Journal of Dermatology Original Article

The expression of cell adhesion molecule 1 and its splicing variants in Sézary cells and cell lines from cutaneous T-cell lymphoma

Mari Yamaguchi[1], Shin Morizane[1]*, Toshihisa Hamada[1], Tomoko Miyake[1], Makoto Sugaya[2], Hiroaki Iwata[3], Kazuyasu Fujii[4], Rie Haramoto-Shiratsuki[5], Yuki Nakagawa[1], Mayumi Miura[6], Koichi Ohshima[6], Kazuhiro Morishita[7], Takahide Takahashi[8], Masahide Imada[8,9], Ken Okada[8], Jiro Uehara[10], Junko Sowa-Osako[11] and Keiji Iwatsuki[1]

[1]Departments of Dermatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

[2]Department of Dermatology, Faculty of Medicine, University of Tokyo, Tokyo, Japan

[3]Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

[4]Department of Dermatology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan

[5]Department of Dermatology, Shimane University Faculty of Medicine, Izumo, Japan

[6]Department of Pathology, Kurume University School of Medicine, Kurume, Japan

[7]Division of Tumor and Cellular Biochemistry, Department of Medical Sciences, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan

[8]Division of Medical Support, Okayama University Hospital, Okayama, Japan

[9]Central Clinical Laboratory, Kawasaki Medical School Hospital, Okayama, Japan

[10]Department of Dermatology, Asahikawa Medical University, Asahikawa, Japan

[11]Department of Dermatology, Osaka City University Graduate School of Medicine, Osaka, Japan

Address correspondence to

Keiji Iwatsuki, M.D., Ph.D.

Department of Dermatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. 2-5-1, Shikata-cho, Kita-ku, Okayama, 700-8558, Japan

Phone: +81-86-235-7282, Fax: +81-86-235-7283

E-mail: keijiiwa@cc.okayama-u.ac.jp

Short title

CADM1 expression in Sézary syndrome

Abbreviations

cell adhesion molecule-1 (CADM1), tumor suppressor lung cancer-1 (TSLC1), Sézary syndrome (SS), mycosis fungoides (MF), adult T-cell leukemia/lymphoma (ATLL), anaplastic large cell lymphoma (ALCL), C-C chemokine receptor type 4 (CCR4), human T-cell leukemia virus 1 (HTLV-1), peripheral blood mononuclear cell (PBMC), cutaneous T-cell lymphoma (CTCL), diffuse large B-cell lymphoma (DLBCL), enzyme-linked immunosorbent assay (ELISA), reverse transcriptase-polymerase chain reaction (RT-PCR)

Abstract; 242 words (limit: 250 words)

Main article; 3087 words (limit: 6,000 words)

ABSTRACT

Cell adhesion molecule 1 (CADM1) is aberrantly expressed by T-cell neoplasms such as adult T-cell leukemia/lymphoma (ATLL) and mycosis fungoides (MF). We studied the expression of CADM1 and its splicing variants in Sézary syndrome (SS), MF, other cutaneous T-cell lymphoma (CTCL), and cell lines derived from T- and B-cell lymphomas. Soluble CADM1 was measured in the patients' sera. CADM1+ cells in the blood and skin lesions were examined by flow cytometry and immunostaining, respectively. Soluble CADM1 was measured by ELISA, and the splicing variants of *CADM1* transcripts were determined by reverse transcriptase-polymerase chain reaction, followed by sequencing. As a result, circulating CADM1+ cells were significantly increased in 7 of 10 patients with SS, ranging from 7.9% to 74.5% of the CD3+CD4+ fractions (median; 33.7%) (cut off value; 6.5%). The percentages of CADM1+ cells were usually less than those of circulating Sézary cells. CADM1 was expressed, to various degrees, in 6 of 9 T-cell lines derived from SS, MF, ATLL, and anaplastic large cell lymphoma (ALCL), but negative in B-cell lymphoma-derived cell lines. CADM1+ cells were present in the skin infiltrates of MF, SS, ATLL and ALCL. Serum levels of soluble CADM1 were not significantly elevated in SS/MF. Three major splicing variants of CADM1 expressed by neoplastic T cells contained different

71 combinations of the exons 7, 8, 9 and 11, including a putative oncogenic variant
72 composed of exons 7-8-9-11. In conclusion, CADM1 is frequently expressed in Sézary
73 cells and cell lines from CTCL.
74

75 **Keywords**

76 CADM1, Mycosis fungoides, Sézary syndrome, splicing variant, T-cell lines,
77

INTRODUCTION

Cell adhesion molecule 1 (CADM1), a member of the immunoglobulin superfamily of cell adhesion molecules (IgCAM) encoded on chromosome 11q23.2, has been designated with a variety of different names because of its multiple functions: TSLC1 (tumor suppressor in non-small cell lung cancer 1) , [1] IGSF4 (immunoglobulin superfamily 4), [2] RA175 mRNA, [3] SynCAM (synaptic cell adhesion molecule), [4] and Necl-2 (nectin-like molecules). [5] CADM1 expression has been observed in the human lung, brain, testis, and various epithelial tissues including skin, and has been shown to function in cell-cell adhesion through the homophilic binding of its ectodomains between the adjacent cells. [6]

The absence of CADM1 expression was first shown to be a prognostic indicator in non-small cell lung cancer, [1] and it was subsequently shown that methylation of the CADM1 promoter inhibits CADM1 expression in various cancers. [7] In contrast, small cell lung cancer expressed a unique CADM1 splicing variant composed of exons 7-8-9-11. [8] It has been shown that this splicing has the ability to enhance tumorigenesis, whereas other splicing variants lacking exon 9 may function as tumor suppressors. [7,8] Although no expression of CADM1 mRNA was detected in normal CD4+ T-cells, molecular and flow cytometric studies revealed the expression of surface CADM1 in adult T-cell leukemia/lymphoma (ATLL) cells. [9,10] Therefore, CADM1 may play dual

roles in human oncogenesis: as a tumor suppressor in epithelial cancers, and as an oncoprotein in small cell lung cancer and T-cell malignancies such as ATLL. In cases of ATLL, it has been reported that the presence of circulating CADM1+ T-cells with a CD7dim+/CD7- phenotype is associated with overt or progressive disease. [10]

In the present study, we detected the expression of CADM1 in leukemic cells of patients with Sézary syndrome (SS) and in infiltrating cells in cutaneous lesions of SS and mycosis fungoides (MF). Since ADAM10 (a disintegrin and metalloproteinases 10) cleaves the ectodomain of CADM1 to form soluble CADM1 [11], we measured soluble CADM1 in the patients' sera, and examined the possibility of being a biomarker for SS, MF, and other CTCL. We determined the splicing variant of CADM1 expressed by circulating Sézary cells, T-cell lines and cultured human epidermal keratinocytes.

Here, we report that CADM1 was expressed by various types of CTCL including SS, MF, ATLL, and cell lines derived from T-cell lymphomas. We detected three major splicing variants of CADM1 in SS, one of which was a putative oncogenic variant as observed in small cell lung cancer.

MATERIALS AND METHODS

Patient's samples

PBMCs were obtained from ten SS patients whose clinical data are summarized in Table 1. In one SS patient (case 1), four blood samples were obtained in different occasions: before and during the treatment with oral etoposide, 100 mg/week for 7 years. And in another one (case 9), three samples were collected before and during the treatment with interferon- γ . Control PBMCs were also obtained from 25 patients with MF, three with non-MF/SS cutaneous T-cell lymphomas (CTCL), two with diffuse large B-cell lymphoma (DLBCL), eight with inflammatory skin diseases with erythroderma, and 19 healthy volunteers.

Cutaneous biopsy specimens were obtained from patients with SS (n=3), MF(n=8), ATLL (n=3) and ALCL (n=5) for diagnostic use, and the rest of them were used for immunostaining. Serum samples from patients with SS (n=7), MF (n=21), ATLL (n=6), ALCL (n=6), and healthy volunteers (n=69) were used for the measurement of soluble CADM1 by ELISA. The present study was approved by the Institutional Review Board (IRB) of Okayama University Hospital (No. 1802-006 and Genome No. 319). Informed consent was obtained from all blood and tissue donors according to the Helsinki Declaration.

Cell lines

We used thee MF/SS cell lines (Hut78, Myla and MJ), one ATL cell line (TL-SU), one non-MF/SS cutaneous T-cell lymphoma line (HH), four ALCL cell lines (SU-DHL-1, Karpas299, SR786, and SUP-M2), and six B-cell lines (Raji, Akata, N83-1, BJ-AB, IB4, and LCL-TT).

