REGULAR ARTICLE

blood advances

Proteomic profiling of HTLV-1 carriers and ATL patients reveals sTNFR2 as a novel diagnostic biomarker for acute ATL

Carmina Louise Hugo Guerrero,[1] Yoshiko Yamashita,[2] Megumi Miyara,[3] Naoki Imaizumi,[4] Megumi Kato,[1] Shugo Sakihama,[5] Masaki Hayashi,[6] Takashi Miyagi,[7] Kaori Karimata,[7] Junnosuke Uchihara,[8] Kazuiku Ohshiro,[9] Junpei Todoroki,[10] Sawako Nakachi,[11] Satoko Morishima,[11] Kennosuke Karube,[5] Yuetsu Tanaka,[12] Hiroaki Masuzaki,[11] and Takuya Fukushima[1]

[1]Laboratory of Hematoimmunology, Graduate School of Health Sciences, University of the Ryukyus, Nishihara, Japan; [2]AI Drug Development Division, NEC Corporation, Tokyo, Japan; [3]Department of Health and Nutrition, Faculty of Health and Nutrition, Okinawa University, Naha, Japan; [4]Laboratory of Molecular Genetics, Graduate School of Health Sciences, and [5]Department of Pathology and Cell Biology, Graduate School of Medicine, University of the Ryukyus, Nishihara, Japan; [6]Department of Hematology, Nakagami Hospital, Okinawa City, Japan; [7]Department of Hematology, Heart Life Hospital, Nakagusuku, Japan; [8]Department of Hematology, Naha City Hospital, Naha, Japan; [9]Department of Hematology, Okinawa Prefectural Nambu Medical Center and Children's Medical Center, Haebaru, Japan; [10]Department of Hematology, Chubu Tokushukai Hospital, Nakagami, Japan; and [11]Division of Endocrinology, Diabetes, and Metabolism, Hematology, Rheumatology (Second Department of Internal Medicine), Graduate School of Medicine, and [12]Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Nishihara, Japan

Key Points

- A distinct sTNFR2 range is observed in acute ATL patients (10-60 ng/mL) vs ACs (1-8 ng/mL) and those in remission (2-9 ng/mL).
- High cell surface expression of TNFR2 on acute ATL cells suggests the clinical utility of sTNFR2 as a diagnostic biomarker for acute ATL.

Adult T-cell leukemia/lymphoma (ATL) is a human T-cell leukemia virus type 1 (HTLV-1)–associated T-cell malignancy with generally poor prognosis. Although only ~5% of HTLV-1 carriers progress to ATL, early diagnosis is challenging because of the lack of ATL biomarkers. In this study, we analyzed blood plasma profiles of asymptomatic HTLV-1 carriers (ACs); untreated ATL patients, including acute, lymphoma, smoldering, and chronic types; and ATL patients in remission. Through SOMAscan, expression levels of 1305 plasma proteins were analyzed in 85 samples (AC, n = 40; ATL, n = 40; remission, n = 5). Using gene set enrichment analysis and gene ontology, overrepresented pathways in ATL vs AC included angiogenesis, inflammation by cytokines and chemokines, interleukin-6 (IL-6)/JAK/STAT3, and notch signaling. In selecting candidate biomarkers, we focused on soluble tumor necrosis factor receptor 2 (sTNFR2) because of its active role in enriched pathways, extreme significance (Welch's t test $P < .00001$), high discrimination capacity (area under the curve >0.90), and novelty in ATL research. Quantification of sTNFR2 in 102 plasma samples (AC, n = 30; ATL, n = 68; remission, n = 4) using enzyme-linked immunosorbent assay showed remarkable elevations in acute ATL, at least 10 times those of AC samples, and return of sTNFR2 to AC state levels after achieving remission. Flow cytometry and immunostaining validated the expression of TNFR2 in ATL cells. No correlation between sIL-2 and sTNFR2 levels in acute ATL was found, suggesting the possibility of sTNFR2 as an independent biomarker. Our findings represent the first extensive blood-based proteomic analysis of ATL, suggesting the potential clinical utility of sTNFR2 in diagnosing acute ATL.

Introduction

Adult T-cell leukemia/lymphoma (ATL) is a mature T-cell neoplasm associated with human T-cell leukemia virus type 1 (HTLV-1).[1-4] The classification of ATL into acute, lymphoma, chronic, and smoldering clinical subtypes was proposed based on prognostic factors, clinical features, and natural history of the disease.[5] The "Revised Adult T-Cell Leukemia-Lymphoma International Consensus Meeting Report" also includes a recently proposed variant of the lymphoma type ATL called the

Submitted 2 January 2020; accepted 23 February 2020; published online 20 March 2020. DOI 10.1182/bloodadvances.2019001429.

Presented in part as an oral presentation at the 61st annual meeting of the American Society of Hematology, Orlando, FL, 9 December 2019 (oral abstract 0660).

For all original data, please contact the corresponding author, Takuya Fukushima (e-mail: fukutaku@med.u-ryukyu.ac.jp).

The full-text version of this article contains a data supplement.

extranodal primary cutaneous variant,[6] which has a fatal clinical course and is considered aggressive ATL.[7] Patients with aggressive ATL (ie, acute, lymphoma, and unfavorable chronic types) are frequently treated with intensive multidrug chemotherapy with or without mogamulizumab, a defucosylated anti–CC chemokine receptor 4 monoclonal antibody. Aggressive ATL typically has a very poor prognosis, with a median survival time of 8 to 10 months. Median survival time with the VCAP-AMP-VECP regimen (ie, vincristine, cyclophosphamide, doxorubicin, and prednisone [VCAP]; doxorubicin, ranimustine, and prednisone [AMP]; and vindesine, etopside, carboplatin, and prednisone [VECP]), which showed the best results for chemotherapy in patients with untreated aggressive ATL in the phase 3 Japan Clinical Oncology Group (JCOG) 9801 trial (1998-2003), was only 13 months.[8] Indolent ATL (ie, favorable chronic and smoldering types) generally progresses slowly, and it is therefore recommended that patients undergo monitoring through watchful waiting or treatment with interferon-α and zidovudine.[9] However, most patients with indolent ATL will eventually die after progression to aggressive ATL during the chronic course of illness; the prognosis is not good, and there is no plateau phase in the survival curve.[10]

HTLV-1 is a retrovirus currently endemic in southwest Japan, sub-Saharan Africa, South America, the Caribbean, parts of the Middle East, and Australo-Melanesia; the estimated prevalence of infection is 10 to 20 million worldwide.[11-15] HTLV-1 generally does not cause clinical features in a majority of infected individuals; in fact, ~95% of HTLV-1 carriers remain asymptomatic throughout their lives.[15] The lifetime risk of developing ATL in HTLV-1 carriers in Japan is ~6% to 7% for men and ~2% to 3% for women, with 1000 new ATL cases diagnosed each year.[12]

Because of the poor prognosis of ATL, it is vital to identify HTLV-1 carriers at high risk of developing ATL to establish early interventional treatment methods. A nationwide prospective study of 1218 asymptomatic HTLV-1 carriers (ACs) in Japan revealed that none developed ATL among those with a baseline proviral load lower than ~4 copies per 100 peripheral blood mononuclear cells (PBMCs); higher proviral load, advanced age, family history of ATL, and first opportunity for HTLV-1 testing during treatment for another disease not related to HTLV-1 were independent risk factors for the progression of ATL in multivariate Cox analyses.[16] In Japan, median age at diagnosis of ATL was reported as 68 years (range, 34-100 years),[17] which is older than that in other ATL endemic areas such as the Caribbean (median, 54 years; range, 28-87 years).[18] Geographical differences in the age at ATL onset may be explained not only by genetic factors, but also by several epigenetic and environmental factors that begin to have an effect during the 60-year latency period.[12] Many of the epigenetic factors involved in the progression of ATL remain unknown, prompting us to explore the less researched field of ATL epigenetics: proteomics.

