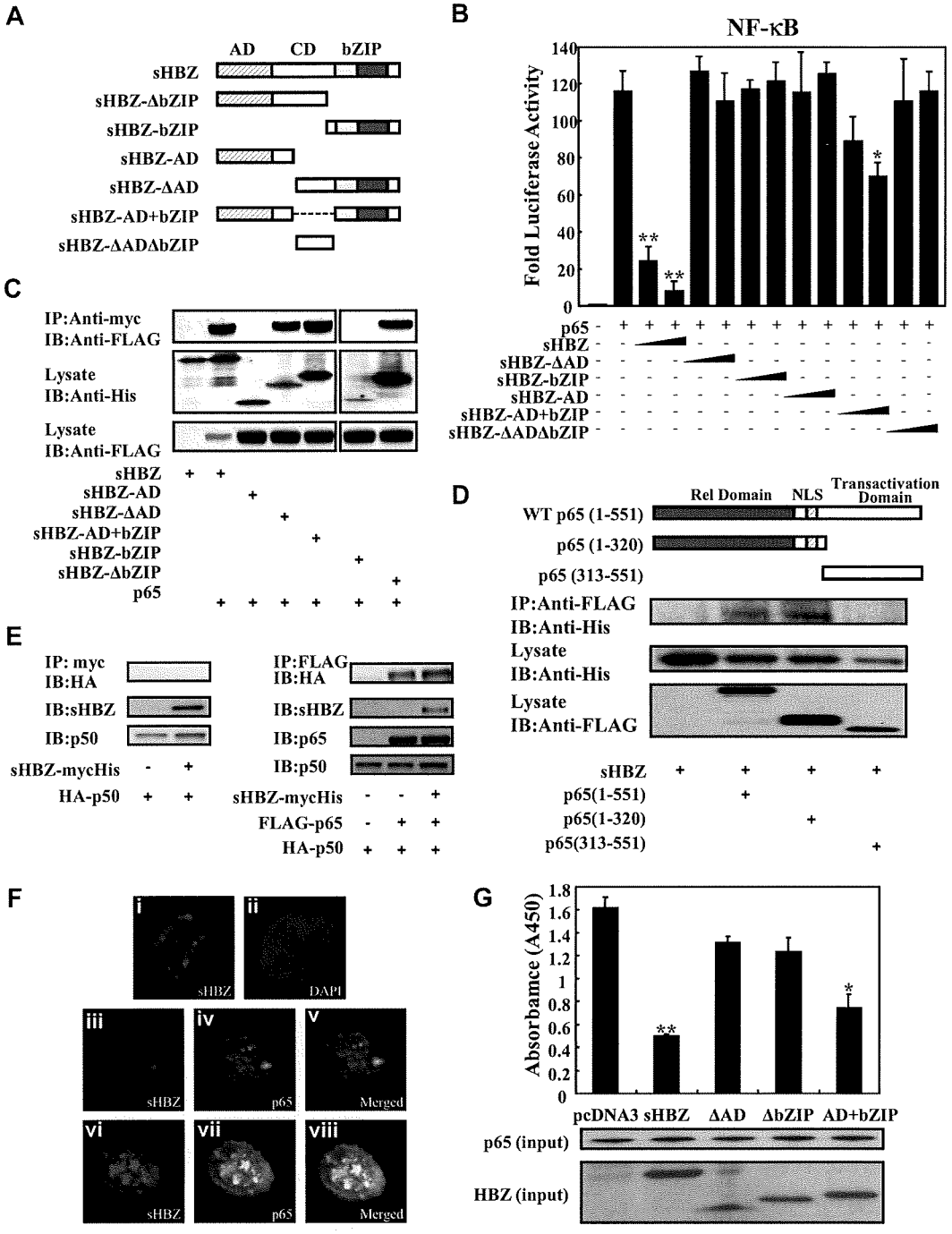

Figure 4. Domains of sHBZ responsible for suppression of NF-κB p65. (A) Schematic diagram of HBZ and its mutants used in this study. Characteristic domains of HBZ are indicated as follows: activation domain (AD), central domain (CD) and basic leucine zipper domain (bZIP). (B) Analysis of HBZ deletion mutants for the effect on p65-mediated NF-κB activation. Jurkat cells were cotransfected with κB-Luc and phRL-TK, with or without 1 μg of pCMV-Tag 2-p65, and with 1 or 5 μg pME18Sneo-sHBZ or sHBZ mutant. After 48 hours, luciferase levels were measured. $^*P < .05$; $^{**}P < .01$. Error bars represent SD. (C) Determination of the region of HBZ responsible for the interaction with p65. 293FT cells were transfected with the indicated mycHis-sHBZ mutants along with the FLAG-p65 vector. Cell lysates were subjected to immunoprecipitation (IP) using anti–c-Myc followed by immunoblotting (IB) using anti-FLAG. The expression levels of p65 and sHBZ mutants were detected. (D) Mapping the region of the p65 protein necessary for the interaction with sHBZ. The schema of p65 deletion mutants has been shown. The locations of the Rel homology domain, the nuclear localization signal (NLS), and the transactivation domain are indicated. 293FT cells were transfected with mycHis-sHBZ along with full-length or mutant FLAG-p65. At 48 hours after transfection, total cell lysates were subjected to IP using anti-FLAG followed by IB using anti-His. (E) HBZ did not influence p65/p50 interaction. 293FT cells were transfected with the indicated expression vectors. Cell lysates were subjected to IP using anti–c-Myc or anti-FLAG followed by IB using anti-HA. The expression levels of p65, p50, and sHBZ were detected. (F) sHBZ colocalizated with p65 in the cell nucleus. HeLa cells were transfected with mycHis-sHBZ together with (panels iii-viii) or without (panels i,ii) FLAG-p65. sHBZ was detected using anti-MYC Cy3 antibody (panels i,iii,vi). p65 was detected using anti–Flag-biotin and secondary Streptavidin-Alexa 488 antibody (panels iv,vii). The overlay of sHBZ and p65 is shown (panels v,viii). DAPI (4,6 diamidino-2-phenylindole) was used to counterstain the nucleus (panel ii). (G) sHBZ decreased p65 DNA binding capability. 293FT cells were transfected with FLAG-p65 together with either mycHis-sHBZ or one of its mutants. Cell lysates were subjected to the enzyme-linked immunosorbent assay (ELISA)–based NoShift assay to measure the DNA binding capability of p65. The absorbance at 450 nm indicated the binding ability of p65 (top panel). The bottom panel shows the amount of p65 and sHBZ in the 20% of input for analysis. $^*P < .05$; $^{**}P < .01$. Error bars represent SD.

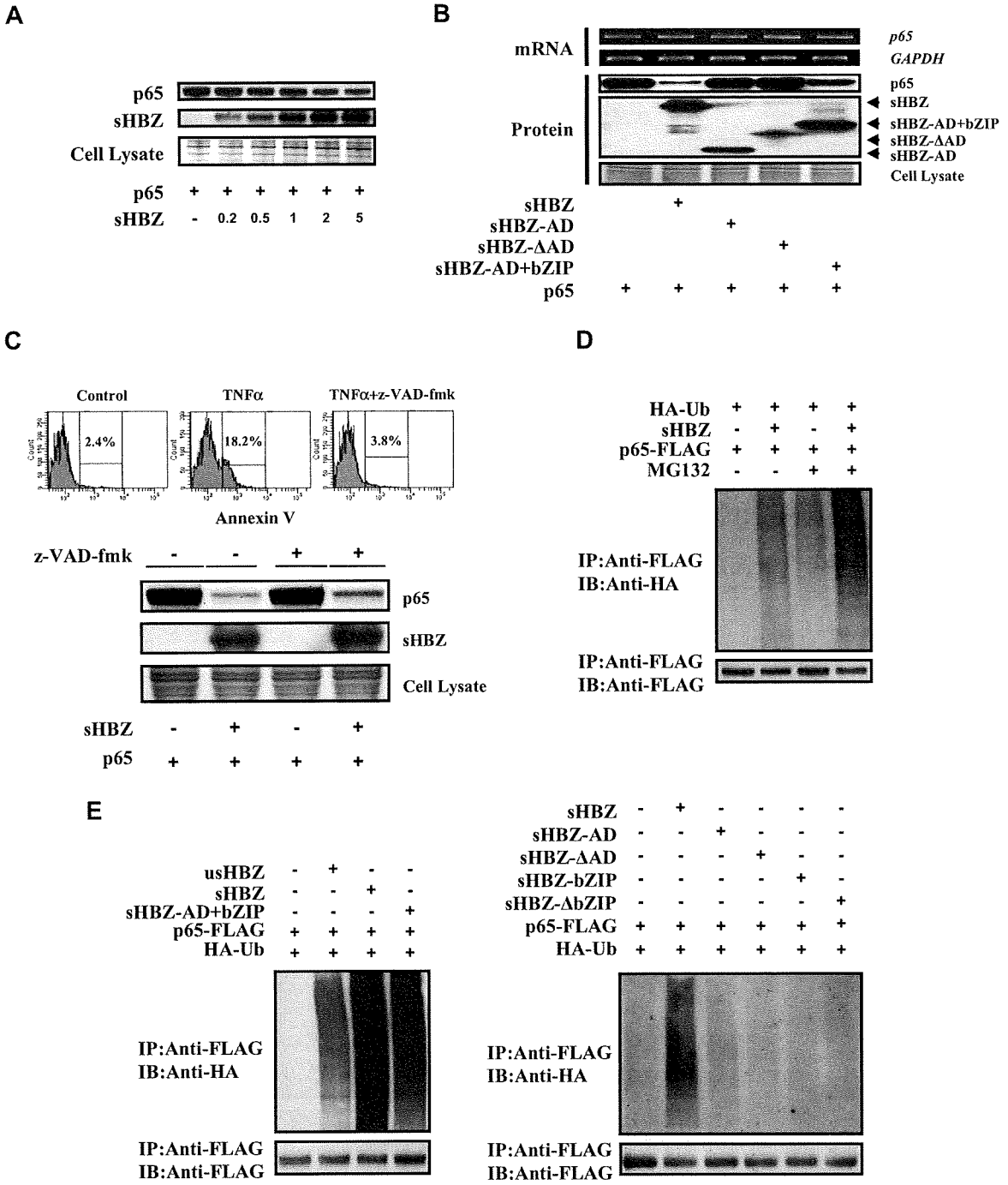

Figure 5. sHBZ promotes p65 degradation through a ubiquitination-dependent pathway. (A) sHBZ repressed the level of p65 in a dose-dependent manner. 293FT cells were transfected with 1 μg pEF-p65 and various amounts of mycHis-sHBZ (0.2, 0.5, 1, 2, and 5 μg). After 36 hours, the cell lysates were subjected to immunoblotting. (B) Activation and leucine-zipper domains of sHBZ were necessary for suppression of p65. 293FT cells were transfected with 50 ng pEF-p65 and 250 ng either mycHis-sHBZ or its mutants. At 36 hours after transfection, the level of *p65* mRNA was analyzed by semiquantitative RT-PCR. The levels of *GAPDH* mRNA are shown as internal control (top panel). Whole cell lysates were subjected to immunoblotting (bottom panel). (C) sHBZ-mediated suppression of p65 protein is caspase independent. Top panel: 293FT cells were transfected with FLAG-p65 together with mycHis-sHBZ. The caspase inhibitor z-VAD-fmk was added 2 hours before transfection. At 48 hours after transfection, cell lysates were subjected to immunoblotting. Bottom panel: Jurkat cells were cultured in the presence of indicated drugs for 24 hours. Cell death was analyzed by annexin V staining. (D,E) sHBZ accelerated the ubiquitination of p65. 293FT cells were transfected with FLAG-p65, HA-ubiquitin, and either mycHis-sHBZ or its mutants. After 24 hours, cells were treated with or without MG132 for 12 hours. Cell lysates were subjected to IP using anti-FLAG followed IB using anti-HA.

