULAR) improvement criteria.
B, Disease activity of HTLV-1 positive (n= 10) and negative (n= 20) patients with RA according to EULAR improvement criteria.
C, Changes in the levels of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and disease activity score in 28 joints (DAS28) at 3 months (3M) after anti-TNF therapy.
HDA: high disease activity, MDA: moderate disease activity, LDA: low disease activity, REM: remission.
HTLV-1: human T- lymphotropic virus type 1.

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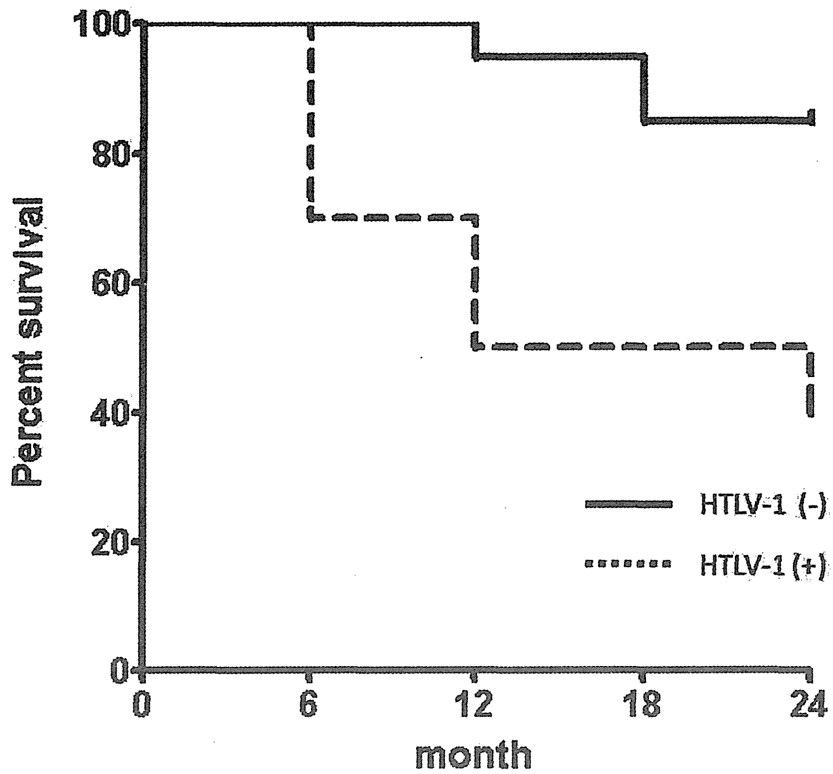

Continuation rate of anti-tumor necrosis factor therapy in human T- lymphotropic virus type 1 positive (n= 10) and negative (n= 20) patients with rheumatoid arthritis.
HTLV-1: human T-cell lymphotropic virus type 1.

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ORIGINAL ARTICLE

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

S Nakahata[1], Y Saito[1], K Marutsuka[2], T Hidaka[3], K Maeda[3,4], K Hatakeyama[5], T Shiraga[1,6], A Goto[1], N Takamatsu[1], Y Asada[5], A Utsunomiya[7], A Okayama[8], Y Kubuki[3], K Shimoda[3], Y Ukai[9], G Kurosawa[9] and K Morishita[1]

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 (*Sac*I site of 5′-LTR to *Sac*I 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)) × 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