Plasma proteins play key roles in various biological processes, such as signaling, transport, growth, repair, and defense mechanisms, which are often dysregulated in disease states. Analyses of plasma proteins prove valuable for developing disease biomarkers with potential clinical utility, because plasma and other components of blood remain the predominant specimens for routine analysis. Previously, a high-throughput mass spectrometric plasma protein analysis of patients with HTLV-1–associated myelopathy was performed to describe the proteomic content of extracellular vesicles in comparison with the AC state.[19] However, comparative proteomic profiling for ACs and ATL patients has not yet been performed. To further understand the biological processes in the progression of ATL, we explored the disease, remission, and carrier states with a proteomic approach. In this study, we aimed to identify potential protein biomarkers for: (1) the onset of ATL by comparing plasma profiles of ACs vs ATL patients; (2) the progression of ATL to its aggressive forms by comparing plasma profiles of acute, lymphoma, smoldering, and chronic types; and (3) the achievement of remission or response to chemotherapy in ATL patients by comparing plasma profiles of pre- and postremission states. Here, we show the discovery of soluble tumor necrosis factor receptor 2 (sTNFR2) as a novel and promising diagnostic biomarker for acute ATL.

Methods

Patients and specimens

Samples were obtained from the following: (1) ACs confirmed with anti-HTLV–1 antibodies through the particle-agglutination method; (2) ATL patients diagnosed based on the criteria proposed by the JCOG[5] and confirmed with monoclonally integrated HTLV-1 proviral genome using the Southern blot hybridization method, as described previously[20]; and (3) previously diagnosed ATL patients achieving complete remission for >4 weeks, where remission was judged based on a modified version[21] of the World Health Organization response criteria.[22] Samples were procured from 7 institutions in Okinawa Prefecture, Japan (University of the Ryukyus Hospital, Heart Life Hospital, Nakagami Hospital, Naha City Hospital, Nanbu Medical Center, Chubu Tokushukai Hospital, and Kariyushi Hospital) from November 2016 to November 2019. Plasma and PBMCs were separated from blood samples and stored at −80°C until use. Lymph node and subcutaneous skin lesions from ATL patients were also harvested.

Proteomic profiling

Expression levels of 1305 proteins from cryopreserved plasma samples (1 mL each) were determined. The SOMAscan assay utilizes new-generation protein capture slow off-rate modified aptamer (SOMAmer) reagents, which are modified nucleotides with amino acid– or protein-like side chains that have affinities for proteins and, at the same time, are recognizable by DNA hybridization probes and measurable by fluorescence.[23,24] The 1305 proteins analyzed were listed using their protein coding gene IDs (eg, TNFα, TNFR1, and TNFR2 are reflected as TNF, TNFRSF1A, and TNFRSF1B, respectively).

Statistical considerations

Significance levels of proteins in ACs vs ATLs and ATLs vs remissions were calculated using Welch's *t* test, and discrimination capacities were determined by calculating the area under the receiver operating characteristic curve (AUC). We illustrated elevations and decreases in AC, ATL, and remission states using box-and-whisker and dot plots. All statistical analyses were performed using Python 3.6.2 (Python Software Foundation, Beaverton, OR) with the following packages: Statsmodels 0.8.0 for *t* tests and Matplotlib 2.0.2 for box-and-whisker plots.

Pathway enrichment

To generate heatmap visualizations and determine significantly enriched pathways among various parameters, such as ATL/

AC/remission states, age, and ATL subtypes, we used gene set enrichment analysis (GSEA version 3.0; Broad Institute; Molecular Signatures Database: hallmarks and curated gene sets) with a false discovery rate cutoff of <25%, run at 1000 permutations. We also determined overrepresented pathways in ATL states using gene ontology (GO PANTHER Pathways) by inputting proteins categorized as extremely significant. Protein names in the heatmaps are also reflected as protein coding gene IDs.

Plasma protein concentrations

To determine plasma protein concentration levels of selected candidate biomarkers, the following enzyme-linked immunosorbent assay (ELISA) kits were sourced: sTNFα, sTNFR1, and sTNFR2 (R&D Systems, Minneapolis, MN) and sTNFRSF8 (RayBiotech, Peachtree Corners, GA).

Flow cytometric analysis

We proceeded with flow cytometry to determinne if elevations in plasma levels of sTNFR2 were correlated with ATL cell surface expression of TNFR2. We performed flow cytometry with the BD FACSCalibur cell analyzer, and data were analyzed with CellQuest Pro software (BD Biosciences, San Jose, CA). Briefly, cryopreserved PBMCs were retrieved by rapid thawing of cryotubes in a 37°C water bath, Fc-blocked with 2 ng/mL of human immunoglobulin G and resuspended in fluorescence-activated cell sorting buffer (phosphate-buffered saline containing 2% fetal calf serum and 0.1% sodium azide), and then washed. Cell surface staining was performed on ice for 30 minutes using the following antibody combinations: fluorescein isothiocyanate–conjugated rabbit anti-SynCAM TLSC1/CADM1 monoclonal antibody (MBL Co., Ltd, Nagoya, Japan), PE-cyanine7 mouse anti-human CD7 (BD Biosciences), Alexa Fluor 647 anti-human CD4 antibody (Biolegend, San Diego, CA), and phycoerythrin-conjugated anti-human TNFR2 (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were then washed and resuspended in 1% paraformaldehyde in phosphate-buffered saline (Wako Pure Chemical Industries, Ltd, Osaka, Japan). The gating strategy for selecting $CD4^+$ T-cell lymphocyte subpopulations is shown in in Figure 3A. Because CADM1 expression is a marker for aggressive ATL and stepwise down-regulation of CD7 is closely associated with clonal expansion of HTLV-1–infected cells in ATL,[25] we also plotted CD4 against CD7 and TNFR2 against CADM1.

Immunostaining

Immunostaining with polyclonal antibodies against TNFR2 (Enzo Life Sciences, Farmingdale, NY) for tissue specimens including lymph nodes and subcutaneous skin lesions obtained from ATL patients was performed with heat-mediated antigen retrieval of formalin-fixed paraffin-embedded sections of the samples.

mRNA expression

Finally, to determine TNFR2 messenger RNA (mRNA) expression in ACs and ATL patients, semiquantitative reverse transcription polymerase chain reaction was performed. Total cellular RNA from PBMCs was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription of 500 ng of RNA to complementary DNA was carried out using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA), with oligo$(dT)_{20}$ primer. Real-time polymerase chain reaction was completed on StepOnePlus (Applied Biosystems, Foster City, CA) using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA). mRNA expression of TNFR2 was calculated using the $2^{-\Delta\Delta CT}$ method. TaqMan primer-probe sets human TNFR2 (Hs00153550_m1) and housekeeping gene β-actin (Hs01060665_g1) were used in this assay (Thermo Fisher Scientific).