Flow cytometric analysis

In addition to our routine panel of conjugated antibodies for flow cytometry, including anti-CD3, CD4, CD7, CD8, CD25, CD30, CD45 and HLA-DR antibodies (Beckman Coulter Inc., CA, U.S.A.), PBMCs were stained with phycoerythrin (PE)-conjugated anti-TSLC1/CADM1 antibody (polyclonal, rabbit, bs-6026R) and allophycocyanin (APC)-conjugated anti-CCR4 antibody (mouse, L291H4). Navios instrument was used for all multicolor flow cytometry, and data were analyzed using Kaluza software (Beckman Coulter Inc., CA, U.S.A.).

Cell activation by CD3/CD28 stimulation

PBMCs from healthy volunteers were stimulated with CD3/CD28-coating beads (Thermo Scientific Inc., MA, U.S.A.) according to the manufacturer's protocol. After 48

151 h stimulation, the PBMCs were processed for flow cytometry as described above.

152

153 *Immunohistochemistry*

154 Formalin-fixed, paraffin-embedded tissue sections were used for immunostaining. After
155 deparaffinization and peroxidase-blocking, the sections were stained with mouse
156 monoclonal anti-human CD194 (CCR4) antibody (Becton, Dickinson and Company
157 Inc., NJ, U.S.A.), or with chicken monoclonal anti-SynCAM/TSLC1/CADM1 antibody
158 (MBL, Nagoya, Japan). Slides were then incubated with the ChemMate Envision
159 polymer (DAKO Japan, Tokyo) or with biotinylated anti-chicken IgY (Immuno HRP
160 DAB kit, Immuno Bio Science Corp., WA, U.S.A.). The target proteins were detected
161 using diaminobenzidine tetrahydrochloride (DAB) solution.

162

163 *Reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing*

164 Total RNA was extracted from cell lines and converted to complementary DNA. The
165 PCR was carried out for 33 cycles of denaturation at 94°C, annealing at 64°C, and
166 extension at 72°C. The primer sequences used were as follows: CADM1 forward, 5'-
167 GTGATGGTAACTTGGGTGAGAGTC-3'; CADM1 reverse, 5'-
168 CCAGAATGATGAGCAAGCACAG-3'. The PCR products were fractionated on 2%

agarose gels and visualized by ethidium bromide staining. The products of target bands from gel were sequenced using an Applied Biosystems 310 Genetic Analyzer (Applied Biosystems, CA, U.S.A.). The results were read by ApE (v2.0.49) (free software by M. Wayne Davis), and compared with the database registered in NCBI BLAST (Basic Local Alignment Search Tool).

Enzyme-linked immunosorbent assay (ELISA)

Soluble forms of CADM1 in patients' sera and the culture supernatants were measured by a sandwich ELISA using phage anti-human CADM1 antibody (Institute for Antibodies Co., Ltd., Nagoya, Japan; phage 035-212) for the capture antibody, and peroxidase-labeled anti-CADM1 antibody (MBL Co., Ltd., Nagoya, Japan; chicken IgY, Clone3E1) for the second antibody. For quantification of soluble CADM1, calibration curve was made using a standard sample with known concentration (the recombinant protein in soluble form from CADM1-transfected HEK293 cells).

Statistical analysis

Statistical analyses were conducted with GRAPHPAD PRISM, version 4.03 (GraphPad, La Jolla, CA, U.S.A.) and IBM SPSS Statistics 22.0 (IBM, Tokyo, Japan). Mann-

187 Whitney U test and Spearman rank correlation coefficient were used for statistical

188 analysis; P-values < 0.05 were considered significant.

189

RESULTS

CADM1 expression in circulating Sézary cells

Flow cytometric analysis revealed a background level of CADM1+ cells in the PBMCs, ranging from 0.0% to 4.5% (mean: 1.66±1.62%) in the healthy subjects, and from 0.1% to 2.4% (mean: 0.97±1.39%) in the disease control group with non-lymphoma skin disorder. Based on these data, a significant increase of CADM1+ cells was considered to be an increase to 6.5% or more of the CD3+CD4+ fraction (the mean + 3SD in the normal individuals: n=19).

Ten SS patients were enrolled in the present study: all patients met the B2 criteria for SS upon initial diagnosis, i.e., ≥1,000 μL^{-1} Sézary cells with positive clones, or either CD4/CD8 ≥10, CD4+CD7- cells ≥40% or CD4+CD26- cells ≥30%.(12) Ten blood samples obtained from the ten SS patients for the first examination contained CADM1+ cells that accounted for 0.4% to 74.5% (median; 22.4%) of the CD3+CD4+ cell fraction (Table 1, Fig. 1a, b). Of the 15 samples tested, seven samples from seven patients (sample No. 1-1, 2, 3, 4, 5, 6 and 7 in Table 1) exhibited significantly increased percentages (≥6.5%) of CADM1+ cells in the CD3+CD4+ fraction, ranging from 7.9% to 74.5% (median; 33.7%) (Fig. 1a, c). The remaining eight samples (No. 1-2, 1-3, 1-4, 8, 9-1, 9-2, 9-3, and 10) obtained from four patients with SS showed a background level (<6.5%) of CADM1+ cells, ranging from 0.4% to 4.8% of the CD3+CD4+ fraction.

209

210 ***CADM1 expression in other cutaneous lymphomas, activated T-cells, and***

211 ***inflammatory skin diseases***

212 In 24 of 25 patients with MF, the percentages of CADM1+ cells were not significantly

213 increased (<6.5% CADM1+ cells) in the PBMCs (Fig. 1b). The one exceptional patient

214 with MF, stage IVB, had a slightly increased CD4/CD8 ratio (3.3%) with 8.8%

215 CADM1+ cells in the CD3+CD4+ fraction, although no abnormal T cells were

216 detectable in blood smear or by flow cytometry. No significant increase of CADM1+

217 cells was observed in the PBMCs from two patients with DLBCL, eight patients with

218 inflammatory skin diseases, including atopic dermatitis (n=6) and generalized drug

219 eruption (n=2), or in the 19 healthy volunteers (Fig. 1b).

220

221 CD3/CD28-stimulated normal T-cells showed no increase of CADM1+ cells in the

222 CD3+ fraction (n=12, mean 2.9±1.8%) (p=0.0091, Mann-Whitney U test) (Fig. 2a),

223 although HLA-DR and CD25 were induced after stimulation (Fig. 2b). Therefore,

224 CADM1 was not inducible in T-cells by CD3/CD28 stimulation.

225 Among the cell lines examined, CADM1+ cells were observed in the T-cell lines

226 ranging from 1.3% to 22.4% (n=9, mean 11.0±8.3%) (Fig. 2a, c). Six of 9 T-cell lines

227 contained CADM1+ cells over the cut-off value (6.5%), including all three T-cell lines
228 derived from MF/SS (Myla, MJ and Hut78), one from ATLL (TL-SU), one from non-
229 MF/SS CTCL (HH), and one from ALCL (SR-786) (Fig. 2a). The remaining three T-cell
230 lines derived from ALCL contained CADM1+ cells below the cut-off value. No increase
231 of CADM1+ cells was observed in all B-cell lines examined (n=6, mean 0.87±0.95%)
232 (p=0.0016) (Fig. 2a). Therefore, CADM1 was expressed in some neoplastic T-cell lines
233 selectively, and not induced in the neoplastic B-cell lines or CD3/CD28-stimulated T
234 cells.
235
236 ***A phenotype of CADM1+ Sézary cells***
237 In addition to cytological examination in the blood smear, the percentages of Sézary
238 cells was estimated by two indicators in the present study: CD3+CD4+ CD7dim+/CD7-
239 cells, and CD3+CD4+ CD26dim+/CD26- cells by flow cytometry. The percentages of
240 CADM1+ cells did not always correspond to those of Sézary cells determined by
241 cytological criteria, CD4+CD7dim+/CD7- cells or CD4+CD26dim+/CD26- cells (Fig.
242 1c). In our series of patients (n=10), the percentages of CD4+CD7dim+/CD7- cells were
243 higher than those of CADM1+ cells, except for a few patients (case 3 in Table 1).

Since CCR4 is usually expressed by Sézary cells, we compared the co-expression of CADM1 and CCR4 in the CD3+CD4+CD7dim+/CD7- fractions. In all six patients (eight samples) studied, the percentages of CADM1+ cells were lower than those of CCR4+ cells: 0.3% (case 1-3 and 1-4), 49.0% (case 4), 39.5% (case 5) 16.4% (case6), 50.2% (case 8), 5.1% (case 9-2) and 21.8% (case 9-3) of the CCR4+ cells, respectively.

