Overexpression of enhancer of zeste homolog 2 with trimethylation of lysine 27 on histone H3 in adult T-cell leukemia/lymphoma as a target for epigenetic therapy

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ABSTRACT

Background
Enhancer of zeste homolog 2 is a component of the Polycomb repressive complex 2 that mediates chromatin-based gene silencing through trimethylation of lysine 27 on histone H3. This complex plays vital roles in the regulation of development-specific gene expression.

Design and Methods
In this study, a comparative microarray analysis of gene expression in primary adult T-cell leukemia/lymphoma samples was performed, and the results were evaluated for their oncogenic and clinical significance.

Results
Significantly higher levels of Enhancr of zeste homolog 2 and RING1 and YY1 binding protein transcripts with enhanced levels of trimethylation of lysine 27 on histone H3 were found in adult T-cell leukemia/lymphoma cells compared with those in normal $CD4^+$ T cells. Furthermore, there was an inverse correlation between the expression level of Enhancer of zeste homolog 2 and that of miR-101 or miR-128a, suggesting that the altered expression of the latter miRNAs accounts for the overexpression of the former. Patients with high Enhancer of zeste homolog 2 or RING1 and YY1 binding protein transcripts had a significantly worse prognosis than those without it, indicating a possible role of these genes in the oncogenesis and progression of this disease. Indeed, adult T-cell leukemia/lymphoma cells were sensitive to a histone methylation inhibitor, 3-deazaneplanocin A. Furthermore, 3-deazaneplanocin A and histone deacetylase inhibitor panobinostat showed a synergistic effect in killing the cells

Conclusions
These findings reveal that adult T-cell leukemia/lymphoma cells have deregulated Polycomb repressive complex 2 with over-expressed Enhancer of zeste homolog 2, and that there is the possibility of a new therapeutic strategy targeting histone methylation in this disease.

Key words: adult T-cell leukemia/lymphoma, human T-cell leukemia virus type-1, Enhancer of zeste homolog 2, H3K27me3.

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The online version of this article has a Supplementary Appendix.

Introduction

The Polycomb group (PcG) proteins play critical roles in the regulation of development by repressing specific sets of developmental genes through chromatin modification.[1] They form two distinct multimeric complexes, Polycomb repressive complex 1 (PRC1) and PRC2, which bind to polycomb responsive elements (PRE), repress genes required for cell differentiation, and maintain pluripotency and self-renewal of embryonic stem cells and hematopoietic stem cells.[2,3] PRC2 consists of Enhancer of zeste homolog 2 (EZH2), which has histone methyltransferase activity, suppressor of zeste 12 (SUZ12), and embryonic ectoderm development (EED), which is required to maintain the integrity of PRC2.[1,4] Sequence-specific DNA binding protein YY1, which recognizes PRE, interacts with EED and recruits PRC2 to a specific chromatin domain to be repressed.[5] EED interacts with histone deacetylase (HDAC) proteins, HDAC1 and HDAC2, and the histone binding proteins RBBP4 (RbAp48) and RBBP7 (RbAp46).[6] PRC2 thus also participates in histone deacetylation. EZH2, as a part of the PRC2 complex, not only methylates histone but also serves as a recruitment platform for DNA methyltransferases that methylate the promoter regions of target genes, which is another mechanism of gene repression.[7] The more diverse complex PRC1 consists of HPC family proteins that mediate chromatin association, HPH family proteins, RING, BMI1, and others.[1] PRC2 initiates trimethylation of lysine 27 on histone H3 (H3K27me3) and, to a lesser extent, lysine 9 of histone H3.[8] PRC1 recognizes H3K27me3 through the chromodomain of the HPC and maintains the trimethylation. There are a number of reports indicating that such epigenetically mediated transcriptional silencing is associated with cancer development.[1,9] Among these, oncogenic roles of over-expressed EZH2 have been studied in a variety of tumors.[10]

Adult T-cell leukemia/lymphoma (ATL) is a neoplasm of mature CD4+ T-cell origin, etiologically associated with human T-cell leukemia virus type-1 (HTLV-1).[11,12] Its clinical behavior differs among patients and is subclassified into four subtypes: smoldering type and chronic type as indolent subtypes, and acute type and lymphoma type as aggressive subtypes.[13] Inactivation of tumor suppressor genes is one of the key events in development and progression, and there is a strong accumulation of *p14ARF/p15INK4B/p16INK4A* gene deletion/methylation or *p53* gene mutations in aggressive subtypes (>60%).[14-20] In the present study, for further investigation of the oncogenesis of ATL, we performed a comparative microarray analysis of gene expression in primary ATL samples. ATL cells expressed significantly higher levels of *EZH2* and *RYBP* (RING1 and YY1 binding protein) transcripts than CD4+ T cells from healthy volunteers. Moreover, acute-type ATL cells showed significantly higher levels of these transcripts than chronic-type ATL cells, suggesting that deregulation of PcG proteins plays a crucial role not only in the development but also in the progression of ATL. In addition, ATL samples were strongly positive for H3K27me3, and were sensitive to 3-deazaneplanocin A (DZNep), a histone methylation inhibitor.[21-23] It has recently been shown that HDAC inhibitor panobinostat (PS, also known as LBH589) depletes the levels of EZH2, SUZ12, and EED and induces apoptotic death in leukemia cells.[24] Deregulation of PcG protein genes with over-expressed EZH2 in ATL cells suggests that ATL is one of the appropriate target diseases for such epigenetic therapy.