Table 1. Number of plasma proteins with significantly different levels in HTLV-1 vs ATL

P	Interpretation	No. of proteins	Cumulative frequency
<.00001	Extremely significant	176	176
.00001 ≤ and < .0001		58	234
.0001 ≤ and < .001		99	333
.001 ≤ and < .01	Very significant	149	482
.01 ≤ and < .05	Significant	149	631
≥.05	Not significant	674	1305

Ethical considerations

All samples and information of stored cryopreserved samples were obtained under prior comprehensive consent for storage and use in correlative studies after receiving approval from the institutional review board of each institution in accordance with the Declaration of Helsinki. The research plan was approved by the institutional review board of the University of the Ryukyus and NEC Corporation Japan.

Results

A total of 85 plasma samples (ACs, n = 40; ATLs, n = 40; remissions, n = 5) were selected for protein analysis using the SOMAscan assay. Demographic and clinical information of ATL patients is provided in supplemental Table 1. We categorized differences in protein levels in ACs vs ATLs as extremely significant, very significant, significant, and nonsignificant (Table 1) and listed the top 10 elevated or decreased proteins and their functions (Table 2). We also determined significance levels of 11 proteins overrepresented in ATLs vs remissions (Table 3) and listed these proteins with their functions (Table 4). The complete list of all 1305 plasma proteins measured is shown in supplemental Table 2, which includes *P* values and AUC values. We depicted the differences in plasma protein expression levels of some of the candidate biomarkers in AC vs ATL states, as well as in ATL vs remission states, in box-and-whisker plots and dot plots, as shown in Figure 1.

Enriched pathways and heatmap visualization

Heatmap visualization and pathway enrichment analysis for the following phenotype comparisons were generated via GSEA: (1) ATL vs AC, (2) ATL patients age <70 vs ≥70 years, (3) acute vs AC, (4) acute vs nonacute (including lymphoma, smoldering, and chronic), (5) acute vs chronic, (6) acute vs lymphoma, (7) acute vs smoldering, (8) chronic vs AC, (9) chronic vs lymphoma, (10) chronic vs smoldering, (11) lymphoma vs AC, (12) lymphoma vs smoldering, (13) smoldering vs AC, (14) preremission ATL vs remission (same patients followed up), (15) preremission ATL vs ATL (patients who did not achieve remission), (16) remission vs ATL (patients who did not achieve remission), and (17) AC vs remission

Table 2. Top 10 proteins with significantly different levels in HTLV-1 vs ATL

	Protein name	Elevation (up) or decrease (down)	Protein function	Protein coding gene ID	UniProt	*P*	AUC
1	Coagulation factor Xa	Up in ATL	Blood coagulation	F10	P00742	1.82E-16	0.99
2	Coagulation factor X	Up in ATL	Blood coagulation	F10	P00742	4.13E-16	0.99
3	Cadherin-6	Down in ATL	Cell adhesion	CDH6	P55285	9.32E-14	0.93
4	Insulin-like growth factor binding protein 4	Up in ATL	IGF regulation	IGFBP4	P22692	2.90E-12	0.91
5	Lysozyme C	Up in ATL	Hydrolysis	LYZ	P61626	5.65E-12	0.89
6	BDNF/NT-3 growth factor receptor	Down in ATL	Neuronal development and regulation	NTRK2	Q16620	1.70E-11	0.88
7	Granulins	Up in ATL	Cell growth regulation	GRN	P28799	2.73E-11	0.91
8	RAS GTPase-activating protein 1	Down in ATL	Cell proliferation and differentiation	RASA1	P20936	2.77E-11	0.95
9	Interleukin-18-binding protein	Up in ATL	Cell immunity induction	IL18BP	O95998	5.03E-11	0.93
10	TNFR2	Up in ATL	Apoptosis modulation and signaling	TNFRSF1B	P20333	6.82E-11	0.92

(supplemental Figure 1A-Q for heatmap visualizations of each phenotype comparison; supplemental Tables 3-19 for enriched pathways). Overrepresented pathways in ATL vs AC, as generated by gene ontology, included inflammation by cytokine and chemokine signaling, IL signaling pathway, notch signaling, angiogenesis pathway, p53 pathway by glucose deprivation, blood coagulation, and ras pathway, among others (supplemental Figure 2).

Plasma protein levels of sTNFα and TNF superfamily receptors

Proteins with active roles in the enriched pathways, extreme significance levels ($P < .00001$), and high discrimination capacities (AUC >0.90) were deemed candidate biomarkers in this study. Among the candidate biomarkers, we focused on sTNFR1, sTNFR2, and sTNFRSF8, which are receptors belonging to a superfamily of proteins: the TNF superfamily. We also included TNFα in our analysis, because it is the ligand of TNFR1 and TNFR2. For sTNFR2, a total of 102 plasma samples (AC, n = 30; ATL, n = 68 [acute, n = 33; lymphoma, n = 9; chronic, n = 12; smoldering, n = 14], and remission, n = 4) were confirmed using ELISA. A remarkable increase in sTNFR2 could be seen in ATL patients vs ACs ($P < .001$), especially in patients with acute ATL vs ACs (range, 10-60 ng/mL vs 1-8 ng/mL; $P < .001$; Figure 2A). A significant increase in sTNFR2 could also be seen in lymphoma ATL ($P < .01$); however, the values were quite varied, with some patients having low sTNFR2 levels (range, 1.5-32.7 ng/mL). Significantly higher sTNFR2 levels were found in acute ATL patients compared with those with other ATL subtypes. Interestingly, sTNFR2 levels of ACs and ATL patients who achieved remission were similar (range, 2-9 ng/mL in patients in remission). For sTNFR1, sTNFα, and sTNFRSF8, we tested 38 plasma samples (AC, n = 9; ATL, n = 29 [acute, n = 13; lymphoma, n = 6; chronic, n = 6; smoldering, n = 4]) using ELISA. Significant increases in sTNFR1 were apparent in ATL patients compared with ACs, especially in acute ATL patients (Figure 2B). Regarding sTNFα protein levels, no significant differences were found between ATL patients vs ACs (Figure 2C). For sTNFRSF8, significant increases in acute ATL patients vs ACs, as well as in acute vs chronic ATL patients, can be seen (Figure 2D). Although elevated levels of sTNFR1 and sTNFRSF8 in acute ATL patients were also apparent, the cutoff point is not as distinct as that of sTNFR2. sTNFR2 concentration values for all samples can be found in supplemental Table 20.

Cell surface expression of TNFR2 on PBMCs of acute ATL cells

Of the proteins tested using ELISA, we focused on TNFR2 because of its remarkable and consistent increase in acute ATL patients. Flow cytometric analysis of PBMCs from 12 samples (AC, n = 6; acute ATL, n = 6) showed significantly higher $TNFR2^{+}CADM1^{+}$ populations in acute ATL patients (mean, 54.9%; range, 33.3%-80.3%) compared with ACs (mean, 26.5%; range, 24.7%-28.9%; Figure 3B-D). Plasma sTNFR2 levels and TNFR2 cell surface expression levels were positively correlated, with a correlation coefficient of 0.63 (Figure 3E).

mRNA expression of TNFR2 on HTLV-1 carriers and ATL patients

TNFR2 mRNA expression analysis of RNA extracted from 12 PBMCs (AC, n = 3; acute ATL, n = 9) showed no significant increase in ACs vs ATL patients (supplemental Figure 3).

