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BCL11B is frequently downregulated in HTLV-1-infected T-cells through Tax-mediated proteasomal degradation

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ABSTRACT

Human T-cell leukemia virus type 1 (HTLV-1) is a causative agent of adult T-cell leukemia-lymphoma (ATLL). The HTLV-1-encoded protein Tax plays important roles in the proliferation of HTLV-1-infected T-cells by affecting cellular proteins. In this study, we showed that Tax transcriptionally and post-transcriptionally downregulates the expression of the tumor suppressor gene *B-cell leukemia/lymphoma 11B (BCL11B)*, which encodes a lymphoid-related transcription factor. *BCL11B* expression was downregulated in HTLV-1-infected T-cell lines at the mRNA and protein levels, and forced expression of BCL11B suppressed the proliferation of these cells. The proteasomal inhibitor MG132 increased BCL11B expression in HTLV-1-infected cell lines, and colocalization of Tax with BCL11B was detected in the cytoplasm of HTLV-1-infected T-cells following MG132 treatment. shRNA knock-down of *Tax* expression also increased the expression of BCL11B in HTLV-1-infected cells. Moreover, we found that Tax physically binds to BCL11B protein and induces the polyubiquitination of BCL11B and proteasome-dependent degradation of BCL11B. Thus, inactivation of *BCL11B* by Tax protein may play an important role in the Tax-mediated leukemogenesis.

1. Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is a retrovirus that causes adult T-cell leukemia-lymphoma (ATLL) in 5% of individuals with HTLV-1 infection. This virus is endemic in various regions, including Japan, Africa, the Caribbean islands, Central America, and South America [1]. The virus expresses the Tax and HBZ proteins, which are fundamental for transformation and proliferation of HTLV-1-infected T-cells. Tax not only activates HTLV-1 gene expression but also influences cellular gene expression during the early stages of leukemogenesis. HBZ protein is continuously expressed in HTLV-1-infected T-cells and ATLL cells and implicated in ATLL pathogenesis [2]. Tax has been identified as an important protein in HTLV-1 pathogenesis as a potent activator of a variety of transcriptional pathways, such as cyclic AMP-responsive element-binding protein (CREB), CREB binding protein (CBP)/p300, and chromatin remodeling, and a variety of signaling pathways, such as the serum response factor, NF-κB, and JAK/STAT pathways [3,4]. Moreover, Tax interacts with several host cell proteins to modify their protein stability and functions, such as the IκB kinase (IKK) complex in the NF-κB pathway and tumor suppressors, such as Forkhead Box O4 (FoxO4) or retinoblastoma protein (RB) [5–7]. Tax can be localized in both the cytoplasm and the nucleus, which results in modulation of transcription or epigenesis and cytoplasmic signaling pathways [8].

ATLL development requires additional genetic or epigenetic events in addition to HTLV-1 infection, which are believed to be important for monoclonal proliferation of ATLL cells. We previously performed an integrative genomic analysis of ATLL cells from acute-type ATLL patients and identified four frequent chromosomal breakpoint cluster regions, including chromosome 14q32 near the *B-cell leukemia/lymphoma 11B (BCL11B)* gene [9]. Human *BCL11B* belongs to the BCL11 gene family and encodes a C2H2-type zinc finger transcription factor that was initially reported as CTIP2 (COUP-TF interacting protein 2) in the brain because of its interaction with the chicken ovalbumin upstream promoter [10]. BCL11B is highly expressed in T-cells and is required for T-cell proliferation, development, and survival [11]. BCL11B acts as a haploinsufficient tumor suppressor in acute lymphoblastic leukemia (ALL) [12], and dysfunctional *BCL11B* alleles influenced the susceptibility of thymic lymphoma in gamma-radiated mice [13]. In an initial report of

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ATLL, elevated expression of *BCL11B* was noted in acute-type ATLL patients [14]. However, two recent studies have reported that *BCL11B* is downregulated in HTLV-1-positive T-cell lines [15,16]. The second report showed that ectopic expression of Tax in the Jurkat T-ALL cell line downregulated *BCL11B* mRNA expression through an unidentified mechanism [16]; however, the third report showed that there was no association between the mRNA expression levels of *BCL11B* and *Tax* in HTLV-1-positive T-cell lines [15]. Therefore, it is still unclear whether the expression of *BCL11B* is deregulated in HTLV-1-infeccted T-cells and whether the downregulation of *BCL11B* contributes cooperatively to HTLV-1-infected cells with Tax expression.

Here, we show that the mRNA and protein levels of *BCL11B* are downregulated in HTLV-1-infected cell lines, and these changes are mediated primarily by Tax expression via transcriptional and post-transcriptional mechanisms in almost all HTLV-1-infected T-cell lines and Tax-immortalized T-lymphocytes analyzed. In the post-transcriptional modification, Tax directly binds to BCL11B and targets it for ubiquitin-dependent proteasomal degradation in the cytoplasm of HTLV-1-infected T-cells. We further showed that ectopic expression of BCL11B in HTLV-1-infected T-cells significantly inhibited the proliferative capacities of the HTLV-1-infected T-cells. Given that Tax inhibits the function of different tumor suppressor genes, including the *RB* gene, by proteasomal degradation, the inactivation of *BCL11B* by Tax in HTLV-1-infected T-cells might be relevant for the Tax-driven proliferation of HTLV-1-infected T cells.

