Mortality and risk of progression to adult T cell leukemia/lymphoma in HTLV-1–associated myelopathy/tropical spastic paraparesis

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Human T cell leukemia virus 1 (HTLV-1) causes the functionally debilitating disease HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) as well as adult T cell leukemia lymphoma (ATLL). Although there were concerns that the mortality of HAM/TSP could be affected by the development of ATLL, prospective evidence was lacking in this area. In this 5-y prospective cohort study, we determined the mortality, prevalence, and incidence of ATLL in 527 HAM/TSP patients. The standard mortality ratio of HAM/TSP patients was 2.25, and ATLL was one of the major causes of death (5/33 deaths). ATLL prevalence and incidence in these patients were 3.0% and 3.81 per 1,000 person-y, respectively. To identify patients at a high risk of developing ATLL, flow cytometry, Southern blotting, and targeted sequencing data were analyzed in a separate cohort of 218 HAM/TSP patients. In 17% of the HAM/TSP patients, we identified an increase in T cells positive for cell adhesion molecule 1 (CADM1), a marker for ATLL and HTLV-1–infected cells. Genomic analysis revealed that somatic mutations of HTLV-1–infected cells were seen in 90% of these cases and 11% of them had dominant clone and developed ATLL in the longitudinal observation. In this study, we were able to demonstrate the increased mortality in patients with HAM/TSP and a significant effect of ATLL on their prognosis. Having dominant clonal expansion of HTLV-1–infected cells with ATLL-associated somatic mutations may be important characteristics of patients with HAM/TSP who are at an increased risk of developing ATLL.

HTLV-1 | HAM/TSP | ATLL | prognosis | SMR

Human T cell leukemia virus 1 (HTLV-1) infects at least 10 million people worldwide (1, 2) and causes severe morbidity and mortality in some of the those who develop HTLV-1–associated diseases including HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) (3, 4), a progressive neurological disease, and adult T cell leukemia/lymphoma (ATLL) (5), an aggressive mature T cell malignancy (6). Understanding the impact of HTLV-1 infection on global health is critical for developing a comprehensive management strategy to tackle HTLV-1 (6, 7). Approximately 2 to 5% of HTLV-1–infected patients develop ATLL and the negative impact of ATLL transformation on the survival of those HTLV-1–infected patients is substantial. The 2008 World Health Organization Classification of Tumors of Hematopoietic and Lymphoid Tissues categorized ATLL into four subtypes, composed of the smoldering,

Significance

HTLV-1 manifests many diseases, which cause morbidity and mortality in 5∼10% of infected individuals, including the fatal adult T cell leukemia/lymphoma (ATLL) and debilitating myelopathy (HAM/TSP). However, the rarity of these diseases had made it prohibitory to conduct large-scale prospective observational studies. This work enabled calculating the standard mortality ratio of HAM/TSP patients and also identified ATLL as one of the major causes of death among these patients. We also identified the features that lead HAM/TSP patients to develop ATLL: having dominant clonal expansion of HTLV-1–infected cells with ATLL-associated somatic mutations. Furthermore, this manuscript describes genomic changes occurring in HAM/TSP patients at the actual time of their ATLL transformation.

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Competing interest statement: M.N. reports personal fees from AstraZeneca and grants from Tempus, outside the submitted work. E.I. reports personal fees from Merck Bio Pharma Japan, outside the submitted work. Y.Y. reports grants from Japan Agency for Medical Research and Development, grants from Ministry of Health, Labour and Welfare, and Japan Society for the Promotion of Science KAKENHI during the conduct of the study; grants from Daiichi Sankyo Co., Ltd.; grants from ONO Pharmaceutical Co., Ltd.; and grants from Kyowa Hakko Kirin, Japan, outside the submitted work; In addition, Y.Y. and N.A. have a patent "Medicine for Treating or Preventing HTLV-I-Related Myelopathy, and Method for Forecasting the Effect of Antibody Therapy for Patient of HTLV-I-Related Myelopathy" (Japan Patent 5552630 licensed to St. Marianna University School of Medicine). Y.Y. has a patent "Therapeutic Method and Medicament for HTLV-1-Associated Myelopathy (HAM)" (Japan Patent 6310845, US9,642,910, AUS2013285970 licensed to St. Marianna University School of Medicine, Kyowa Hakko Kirin, Japan); and a patent "Preventive or Therapeutic Agent for HTLV-1-Associated Myelopathy Using Low-Dose Anti-CCR4 Antibody" (Japan Patent 6430082 licensed to St. Marianna University School of Medicine, Kyowa Hakko Kirin, Japan). All other authors have no competing interests to disclose.

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chronic, lymphomatous, and acute subtypes (8). For smoldering and chronic ATLL, median survival is estimated to be ~30 to 55 mo (9), whereas survival is estimated to be 10 mo for the lymphomatous and 8 mo for the acute subtype, respectively (9).

Of HTLV-1–infected patients, 0.25 to 3.8% develop HAM/TSP. HAM/TSP is a debilitating condition for affected patients causing a spectrum of symptoms including motor deficits, incontinence, constipation, back pain, and sexual dysfunction frequently impairing their quality of life (10). However, unlike ATLL, which has a poor prognosis, there is some controversy concerning the possible negative prognostic impact that HAM/TSP may have toward survival. Because of the lack of age-matched prospective studies for this population, reliable data on the survival outcomes of patients with HAM/TSP have been extremely limited. Although there are some case reports of patients with HAM/TSP developing ATLL (11–13), no large-scale prospective cohort studies have been conducted on patients with HAM/TSP, nor has the risk of ATLL transformation been evaluated on these patients.