Discussion

In this study, we have demonstrated a novel approach in searching for ATL biomarkers through the use of a SOMAmer-based high-throughput proteomic assay, followed by an extensive proteomic pathway analysis and confirmed using standard laboratory techniques such as ELISA, flow cytometry, and immunostaining. This approach led us to the discovery of candidate biomarkers belonging to the TNF receptor superfamily, namely sTNFR1, sTNFR2, and sTNFRSF8. Among them, sTNFR2 was deemed to be the most prominent in comparing AC vs ATL. Plasma protein analysis of sTNFR2 showed: (1) remarkable and distinct elevations in acute ATL (10-60 ng/mL), approximately at least 10 times higher than levels in ACs (1-9 ng/mL) and reference values of healthy controls[26] (1-5 ng/mL), and (2) a return of sTNFR2 to levels similar to carrier

Table 3. Number of significantly different proteins in remission state

HTLV-1 and ATL $P < .00001$ (176 proteins)		Remission and ATL					
		<.00001	<.0001	<.001	<.01	<.05	≥.05
Remission and HTLV-1	≥.05	11	5	15	24	20	32

We identified a total of 75 proteins with significantly different expression levels in postremission vs ATL, which were also not significantly different in remission vs HTLV-1, to determine which proteins returned to the HTLV-1 carrier state after achieving remission.

Table 4. Expression levels of 11 proteins that returned to HTLV-1 state after achieving remission in ATL patients with $P > .00001$

	Protein name	Elevation (up) or decrease (down)	Protein function	Protein coding gene	UniProt	P
1	Soluble L-selectin	Down in remission	Leukocyte-endothelial cell adhesion	SELL	P14151	9.98E-8
2	Cell adhesion molecule 1	Down in remission	Cell adhesion	CADM1	Q9BY67	1.26E-7
3	TNFRSF8	Down in remission	Regulation of cell growth, activated lymphoblast transformation	TNFRSF8	P28908	1.62E-7
4	LDL receptor related protein 8	Down in remission	Cholesterol transport receptor	LRP8	Q14114	6.03E-7
5	Lymphocyte activation gene	Down in remission	Lymphocyte activation	LAG3	P18627	6.23E-7
6	Tissue inhibitor of metallopeptidase-1	Down in remission	Promotion of erythroid progenitor stem cell proliferation	TIMP1	P01033	9.17E-7
7	Angiopoietin-2	Down in remission	Vascular remodeling, proliferation	ANGPT2	O15123	8.05E-06
8	T-lymphocyte surface antigen Ly-9/CD229	Down in remission	Innate immune response	LY9	Q9HBG7	1.07E-6
9	Deoxycytidine-triphosphatase 1	Down in remission	DNA replication	DCTPP1	Q9H773	1.14E-6
10	T-cell cytokine receptor (WSX-1) TCCR	Down in remission	Induction of Th-1 type immune response	IL27RA	Q6UWB1	2.99E-6
11	Ephrin A4	Down in remission	Metastasis	EFNA4	P52798	4.51E-6

state levels after achieving remission (2-8 ng/mL). Flow cytometric analysis showed higher cell surface expression of TNFR2 in ATL patients vs ACs (Figure 3B-D), and sTNFR2 concentration levels correlated with cell surface TNFR2 expression (Figure 3E). TNFR2 was immunohistochemically positive in atypical lymphoid cells (Figure 4A), as well as in skin infiltrates (Figure 4B), both from tumor cells of patients diagnosed with lymphoma ATL. However, the circulating levels of sTNFR2 among lymphoma ATL patients seemed to vary, and the range of concentration values was not as distinct compared with the range in acute ATL patients. These results indicate the potential of sTNFR2 as a clinically useful diagnostic biomarker for acute ATL.

TNFR1, TNFR2, and TNFRSF8, which are all receptors of the TNF superfamily, consist of proteins that are activated by various TNF-like cytokine ligands, including TNFα itself and lymphotoxin-α.[27] In contrast to TNFR1, TNFR2 lacks the death domain and recruits TNFR-associated factor 2, which promotes cell activation, proliferation, and survival,[28,29] likely via NF-κB activation. Previously, a comprehensive molecular analysis of ATL by Kataoka et al[30,31] described NF-κB activation and multiple gene mutations in the ATL state to have a substantial impact on ATL progression. Of note, some of the overrepresented proteins found in the present study corresponded with some of the mutated protein coding genes in the aforementioned molecular analysis, including FYN and VAV1. In our analysis, FYN and VAV1 both have decreased expression levels in ATL patients vs ACs (supplemental Figure 1A). Another NF-κB–activating TNF superfamily receptor significantly overrepresented in this study was TNFRSF4 (OX40; supplemental Table 2). Interestingly, high levels of soluble OX40 has recently been associated with acute

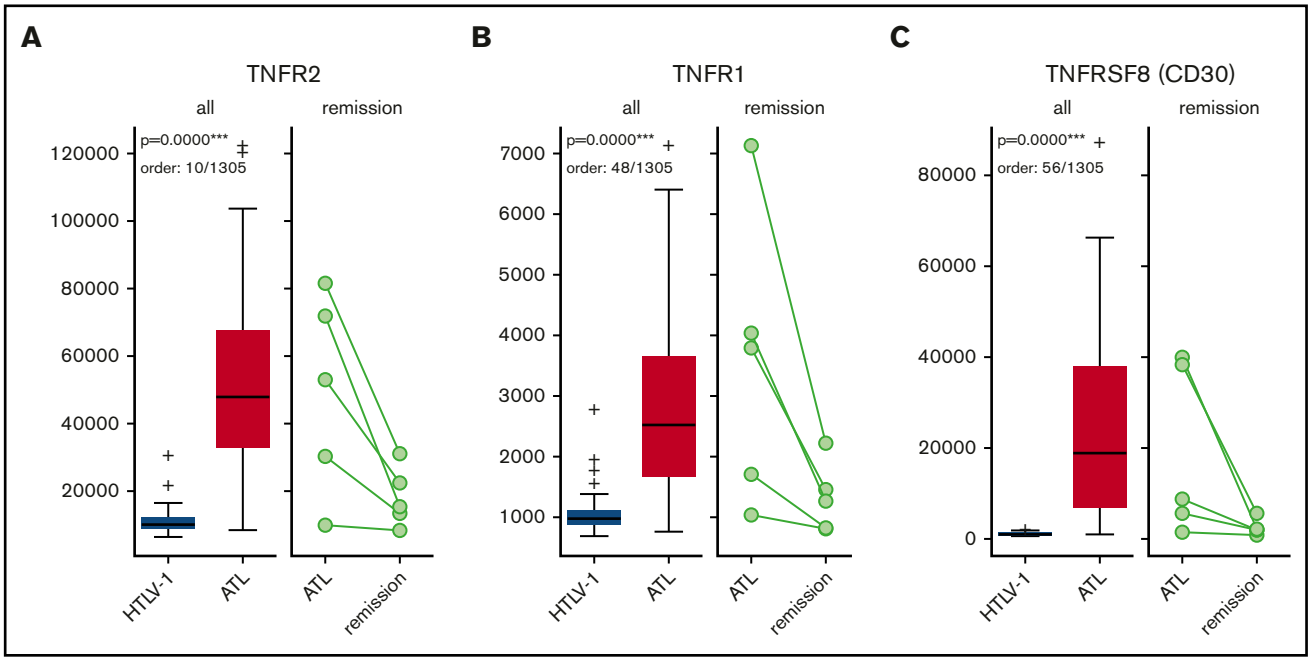

Figure 1. Box-and-whisker plots for sTNFR1, sTNFR2, and sTNFRSF8 in HTLV-1 ACs vs ATL patients and ATL patients vs those in remission. Numbers on the left side are in relative fluorescence units. sTNFR1 (A), sTNFR2 (B), and sTNFRSF8 (C).