2. Material and methods

2.1. Cell lines

The human T-ALL-derived Jurkat and human cutaneous T-cell lymphoma (CTCL)-derived HUT78 are HTLV-1-negative T-cell lines. HUT102, MT2, MT4, and SLB1 are human T-cell lines transformed by HTLV-1 infection. JPX-9 is a clone of Jurkat cells stably transfected by the *Tax* gene driven by the $ZnCl_2$-inducible *metallothionein* promoter [17], and was kindly provided by Dr. K Sugamura (Tohoku University School of Medicine, Japan). Tax-immortalized human T-lymphocytes were kind gifts from Dr. M. Hijikata (Kyoto University, Japan) [18,19]. Jurkat was obtained from the Fujisaki Cell Center, Hayashibara Biochemical Laboratories (Okayama, Japan). HUT78 was purchased from the American Type Culture Collection (ATCC Manassas, VA, USA). HUT102, MT2, MT4, and SLB1 were kind gifts from Dr. H. Iha (Oita University, Japan). These cell lines were cultured in RPMI 1640 medium (Wako Pure Chemicals, Osaka, Japan) in the presence of 10% fetal bovine serum (FBS) and antibiotics. Tax-immortalized T-cells were maintained in AIM-V medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 50 JRU/ml recombinant human interleukin-2 (Takeda, Osaka, Japan), and 0.05 mM 2-mercaptoethanol. HEK293T is a human embryonic kidney cell line and was maintained in Dulbecco's modified Eagle's medium (Wako Pure Chemicals) supplemented with 10% FBS and antibiotics.

2.2. Plasmids

To generate the Flag-BCL11B expression vector, complementary DNA fragments encoding *BCL11B* (NM_138576.3) were generated by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA of HEK293T and subcloned into the p3XFlag-myc-CMV-26 vector (Sigma-Aldrich, St. Louis, MO, USA). To generate the GFP-BCL11B expression vector, the open reading frame (ORF) of *BCL11B* was excised from the FLAG-BCL11B expression vector and subcloned into the pEGFP-C1 vector (Clontech, Mountain View, CA, USA). To generate the Tax construct, the ORF of *Tax* was amplified by PCR using pCG-Tax [20] (a kind gift from Dr. J. Fujisawa, Kansai Medical University, Japan) as a template and subcloned into p3XFlag-myc-CMV-26. To construct the short hairpin RNA (shRNA) expression vector targeting *Tax*, the following oligonucleotides were annealed and inserted into the RNAi-Ready pSIREN-RetroQ-ZsGreen vector (Clontech): 5′ GCAGATGACAATGACCATGA 3'. A control shRNA vector targeting luciferase (shLuc) was purchased from Clontech.

2.3. RT-PCR analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized from 1 μg of extracted RNA using an RNA-PCR kit (TaKaRa Bio Inc., Tokyo, Japan). Real-time RT-PCR was performed using GeneAce SYBR qPCR Mix α (Nippon Gene, Tokyo, Japan) and the StepOne Real-Time PCR System (Applied Biosystems Foster City, CA, USA). The quantity of mRNA was normalized to the level of *β-actin*. The primers used were as follows:

β-actin forward, 5′-TCCTTCTGCATCCTGTCGGC-3′,
β-actin reverse, 5′-AAGAGATGGCCACGGCTGCT-3';
BCL11B forward, 5′-GGCGATGCCAGAATAGATGCCG-3′,
BCL11B reverse, 5′-CCAGGCCACTTGGCTCCTCTAT-3';
Tax forward, 5′-CTCTGGGGGACTATGTTCGGCC-3′,
Tax reverse, 5′-GTACATGCAGACAACGGAGCCT-3'.

2.4. Western blot analysis and antibodies

Western blot analyses were performed as described elsewhere [21]. Densitometry analysis was performed using a LAS-3000 imaging system (Fujifilm, Tokyo, Japan). The primary antibodies used were anti-BCL11B (DNF1; Cell Signaling Technology, Beverly, MA, USA), anti-TAX (Y-7) (a kind gift from Dr. Y. Tanaka, University of Ryukyus, Japan), anti-FLAG (Sigma-Aldrich, M2), anti-GFP (598; MBL, Nagoya, Japan), anti-histone H3 (D1H2; Cell Signaling Technology), anti-Ubiquitin (6C1; Sigma-Aldrich), and anti-β-actin (AC-15; Sigma-Aldrich).

2.5. Immunofluorescence staining

Immunofluorescence staining was performed as described elsewhere [21]. Fluorescence signals were detected using a confocal laser-scanning microscope (LSM 700; Carl Zeiss Microscopy, Jena, Germany). The antibodies used were anti-BCL11B (DNF1), anti-TAX (Y-7), Alexa Fluor-546 goat anti-mouse IgG (Invitrogen), and Alexa Fluor-488 goat anti-rabbit IgG (Invitrogen). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

2.6. Immunoprecipitation

The JPX-9 cell line was treated with 1.25 μM MG132 (Life-Sensors, Inc., Malvern, PA, USA) and 120 μM $ZnCl_2$ (Nacalai Tesque, Kyoto, Japan) for 12 h. HEK293T cells were transfected with the expression constructs using HilyMax (DOJINDO, Kumamoto, Japan) according to the manufacturer's protocol. The cells were solubilized in RIPA buffer containing 50 mM Tris-HCl pH 7.8, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, and 1 mM EDTA supplemented with protease inhibitor cocktail (Sigma-

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