We thus asked whether patients with HAM/TSP are under increased risk of developing ATLL. Previous prospective studies demonstrated that peripheral blood HTLV-1 proviral load (PVL) at or more than 4% and clonal expansion of HTLV-1–infected cells reflect a high risk of ATLL transformation in HTLV-1 carriers (14, 15). Although patients with HAM/TSP have increased HTLV-1 PVL compared with that of HTLV-1 carriers (16), whether or not they are at increased risk of developing ATLL has never been investigated. We therefore investigated whether patients with HAM/TSP display genetic characteristics that are commonly seen in patients with ATLL such as the recently reported somatic mutations (17). For this, we have taken full advantage of the Japanese nationwide HAM/TSP patient registry (HAM-net) (18). To our surprise, we observed that ATLL was the major cause of death among HAM/TSP patients. We also saw two patients with HAM/TSP who later on developed ATLL during the course of our study, in which we were able to track the clonal expansion and somatic mutations before and after they developed ATLL. With prospective and longitudinal data, this cohort study presents valuable evidence on the mortality of patients with HAM/TSP. The current study also prompted us to realize the importance of establishing a methodology to identify patients with HAM/TSP who are at an increased risk of developing ATLL, so that we could ultimately devise a preventative strategy for those at risk.

Methods

Study Design and Participants. We designed a prospective study to determine the mortality, prevalence, and incidence of ATLL in patients with HAM/TSP. These variables were calculated using our nationwide HAM-net (18) (https://www.umin.ac.jp/ctr/index.htm; UMIN000028400). This nationwide registry recruits HAM/TSP patients throughout Japan. After diagnostic documents were confirmed centrally, the study coordinator contacted the patients annually. Patient data collected between April 2012 and March 2018 were analyzed. At the time of data analysis, the registry included 527 patients diagnosed with HAM/TSP throughout Japan. To identify HAM/TSP patients at a high risk of developing ATLL, a separate cohort of 218 patients with HAM/TSP consented to peripheral blood sample testing, which was used for flow cytometry, Southern blotting, and genomic analysis. The study protocols were approved by the St. Marianna University School of Medicine Institutional Review Board (#2044 and #1646). All patients provided written informed consent.

Outcomes. *SI Appendix*, Fig. S1 shows the number of patients included in each analysis (*SI Appendix*, Fig. S1). The standardized mortality ratio (SMR) was calculated using data from 487 patients with confirmed follow-up. The 2015 Japanese National Population Census was used as the standardized population for SMR calculation. ATLL prevalence was determined from 527 patients with HAM/TSP. ATLL incidence was determined from 479 patients with HAM/TSP who had confirmed follow-ups, excluding eight patients who were diagnosed with ATLL prior to study enrollment. ATLL incidence rates were calculated by dividing the number of patients newly diagnosed with ATLL by the observation period.

PVL, Flow Cytometry, and Southern Blotting. Peripheral blood mononuclear cells (PBMCs) separated by Ficoll-based density gradient centrifugation was used to measure the PVL and for flow-cytometric analyses. The separated PBMCs were frozen in cryopreserving fluid and stored in liquid nitrogen until analysis. PBMC PVL was measured using previously reported methods (19) and compensated using standard reference material (20).

Flow cytometry analysis was performed using previously reported methods (21) to detect the expression levels of cell adhesion molecule 1 (CADM1) and CD7 in $CD3^+CD4^+$ cells from 218 patients with HAM/TSP who had not developed ATLL. Certain expression patterns of CADM1, a marker for HTLV-1–infected cells, and CD7 have been suggested to reflect HTLV-1–infected cells progressing to ATLL cells (22, 23). For example, $CADM1^+CD7^{neg}$ cells have been reported to be enriched with clonally expanded cells and abnormal lymphocytes (ATLL cells) (23). We hypothesized from previous studies that patients with flow cytometry analysis demonstrating more than 25% of $CD4^+$ cells to be positive for CADM1 with more than 50% of $CADM1^+$ cells being negative for CD7 ($CADM1^+CD7^{dim}$ < $CADM1^+CD7^{neg}$; $CADM1^+CD7^{neg}$ dominant pattern) could be at an increased risk for progression to ATLL (22, 23).

As Firouzi et al. (15) reported, HTLV-1 carriers who progressed to ATLL exhibited monoclonal expansion, which highlights the importance of clonality of HTLV-1–infected T cells as a risk indicator for ATLL development. To examine the clonal expansion of HTLV-1–infected cells, we performed Southern blot analysis of PBMCs as reported previously (24).

Genomic Analysis. First, PBMCs from 21 HAM/TSP patients including longitudinal samples from two patients with HAM/TSP pre- and post-ATLL progression were sorted into HTLV-1–infected ($CD4^+CADM1^+$) or HTLV-1 noninfected ($CD4^+CADM1^-$) enriched cells by fluorescence-activated cell sorting using ARIA III (BD Biosciences). Then, targeted DNA sequencing of both cell populations was performed to detect single-nucleotide variants (SNVs). Target capture was conducted using the SureSelect Target Enrichment System (Agilent Technologies). The sequence data were obtained using an HiSeq2500 sequencer (Illumina) with 100-bp paired-end reads. *SI Appendix*, Table S1 lists the 240 target genes, which include 50 genes that were previously reported to be frequently associated with ATLL (17) as well as 190 genes frequently mutated in hematologic and solid malignancies as deposited in the COSMIC database (25) and differentially expressed in ATLL based on expression array dataset GSE33615 (*SI Appendix*, Table S1) (26). The sequenced data were aligned to the human reference genome hg38 by BWA (version 0.7.15) software. The PCR duplicates were removed using Picard (version 2.92) and SAMtools (version 1.2) software (27). The somatic mutation candidates were called using MuTect2 from GATK (version 4.0.12) software (28) and annotated with ANNOVAR (29). Candidate mutations with 1) ≥5 variant reads in tumor samples, 2) a VAF in tumor samples ≥0.01, 3) read depth ≥200, and 4) tumor variant/normal variant ratio ≥2, were adopted and further filtered by excluding synonymous SNVs.

Table 1. Standardized mortality rate of patients from the HAM-net registry (n = 487)

	No. of patients	No. of deaths	Observation period, person-y	CMR	Expected no. of deaths in standard population	SMR
Total	487	33	1,881.5	17.54	14.69	2.25 (1.57–3.20*)
Male	123	14	467.3	29.96	6.67	2.10 (1.19–3.61*)
Female	364	19	1,414.2	13.44	8.01	2.37 (1.47–3.78*)

CMR, crude mortality rate; SMR, standardized mortality ratio.
*95% CI.