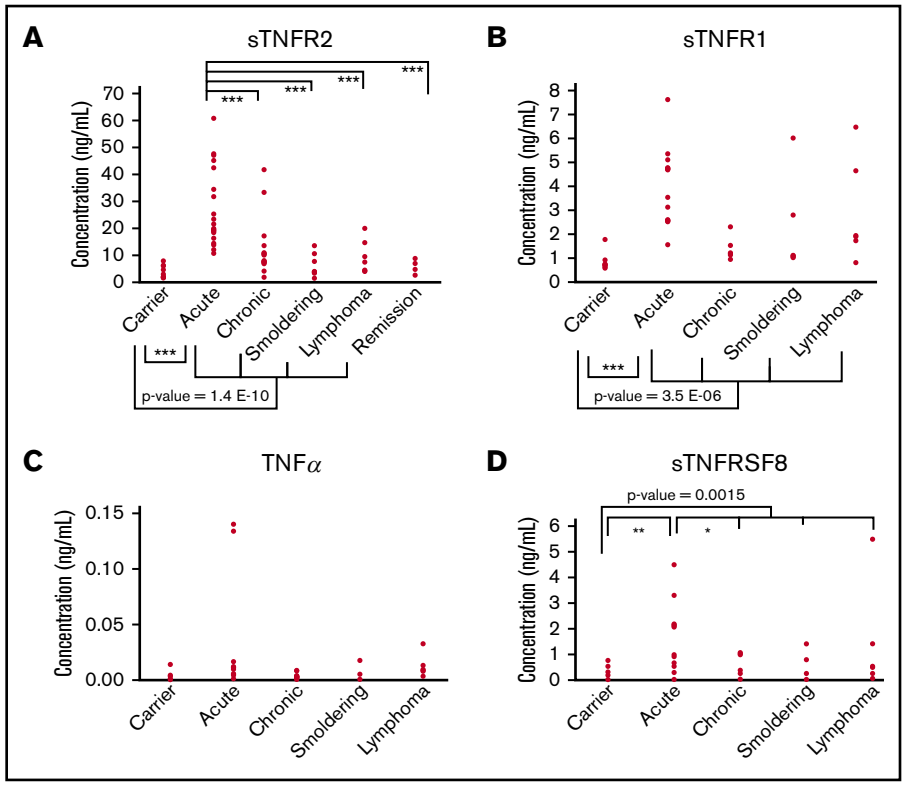

Figure 2. Plasma protein levels of TNF and TNF superfamily receptor proteins in HTLV-1 ACs, ATL patients, and patients in remission. sTNFR2 (n = 102) (A), sTNFR1 (n = 38) (B), TNFα (n = 38) (C), and sTNFRSF8 (n = 38) (D). Statistically significant *P* values in ACs vs ATL patients are shown. **P* < .05, ***P* < .01, ****P* < .001.

ATL patients,[32] and shedding of OX40 is speculated to be involved in skin infiltration of leukemic ATL cells.[33,34] Other overrepresented proteins in our analysis, such as CADM1, L-selectin, VCAM1, NOTCH1, β2M, and CD163 (elevated in ATL), as well as SPARC (decreased in ATL), have been previously implicated in ATL by virtue of gene mutation, protein deregulation, or gene network–based analyses,[31,35-42] indicating the reproducibility of our proteomic profiling.

The TNFα-TNFR2 interaction has been associated with inflammatory conditions such as rheumatoid arthritis, allergies, and autoimmune diseases such as psoriasis.[43] In recent years, TNFR2 has been considered a novel target for cancer immunotherapy because it can be found on the surface of potent regulatory T cells and is aberrantly expressed in various human tumor cells.[44] TNFR2 expression has also been associated in patients with acute myeloid leukemia[45] and Sèzary syndrome.[46] A recent study revealed that peripheral T-cell non-Hodgkin lymphoma patients with high levels of circulating TNFR2 (≥2.16 ng/mL) had a twofold increased relative risk for shorter overall survival.[47] Although sTNFR2 levels seem to be associated with other non-ATL peripheral T-cell lymphomas, sTNFR2 levels among acute ATL patients enrolled in our study seemed to be more prominently elevated, with concentration values ~9 times higher than those of the patients in the aforementioned study (18.7 vs 2.16 ng/mL).

It is worth noting that TNFR2 is highly shed from the cell surface after binding with TNFα, and this TNFα-TNFR2 complex separates immediately in plasma. Because neither TNFα plasma levels nor TNFR2 mRNA expression levels in ACs vs ATL patients were significantly different, we are considering the possibility of TNFR2 binding to a ligand other than TNFα or TNF receptor shedding. To shed light on the mechanisms of TNFR2 expression, we have currently initiated an analysis of plasma lymphotoxin-α levels and ADAM17, a sheddase involved in the shedding of TNFR2.[48,49] Interestingly, TNFR1, TNFR2, and TNFRSF8 are all substrates of ADAM17.[50] TNFRSF8, sometimes referred to as CD30, has been previously associated with ATL, in both cell expression studies[51] and plasma protein analyses[52]; however, in this study, some acute ATL patients had low levels of sTNFRSF8, similar to levels in ACs (Figure 2D), in contrast to sTNFR2, where a range of values for acute ATL could be established.

Our pathway analysis revealed enrichment of IL-2 STAT5 signaling, IL6/JAK/STAT3 signaling, epithelial mesenchymal transition, interferon-γ response, and angiogenesis pathways (supplemental Table 3). One of the roles of the HTLV-1 Tax oncoprotein is the induction of cytokines, including IL-2, which has long been known for its role in ATL leukemogenesis.[53] We compared sTNFR2 levels among acute ATL patients with sIL-2R levels obtained from existing patient clinical data and found no correlation (Pearson's coefficient $r = 0.23$), suggesting the possibility of sTNFR2 as an independent biomarker for acute ATL.