Design and Methods

Sample preparation

This study was approved by the ethics committees of Nagasaki University, and all clinical samples were obtained after written informed consent was provided. The diagnosis of ATL was confirmed by the monoclonal integration of HTLV-1 proviral DNA in the genomic DNA of leukemia cells. Peripheral blood mononuclear cells (PBMCs) were obtained from ATL patients (acute type 22 cases, chronic type 19 cases) and healthy adult volunteers by density gradient centrifugation using Lympho-prep (AXIS SHIELD, Oslo, Norway). For enrichment of ATL cells, CD4+ cells were purified from the PBMCs by the magnetic bead method (CD4 MicroBeads, Miltenyi Biotec, Auburn, CA, USA) as described elsewhere.[25] Besides these samples for microarray analysis, we prepared another set of samples for quantitative real-time RT-PCR (qRT-PCR) and Western blotting (25 ATL patients, 13 HTLV-1 carriers, and 12 healthy adults) to confirm the results of microarray analysis. We also used formalin-fixed, paraffin-embedded lymph nodes from 7 patients with lymphoma-type ATL and 5 patients with follicular lymphoma for immunohistochemical analysis.

ATL cell lines used in this study, SO4, ST1, KK1, KOB, and LM-Y1, were established from respective patients in our laboratory and have been confirmed to be of primary ATL cell origin.[26] Cells were maintained in RPMI1640 medium supplemented with 10% FBS and 100 Japan reference units of recombinant interleukin-2 (rIL-2) (kindly provided by Takeda Pharmaceutical Company, Ltd., Osaka, Japan). We also used HTLV-1-infected T-cell lines MT2 and HuT102 and acute T-lymphoblastic leukemia cell lines Jurkat and MOLT4, which were maintained without rIL-2.

DNA microarray analysis

RNA was prepared from purified CD4+ T cells, and subjected to hybridization to HGU133A & B microarray containing 44,760 probe sets for human genes (Affymetrix, Santa Clara, CA, USA) as described previously.[25,27] The mean expression intensity of the internal positive control probe sets (*http://www.affymetrix.com/support/technical/mask_files.affx*) was set to 500 units in each hybridization, and the fluorescence intensity of each test gene was normalized accordingly. All HGU133A & B microarray data are available from the Gene Expression Omnibus website (*http://www.ncbi.nlm.nih.gov/geo*) under the accession number GSE1466.

Quantitative real-time RT-PCR

For confirmation of the results of microarray analysis, we performed quantitative real-time RT-PCR (qRT-PCR) for PcG protein genes. Total RNA was prepared using Isogen (Wako, Osaka, Japan). After removal of contaminated DNA with DNase (Message Clean kit; GenHunter, Nashville, TN, USA), cDNA was constructed from 1 μg of total RNA using the SuperScript III RT-PCR System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Primers and TaqMan probes labeled with TAMRA dye at the 3' end and FAM at the 5' end are listed in *Online Supplementary Table S1*. The mRNA levels for PcG family proteins and porphobilinogen deaminase (PBGD) were measured from a cDNA template using a LightCycler480 PCR System (Roche Diagnostics, Mannheim, Germany). Briefly, reactions were performed in a 20 μL volume with 5 μL (25 ng) of cDNA, 0.5 μM PCR primers, 0.1 μM TaqMan probes, and 10 μL of LightCycler

480 probes Master Mix (Roche Diagnostics). The PCR program consisted of 95°C for 5 min followed by 50 cycles of 95°C for 10 sec and 60°C for 30 sec. After 50 cycles, the absolute amounts of PcG protein mRNA and *PBGD* mRNA were interpolated from the standard curves generated by the dilution method using plasmids derived from a clone transfected with pTAC-1 Vector (BioDynamics Laboratory Inc., Tokyo, Japan) containing amplicons from the PcG family protein and PBGD genes, respectively. To normalize these results for variability in concentration and integrity of RNA and cDNA, the *PBGD* gene was used as an internal control in each sample.