Table 2. Use of oral steroids prior to ATLL progression in HAM/TSP patients (n = 527)

ATLL progression	No. of patients (%)	Use of oral steroids prior to ATLL			
		Yes	No	Unknown	Total
No	*n*	350	148	13	511
	%	68.5%	29.0%	2.5%	100.0%
Yes	*n*	8	7	1	16
	%	50.0%	43.8%	6.3%	100.0%
Total	*n*	360	154	13	527
	%	68.3%	29.2%	2.5%	100.0%

For patients who developed ATLL, data on steroid use prior to ATLL were recorded. For patients who did not develop ATLL during the observation period, data on steroid use data prior to study enrollment were recorded. Fisher's exact test, $P = 0.1425$.

The clonality analysis of HTLV-1–infected T cells was also performed by high-throughput sequencing based mapping of proviral integration sites (15). To designate the virus integration site, the sequences spanning the viral and human genomes were identified and their junction points were extracted by as the soft-clipped read using the perl script and then validated by using Blastn, version 2.6.0+.

Statistical Analysis. Using Fisher's exact and Mann–Whitney *U* tests, we compared the characteristics of patients with and without ATLL development. Patients who were lost to follow-up were censored at the time of last contact. Statistical analysis was performed with IBM SPSS Statistics (version 22) (IBM) and R (version 3.4.2) software (Free Software Foundation). Statistical tests were two-sided, with a type I error set at an α of 0.05.

Data and Materials Availability. The sequencing data reported in this paper have been deposited in the National Bioscience Database Center (NBDC) Human Database, https://humandbs.biosciencedbc.jp/ (accession no. JGAS00000000226).

Results and Discussion

Given the rarity of HAM/TSP, conducting large-scale prospective observational studies on this disease has been very challenging. Thus, reliable estimates of mortality and ATLL transformation rates in patients with HAM/TSP and the effect of ATLL on HAM/TSP prognosis have not been previously described. In this study, during the 5-y follow-up period, the HAM-net detected 33 deaths. The median age at death was 72.0 y (men, 72.5; women, 69.0). The SMR of HAM/TSP patients was 2.25 (95% CI, 1.57 to 3.20), reflecting the poor prognosis of HAM/TSP patients compared with that of the age-matched general population (Table 1). Based on the metaanalysis by Manouchehrinia et al. (30), the SMR for multiple sclerosis, a similarly debilitating but a neurological disorder perhaps better known than HAM/TSP, was 2.80 (95% CI, 2.74 to 2.87), which was much the same to the SMR of HAM/TSP. Of note, the poor prognosis of patients with HAM/TSP has been reported from Brazil as well (31).

Of the 33 deaths during the study period, the causes of death were ATLL (five cases) followed by pneumonia, aspiration pneumonitis, and heart failure, which were reported to be four cases each (*SI Appendix*, Table S2). To our surprise, ATLL was numerically the most common cause of death in patients with HAM/TSP in our study, and the numbers were comparable to much more commonly seen complications/comorbidities, such as pneumonia, aspiration pneumonitis, and heart failure. This finding highlights the major impact of ATLL on the prognosis of patients with HAM/TSP, as survival is projected to be ~8 to 10 mo with aggressive ATLL (9).

Since the significant impact of ATLL on HAM/TSP prognosis was demonstrated, determining the prevalence and incidence of ATLL transformation in patients with HAM/TSP would be a crucial step in understanding this disease. *SI Appendix*, Table S3 shows the baseline characteristics of HAM/TSP patients from the HAM-net (*SI Appendix*, Table S3). The prevalence of ATLL in HAM/TSP patients was ~3.0% (16/527). Between patients with and without ATLL development, there were no significant differences in the median age at enrollment or median age of HAM/TSP onset, median years from symptoms to diagnosis, duration of disease, motor dysfunction measured by the Osame Motor Disability Score (*SI Appendix*, Table S3), and prior steroid treatment (Table 2). During the prospective 5-y observation period, seven patients were newly diagnosed with ATLL (averaged age of diagnosis was 64.6), suggesting an ATLL incidence in HAM/TSP patients of around 3.81 per 1000 person-y (Table 3). The incidence of aggressive ATLL (four acute and two lymphoma subtype) in HAM/TSP patients was calculated as 3.26 per 1,000 person-y. The mean survival time for the seven patients diagnosed with ATLL was 1.19 y, with the indolent type at 3.84 y and the aggressive type at 0.74 y. Although direct comparisons have certain limitations, the incidence of aggressive ATLL in HTLV-1 carriers is around 0.6 to 1.3 per 1,000 person-y (32), which suggests that the ATLL incidence in patients with HAM/TSP observed in this study is rather high. At least in Japan, this study suggests that patients with HAM/TSP are at increased risk for developing ATLL, and therefore, careful and longitudinal surveillance would be recommended in these patients. It would be of utmost importance to evaluate for the potential concurrent diagnosis of ATLL as patients with HAM/TSP are often offered immunosuppressive therapy such as steroids, which may delay true therapy for ATLL. While this prospective large-scale cohort study is limited to data essentially originating from Japan, there are, however, reports of ATLL diagnosis in patients with HAM/TSP from around the world, including three concurrent diagnosis of ATLL from 63 patients with HAM/TSP reported from the United States (11), one ATLL transformation from 48 patients with HAM/TSP reported from United Kingdom (12), and a report of concurrent ATLL diagnosis in a patient with HAM/TSP from Brazil (13). Therefore, it would be critical to determine the risks of ATLL transformation in patients with HAM/TSP in other countries and areas around the world outside of Japan as well, and we believe that multicenter analysis of HAM/TSP patient cohorts of different ethnic and genetic diversities needs to be conducted.