CADM1+ cells in skin lesions of cutaneous T-cell lymphomas

In the cutaneous lesions of MF/SS at stages IIA (n=1), IIB (n=4), IIIA (n=2), IVA1 (n=2) and IVA2 (n=2), CADM1+ cells were present in the dermal and epidermal infiltrates to various degrees (Fig. 3). Cutaneous lesions of ATLL (n=3) and primary cutaneous ALCL (n=5) also contained CADM1+ cell in the infiltrates (Fig. 3). Since neoplastic T cells of MF, SS and ATLL are known to express CCR4, we examined the ratios of CADM1+ cells among CCR4+ cells in the skin lesions, excluding the inflammatory cell infiltrates as much as possible. Similar to the cases of ATLL (n=3), the numbers of CADM1+ cells were less than those of CCD4+ cells in the skin lesions of SS (n=3) and MF (n=8), except for one MF case (Fig. 3, Fig. S1). There was no clear difference in CADM1 expression by the stages of illness. By contrast, atypical lymphoid cells of the primary cutaneous ALCL (n=5), which is usually not related to CCR4 expression, were positive

for CADM1 without CCR4 expression to various degrees. When compared with the percentages of CADM1+ cells between the skin infiltrates and peripheral blood in the same patient with SS (case 1 in Table 1), only a small percentage of CADM1+ cells (approximately 18%) was observed among CCR4+ cells in the dermis, although 74.5% of CADM1+ cells were present in the CD3+CD4+ fraction in the blood.

Splicing variants of CADM1 mRNA

The RT-PCR amplification revealed that cDNA generated from mRNA extracted from a Sézary cell line (Hut78) contained three major PCR products with the respective molecular sizes of 368, 332, and 257 bp, respectively. A direct sequencing study revealed that the three PCR products were composed of exons 7, 8, 9 and 11 of *CADM1* (368-bp product), exons 7,8 and 11 (332-bp product), and exons 7 and 11 (257-bp product) (NCBI, BLAST data) (Fig. 4a-c).

Cultured normal human keratinocytes also showed three PCR products corresponding to those observed in Sézary cells (Fig. 4b, c). Therefore, one Sézary cell line (Hut78) and cultured normal human keratinocytes expressed the same combination of exons of *CADM1*. Freshly isolated Sézary cells (cases 4 and 6 in Table 1) expressed the two major isoforms composed of exons 7, 8, 9 and 11, and exons 7, 8, and 11,

respectively. Other T-cell lines from MF, ATLL and ALCL also expressed splicing isoforms with a different combination of the above-mentioned three variants (Fig. 4b). No *CADM1* mRNA was expressed in the three B-cell lymphoma cell lines examined (N83-1, BJ-AB and Raji).

Comparison of CADM1 expression with hematological markers and clinical courses

When CADM1 expression was compared with hematological markers having prognostic significance, such as lactate dehydrogenase (LDH) and soluble IL-2 receptors (sIL-2R), there were weak correlations between the percentages of CADM1+ cells in the peripheral blood and the serum levels of LDH or soluble IL-2 receptor (sIL-2R) (Fig. 5).

In our series of ten patients with SS, four of five patients whose CADM1+ cells made up more than 20% (median value of CADM1+ cells; 22.4%) of the CD3+CD4+ fraction died of SS-related complications in a 7-year follow-up period (Fig. S2).

Soluble form of CADM1 in patients' sera

In order to evaluate the shedding of CADM1 ectodomains, we measured soluble CADM1 in the sera of SS patients by ELISA. Two of six serum samples from ATLL

298 patients contained extremely high levels of soluble CADM1, i.e., 2913.6 and 2132.4 ng
299 mL^{-1}, but the other four ATLL samples and five samples obtained from patients with SS
300 at different interval (cases 1, 2 and 3 in Table 1) showed low concentrations of soluble
301 CADM1 (below 400 ng mL^{-1}) (Fig. 6). No difference was observed in the serum levels
302 of soluble CADM1 between SS and the other CTCL excluding ATLL (196.8±89.5 vs.
303 198.2±92.2 ng mL^{-1}).
304

Discussion

Similar to the previous observations in overt ATLL, [13] we herein detected circulating CADM1+ cells in a group of patients with SS. Neoplastic cells of ATLL and SS share a cytological profile: a convoluted or flower-like nucleus and a CD3+CD4+CCR4+ phenotype. In addition to these cytological similarities, our observations indicate that CADM1 expression is also shared by both cell types. The expression of CADM1, however, was not specific for leukemic cells of ATLL, SS and MF because its expression was detected in infiltrating cells in ALCL and cell line cells from CTCL. The expression of CADM1 was not restricted to the type 2 helper T cells (Th2) or regulatory T cells (Treg), both of which are positive for CCR4, but could be induced in the other cell types negative for CCR4, as shown in cases of ALCL. It is intriguing to note that the B-cell lines used for the present study did not express CADM1.

The detection of a CADM1+CD7dim+/CD7- fraction has been used to predict the progressive form of ATLL. [9,13] In our small series of SS patients, CADM1 expression was observed in patients with progressive SS: four of five SS patients harboring CADM1+ cells that made up over 20% of the CD3+CD4+ fraction died of progressive SS within 1.5 year after the appearance of more than 20% of CADM1+ cells. Similar to our observations, recent reports have described that CADM1 is expressed in the

323 cutaneous infiltrates of MF, and its expression might be related to the poor prognosis.
324 [14,15] Therefore, further cohort study is required to address whether CADM1 expression
325 is a marker of progressive MF and SS.

326 Our study indicates that the expression of CADM1 is not a simple activation marker,
327 because CD3/CD28 stimulation of normal human lymphocytes induced CD25 and
328 HLA-DR expression, but did not induce CADM1. It has been postulated that CADM1
329 expression is induced by HTLV-1-encoded gene products such as Tax and HBZ in
330 ATLL. [13,16] But this scenario cannot explain the fact that CADM1 is expressed by
331 Sézary cells without HTLV-1 infection.

332 Sézary cells are characterized by the expression of CCR4 and the loss or diminished
333 expression of CD7 and/or CD26. [17] Our present flow cytometric study revealed that
334 the percentages of CADM1+ cells did not necessarily correspond to those of Sézary
335 cells determined by cytological criteria, or a CD4+CD7dim+/CD7- or a
336 CD4+CD26dim+/CD26- phenotype.

337 In addition to the loss or diminished expression of CD7 and CD26, recent studies
338 described expression of CD158k/ KIR3DL2, CD164, and the central memory T-cell
339 phenotype (CD27+CD45RA- CD45RO+) as characteristic features of Sézary cells. [18-21]
340 Various novel gene alterations have been reported in Sézary cells: *MYC* gain and *MNT*

loss, up-regulation of *DNM3*, *TWIST1*, *EPHA4* and *PLS3*, and down-regulation of *STAT4*. [22] Whole exome and RNA sequencing revealed a complex genomic landscape of somatic copy number variations and fusion genes possibly related to the pathogenesis of SS. [23]

Concerning the oncogenic isoform of CADM1, Kikuchi *et al.* reported that normal human lung cDNA reveals a single major isoform composed of the exons 7-8 -11 (the 332 bp in Fig. 4a), whereas small cell lung cancer expresses another splicing variant containing the exons 7-8-9-11 (the 368 bp in Fig. 4a). The authors' experimental data suggest that this variant is associated with the malignant features of small cell lung carcinoma, as is observed in progressive ATLL[8]. In our study, Sézary cells and T-cell lines including Hut78, TL-SU and SUP-M2 expressed the 368bp variant composed of exons 7-8-9-11 to various degrees.

Our study indicates that cultured normal human keratinocytes showed the three major splicing variants of CADM1, identical to those observed in Sézary cells. Furthermore, a previous report has described that the extracellular domains of CADM1 interact with integrin $\alpha 6\beta 4$ in hemidesmosomes[24]. These observations suggest the possible involvement of CADM1 in epidermotropic infiltration of neoplastic T-cells via the homophilic and heterophilic binding of CADM1 to epidermal keratinocytes and

359 basement membrane zone.

360 In conclusion, CADM1 is frequently expressed by neoplastic cells in SS and MF,

361 and T-cell lines from CTCL. The pathogenic properties of CADM1 in the progression of

362 CTCL remain to be answered.

363

Acknowledgments

We thank Michinori Aoe, Department of Laboratory Medicine, Okayama University Hospital, Okayama, and Hiroko Katayama, Departments of Dermatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, for support in this project.

Conflict of Interest

The authors state no conflict of interest.

Funding: None

References

1. Kuramochi M, Fukuhara H, Nobukuni T, et al. TSLC1 is a tumor-suppressor gene in human non-small-cell lung cancer. Nature genetics. 2001;**27**(4):427-30.

2. Gomyo H, Arai Y, Tanigami A, et al. A 2-Mb sequence-ready contig map and a novel immunoglobulin superfamily gene IGSF4 in the LOH region of chromosome 11q23.2. Genomics. 1999;**62**(2):139-46.

