

Human T-Cell Leukemia Virus Type I–Mediated Repression of PDZ-LIM Domain-Containing Protein 2 Involves DNA Methylation But Independent of the Viral Oncoprotein Tax¹

Pengrong Yan^{*,†,2}, Zhaoxia Qu^{*,†,2}, Chie Ishikawa[‡], Naaki Mori[‡] and Gutian Xiao^{*,†}

*University of Pittsburgh Cancer Institute, University of Pittsburgh Medical Center, Pittsburgh, PA 15213, USA; †Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA; ‡Division of Molecular Virology and Oncology, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan

Abstract

Human T-cell leukemia virus type I (HTLV-I) is the etiological agent of adult T-cell leukemia (ATL). Our recent studies have shown that one important mechanism of HTLV-I–mediated tumorigenesis is through PDZ-LIM domain-containing protein 2 (PDLIM2) repression, although the involved mechanism remains unknown. Here, we further report that HTLV-I–mediated PDLIM2 repression was a pathophysiological event and the PDLIM2 repression involved DNA methylation. Whereas DNA methyltransferases 1 and 3b but not 3a were upregulated in HTLV-I–transformed T cells, the hypomethylating agent 5-aza-2'-deoxycytidine (5-aza-dC) restored PDLIM2 expression and induced death of these malignant cells. Notably, the PDLIM2 repression was independent of the viral regulatory protein Tax because neither short-term induction nor long-term stable expression of Tax could downregulate PDLIM2 expression. These studies provide important insights into PDLIM2 regulation, HTLV-I leukemogenicity, long latency, and cancer health disparities. Given the efficient antitumor activity with no obvious toxicity of 5-aza-dC, these studies also suggest potential therapeutic strategies for ATL.

Neoplasia (2009) 11, 1036–1041

Introduction

Human T-cell leukemia virus type I (HTLV-I) infection causes an aggressive and fatal CD4⁺ T-cell malignancy termed *adult T-cell leukemia* (ATL) in approximately 2% to 5% virus carriers after an extensive latency period. Although the detailed mechanisms of ATL development remain ill defined, it seems clear that the early stage of HTLV-I–mediated leukemogenesis is largely mediated by its encoded regulatory protein Tax [1,2]. Tax is able to transform many different types of cells *in vitro* and induce tumors in mice. Conversely, the HTLV-I genome without Tax loses its original oncogenic ability.

The Tax oncoprotein exerts its transforming action largely through deregulation of cellular transcription factors that are critical for cell growth and division, such as nuclear factor κ B (NF- κ B) [1,2]. The mechanisms by which Tax deregulates the NF- κ B signaling for tumorigenesis have been well studied. In the cytoplasm, Tax recruits the inhibitor of NF- κ B (I κ B) kinase (IKK) complex into specific compartments for IKK activation [3,4], resulting in degradation of I κ B and subsequent nuclear translocation of NF- κ B factors including p65, also known as RelA, the prototypic member of NF- κ B [5]. In the nucleus, Tax recruits

p65 and other cellular transcriptional components into interchromatin granules to form discrete transcriptional hot spots termed as *Tax nuclear bodies* for full NF- κ B transcriptional activation [6,7].

Contrast to the mechanisms by which Tax hijacks cellular signaling to initiate malignant transformation leading to the development of

Abbreviations: 5-aza-dC, 5-aza-2'-deoxycytidine; ATL, adult T-cell leukemia; DNMT1, DNA methyltransferase 1; DNMT3a, DNA methyltransferase 3a; DNMT3b, DNA methyltransferase 3b; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HTLV-I, human T-cell leukemia virus type I; MEF, mouse embryonic fibroblast; PDLIM2, PDZ-LIM domain-containing protein 2

Address all correspondence to: Gutian Xiao, Hillman Cancer Center Research Pavilion, 5117 Centre Ave, Pittsburgh, PA 15213. E-mail: xiaog2@upmc.edu

¹This study was supported in part by National Institutes of Health/National Cancer Institute grant R01 CA116616, Hillman Innovative Cancer Research Award and Molecular Virology Research Award of the University of Pittsburgh Cancer Institute to G. Xiao.