genes was analyzed by semiquantitative RT-PCR (Figure 7A). Expression of sHBZ was associated with suppression of some selected target genes, which are normally up-regulated after PMA/ionomycin treatment, such as *IL-8*, *IFN-γ*, *IL2RA*, *IRF4*, *VCAM-1*, and *VEGF*. Because PMA/ionomycin treatment activates NF-κB mainly by the classical pathway, this observation indicates that HBZ expression modulates the transcription of genes activated by the classical pathway. However, overexpression of *sHBZ* gene might influence these results. Indeed, the level of sHBZ mRNA in sHBZ expressing Jurkat cells was much higher than that in an ATL cell line, MT-4 (Figure 7B). However, the level of sHBZ expression in MT-4 cells is comparable to that of $CD4^+$ T cells from

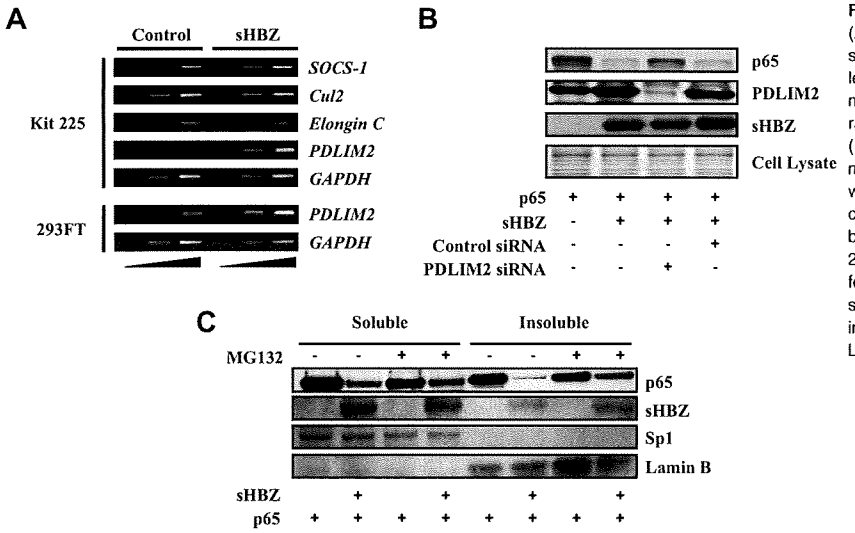

Figure 6. HBZ up-regulates expression of *PDLIM2* gene. (A) sHBZ up-regulates *PMLIM2*. Total RNA was extracted from sHBZ-expressing or control Kit 225 and 293FT cells. The levels of *SOCS-1*, *Cul2*, *Elongin C*, *PDLIM2*, and *GAPDH* mRNA were measured by semiquantitative RT-PCR. The ramp on the left represented an increasing PCR cycle number. (B) Reducing PDLIM2 expression by siRNA recovered sHBZ-mediated suppression of p65. 293FT cells were transfected with expression vectors together with PDLIM2 siRNA or control siRNA. Protein expression was analyzed by western blotting. (C) sHBZ induced the degradation of insoluble p65. 293FT cells, untreated or treated with MG132, were transfected with mycHis-sHBZ along with FLAG-p65. After 48 hours, soluble and insoluble nuclear fractions were subjected to immunblotting. The expression levels of p65, sHBZ, Sp1, and Lamin B were detected.

HBZ-transgenic mice. In this transgenic mouse, sHBZ gene is expressed by a mouse CD4-specific promoter/enhancer/silencer. To investigate HBZ-mediated suppression of genes activated by the classical NF-κB pathway in vivo, we studied the expression of p65-specific target genes in thymus $CD4^+$ cells from sHBZ transgenic mice. After stimulating the cells with PMA/ionomycin, expressions of *IL2RA*, *IRF4,* and *VCAM-1* genes were suppressed in sHBZ transgenic mice as observed in sHBZ transfected Jurkat cells (Figure 7C).

Discussion

Activation of the NF-κB signaling pathway, which has been reported in various cancer cells, plays an important role in the development and progression of tumor cells.[46,47] In oncogenesis by HTLV-1, the *tax* gene has been extensively studied. Tax can activate various transcription pathways and functionally inhibit p53 and MAD1.[7] Furthermore, various tumors have been observed in *tax* gene transgenic animals, depending on the promoter used.[48,49] These findings show the oncogenic potential of Tax in vivo. Although Tax can transform Rat-1 cells in vitro, a Tax mutant lacking the ability to activate NF-κB lost its transforming activity, indicating that NF-κB activation is indispensable for Tax mediated transformation.[18] Tax can activate NF-κB by both the classical and alternative pathways via its interactions with IKKγ[20] and p100.[22] Although they are often activated concurrently, the classical and alternative NF-κB pathways have distinct regulatory functions. Accumulating evidence suggests that the alternative NF-κB pathway is more important in several cancers.[37] It has been reported that the classical and alternative NF-κB pathways differentially control genes with anti-apoptotic functions in lymphoma cell lines.[50] In transformation by Tax, it has been reported that the alternative pathway is critical.[51] This study demonstrates the selective suppression of the classical NF-κB pathway by HBZ, a phenomenon that selectively modulates NF-κB activation by Tax. In many ATL cells, Tax is not expressed, while the *HBZ* gene is expressed in all ATL cases. Even in ATL cells without Tax expression, NF-κB is constitutively activated.[52] Recently, elevated expression of NIK has been reported in ATL cells.[53] Because NIK activates both the classical and alternative NF-κB pathways, HBZ might modulate the classical NF-κB pathway even in the absence of Tax, leading to predominant activation of alternative pathway, and perhaps to oncogenesis.

The classical NF-κB pathway is still potently activated by Tax in the HTLV-1 transformed T-cell lines, such as MT-4 and MT-2, regardless of HBZ expression. Because these cell lines express large amount of Tax, it is likely that HBZ does not have strong suppressive effect on classical pathway of NF-κB due to excess Tax expression. However, the effect of HBZ on the classical NF-κB pathway may be more pronounced when the level of Tax expression is down-regulated or silenced as in chronically infected T cells in infected people and ATL cells.

Many viruses have developed strategies to manipulate NF-κB signaling through the use of multifunctional viral proteins. For example, the HIV-1 encoded Tat protein enhances NF-κB mediated LTR activation while HIV-1 Nef induces the expression of NF-κB inhibitor, IκBα, to suppress this pathway.[54] In Epstein-Barr virus, the LMP-2 viral protein activates the NF-κB pathway by the recruitment of cellular adaptor proteins, TNF receptor–associated factor families and TNF receptor–associated death domain, to the C-terminal domain. Like HBZ, the EBV bZIP protein inhibits the classical NF-κB pathway through interacting with p65.[28] Similar suppression of NF-κB has been reported for other viruses, including African swine fever virus, hepatitis C virus, and human herpesvirus-8. These findings show that NF-κB suppressive activities are common among different viruses, suggesting that these activities are important for viral infection. In this regard, it is noteworthy that transcription of the *IFNγ* and *IRF4* genes, which is induced by the classical pathway, is suppressed by HBZ as shown in this study. A virus might facilitate escape from the host immune system by suppressing the classical NF-κB pathway in such a manner.

Viruses have evolved to sneak through the innate and adaptive antiviral response both at the cellular and whole organism levels, for survival and successful spread of infection. One mechanism used by some viruses to avoid immune surveillance is to control the level of cellular transcription factors by sorting them for degradation through ubiquitination. In all instances of which we are currently aware, this modulation process takes place at the level of E3 ligase, that is, at the step where the substrate specificity is critically defined. Some viral proteins act as E3 ligases, and others redirect host ubiquitin E3 ligases to target new substrate proteins. For example, the E6 oncoprotein of human papilloma virus binds

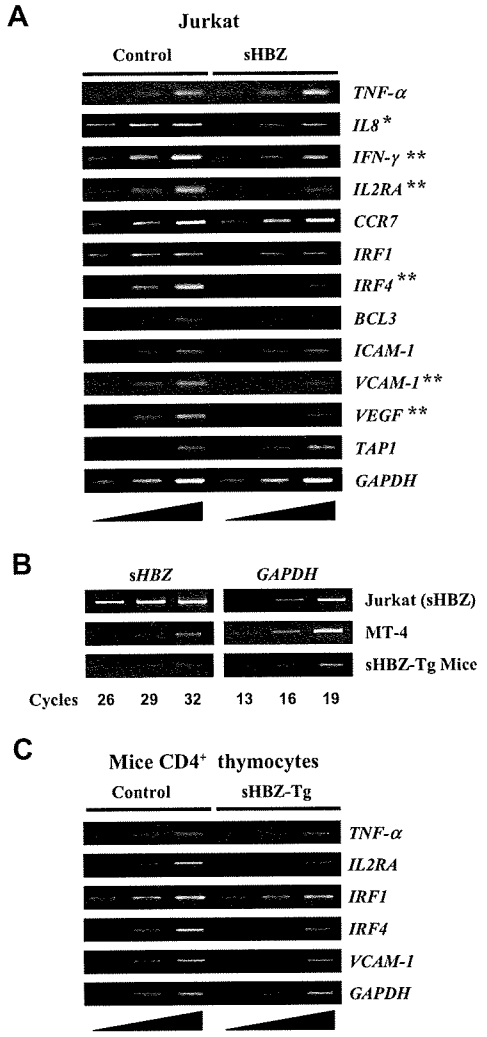

Figure 7. Suppressed expression of selected classical NF-κB target genes in vitro and in vivo by sHBZ. (A) Transcriptional changes of selected classical NF-κB target genes in sHBZ-expressing Jurkat cells. After stimulating the cells with PMA plus ionomycin, the levels of *TNF-α*, *IL-8*, *IFN-γ*, *IL2RA*, *CCR7*, *IRF1*, *IRF4*, *BCL3*, *ICAM-1*, *VCAM-1*, *VEGF*, *TAP1*, and *GAPDH* mRNA were analyzed by increasing cycles of semiquantitative RT-PCR, represented by the ramp on the left. (B) Comparison of the *HBZ* gene transcripts in an ATL cell, MT-4, in HBZ-transfected Jurkat cells, and in CD4+ thymocytes from *HBZ* transgenic mice. All samples were amplified over the same number of PCR cycles as shown. (C) Transcriptional changes of selected classical NF-κB target genes in CD4+ thymocytes from sHBZ transgenic mice. After stimulating the cells with PMA plus ionomycin, the levels of *TNF-α*, *IL2RA*, *IRF1*, *IRF4*, *VCAM-1*, and *GAPDH* mRNA were analyzed by increasing cycles of semiquantitative RT-PCR, represented by the ramp on the left. $^{*}P < .05$; $^{**}P < .01$.

the tumor suppressor p53 through its interaction with another cellular protein, E6-associated protein, leading to the degradation of p53 via the ubiquitin-mediated pathway.[55] VIF encoded by HIV-1 connects APOBEC3G and APOBEC3F as a substrate to the multisubunit E3 ligase for polyubiquitination and degradation.[56] Our study is the first to report that a viral protein enhances expression of the cellular ubiquitin E3 ligase, PDLIM2, resulting in the degradation of p65. In T cells, PDLIM2 can interact with STAT and p65 transcription factors and promote their polyubiquitination and subsequent degradation, thereby negatively regulating STAT and NF-κB–dependent signaling.[41,57] We have not yet clarified whether HBZ can negatively regulate the JAK/STAT pathway. Because PDLIM2 is expressed not only in T cells but also in innate immune cells,[57] we speculate that the positive effect of HBZ on PDLIM2 expression might also influence T-cell proliferation and immune responses.