For the quantitative PCR for microRNAs (miRNAs), miR-101, miR-26a, and miR-128a, 10 ng of total RNA (containing miRNA) was used. RT reaction and real-time quantification were performed using TaqMan MicroRNA RT kit and TaqMan MicroRNA assays (hsa-miR-26a, assay ID 000405; hsa-miR-101, assay ID 002253; hsa-miR-128a, assay ID 002216; RNU6B, assay ID 001093) (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. Each PCR reaction mixture contained 10 μL of LightCycler 480 probes Master Mix, 4 μL of nuclease-free water, 1 μL of 20X specific PCR primer, and 5 μL of RT product. The thermal cycler was programmed as follows: 95°C for 5 min, 40 cycles of 95°C for 15 sec, and 60°C for 60 sec. Using the comparative CT method, we used an endogenous control (RNU6B) to normalize the expression levels of target micro-RNA by correcting differences in the amount of RNA loaded into qPCR reactions.

Western blot analysis and antibodies

Western blot analysis was performed as described previously.[28] The analysis was performed using antibodies to EZH2 and Histone H3 (Cell Signaling Technology, Danvers, MA, USA), phospho EZH2 (Ser21) (Bethyl Laboratories, Montgomery, TX, USA), H3K27me3, dimethylated H3K27 (H3K27me2), monomethylated H3K27 (H3K27me1) (Millipore, Temecula, CA, USA), and β-actin (Sigma, St. Louis, MO, USA).

Immunohistochemistry

Immunohistochemical staining for EZH2 and H3K27me3 was performed on formalin-fixed, paraffin-embedded lymph node samples from lymphoma-type ATL patients and follicular lymphoma patients as a control. The deparaffinized slides were pretreated with DAKO Target Retrieval Solution, pH 9 (DAKO Japan, Tokyo, Japan), and heated in a water bath at 95°C for 40 min. For all stains, the endogenous peroxidase was quenched using 3% H_2O_2 for 15 min. Sections were then placed in 0.5% non-fat dry milk for 30 min at room temperature. The primary antibodies used were anti-EZH2 antibody (BD Biosciences, San Jose, CA, USA) and anti-H3K27me3 antibody (Cell Signaling Technology, Boston, MA, USA), and were applied at 1:50 dilution and 1:100 dilution, respectively. They were allowed to react for 1 h at room temperature, and then the DAKO EnVision™ + Dual Link System-HRP (DAKO Japan, Tokyo, Japan) was applied using diaminobenzidine as the chromogen, following the manufacturer's protocol.

Sensitivity of adult T-cell leukemia/lymphoma cell lines to DZNep and PS (LBH589)

DZNep was synthesized by one of the authors (VEM). Cells were treated with different concentrations of DZNep for 72 h and the cell proliferation status was evaluated by an MTS assay using a Cell Titer 96® AQueos Cell Proliferation Assay kit (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. To analyze the synergistic effect of combined treatment with DZNep and PS (LBH589) (kindly provided by Novartis Pharma AG, Basel, Switzerland), cells were treated with DZNep (0.3-5.0 μM) and PS (LBH589) (3-50 nM) for 48 h. After the cell proliferation status was evaluated by an MTS assay, the combination index (CI) for each drug combination was obtained by determining the median dose effect of Chou and Talalay using the CI equation within the commercially available software Calcusyn (Biosoft).[29] CI<1, CI=1, and CI>1 indicate synergism, additive effect, and antagonism, respectively. Cell viability represents the value relative to that of the control culture without these agents.

Results

Microarray analysis shows increased EZH2 and/or RYBP transcripts in adult T-cell leukemia/lymphoma cells