²These authors contribute equally.

Received 11 May 2009; Revised 15 June 2009; Accepted 16 June 2009

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DOI 10.1593/neo.09752

ATL, the molecular studies on how HTLV-I/Tax is regulated by cellular factors are still lacking. Recently, we have identified the PDZ-LIM domain-containing protein 2 (PDLIM2) as a potent suppressor of Tax [8]. PDLIM2 not only promotes Tax polyubiquitination but also binds to and shuttles Tax from its functional sites including the perinuclear structures and Tax nuclear foci into the nuclear matrix for proteasomal degradation. Accordingly, PDLIM2 prevents downstream signaling and subsequent tumorigenicity of HTLV-I/Tax. Interestingly, PDLIM2 is conversely repressed in various HTLV-I-transformed cells at the RNA level. These studies clearly suggest that PDLIM2 repression is one important mechanism of HTLV-I-mediated tumorigenesis. However, whether the PDLIM2 repression occurs under pathophysiological conditions and how HTLV-I represses PDLIM2 expression still remain unknown.

Here, we further report that HTLV-I repressed PDLIM2 expression in primary ATL cells freshly isolated from patients and established ATL cell line. However, the PDLIM2 repression was independent of the viral oncoprotein Tax because neither short-term induction nor long-term stable expression of Tax could downregulate PDLIM2 expression. Instead, the HTLV-I-mediated PDLIM2 repression involved DNA methylation. Whereas DNA methyltransferases 1 (DNMT1) and 3a (DNMT3b) but not 3a (DNMT3a) were upregulated in HTLV-I-transformed T cells, the hypomethylating agent 5-aza-2'-deoxycytidine (5-aza-dC) restored PDLIM2 expression. Interestingly, we also find that in association with the reactivation of PDLIM2, 5-aza-dC was able to induce death of HTLV-I-transformed T cells in a dose-dependent manner. These studies not only provide important insights into PDLIM2 regulation and HTLV-I leukemogenicity but also suggest potential therapeutic strategies for ATL.

Materials and Methods

Reagents

Tax antibody was generated as described [9]. Fetal bovine serum (FBS), doxycycline, and 5-aza-dC were from Hyclone Laboratories (Logan, UT) and Sigma (St. Louis, MO), respectively.

Cell Culture

Jurkat and HTLV-I-transformed cell lines were maintained in suspension in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-glutamine [10,11]. Mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's modified Eagle medium supplemented with 10% FBS and 2 mM L-glutamine [12–14].

Induction of Tax in Jurkat-Inducible Cell Line

For Tax induction, Jurkat-TetOn-inducible cells maintained in 10% tetracycline-free FBS were treated with 0.1 $\mu\text{g/ml}$ doxycycline for the indicated time as described before [15]. The cells were then lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% (wt/vol) Na-deoxycholate, 1% (vol/vol) NP-40, 1 mM DTT) for whole lysate extraction (for immunoblot analysis) or in TRIzol reagent (Invitrogen, Carlsbad, CA) for RNA extraction (for reverse transcription-polymerase chain reaction [RT-PCR]).

Clinical Samples

With informed consent according to the Helsinki Declaration, peripheral blood mononuclear cells (PBMCs) were obtained from five patients with acute-type ATL (nos. 1-5) and two healthy volunteers (nos. 1 and 2). The diagnosis of ATL was established hematologically, and monoclonal HTLV-I provirus integration into the genome was confirmed by Southern blot hybridization in all cases (data not shown). Subtypes of ATL were defined as described before [16,17]. Mononuclear cells were isolated from heparinized venous blood samples by Ficoll Paque gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden). Each patient sample contained more than 90% leukemic cells at the time of analysis. All samples were collected at the time of admission to hospital before the patients started chemotherapy. The normal PBMC control nos. 3 and 4 were purchased from the Biologic Specialty Corporation (Colmar, PA).