There are 2 transcripts of the *HBZ* gene. The transcript of the *sHBZ* gene is more abundant than that of usHBZ as reported.[14] Inhibitory effect of sHBZ on Tax mediated transcription from 5′LTR was much stronger than that of usHBZ, and sHBZ has a much longer half-life than usHBZ. Therefore, the protein level of sHBZ is much higher than that of usHBZ,[32] which leads to stronger inhibitory activity of the classical NF-κB pathway as shown in this study. Taken together, sHBZ, rather than usHBZ, is more important in HTLV-1 infected cells.

As shown in this study, HBZ downmodulates the classical NF-κB pathway by 2 mechanisms, (1) inhibition of DNA binding by p65 and (2) enhanced degradation of p65, leading to decreased expression of some RelA specific target genes. Such a function of HBZ in cooperation with Tax-mediated activation might be beneficial for proliferation of infected cells and oncogenesis. Further studies are necessary to clarify the significance of HBZ in proliferation of infected cells and oncogenesis.

Acknowledgments

This work was supported by a Grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan, a grant from the Uehara Memorial Foundation, a grant from the Naito Foundation, and a grant from the Sumitomo Foundation to M.M.

Authorship

Contribution: T.Z., J.Y., Y.S., M.N., M.F., and M.M. designed the research; T.Z., J.Y., Y.S., and M.T. performed the research; T.Z., M.N., M.F., and M.M. analyzed the data; and T.Z., J.Y., M.N., M.F., and M.M. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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In vivo expression of the HBZ gene of HTLV-I correlates with proviral load, inflammatory markers and disease severity in HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP)

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Published: 19 February 2009

Retrovirology 2009, **6**:19 doi:10.1186/1742-4690-6-19

Received: 27 November 2008
Accepted: 19 February 2009

This article is available from: http://www.retrovirology.com/content/6/1/19

Abstract

Background: Recently, human T-cell leukemia virus type I (HTLV-I) basic leucine zipper factor (HBZ), encoded from a minus strand mRNA was discovered and was suggested to play an important role in adult T cell leukemia (ATL) development. However, there have been no reports on the role of HBZ in patients with HTLV-I associated inflammatory diseases.

Results: We quantified the HBZ and tax mRNA expression levels in peripheral blood from 56 HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients, 10 ATL patients, 38 healthy asymptomatic carriers (HCs) and 20 normal uninfected controls, as well as human leukemic T-cell lines and HTLV-I-infected T-cell lines, and the data were correlated with clinical parameters. The spliced HBZ gene was transcribed in all HTLV-I-infected individuals examined, whereas tax mRNA was not transcribed in significant numbers of subjects in the same groups. Although the amount of HBZ mRNA expression was highest in ATL, medium in HAM/TSP, and lowest in HCs, with statistical significance, neither tax nor the HBZ mRNA expression per HTLV-I-infected cell differed significantly between each clinical group. The HTLV-I HBZ, but not tax mRNA load, positively correlated with disease severity and with neopterin concentration in the cerebrospinal fluid of HAM/TSP patients. Furthermore, HBZ mRNA expression per HTLV-I-infected cell was decreased after successful immunomodulatory treatment for HAM/TSP.

Conclusion: These findings suggest that *in vivo* expression of HBZ plays a role in HAM/TSP pathogenesis.

Background

Human T-cell lymphotropic virus type 1 (HTLV-1) is a replication-competent human retrovirus [1,2] which is associated with adult T-cell leukemia (ATL) [3,4] and with a slowly progressive neurological disorder HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [5,6]. In HTLV-1 infection, approximately 5% develop ATL [7] and another 2%-3% develop chronic inflammatory diseases involving the central nervous system (HAM/TSP), the eyes [8], the lungs [9], the joints [10], or the skeletal muscles [11]; most infected individuals, however, remain healthy in their lifetime (healthy asymptomatic carriers: HCs). Although the factors that cause these different manifestations of HTLV-1 infection are not fully understood, previous population association studies suggested that both viral and host genetic factors influence the outcome of infection [12].

Among several HTLV-1 genes, a transcriptional activator Tax encoded in the pX region is thought to play a central role in immortalization, oncogenesis and inflammation through its pleiotropic activity [13]. In HAM/TSP patients, it has been reported that several cytokines, chemokines and matrix metalloproteinases transactivated by Tax protein such as tumor necrosis factor-α (TNF-α) [14], monocyte chemoattractant protein-1 (MCP-1) [15] and matrix metalloproteinase (MMP)-9 [16] are overexpressed in the infiltrating mononuclear cells in the patients' spinal cords. In addition, a previous report from the United States suggested that the level of HTLV-1 tax mRNA expression in HTLV-1-infected cells (mRNA/DNA ratio) was significantly higher in HAM/TSP patients than HCs, and this finding correlated with the HTLV-1 proviral load, Tax-specific CD8+ T cell frequency and disease severity of the patients [17]. A report from Japan also indicated that HTLV-1 tax mRNA expression was higher in HAM/TSP than HCs, although the mRNA/DNA ratio was similar between both groups [18]. These results suggest an important role of Tax in the induction of HAM/TSP.

It has been reported that among fresh leukemic cells isolated from ATL patients, about 60% of cases do not express the tax transcript [19]. In tax transgenic mouse models, the mice develop a wide range of tumors such as neurofibrosarcomas, mesenchymal tumors, and mammary adenomas, or even skeletal abnormalities including osteolytic bone metastases [20-27]; however, no leukemias or lymphomas were identified except in three models, which used respectively the granzyme B promoter [28], Lck proximal promoter [29] and Lck distal promoter [30]. These findings suggest that Tax is required for malignant transformation but not essential for the maintenance of leukemic cells *in vivo*. Recently, a novel basic leucine zipper protein encoded by the complementary strand of the HTLV-1 genome, named HTLV-1 basic leucine zipper factor (HBZ), was characterized [31]. HBZ is expressed in all ATL cells [32], promotes proliferation of T-lymphocytes in its RNA form [32], suppresses Tax-mediated transactivation through the 5' LTR [31,33], promotes CD4+ T-lymphocyte proliferation in transgenic mice [32], and enhances infectivity and persistence in HTLV-1-inoculated rabbits [34].

In this study, we investigated whether HTLV-1 HBZ mRNA expression is associated with clinical and laboratory markers reported in HAM/TSP patients, including HTLV-1 proviral load, neopterin concentration in cerebrospinal fluid (CSF), and motor disability score. In addition, to confirm the previous observations [17,18], we have also investigated the tax mRNA expression in ATL patients, HAM/TSP patients, and HCs by using the same technology but in a larger number of subjects.

Methods

Patients and cells

Human leukemic T-cell lines (Jurkat, MOLT-4, and CEM) and HTLV-1-infected T-cell lines (C5/MJ, SLB1, HUT102, MT-1, MT-2, and MT-4) were cultured in RPMI 1640 medium supplemented with 10% FCS. The diagnosis of HAM/TSP was done in accordance with World Health Organization criteria [35]. The diagnosis of ATL was made on the basis of clinical features, hematological characteristics, serum antibodies against HTLV-1 antigens, and detection of the HTLV-1 viral genome inserted into leukemia cells by Southern blot hybridization. All the PBMC samples used in this study were collected prior to treatment by a Histopaque-1077 (Sigma) density gradient centrifugation, washed and stored in liquid nitrogen until use. This research was approved by the institutional review boards of the authors' institutions, and informed consent was obtained from all individuals.

Quantification of HTLV-I proviral load, tax and HBZ mRNA expression, anti-HTLV-I antibody titers and neopterin concentration in cerebrospinal fluid

RNA was extracted from PBMCs using RNeasy Mini Kit with on-column DNase digestion (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using TaqMan Gold RT-PCR Kit (Applied Biosystems, Tokyo, Japan). For cDNA synthesis from extracted mRNA, 2 μg total RNA, 10 μl 10×TaqMan RT buffer, 22 μl $MgCl_2$ (25 mM), 20 μl dNTPs mixture (at a final concentration of 500 μM each), 5 μl random hexamers (50 μM), 2 μl RNase inhibitor (20 U/μl), and 2.5 μl (50 U/μl) Moloney murine leukemia virus reverse transcriptase were added to a total volume of 100 μl. Samples were incubated at 25°C for 10 minutes and 48°C for 30 minutes, and reactions were stopped by heating to 95°C for 5 minutes. Genomic DNA was extracted from the frozen PBMCs by QIAamp blood kit

(QIAGEN, Tokyo, Japan). We, then, carried out a real time quantitative PCR using ABI Prism 7900 HT Fast Real-Time PCR System (Applied Biosystems) to examine the HTLV-1 proviral load [36] and tax mRNA expression [17] in PBMCs or HTLV-1 infected cell lines as reported previously. The amount of the HTLV-1 proviral load was calculated using β-actin as an internal control through the following formula: copy number of HTLV-1 tax per cell = [(copy number of tax)/(copy number of β-actin/2)]. The sequences of primers for HTLV-1 provirus were as follows: 5'-CAA ACC GTC AAG CAC AGC TT-3' and 5'-TCT CCA AAC ACG TAG ACT GGG T-3', and the probe was 5'-TTC CCA GGG TTT GGA CAG AGT CTT CT-3'. HBZ mRNA expression levels were also quantified by real time quantitative PCR using the same method for tax mRNA [17]. Namely, serially diluted cDNA from HTLV-1 infected MT-2 cells was used for generating standard curves for the value of HTLV-1 tax or HBZ mRNA and hypoxanthine ribosyl transferase (HPRT) mRNA, and the relative HTLV-1 tax or HBZ mRNA load was calculated by the following formula: HTLV-1 tax mRNA load = value of tax/value of HPRT. HTLV-1 HBZ mRNA load = value of HBZ/value of HPRT. We used aliquots of the same standard MT-2 cDNA preparation for all assays and the correlation values of standard curves were always more than 99%. The sequences of primers for tax mRNA detection were as follows: 5'-ATC CCG TGG AGA CTC CTC AA-3' and 5'-ATC CCG TGG AGA CTC CTC AA-3', and the probe was 5'-TCC AAC ACC ATG GCC CAC TTC CC-3'. The sequences of primers for HBZ mRNA detection were as follows: 5'-AGA ACG CGA CTC AAC CGG-3' and 5'-TGA CAC AGG CAA GCA TCG A-3', and the probe was 5'-TGG ATG GCG GCC TCA GGG CT-3'. As the probes for tax and HBZ mRNA surrounded the splice junction site of each mRNA, we detected HBZ splicing isoform, which is the most abundant HBZ transcript and contributed significantly to HBZ protein synthesis [37-39], but not unspliced form in this study. We used the HPRT primers and probe set (Applied Biosystems) for internal calibration. The tax and HBZ probes were labeled with fluorescent 6-carboxyfluorescein (FAM) (reporter) at the 5' end and fluorescent 6-carboxy tetramethyl rhodamine (TAMRA) (quencher) at the 3' end. All assays were performed in triplicate. The sensitivity of our real-time RT-PCR assay was determined using MT-2 cells diluted serially with PBMCs from a healthy uninfected donor. The HTLV-1 mRNA signal (both tax and HBZ) could be detected in a dose-dependent manner with a sensitivity limit as low as one MT-2 cell in 10^6 PBMCs. Neopterin levels were evaluated by HPLC with fluorometric detection methods as described previously [40]. Serum HTLV-1 antibody titers were determined by a particle agglutination method (Serodia-HTLV-1®, Fujirebio, Japan).

