Human T-cell lymphotropic virus *HBZ* and *Tax* mRNA expression are associated with specific clinicopathological features in adult T-cell leukemia/lymphoma

Authors :

Kyohei Yamada[1], Hiroaki Miyoshi[1],Noriaki Yoshida[1,2], Joji Shimono[1,3], Kensaku Sato[1], Kazutaka Nakashima[1], Mai Takeuchi[1], Fumiko Arakawa[1], Naoko Asano[1,4], Eriko Yanagida[1], Masao Seto[1], and Koichi Ohshima[1]

Affiliations:

[1] Department of Pathology, Kurume University School of Medicine, Kurume, Japan;

[2] Department of Clinical Studies, Radiation Effects Research Foundation, Hiroshima, Japan;

[3] Department of Hematology, Hokkaido University Faculty of Medicine, Sapporo, Japan;

[4] Department of Molecular Diagnostics, Nagano Prefectural Shinshu Medical Center, Suzaka, Japan

Short running title:

Expression of HBZ and Tax mRNA in ATLL

Corresponding author:

Hiroaki Miyoshi, MD, PhD

Department of Pathology, School of Medicine, Kurume University

Asahimachi 67, Kurume, Fukuoka, 830-0011, Japan

Phone: +81-942-31-7547, Fax: +81-942-31-0342

E-mail: miyoshi_hiroaki@med.kurume-u.ac.jp

Abstract

Adult T-cell leukemia/lymphoma (ATLL) is caused by human T-cell leukemia virus type 1 (HTLV-1). HTLV-1-associated mRNA, including *HBZ* and *tax,* is deeply involved in the pathogenesis of ATLL. Using 88 ATLL tissue samples, we performed *in situ* mRNA analysis of *HBZ* and *tax*, and investigated its association with clinicopathological characteristics of ATLL. The median value of *HBZ* signals (/ 1000 ATLL cells) was 795.2 (range: 0.4-4013.1) and of *tax* signals (/ 1000 ATLL cells) was 5.1 (range: 0.1-891.2). The low expression *HBZ* group displayed significant increase in the number of skin lesion ($P = .0283$). The high expression *tax* group displayed significant increase in the number of PD-1-positive tumor-infiltrating lymphocytes ($P < .0001$). In addition, we identified patients with very high expression of *tax* signals (400 or more signals/ 1000 ATLL cells). These patients displayed significant reductions in the expression of HLA class I ($P = .0385$) and β2M ($P = .0124$). Moreover, these patients displayed significantly poor overall survival (median survival time [MST] 7.7 months, 95% confidence interval [CI] [4.7-NA]), compared with the survival in patients with less than 400 *tax* signals (MST 22.6 months, 95% CI [13.7-41.7]) ($P = .0499$). These results suggest that Tax-mediated treatment of ATLL should be performed carefully in the high expression *tax* group. More detailed studies could elucidate the clinicopathological significance of *HBZ* and *tax* mRNA expressions in ATLL.

Introduction

Adult T-cell leukemia/lymphoma (ATLL) is defined as a mature T-cell neoplasm caused by human T-cell leukemia virus type 1 (HTLV-1).[1] ATLL has a long incubation period, and only less than 5% of HTLV-1 carriers develop ATLL in their lifetime.[1,2] According to Shimoyama,[3] ATLL is classified into four clinical subtypes: acute, lymphoma, smoldering, and chronic type. Of the four clinical subtypes, acute, lymphoma, and chronic types, are especially known to have poor prognosis.[4,5]

Previous studies with deep sequencing have revealed that most HTLV-1-infected T cells contain a single copy of integrated HTLV-1 provirus[6] and that each host contains a large number (often 10^4-10^5) of distinct HTLV-1-infected T-cell clones.[6,7] Moreover, the integration sites of the provirus could be involved in clone selection and clinical subtype.[7]

Like other complex retroviruses, HTLV-1 provirus encodes long terminal repeat (LTR) on both 5' and 3' sides, and structural genes (*gag*, *pol*, *env*) downstream of 5'LTR.[8,9] Furthermore, regulatory genes (*tax* and *rex*) and accessory genes (*p12*, *p13*, *p30,* and *HTLV-1 bZIP factor* (*HBZ*)) are encoded in pX region.[8,9] Of these viral genes, *HBZ* is only encoded on the minus strand of the provirus and transcribed from 3'LTR. On the other hand, other viral genes including *tax* are encoded on the plus strand and transcribed from 5'LTR.[8,9]

Tax plays an important role in the early stages of tumorigenesis through various mechanisms.[10-16] It is considered to be involved in tumorigenesis *in vivo*[17]; however, the expression of *tax* in ATLL could be suppressed due to genetic and epigenetic alterations.[18-21] While *tax* mRNA is frequently undetectable in ATLL cells, it has been reported that *HBZ* mRNA is expressed in almost all ATLL cells.[22] *HBZ* suppresses the Tax-mediated transcription from 5'LTR by interacting with CREB-2.[23] On the other hand, *HBZ* promotes cell proliferation and migration,[22,24-26] and induces T-cell lymphoma *in vivo.*[27] Interestingly, *HBZ* has different functions as RNA and protein.[28]

From these results, both *HBZ* and *tax* are considered to be deeply associated in the pathogenesis of ATLL. However, there are scant data concerning HTLV-1-related mRNA including *HBZ* and *tax* in human formalin-fixed, paraffin-embedded (FFPE) tissue samples. In this study, we detected *HBZ* and *tax* mRNA on FFPE tissue samples

using *in situ* hybridization (ISH), to investigate their association with clinicopathological characteristics in ATLL patients.

Materials and methods

Patients and samples

In this study, 88 biopsy samples from newly diagnosed ATLL patients were examined, which are included in our previous studies[29-31]. Tissue microarrays (TMAs) including all 88 samples were created with a 2-mm core diameter. Each sample was reviewed according to the World Health Organization classification[1] by two experienced hematopathologists (H. M. and K. O.). The use of patient materials and clinical information was approved by the Research Ethics Committee of Kurume University and was in accordance with the Declaration of Helsinki.

In situ mRNA analysis

ISH was performed on FFPE samples using RNAscope 2.5 HD Reagent Kit-BROWN [Advanced Cell Diagnostics (ACD), Hayward, CA] according to the manufacturer's protocols. In brief, 2.5-μm-thick sections from the TMA samples were created. All sections were baked at 60°C for 1 hour and deparaffinized; then other pretreatments were performed appropriately. Hybridization was performed at 40°C for 2 hours using HybEZ hybridization oven (ACD). *HBZ*-specific probe (ACD) and *tax*-specific custom-designed probe (ACD, targeting 7394-7812 of U19949.1) were used. Hs-PPIB (ACD) and DapB (ACD) were used for positive and negative control of ISH assay, respectively (supplemental figure 1). ISH of *HBZ* and *tax* was validated using MT-4 (HTLV-1-immortalized cell line) and Jurkat (T-cell acute lymphoblastic leukemia cell line). Amplification and detection of signals were performed properly, and then

hematoxylin was used for counterstain. All samples were scanned by using Aperio ScanScope AT2 (Leica Biosystems, Vista, CA, USA). Dot-like signals were counted at high magnification (40 diameters) on 10 randomly selected fields. The number of signals per 1000 ATLL cells were calculated. High expression was indicated when not less than the median value of *HBZ* or *tax* signals was stained.

Immunohistochemical analysis

Immunohistochemistry (IHC) was performed as previously reported[29-31]. The antibodies used for IHC targeted CD4, CD30, Ki-67 (MIB-1), CCR4, FoxP3, GATA3, IRF4, HLA class I, β2 microglobulin (β2M), HLA class II, PD-1, and PD-L1

(Supplemental Table 1). As previously reported[29-31], neoplastic PD-L1 (nPD-L1) was considered positive when 50% or more neoplastic cells were stained. PD-L1-positive nonmalignant stromal cells were counted as microenvironmental PD-L1 (miPD-L1), and miPD-L1 was considered as positive when 10 or more nonmalignant stromal cells were stained per high power field (HPF)[29-31]. Other than PD-L1, it was considered as positive when 30% or more neoplastic cells were stained [29-31]. PD-1-positive tumor infiltration lymphocytes (TILs) were counted in up to 5 representative HPFs, and the average and median values were calculated as previously reported[29-31].

Statistical analysis

Clinicopathological characteristics of ATLL patients were compared by Fisher's exact test (2-sided), Mann-Whitney's U test and Spearman's rank correlation analysis. Overall survival (OS) was defined as the time from the day of diagnosis to the day of death or last follow up. Progression-free survival (PFS) was defined as the time from the day of diagnosis to the day of first progression or death. OS and PFS were estimated using the Kaplan-Meyer method and compared using log-rank test. Univariate and multivariate analyses for survival time were performed using the Cox proportional regression model. $P < .05$ was considered as statistically significant. All statistical analyses were performed by EZR ver. 1.32[32].

Results

Clinicopathological characteristics of ATLL patients

Table 1 summarizes the clinicopathological characteristics of 88 newly diagnosed ATLL patients. The median age was 66 years (range: 35-85), including 54 men and 34 women. The median follow-up period was 12.1 months (range: 0-105.2). Complete response (CR) or complete response unconfirmed (CRu) was achieved in 29% (22/76) of patients. According to Shimoyama classification[3], 2% (1/61); 44% (31/71); and 55% (39/71) of patients were of smoldering, acute and lymphoma types, respectively. No patient was of chronic type in this study. High or high-intermediate scores of international prognostic index (IPI) were observed in 56% (46/82) of patients. High scores of Japan Clinical Oncology Group prognostic index (JCOG-PI) were observed in 39% (33/84) patients.

Regarding IHC of tumor-immunity-related proteins, patients were positive for both HLA class I and β2M for membrane (39% [30/78]); HLA class II (34% [28/82]); neoplastic PD-1 (nPD-1) (16% [14/87]); and nPD-L1 (5% [4/87]). PD-1-positive TIL

ranged from 0 to 187.0 counts/HPF with an average value of 7.4 counts/HPF and with a median value of 0 count/HPF. miPD-L1 ranged from 0 to 171.0 counts/HPF with an average value of 27.2 counts/HPF and with a median value of 22.0/HPF.

In situ hybridization of *HBZ* and *tax* mRNA

HBZ and *tax* mRNA were evaluated by ISH for 88 ATLL samples shown in Table 1. Figure 1 shows the histogram and scatter plot of *HBZ* and *tax* mRNA. *HBZ* signals ranged from 0.4-4013.1 /1000 ATLL cells, with an average value of 916.7 /1000 ATLL cells and with a median value of 795.2 /1000 ATLL cells. *Tax* signals ranged from 0.1-891.2 /1000 ATLL cells, with an average of 80.4 /1000 ATLL cells, and a median of 5.1 /1000 ATLL cells. Both *HBZ* and *tax* signals showed extremely high expression in a few cases. There was no significant correlation between *HBZ* and *tax* signals (ρ = - .00367, P = .973; Figure 1C). Representative samples of *HBZ* and *tax* signals were presented in Figure 2A and 2B, respectively. Target-specific signals showed dot-like pattern and were observed in ATLL cells.

Clinicopathological comparison according to the expression of *HBZ* mRNA

HBZ has been known to be expressed in all ATLL cells. However, the clinicopathological characteristics due to the difference in the expression level have remained unknown. Therefore, we showed a clinicopathological comparison between the high expression group and the low expression group of *HBZ* (Table 2). Notably, the low-expression group of *HBZ* displayed significant increases in skin lesions (P = .0283), Ann Arbor stage III or IV (P = .00696) and IPI high-intermediate or high (P = .0461). Small or medium cell variants, compared with other variants, were significantly more frequent in the low-expression group of *HBZ* (P = .000771).