We have revealed that the development of ATLL plays an important role in determining the prognosis of patients with HAM/TSP. The survival outcomes of those with aggressive ATLL remain dismal (9). This was also demonstrated by our cohort of HAM/TSP patients who subsequently developed ATLL. We therefore hypothesized that by identifying HAM/TSP patients who are at an increased risk for developing ATLL ahead of time, we may be able to offer preventive therapy that may ultimately improve survival outcomes in these patients prior to the actual onset of ATLL. In HTLV-1 carriers, risk factors for ATLL development include family history, high HTLV-1 PVL levels (14), and clonal expansion of HTLV-1–infected T cells (15). There is limited evidence for the identification of patients

Table 3. ATLL incidence and incidence rates in HAM/TSP patients (n = 479)

	Male	Female	Total
No. at risk	121	358	479
Average observation period, y	3.77	3.87	3.84
Median observation period, y	4.90	4.93	4.93
Observation, person-y	455.6	1,383.8	1,839.5
ATLL incidence during observation	3	4	7
ATLL incidence rates, 1,000 person-y	6.58	2.89	3.81

Table 4. Flow cytometry analysis results for samples from patients with HAM/TSP (n = 218)

	Flow cytometry analysis	
	No. of patient samples	Percentage, %
No. of patients with HAM/TSP	218	100
CADM1$^+$ ≥ 25%	37	17
CADM1$^+$CD7^{dim} > CADM1$^+$CD7^{neg}	30	13.8
CADM1$^+$CD7^{dim} < CADM1$^+$CD7^{neg}	7	3.2

with HAM/TSP who are at high risk for developing ATLL. We hypothesized that the increased incidence of ATLL in patients with HAM/TSP could be related to the emergence of an HTLV-1–infected cell population that is more prone to leukemogenesis. Recently, flow cytometry analysis has captured attention as a unique method to detect the emergence of such HTLV-1–infected cell population thought to be at increased risk for leukomogenesis (22, 23, 33). To evaluate the risk of ATLL transformation in HAM/TSP patients without concurrent diagnosis of ATLL, we performed flow cytometry analysis of PBMC samples from a separate cohort of 218 HAM/TSP patients. Out of the 218 patients, 37 patients (17%) had more than 25% of CD4$^+$ cells positive for CADM1 (Table 4), reflecting the high prevalence of HTLV-1–infected cells in the HAM/TSP population as reported previously (16). In our analysis, 7 of the 37 cases had increased numbers of CADM1$^+$CD7^{neg} cells (CADM1$^+$CD7^{neg} dominant pattern) (Table 4). Fig. 1 shows the various patterns from flow cytometry analysis. HAM/TSP patients typically presented CADM1$^+$ cells with CD7^{dim} > CD7^{neg} without clonal bands on Southern blotting (Fig. 1*A*). Data from a HAM/TSP patient with clonal bands who developed ATLL 13 mo later are shown in Fig. 1*B*. Fig. 1*C* presents data from a HAM/TSP patient who has CADM1$^+$ cells with CD7^{dim} > CD7^{neg} pattern and is positive for clonal bands. Fig. 1*D* shows the flow cytometry pattern of the patient from Fig. 1*C* at the time of ATLL development, which was collected at 28 mo from the time point of Fig. 1*C*. Upon ATLL development, the flow cytometry pattern changed to CADM1$^+$CD7^{neg} dominant pattern (Fig. 1*D*).

Next, we analyzed the clonal expansion of HTLV-1–infected T cells in HAM/TSP patients. Of the 37 patients with HAM/TSP who had more than 25% of CD4$^+$ cells positive for CADM1, 27 had enough samples for further Southern blotting analysis. Of these patients, nine had the clonal band of HTLV-1 (Table 5). Six out of seven (85.7%) cases with the CADM1$^+$CD7^{neg} dominant pattern had a clonal band consistent with findings from Kobayashi et al. (23). Interestingly, clonal expansion of HTLV-1–infected cells on Southern blotting was still observed with the alternate pattern of increased CADM1$^+$CD7^{dim} cells; however, the rates were lower (3/20). Importantly, one (HAM#25) out of