Clinical evaluation

Motor dysfunction seen in HAM/TSP patients was evaluated by clinical neurologists according to the Osame Motor Disability Score (OMDS) [41], which grades motor dysfunction from zero (normal walking and running) to 13 (complete bedridden) as follows: 1 = normal gait but runs slow; 2 = abnormal gait; 3 = abnormal gait and unable to run; 4 = need support while using stairs; 5 = need one hand support in walking; 6 = need two hands support in walking; 7 = need two hands support in walking but is limited to 10 m; 8 = need two hands support in walking but is limited to 5 m; 9 = unable to walk but able to crawl on hands and knees; 10 = crawls with hands; 11 = unable to crawl but can turn sideways in bed; 12 = unable to turn sideways but can move the toes. We have used OMDS throughout our previous studies [41-43] because this is a neurological measure of disability weighted toward ambulation and was specifically developed to evaluate motor dysfunction seen in HAM/TSP patients. It is therefore more suitable for evaluating HAM/TSP motor symptoms than the widely used EDSS [44]. The laboratory data were examined by an investigator who was not involved in the patients' clinical care, and the neurologists who made the clinical evaluation did not have access to the laboratory data.

Statistical analysis

The Mann-Whitney U test was used to compare data between two groups. Correlations between variables were examined by Spearman rank correlation analysis. Values of $p < 0.05$ were considered statistically significant.

Results

HTLV-I tax and HBZ mRNA load in HAM/TSP, ATL and HCs

A total of 56 HAM/TSP patients, 10 ATL patients and 38 HCs completed the evaluation. Twenty normal uninfected healthy controls (NCs) were used as negative controls. The HTLV-1 proviral load in this study represents the copy number of HTLV-1 tax per cell (for HTLV-1 infected cell lines) or PBMC (for HAM/TSP, ATL and HCs) (Table 1). Therefore, the HTLV-1 proviral load represents the population of infected cells in PBMCs when one cell harbors one provirus. However, since recent data by Kamihira et al. indicated that 43 out of 321 ATL specimens (17.8%) showed two or more bands by Southern blot analysis after *EcoRI* digestion [45], we reviewed the Southern blot data of our 10 ATL patients. As a result, two distinct bands of over 9 kb were observed in *EcoRI* digestion in samples from two ATL patients, indicating at least the biclonal integration of HTLV-1 proviral DNA. The incidence of multibands in our cases (two out of ten: 20%) was comparable with the data by Kamihira et al. (17.8%). The

Table 1: HTLV-I mRNA load, proviral load and mRNA/DNA ratio in HTLV-I – infected individuals and T-cell lines.

Cell line	HBZ mRNA[a]	tax mRNA[b]	Proviral load[c]	HBZ mRNA/DNA[d]	tax mRNA/DNA[e]
C5/MJ	13.3	0.062	8.1	1.64	0.0076
HUT102	1.2	26.35	19.3	0.063	1.37
MT1	25.2	0.011	7.1	3.56	0.0015
MT2	7.8	1.24	16.2	0.48	0.077
MT4	2.4	1.71	12.6	0.19	0.135
SLB1	25.8	87.4	115.5	0.22	0.756
HAM/TSP*	0.74 (0.023–33.50)	0 (0–0.041)	0.051 (0.0008–0.41)	19.10 (0.81–273.45)	0 (0–0.32)
HCs*	0.15 (0.0013–6.42)	0 (0–0.000078)	0.0089 (0.0001–0.10)	16.67 (0.21–7358.91)	0 (0–0.11)
ATL*	31.43 (5.93–225.64)	0.000018 (0–0.59)	1.14 (0.25–2.88)	24.04 (13.77–135.83)	0 (0–0.29)

*The results represent the median and range (n = 56 for HAM/TSP, n = 38 for HCs and n = 10 for ATL)
[a]HTLV-I HBZ mRNA load = value of HBZ/value of HPRT
[b]HTLV-I tax mRNA load = value of tax/value of HPRT
[c]Proviral load: HTLV-I tax copy number per cell
[d]HBZ mRNA/DNA ratio = HTLV-I HBZ mRNA load/Proviral load
[e]tax mRNA/DNA ratio = HTLV-I tax mRNA load/Proviral load

number of HTLV-1 proviral load in MT-2 cells measured by our quantitative PCR method (16.2 copies/cell) was also comparable with the previous report (12.6 copies/cell) [46].

The HTLV-1 proviral load was significantly greater in HAM/TSP patients (median 0.051, range 0.0008–0.41) than HCs (median 0.0089, range 0.0001–0.10) (P = 0.000011, Mann Whitney U test, Table 1). The HTLV-1 HBZ mRNA level was highest in ATL, medium in HAM/TSP, and lowest in HCs with statistical significance (Table 1 and Figure 1A). It is noteworthy that we could detect HTLV-1 HBZ gene transcripts in all infected individuals tested. Interestingly, there were three cases with extremely high data of HBZ mRNA in HCs (Figure 1C). Since recent report by Shimizu et al. indicated that HTLV-1-specific T-cell responsiveness widely differed among HTLV-1 carriers [47], these extremely high data of HBZ mRNA might be explained by immunological diversity observed in HCs. In contrast, although the HTLV-1 tax mRNA levels in ATL patients was significantly higher than HCs (p = 0.014, Mann-Whitney U test), the HTLV-1 tax mRNA levels between HCs-HAM/TSP and HAM/TSP-ATL did not reach statistical difference (Figure 1B). We could not detect any HTLV-1 tax and HBZ mRNA expression in any of the 20 NCs and 3 uninfected human leukemic T-cell lines (Jurkat, MOLT-4, and CEM) tested (data not shown).

Comparison of HTLV-I tax and HBZ mRNA load with HTLV-I proviral load

To test whether higher HBZ mRNA levels reflect higher proviral load, we adjusted the tax or HBZ mRNA load (i.e. value of tax or HBZ/value of HPRT) by the HTLV-1 proviral load (i.e. HTLV-1 tax copy number per cell). As a result, neither tax nor the HBZ mRNA/DNA ratio differed significantly between each clinical group (i.e. HAM/TSP-HCs, HAM/TSP-ATL and HCs-ATL) (figure 1C, D). Interestingly, although both HTLV-1 proviral load and tax mRNA/DNA ratio were higher in HTLV-1-infected cell lines (C5/MJ, SLB1, HUT102, MT-1, MT-2, and MT-4) than PBMCs, HBZ mRNA/DNA ratio was even higher in PBMCs than HTLV-1-infected cell lines (Table 1). Consistent with the previous observations that HBZ suppresses Tax mediated transactivation through the 5' LTR [31,33,48], HBZ mRNA load tended to be higher in cell lines with lower tax mRNA load, and indeed HBZ mRNA/DNA ratio was inversely correlated with tax mRNA/DNA ratio in 6 HTLV-1-infected cell lines (Spearman's rank correlation coefficient r = -0.943, P = 0.035) (Table 1 and data not shown), although such correlation was not observed between HBZ and tax mRNA/DNA ratio in PBMCs from HAM/TSP patients, ATL patients, HCs and all groups combined (data not shown). As shown in Figure 2, the HTLV-1 HBZ mRNA load was significantly correlated with HTLV-1 proviral load in HAM/TSP patients (P = 0.0005, r = 0.470 by Spearman rank correlation analysis), HCs (P = 0.0013, r = 0.528) and all groups combined (P < 0.000001, r = 0.686), but not in ATL patients (P = 0.300, r = 0.345). The tax mRNA load was correlated with the HTLV-1 proviral load in HCs (P = 0.045, r = 0.444), ATL patients (P = 0.045, r = 0.673), and all groups combined (P < 0.01, r = 0.365), but not in HAM/TSP patients (P = 0.411, r = 0.210).