3. Urase K, Soyama A, Fujita E, Momoi T. Expression of RA175 mRNA, a new member of the immunoglobulin superfamily, in developing mouse brain. Neuroreport. 2001;**12**(15):3217-21.

4. Biederer T, Sara Y, Mozhayeva M, et al. SynCAM, a synaptic adhesion molecule that drives synapse assembly. Science (New York, NY). 2002;**297**(5586):1525-31.

5. Shingai T, Ikeda W, Kakunaga S, et al. Implications of nectin-like molecule-2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM1 in cell-cell adhesion and transmembrane protein localization in epithelial cells. The Journal of biological chemistry. 2003;**278**(37):35421-7.

6. Masuda M, Yageta M, Fukuhara H, et al. The tumor suppressor protein TSLC1 is involved in cell-cell adhesion. The Journal of biological chemistry. 2002;**277**(34):31014-9.

7. Murakami Y. Involvement of a cell adhesion molecule, TSLC1/IGSF4, in human oncogenesis. Cancer Sci. 2005;**96**(9):543-52.

393 8. Kikuchi S, Iwai M, Sakurai-Yageta M, et al. Expression of a splicing variant of the
394 CADM1 specific to small cell lung cancer. Cancer Sci. 2012;**103**(6):1051-7.
395 9. Sasaki H, Nishikata I, Shiraga T, et al. Overexpression of a cell adhesion molecule,
396 TSLC1, as a possible molecular marker for acute-type adult T-cell leukemia. Blood.
397 2005;**105**(3):1204-13.
398 10. Kobayashi S, Nakano K, Watanabe E, et al. CADM1 expression and stepwise
399 downregulation of CD7 are closely associated with clonal expansion of HTLV-I-infected cells
400 in adult T-cell leukemia/lymphoma. Clinical cancer research : an official journal of the
401 American Association for Cancer Research. 2014;**20**(11):2851-61.
402 11. Mimae T, Hagiyama M, Inoue T, et al. Increased ectodomain shedding of lung
403 epithelial cell adhesion molecule 1 as a cause of increased alveolar cell apoptosis in
404 emphysema. Thorax. 2014;**69**(3):223-31.
405 12. Olsen EA, Whittaker S, Kim YH, et al. Clinical end points and response criteria in
406 mycosis fungoides and Sezary syndrome: a consensus statement of the International
407 Society for Cutaneous Lymphomas, the United States Cutaneous Lymphoma Consortium,
408 and the Cutaneous Lymphoma Task Force of the European Organisation for Research and
409 Treatment of Cancer. Journal of clinical oncology : official journal of the American Society of
410 Clinical Oncology. 2011;**29**(18):2598-607.

411 13. Nakahata S, Saito Y, Marutsuka K, et al. Clinical significance of
412 CADM1/TSLC1/IgSF4 expression in adult T-cell leukemia/lymphoma. Leukemia.
413 2012;**26**(6):1238-46.
414 14. Mashima E, Sawada Y, Yamaguchi T, et al. A high expression of cell adhesion
415 molecule 1 (CADM1) is an unfavorable prognostic factor in mycosis fungoides. Clinical
416 immunology (Orlando, Fla). 2018;**193**:121-2.
417 15. Yuki A, Shinkuma S, Hayashi R, et al. CADM1 is a diagnostic marker in early-
418 stage mycosis fungoides: Multicenter study of 58 cases. Journal of the American Academy of
419 Dermatology. 2018.
420 16. Pujari R, Hunte R, Thomas R, et al. Human T-cell leukemia virus type 1 (HTLV-1)
421 tax requires CADM1/TSLC1 for inactivation of the NF-kappaB inhibitor A20 and
422 constitutive NF-kappaB signaling. PLoS pathogens. 2015;**11**(3):e1004721.
423 17. Kim EJ, Hess S, Richardson SK, et al. Immunopathogenesis and therapy of
424 cutaneous T cell lymphoma. The Journal of clinical investigation. 2005;**115**(4):798-812.
425 18. Bagot M, Moretta A, Sivori S, et al. CD4(+) cutaneous T-cell lymphoma cells
426 express the p140-killer cell immunoglobulin-like receptor. Blood. 2001;**97**(5):1388-91.
427 19. Campbell JJ, Clark RA, Watanabe R, Kupper TS. Sezary syndrome and mycosis
428 fungoides arise from distinct T-cell subsets: a biologic rationale for their distinct clinical

429 behaviors. Blood. 2010;**116**(5):767-71.

430 20. Poszepczynska-Guigne E, Schiavon V, D'Incan M, et al. CD158k/KIR3DL2 is a new

431 phenotypic marker of Sezary cells: relevance for the diagnosis and follow-up of Sezary

432 syndrome. The Journal of investigative dermatology. 2004;**122**(3):820-3.

433 21. Wysocka M, Kossenkov AV, Benoit BM, et al. CD164 and FCRL3 are highly

434 expressed on CD4+CD26- T cells in Sezary syndrome patients. The Journal of investigative

435 dermatology. 2014;**134**(1):229-36.

436 22. Boonk SE, Zoutman WH, Marie-Cardine A, et al. Evaluation of Immunophenotypic

437 and Molecular Biomarkers for Sezary Syndrome Using Standard Operating Procedures: A

438 Multicenter Study of 59 Patients. The Journal of investigative dermatology.

439 2016;**136**(7):1364-72.

440 23. Prasad A, Rabionet R, Espinet B, et al. Identification of Gene Mutations and

441 Fusion Genes in Patients with Sezary Syndrome. The Journal of investigative dermatology.

442 2016;**136**(7):1490-9.

443 24. Mizutani K, Kawano S, Minami A, Waseda M, Ikeda W, Takai Y. Interaction of

444 nectin-like molecule 2 with integrin alpha6beta4 and inhibition of disassembly of integrin

445 alpha6beta4 from hemidesmosomes. The Journal of biological chemistry.

446 2011;**286**(42):36667-76.

SUPPORTING INFRMATION

Figure S1. CADM1+ cells in the skin lesions of MF/SS, ATLL, and ALCL (all data)

Figure S2. Outcomes of SS patients during the observation periods, and the absolute numbers of CADM1+ cells on diagnosis

FIGURE LEGENDS

Figure 1. CADM1+ cells in peripheral blood

(a) CADM1+ cells in the CD3+CD4+ fraction of PBMCs were analyzed by flow cytometry. **(b)** The percentages of CADM1+ cells of the CD3+CD4+ fraction in PBMCs are shown. **(c)** The absolute numbers of CADM1+ cells the CD3+CD4+ fraction in PBMCs are shown.

Figure 2. CADM1 expression by T-cell line cells and activated PBMCs

(a) The CADM1+ cells were significantly higher in T cell lines than those in the B-cell ones ($p=0.0016$) and the activated PBMCs ($p=0.0091$). (Mann-Whitney U test) **(b)** CD3/CD28-activated PBMCs from healthy controls begin to express activation markers such as CD25 and/or HLA-DR, but do not express CADM1. **(c)** CADM1 expression in MF/SS-derived cell lines (Myla, Hut78 and MJ), and non-MF/SS CTCL-derived one (HH).

Figure 3. CADM1+ cells in the skin lesions of MF/SS, ATLL, and ALCL

The representative cases show that CADM1+ cells are present among CCR4+ cells in the dermal and epidermal infiltrates in MF/SS and ATLL. The expression of CADM1+

cells are also observed in the skin infiltrates of primary cutaneous ALCL. (See all the data of immunostaining in Fig. S1.)

Figure 4. Splicing variants of CADM1 expressed by Sezary cells, T-cell lines, and normal human epidermal keratinocytes

(a) Possible splicing variants of *CADM1* mRNA (Kikuchi et al, 2012). **(b)** One Sézary cell line (Hut78) and cultured normal human keratinocytes (NHEK) express the identical splicing variants of CADM1. Other T-cell lines also express splicing isoforms with a different combination. No *CADM1* mRNA was expressed in B-cell lymphoma cell lines (N83-1, BJ-AB and Raji). **(c)** Three major PCR products expressed by a SS cell line (Hut78) are composed of exons 7, 8, 9 and 11 of *CADM1* for the 368 bp, exons 7,8 and 11 for the 332bp, and exons 7 and 11 for the 257 bp.(NBCI, BLAST)

Figure 5. Comparison of CADM1 expression with hematologic markers, LDH and sIL-2R

(a) The percentages of CADM1+ cells in the peripheral blood tend to correlate with the serum levels of LDH or sIL-2R. (Spearman's rank correlation coefficient) **(b)** Comparison of hematological markers between the alive and dead cases (Mann-

Whitney U test). All four SS patients with fatal outcome in the follow-up period had more than 20% of CADM1+ cells (See Figure S2).