HBZ is known to be closely associated with immune-suppressive phenotypes of HTLV-1 infected cells[33]. Therefore, we evaluated the expression level of *HBZ* and the phenotypes of ATLL. HBZ-Transgenic mice and in vitro analysis showed that *HBZ* induces *Foxp3* expression[33], however, no significant association was observed between the expression level of *HBZ* and FOXP3 expression in this study (Table 2). In addition, there was no difference between the expression level of *HBZ* and the other protein expression (Table 2). However, by comparing each protein expression frequency with the expression level of *HBZ*, weak but significant correlation was observed between *HBZ* and IRF4 (ρ = .326; P = .0288), between *HBZ* and nPD-L1 (ρ = - .264; P = .0135), and between *HBZ* and miPD-L1 (ρ = -.223; P = .038) (Figure 3).

Clinicopathological comparison according to the expression of *tax* mRNA

Regarding the expression level of *tax*, we first performed clinicopathological comparison between the high expression group and the low expression group of *tax* (Table 3). Notably, the high-expression group of *tax* displayed significant increases in lactate dehydrogenase (LDH) activity ($P = .00209$), splenomegaly ($P = .00721$), bone marrow (BM) involvement (P =.0295), CD30 positivity (P = .00434), nPD-1 positivity ($P = .0385$) and PD-1-positive TIL ($P < .0001$). In addition, the high-expression group of *tax* displayed significant reductions in male ($P = .0481$) and radiation ($P = .0298$).

Subsequently, we compared each protein expression frequency with the expression level of *tax*; then, weak but significant correlation was observed between *tax* and age ($\rho = .225$; $P = .00352$), between *tax* and CD30 ($\rho = .303$; $P = .00435$), between *tax* and GATA3 ($\rho = .237$; $P = .0263$), between *tax* and ß2M for membrane ($\rho = -.281$; $P = .0298$), between *tax* and nPD-1 ($\rho = .259$; $P = .0156$), between *tax* and nPD-L1 ($\rho = .297$; $P = .00527$), and between *tax* and PD-1-positive TIL ($\rho = .397$; $P = .000138$) (Figure 4).

OS in ATLL patients according to the expression of *HBZ* and *tax* mRNA

We evaluated the association between the expression level of *HBZ* and prognosis, however, there was no significant difference in OS between the high expression group and the low expression group of *HBZ* (Log-rank $P = .834$; supplemental figure 2A). Likewise, there was no significant difference in OS between the high expression group and the low expression group of *tax* (Log-rank $P = .365$; supplemental figure 2B).

Clinicopathological characteristics and OS in ATLL patients with extremely high expression of *tax* mRNA

Tax is not expressed in many ATLL patients[18-21]. However, Tax has been reported as a viral oncoprotein[10-16]; it is highly immunogenic and can be the target for cytotoxic T lymphocytes (CTLs)[34-40]. In this study, we identified seven patients with extremely high expression of *tax* (not less than 400 signals /1000 ATLL cells). Among these pathological characteristics, only 1/7 patients (14%) were positive for HLA class I for membrane and no patient was positive for ß2M for membrane. The group of 400 or more *tax* signals displayed significant reductions in the membranous positivity of HLA class I and ß2M compared with the group of less than 400 *tax* signals ($P = .0385$, .0124, respectively) (Table 4). The group of 400 or more *tax* signals also displayed significant increases in LDH activity ($P = .0170$) and splenomegaly ($P = .0326$). These group (n=6,

median survival time [MST] 7.7 months, 95% confidence interval [CI] [4.7-NA]) had significantly inferior OS compared with the group with less than 400 *tax* signals (n=78, MST 22.6 months, 95%CI [13.7-41.7]), (P = .0499; Figure 5).

Prognostic factors in ATLL patients

We analyzed the prognostic factors affecting OS and PFS in ATLL patients (Supplemental Table 3 and 4). In OS (n=74), univariate analyses identified the following variables as prognostic factors: age over 70 [hazard ratio(HR), 2.196; 95%CI, 1.209-3.986; P = 0.00974), miPD-L1 expression (HR, 0.545; 95%CI, 0.307-0.970; and P = 0.0390), HLA class I and ß2M expression (HR, 0.0386; 95%CI, 0.253-0.964; and P = 0.0386) and HLA class II expression (HR, 0.398; 95%CI, 0.205-0.772; and P = 0.00640). Multivariate analyses revealed that age over 70 remained a significant prognostic factor (HR, 2.262; 95%CI, 1.171-4.369; and P = 0.0150). In PFS (n=74), univariate analyses identified *tax* signals not less than 400 as a prognostic factor (HR, 12.570; 95%CI, 2.186-72.340; and P = 0.00457), but was not remain as a significant prognostic factor in multivariate analyses (P = 0.158).

Discussion

In this study, we demonstrated three new findings on FFPE samples in ATLL patients as follows: (i) some patients showed low expression of *HBZ*; (ii) some clinicopathological characteristics including antitumor immunity were significantly associated with expression of *HBZ* and *tax*; and (iii) the extremely high-expression group of *tax* was significantly associated with loss of HLA class I/ß2M and poor prognosis.

In our *in situ* mRNA analysis, the median value of *HBZ* signals was about 800/1000 ATLL cells with low-expression group of *HBZ* observed. Satou et al reported that *HBZ* mRNA was universally expressed in almost all ATLL cells[22]. *HBZ* mRNA suppresses cell apoptosis via transcription of *survivin*[25] and promotes cell proliferation and migration[22,24-26]. On the other hand, HBZ protein has different functions from *HBZ* mRNA; *HBZ* protein promotes transcription of immunity-related genes including *FoxP3*, interleukin 2 (*IL-2),* and *IL-10*; and promotes cell proliferation and apoptosis[28]. In this study, it cannot be completely ruled out that the low-expression group of *HBZ* was observed due to RNA degradation in FFPE samples[41]. However, a recent study with integrated molecular analysis reported that some ATLL patients displayed low expression of *HBZ* [42]. Furthermore, another study reported that *HBZ* mRNA expression

was suppressed in some HTLV-1-infected cell lines and fresh ATLL cells[43].

This study revealed that the high-expression group of *HBZ* displayed significant reduction in skin lesion, progressive Ann Arbor stage and HI or more risk of IPI; these results were different from the previous studies with model mice[24,27]. Sato et al reported that skin lesion of CD4-positive T-cells increased in the transgenic (Tg) mice expressing *HBZ* on CD4-positive T-cells, compared to non-Tg mice[27]. These results are suggested to be derived from *HBZ*-mediated cell proliferation *in vitro* and *in vivo*[22,24-27], and from *HBZ*-mediated migration by inducing CCR4 via GATA3[24]. However, no significant differences were observed between GATA3, CCR4, *HBZ* and organ infiltration in this study (data not shown). More detailed studies of *HBZ* expression will validate the association between *HBZ* expression and biology in ATLL tissue samples.

Unlike *HBZ*, the median value of *tax* signals was around 5/1000 ATLL cells in the present study: *tax* expression was suppressed in most patients. However, a small number of patients displayed extremely high expression of *tax*. A recent study showed that most patients displayed low expression of *tax* and that 1/57 (1.8%) ATLL patients displayed high expression of *tax*[42]. In this study, the high-expression group of *tax* displayed significant increases in splenomegaly and BM involvement. Tax has various functions[11], including activation of the transcription of HTLV-1-related genes from 5'LTR [10], collapse of cell cycle checkpoint[12], activation of nuclear factor kappa B (NF-κB)[13,14], and inducing genomic instability and chromosomal aneuploidy via TAX1BP2[15] and RanBP1[16]. Especially, NF-κB was activated in *tax*-Tg mice with ATLL-like phenotype[17]. Splenomegaly and infiltration into many organs including BM and spleen are observed in *tax*-Tg mice and severe combined immunodeficiency mice transferred lymphoma cells from *tax*-Tg mice[17]. For the first time, we found the association between *tax* and splenomegaly, and between *tax* and BM involvement in ATLL tissue samples. More detailed studies of *tax* expression are needed to elucidate the mechanism of these associations. In addition, 5'-LTR deletion[18] and epigenetic alterations including DNA methylation of 5'-LTR[19-21] may occur in the patients with low expression of *tax*. Further studies are needed to validate biological significance of *tax* expression level in ATLL tissue samples.

In this study, weak but significant positive correlation was observed between *tax* and nPD-1, between *tax* and nPD-L1, and between *tax* and PD-1-positive TIL. Moreover, the group with 400 or more signals of *tax* displayed poor prognosis and reductions in HLA

class I, ß2M or both for membrane ($HLA^{m+}ß2M^{m+}$). However, 400 or more signals of *tax* did not have the prognostic significance in multivariate analysis. Tax is most immunogenic of HTLV-1-associated molecules[34-39] and Tax-specific CTL response was activated in some ATLL patients who achieved complete response after allogeneic hematopoietic stem cell transplantation[40]. Tax-specific CTLs are being applied clinically[44], however, "T-cell exhaustion" [45,46] is induced due to PD-1 expression on Tax-specific CTLs[47]. CTLs recognize HLA class I/ß2M [48]. The mechanism of HLA class I/ß2M loss in this study is suggested to be hypermethylation, loss-of-function mutations and copy number deletion of HLA class I/ß2M genes[42]. We have previously reported that ATLL patients with $HLA^{m+}ß2M^{m+}$ is significantly associated with high expression of miPD-L1 and a good prognosis compared to patients in other groups.[30] There was no significant association with miPD-L1 expression in this study. Various numbers of PD-1-TIL were observed in this study (supplemental figure 3), and we also analyzed the prognostic significance of PD-1-TIL positivity; there were no significant differences (data not shown). Mahgoub et al have reported that sporadic and transient Tax expression by various cytotoxic stresses in cooperation with HBZ is critical for survival of the whole population.[49] We calculated *tax*/*HBZ* ratio (supplemental figure 4) and investigated the clinicopathological features; there were no new significant differences (data not shown). In this study, we showed for the first time that the high expression of *tax* is important in evading antitumor immunity including loss of HLA class I/ß2M in ATLL tissue samples. Furthermore, the high expression of *tax* may be associated with poor prognosis, so Tax-targeted treatment such as Tax-targeted vaccine therapy[44] should be performed carefully. Comprehensive analysis with tissue microenvironment including various immune cells is necessary for elucidating the biological significance of *tax* expression in antitumor immunity.

Weak but significant positive correlation was observed in *HBZ* and IRF4 in the present study. *HBZ* suppresses gene expression downstream of the classical pathway of NF-κB including IRF4 due to inhibiting the binding of p65 to DNA and inducing the degradation of p65[50]. However, in ATLL patients, that gain-of-function mutations are highly enriched for T-cell receptor/NF-κB signaling, including IRF4[42]. Moreover, a recent study with functional screening in ATLL cell lines revealed that *HBZ* promoted the expression of basic leucine zipper ATF-like transcription factor 3 (BATF3) and its downstream targets including IRF4, and that both BATF3 and IRF4 were necessary for the gene expression and proliferation of ATLL[51]. Further studies are expected to clarify the detailed mechanism of *HBZ* and IRF4 expression in ATLL.

There were some limitations in the present study. First, this study was conducted only with patients diagnosed by biopsy samples. Because previous HTLV-1-clonality analysis suggests that HTLV-1 clone size is different in peripheral blood and in lymph nodes[52], further studies are needed to validate our results, using all clinical subtypes of ATLL patients diagnosed by various organ samples. Second, this study is conducted only with mRNA regarding *HBZ* and *tax*, and is not conducted with gene mutation analysis. More detailed studies conducted with integrated analysis including DNA, RNA and protein on ATLL tissue samples are required. Last, sample size is relatively small in this study although several statistical differences were found in the present study. Further large-scale studies are needed to confirm our results.