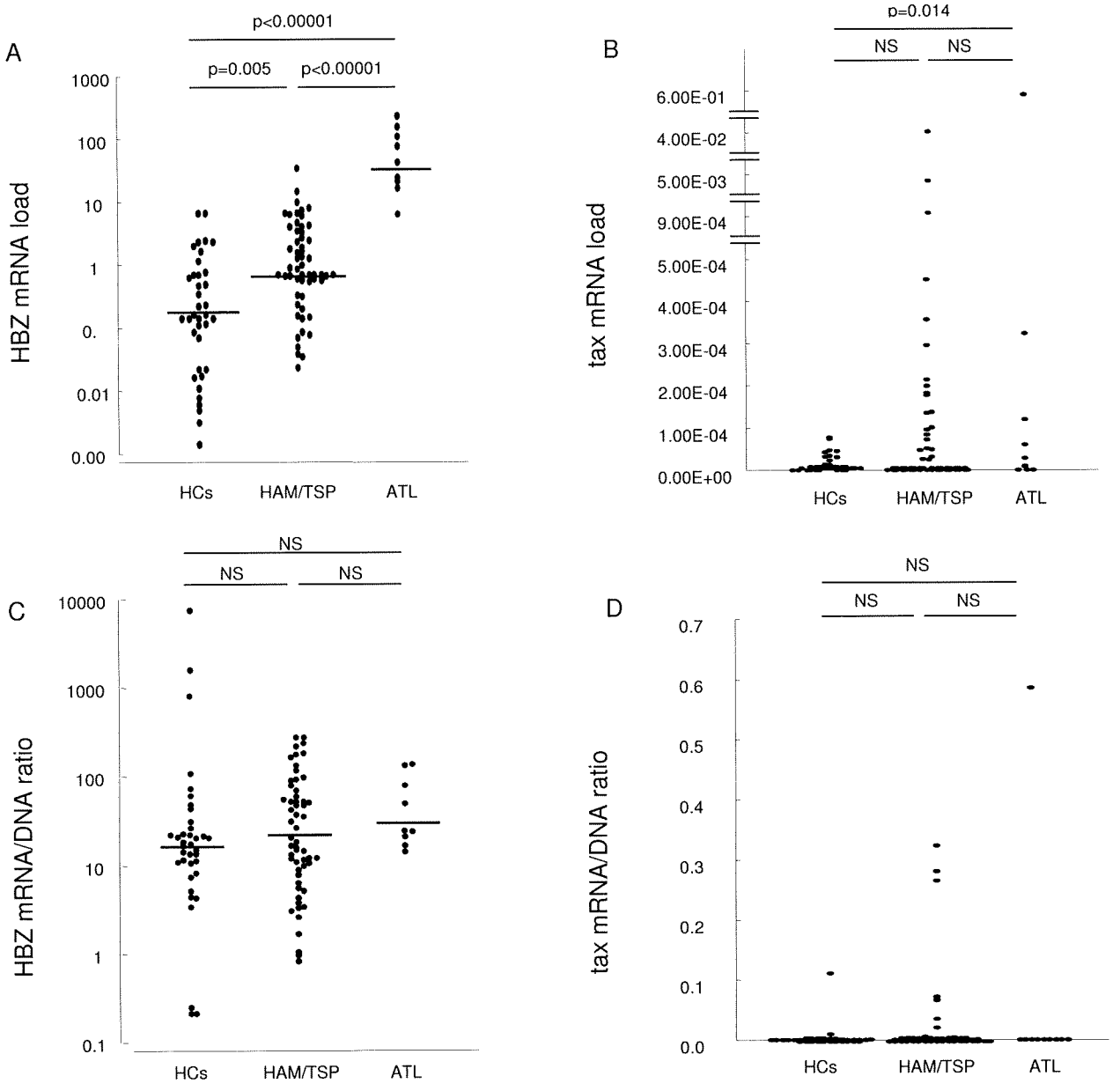

Figure 1

HTLV-I tax and HBZ mRNA load in patients with HAM/TSP, ATL and asymptomatic HTLV-I carriers. A. HTLV-I HBZ mRNA load was highest in ATL, medium in HAM/TSP, and lowest in HCs. B. The HTLV-I tax mRNA load between HCs and HAM/TSP, HAM/TSP and ATL did not reach statistical significance, although the HTLV-I tax mRNA load in ATL patients was significantly higher than HCs ($p = 0.014$, Mann Whitney U test). C and D. To normalize the HTLV-I tax or HBZ mRNA expression level per provirus, the mRNA/DNA ratio was calculated by dividing the HTLV-I tax or HBZ mRNA load by the HTLV-I proviral load. Neither the HBZ (C) nor the tax (D) mRNA/DNA ratio differed significantly between each clinical group (HAM/TSP – HCs, HAM/TSP – ATL, HCs – ATL). The zero value of tax gene transcripts was observed in 60.7% of HAM/TSP patients (34 out of 56), 71.1% of HCs (27 out of 38) and 30.0% of ATL patients (3 out of 10). The medians are represented by horizontal lines and the statistical differences between them were calculated with a Mann Whitney U test.

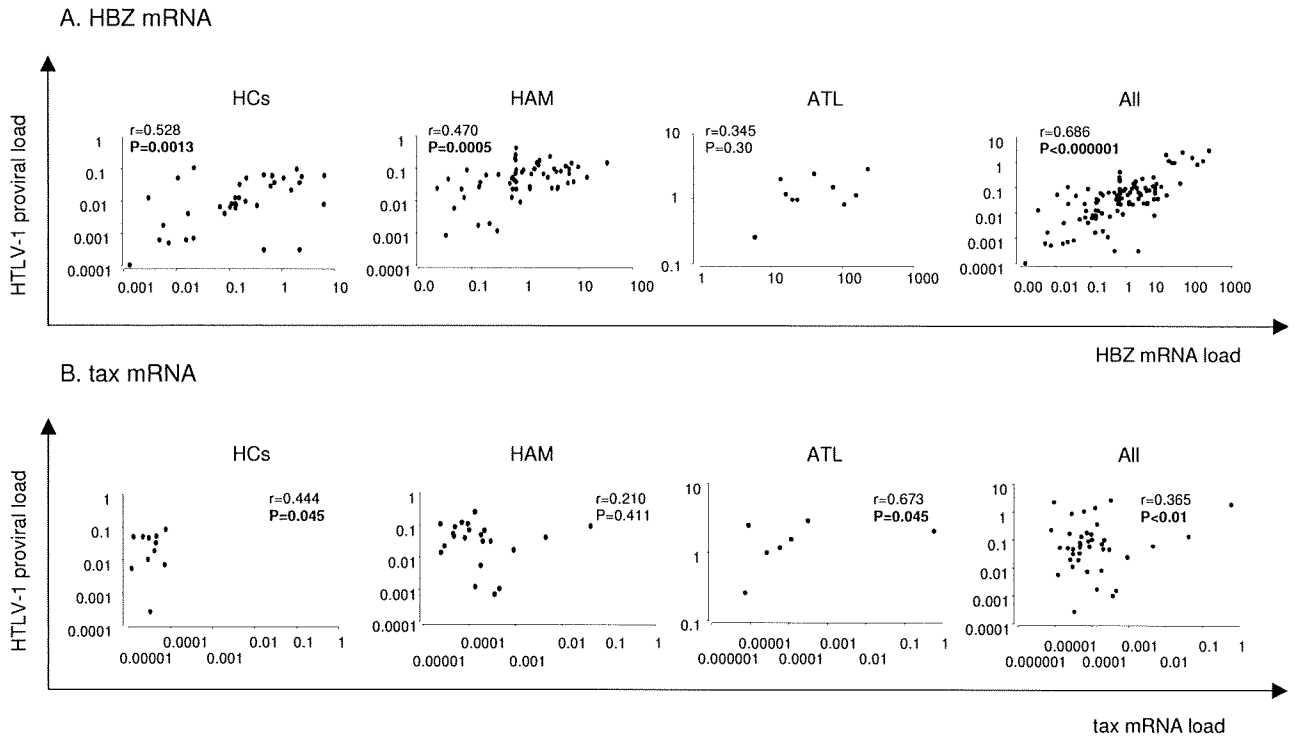

Figure 2
Correlation between HTLV-I proviral load and HTLV-I mRNA load in HTLV-I infected individuals. A. The HTLV-I HBZ mRNA load was significantly correlated with HTLV-I proviral load in HAM/TSP patients alone (P = 0.0005, r = 0.470 by Spearman rank correlation analysis), HCs alone (P = 0.0013, r = 0.528) and all groups combined (P < 0.000001, r = 0.686) but not in ATL patients (P = 0.300, r = 0.345). B. The tax mRNA load correlated with the HTLV-I proviral load in HCs (P = 0.045, r = 0.444), ATL patients (P = 0.045, r = 0.673) and both group combined (P < 0.01, r = 0.365) but not in HAM/TSP patients (P = 0.411, r = 0.210). The zero value of tax gene transcripts did not appear in the figures. Correlations were examined by Spearman rank correlation analysis.

Comparison of HBZ mRNA load with tax mRNA load among HTLV-I infected individuals in different clinical status

To investigate the mutual expression status of HBZ and tax mRNA in different clinical status, we calculated the ratio of HBZ mRNA/tax mRNA in 22 HAM/TSP patients, 11 HCs and 7 ATL patients, who express both tax and HBZ mRNA in PBMCs. HTLV-1 tax mRNA was not expressed in 60.7% (34 out of 56) of HAM/TSP patients, 71.1% (27 out of 38) of HCs and 30.0% (3 out of 10) of ATL patients, whereas HTLV-1 HBZ mRNA was expressed in all the infected individuals tested. As shown in figure 3, HBZ mRNA/tax mRNA ratio in PBMCs was significantly increased in ATL patients than HAM/TSP patients and HCs (P = 0.013 and 0.0051, Mann-Whitney U test, respectively), indicating very high HBZ transcript levels relative to tax, especially in ATL patients.

Correlation of HTLV-I HBZ mRNA load with CSF neopterin concentration and disease severity in HAM/TSP patients

To investigate the relationship between HTLV-1 mRNA load and various laboratory markers, HTLV-1 proviral load, CSF neopterin concentration and anti-HTLV-1 antibody titers were quantified and compared with motor dysfunction of HAM/TSP patients. Since neopterin is a low molecular weight pteridine compound released from macrophages upon stimulation with γ-interferon secreted by activated T cells, the measurement of neopterin concentrations in body fluids like blood serum, CSF or urine provides information about cellular immune activation in humans under the control of type 1 T helper cells [49]. As shown in table 2, we showed that the CSF neopterin level, which was positively correlated with proviral load, was also positively correlated with the HBZ mRNA load in HAM/TSP patients (Spearman's rank correlation coefficient P = 0.0052, r = 0.437). However, such a correlation was not observed between neopterin and HTLV-1 tax mRNA load (P = 0.544, r = 0.228). Motor dysfunction evaluated by OMDS significantly correlated with HTLV-1 HBZ mRNA load (P = 0.023, r = 0.328), but again not with HTLV-1 tax mRNA load (P = 0.401, r = 0.241).

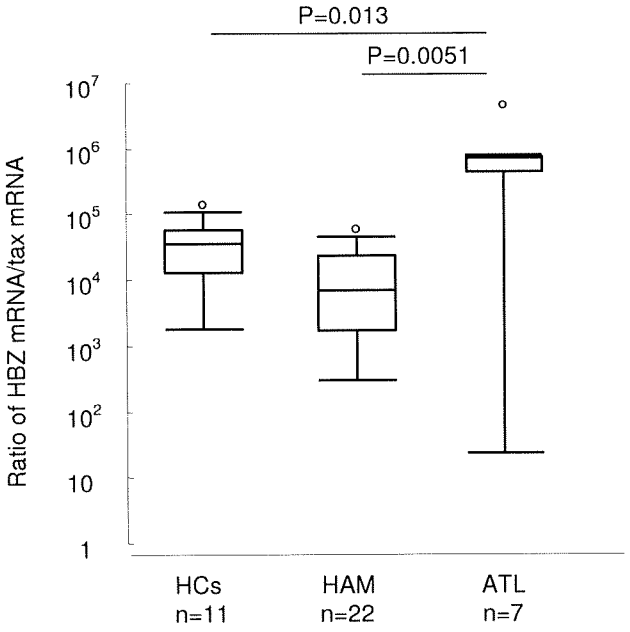

Figure 3
Comparison of HBZ mRNA load with tax mRNA load among HTLV-I infected individuals in different clinical status. The ratio of HBZ mRNA/tax mRNA was significantly increased in ATL patients (median 700,512.24, range 23.11 – 4,308,413.02) than HAM/TSP patients (median 4,932.41, range 295.63–56,082.14) or HCs (median 35,602.96, range 1,804.77–137,999.33). The statistical differences between groups were calculated with a Mann Whitney U test.

HBZ mRNA load and HBZ mRNA/DNA ratio in PBMCs was decreased in HAM/TSP patients after effective IFN-treatment

Finally, to determine whether HTLV-1 mRNA load and mRNA/DNA ratio are associated with clinical improvement, we measured the HTLV-1 (both tax and HBZ) mRNA load and mRNA/DNA ratio before, during, and after interferon-alpha (IFN-α) treatment in four HAM/TSP patients who received 4 weeks of daily administration. Three million international units (IU) of IFN-α (human lymphoblastoid interferon-HLBI, Sumiferon® by Sumitomo Pharmaceutical Co., Osaka, Japan) were administrated per intramuscular injection. Two patients (HAM1 and 2) showed marked clinical improvement with the changes of the OMDS, whereas two patients (HAM3 and 4) did not show clinical improvement (without the changes of the OMDS) (Additional file 1). The HBZ mRNA load and mRNA/DNA ratio was decreased after IFN-α treatment in two patients who showed clinical improvement, whereas the HBZ mRNA load and mRNA/DNA ratio was stable during the treatment in two patients without clinical improvement (Additional file 1 and Figure 4). In contrast, the tax mRNA load and mRNA/DNA ratio did not show such a clear correlation with clinical improvement.