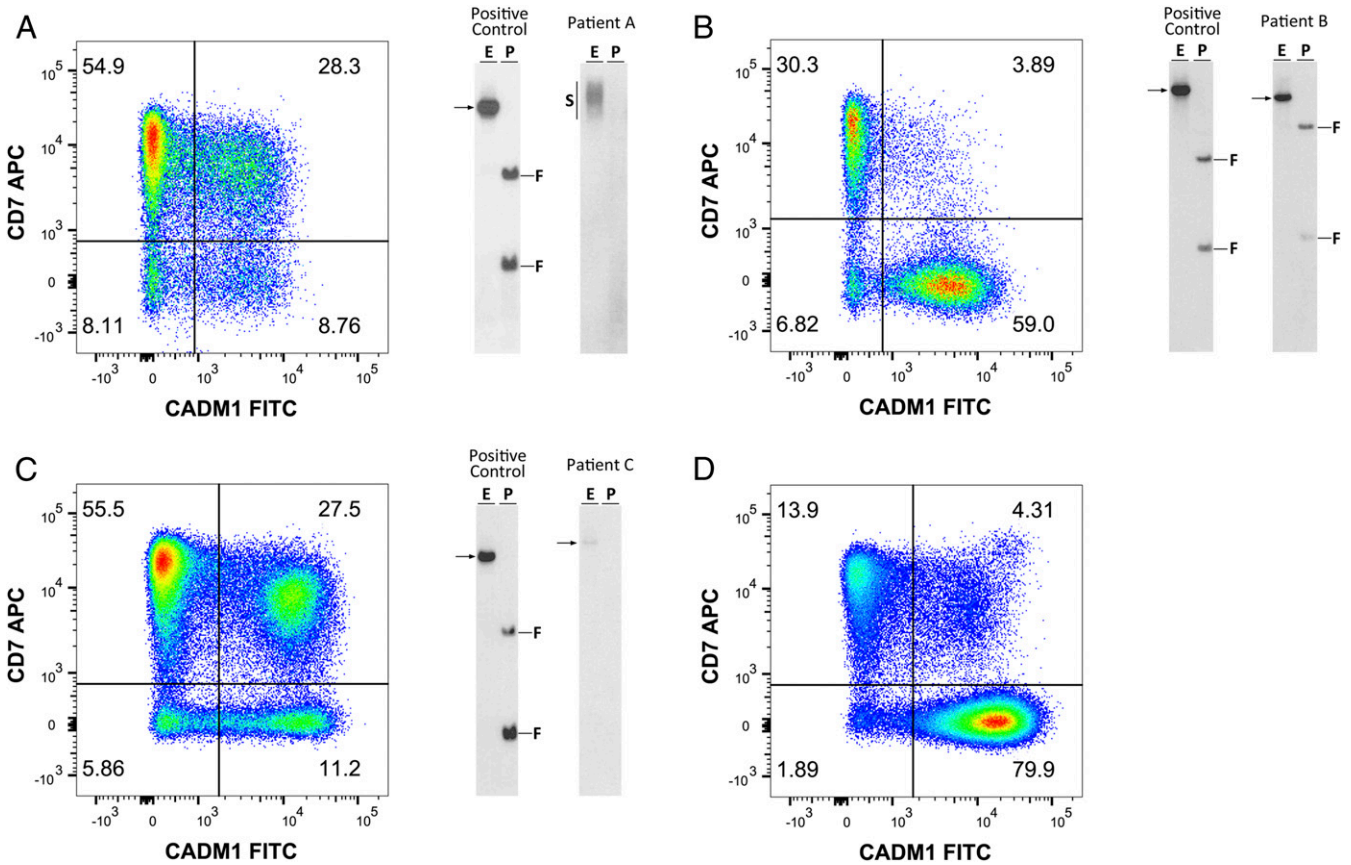

Fig. 1. Representative expression patterns of CADM1/CD7 and Southern blot analysis in patients with HAM/TSP. Each *Left* panel shows a representative flow cytometric plot of CADM1 and CD7 expression in CD4$^+$ T cells among PBMCs. Each *Right* panel shows a Southern blot analysis using the HTLV-1 probe. Patients with HAM/TSP tend to have the flow cytometry pattern (CADM1$^+$CD7^{dim} > CADM1$^+$CD7^{neg}) shown in *A* with a smear Southern blot pattern. *B* and *C* represent patients with HAM/TSP at a potential risk for ATLL who have a positive clonal band. *D* shows the flow-cytometric pattern of the patient in *C* who developed ATLL after 2 y and 4 mo. The arrows point to the major clone. E, EcoRI digestion; F, flanking band; P, PstI digestion; S, smear. (*A*) HAM/TSP, (*B* and *C*) HAM/TSP at potential risk for ATLL, and (*D*) both HAM/TSP and ATLL.

Table 5. PVL, Southern blotting, next-generation sequencing, and mutation target sequencing results in HAM/TSP patients with more than 25% of CD4$^+$ cells positive for CADM1 (n = 27)

HAM ID no.	PVL in PBMC	% in CD4$^+$			Monoclonal band by Southern blot	% of major clone by NGS	Mutation by target sequence
		CADM1$^+$	CADM1$^+$CD7^{dim}	CADM1$^+$CD7^{neg}			
CD7^{dim} < CD7^{neg}							
HAM1	3.89	63.90	5.04	58.86	+	41.26%	+
HAM2	2.31	27.13	12.81	14.32	—	2.25%	+
HAM3	12.93	67.28	33.97	33.31	+	10.70%	+
HAM4	14.79	43.82	9.29	34.53	+	NT	NT
HAM5	10.26	36.16	12.6	23.56	+	NT	NT
HAM6	12.78	35.40	13.32	22.08	+	10.52%	+
HAM7	21.57	42.92	20.04	22.88	+	9.92%	+
CD7^{dim} > CD7^{neg}							
HAM8	25.50	33.99	26.71	7.28	—	1.16%	—
HAM9	9.52	39.49	31.68	7.81	—	2.75%	+
HAM10	6.11	30.69	23.00	7.69	—	3.44%	+
HAM11	10.83	30.22	22.36	7.86	—	4.02%	+
HAM12	8.10	26.98	18.51	8.47	—	2.36%	+
HAM13	6.78	33.17	21.72	11.45	—	NT	NT
HAM14	14.41	48.19	26.35	21.84	—	3.56%	+
HAM15	12.39	36.79	28.21	8.58	—	2.89%	+
HAM16	14.33	42.38	26.52	15.86	—	3.38%	+
HAM17	12.44	30.98	24.12	6.86	—	2.89%	+
HAM18	9.31	30.46	21.03	9.43	—	3.53%	+
HAM19	17.13	38.60	28.52	10.08	—	NT	NT
HAM20	10.49	30.87	20.42	10.45	—	4.34%	+
HAM21	17.54	52.60	37.77	14.83	—	NT	NT
HAM22	5.80	29.73	26.98	2.75	+	NT	NT
HAM23	20.26	37.80	28.65	9.15	—	4.04%	+
HAM24	13.27	26.75	19.09	7.66	—	2.39%	—
HAM25	9.99	34.53	25.19	9.34	+	43.06%	+
HAM26	12.70	33.32	21.07	12.25	—	14.56%	+
HAM27	22.17	55.65	44.48	11.17	+	72.96%	+

NGS, next-generation sequencing; NT, not tested; PVL, proviral load.

these three cases subsequently developed ATLL, and upon progression, the identical infected clones transformed from CADM1$^+$ CD7^{dim} to CADM1$^+$ CD7^{neg} (Fig. 1 *C* and *D*). These results suggest that, although CD7 down-regulation may correlate with clonal expansion and progression to ATLL, the use of CD7 as a predictive marker for progression to ATLL has insufficient evidence and patients with increased CD7^{dim} may still be at risk for ATLL progression. This prompted us to use an alternative approach to identify more relevant biomarkers of ATLL transformation. Considering this, we thus utilized the target capture sequencing to further examine these HTLV-1–infected cells for somatic mutations. Although ATLL cells have been shown to accumulate multiple genomic mutations (17), there is no literature on somatic mutations in the HTLV-1–infected cells of patients with HAM/TSP and how the accumulation of such mutations may influence ATLL progression prior to its onset is unclear.