RT-PCR Analysis

Total RNA was prepared with TRIzol reagent, and complementary DNA was generated with SuperScript II reverse transcriptase (Invitrogen),

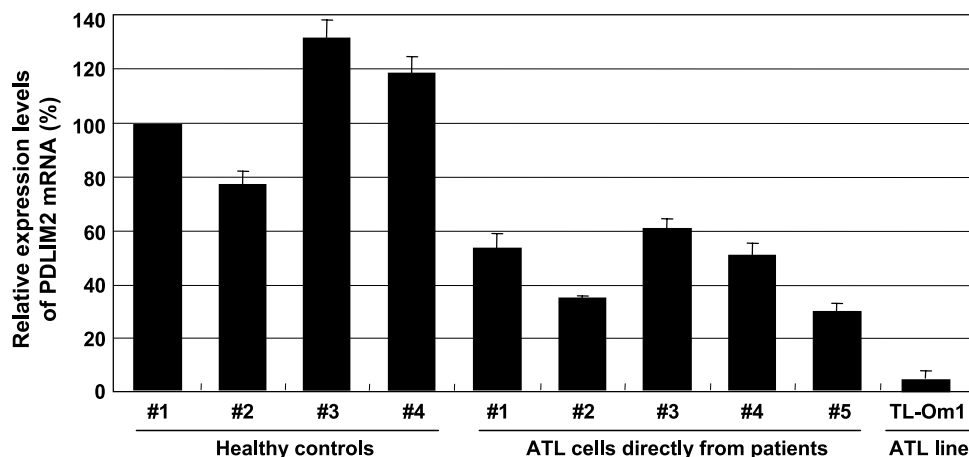


Figure 1. HTLV-I represses PDLIM2 expression in primary ATL cells. The relative levels of PDLIM2 mRNA in PBMCs directly from ATL patients or established ATL cell line TL-Om1 were analyzed by real-time PCR and normalized according to GAPDH mRNA level and represented as percentile in cells from healthy control no. 1 (arbitrarily set as 100). Data presented are the mean \pm SD ($n = 3$).

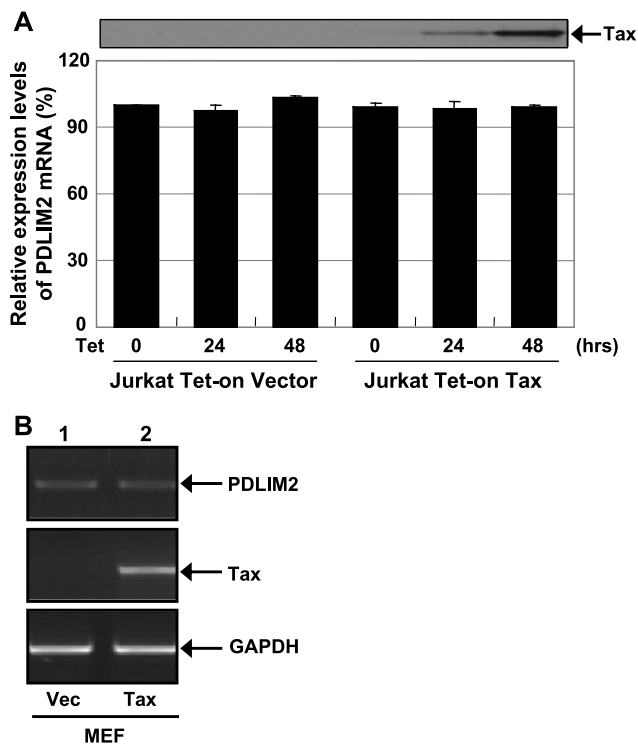


Figure 2. HTLV-I represses RNA expression of PDLIM2 independent of Tax. (A) Tax-inducible Jurkat cells were mock-treated or treated with doxycycline for the indicated times followed by real-time PCR. The induction of Tax protein was analyzed by direct immunoblot analysis. (B) MEF cells stably expressing Tax or an empty vector were used for RT-PCR to check expressions of the indicated genes.