Discussion

In this study, we demonstrated that there was a statistically significant difference in the HTLV-1 HBZ mRNA load, but not tax mRNA load, in PBMCs between HAM/TSP patients and HCs. This is probably because tax mRNA was not expressed in significant numbers of individuals tested (60.7% of HAM/TSP patients, 34 out of 56; 71.1% of HCs, 27 out of 38; 30.0% of ATL patients, 3 out of 10), whereas HTLV-1 HBZ mRNA was expressed in all the infected individuals tested. There was also a statistically significant correlation between HTLV-1 HBZ mRNA load and HTLV-1 proviral load both in HAM/TSP patients and HCs, whereas tax mRNA load correlated with the HTLV-1 proviral load only in HCs but not in HAM/TSP patients. Recently, Usui et al. reported a similar observation [37]. Namely, HBZ spliced isoform mRNA was detectable in samples from most HCs and ATL patients, and was significantly correlated with the HTLV-1 proviral load. These results indicate that the regulation of HBZ mRNA expression is different from that of tax mRNA. It seems likely that HBZ mRNA is near-equally expressed by all provirus-positive cells despite different clinical status, while tax

Table 2: Results of rank correlation test between clinical and virological parameters.

	Proviral load		HBZ mRNA[a]		tax mRNA[b]		HBZ mRNA/DNA[c]		tax mRNA/DNA[d]	
	r	p	r	p	r	p	r	p	r	p
OMDS	0.169	0.285	0.328	**0.023**	0.241	0.401	0.252	0.091	0.257	0.300
Neopterin in CSF	0.512	**0.001**	0.437	**0.0052**	0.228	0.544	0.121	0.442	0.211	0.608
Serum Ab	0.117	0.431	0.185	0.194	0.234	0.333	0.102	0.497	0.248	0.279
CSF Ab	0.071	0.639	0.042	0.801	-0.0029	0.322	-0.046	0.690	0.0025	0.345

OMDS: Osame Motor Disability Scale for HAM/TSP
[a]HTLV-I HBZ mRNA load = value of HBZ/value of HPRT
[b]HTLV-I tax mRNA load = value of tax/value of HPRT
[c]HBZ mRNA/DNA ratio = HTLV-I HBZ mRNA load/Proviral load
[d]tax mRNA/DNA ratio = HTLV-I tax mRNA load/Proviral load

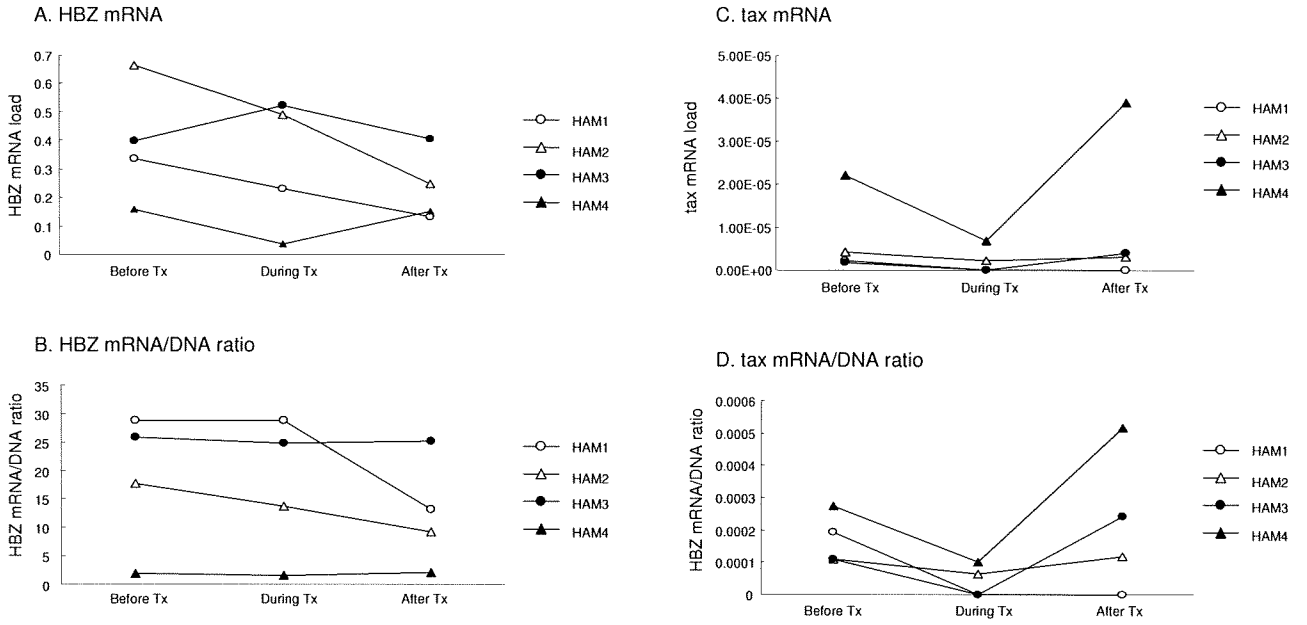

Figure 4

HBZ mRNA load and HBZ mRNA/DNA ratio in PBMCs were decreased in HAM/TSP patients after effective IFN-α treatment. To investigate whether HTLV-I mRNA load and mRNA/DNA ratio are associated with clinical improvement, we measured the HBZ mRNA/DNA ratio in four HAM/TSP patients who received 4 weeks of daily IFN-α administration (three million international units of IFN-α per one intramuscular injection). Two HAM/TSP patients with clinical improvement in Osame Motor Disability Score (OMDS) (HAM1 and 2) showed decreased HBZ mRNA load and HBZ mRNA/DNA ratio during the IFN-α treatment, whereas two HAM/TSP patients without clinical improvement in OMDS (HAM3 and 4) showed stable HBZ mRNA load and HBZ mRNA/DNA ratio during the IFN-α treatment. In contrast, the tax mRNA load and tax mRNA/DNA ratio did not show such a clear correlation with clinical improvement.

mRNA expression levels are variable in different clinical status.

When HTLV-1 tax or HBZ mRNA load was adjusted with HTLV-1 proviral DNA load (i.e. calculate mRNA/DNA ratio), the amount of tax and HBZ mRNA expressed per provirus was not significantly different between HAM/TSP patients and HCs, suggesting that the higher HTLV-1 proviral load seen in HAM/TSP patients caused higher HTLV-1 HBZ mRNA expression. This is consistent with our previous study using different methods for mRNA and DNA quantification [18], but differed from a previous American study using exactly the same methods, which showed significantly higher mRNA/DNA ratio in HAM/TSP patients than HCs [17]. In contrast to the previous study, which showed significant correlation between disease severity in HAM/TSP patients and both HTLV-1 tax mRNA load and mRNA/DNA ratio [17], we could not find such a correlation between clinical parameters of HAM/TSP patients including disease severity and both HTLV-1 tax mRNA load and mRNA/DNA ratio (Table 2). As we have already confirmed and reported the same levels of Tax protein expression in HTLV-1-infected PBMCs between HAM/TSP patients and HCs in the same cohort [50], the observed discrepancy may be due to the differences of a number of host genetic and virologic factors in HTLV-1 infected individuals, including differences in HLA haplotypes [51-53], differences in the amount of soluble suppressive factors and CD8+ T-cell responses, and differences in HTLV-1 tax genomic sequences [54]. As a recent report indicated that HTLV-I infection was associated with activated T-cell immunity in Jamaicans but with diminished T-cell immunity in Japanese persons [55], the interaction between different genes and/or environmental factors is also likely to contribute to the observed differences between the two populations. Namely, genetic resistance to infectious diseases that is formed by complex host genetic effects might be complicated further by pathogen diversity and environmental factors.

Another important observation is that the amount of HTLV-1 HBZ mRNA expression per provirus was more than a thousand times higher than tax mRNA expression both in HAM/TSP patients and HCs. Surprisingly, the amount of HTLV-1 HBZ mRNA expression per provirus was even higher in HTLV-1-infected PBMCs than in

infected cell lines, whereas tax mRNA expression was significantly higher in cell lines than infected PBMCs. Since HBZ suppresses Tax-mediated viral transcription [31], the abundant expression of HBZ mRNA in HTLV-1-infected PBMCs will be one of the molecular mechanisms involved in viral latency by suppressing HTLV-1 transcription and Tax expression, which may be a significant advantage to the virus in the infected cell by preventing its detection through a CTL response. Since we and others [37] found that down-regulation of tax mRNA (higher HBZ mRNA/tax mRNA ratio) was characteristic of primary ATL cells, imbalanced expression between HBZ and tax may induce the outgrowth of HTLV-1-transformed T cell and increase the risk of ATL, which is associated with a Tax-low or -negative phenotype.

We also found that the HTLV-1 HBZ mRNA load significantly correlated with the neopterin concentrations in CSF of HAM/TSP patients. Since neopterin levels in CSF have been used as an immunologic marker for monitoring disease activity and treatment efficacy of HAM/TSP [40,42,56], the quantitative analysis of HTLV-1 HBZ mRNA might also be used to monitor HAM/TSP disease activity. As expected, motor dysfunction of HAM/TSP patients evaluated by the OMDS score significantly correlated with HTLV-1 HBZ mRNA load ($P = 0.023$) but not with HTLV-1 tax mRNA load ($P = 0.401$). The correlation between HBZ mRNA load and two independent clinical parameters reflecting disease activities strongly suggest its stronger relevance than both tax mRNA and proviral load for HAM/TSP pathogenesis. This is further supported by the data that both HBZ mRNA load and HBZ mRNA/DNA ratio were decreased in HAM/TSP patients after effective IFN-α treatment. Collectively, our results suggest that higher HTLV-1 HBZ mRNA load may have relative prognostic value for the assessment of disease progression and could also be used as a surrogate marker to predict long-term outcome in HAM/TSP patients.

In summary, we showed that spliced HBZ gene was transcribed in all the HTLV-1 infected individuals examined, whereas tax mRNA was not transcribed in more than half in the same groups. Moreover, our data demonstrated a significant correlation between HTLV-1 HBZ mRNA load and HTLV-1 proviral load, neopterin concentrations in CSF and motor disability seen in HAM/TSP patients, indicating that HTLV-1 HBZ mRNA load may be a valid predictor of disease progression. Our present findings suggest that HTLV-1 HBZ mRNA expression plays a role not only in ATL, but also in the pathogenesis of the HTLV-1-associated inflammatory disease HAM/TSP.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MS designed and performed the experiments, analyzed the data, and wrote the paper; TM and KA provided clinical samples and assembled clinical database. YS and JY provided clinical samples and performed experiments. KS performed experiments, analyzed and interpreted data. MM made contribution to the conception and design of the study. YO contributed to obtaining funding and gave advice.

Additional material

Additional file 1

Changes in HBZ mRNA load and HBZ mRNA/DNA ratio in PBMCs of HAM/TSP patients after IFN- treatment.

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Acknowledgements

We are grateful to the staff and blood donors of Kagoshima University Hospital. We also thank Dr. Ryuji Kubota for providing the clinical samples, Prof. Masahiro Fujii of Niigata University for the gift of HTLV-I-infected T-cell lines (C5/MJ, SLB1, and MT-4), and Ms. Sumie Saito of Kanazawa Medical University for technical assistance. This work was supported by the Ministry of Health, Labor and Welfare, Japan (Neuroimmunological Disease Research Committee Grant to Y.O.); Takeda Science Foundation (to M.S.); Kanazawa Medical University (Grants H2007-11, H2008-11, C2008-2, and S2008-8 to M.S.).