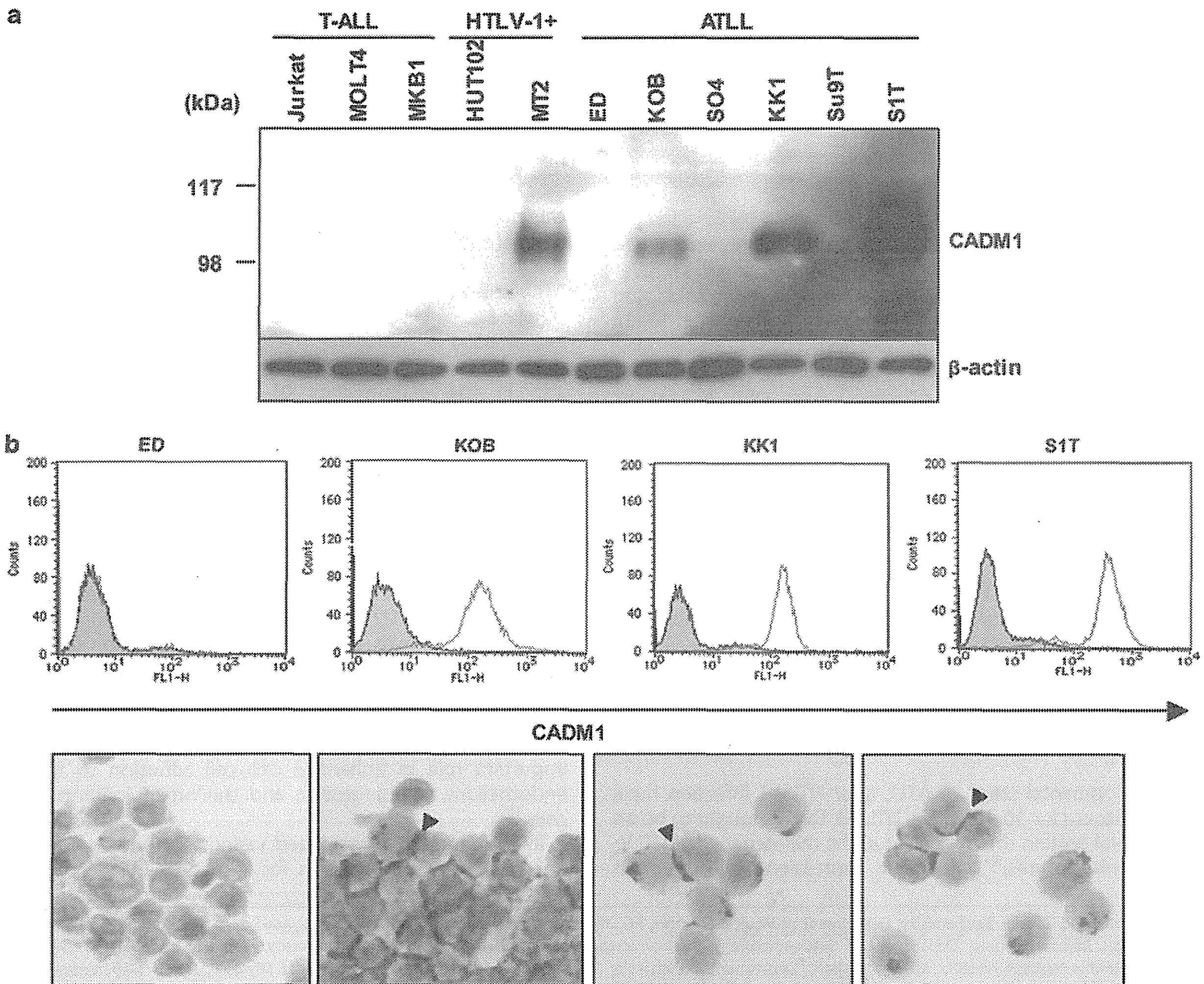

Figure 1. High CADM1 expression in ATLL analyzed by immunoblot, flow cytometry (FMC) and immunohistochemical staining (IHC). **(a)** Immunoblot analysis was performed on a series of T-lymphoid leukemia cell lines (three T-ALL, T-acute lymphoid leukemias; two HTLV-1 +, HTLV-1-infected cell lines; six 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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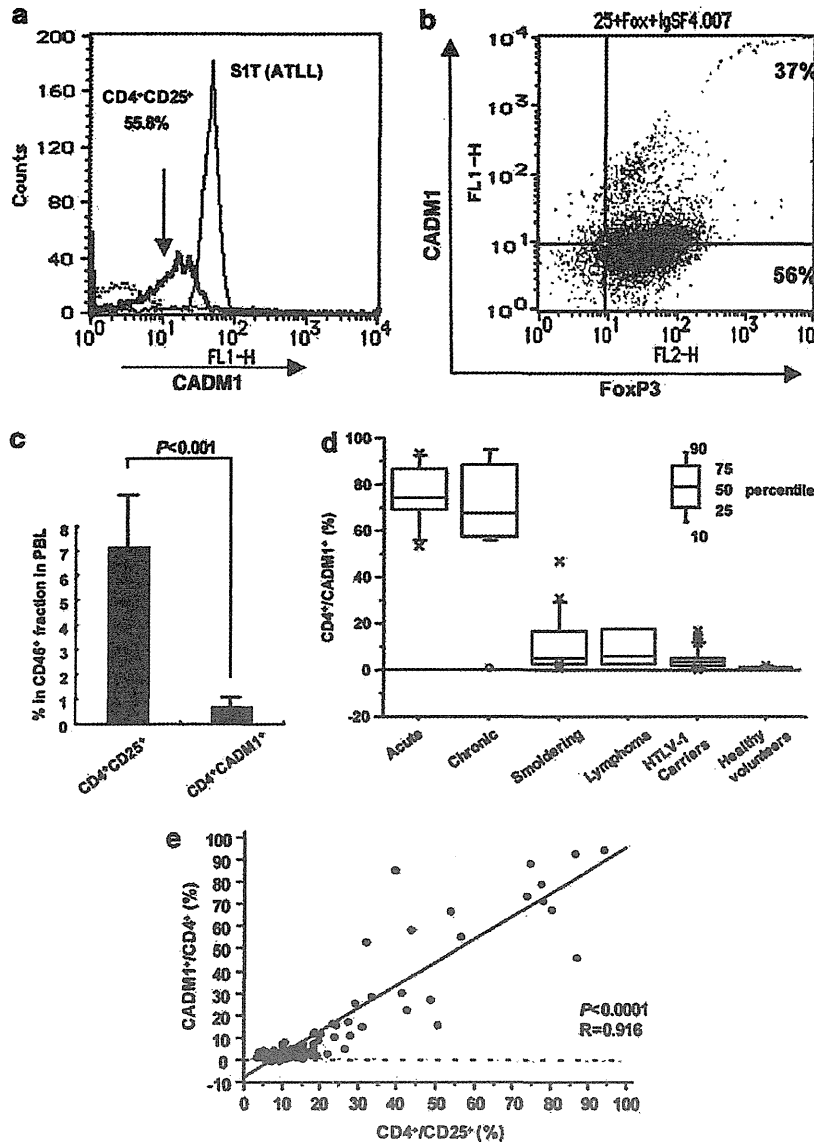

Figure 2. Flow cytometric analysis of CADM1 in T-reg lymphocytes, ATLL cells and HTLV-1-infected T cells. (a) Flow cytometric analysis of CADM1 expression in the CD4$^+$CD25$^+$ fraction from peripheral T lymphocytes. Each sample was stained with an Alexa 488-labeled anti-CADM1 antibody. The S1T-ATLL 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-2Rα are correlated with the prognosis

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