Figure 6. Serum levels of soluble form of CADM1

Two of six serum samples from ATLL patients contained extremely high levels of soluble CADM1, but other samples including 5 serum samples from patients with Sézary syndrome showed low concentrations of soluble CADM1. n.s.: not significant. (Mann-Whitney U test)

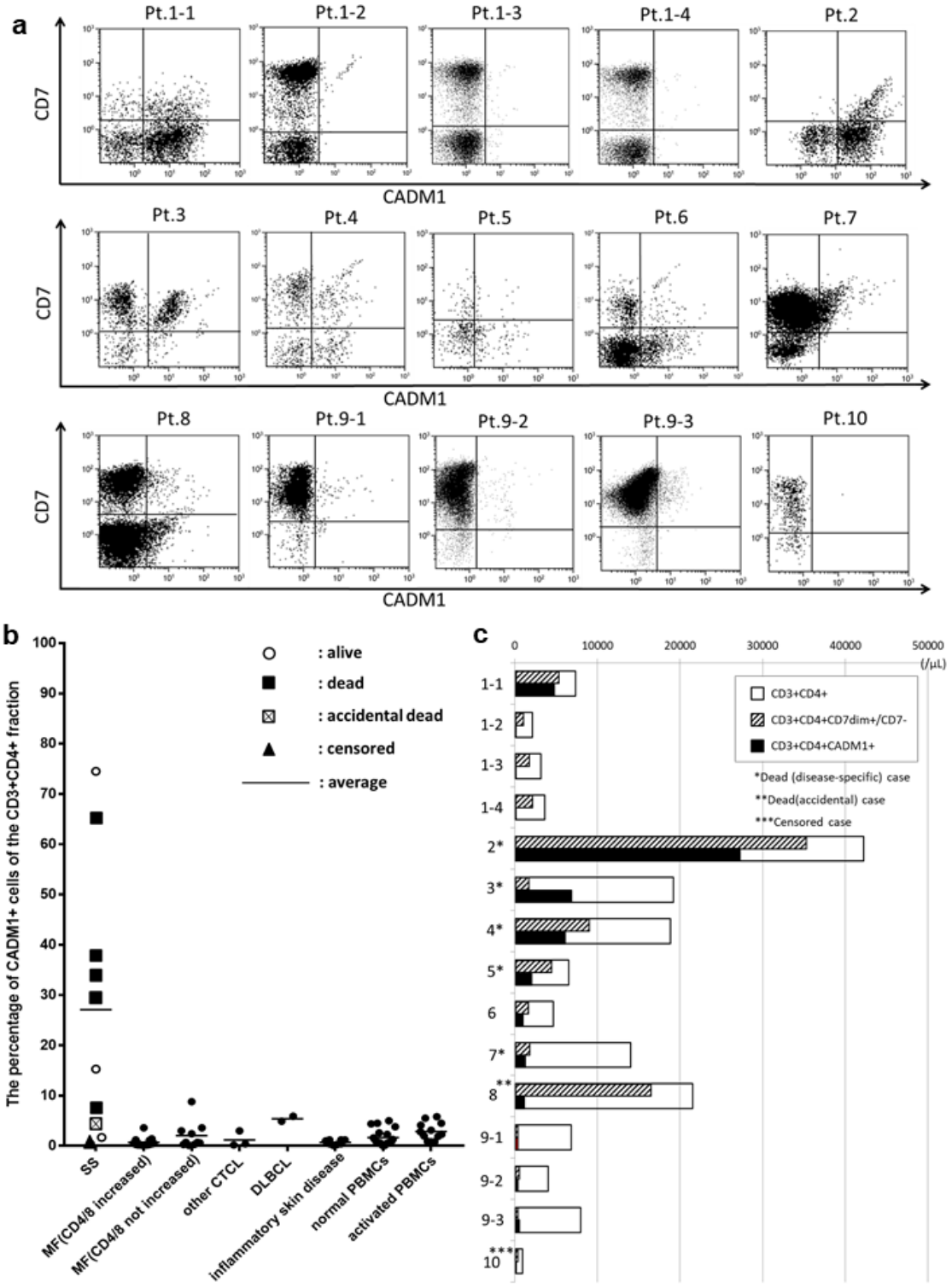
Table 1. Clinical data of the SS patients

Table 1. Clinical characteristics of SS patients

No.	Age/ Sex	Stage	WBC, /μL	Aty-Ly, /μL (%)	LDH, IU/L	sIL-2R, U/mL	CD4/8 ratio	CD3+CD4+ fraction		Treatment	Observation period	Outcome
								CADM1+ %	CD7+dim %			
1-1	69/M	Stage4A1 (T4N1M0B2)	8040	3256 (40.5)	366	1739	19.3	74.5	84	Photo.	7y	alive
1-2	75/M	Stage4A1 (T4N1M0B2)	2580	232 (9.0)	296	481	6.4	0.9	48.8	MC (VP16)		
1-3	75/M	Stage4A1 (T4N1M0B2)	3700	56 (1.5)	342	737	11.2	0.5	55.6	MC (VP16)		
1-4	76/F	Stage4A1 (T4N1M0B2)	4170	167 (4.0)	306	582	13.5	0.5	64.8	MC (VP16)		
2	62/F	Stage4A1 (T4N3M0B2)	54110	23538 (43.5)	1347	11385	18.4	65.1	83.8	PC (CHP-based)	3y+10m	dead
3	76/F	Stage4A2 (T4N3M0B2)	20510	3434 (65.6)	375	1184	98	38	9.6	MC (VP16 / HDACi)	1y+2m	dead
4	58/M	Stage4A1 (T4N3M0B2)	20800	11024 (53.0)	322	26100	0.6	33.7	49.2	PC	11m	dead
5	89/F	Stage4	12000	0 (0)	511	7077	98	29.4	82.8	Oral corticosteroid	9y	dead
6	64/F	Stage4A2 (T4N3M0B2)	5410	0 (0)	380	413	3.9	15.3	34.7	PC (CHOP) MC (HDACi)	5y+1m	alive
7	74/M	Stage4A1	15900	9302 (58.5)	452	2753	28.7	7.9	11.1	MC (VP16)	2y+11m	dead
8	72/F	Stage4A2 (T4N3M0BX)	24000	3472 (14)	298	3584	15.4	4.8	74.6	PC (CHOP) MC (HDACi)	5m	dead
9-1	56/M	Stage4A2 (T4NxM0B2)	8860	1063 (12)	319	1033	9.6	1.7	5.1	Oral corticosteroid	4y+1m	alive
9-2	56/M	Stage4A2 (T4NxM0B2)	6960	522 (7.5)	365	-	2.6	2.2	1.4	IFN - γ		
9-3	57/M	Stage4A2 (T4NxM0B2)	8540	1153 (13.5)	180	1271	21	2.0	17.8	IFN - γ		
10	54/M	Stage4A2	6000	120 (2.0)	88	838	0.6	0.4	17.8	PC (CHOP, ICE)	2y+6m	censored

Abbreviations: WBC, white blood cell; Aty-Ly, atypical lymphocyte; LDH, lactate dehydrogenase; sIL-2R; soluble interleukin-2 receptor; CD, cluster of differentiation; CADM, cell adhesion molecule; Photo., phototherapy; MC, monochemotherapy; PC, polychemotherapy; VP16, etoposide; HDACi, histone deacetylase inhibitor; IFN -γ, intravenous interferon-γ; C, cyclophosphamide; H, doxorubicin (hydroxydaunomycin); O, vincristine; P, prednisolone; I, ifosfamide; C, carboplatin; E, etoposide; y, year(s); m, month(s).