In conclusion, we demonstrated for the first time that the expression of *HBZ* and *tax* mRNA is associated with clinicopathological characteristics including antitumor immunity on ATLL tissue samples. These results suggested that detailed *in situ* mRNA analysis on FFPE samples may identify the association between the expression of HTLV-1-related mRNA and biology. Multidisciplinary analysis using DNA, RNA, and protein of ATLL and HTLV-1 is necessary for more-detailed analysis of the pathophysiology and for applying the treatment and prevention of ATLL.

Acknowledgements

All authors thank Mayumi Miura, Kanoko Miyazaki, Chie Kuroki, and Kaoruko Nagatomo for their technical assistance.

Disclosure of Conflicts of Interest

No relevant conflicts of interest to declare.

Supplementary information is available at Modern Pathology's website.

References

1. Ohshima K, Jaffe ES, Yoshino T, Siebert R. Adult T-cell Leukemia/Lymphoma. In: Sweldrow SH, Campo E, Harris NL, et al. World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues. Revised 4th ed. Lyon: IARC Press, 2017. p.363-367.
2. Tajima K, Hinuma Y. Epidemiology of HTLV-I/II in Japan and the World. In: Takatsuki K, Hinuma Y, Yoshida M editors. Advances in adult T-cell leukemia and HTLV-I research (Gann Monograph on Cancer Research). Tokyo: Japan Scientific

Societies Press, 1992. p.129-149.
3. 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;79:428-437.
4. Katsuya H, Ishitsuka K, Utsunomiya A, Hanada S, Eto T, Moriuchi Y, et al. Treatment and survival among 1594 patients with ATL. Blood. 2015;126:2570-2577.
5. Takasaki Y, Iwanaga M, Imaizumi Y, Tawara M, Joh T, Kohno T, et al. Long-term study of indolent adult T-cell leukemia-lymphoma. Blood. 2010;115:4337-4343.
6. Cook LB, Rowan AG, Melamed A, Taylor GP, Bangham CR. HTLV-1-infected T cells contain a single integrated provirus in natural infection. Blood. 2012;120:3488-3490.
7. Gillet NA, Malani N, Melamed A, Gormley N, Carter R, Bentley D, et al. The host genomic environment of the provirus determines the abundance of HTLV-1-infected T-cell clones. Blood. 2011;117:3113-3122.
8. Matsuoka M, Jeang KT. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. Nat Rev Cancer. 2007;7:270-280.
9. Bangham CRM, Matsuoka M. Human T-cell leukaemia virus type 1: parasitism and pathogenesis. Philos Trans R Soc Lond B Biol Sci. 2017;372.
10. Nyborg JK, Egan D, Sharma N. The HTLV-1 Tax protein: revealing mechanisms of transcriptional activation through histone acetylation and nucleosome disassembly. Biochim Biophys Acta. 2010;1799:266-274.
11. Grassmann R, Aboud M, Jeang KT. Molecular mechanisms of cellular transformation by HTLV-1 Tax. Oncogene. 2005;24:5976-5985.
12. Iwanaga R, Ohtani K, Hayashi T, Nakamura M. Molecular mechanism of cell cycle progression induced by the oncogene product Tax of human T-cell leukemia virus type I. Oncogene. 2001;20:2055-2067.
13. Suzuki T, Hirai H, Yoshida M. Tax protein of HTLV-1 interacts with the Rel homology domain of NF-kappa B p65 and c-Rel proteins bound to the NF-kappa B binding site and activates transcription. Oncogene. 1994;9:3099-3105.
14. Murakami T, Hirai H, Suzuki T, Fujisawa J, Yoshida M. HTLV-1 Tax enhances NF-kappa B2 expression and binds to the products p52 and p100, but does not suppress the inhibitory function of p100. Virology. 1995;206:1066-1074.
15. Ching YP, Chan SF, Jeang KT, Jin DY. The retroviral oncoprotein Tax targets the coiled-coil centrosomal protein TAX1BP2 to induce centrosome overduplication. Nat Cell Biol. 2006;8:717-724.

16. Peloponese JM, Jr., Haller K, Miyazato A, Jeang KT. Abnormal centrosome amplification in cells through the targeting of Ran-binding protein-1 by the human T cell leukemia virus type-1 Tax oncoprotein. Proc Natl Acad Sci USA. 2005;102:18974-18979.
17. Hasegawa H, Sawa H, Lewis MJ, Orba Y, Sheehy N, Yamamoto T, et al. Thymus-derived leukemia-lymphoma in mice transgenic for the Tax gene of human T-lymphotropic virus type I. Nat Med. 2006;12:466-472.
18. Tamiya S, Matsuoka M, Etoh K, Watanabe T, Kamihira S, Yamaguchi K, et al. Two types of defective human T-lymphotropic virus type I provirus in adult T-cell leukemia. Blood. 1996;88:3065-3073.
19. Koiwa T, Hamano-Usami A, Ishida T, Okayama A, Yamaguchi K, Kamihira S, et al. 5'-long terminal repeat-selective CpG methylation of latent human T-cell leukemia virus type 1 provirus in vitro and in vivo. J Virol. 2002;76:9389-9397.
20. Takeda S, Maeda M, Morikawa S, Taniguchi Y, Yasunaga J, Nosaka K, et al. Genetic and epigenetic inactivation of tax gene in adult T-cell leukemia cells. Int J Cancer. 2004;109:559-567.
21. Taniguchi Y, Nosaka K, Yasunaga J, Maeda M, Mueller N, Okayama A, et al. Silencing of human T-cell leukemia virus type I gene transcription by epigenetic mechanisms. Retrovirology. 2005;2:64.
22. Satou Y, Yasunaga J, Yoshida M, Matsuoka M. HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells. Proc Natl Acad Sci USA. 2006;103:720-725.
23. Gaudray G, Gachon F, Basbous J, Biard-Piechaczyk M, Devaux C, Mesnard JM. The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription. J Virol. 2002;76:12813-12822.
24. Sugata K, Yasunaga J, Kinosada H, Mitobe Y, Furuta R, Mahgoub M, et al. HTLV-1 viral factor HBZ induces CCR4 to promote T-cell migration and proliferation. Cancer Res. 2016;76(17):5068-5079.
25. Kinosada H, Yasunaga J, Shimura K, Miyazato P, Onishi C, Iyoda T, et al. HTLV-1 bZIP factor enhances T-cell proliferation by impeding the suppressive signaling of co-inhibitory receptors. PLoS Pathog. 2017. https://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1006120
26. Kinosada H, Yasunaga J, Shimura K, Miyazato P, Onishi C, Iyoda T, et al. Correction: HTLV-1 bZIP factor enhances T-cell proliferation by impeding the suppressive signaling of co-inhibitory receptors. PLoS Pathog. 2017.

https://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1006228
27. Satou Y, Yasunaga J, Zhao T, Yoshida M, Miyazato P, Takai K, et al. HTLV-1 bZIP factor induces T-cell lymphoma and systemic inflammation in vivo. PLoS Pathog. 2011. https://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1001274
28. Mitobe Y, Yasunaga J, Furuta R, Matsuoka M. HTLV-1 bZIP factor RNA and protein impart distinct functions on T-cell proliferation and survival. Cancer Res. 2015;75:4143-4152.
29. Miyoshi H, Kiyasu J, Kato T, Yoshida N, Shimono J, Yokoyama S, et al. PD-L1 expression on neoplastic or stromal cells is respectively a poor or good prognostic factor for adult T-cell leukemia/lymphoma. Blood. 2016;128:1374-1381.
30. Asano N, Miyoshi H, Kato T, Shimono J, Yoshida N, Kurita D, et al. Expression pattern of immunosurveillance-related antigen in adult T cell leukaemia/lymphoma. Histopathology. 2018;72:945-954.
31. Takeuchi M, Miyoshi H, Asano N, Yoshida N, Yamada K, Yanagida E, et al. Human leukocyte antigen class II expression is a good prognostic factor of adult T-cell leukemia/lymphoma. Haematologica. 2019;104:1626-1632.
32. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 2013;48:452-458.
33. Yasunaga J, Matsuoka M. Oncogenic spiral by infectious pathogens: Cooperation of multiple factors in cancer development. Cancer Sci. 2018;109:24-32.
34. Jacobson S, Shida H, McFarlin DE, Fauci AS, Koenig S. Circulating CD8+ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease. Nature. 1990;348:245-248.
35. Kannagi M, Harada S, Maruyama I, Inoko H, Igarashi H, Kuwashima G, et al. Predominant recognition of human T cell leukemia virus type I (HTLV-I) pX gene products by human CD8+ cytotoxic T cells directed against HTLV-I-infected cells. Int Immunol. 1991;3:761-767.
36. Parker CE, Daenke S, Nightingale S, Bangham CR. Activated, HTLV-1-specific cytotoxic T-lymphocytes are found in healthy seropositives as well as in patients with tropical spastic paraparesis. Virology. 1992;188:628-636.
37. Elovaara I, Koenig S, Brewah AY, Woods RM, Lehky T, Jacobson S. High human T cell lymphotropic virus type 1 (HTLV-1)-specific precursor cytotoxic T lymphocyte frequencies in patients with HTLV-1-associated neurological disease. J Exp Med. 1993;177:1567-1573.
38. Pique C, Ureta-Vidal A, Gessain A, Chancerel B, Gout O, Tamouza R, et al. Evidence for the chronic in vivo production of human T cell leukemia virus type I

Rof and Tof proteins from cytotoxic T lymphocytes directed against viral peptides. J Exp Med. 2000;191:567-572.
39. Kannagi M, Ohashi T, Harashima N, Hanabuchi S, Hasegawa A. Immunological risks of adult T-cell leukemia at primary HTLV-I infection. Trends Microbiol. 2004;12:346-352.
40. Harashima N, Kurihara K, Utsunomiya A, Tanosaki R, Hanabuchi S, Masuda M, et al. Graft-versus-Tax response in adult T-cell leukemia patients after hematopoietic stem cell transplantation. Cancer Res. 2004;64:391-399.
41. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version). Arch Pathol Lab Med. 2010;134:e48-72.
42. Kataoka K, Nagata Y, Kitanaka A, Shiraishi Y, Shimamura T, Yasunaga J, et al. Integrated molecular analysis of adult T cell leukemia/lymphoma. Nat Genet. 2015;47:1304-1315.
43. Suemori K, Fujiwara H, Ochi T, Ogawa T, Matsuoka M, Matsumoto T, et al. HBZ is an immunogenic protein, but not a target antigen for human T-cell leukemia virus type 1-specific cytotoxic T lymphocytes. J Gen Virol. 2009;90:1806-1811.
44. Suehiro Y, Hasegawa A, Iino T, Sasada A, Watanabe N, Matsuoka M, et al. Clinical outcomes of a novel therapeutic vaccine with Tax peptide-pulsed dendritic cells for adult T cell leukaemia/lymphoma in a pilot study. Br J Haematol. 2015;169:356-367.
45. Wherry EJ. T cell exhaustion. Nat Immunol. 2011;12:492-499.
46. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol. 2015;15:486-499.
47. Masaki A, Ishida T, Suzuki S, Ito A, Narita T, Kinoshita S, et al. Human T-cell lymphotropic/leukemia virus type 1 (HTLV-1) Tax-specific T-cell exhaustion in HTLV-1-infected individuals. Cancer Sci. 2018;109:2383-2390.
48. Wieczorek M, Abualrous ET, Sticht J, Alvaro-benito M, Stolzenberg S, Noe Frank, et al. Major histocompatibility complex (MHC) class I and MHC class II proteins: conformational plasticity in antigen presentation. Front Immunol. 2017;8:292.
49. Mahgoub M, Yasunaga J, Iwami S, Nakaoka S, Koizumi Y, Shimura K, et al. Sporadic on/off switching of HTLV-1 Tax expression is crucial to maintain the whole population of virus-induced leukemic cells. Proc Natl Acad Sci USA. 2018. https://www.pnas.org/content/115/6/E1269.long

50. Zhao T, Yasunaga J, Satou Y, Nakao M, Takahashi M, Fujii M, et al. Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-kappaB. Blood. 2009;113:2755-2764.
51. Nakagawa M, Shaffer AL, 3rd, Ceribelli M, Zhang M, Wright G, Huang DW, et al. Targeting the HTLV-I-regulated BATF3/IRF4 transcriptional network in adult T cell leukemia/lymphoma. Cancer Cell. 2018. https://www.sciencedirect.com/science/article/pii/S153561081830271X?via%3Dihub
52. Bangham CR, Cook LB, Melamed A. HTLV-1 clonality in adult T-cell leukaemia and non-malignant HTLV-1 infection. Semin Cancer Biol. 2014;26:89-98.