followed by normal RT-PCR or real-time RT-PCR as described we before [18,19]. Primer pairs for RT-PCR were as follows: mouse PDLIM2, forward 5'-GACAGCCAGTCTTCCCAGAG and reverse 5'-TCTCACAGGTGTGGAGCTTG; Tax, forward 5'-CACCTGTCCAGAGCATCAGA and reverse 5'-CGCTTGTAGGGAACATTGGT; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-CACAGTCCATGCCATCACTG and reverse 5'-CTTACTCCTTGAGGCCATG. Primer pairs for real-time PCR were as follows: human PDLIM2, forward 5'-GCCCATCATGGTACTAAGG and reverse 5'-ATGGCCACGATTATGTCTCC; human β -actin, forward 5'-ATCAAGATCATTGCTCCTCCT and reverse 5'-GAGAGCGAGGCCAGGATGGA; human GAPDH, forward 5'-GCAAATTCATGGCACCCTG and reverse 5'-TCGCCCACTTGA TTTTGG; human DNMT1, forward 5'-GGTCTCTCCTCCTGGAGAATGTC and reverse 5'-GGGCCACGCCGTAAGT; human DNMT3a, forward 5'-GCCTCAATGTTACCCTGGAA and reverse 5'-CAGCAGATGGTGCAGTAGGA; human DNMT3b, forward 5'-CCCATTTCGAGTCTGTTCATT and reverse 5'-GGTTCACACAGCAATGGACT.

Results

PDLIM2 Is Repressed in Primary ATL Cells

Our recent studies showed that PDLIM2 is repressed at the RNA level in various T-cell lines *in vitro* transformed by HTLV-I [8]. To further substantiate the findings, we examined messenger RNA (mRNA) levels of PDLIM2 in primary ATL cells. Indeed, the expression levels of

PDLIM2 were much lower in primary ATL cells directly from patients or established ATL cell line TL-Om1 compared with that in healthy control cells (Figure 1). These results indicated that PDLIM2 down-regulation is an HTLV-I pathophysiological event, further supporting our original finding that PDLIM2 repression is one important mechanism of HTLV-I-mediated tumorigenesis [8].