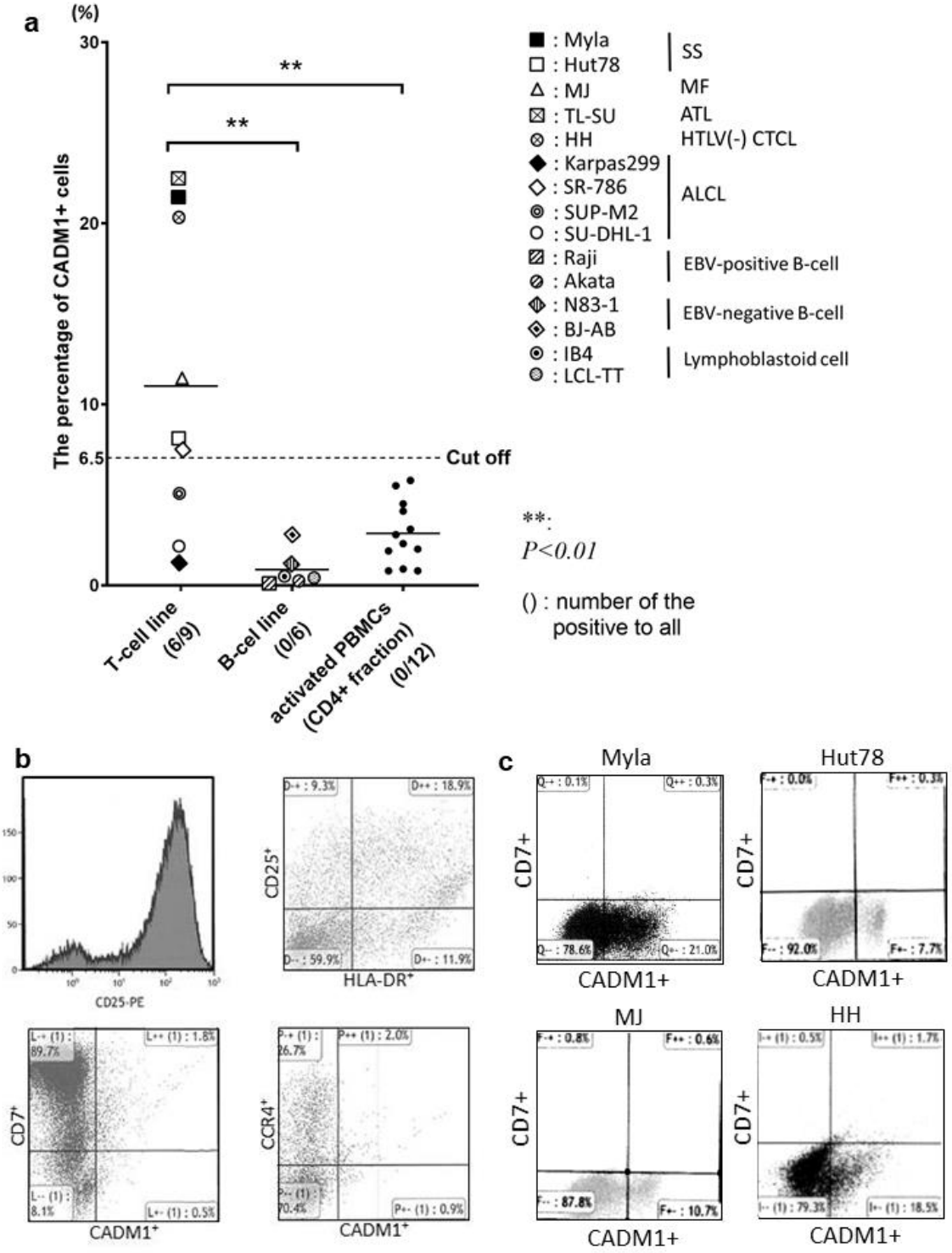
503 **Figure 1.**



504

505

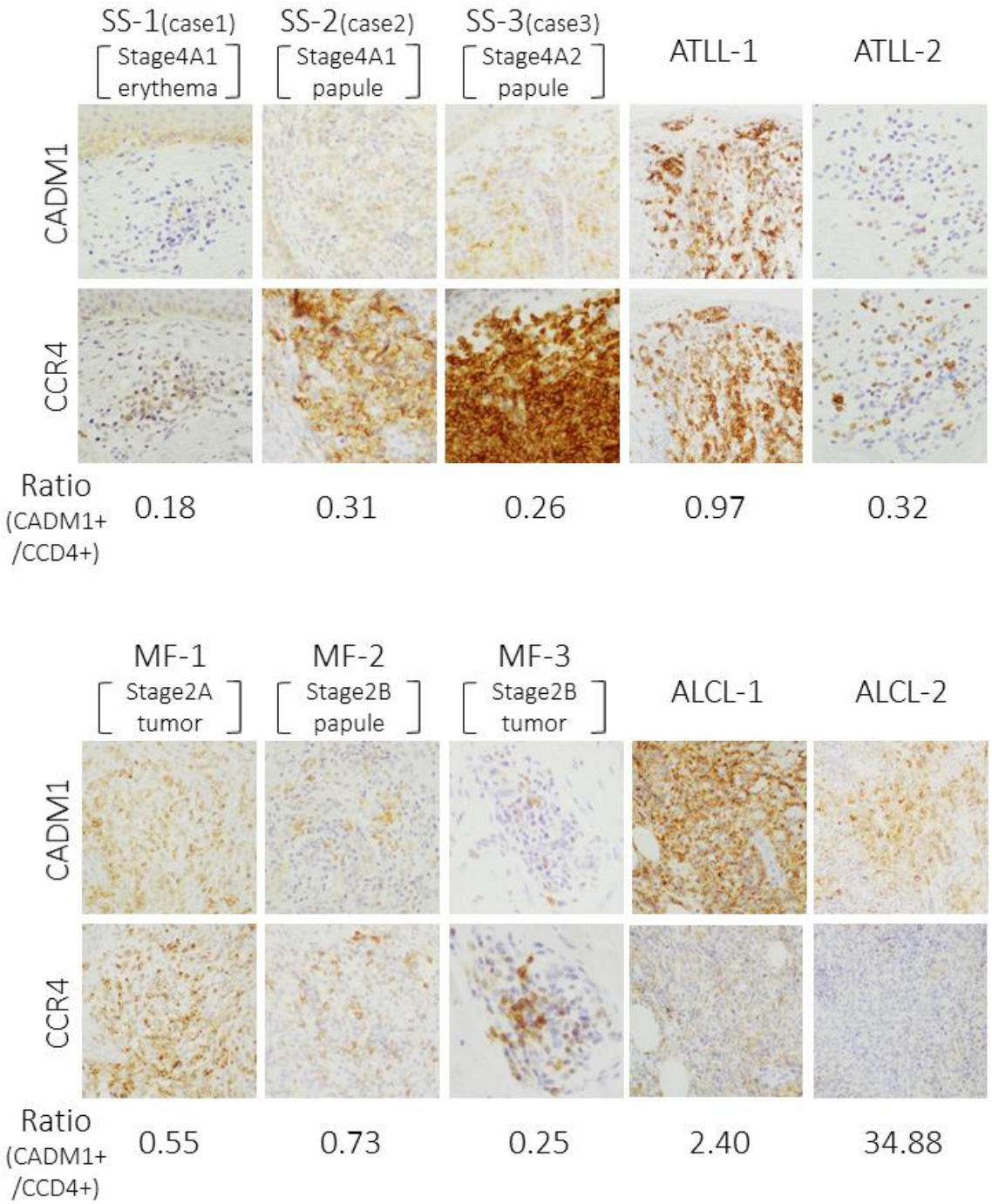
506 **Figure 2.**



507

508

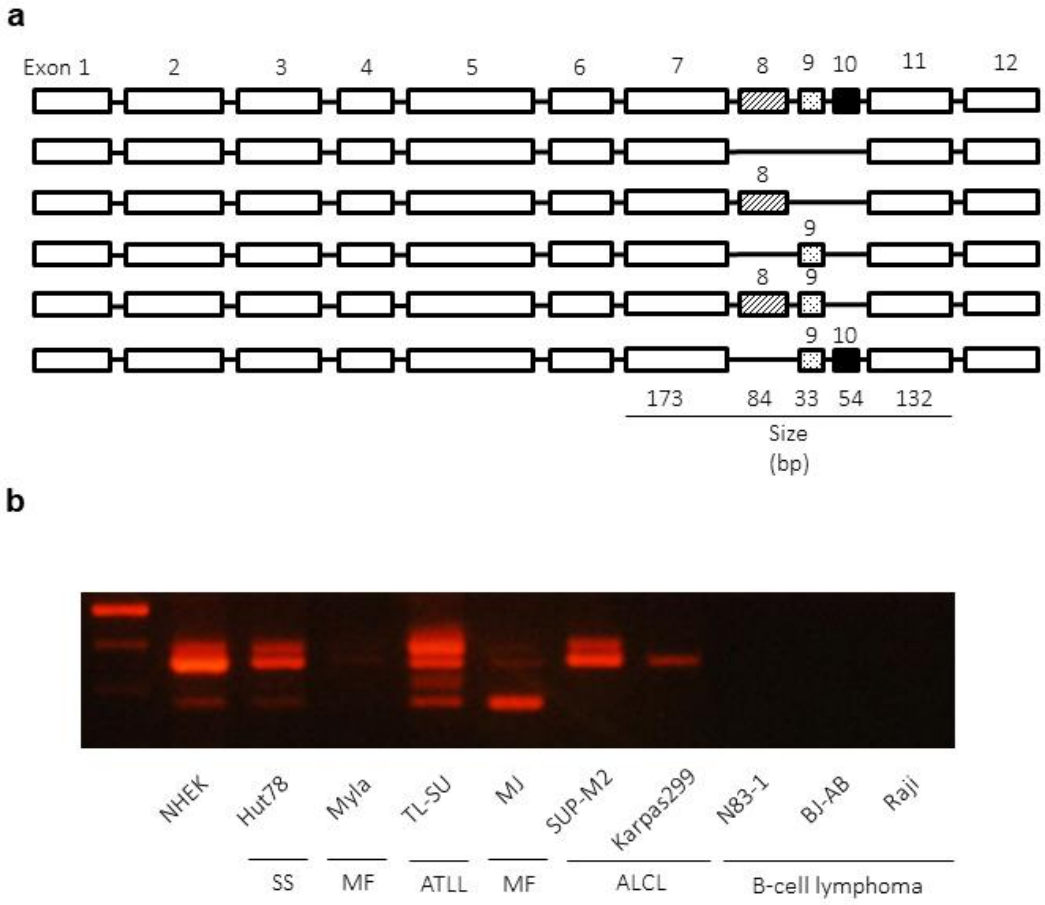