Figure legends

Figure 1.

Distribution of *HBZ* and *tax* mRNA signals. (A) Histogram of HBZ mRNA signals. The median value of *HBZ* signals (/1000 ATLL cells) was 795.2 (range: 0.4-4013.1). (B) The median value of *tax* signals (/1000 ATLL cells) was 5.1 (range: 0.1-891.2). (C) Scatter plot of *HBZ* and *tax* mRNA signals. There was no significant correlation between *HBZ* and *tax* signals (ρ = - .00367, P = .973)

Figure 2.

Representative results of ISH. (A) ISH of *HBZ* mRNA. Target-specific signals showed dot-like pattern. The signals were expressed in a large number of ATLL cells, with some cluster formation (arrow). (B) ISH of *HBZ* mRNA. 1 or 2 signals were expressed per ATLL cell (arrow). (C) ISH of *HBZ* mRNA. A few ATLL cells expressed the signals, and the intensity of some signals was weak (arrow). (D) ISH of *tax* mRNA. At most 1 or 2 signals were expressed per ATLL cell, and the intensity of many signals was weak (arrow). (E) ISH of *tax* mRNA. The signals were expressed only in a small number of ATLL cells (arrow). Original magnification is ×1000 for all panels.

Figure 3.

Representative correlation between *HBZ* and clinicopathological findings. (A) *HBZ* and IRF4 (ρ = .326; P = .0288). (B) *HBZ* and miPD-L1 (ρ =-.223; P = .0380). (C) *HBZ* and nPD-L1 (ρ =- .264; P = .0135).

Figure 4.

Representative correlation between *tax* and clinicopathological findings. (A) *tax* and Age (ρ = .225; P = .0352). (B) *tax* and CD30 (ρ = .303; P = .00435). (C) *tax* and GATA3 (ρ = .237; P = .0263). (D) *tax* and β2M (ρ =- .281; P = .0298). (E) *tax* and nPD-1 (ρ = .259; P = .0156). (F) *tax* and nPD-L1 (ρ = .297; P = .00527). (G) *tax* and PD-1-positive TIL (ρ = .397; P = .000138).

Figure 5.

OS of ATLL between the two *tax* expression groups. The group of 400 or more *tax* signals displayed significant inferior OS compared to compared with the group of less than 400 *tax* signals (P = .0499).

Figure 1

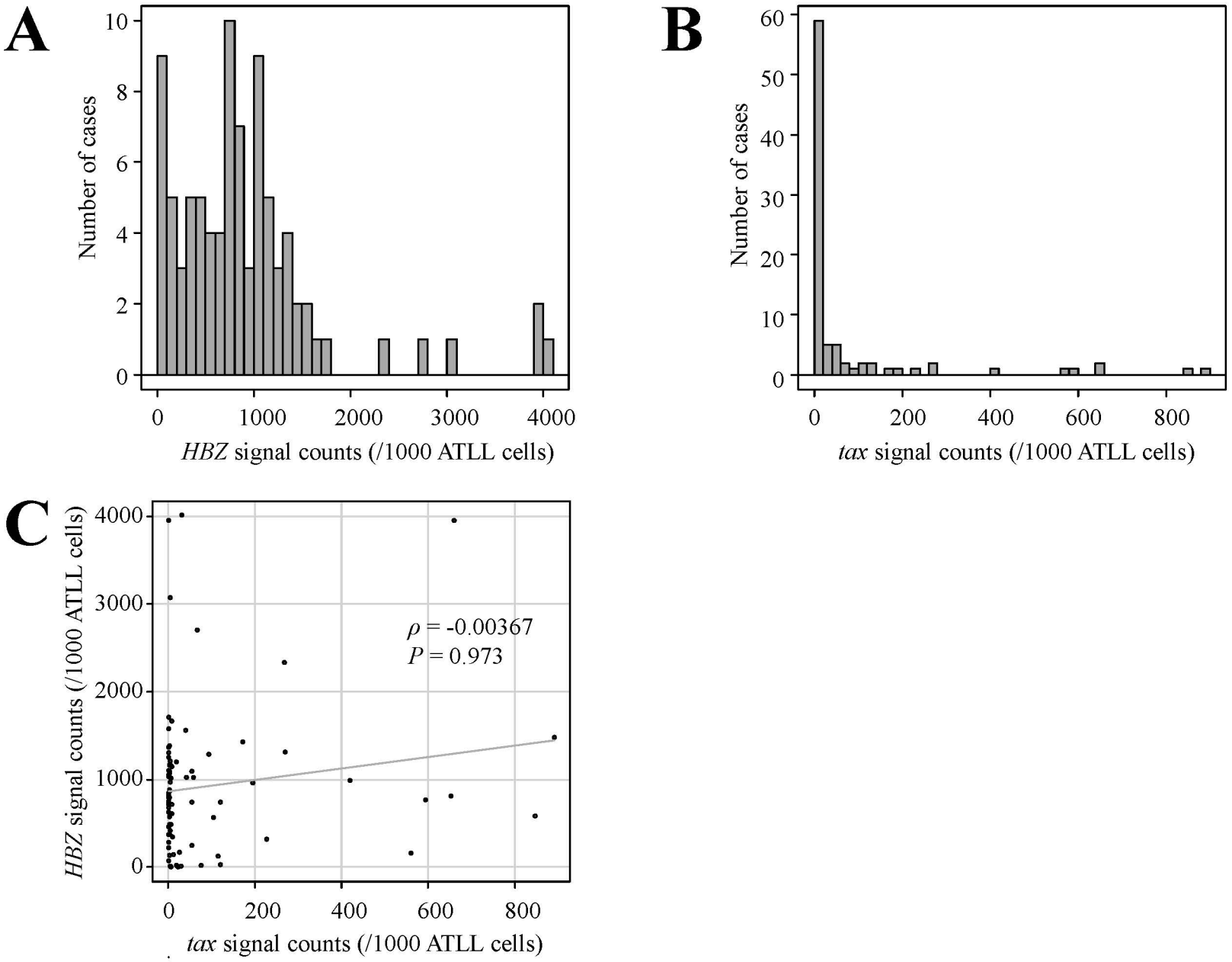

A
Number of cases
0
2
4
6
8
10
0
1000
2000
3000
4000
HBZ signal counts (/1000 ATLL cells)
B
Number of cases
0
10
20
30
40
50
60
0
200
400
600
800
tax signal counts (/1000 ATLL cells)
C
HBZ signal counts (/1000 ATLL cells)
0
1000
2000
3000
4000
ρ = -0.00367
P = 0.973
0
200
400
600
800
tax signal counts (/1000 ATLL cells)