Among other cytokine receptors, IL-6 receptor subunit α was also an overrepresented protein in ATL, possibly having a role in activation of the JAK/STAT3 pathway, which is known to be aberrantly activated in hematopoietic malignancies and solid tumors.[54-56] Older studies have shown that elevated levels of IL-6 are linked with inflammatory responses and poor general status among ATL patients.[57] In HTLV-1–infected T cells, both IL-6 and IL-6 receptor have been reported to be elevated and associated with Tax activity.[58]

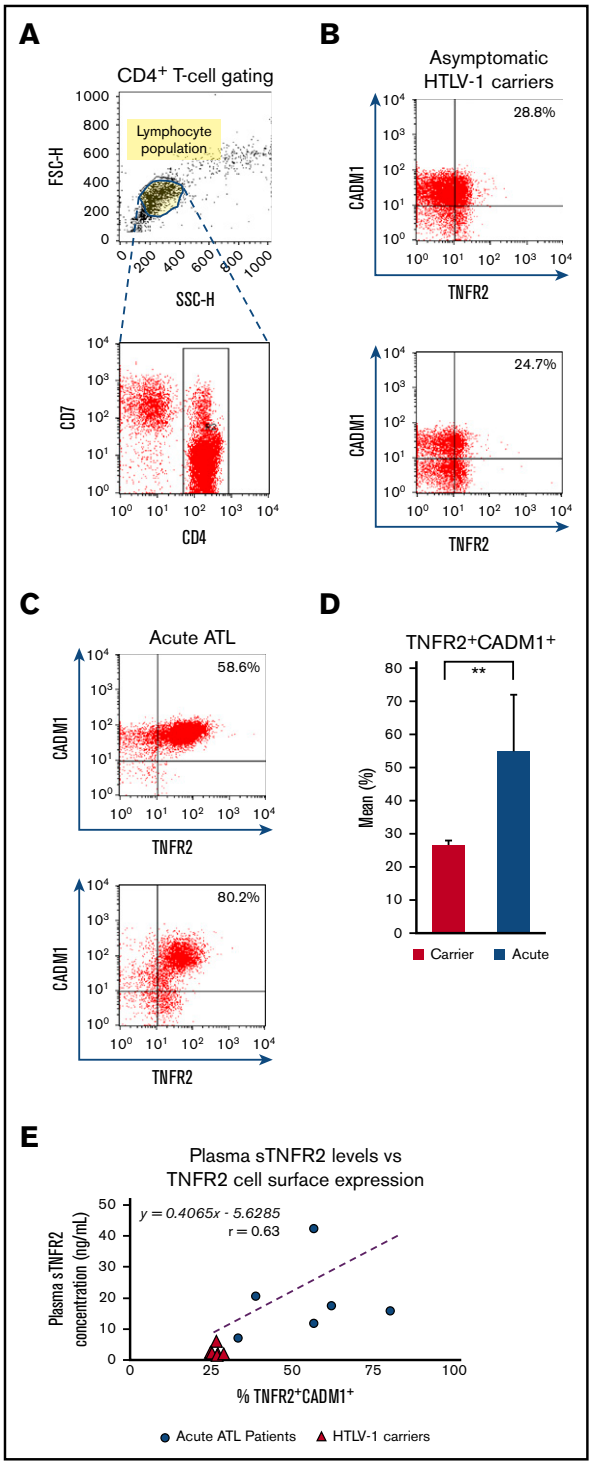

Figure 3. TNFR2 marker expression in CD4$^+$ T cells in HTLV-1 ACs vs ATL patients. (A) CD4$^+$ T-cell lymphocyte gating strategy. (B) TNFR2$^+$CADM1$^+$ asymptomatic HTLV-1 carriers. (C) TNFR2$^+$CADM1$^+$ T-cell expression among acute ATL patients. (D) Percentage of TNFR2 expression in CADM1$^+$ (up) and CD4$^+$ T cells (down). (E) Correlation of plasma sTNFR2 concentration and cell surface TNFR2$^+$CADM1$^+$ T-cell expression (n = 12 [AC, n = 6; acute ATL, n = 6]). $^{**}P < .01$.

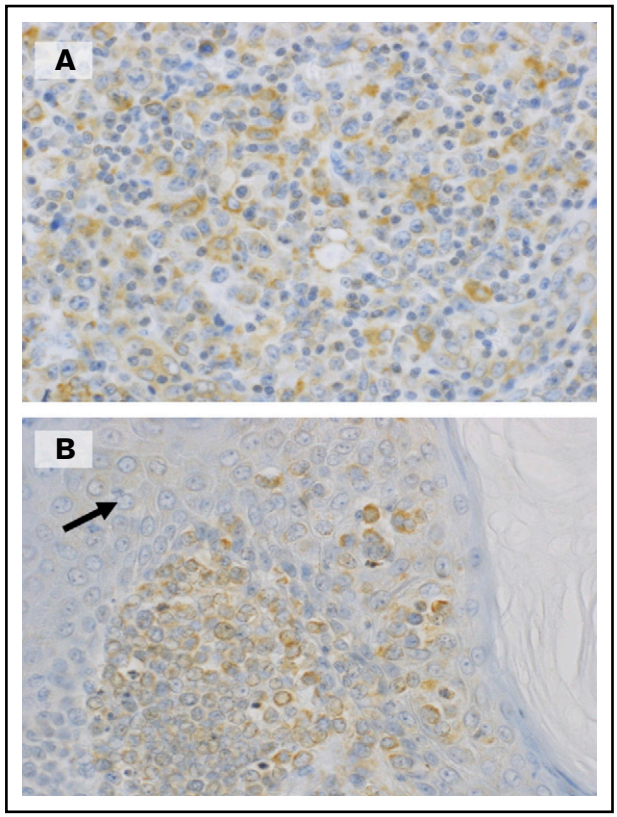

Figure 4. Expression of TNFR2 in tumor cells from ATL patients. (A) A biopsied lymph node shows numerous atypical large lymphoid cells positive for TNFR2. (B) In this skin lesion, infiltrating tumor cells were positive for TNFR2, whereas epithelial cells (arrow) were negative. (A-B) Original magnification ×400, immunostaining with polyclonal antibodies against TNFR2.

As for other candidate biomarkers, overrepresentation of various angiogenic factors (osteopontin, thrombospondin 2, TIMP1, angiopoietin 2, neuropilin 1, and follistatin-like 1) in ATL patients contributes to the enrichment of the angiogenesis pathway. Existing antiangiogenic treatments such as bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor, have been studied in ATL mice models, in which combination with CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) therapy led to significantly prolonged overall survival.[59] However, protein expression of vascular endothelial growth factor was not significantly different in ATL patients vs ACs in our study. This suggests that the angiogenic process in tumor cell promotion and metastasis for ATL cells may be a contribution of not only a single factor but rather many angiogenic factors that may replace the function of others in times of aberration. The roles of the abovementioned angiogenic factors in ATL progression should be further investigated.

Because acute ATL variants generally have a more rapid progression than other subtypes, we compared acute ATL patients with nonacute ATL patients (supplemental Figure 1D; supplemental Table 6). In comparing each subtype with AC (supplemental Tables 5, 10, 13, and 15), we showed elevations of different sets of cell adhesion molecules: CADM1, L-selectin, VCAM-1, and E-selectin for acute types; ICAM-1 for lymphoma

types; L-selectin and CADM1 for chronic types; and CADM1 and ALCAM for smoldering types. Additional analyses of the proteomic profiles of all ATL subtypes are needed to provide more accurate subclassifications and elucidate the nature of ATL progression specific to each ATL subtype.