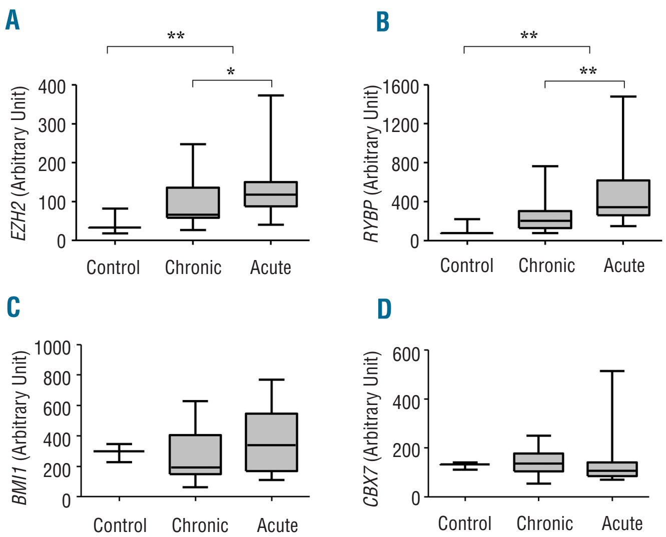
In a comparative microarray analysis of primary ATL samples, we focused on investigating PcG protein genes, *EZH2*, *RYBP*, *BMI-1*, and *CBX7*, in the present study because ATL cells show many aberrantly hypermethylated DNA sequences.[30] ATL cells expressed significantly higher levels of *EZH2* and *RYBP* transcripts than $CD4^+$ T cells from healthy adults (Figure 1A and B). In addition, there was a difference between ATL subtypes in these expressions, and cells from the acute type showed significantly higher levels of these transcripts than the cells from the chronic type. When patients were separated into two groups consisting of those with high expression and those with low expression, the group with high *EZH2* or high *RYBP* transcript showed significantly shorter survival than the respective low-expression groups (Figure 1E and F), indicating that high *EZH2* and/or *RYBP* expression is associated with aggressive clinical behavior. Convincingly, there was a trend toward accumulation of acute-type ATL in the high *EZH2* or the high *RYBP* expression group: 14 cases of acute type and 6 cases of chronic type in the high *EZH2* group, 7 cases of acute type and 13 cases of chronic type in the low *EZH2* group, 14 cases of acute type and 6 cases of chronic type in the high RYBP group, and 7 cases of acute type and 13 cases of chronic type in the low RYBP group. BMI1 is known to down-regulate the expression of *p14ARF/p16INK4A* and lead to neoplastic transformation.[31] Chromobox 7 (CBX7), a component of the PRC1, is also known to repress the transcription of *p14ARF/p16INK4A*.[32] Since inactivation of *p14ARF/p15INK4B/p16INK4A* genes is one of the key events in ATL progression, expression of *BMI-1* and/or *CBX7* transcript was expected to be elevated in acute-type ATL cells. There was, however, no difference in these expressions between ATL subtypes or even between ATL cells and normal $CD4^+$ T cells (Figure 1C and D). There was no difference in survival for different *BMI-1* or *CBX7* expression levels (Figure 1G and H).

Confirmation of increased EZH2 and/or RYBP transcripts by quantitative real-time RT-PCR

For confirmation of the results of microarray analysis, we quantified the transcripts of the PcG protein genes including *EZH2* and *RYBP* by qRT-PCR using another set of samples from ATL patients, healthy adults, HTLV-1 carriers, and hematologic cell lines including ATL cell lines. In accordance with the results of microarray analysis, *EZH2* and *RYBP* transcripts were increased in primary ATL cells compared with those in the cells from healthy adults and HTLV-1 carriers, with statistically significantly higher values in *EZH2* in terms of both absolute copy number per 25 ng of total RNA and normalized expression level (*Online Supplementary Figure S1A, a, B, b*). *RBBP4* was significantly higher in primary ATL cells than in the cells from healthy adults and HTLV-1 carriers in terms of normalized expression level (*Online Supplementary Figure S1 C, c*). In contrast, there was no difference in *BMI1*, *YY1*, and *EED* expressions among these groups, although some patients showed very high *BMI1* expression (*Online Supplementary Figure S1D, d, E, e, F, f*). Similarly to primary ATL cells, some ATL cell lines showed high *EZH2* expression in terms of absolute copy number per 25 ng of total RNA (*Online Supplementary Figure S1A*).

EZH2 protein expression with trimethylation of H3K27 is characteristic in adult T-cell leukemia/lymphoma cells

We then examined EZH2 and RYBP at the protein level by Western blotting. A 98-kDa band for EZH2 protein and a 32-kDa band for RYBP protein were detected in all primary ATL samples irrespective of subtype, but they were hardly detected in cells from healthy adults and HTLV-1 carriers (Figure 2A, *Online Supplementary Figure S2*, and *data not shown*). ATL cell lines and acute T-lymphoblastic leukemia cell lines also showed intense EZH2 bands. The serine-threonine kinase Akt phosphorylates EZH2 at serine 21 and suppresses its methyltransferase activity by impeding EZH2 binding to histone H3, which results in a decrease in lysine 27 trimethylation.[33] EZH2 of ATL cells was not phosphorylated and was in its active form (Figure 2A). In fact, most primary ATL samples showed the band for H3K27me3, while the cells from healthy adults lacked the band (Figure 2B). As it is known that EZH2 plays a crucial role in trimethylation but not in dimethylation or monomethylation, the bands for H3K27me2 and H3K27me1 were detected in all samples examined, but the band for H3K27me3 was limited in primary ATL cells and ATL cell lines LMY1 and KOB that showed an intense EZH2 band with a faint phosphorylated EZH2 band (Figure 2A and B). In contrast, EZH2 was strongly phosphorylated in ATL cell lines ST1, SO4, KK1, and acute T-lymphoblastic leukemia cell lines Jurkat and MOLT4, and these cell lines hardly showed the band for H3K27me3. Collectively, these results indicate that ATL cells express