Of the 27 cases (where we had enough samples for Southern blotting), 21 had enough samples for genomic analysis with targeted deep sequencing for SNV detection. Of these cases, 5 had the CADM1$^+$ CD7^{neg} dominant pattern and 16 had the alternate pattern CADM1$^+$CD7^{dim} (CD7^{dim} > CD7^{neg}). Clonally expanded nonsynonymous mutations were identified in 19/21 cases (Table 5). The mutated genes of these 19 cases are summarized in *SI Appendix*, Table S4 (*SI Appendix*, Table S4). Of these cases, two subsequently developed acute ATLL; HAM #1 at 13 mo and HAM #25 at 28 mo following their study enrollment. Both cases that subsequently developed ATLL had somatic mutations frequently reported in ATLL such as *PLCG1*, *POT1*, *TET2*, and *GATA3* at high VAF (variant allele frequency) levels, suggesting that having these "high-risk" somatic mutations previously reported in ATLL (17) prior to the actual ATLL onset may play a role in ATLL progression. Importantly, these high-risk mutations were also seen in HAM/TSP cases who had not developed ATLL (HAM#2, FAS, CCR4; HAM#3, POT1; HAM#6, CCR4, PTPRC, GATA3; HAM#7, EP300, CARD11, PLCG1; HAM#11, CBLB, RELA; HAM#12, SETD2; HAM#15, TNFAIP3; HAM#17, EP300; HAM#18, HLA-B; HAM#20, SETD2; HAM#23, HLA-4; HAM#26, IRF2BP2; and HAM#27, GATA3, VAV1 and PRKCB). Most had low VAF levels with the exception of HAM#26 and HAM#27 (*SI Appendix*, Table S4). These two patients had high VAF levels with dominant clones as shown in Fig. 2 and should be monitored for progression to ATLL with extra caution.

Investigating further the function of genes that were found to be mutated may also be crucial in understanding the pathogenesis of ATLL. *MRPL37* and *GPR39*, the top two somatic mutations found in HAM#18, were both protein-coding genes. While *MRPL37* is involved in mitochondrial translation, organelle biogenesis, and maintenance, related pathways for *GPR39* include peptide ligand-binding receptors and signaling by G-protein–coupled receptors. *KPRP*, *FAT1*, and *RECQL4*, the top three somatic mutations found in HAM#26, were also all protein-coding genes. *KPRP* encodes a proline-rich skin protein possibly involved in keratinocyte differentiation. *FAT1* encodes a tumor suppressor essential for controlling cell proliferation during *Drosophila* development. The protein encoded by *RECQL4* is a DNA helicase that belongs to the RecQ helicase family, and thus *RECQL4*

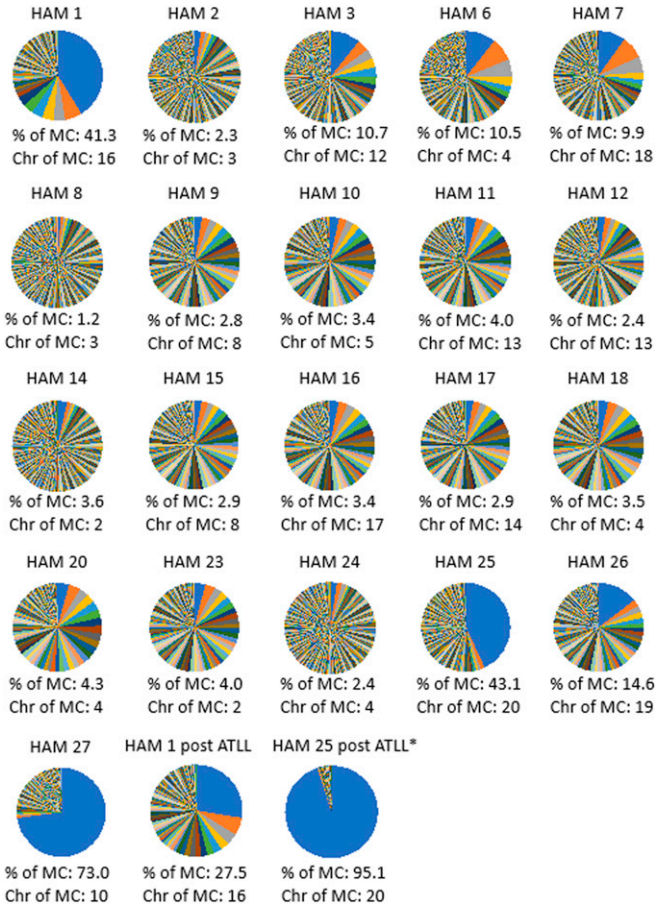

Fig. 2. Clonality analysis of HTLV-1–infected cells using next-generation sequencing. The pie charts show the relative frequency of each integrations site ($n = 27$). For example, the percentage of the major clone (shown in blue) found in HAM1 patient was 41.3% and was located on chromosome 16. *For HAM25 post ATLL, the cells for analysis were gated for CADM1-positive, CD7-negative cells. Chr of MC, chromosomal location of the major clone.

is related to the DNA damage pathway. On the other hand, HAM#1, who developed ATLL, had high VAF levels of somatic mutations directly related to the T cell receptor/NF-κβ signaling pathway (*PLCG1*), while this was not seen in HAM#25, who also subsequently developed ATLL. While it is possible to speculate that some patients with high VAF levels of somatic mutations (17) had not yet developed ATLL as these mutations (i.e., *MRPL37* and *GPR39* in HAM#18 and *KPRP*, *FAT1*, and *RECQL4* in HAM#26) may not directly affect pathways important to ATLL, further follow-up research would still be recommended as many of the patients with HAM/TSP appear to have these somatic mutations related to T cell receptor/NF-κβ signaling pathway (*PLCG1*, *PRKCB*, *CARD11*, *VAV1*), transcription factors (*IRF4*, *IKZF2*, *GATA3*), as well as chemokine receptors (*CCR4*, *CCR7*) and molecules related to immune surveillance (*HLA-A/B*, *FAS*), although currently at lower VAF levels.