Figure 2

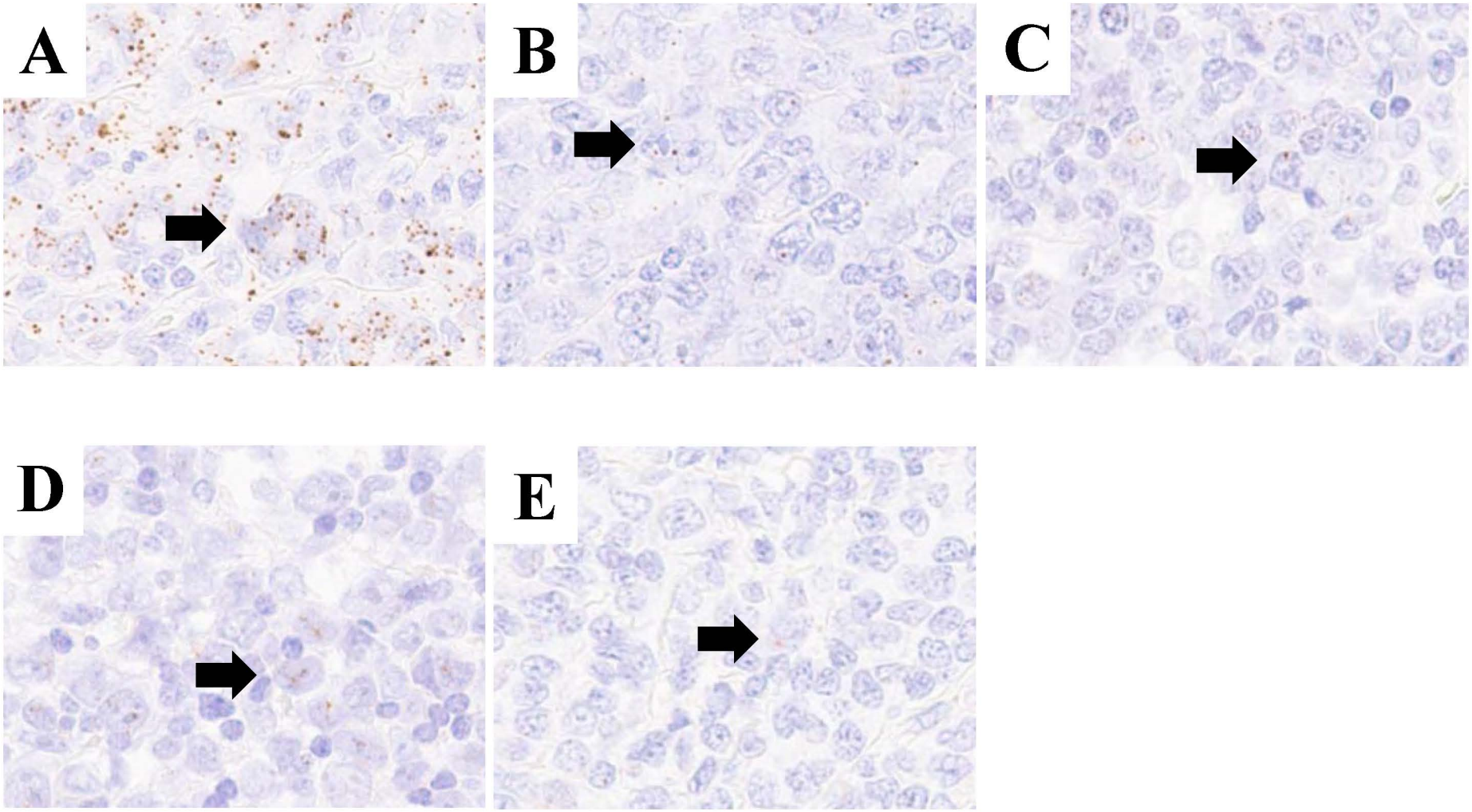


Figure 3

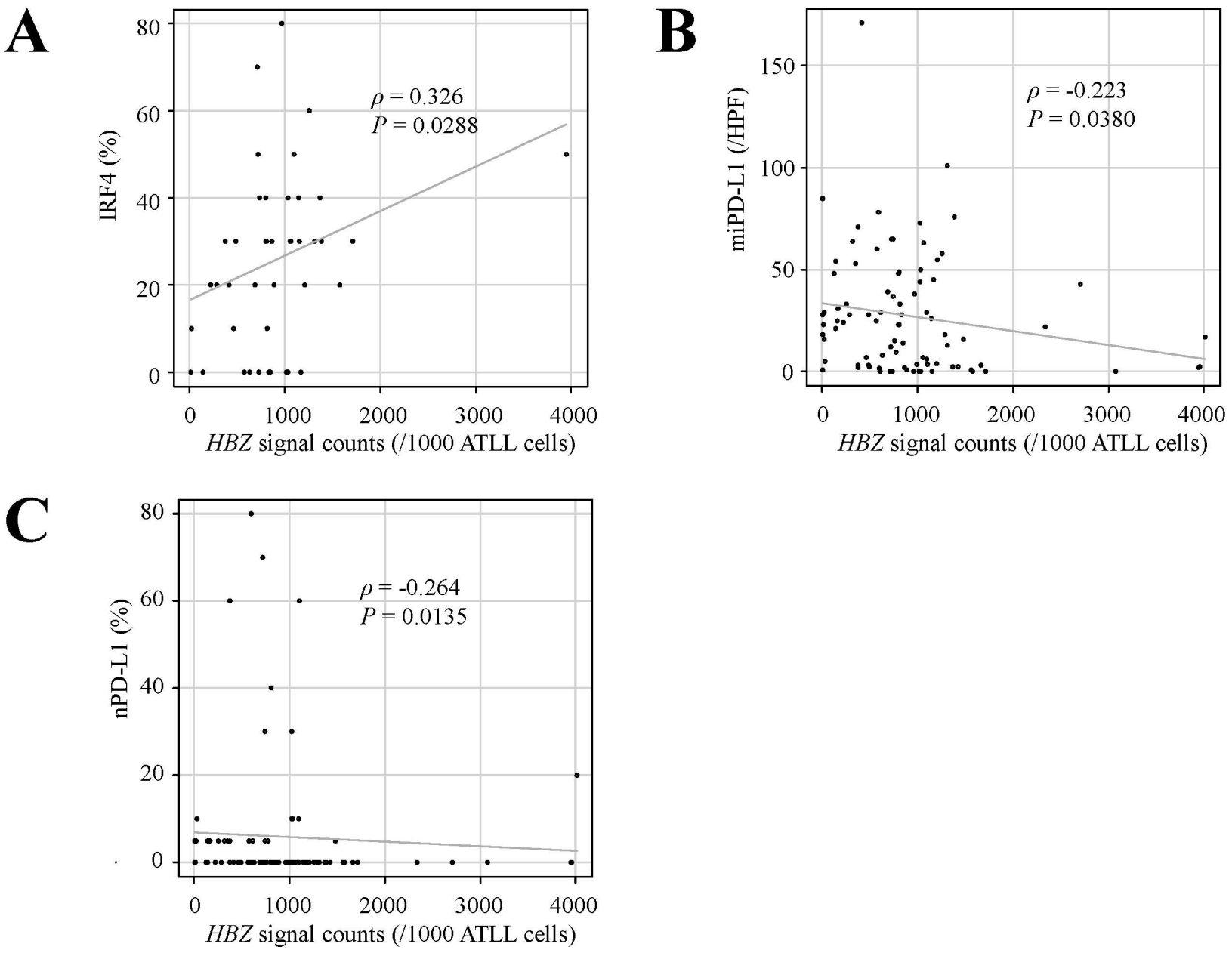

A
IRF4 (%)
80
60
40
20
0
ρ = 0.326
P = 0.0288
0
1000
2000
3000
4000
HBZ signal counts (/1000 ATLL cells)
B
miPD-L1 (/HPF)
150
100
50
0
ρ = -0.223
P = 0.0380
0
1000
2000
3000
4000
HBZ signal counts (/1000 ATLL cells)
C
nPD-L1 (%)
80
60
40
20
0
ρ = -0.264
P = 0.0135
0
1000
2000
3000
4000
HBZ signal counts (/1000 ATLL cells)

Figure 4

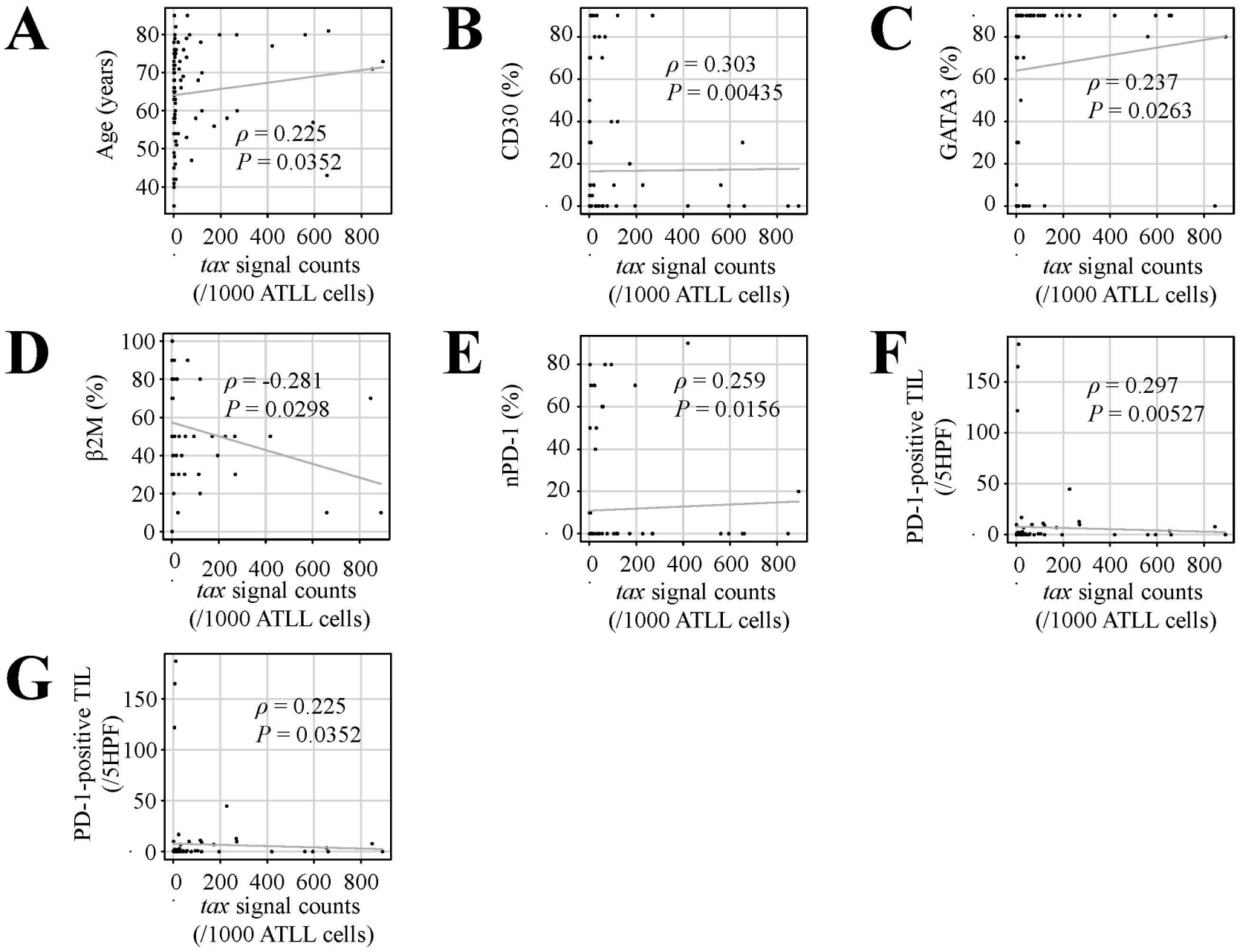
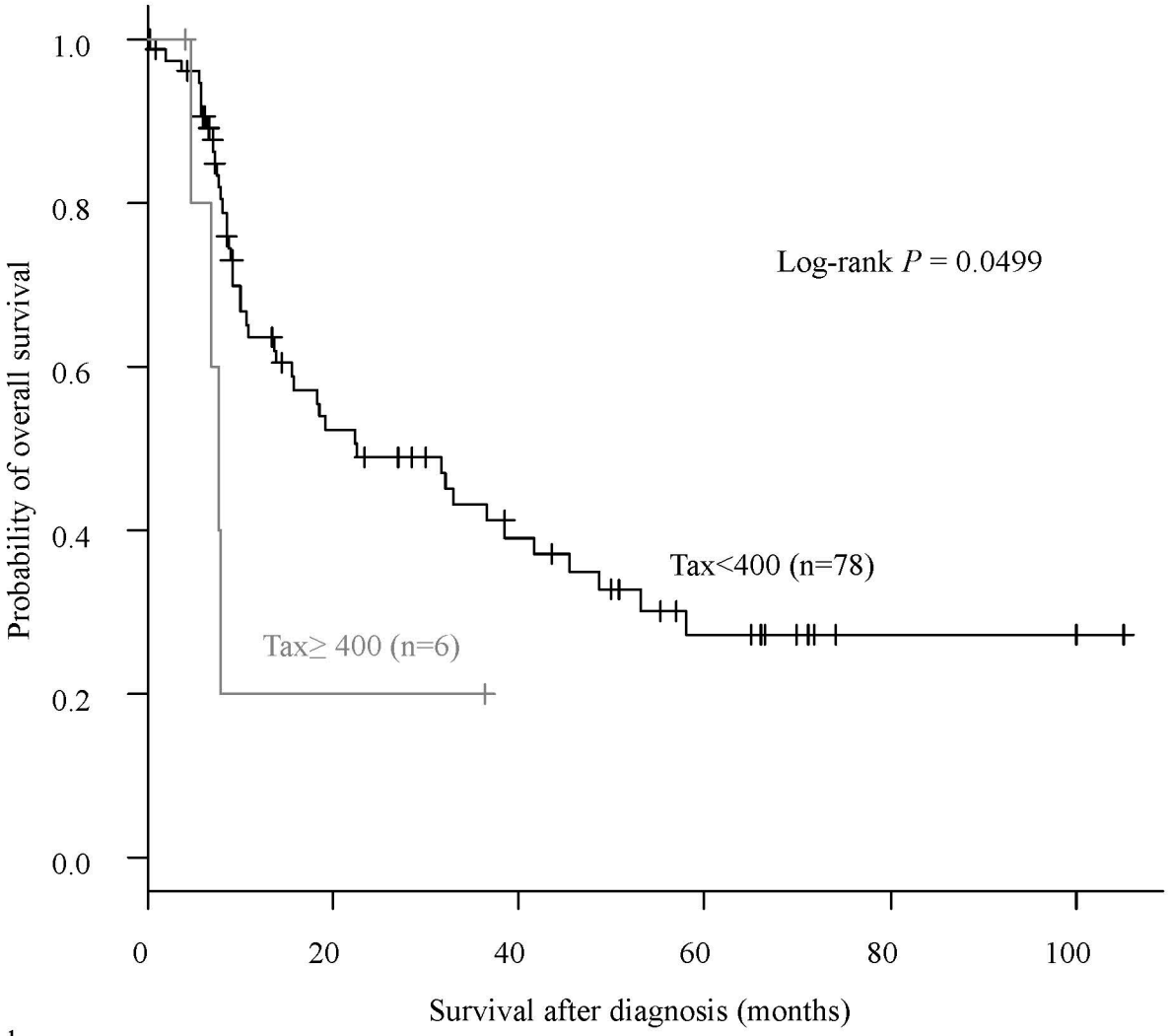

A
Age (years)
ρ = 0.225
P = 0.0352
tax signal counts
(/1000 ATLL cells)
B
CD30 (%)
ρ = 0.303
P = 0.00435
tax signal counts
(/1000 ATLL cells)
C
GATA3 (%)
ρ = 0.237
P = 0.0263
tax signal counts
(/1000 ATLL cells)
D
β2M (%)
ρ = -0.281
P = 0.0298
tax signal counts
(/1000 ATLL cells)
E
nPD-1 (%)
ρ = 0.259
P = 0.0156
tax signal counts
(/1000 ATLL cells)
F
PD-1-positive TIL
(/5HPF)
ρ = 0.297
P = 0.00527
tax signal counts
(/1000 ATLL cells)
G
PD-1-positive TIL
(/5HPF)
ρ = 0.225
P = 0.0352
tax signal counts
(/1000 ATLL cells)