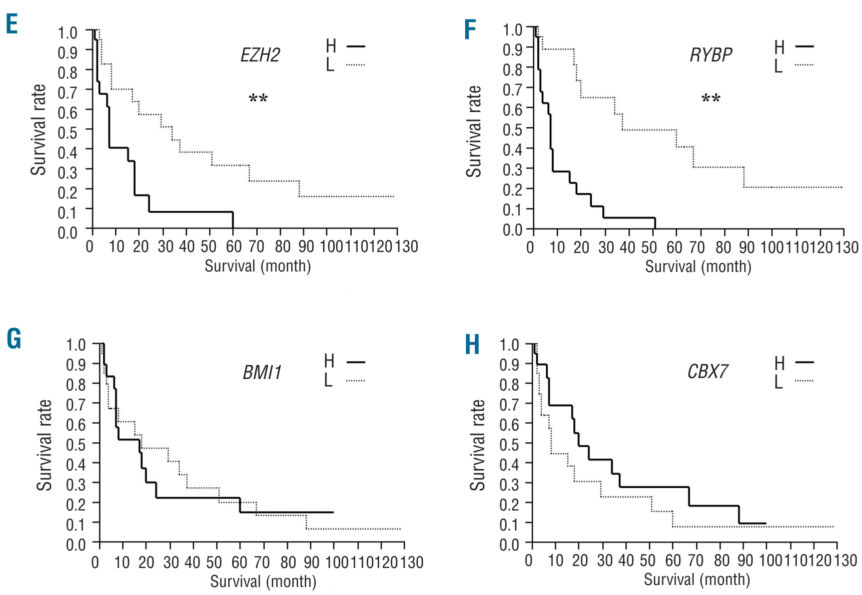


Figure 1. Microarray analysis of gene expression in primary ATL cells. (A-D) Expression levels of PcG protein genes were compared among normal CD4+ T cells (Control), chronic ATL cells (Chronic), and acute ATL cells (Acute), and results of ***EZH2*** (A), ***RYBP*** (B), ***BMI1*** (C), and ***CBX7*** (D) are demonstrated in box plots. ATL cells showed significantly higher levels of ***EZH2*** and ***RYBP*** transcripts than normal CD4+ T cells (Mann-Whitney's U test), with a higher expression in the acute type than in the chronic type (Mann-Whitney's U test) (A, B). In contrast, there was no statistical difference in the level for ***BMI1*** or ***CBX7*** among these groups (C, D). (E-H) Overall survival curves for ATL patients separated into two groups consisting of those with high expression (H, n=20) and low expression (L, n=20) for ***EZH2*** (E), ***RYBP*** (F), ***BMI1*** (G), or ***CBX7*** (H) are shown. Patients with high ***EZH2*** or high ***RYBP*** expression showed significantly shorter survival than those in corresponding low expression groups (log rank test) (E, F). There was no difference in survival for different ***BMI1*** or ***CBX7*** expressions (G, H). H: high expression group (bold line), L: low expression group (thin dotted line). $*P<0.05$, $**P<0.01$

functionally active EZH2, and as a result, their H3K27 are trimethylated, and that ATL cell lines LMY1 and KOB preserve this characteristic of primary ATL cells.

Immunohistochemical confirmation of the expression of EZH2 and H3K27me3 in lymph nodes

We next used lymph nodes from lymphoma-type ATL patients for immunohistochemical evaluation of EZH2 expression and H3K27me3. In agreement with the results of Western blotting, all ATL lymph nodes from 7 patients were strongly positive for both EZH2 and H3K27me3 without exception in their nuclear staining (*Online Supplementary Figure S3* and *data not shown*), suggesting that overexpression of EZH2 with H3K27me3 is a common feature of ATL cells irrespective of ATL subtypes. In contrast, in lymph nodes from 5 follicular lymphoma patients, only a few cells were positive for EZH2 with some variation among patients and most cells were negative for H3K27me3 (*Online Supplementary Figure S3* and *data not shown*).