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Journal of General Virology (2009), 90, 1806–1811 DOI 10.1099/vir.0.010199-0

Short Communication

HBZ is an immunogenic protein, but not a target antigen for human T-cell leukemia virus type 1-specific cytotoxic T lymphocytes

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Received 10 January 2009
Accepted 21 April 2009

Recently, HBZ has been reported to play an important role in the proliferation of adult T-cell leukaemia (ATL) cells and might be a target of novel therapy for ATL. To develop a novel immunotherapy for ATL, we verified the feasibility of cellular immunotherapy targeting HBZ. We established an HBZ-specific and HLA-A*0201-restricted cytotoxic T lymphocyte (CTL) clone. Detailed study using this CTL clone clearly showed that HBZ is certainly an immunogenic protein recognizable by human CTLs; however, HBZ-specific CTLs could not lyse ATL cells. Failure of HBZ-specific CTLs to recognize human T-cell leukemia virus type 1 (HTLV-1)-infected cells might be due to a low level of HBZ protein expression in ATL cells and resistance of HTLV-1-infected cells to CTL-mediated cytotoxicity. Although HBZ plays an important role in the proliferation of HTLV-1-infected cells, it may also provide a novel mechanism that allows them to evade immune recognition.

Adult T-cell leukaemia (ATL) is a neoplasm of peripheral T lymphocytes generated by a human retrovirus, human T-cell leukemia virus type 1 (HTLV-1) (Satou & Matsuoka, 2007). The prognosis of ATL is very poor despite intensive chemotherapy, and the current mean survival time of patients with aggressive ATL is less than 1 year. Recently, however, longer survival than that achieved solely by chemotherapy has been achieved in ATL patients after allogeneic haematopoietic stem cell transplantation (HSCT) (Utsunomiya *et al.*, 2001; Fujiwara *et al.*, 2008). The clinical effect of allogeneic HSCT is thought to be mediated mainly by the anti-HTLV-1 immune response. Because Tax-specific cytotoxic T lymphocytes (CTLs) are frequently detected in peripheral blood of patients with ATL who have undergone allogeneic HSCT (Harashima *et al.*, 2004), immunotherapy targeting Tax might be a promising strategy for treatment of ATL. However, universal clinical application of Tax-targeted immunotherapy seems unlikely because *tax* mRNA is detected in only about 40% of ATL cases (Taylor& Matsuoka, 2005). Therefore, identification of a novel target antigen recognized by CTLs and directed against ATL cells is desirable.

Recently, mRNA encoding an open reading frame in the minus strand of the HTLV-1 provirus has been identified (Gaudray *et al.*, 2002). This mRNA encodes HBZ (HTLV-1 bZIP factor), a protein that contains an N-terminal transcriptional activation domain and a leucine zipper motif at its C terminus. HBZ was found to inhibit Tax-mediated transactivation of viral transcription from the 5′LTR by interaction with cellular factors of the JUN and ATF/CREB families (Basbous *et al.*, 2003; Lemasson *et al.*, 2007). The *HBZ* transcript is reportedly detectable in all ATL cases without exception (Taylor& Matsuoka, 2005). Importantly, it has been reported that downregulation of HBZ results in inhibition of ATL cell growth, and that conversely, the expression of HBZ in human T-cell lines promotes their proliferation (Satou *et al.*, 2006). These data strongly suggest that HBZ plays an important role in the proliferation of ATL cells, and might be a universal target of novel therapy for ATL. On the basis of this concept, we attempted to verify the feasibility of cellular immunotherapy for ATL targeting HBZ.

Approval for this study was obtained from the institutional review board of Ehime University Hospital. Written informed consent was provided by all patients. HTLV-1-infected cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS and with or without 10 U IL-2 ml^{-1}. Four 9 aa peptides derived from the HBZ

sequence, which were predicted to bind with high affinity to the HLA-A*0201 molecule, were designed by computer algorithms available at the BioInformatics & Molecular Analysis Section (BIMAS) website (http://www-bimas.cit.nih.gov/molbio/hla_bind/) and SYFPEITHI website (http://www.syfpeithi.de/home.htm), as described previously (Suemori *et al.*, 2008). The amino acid sequences of the synthetic peptides were LVEELVDGL (HBZ_{19-27}), GLLSLEEEL (HBZ_{26-34}), AVLDGLLSL (HBZ_{42-50}) and KLLQEKEDL ($HBZ_{181-189}$), and their binding affinities for the HLA-A*0201 molecule were evaluated by an HLA stabilization assay, as reported previously (Kuzushima *et al.*, 2001). We attempted to generate HBZ peptide-specific CTLs from HLA-*A0201-positive individuals by stimulating $CD8^+$ T lymphocytes with peptide-loaded autologous dendritic cells, as reported previously (Ohminami *et al.*, 2000). The epitope specificity, HLA restriction, and cytotoxic activity of the induced CTLs were determined by standard ^{51}Cr-release assays, as reported previously (Yasukawa *et al.*, 1999; Suemori *et al.*, 2008).

Using TaqMan assay reagent target kits (Applied Biosystems), quantitative real-time PCR (QRT-PCR) for *HBZ* mRNA and *tax* mRNA was performed in accordance with the manufacturer's instructions. Expression levels of HBZ protein were determined by Western blotting using anti-HBZ serum, which was produced by immunizing rabbits with purified six-His-tagged HBZ polypeptide corresponding to the bZIP domain of HBZ, as reported previously (Gaudray *et al.*, 2002). Tetramer assays were performed by flow cytometry with a tetramer comprising HBZ_{26-34} peptide and HLA-A*0201 molecule with genetically altered $\alpha 3$ domain, and Tax_{11-19} (LLFGYPVYV) peptide and HLA-A*0201 molecules (Medical & Biological Laboratories), as reported previously (Azuma *et al.*, 2004).

Among the four synthetic HBZ peptides, HBZ_{26-34}, HBZ_{42-50}, and $HBZ_{181-189}$ appeared to bind strongly to the HLA-A*0201 molecule (data not shown). We tried to generate HBZ-specific CTLs by stimulating peripheral blood lymphocytes with these peptides. Consequently, an HBZ_{26-34} peptide-specific CTL line, designated HBZ-1, was established from an HLA-A*0201-positive individual. HBZ-1 was able to lyse an autologous B-lymphoblastoid cell line (LCL) loaded with a low concentration (10^{-4} μmol l^{-1}) of HBZ peptide (Fig. 1a), demonstrating that HBZ-1 might express the high-affinity T-cell receptor (TCR). Moreover, direct sequencing of the HBZ-1 *TCR* β-*chain* gene revealed that the HBZ-1 cell line appeared to carry Vβ10-3*01/D1*01/J1-5*01. In addition, all HBZ-1 cells were brightly stained with HLA-A*0201/HBZ_{26-34} tetramer (Fig. 1b), indicating that this cell line is a CTL clone derived from a single HBZ_{26-34}-specific T cell. As shown in Fig. 1(c), HBZ-1 exhibited cytotoxicity against an HBZ_{26-34} peptide-loaded but not peptide-unloaded autologous B-LCL, HLA-A*0201-positive allogeneic LCL, and an *HLA-A*0201* gene-transduced C1R cell line (C1R-A*0201: kindly provided by Dr A. John Barrett). HBZ-1 did not show any cytotoxicity against HBZ_{26-34} peptide-loaded HLA-A2-negative allogeneic LCLs. Autologous LCLs loaded with other HLA-A*0201-binding peptides were not lysed by HBZ-1 (data not shown). The CD107a assay demonstrated that HBZ-1 showed granule exocytosis upon recognition of HBZ peptide-loaded but not HIV-gag peptide-loaded HLA-A*0201-positive B-LCL (Fig. 1d). In addition, the cytotoxicity of HBZ-1 against HBZ peptide-loaded B-LCL appeared to be significantly abrogated by the Ca^{2+}-chelating agent EGTA and an inhibitor of vacuolar-type H^+-ATPase, concanamycin A (data not shown). These data strongly suggest that the cytotoxicity of HBZ-1 is mediated through the granule exocytosis pathway.

Mean expression levels of *HBZ* mRNA and *tax* mRNA in HTLV-1 infected cell lines ($n=8$), freshly isolated ATL cells ($n=4$), and peripheral blood mononuclear cells (PBMCs) of HTLV-1 carriers ($n=4$) were measured by QRT-PCR. As reported previously (Satou *et al.*, 2006), *HBZ* mRNA was detected in all HTLV-1-infected cell lines and freshly isolated ATL cells examined, and *tax* mRNA was detected in all HTLV-1-infected cell lines and half of the ATL cases (data not shown).

Next, we examined the cytotoxicity of HBZ-1 against HTLV-1-infected cells. Unexpectedly, HBZ-1 failed to lyse HLA-A*0201-positive HTLV-1-infected cell lines or freshly isolated ATL cells (Fig. 2a). We further examined whether HBZ is an immunogenic protein that can be processed within cells, and whether HBZ-derived peptides can be presented on the cell surface in context with the HLA class I molecule. To address this issue, the *HBZ* gene was transfected into *HLA-A*0201* gene-transfected K562 (K562-A*0201: kindly provided by Dr Marieke Griffioen) and C1R (C1R-A*0201) cell lines and their susceptibility to HBZ-1-mediated cytotoxicity was examined. As shown in Fig. 2(a), HBZ-1 exerted cytotoxicity against the *HBZ* gene-transfected K562-A*0201 and C1R-A*0201 cell lines. HBZ-1 did not show any cytotoxicity against empty vector-transfected cell lines. These data indicate that HBZ_{26-34} peptide can be produced by processing HBZ protein in the cells, and can be expressed on the cell surface in context with HLA-A*0201 molecules.

We further addressed the issue of why HTLV-1-infected HLA-A*0201-positive cells cannot be recognized by HBZ-specific CTLs even though *HBZ* mRNA is expressed in HTLV-1-infected cells. Western blotting for HBZ protein expression in various cells is shown in Fig. 2(b). As expected, HBZ protein was detected in abundance in *HBZ* gene-transfected K562-A*0201 and C1R-A*0201 cell lines. In contrast, HBZ protein was scarcely detectable in HTLV-1-infected cell lines and freshly isolated ATL cells. These findings strongly suggest that the amount of HBZ protein produced in HTLV-1-infected T lymphocytes is insufficient for recognition by HBZ-specific CTLs. Interestingly, we found that there was no correlation between the levels of expression of *HBZ* mRNA and HBZ protein in the samples. These data suggest that the efficiency of *HBZ* mRNA translation into HBZ protein depends on cell type, and that