As for remission states, the decrease in sTNFR2 levels suggests that sTNFR2 may prove useful in monitoring chemotherapy sensitivity or progress in achieving remission. However, analyzing sTNFR2 levels in various treatment strategies using a larger sample is necessary to confirm whether sTNFR2 can be used as a chemotherapy-sensitivity marker.

The role of TNFR2 in ATL pathogenesis is yet to be explored and may prove to be pivotal in ATL research. With the developments in proteomic research and technology, it will be vital to analyze proteins for the improvement of diagnosis and treatment of disease, in the hope of improving patient survival outcomes.

Acknowledgments

The authors thank all the patients who participated in this study, as well as the staff of Kariyushi Hospital, Ishigaki, Okinawa, for their contributions.

This work is partially supported by funds from the Advanced Medical Research Center, Faculty of Medicine, University of the Ryukyus.

Authorship

Contribution: T.F. designed the study; C.L.H.G. and T.F. wrote the manuscript; C.L.H.G. and Y.Y. had access to the raw data and analyzed the data; C.L.H.G., Y.Y., M.K., N.I., and T.F. conceptualized the visual abstract; C.L.H.G., M.M., N.I., and S.S. prepared samples and managed the data; C.L.H.G., M.K., and N.I. performed ELISA; M.K. and Y.T. performed flow cytometry; K. Karube performed immunostaining; K. Karube and Y.T. contributed reagents, materials, and analysis tools; N.I. performed mRNA analysis; M.H., T.M., K. Karimata, J.U., K.O., J.T., S.N., S.M., H.M., and T.F. managed the patients and collected samples; T.F. had full access to all the data in this study and had final responsibility for the decision to submit for publication; and all authors reviewed the manuscript.

Conflict-of-interest disclosure: T.F. and Y.Y. have patent rights related to findings from this study. NEC Corporation has a patent for the proteomic data (patent 2019-021539), and the University of the Ryukyus has a patent for data regarding sTNFR2 as an ATL biomarker (patent 2019-200986). The remaining authors declare no competing financial interests.

ORCID profiles: C.L.H.G., 0000-0002-0283-0775; K. Karube, 0000-0002-1205-858X; T.F., 0000-0001-9422-2875.

Correspondence: Takuya Fukushima, Laboratory of Hematoimmunology, Graduate School of Health Sciences, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa, 903-0215 Japan; e-mail: fukutaku@med.u-ryukyu.ac.jp.

References

1. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood*. 1977;50(3):481-492.
2. Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci USA*. 1980;77(12):7415-7419.
3. Hinuma Y, Nagata K, Hanaoka M, et al. Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. *Proc Natl Acad Sci U S A*. 1981;78(10):6476-6480.
4. 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 U S A*. 1982;79(6):2031-2035.
5. Shimoyama M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984-87). *Br J Haematol*. 1991;79(3):428-437.
6. Tsukasaki K, Imaizumi Y, Tokura Y, et al. Meeting report on the possible proposal of an extranodal primary cutaneous variant in the lymphoma type of adult T-cell leukemia-lymphoma. *J Dermatol*. 2014;41(1):26-28.
7. Cook LB, Fuji S, Hermine O, et al. Revised adult T-cell leukemia-lymphoma international consensus meeting report. *J Clin Oncol*. 2019;37(8):677-687.
8. Tsukasaki K, Utsunomiya A, Fukuda H, et al; Japan Clinical Oncology Group Study JCOG9801. VCAP-AMP-VECP compared with biweekly CHOP for adult T-cell leukemia-lymphoma: Japan Clinical Oncology Group Study JCOG9801. *J Clin Oncol*. 2007;25(34):5458-5464.
9. Bazarbachi A, Plumelle Y, Carlos Ramos J, et al. Meta-analysis on the use of zidovudine and interferon-alfa in adult T-cell leukemia/lymphoma showing improved survival in the leukemic subtypes. *J Clin Oncol*. 2010;28(27):4177-4183.
10. Takasaki Y, Iwanaga M, Imaizumi Y, et al. Long-term study of indolent adult T-cell leukemia-lymphoma. *Blood*. 2010;115(22):4337-4343.
11. Gessain A, Cassar O. Epidemiological aspects and world distribution of HTLV-1 infection. *Front Microbiol*. 2012;3:388.
12. Iwanaga M, Watanabe T, Yamaguchi K. Adult T-cell leukemia: a review of epidemiological evidence. *Front Microbiol*. 2012;3:322.
13. Yoshida M, Seiki M, Yamaguchi K, Takatsuki K. Monoclonal integration of human T-cell leukemia provirus in all primary tumors of adult T-cell leukemia suggests causative role of human T-cell leukemia virus in the disease. *Proc Natl Acad Sci U S A*. 1984;81(8):2534-2537.
14. Proietti FA, Carneiro-Proietti ABF, Catalan-Soares BC, Murphy EL. Global epidemiology of HTLV-I infection and associated diseases. *Oncogene*. 2005;24(39):6058-6068.
15. Gonçalves DU, Proietti FA, Ribas JGR, et al. Epidemiology, treatment, and prevention of human T-cell leukemia virus type 1-associated diseases. *Clin Microbiol Rev*. 2010;23(3):577-589.

16. Iwanaga M, Watanabe T, Utsunomiya A, et al; Joint Study on Predisposing Factors of ATL Development investigators. 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*. 2010;116(8): 1211-1219.

17. Nosaka K, Iwanaga M, Imaizumi Y, et al. Epidemiological and clinical features of adult T-cell leukemia-lymphoma in Japan, 2010-2011: a nationwide survey. *Cancer Sci*. 2017;108(12):2478-2486.

18. Zell M, Assal A, Derman O, et al. Adult T-cell leukemia/lymphoma in the Caribbean cohort is a distinct clinical entity with dismal response to conventional chemotherapy. *Oncotarget*. 2016;7(32):51981-51990.

19. Jeannin P, Chaze T, Giai Gianetto Q, et al. Proteomic analysis of plasma extracellular vesicles reveals mitochondrial stress upon HTLV-1 infection. *Sci Rep*. 2018;8(1):5170.

20. Kamihira S, Sugahara K, Tsuruda K, et al. Proviral status of HTLV-1 integrated into the host genomic DNA of adult T-cell leukemia cells. *Clin Lab Haematol*. 2005;27(4):235-241.

21. Yamada Y, Tomonaga M, Fukuda H, et al. A new G-CSF-supported combination chemotherapy, LSG15, for adult T-cell leukaemia-lymphoma: Japan Clinical Oncology Group study 9303. *Br J Haematol*. 2001;113(2):375-382.

22. World Health Organization. WHO Handbook for Reporting Results of Cancer Treatment. Geneva, Switzerland: World Health Organization; 1979.

23. Gold L, Ayers D, Bertino J, et al. Aptamer-based multiplexed proteomic technology for biomarker discovery. *PLoS One*. 2010;5(12):e15004.

24. SomaLogic. SOMAscan Proteomic Assay: Technical White Paper. Boulder, CO: SomaLogic; 2015.

25. Kobayashi S, Nakano K, Watanabe E, et al. CADM1 expression and stepwise downregulation of CD7 are closely associated with clonal expansion of HTLV-I-infected cells in adult T-cell leukemia/lymphoma. *Clin Cancer Res*. 2014;20(11):2851-2861.