Figure 5



Number at risk

Tax<400	78	32	19	9	2	1
Tax≥ 400	6	1	0	0	0	0

Table 1. Clinicopathological characteristics of ATLL patients

Characteristics	No. (n=88)	%
Age, years, median (range)	66 (35-85)	-
Sex, male/female	54/34	61/39
ECOG PS, 2-4	26/85	31
Shimoyama classification		
Smoldering type	1/71	1
Acute type	31/71	44
Lymphoma type	39/71	55
B symptoms	28/84	33
Elevated LDH	48/85	56
Elevated CRP	48/83	58
Hypercalcemia	11/84	13
Skin lesion	16/85	19
Hepatomegaly	3/85	4
Splenomegaly	10/85	12
Ann Arbor Stage, III or IV	70/84	83
Peripheral blood involvement	40/83	48
Bone marrow involvement	18/71	25
IPI, high-intermediate or more	46/82	56
JCOG-PI, high	33/84	39
WBC, ×10^9/L, average, median (range)	7.8, 6.5 (1.2-35.7)	-
Hemoglobin, g/dL, average, median (range)	12.7, 13.0 (7.8-16.6)	-
Platelets, ×10^6/L, average, median (range)	22.7, 21.0 (1.9-84.0)	-
Treatment		
Chemotherapy	74/79	94
Radiation	9/84	11
Transplantation	12/80	15
No chemotherapy	7/83	8
CR or CR(u)	22/76	29
Morphological variant		
Small cell variant	2	2
Medium cell variant	15	17
Large cell variant	36	41
Hodgkin-like variant	1	1
Anaplastic variant	6	7
Pleomorphic variant	28	32
Immunohistochemistry		
CD4 positive	35/42	85
CD30 positive	20/87	23
CCR4 positive	83/88	93
FoxP3 positive	20/88	23
GATA3 positive	69/88	79
IRF4 positive	25/45	56
HLA class I positive	43/77	57
β2M positive	35/78	45
HLA class I and β2M, positive	30/78	39
HLA class II positive	28/82	34
nPD-1positive	14/87	16
nPD-L1positive	4/87	5
miPD-L1 positive	33/87	38
PD-1-positive TIL, counts/5HPF, average, median (range)	7.4, 0, (0-187.0)	-
miPD-L1, counts/HPF, average, median (range)	27.2, 22.0 (0-171.0)	-
MIB-1 index (%), average, median (range)	53.1, 65.0 (0-90.0)	-
***In situ* mRNA analysis**		
HBZ, average, median (range)	916.7, 795.2 (0.4-4013.1)	-
tax, average, median (range)	80.4, 5.1 (0.1-891.2)	-

ATLL, Adult T-cell leukemia/lymphoma; β2M, β2 microglobulin; CRP, C-reactive

protein; CR, complete response/remission; CR(u), uncertain complete response/remission; ECOG PS, Eastern Cooperative Oncology Group performance status; HBZ, HTLV-I bZIP factor; HLA, human leukocyte antigen; IPI, international prognostic index; JCOG-PI, Japan Clinical Oncology Group prognostic index; LDH, lactate dehydrogenase; mPD-L1, microenvironmental programmed cell death ligand-1; nPD-1, neoplastic programmed cell death 1; WBC, white blood cell.

Table 2. Comparison of clinicopathological characteristics and HBZ mRNA expression

	HBZ high expression (n=44)		HBZ low expression (n=44)		
Characteristics	**No.**	**%**	**No.**	**%**	***P***
Sex, male/female	27/17	61/39	27/17	61/39	1.000*
Age, years, median (range)	66 (41–85)		68 (35–82)		0.897†
ECOG PS, 2–4	11/43	26	15/42	36	0.353*
Shimoyama classification					
Smoldering type	1/38	3	0/33	0	
Acute type	16/38	42	15/33	45	[1.000*]
Lymphoma type	21/38	55	18/33	42	
B symptoms	15/43	35	13/41	32	0.819*
Elevated LDH	24/43	56	24/42	57	1.000*
Elevated CRP	27/43	63	21/40	53	0.380*
Hypercalcemia	7/43	16	4/43	9	0.521*
Skin lesion	4/43	9	12/42	30	0.0283*
Hepatomegaly	1/43	2	2/42	5	0.616*
Splenomegaly	5/43	12	5/42	12	1.000*
Ann Arbor Stage, III or IV	31/43	72	39/41	95	0.00696*
Peripheral blood involvement	17/43	40	24/40	60	0.0802*
Bone marrow involvement	9/37	24	9/34	26	1.000*
IPI, high-intermediate or more	20/44	45	26/38	68	0.0461*
JCOG-PI, high	16/43	37	17/41	41	0.824*
WBC, ×10^9/L, median (range)	6.5 (1.2–15.4)		7.1 (2.4–35.7)		0.187†
Hemoglobin, g/dL, median (range)	12.7 (9.1–16.6)		13.2 (7.8–15.3)		0.857†
Platelets, ×10^6/L, median (range)	21.2 (1.9–84.0)		20.4 (2.7–53.8)		0.589†
Treatment					
Chemotherapy	41/42	98	33/37	89	0.180*
Radiation	7/43	16	2/41	5	0.157*
Transplantation	9/43	21	4/39	10	0.234*
No chemotherapy	1/43	2	6/40	15	0.052*
CR or CR(u)	12/40	30	10/36	28	1.000*
Morphological variant					0.000771‡
Small cell variant	0	0	2	5	
Medium cell variant	2	5	13	30	
Large cell variant	23	52	13	30	
Hodgkin like variant	0	0	1	2	
Anaplastic variant	5	11	1	2	
Pleomorphic variant	14	32	14	32	
Immunohistochemistry					
CD4, positive	17/19	89	18/23	78	0.428*
CD30, positive	12/44	27	8/43	19	0.446*
CCR4, positive	41/44	93	42/44	95	1.000*
FoxP3, positive	8/44	18	12/44	27	0.446*
GATA3, positive	38/44	86	31/44	70	0.119*
IRF4, positive	16/27	59	9/20	45	0.239*
HLA class I positive	21/37	57	22/40	55	1.000*
β 2M positive	16/38	42	19/40	48	0.656*
HLA class I and β 2M, positive	13/38	34	17/40	43	0.492*
HLA class II, positive	14/42	33	14/40	35	1.000*
nPD-1, positive	9/43	21	5/43	12	0.383*
nPD-L1, positive	1/44	2	3/43	7	0.360*
miPD-L1 positive	24/44	55	30/43	70	0.186*
PD-1-positive TIL, counts/5HPF, average, median (range)	1.5, 0 (0–13)		13.4, 0 (0–187)		0.521†
miPD-L1, counts/HPF, average, median (rage)	23.2, 15 (0–101)		31.2, 25 (0–171)		0.149†
MIB-1 index (%), average, median (range)	61, 70 (0–90)		47, 50 (0–90)		0.137†

Abbreviations are explained in Table 1.

*Fisher's exact test; values in brackets show P value between the acute type and lymphoma type.

†Mann-Whitney's U test

‡Fisher's exact test; values in brackets show P value between the small/medium cell variant and others.

Table 3. Comparison of clinicopathological characteristics and Tax mRNA expression

	Tax high expression (n=44)		Tax low expression (n=44)		
Characteristics	**No.**	**%**	**No.**	**%**	***P***
Sex, male/female	22/22	50/50	32/44	72/28	0.0481*
Age, years, median (range)	69 (42–85)		66 (35–85)		0.201†
ECOG PS, 2–4	12/42	30	14/43	33	0.815*
Shimoyama classification					
Smoldering type	1	3	0	0	
Acute type	11	37	20	49	[0.466*]
Lymphoma type	18	60	21	51	
B symptoms	10/41	24	18/43	42	0.108*
Elevated LDH	31/42	74	17/43	40	0.00209*
Elevated CRP	23/42	55	25/41	61	0.658*
Hypercalcemia	5/42	12	6/42	14	1.000*
Skin lesion	9/42	21	7/43	16	0.589*
Hepatomegaly	2/42	5	1/43	2	0.616*
Splenomegaly	9/42	21	1/43	2	0.00721*
Ann Arbor Stage, III or IV	33/41	80	37/43	86	0.566*
Peripheral blood involvement	20/41	49	21/42	50	1.000*
Bone marrow involvement	12/31	39	6/40	15	0.0295*
IPI, high–intermediate or more	26/40	65	20/42	48	0.126*
JCOG–PI, high	15/42	36	18/42	43	0.655*
WBC, ×10^9/L, median (range)	6.4 (3.0–24.3)		6.9 (1.2–35.7)		0.732†
Hemoglobin, g/dL, median (range)	13.1(7.8–16.6)		12.8 (8.3–15.9)		0.951†
Platelets, ×10^6/L, median (range)	19.2 (2.2–84.0)		22.1 (1.9–48.4)		0.419†
Treatment					
Chemotherapy	36/39	92	38/40	95	0.675*
Radiation	1/41	2	8/43	19	0.0298*
Transplantation	7/39	18	6/43	14	0.764*
No chemotherapy	3/40	8	4/43	9	0.616*
CR or CR(u)	11/37	30	11/39	28	1.000*
Morphological variant					0.590‡
Small cell variant	1	2	1	2	
Medium cell variant	9	20	6	14	
Large cell variant	15	34	21	48	
Hodgkin–like variant	0	0	1	2	
Anaplastic variant	4	9	2	5	
Pleomorphic variant	15	34	13	30	
Immunohistochemistry					
CD4 positive	31/38	82	4/0	100	1.000*
CD30 positive	16/44	36	4/43	9	0.00434*
CCR4 positive	43/44	98	40/44	91	0.360*
FoxP3 positive	12/44	27	8/44	18	0.446*
GATA3 positive	36/44	82	33/44	75	0.605*
IRF4 positive	4/6	67	21/39	54	0.678*
HLA class I positive	20/40	50	23/37	62	0.360*
β 2M positive	17/41	42	18/37	49	0.649*
HLA class I and β 2M, positive	13/41	32	17/37	46	0.246*
HLA class II, positive	17/41	41	11/41	27	0.244*
nPD–1, positive	11/44	25	3/43	7	0.0385*
nPD–L1, positive	2/44	5	2/43	5	1.000*
miPD–L1 positive	28/44	64	26/43	60	0.827*
PD–1–positive TIL, counts/5HPF, average, median (range)	14.3, 0.5 (0–187.0)		1.5, 0 (0–10.0)		<0.0001†
miPD–L1, counts/HPF, average, median (rage)	25.0, 18.0 (0–85.0)		29.3, 23.0 (0–171.0)		0.922†
MIB–1 index (%), average, median (range)	51, 60 (0–90)		73, 70 (70–80)		0.241†

Abbreviations are explained in Table 1.

*Fisher's exact test; values in brackets show P value between the acute type and lymphoma type.

†Mann–Whitney's U test

‡Fisher's exact test; values in brackets show P value between the small/medium cell variant and others.