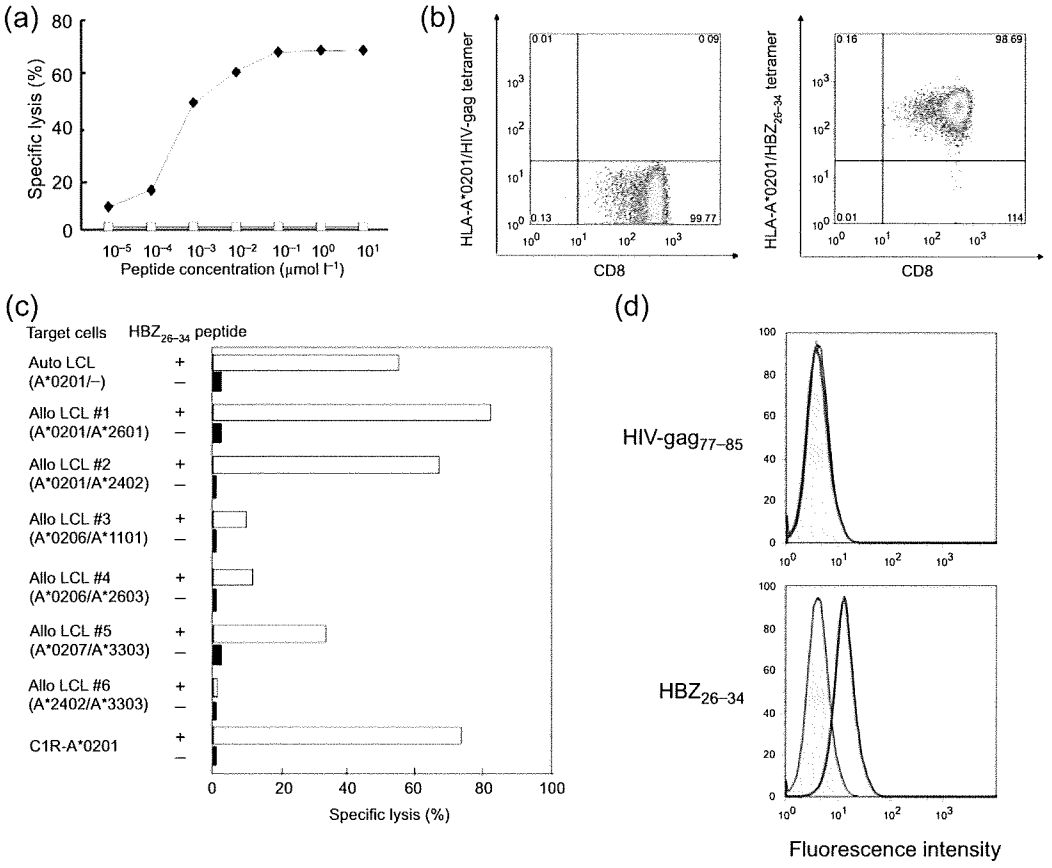

Fig. 1. Establishment of an HLA-A*0201-restricted and HBZ_{26-34} peptide-specific CTL line, HBZ-1. (a) HBZ peptide-specific and concentration-dependent cytotoxicity of HBZ-1. HBZ_{26-34}-specific CTLs were generated and their cytotoxicity against HBZ_{26-34} peptide-loaded autologous B-LCL (◆) and HBZ_{26-34} peptide-loaded HLA-A*0201-negative allogeneic B-LCL (□) was determined by ^{51}Cr-release assays at an effector : target cell (E : T) ratio of 5 : 1. Target cells were preincubated with and without HBZ_{26-34} peptide at various concentrations for 1 h. (b) Tetramer assay for HBZ-1 cell line. HBZ-1 cells were stained with HLA-A*0201/HBZ_{26-34} tetramer, but not with HLA-A*0201/HIV-gag_{77-85} tetramer. (c) Cytotoxicity of HBZ-1 against various cells. The cytotoxicity of the CTL line designated HBZ-1 against various LCLs and *HLA-A*0201* gene-transfected cells (C1R-A*0201), which were loaded or unloaded with HBZ_{26-34} peptide, was determined by 4 h ^{51}Cr-release assays at an E : T ratio of 5 : 1. Experiments were performed three times and representative data are shown. (d) Granular exocytosis of HBZ-1 upon antigen recognition. Granular exocytosis of HBZ-1 upon target antigen recognition was determined by CD107a assay. Granular exocytosis of HBZ-1 cells was detected when they were stimulated with HBZ_{26-34} peptide-loaded, but not irrelevant HLA-A*0201-binding HIV-gag_{77-85} peptide-loaded autologous B-LCL. Grey zones show negative control without peptide.

HBZ mRNA might be inefficiently translated in T lymphocytes.

Previous reports have shown that mature T lymphocytes are relatively resistant to CTL-mediated cytotoxicity in comparison with other cell types (Jiang *et al.*, 1990; Muller & Tschopp, 1994). We have also reported that the sensitivity of various kinds of tumour to tumour-associated antigen-specific CTLs differs (Azuma *et al.*, 2004). These findings led us to investigate whether HTLV-1-infected T lymphocytes are resistant to cytotoxicity mediated by HBZ-specific CTLs. To address this question, we compared the cytotoxic activities of HBZ-1 against HBZ peptide-loaded B-LCL and HTLV-1-transformed T-cell lines. As shown in Fig. 3, HBZ-1 was not cytotoxic to HTLV-1-transformed T-cell lines loaded with HBZ peptide at low concentrations; however, at these low concentrations, autologous B-LCL was lysed in a dose-dependent manner. At high HBZ peptide concentrations, HLA-A*0201-positive HTLV-1-transformed T-cell lines were lysed by HBZ-1, although cytotoxic activity against HBZ peptide-loaded B-LCL was higher than that against HBZ peptide-loaded T-cell lines.

In this study, we succeeded for the first time in establishing an HBZ peptide-specific CTL clone, and a detailed study using this CTL clone and *HBZ* gene-transfected cells clearly revealed that HBZ_{26-34} is an immunogenic epitope

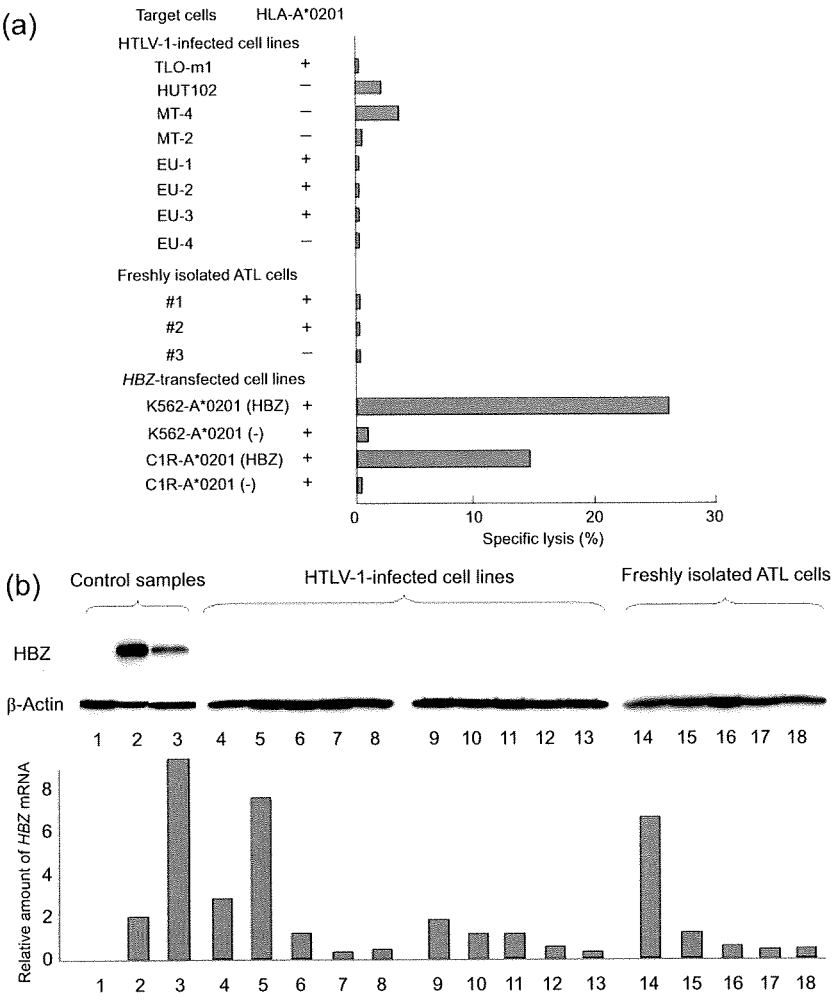

Fig. 2. Susceptibility of ATL cells and HTLV-1-infected cells to HBZ-1-mediated cytotoxicity and expression of *HBZ* mRNA and protein in various cells. (a) Cytotoxicity of the HBZ_{26-34} peptide-specific CTL line HBZ-1 against various cells. The cytotoxicity of HBZ-1 to HLA-A*0201-positive and HLA-A*0201-negative HTLV-1-infected cell lines, freshly isolated ATL cells and *HBZ* gene-transfected and -untransfected K562-A*0201 and C1R-A*0201 cell lines was determined by 4 h ^{51}Cr-release assays at an E:T ratio of 10:1. Experiments were performed three times and representative data are shown. (b) Expression of *HBZ* mRNA and protein in leukaemia cell lines, freshly isolated ATL cells, and normal PBMCs. HBZ protein expression was examined by Western blotting using anti-HBZ antibody and anti-β-actin antibody as the control. Expression levels of *HBZ* mRNA in the cells were determined by QRT-PCR. The expression level of *HBZ* mRNA in MT-4 is shown as 1.0 and the expression levels in samples were calculated relative to this value. 1, PBMCs; 2, *HBZ*-transfected K562-A*0201; 3, *HBZ*-transfected C1R-A*0201; 4, MT-1; 5, MT-2; 6, MT-4; 7, TL-MAT; 8, TLO-m1; 9, EU-1; 10, EU-2; 11, EU-3; 12, EU-4; 13, EU-5; 14, ATL#1; 15, ATL#2; 16, ATL#3; 17, ATL#4; 18, ATL#5.

recognizable by HLA-A*0201-restricted CTLs. However, HBZ-specific CTLs could not lyse HTLV-1-infected cells. In addition, cytokine production by HBZ-specific CTLs in response to stimulation with HTLV-1-infected cells in an HLA-restricted manner could not be detected (data not shown). The hypothesis that downregulation of HLA class I molecules on HTLV-1-infected cells is the cause of unsuccessful recognition of HTLV-1-infected cells by HBZ-1 seems unlikely, because flow cytometry showed strong expression of HLA class I molecules on HTLV-1-infected cells, and HBZ_{26-34} peptide-loaded HLA-A*0201-positive HTLV-1-infected cells were efficiently lysed by HBZ-1 (data not shown).

Because *HBZ* gene-transfected cells abundantly expressing HBZ protein were lysed by HBZ-specific CTLs in an HLA-A*0201-restricted manner, and Western blotting revealed a very low level of HBZ protein expression in HTLV-1-infected cells, we concluded that HBZ protein can certainly be processed in the cells and presented in context with the HLA-A*0201 molecule; however, HBZ-specific CTLs cannot discriminate HTLV-1-infected from HTLV-1-uninfected cells due to the small amount of HBZ protein in the former. Furthermore, HLA-A*0201/HBZ_{26-34} tetramer analysis of freshly isolated PBMCs from HLA-A*0201-positive ATL patients (n=5) and a HLA-A*0201-positive HTLV-1 carrier (n=1) revealed that HBZ-specific CTLs were scarcely detectable in HTLV-1-infected individuals (data not shown). Because PBMCs from HLA-A*0201-positive HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients were not available, tetramer assays for HBZ-specific T-cell immune response in HAM/TSP could not be performed. Although the possibility that HBZ protein could be presented by HTLV-1-infected T lymphocytes of HAM/TSP patients cannot be excluded, these data strongly support our interpretation that HBZ protein cannot be presented by T lymphocytes naturally infected with HTLV-1. Although *HBZ* mRNA is expressed in all ATL cases, and previous studies using overexpression and gene silencing methods have clearly demonstrated the important role of *HBZ* mRNA in proliferation of ATL cells (Satou *et al.*, 2006), the detailed characteristics and functional role of HBZ protein in leukaemogenesis and progression of HTLV-1-infected cells