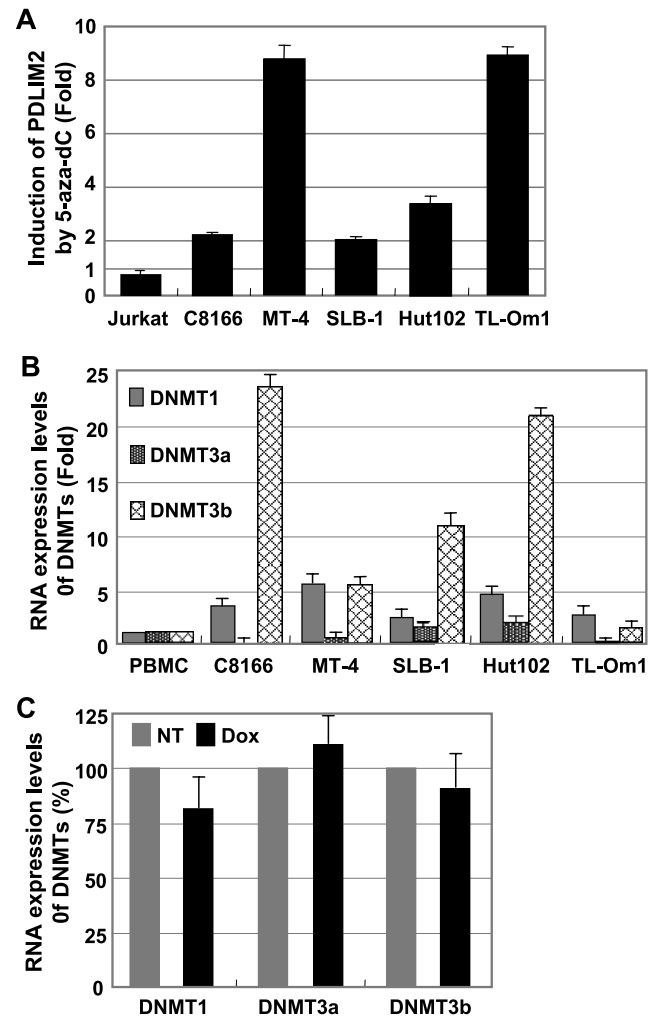


Figure 3. HTLV-I-mediated repression of PDLIM2 involves DNA methylation. (A) The indicated cells were treated with the hypomethylating agent 5-aza-dC for 48 hours followed by real-time PCR. PDLIM2 mRNA were normalized according to β -actin mRNA level and represented as fold induction in mRNA abundance relative to that in the mock-treated sample of each request. Data presented are the mean \pm SD ($n = 3$). (B) RNA expression levels of DNMT1, DNMT3a, and DNMT3b were analyzed in the indicated cell lines by real-time PCR. RNA levels of individual DNMTs in HTLV-I-transformed T cells were normalized according to β -actin mRNA level and represented as fold induction in mRNA abundance relative to that in normal PBMCs (set as 1). Data presented are the mean \pm SD ($n = 3$). (C) Tax-inducible Jurkat cells were mock-treated or treated with doxycycline for 36 hours followed by real-time PCR to check the relative RNA expression levels of DNMT1, DNMT3a, and DNMT3b. RNA levels of individual DNMTs in doxycycline-treated (Dox) cells were normalized according to β -actin mRNA level and represented as percentile of that in the mock-treated (NT) cells (set as 100). Data presented are the mean \pm SD ($n = 3$).

PDLIM2 Repression by HTLV-I Is Independent of Tax

Currently, the mechanism of PDLIM2 regulation, including that by which PDLIM2 expression is repressed by HTLV-I, still remains unknown. To address this important issue, we initially tested whether the Tax oncoprotein is involved, giving its role in gene regulation and cellular transformation. Consistent with our previous studies showing that Jurkat cells, an HTLV-I-negative leukemic T-cell line, express abundant PDLIM2 [8], we found a high expression of PDLIM2 in Tax-inducible Jurkat cells in the absence of Tax expression (Figure 2A). However, Tax induction had no obvious effect on PDLIM2 expression. It should be noted that Jurkat cells would die 72 to 96 hours after Tax induction because of Tax-induced TRAIL and, subsequently, TRAIL-mediated apoptosis [20]. To rule out that the failure of the PDLIM2 repression was not due to short period of Tax expression, we compared the PDLIM2 expression levels in MEF cells stably expressing Tax or an empty vector. It was noteworthy that the Tax MEF cells have acquired the ability to form foci in soft agar and tumor in mice [8]. As shown in Figure 2B, a long period of Tax expression in these transformed cells also failed to downregulate PDLIM2 expression. Thus, it seemed that HTLV-I represses PDLIM2 expression independent of Tax. In agreement with this, PDLIM2 expression is also repressed in primary ATL cells and ATL cell line TL-Om1 (Figure 1), which already lost Tax expression [10,17].

PDLIM2 Repression by HTLV-I Involves DNA Methylation

We then examined the potential role of DNA methylation, one major mechanism responsible for the repression of tumor suppressor genes in neoplastic cells [21]. Interestingly, treatment of the hypomethylating agent 5-aza-dC significantly increased RNA expression of PDLIM2 in T-cell lines either *in vitro* transformed by HTLV-I or established from ATL patients (Figure 3A). Conversely, the treatment of 5-aza-dC led to a slight decrease of PDLIM2 in Jurkat cells. These results suggested that DNA methylation contributes specifically to HTLV-I-mediated repression of PDLIM2.

The methylation of mammalian genomic DNA is mediated by three DNA methyltransferases, namely, DNMT1, DNMT3a, and DNMT3b [21]. Nucleoside analog 5-aza-dC inhibits all three enzymes. To identify the methyltransferase(s) involved in HTLV-I-mediated PDLIM2 repression, we examined their expression levels in

various HTLV-I-transformed T-cell lines including C8166, MT-4, SLB-1, Hut102, and TL-Om1. As shown in Figure 3B, DNMT1 and DNMT3b were upregulated significantly and consistently in all these HTLV-I-transformed T-cell lines, although to different extents. Conversely, DNMT3a was only slightly upregulated in certain cell lines but decreased in others. Thus, it seemed that DNMT1 and DNMT3b but not DNMT3a may be involved in HTLV-I-mediated repression of PDLIM2. In support of this, it is well known that DNMT1 and DNMT3b but not DNMT3a usually cooperate to inactivate tumor suppressor genes in cancer cells but not in nonmalignant cells [21].