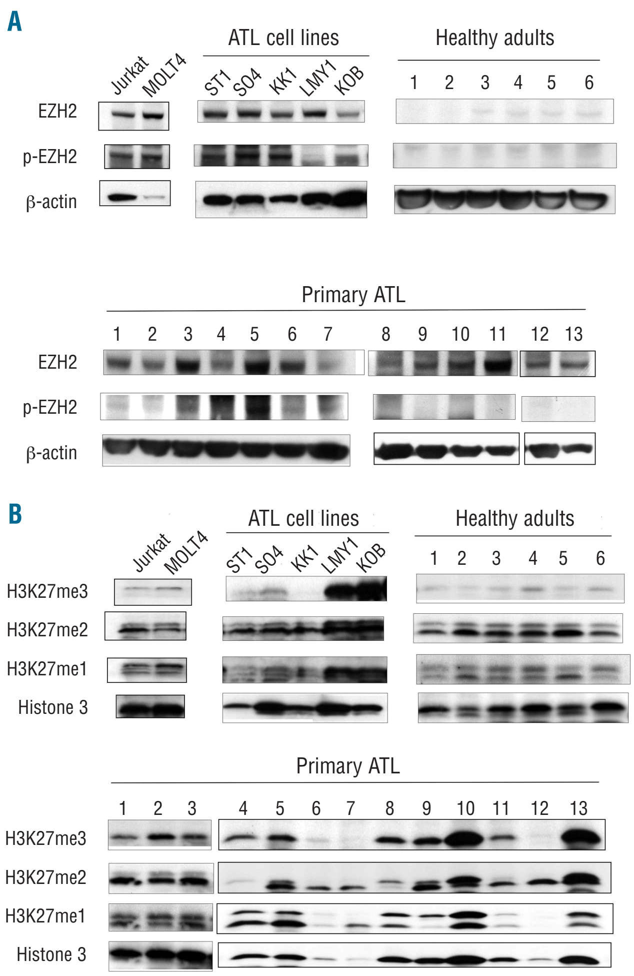


Figure 2. EZH2 protein expression and histone methylation. (A) Western blot analysis for EZH2 protein was performed on primary ATL cells, cells from healthy adults, and ATL cell lines. Primary ATL cells showed a clear 98-kDa band for EZH2 with the absence or presence of faint bands for phosphorylated EZH2 (p-EZH2). Cells from healthy adults hardly showed these bands. ATL cell lines ST1, SO4, and KK1 showed intense bands for both EZH2 and p-EZH2, but LM-Y1 and KOB cells showed intense bands for EZH2 with the absence of a band for p-EZH2. (B) Western blot analysis for histone methylation status was performed. Only primary ATL cells and LM-Y1 and KOB cell lines showed a clear band for H3K27me3, but others hardly showed the band. Bands for H3K27me2, H3K27me1, and histone H3 were observed in almost all samples examined.

Downregulation of miR-101 and miR-128a may be responsible for increased EZH2 expression

So far, more than 700 miRNAs have been identified in humans, and each miRNA regulates multiple target genes. miR-101 and miR-26a have been shown to be negative regulators of *EZH2* expression and are depressed in several types of cancer cells.[34,35] miR-128a is known to be a negative regulator of *BMI1* and has been reported to be involved in glioma cell proliferation.[36] We quantified these miRNAs in primary ATL cells and cells from HTLV-1 carriers to investigate the mechanism of *EZH2* overexpression. ATL cells showed significantly decreased levels of miR-101 and miR-128a compared with the cells from HTLV-1 carriers (Figure 3A and C). Notably, there were significant inverse correlations between *EZH2* expression and miR-101 expression or *EZH2* expression and miR-128a expression (Figure 3D and E), suggesting that decrease of these miRNAs accounts for the overexpression of *EZH2*. Since genomic loss of miR-101 has been reported in prostate cancer,[34] we performed quantitative genomic PCR for miR-101 in two loci, miR-101-1 (chromosome 1p31) and miR-101-2 (chromosome 9p24). Both loci were preserved in all 10 ATL samples examined (*Online Supplementary Figure S4*). The expression of miR-26a did not, in contrast, differ between ATL cells and cells from HTLV-1 carriers (Figure 3B). Unexpectedly, there was no significant correlation between *BMI1* expression and miR-128a expression (Figure 3F).

Adult T-cell leukemia/lymphoma cells are sensitive to DZNep and PS (LBH589)

We first examined the sensitivity of ATL-related cell lines and acute T-lymphoblastic leukemia cell lines to DZNep, an inhibitor of S-adenosylhomocysteine hydrolase, which has recently been shown to decrease the expression of EZH2 and histone methylation.[22,23] DZNep inhibited the proliferation of these cell lines, at concentrations above 0.5 μM (*Online Supplementary Figure S5A*). In contrast, $CD4^+$ T cells from healthy adults as a normal control were resistant to DZNep even at 5 μM. Notably, although DZNep decreased *EZH2* expression in ST1, SO4, and KK1, it did not decrease but rather increased the expression in KOB, results which were confirmed by Western blot (*Online Supplementary Figure S5B and C*). PS (LBH589) is also known to decrease the level of EZH2 in several types of leukemia cells.[24] One hundred nM of PS (LBH589) decreased *EZH2* expression at both transcript and protein levels in ATL cell lines including KOB and LM-Y1, which showed a similar EZH2 expression profile to that of primary ATL cells, namely, high EZH2 expression with low phosphorylated EZH2 and strong H3K27me3 (*Online Supplementary Figure S5D and E*). We next examined whether these agents show a synergistic effect or just an additive effect. As shown in *Online Supplementary Figure S5F* (upper panel), the cell viabilities of LM-Y1 treated with 25 nM PS (LBH589) or 2.5 μM DZNep were 70% and 87%, respectively. A combination of this setting (LBH:DZNep=1:100) markedly decreased the proportion of viable cells (40%) compared with that of cells treated with either agent alone. Similarly, cell viabilities of KOB treated with 25 nM PS (LBH589), 2.5 μM DZNep, or a combination of these agents were 86%, 93%, and 48%, respectively. By calculating CI according to the method of Chou and Talalay,[29] we found a strong synergistic antiproliferative effect in both cell lines (*Online Supplementary Figure S5F*, lower panel).