Table 4. Clinicopathological comparison between *tax* signals ≧ 400 group and *tax* signals < 400 group

	tax signals ≧ 400 (n=7)		*tax* signals < 400 (n=81)		
Characteristics	**No.**	**%**	**No.**	**%**	***P***
Sex, male/female	4/3	57/43	50/31	62/38	1.000*
Age, years, median (range)	73 (43-81)		66 (35-85)		0.217†
ECOG PS, 2-4	3/7	43	23/78	29	0.670*
Shimoyama classification					
Smoldering type	0/4	0	1/67	1	
Acute type	2/4	50	29/67	43	[1.000*]
Lymphoma type	2/4	50	37/67	55	
B symptoms					
Elevated LDH	7/7	100	41/78	53	0.0170*
Elevated CRP	3/7	43	45/76	62	0.448*
Hypercalcemia	2/7	29	9/77	12	0.227*
Skin lesion	2/7	29	14/78	19	0.611*
Hepatomegaly	1/7	14	2/78	3	0.230*
Splenomegaly	3/7	43	7/78	9	0.0326*
Ann Arbor Stage, III or IV	5/6	83	65/78	83	1.000*
Peripheral blood involvement	2/7	29	39/76	51	0.433*
Bone marrow involvement	2/3	67	16/68	24	0.156*
IPI, high-intermediate or more	5/6	83	41/76	54	0.223*
JCOG-PI, high	3/7	43	30/77	39	1.000*
WBC, ×10^9/L, median (range)	6.9 (3.5-10.7)		6.5 (1.2-35.7)		0.994†
Hemoglobin, g/dL, median (range)	13.2 (11.6-15.0)		12.9 (7.8-16.6)		0.603†
Platelets, ×10^6/L, median (range)	18.7 (9.3-84.0)		21.3 (1.9-53.8)		0.482†
Treatment					
Chemotherapy	5/5	100	69/74	97	1.000*
Radiation	0/6	0	9/78	12	1.000*
Transplantation	1/6	17	12/78	15	1.000*
No chemotherapy or radiotherapy	0/6	0	7/77	9	1.000*
CR or CR(u)	0/6	0	22/70	31	0.173*
Morphological variant					0.616††
Small cell variant	0	0	2	2	
Medium cell variant	2	29	13	16	
Large cell variant	2	29	34	42	
Hodgkin like variant	0	0	1	1	
Anaplastic variant	0	0	6	7	
Pleomorphic variant	3	43	25	31	
Immunohistochemistry					
CD4, positive	6/7	86	29/35	83	1.000*
CD30, positive	1/7	14	19/80	23	1.000*
CCR4, positive	7/7	100	76/81	94	1.000*
FoxP3, positive	2/7	29	18/81	22	0.655*
GATA3, positive	6/7	86	63/81	78	1.000*
IRF4, positive	none				
HLA class I positive	1/7	14	42/69	61	0.0385*
β 2M positive	0/7	0	37/71	52	0.0124*
HLA class I and β 2M, positive	0/7	0	30/71	42	0.0394*
HLA class II, positive	1/6	17	27/76	36	0.659*
nPD-1, positive	1/7	14	13/70	19	1.000*
nPD-L1, positive	0/7	0	4/80	5	1.000*
miPD-L1 positive	4/7	57	50/80	63	1.000*
PD-1-positive TIL, counts/5HPF, average, median (range)	1.7, 0 (0-8.0)		7.9, 0 (0-187.0)		0.909†
miPD-L1, counts/HPF, average, median (rage)	26.2, 16.0 (2.5-78.0)		27.2, 22.5 (0-171.0)		0.845†
MIB-1 index (%), average, median (range)	46, 40 (20-80)		55, 70 (0-90)		0.383†

Abbreviations are explained in Table 1.

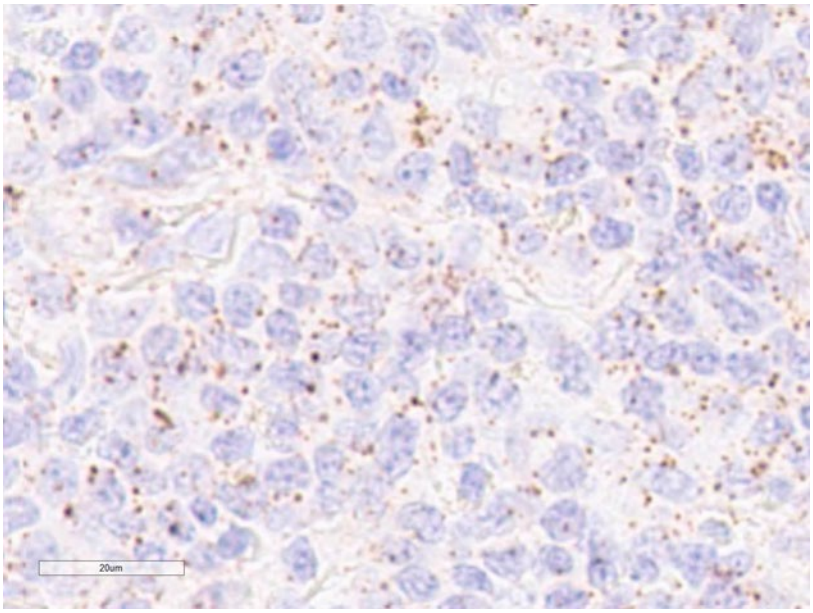
*Fisher's exact test; values in brackets show P value between the acute type and lymphoma type.

†Mann-Whitney's U test

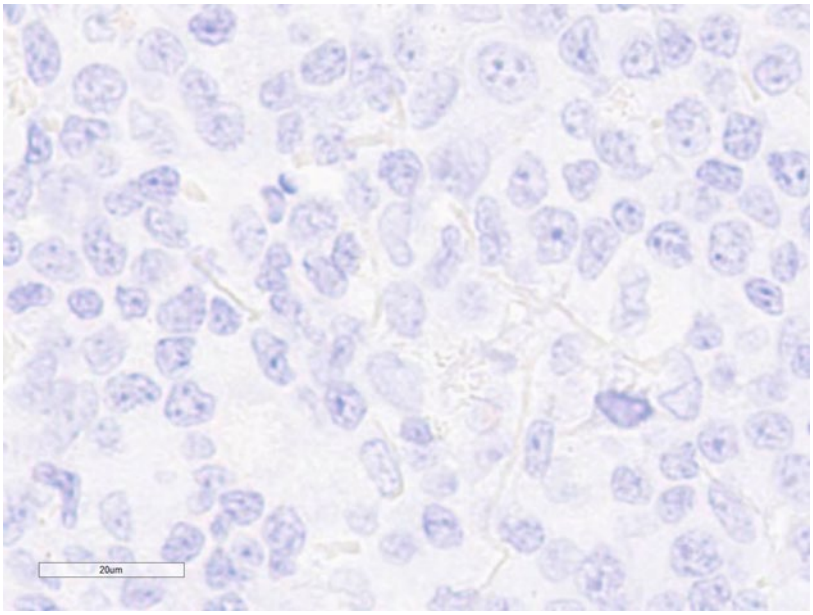
‡Fisher's exact test; values in brackets show P value between the small/medium cell variant and others.

Supplemental Figure 1.

A



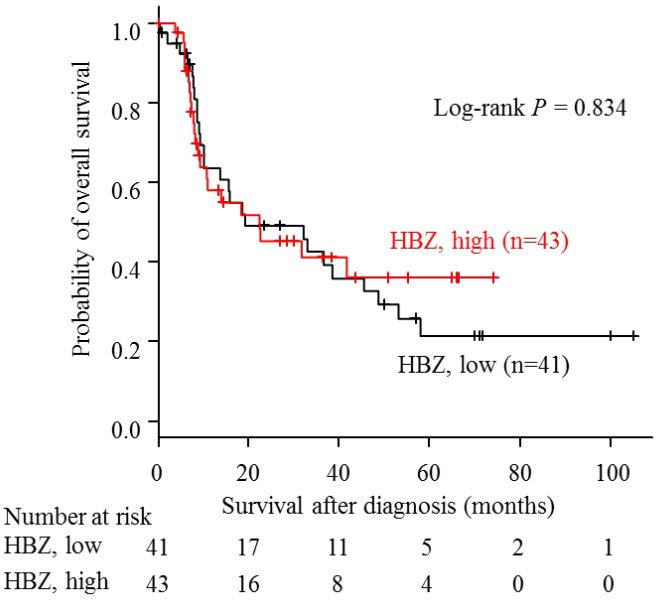
B



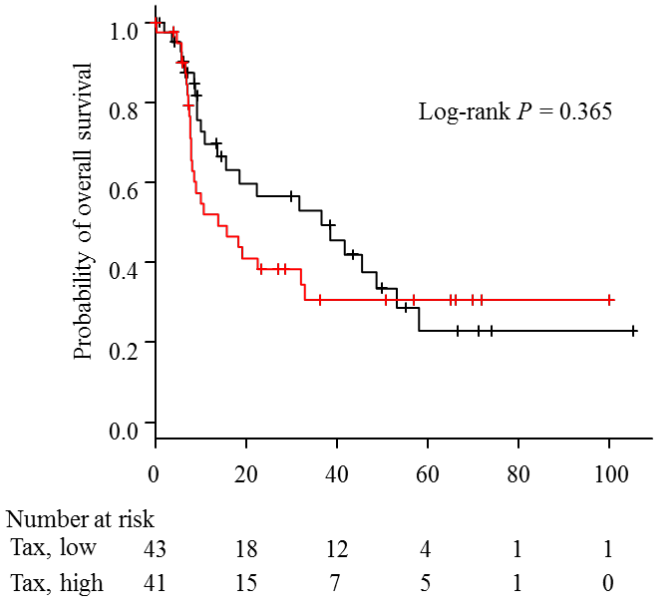
Supplemental Figure 1. Representative results of ISH in ATLL. (A) ISH of PPIB was performed for positive control. (B) ISH of DapB was performed for negative control. Original magnification ×1000 for all panels.

Supplemental Figure 2.

A



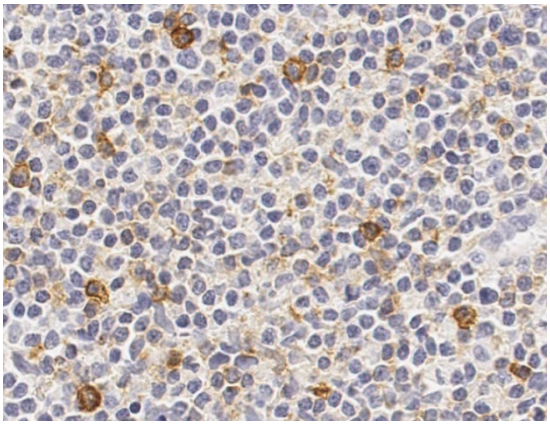
B



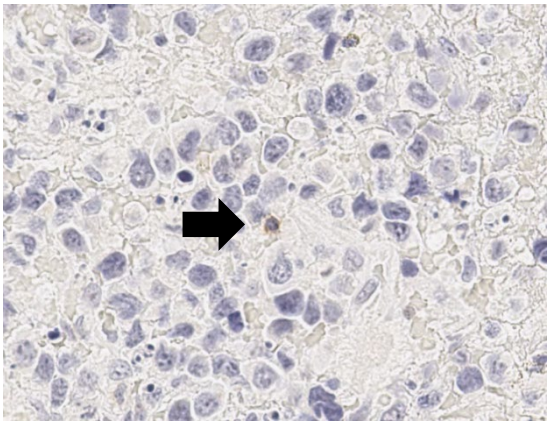
Supplemental Figure 2. OS of ATLL between the two expression groups of *HBZ* and *tax*. (A) There is no significant correlation between high and low expression groups of *HBZ* (Log-lank P = .834). (B) There is no significant correlation between high and low expression groups of *tax* (Log-lank P = .365).

Supplemental Figure 3.