The genomic alterations pre- and post-ATLL transformation in the two patients who developed ATLL during follow-up is of particular interest. The first case (HAM#1), who had the $CADM1^{+}CD7^{neg}$ dominant ($CD7^{dim} < CD7^{neg}$) pattern on flow cytometry analysis, had mutations in *GPR39* (48.2%: VAF), *MGAM* (46.1%), *JPH1* (45.0%), *DDR2* (43.3%), and *PRICKLE2* (30.2%), as well as two separate mutations that were detected in *PLCG1* (45.0% at chromosome 20 41173750 and 42.1% at chromosome 20 41137783). Kataoka et al. (17) have previously described that the *PLCG1* mutation was the most common somatic mutation observed in 36% of patients with ATLL. In this case, proviral integration site analysis demonstrated that leukemic cells had dominant clones identical to those detected in HTLV-1–infected cells prior to ATLL transformation with the emergence of additional mutations such as *KDM6A* (1.7%), *IRF2BP2* (1.7%), *ACAN* (1.7%), and *FLT3* (1.4%) being detected at the time of ATLL transformation at lower VAF (Fig. 2).

The other case (HAM#25), which had the alternate pattern $CADM1^{+}CD7^{dim}$ ($CD7^{dim} > CD7^{neg}$) on flow cytometry analysis, had mutations in *POT1* (39.7%), *DDX3X* (25.8%), *TET2* (21.9%), *GATA3* (1.9% on chromosome 10 8058764 and 1.2% on chromosome 10 8055844), *VAV1* (1.4%), *PRPS1L1* (1.5%), *TSC1* (1.7%), and *CSNK1A1* (1.0%) (*SI Appendix*, Table S4). *POT1* is known to be mutated in 10% of patients with ATLL (17). Along with *TP53* and *CDKN2A*, *POT1* is known to be related to DNA repair and telomere maintenance. In this case, proviral integration site analysis also demonstrated that leukemic cells carried dominant clones identical to that detected prior to transformation (Fig. 2). Furthermore, we identified additional somatic mutations in *CCR7* and deletion in *CDKN2A*. Interestingly, loss of *CDKN2A* function is associated with poor prognosis in ATLL (34). Importantly, the percentage of abnormal lymphocytes in the peripheral blood of these two patients who subsequently developed aggressive ATLL over a short period of time was initially less than 5%, which did not meet the diagnostic criteria for smoldering ATL, but had dominant clonal expansion with somatic mutations that are known to be associated with ATLL. The current diagnostic criteria of smoldering ATLL (which includes pathological evaluation and abnormal lymphocytes over 5%) may not be sufficient to capture these patients. Thus, it would be crucial to accumulate further data on the pathological characterization, including clonality and genomic alterations pre- and post-ATLL transformation samples to consider these features into account when making the diagnosis of ATLL.

By targeting and eradicating HTLV-1–infected cells with clonal expansion prior to the development of deleterious somatic mutations, we may be able to prevent the onset of ATLL in high-risk patients. Recently, mogamulizumab, a humanized anti-CCR4 monoclonal antibody, was shown to significantly reduce the number of HTLV-1–infected cells and ATLL cells (21, 35). We also demonstrated the high CCR4 positivity with 93.48 (±5.18)% in HTLV-1–infected cells from the 37 patients who had more than 25% of $CD4^{+}$ cells positive for CADM1 (*SI Appendix*, Fig. S2). The two cases (HAM#1 and HAM#25, who subsequently developed ATLL) had relatively high levels of CCR4 (shown as red dots in *SI Appendix*, Fig. S2). Further prospective clinical research should evaluate whether the use of therapy targeting ATLL prone cells such as anti-CCR4 therapy can improve the survival outcomes of HAM/TSP and HTLV-1 carriers who are at exceptionally increased risk for developing ATLL.

This study describes genomic changes occurring in patients with HAM/TSP at the actual time of their ATLL transformation, which are findings that, to our knowledge, have never been reported thus far. Since one of the limitations of our study includes the fact that the analyses were not performed based on a single-cell analysis, thereby making it impossible to accurately determine which mutation combination constructed the actual major clone, future studies should include single-cell analysis to overcome this issue. Nevertheless, our study proposes the importance of evaluating patients for high-risk patterns of clonally expanded HTLV-1–infected cells with ATLL-associated genomic alterations and suggests the emergence of these mutated

dominant clones prior to the actual onset of ATLL. Technically, the true definition of high-risk patterns that warrant early intervention must be further evaluated in prospective studies utilizing not only pre- and post-genomic samples of patients who actually transform into ATLL from HTLV-1–infected individuals including HAM/TSP, but also through the long-term follow-up of those who never develop ATLL. In order to determine an accurate cutoff value of the "high" vs. "low" risk, many more sample numbers as well as longer follow-up time are required.

In summary, we report here the mortality, prevalence, and incidence of ATLL in patients with HAM/TSP from prospective cohorts. It would be critical to determine the risks of ATLL transformation in patients with HAM/TSP in other countries and areas around the world outside of Japan as well, and we believe that multicenter analysis of HAM/TSP patient cohorts of different ethnic and genetic diversities needs to be conducted. These studies should not only focus on epidemiology, but also include evidence on the detection and analysis of high-risk patterns of clonally expanded HTLV-1–infected cells with ATLL-associated genomic alterations. This will allow for an early intervention to those patients with high risk of ATLL transformation and will also elucidate the mechanisms leading to leukemic transformation. To achieve these goals, multicenter, international, large-scale prospective studies utilizing pre- and post-ATLL progression samples from HTLV-1 carriers and patients with HAM/TSP are warranted.