26. Kudo N, Yamamori H, Ishima T, et al. Plasma levels of soluble tumor necrosis factor receptor 2 (STNFR2) are associated with hippocampal volume and cognitive performance in patients with schizophrenia. *Int J Neuropsychopharmacol*. 2018;21(7):631-639.

27. MacEwan DJ. TNF ligands and receptors—a matter of life and death. *Br J Pharmacol*. 2002;135(4):855-875.

28. Tartaglia LA, Ayres TM, Wong GHW, Goeddel DV. A novel domain within the 55 kd TNF receptor signals cell death. *Cell*. 1993;74(5):845-853.

29. Faustman D, Davis M. TNF receptor 2 pathway: drug target for autoimmune diseases. *Nat Rev Drug Discov*. 2010;9(6):482-493.

30. Kataoka K, Nagata Y, Kitanaka A, et al. Integrated molecular analysis of adult T cell leukemia/lymphoma. *Nat Genet*. 2015;47(11):1304-1315.

31. Kataoka K, Iwanaga M, Yasunaga JI, et al. Prognostic relevance of integrated genetic profiling in adult T-cell leukemia/lymphoma. *Blood*. 2018;131(2): 215-225.

32. Tanaka Y, Takahashi Y, Tanaka R, Miyagi T, Saito M, Fukushima T. Association of high levels of plasma OX40 with acute adult T-cell leukemia. *Int J Hematol*. 2019;109(3):319-327.

33. Imura A, Hori T, Imada K, et al. The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. *J Exp Med*. 1996;183(5):2185-2195.

34. Kasahara D, Takara A, Takahashi Y, et al. Natural OX40L expressed on human T cell leukemia virus type-I-immortalized T cell lines interferes with infection of activated peripheral blood mononuclear cells by CCR5-utilizing human immunodeficiency virus. *Virol J*. 2013;10:338.

35. Sasaki H, Nishikata I, Shiraga 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(3):1204-1213.

36. Tatewaki M, Yamaguchi K, Matsuoka M, et al. Constitutive overexpression of the L-selectin gene in fresh leukemic cells of adult T-cell leukemia that can be transactivated by human T-cell lymphotropic virus type 1 Tax. *Blood*. 1995;86(8):3109-3117.

37. Ishikawa T, Imura A, Tanaka K, Shirane H, Okuma M, Uchiyama T. E-selectin and vascular cell adhesion molecule-1 mediate adult T-cell leukemia cell adhesion to endothelial cells. *Blood*. 1993;82(5):1590-1598.

38. Weng AP, Ferrando AA, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science*. 2004;306(5694): 269-271.

39. Yu P, Petrus MN, Ju W, et al. Augmented efficacy with the combination of blockade of the Notch-1 pathway, bortezomib and romidepsin in a murine MT-1 adult T-cell leukemia model. *Leukemia*. 2015;29(3):556-566.

40. Asano N, Miyoshi H, Kato T, et al Expression pattern of immunosurveillance-related antigen is a critical prognostic factor of adult T-cell leukemia/ lymphoma [abstract]. Blood. 2016;128(22). Abstract 4135.

41. Niino D, Komohara Y, Kimura Y, et al M2 macrophage infiltration is closely associated with poor prognosis for adult T-cell leukemia/lymphoma (ATLL). Blood. 2011;118(21). Abstract 3672.

42. Ohyashiki JH, Hamamura R, Kobayashi C, Zhang Y, Ohyashiki K. A network biology approach evaluating the anticancer effects of bortezomib identifies SPARC as a therapeutic target in adult T-cell leukemia cells. *Adv Appl Bioinform Chem*. 2008;1:85-98.

43. Croft M, Benedict CA, Ware CF. Clinical targeting of the TNF and TNFR superfamilies. *Nat Rev Drug Discov*. 2013;12(2):147-168.

44. Vanamee É, Faustman DL. TNFR2: a novel target for cancer immunotherapy. *Trends Mol Med*. 2017;23(11):1037-1046.

45. Wang M, Zhang C, Tian T, et al. Increased regulatory T cells in peripheral blood of acute myeloid leukemia patients rely on tumor necrosis factor (TNF)-α-TNF receptor-2 pathway. *Front Immunol*. 2018;9:1274.

46. Torrey H, Khodadoust M, Tran L, et al. Targeted killing of TNFR2-expressing tumor cells and T_{regs} by TNFR2 antagonistic antibodies in advanced Sézary syndrome. *Leukemia*. 2019;33(5):1206-1218.

47. Heemann C, Kreuz M, Stoller I, et al. Circulating levels of TNF receptor II are prognostic for patients with peripheral T-cell non-Hodgkin lymphoma. *Clin Cancer Res*. 2012;18(13):3637-3647.

48. Faustman DL, Davis M. TNF receptor 2 and disease: autoimmunity and regenerative medicine. *Front Immunol*. 2013;4:478.

49. Ward-Kavanagh LK, Lin WW, Šedý JR, Ware CF. The TNF receptor superfamily in co-stimulating and co-inhibitory responses. *Immunity*. 2016;44(5): 1005-1019.

50. Zunke F, Rose-John S. The shedding protease ADAM17: physiology and pathophysiology. *Biochim Biophys Acta Mol Cell Res*. 2017;1864(11): 2059-2070.

51. Takeshita M, Akamatsu M, Ohshima K, et al. CD30 (Ki-1) expression in adult T-cell leukaemia/lymphoma is associated with distinctive immunohistological and clinical characteristics. *Histopathology*. 1995;26(6):539-546.

52. Nishioka C, Takemoto S, Kataoka S, et al. Serum level of soluble CD30 correlates with the aggressiveness of adult T-cell leukemia/lymphoma. *Cancer Sci*. 2005;96(11):810-815.

53. Maeda M, Arima N, Daitoku Y, et al. Evidence for the interleukin-2 dependent expansion of leukemic cells in adult T cell leukemia. *Blood*. 1987;70(5): 1407-1411.

54. Halfter H, Friedrich M, Postert C, Ringelstein EB, Stögbauer F. Activation of Jak-Stat and MAPK2 pathways by oncostatin M leads to growth inhibition of human glioma cells. *Mol Cell Biol Res Commun*. 1999;1(2):109-116.

55. Richards CD. The enigmatic cytokine oncostatin m and roles in disease. *ISRN Inflamm*. 2013;2013:512103.

56. Hermanns HM. Oncostatin M and interleukin-31: cytokines, receptors, signal transduction and physiology. *Cytokine Growth Factor Rev*. 2015;26(5): 545-558.

57. Yamamura M, Yamada Y, Momita S, Kamihira S, Tomonaga M. Circulating interleukin-6 levels are elevated in adult T-cell leukaemia/lymphoma patients and correlate with adverse clinical features and survival. *Br J Haematol*. 1998;100(1):129-134.

58. Horiuchi S, Yamamoto N, Dewan MZ, et al. Human T-cell leukemia virus type-I Tax induces expression of interleukin-6 receptor (IL-6R): shedding of soluble IL-6R and activation of STAT3 signaling. *Int J Cancer*. 2006;119(4):823-830.

59. Mori F, Ishida T, Ito A, et al. Antitumor effects of bevacizumab in a microenvironment-dependent human adult T-cell leukemia/lymphoma mouse model. *Eur J Haematol*. 2014;92(3):219-228.