509 **Figure 3.**



510

511

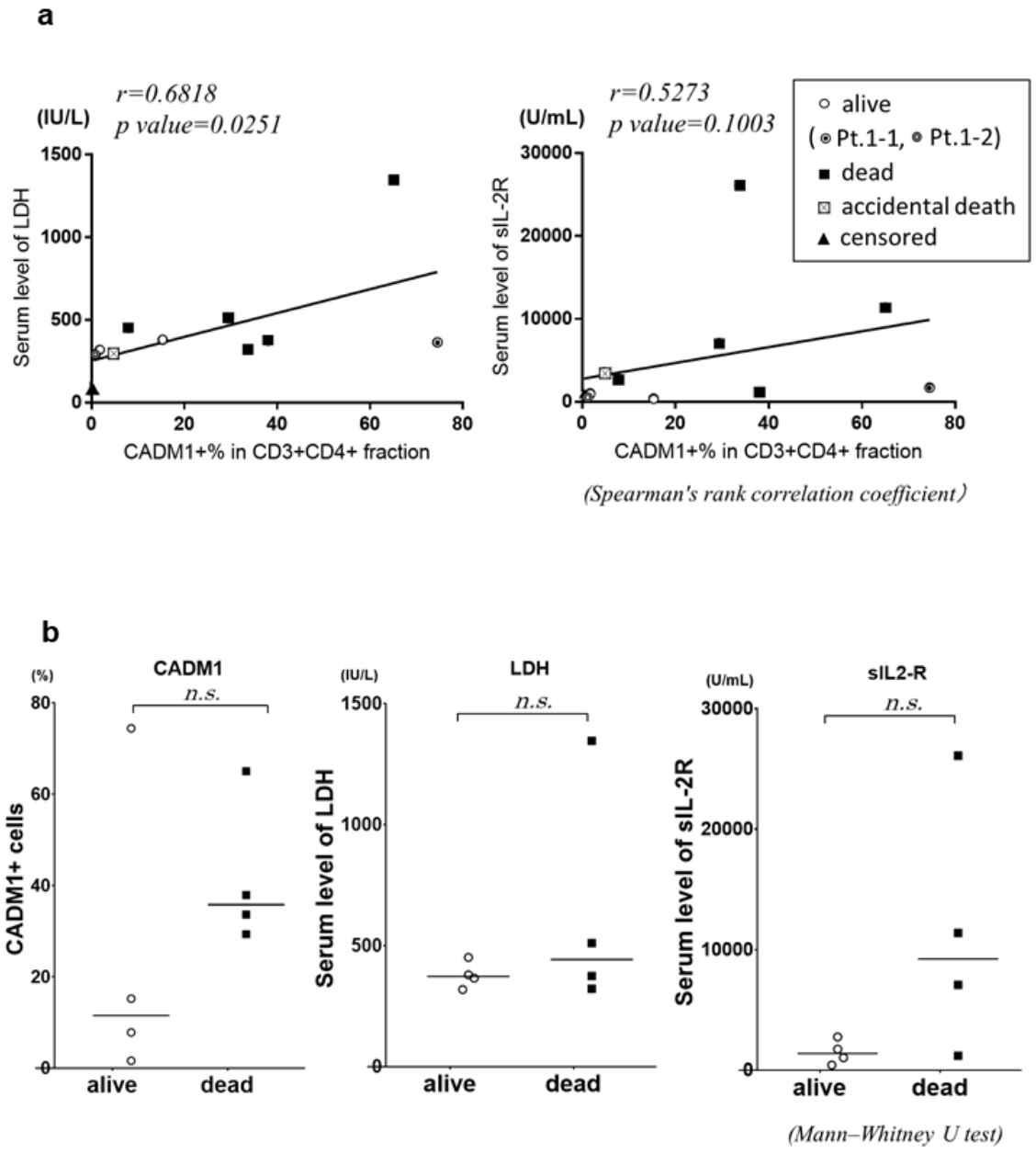
512 **Figure 4.**



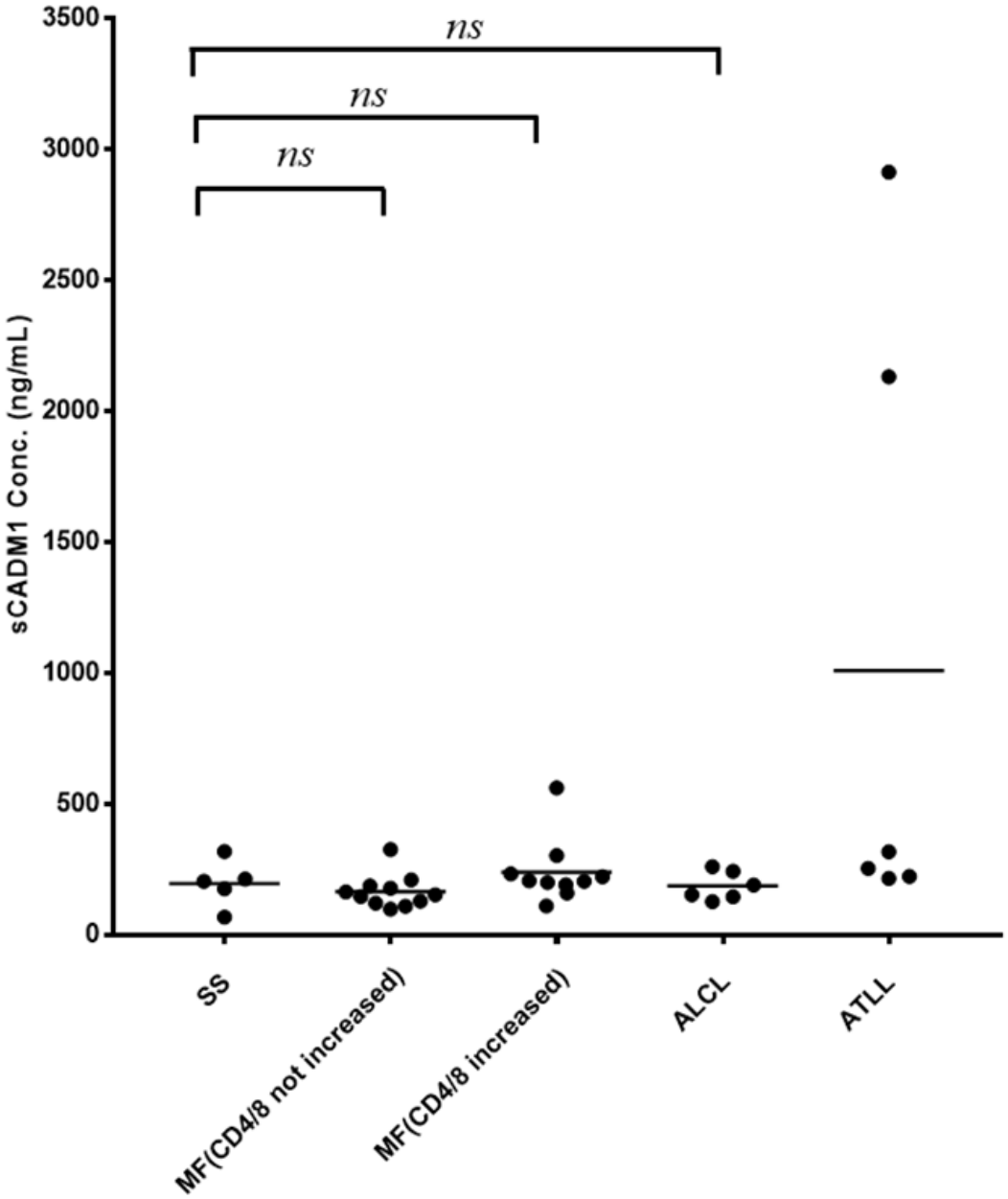
513

514

Figure 5.