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1. B. J. Poiesz *et al*., 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. U.S.A.* **77**, 7415–7419 (1980).
2. A. Gessain, O. Cassar, Epidemiological aspects and world distribution of HTLV-1 infection. *Front. Microbiol.* **3**, 388 (2012).
3. M. Osame *et al*., HTLV-I associated myelopathy, a new clinical entity. *Lancet* **1**, 1031–1032 (1986).
4. A. Gessain *et al*., Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet* **2**, 407–410 (1985).
5. T. Uchiyama, J. Yodoi, K. Sagawa, K. Takatsuki, H. Uchino, Adult T-cell leukemia: Clinical and hematologic features of 16 cases. *Blood* **50**, 481–492 (1977).
6. Y. Tagaya, M. Matsuoka, R. Gallo, 40 years of the human T-cell leukemia virus: Past, present and future. *F1000Res.* **8**, F1000 Faculty Rev 228 (2019).
7. F. Martin, Y. Tagaya, R. Gallo, Time to eradicate HTLV-1: An open letter to WHO. *Lancet* **391**, 1893–1894 (2018).
8. S. H. Swerdlow *et al*., *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues* (IARC, Lyon, France, 2008).
9. H. Katsuya *et al*.; ATL-Prognostic Index Project, Treatment and survival among 1594 patients with ATL. *Blood* **126**, 2570–2577 (2015).
10. C. R. Bangham, A. Araujo, Y. Yamano, G. P. Taylor, HTLV-1-associated myelopathy/tropical spastic paraparesis. *Nat. Rev. Dis. Primers* **1**, 15012 (2015).
11. W. J. Harrington, Jr *et al*., Clinical spectrum of HTLV-I in south Florida. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **8**, 466–473 (1995).
12. F. Martin, A. Fedina, S. Youshya, G. P. Taylor, A 15-year prospective longitudinal study of disease progression in patients with HTLV-1 associated myelopathy in the UK. *J. Neurol. Neurosurg. Psychiatry* **81**, 1336–1340 (2010).
13. D. U. Gonçalves *et al*., Simultaneous occurrence of HTLV-I associated myelopathy, uveitis and smouldering adult T cell leukaemia. GIPH (Interdisciplinary HTLV-I/II Research Group). *Int. J. STD AIDS* **10**, 336–337 (1999).
14. M. Iwanaga *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* **116**, 1211–1219 (2010).
15. S. Firouzi *et al*., Clonality of HTLV-1-infected T cells as a risk indicator for development and progression of adult T-cell leukemia. *Blood Adv.* **1**, 1195–1205 (2017).
16. M. Nagai *et al*., Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: High proviral load strongly predisposes to HAM/TSP. *J. Neurovirol.* **4**, 586–593 (1998).
17. K. Kataoka *et al*., Integrated molecular analysis of adult T cell leukemia/lymphoma. *Nat. Genet.* **47**, 1304–1315 (2015).
18. A. L. G. Coler-Reilly *et al*., Nation-wide epidemiological study of Japanese patients with rare viral myelopathy using novel registration system (HAM-net). *Orphanet J. Rare Dis.* **11**, 69 (2016).
19. Y. Yamano *et al*., Correlation of human T-cell lymphotropic virus type 1 (HTLV-1) mRNA with proviral DNA load, virus-specific $CD8^+$ T cells, and disease severity in HTLV-1-associated myelopathy (HAM/TSP). *Blood* **99**, 88–94 (2002).
20. M. Kuramitsu *et al*., Development of reference material with assigned value for human T-cell leukemia virus type 1 quantitative PCR in Japan. *Microbiol. Immunol.* **62**, 673–676 (2018).
21. T. Sato *et al*., Mogamulizumab (Anti-CCR4) in HTLV-1-associated myelopathy. *N. Engl. J. Med.* **378**, 529–538 (2018).
22. S. Kobayashi *et al*., Advanced human T-cell leukemia virus type 1 carriers and early-stage indolent adult T-cell leukemia-lymphoma are indistinguishable based on CADM1 positivity in flow cytometry. *Cancer Sci.* **106**, 598–603 (2015).
23. S. Kobayashi *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.* **20**, 2851–2861 (2014).
24. M. Yoshida, M. Osame, K. Usuku, M. Matsumoto, A. Igata, Viruses detected in HTLV-I-associated myelopathy and adult T-cell leukaemia are identical on DNA blotting. *Lancet* **1**, 1085–1086 (1987).
25. Z. Sondka *et al*., The COSMIC cancer gene census: Describing genetic dysfunction across all human cancers. *Nat. Rev. Cancer* **18**, 696–705 (2018).
26. M. Yamagishi *et al*., Polycomb-mediated loss of miR-31 activates NIK-dependent NF-κB pathway in adult T cell leukemia and other cancers. *Cancer Cell* **21**, 121–135 (2012).
27. H. Li *et al*.; 1000 Genome Project Data Processing Subgroup, The sequence alignment/map (SAM) format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009).
28. K. Cibulskis *et al*., Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat. Biotechnol.* **31**, 213–219 (2013).
29. K. Wang, M. Li, H. Hakonarson, ANNOVAR: Functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res.* **38**, e164 (2010).
30. A. Manouchehrinia, R. Tanasescu, C. R. Tench, C. S. Constantinescu, Mortality in multiple sclerosis: Meta-analysis of standardised mortality ratios. *J. Neurol. Neurosurg. Psychiatry* **87**, 324–331 (2016).
31. R. M. N. Marcusso *et al*., Dichotomy in fatal outcomes in a large cohort of people living with HTLV-1 in São Paulo, Brazil. *Pathogens* **9**, E25 (2019).
32. K. Arisawa *et al*., Evaluation of adult T-cell leukemia/lymphoma incidence and its impact on non-Hodgkin lymphoma incidence in southwestern Japan. *Int. J. Cancer* **85**, 319–324 (2000).
33. J. Makiyama *et al*., $CD4^+$ $CADM1^+$ cell percentage predicts disease progression in HTLV-1 carriers and indolent adult T-cell leukemia/lymphoma. *Cancer Sci.* **110**, 3746–3753 (2019).
34. K. Kataoka *et al*., Prognostic relevance of integrated genetic profiling in adult T-cell leukemia/lymphoma. *Blood* **131**, 215–225 (2018).
35. T. Ishida *et al*., The CC chemokine receptor 4 as a novel specific molecular target for immunotherapy in adult T-cell leukemia/lymphoma. *Clin. Cancer Res.* **10**, 7529–7539 (2004).