Discussion

EZH2 is a critical component of PRC2, which mediates epigenetic gene silencing through trimethylation of H3K27.[37,38] EED and SUZ12 are also required for the exhibition of methyltransferase activity and for the localization of this complex to target genes.[39] In an analysis of genome-wide H3K27 methylation in aggressive prostate cancer tissues, a significant subset of the target genes were also targets in embryonic stem cells, suggesting that the mechanism for gene silencing used to maintain stem cell renewal is converted into oncogenesis.[40] Ectopic expression of EZH2 is capable of providing a proliferative advantage to primary cells, and its gene locus is amplified in primary tumors.[41] Indeed, increased EZH2 expression has been reported in several types of cancer cells, and its clinical significance is extensively studied in prostate cancer.[42] Amounts of both *EZH2* transcript and EZH2 protein were elevated in metastatic prostate cancer; in addition, clinically localized prostate cancers that express higher concentrations of *EZH2* showed a poorer prognosis. An association of increased EZH2 expression with poor prognosis has also been reported in other solid tumors. Currently, however, there are only limited reports describing EZH2 expression in hematologic malignancies.

In the present study, we showed for the first time that EZH2 was over-expressed in ATL cells, and that the increased EZH2 was not phosphorylated and was in its active form. The increased EZH2 seemed to exhibit histone methyltransferase activity *in vivo*, as supported by the results that ATL cells from both peripheral blood and lymph nodes were strongly positive for H3K27me3. Since EZH2 was almost undetectable in cells from healthy adults and HTLV-1 carriers, it is likely that deregulation of PRC2 caused by over-expressed EZH2 is involved in the early steps of ATL oncogenesis. Meanwhile, ATL patients with high EZH2 expression showed shorter survival than patients with low EZH2 expression, indicating that increased EZH2 also plays a role in the process of ATL progression. It has been reported that genes methylated in cancer cells are specifically packaged with nucleosomes containing H3K27.[43] However, there are only a few studies that actually examined H3K27me3 in primary tumor cells or tissues. In one such study, H3K27me3 expression was unexpectedly lower in breast, ovarian, and pancreatic cancers than in corresponding normal tissues, although it has been reported that there are increased levels of H3K27me3 in breast cancer cell lines.[44,45] We do not have an adequate explanation for these conflicts at present, but there may be some differences in the process of oncogenesis between solid tumors and hematologic malignancies.

The mechanism of the overexpression of EZH2 in tumors remains largely unknown. miRNAs regulate gene expression and play important roles in cellular differentiation and embryonic stem cell development. Recently, two miRNAs, miR-101 and miR-26a, were found to repress *EZH2* expression. The expression of miR-101 decreases in parallel with an increase in *EZH2* expression during progression in prostate tumors.[34] In addition to these miRNAs, we examined miR-128a, which has been shown to repress *BMI1* expression in glioblastoma, because overexpression of *BMI-1* is associated with the development of malignant lymphoma.[31,36] ATL cells showed a decreased level of miR-