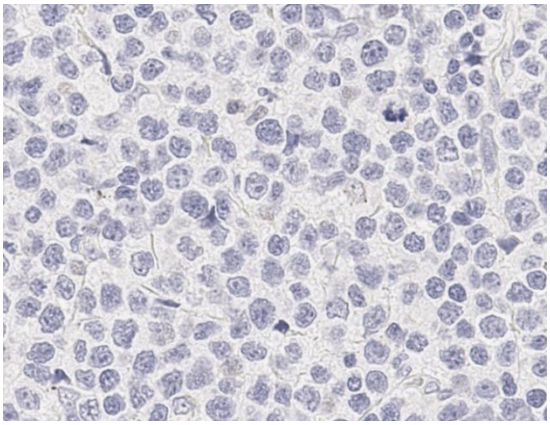
A



B

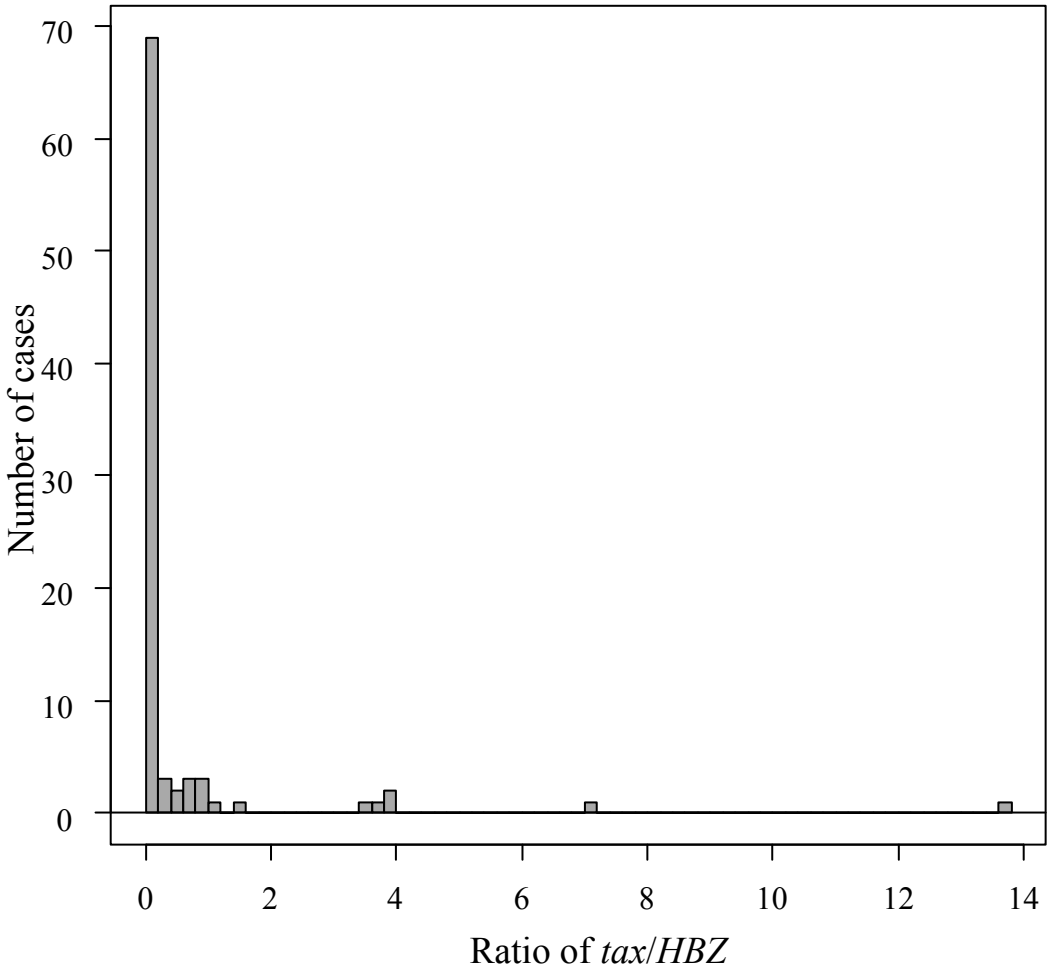


C



Supplemental Figure 3. PD-1-TIL positivity in ATLL patients. (A) A great amount of PD-1-TIL (187.0/5HPF) were observed. (B) A few number of PD-1-TIL (17.0/5HPF) were observed (arrow). (C) No PD-1-TIL (0/5HPF) was observed. Original magnification × 400 for all panels.

Supplemental Figure 4.



Supplemental Figure 4. The ratio of *tax*/*HBZ* in ATLL patients. *Tax*/*HBZ* ratio ranged from 0 to 13.7, with an average value of 0.5 and with a median value of 0.008.

Supplemental Table 1. Antibodies for immunohistochemistry

Antigen	Clone	Source
CD4	SP35	Roche Diagnostics, Tokyo, Japan
CD30	Ber-H2	Dako, Tokyo, Japan
Ki-67	MIB-1	Dako, Tokyo, Japan
CCR4	POTELIGEO TEST	Kyowa Medex, Japan
FoxP3	SP97	Abcam, Tokyo, Japan
GATA3	D13C9	Cell Signaling Technology, Tokyo, Japan
IRF4	MUM1p	Dako, Tokyo, Japan
HLA class I	EMR8-5	Abcam, Tokyo, Japan
ß2M	HPA006361	Sigma-Aldrich, Tokyo, Japan
HLA class II	CR3/43	Dako, Tokyo, Japan
PD-1	NAT105	Abcam, Tokyo, Japan
PD-L1	EPR1161(2)	Abcam, Tokyo, Japan

CCR4, C-C chemokine receptor type 4; FoxP3, Forkhead boxprotein P3; GATA3, GATA binding protein 3; IRF4, Interferon regulatory factor 4; HLA, Human leukocyte antigen; ß2M, ß2-microglobulin; PD-1, Programmed cell death 1; PD-L1, Programmed cell death ligand 1

Supplemental Table 2. Clinicopathological characteristics of ATLL patients

Characteristics	No. (n=88)	%
Age, years, median (range)	66 (35-85)	-
Sex, male/female	54/34	61/39
ECOG PS, 2-4	26/85	31
Shimoyama classification		
Smoldering type	1/71	1
Acute type	31/71	44
Lymphoma type	39/71	55
B symptoms	28/84	33
Elevated LDH	48/85	56
Elevated CRP	48/83	58
Hypercalcemia	11/84	13
Skin lesion	16/85	19
Hepatomegaly	3/85	4
Splenomegaly	10/85	12
Ann Arbor Stage, III or IV	70/84	83
Peripheral blood involvement	40/83	48
Bone marrow involvement	18/71	25
IPI, high-intermediate or more	46/82	56
JCOG-PI, high	33/84	39
WBC, $\times 10^9$/L, median (range)	6.5 (1.2-35.7)	-
Hemoglobin, g/dL, median (range)	13.0 (7.8-16.6)	-
Platelets, $\times 10^6$/L, median (range)	21.0 (1.9-84.0)	-
Treatment		
Chemotherapy	74/79	94
Radiation	9/84	11
Transplantation	12/80	15
No chemotherapy	7/83	8
CR or CR(u)	22/76	29
Morphological variant		
Small cell variant	2	2
Medium cell variant	15	17
Large cell variant	36	41
Hodgkin-like variant	1	1
Anaplastic variant	6	7

Pleomorphic variant	28	32
Immunohistochemistry		
CD4, positive	35/42	85
CD30, positive	20/87	23
CCR4, positive	83/88	93
FoxP3, positive	20/88	23
GATA3, positive	69/88	79
IRF4, positive	25/45	56
HLA class I, positive (membrane)	43/77	57
ß2M, positive (membrane)	35/78	45
HLA class I and ß2M, positive (membrane)	30/78	39
HLA class II, positive	28/82	34
nPD-1, positive	14/87	16
nPD-L1, positive	4/87	5
miPD-L1, positive	33/87	38
PD-1-positive TIL, counts/5HPF, average, median (range)	7.4, 0 (0-187.0)	-
miPD-L1, counts/HPF, average, median (range)	27.2, 22.0 (0-171.0)	-
MIB-1 index (%), average, median (range)	53.1, 65.0 (0-90.0)	-
***In situ* mRNA analysis**		
HBZ, average, median (range)	916.7, 795.2 (0.4-4013.1)	-
tax, average, median (range)	80.4, 5.1 (0.1-891.2)	-

ATLL, Adult T-cell leukemia/lymphoma; ß2M, ß2 microglobulin; CRP, C-reactive protein; CR, complete response/remission; CR(u), uncertain complete response/ remission; ECOG PS, Eastern Cooperative Oncology Group performance status; HBZ, HTLV-I bZIP factor; HLA, human leukocyte antigen; IPI, international prognostic
index; JCOG-PI, Japan Clinical Oncology Group prognostic index; LDH, lactate dehydrogenase; mPD-L1, microenvironmental programmed cell death ligand-1; nPD-1,
neoplastic programmed cell death 1; WBC, white blood cell.

Supplemental Table 3. Prognostic factors affecting overall survival in ATLL patients

	Overall survival			
	Univariate analysis		Multivariate analysis	
Characteristics	HR (95%CI)	*P*	HR (95%CI)	*P*
HBZ signals, net less than 916.7 (median)	0.941 (0.532-1.664)	0.834		
Tax signals, not less than 5.1 (median)	1.300 (0.736-2.295)	0.366		
Tax signals, not less than 400	2.72 (0.960-7.711)	0.0598	2.520 (0.802-7.914)	0.114
Age, over 70	2.196 (1.209-3.986)	0.00974	2.262 (1.171-4.369)	0.015
Sex, male	0.956 (0.536-1.707)	0.879		
Elevated LDH (> normal)	1.374 (0.767-2.461)	0.286		
JCOG-PI, high	1.711 (0.952-3.0750)	0.0726	1.790 (0.960-3.334)	0.067
IPI, high or high-intermediate	1.462 (0.804-2.660)	0.213		
miPD-L1, positive	0.545 (0.307-0.970)	0.039	0.633 (0.316-1.265)	0.195
HLA class I and ß2M, positive (membrane)	0.494 (0.253-0.964)	0.0386	0.660 (0.323-1.334)	0.244
HLA class II, positive	0.398 (0.205-0.772)	0.0064	0.578 (0.253-1.323)	0.195

HR, hazard ratio; CI confidence interval; LDH lactate dehydrogenase; JCOG-PI, Japan clinical oncology group- prognostic index; IPI, international prognostic index; miPD-L1, microenvironmental programmed cell death ligand-1; HLA, human leukocyte antigen; ß2M, ß2 microglobulin

Supplemental Table 4. Prognostic factors affecting progression-free survival in ATLL patients

	Progression-free survival			
	Univariate analysis		Multivariate analysis	
Characteristics	HR (95%CI)	*P*	HR (95%CI)	*P*
HBZ signals, net less than 916.7 (median)	1.109 (0.338-3.640)	0.865		
Tax signals, not less than 5.1 (median)	1.372 (0.416-4.529)	0.604		
Tax signals, not less than 400	12.570 (2.186-72.340)	0.00457	3.901 (0.590-25.790)	0.158
Age, over 70	2.276 (0.690-7.505)	0.177	3.334 (0.742-14.980)	0.116
Sex, male	1.761 (0.466-6.647)	0.404		
Elevated LDH (> normal)	0.781 (0.237-2.575)	0.685		
JCOG-PI, high	2.605 (0.792-8.565)	0.115	4.976 (1.113-22.250)	0.036
IPI, high or high-intermediate	1.708 (0.498-5.856)	0.394		
miPD-L1, positive	0.359 (0.109-1.184)	0.0926	0.564 (0.138-2.313)	0.427
HLA class I and ß2M, positive (membrane)	0.141 (0.0181-1.106)	0.0654	0.096 (0.00815-1.124)	0.062
HLA class II, positive	0.320 (0.0686-1.494)	0.147	1.148 (0.170-7.751)	0.888

HR, hazard ratio; CI confidence interval; LDH lactate dehydrogenase; JCOG-PI, Japan clinical oncology group- prognostic index; IPI, international prognostic index; miPD-L1, microenvironmental programmed cell death ligand-1; HLA, human leukocyte antigen; ß2M, ß2 microglobulin