These results also implied that the up-regulation of DNMT1 and DNMT3b in HTLV-I-transformed T cells is independent of Tax expression because whereas C8166, MT-4, SLB-1, and Hut102 cells still express Tax, the ATL cell line TL-Om1 already loses Tax expression [8,10,17]. To further confirm this, we examined whether Tax induction affects the expression of different DNMTs in Jurkat cells. As shown in Figure 3C, Tax induction had no obvious effect on the expression of all three DNMTs. Taken together, these results suggested that Tax is not involved in the up-regulation of DNMT1 and DNMT3b in HTLV-I-transformed T cells. These studies further supported that PDLIM2 repression by HTLV-I is independent of Tax.

Treatment of 5-aza-dC Leads to Death of HTLV-I-Transformed T Cells

In association with reactivation of PDLIM2, interestingly, the treatment of 5-aza-dC resulted in death of HTLV-I-transformed T cells in a dose-dependent manner (Figure 4). Although 5-aza-dC is unable to further increase PDLIM2 expression in Jurkat cells, it could also induce death of the leukemic cells in a dose-dependent manner. Conversely, normal PBMCs were largely resistant to 5-aza-dC-induced death. These results suggested that 5-aza-dC-induced cancer cell death may involve different target genes. Nevertheless, these results were consistent with the fact that 5-aza-dC is toxic to cancer cells but not normal cells [21,22]. Currently, there is no beneficial treatment for ATL patient except the bone marrow transplantation because of the high resistance of ATL cells to cancer therapy-induced death [23]. These results thus suggested an immediate therapeutic approach for the poorly diagnos-

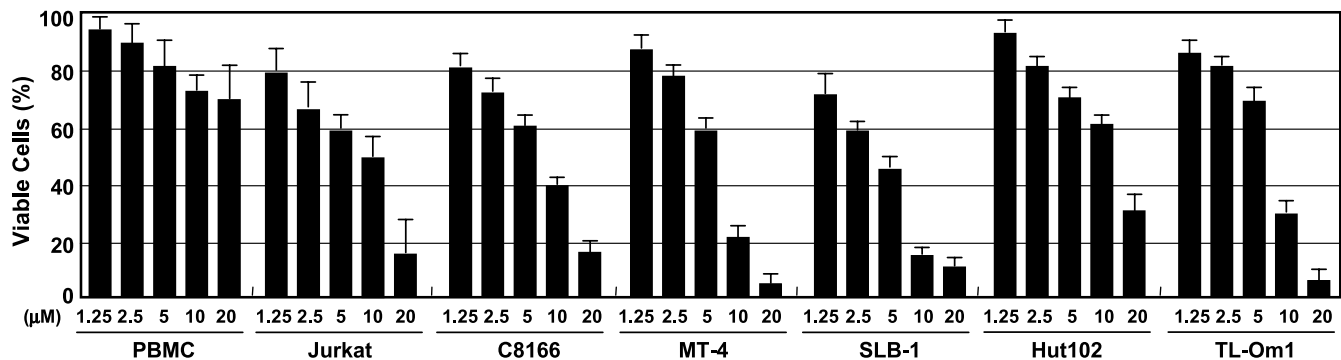


Figure 4. Hypomethylating agent 5-aza-dC induces apoptosis of HTLV-I-transformed T cells in a dose-dependent manner. The indicated cells were treated with increasing doses of 5-aza-dC for 72 hours followed by Trypan blue counting of dead and viable cells. The presented percentiles of viable cells were normalized to the percent viability of mock-treated cells (denoted as 100%). Data presented are the mean \pm SD ($n = 3$).

tic disease ATL, although targets of 5-aza-dC other than PDLIM2 were also involved.