a
r=0.6818
p value=0.0251
(IU/L)
Serum level of LDH
CADM1+% in CD3+CD4+ fraction
r=0.5273
p value=0.1003
(U/mL)
Serum level of sIL-2R
CADM1+% in CD3+CD4+ fraction
alive
(Pt.1-1, Pt.1-2)
dead
accidental death
censored
(Spearman's rank correlation coefficient)
b
CADM1
(%)
CADM1+ cells
n.s.
alive
dead
LDH
(IU/L)
Serum level of LDH
n.s.
alive
dead
sIL2-R
(U/mL)
Serum level of sIL-2R
n.s.
alive
dead
(Mann–Whitney U test)

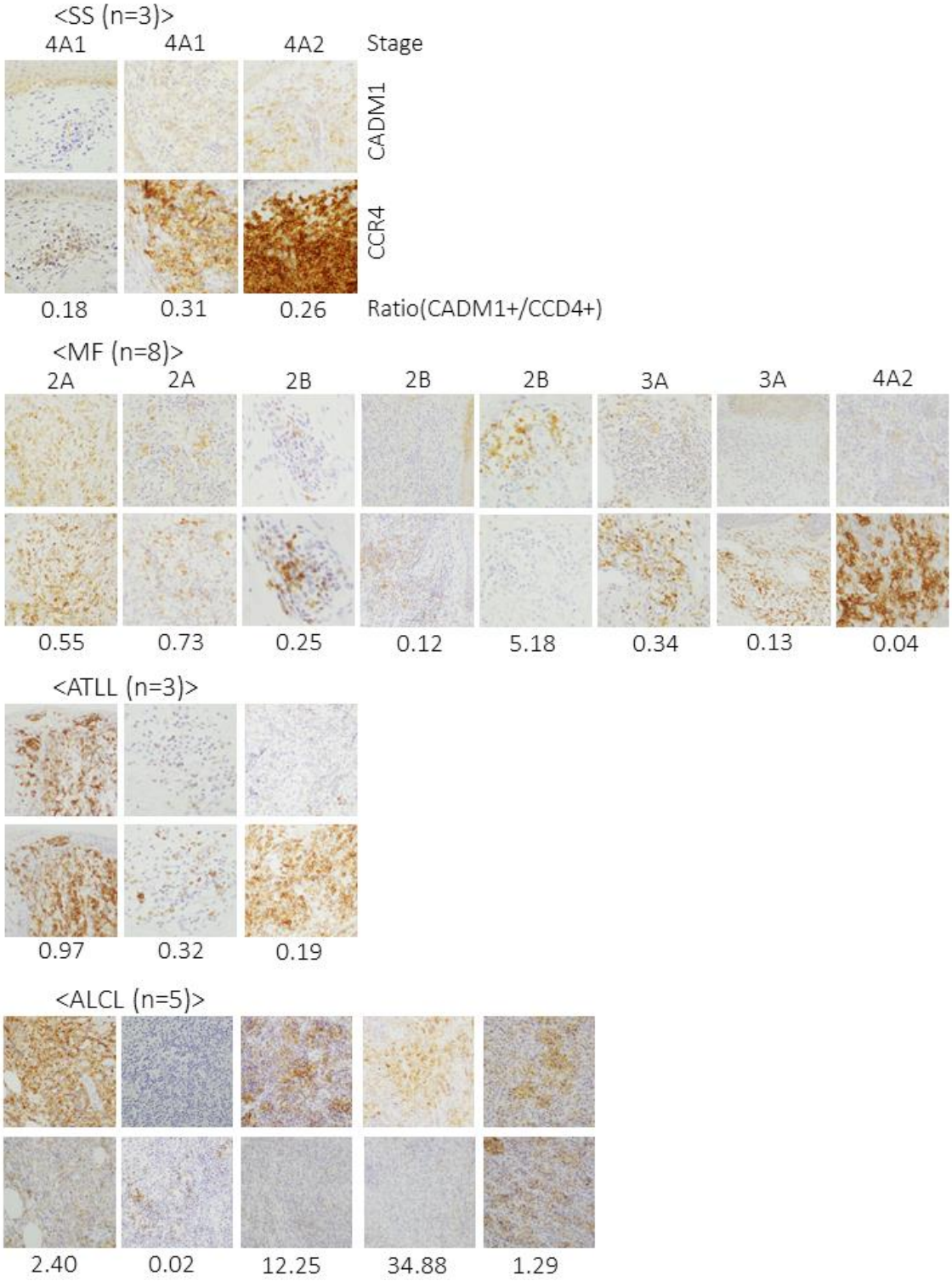
518 **Figure 6.**



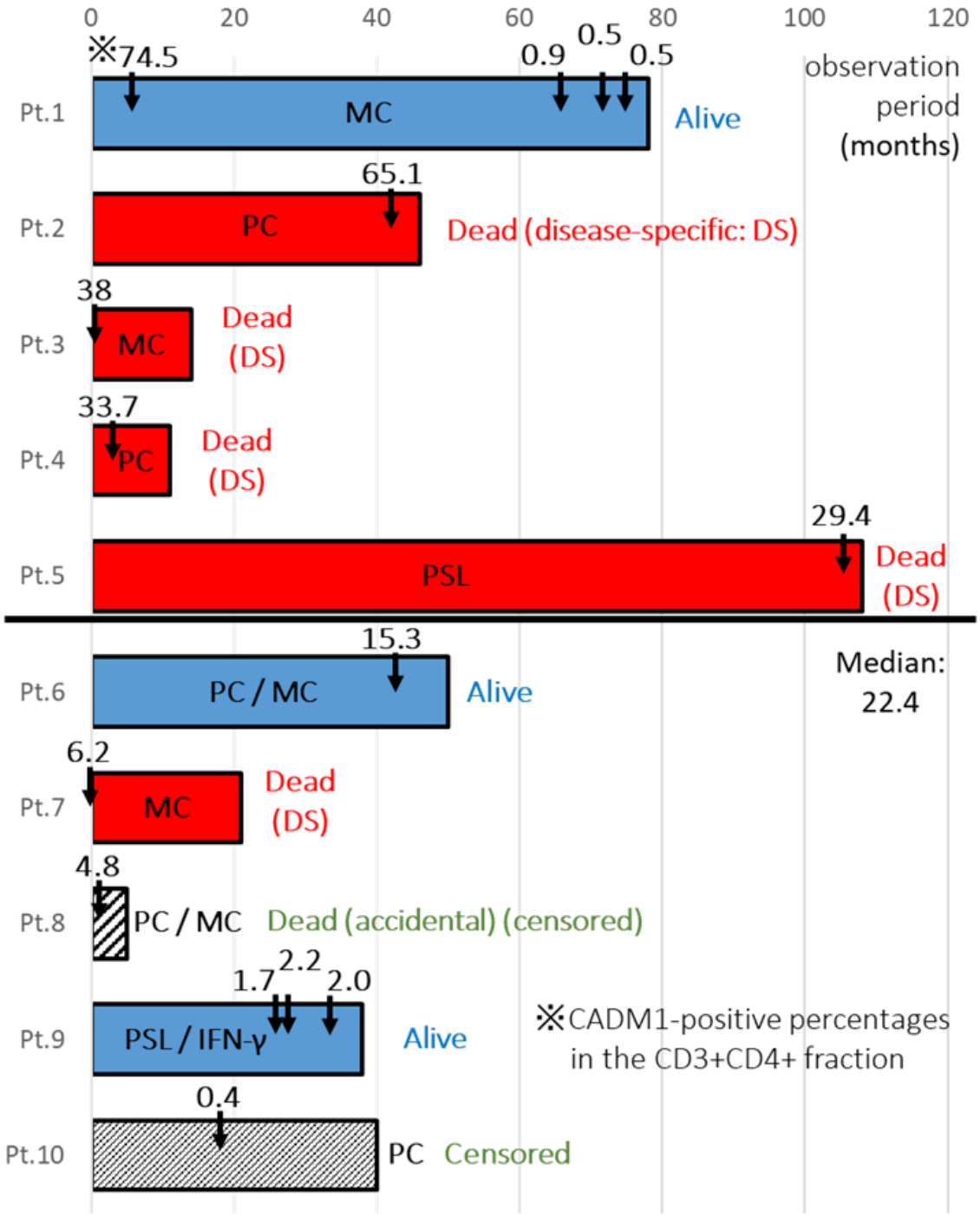
519

520

Figure S1.


<SS (n=3)>
4A1
4A1
4A2
Stage
CADM1
CCR4
0.18
0.31
0.26
Ratio(CADM1+/CCD4+)
<MF (n=8)>
2A
2A
2B
2B
2B
3A
3A
4A2
0.55
0.73
0.25
0.12
5.18
0.34
0.13
0.04
<ATLL (n=3)>
0.97
0.32
0.19
<ALCL (n=5)>
2.40
0.02
12.25
34.88
1.29

524 **Figure S2.**



525