Discussion

Under physiological conditions, PDLIM2 is expressed in most tissues, with the highest expression in the lungs; modest in thymus, spleen, kidney, and testis; and low in the brain, heart, and muscle [24–26]. At the cellular level, PDLIM2 expression is high in various hematopoietic cells including CD4⁺ T cells [26], the targets of HTLV-I. Data presented in this study indicate that PDLIM2 expression was significantly down-regulated in freshly isolated primary ATL cells and established ATL cell line (Figure 1). Given the ability of PDLIM2 in suppressing tumorigenicity of HTLV-I-transformed T cells [8], this study suggests that PDLIM2 repression is one pathophysiologically important mechanism of HTLV-I-mediated ATL.

Our mechanistic studies further suggest that HTLV-I-mediated PDLIM2 repression involves DNA methylation, which may be mediated specifically by DNMT1 and DNMT3b. We have shown that the hypomethylating agent 5-aza-dC could significantly reinduce expression of PDLIM2 in both HTLV-I-transformed T cells and ATL cells (Figure 3). In addition, DNMT1 and DNMT3b, but not DNMT3a, were upregulated significantly and consistently in these malignant T cells (Figure 3). These findings are highly consistent with the cooperative role of DNMT1 and DNMT3b but not DNMT3a in the inactivation of tumor suppressor genes in cancer cells but not in nonmalignant cells [21]. However, it should be noted that the expression levels of DNMTs are correlated generally but imperfectly with the expression levels of PDLIM2 as well as the induction of PDLIM2 by 5-aza-dC in different HTLV-I-transformed T cells. One immediate explanation is that epigenetic regulation is complex in cell and gene contexts. The repression extent of a particular gene not only requires availability of individual DNMTs but also depends on their ratios and the availabilities of many other cellular factors. In this regard, it is well known that in addition to cooperating with each other, DNMTs associate with histone deacetylase 1, histone methyltransferase SUV39H1, and methyl-CpG-binding proteins-domains for gene repression [21].

In association with the reactivation of PDLIM2, 5-aza-dC could efficiently induce death of HTLV-I-transformed T cells and ATL cells. Because there is no obvious toxicity of 5-aza-dC observed in phase 3 clinical trials for patients with myelodysplastic syndromes and acute myelogenous leukemia [22], our studies therefore suggest an immediate therapeutic approach for ATL. This is particularly interesting because HTLV-I-transformed cells are highly resistant to the induction of apoptosis and there is still no actually beneficial treatment other than allogeneic hematopoietic stem cell transplantation for this acute and fatal disease [23]. Clearly, the effect of 5-aza-dC could not attribute only to the reactivation of PDLIM2. Other yet-identified targets of this antitumor drug also play very important roles in the death of HTLV-I-transformed T cells.

Although Tax plays a critical role in the initial phases of HTLV-I-mediated leukemogenesis, there are several pieces of evidence suggesting that HTLV-I represses PDLIM2 expression independent of the key viral regulatory protein. First, PDLIM2 is repressed in Tax-negative ATL cells in addition to Tax-positive HTLV-I-transformed T cells (Figure 1). Second, Tax is dispensable for the specific up-regulation of DNMT1 and DNMT3b in HTLV-I-transformed T cells, although DNA methylation plays an important role in the PDLIM2 repression

in these malignant cells (Figure 3). Most importantly, neither short-term induction nor long-term stable expression of Tax could down-regulate PDLIM2 expression (Figure 2).

In summary, our studies suggested that HTLV-I-mediated repression of PDLIM2 occurs under pathophysiological conditions and that the PDLIM2 repression involves DNA methylation possibly through specific up-regulation of DNMT1 and DNMT3b. Our studies also excluded the role of Tax in the PDLIM2 repression. Given the death of HTLV-I-transformed cells triggered by the antitumor drug 5-aza-dC, these studies not only help understand regulation of PDLIM2, leukemogenicity, long latency and cancer health disparities of HTLV-I but also suggest a direct therapeutic strategy for ATL.

Acknowledgments

The authors thank M. Maeda and S. Yamaoka for MT-4 and TL-Om1 cells and W.C. Greene and E. Harhaj for TetOn Jurkat cells.

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