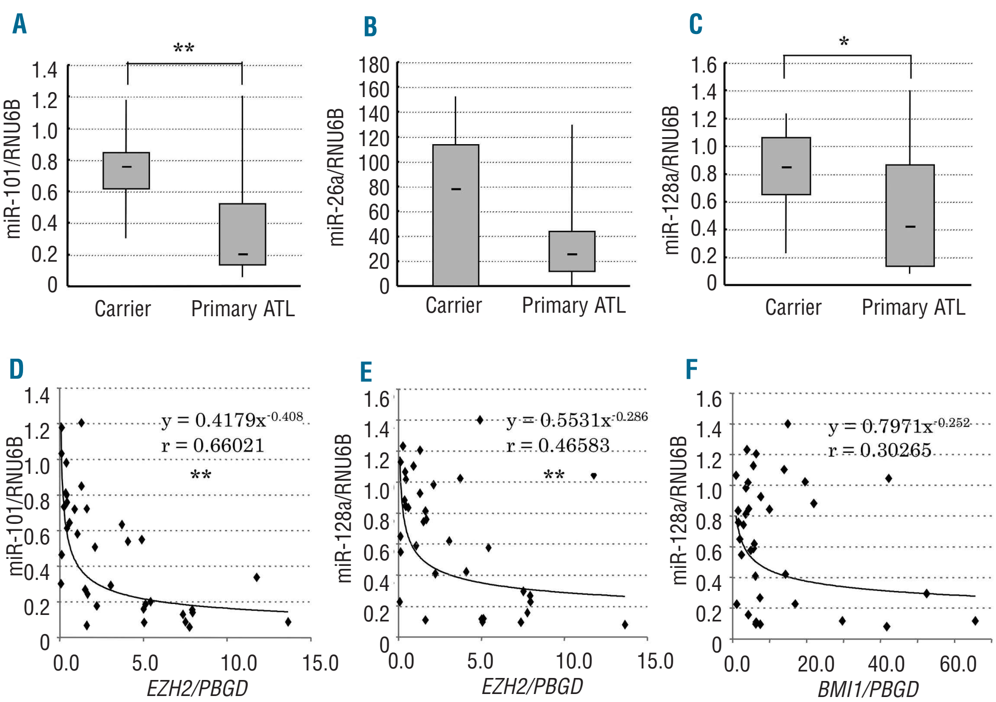


Figure 3. Quantitative real-time RT-PCR for miRNAs. (A-C) Expressions of miR-101 (A), miR-26a (B), and miR-128a (C) were compared between ATL patients and HTLV-1 carriers. Primary ATL cells showed significantly lower levels of miR-101 and miR-128a (Mann-Whitney's U test) compared with the cells from HTLV-1 carriers (A, C). There was no significant difference in miR-26a expression between the two groups (B). (D, E, F) Correlation between miRNA and *EZH2* or *BMI1* expression was examined. There were significant inverse correlations between normalized *EZH2* expression and miR-101 expression (D) or between normalized *EZH2* expression and miR-128a expression (E) (Spearman's correlation coefficient). In contrast, there was no correlation between normalized *BMI1* expression and miR-128a expression (F). *P<0.05, **P<0.01

101 expression compared with the cells from HTLV-1 carriers, which is not caused by genomic loss of the *miR-101* gene, in contrast to prostate cancer.[34] Moreover, there was a clear inverse correlation between *EZH2* expression and miR-101 expression, suggesting that increased *EZH2* is caused by the decrease in miR-101 expression. Although currently there is no report indicating an association of miR-128a with *EZH2* expression, miR-128a showed exactly the same pattern as miR-101, suggesting that the decrease in miR-128a also participates in *EZH2* overexpression in ATL. By analyzing the 3'-UTR sequence of *EZH2*, it has recently been shown that there are two predicted miR-101 target sites and one predicted miR-26a target site in the 3'-UTR of EZH2.[46] We performed a similar analysis and found that there was also a potential target site for miR-128a near one of the miR-101 target sites (*Online Supplementary Figure S6*). miR-26a was not decreased in ATL cells, and there was no correlation between miR-26a expression and *EZH2* expression or miR-128a expression and *BMI1* expression. The association of miR-26a with *EZH2* was found in normal cell differentiation as a physiological phenomenon but not in tumor cells. The miRNAs used to regulate normal development and differentiation may be different from those used for the development of tumors. Another possible explanation for the mechanism of increased *EZH2* expression in ATL is inactivation of *p14ARF/p15INK4B/p16INK4A* tumor suppressor genes, which frequently occurs in ATL.[14,15,19,20] EZH2 is a molecule downstream of the pRB-E2F pathway, and inactivation of these genes allows E2F to be released from pRB, which results in the upregulation of *EZH2* expression.[41] Several recent reports indicate that EZH2 functions to repress the expression of *p14ARF/p15INK4B/p16INK4A*; therefore, increased EZH2 may be used to further decrease the expression of *p14ARF/p15INK4B/p16INK4A*.[47] Since somatic mutations altering EZH2 (Tyr641) have recently been reported in follicular and diffuse large B-cell lymphomas of germinal-center origin,[48] we performed a similar analysis in 10 primary ATL samples. There were however no such mutations (*Online Supplementary Figure S7*).

ATL is quite resistant to antineoplastic agents and the median survival time of those with the aggressive subtypes is only 13 months, even in a recent multicenter clinical trial.[49] Since high EZH2 expression with H3K27me3 seems to be an essential component for the initiation and promotion of cell proliferation in ATL, we searched for the possibility of therapeutic strategies targeting EZH2. We examined the sensitivity of ATL cells to agents that have been shown to inhibit *EZH2* expression and histone methylation. DZNep is a carbocyclic analog of adenosine synthesized more than 20 years ago as an inhibitor of S-adenosylhomocysteine hydrolase, which has therapeutic potential as an anticancer or antiviral drug.[21] DZNep has recently aroused interest for its unique features; it decreases the expressions of *EZH2*, *SUZ12*, and *EED* with inhibition of H3K27 methylation and induces apoptosis in cancer cells but not in normal cells.[22,23] ATL cell lines were sensitive to DZNep and their cell proliferation was attenuated at one-tenth of the concentration used in these studies. More interestingly, DZNep showed no toxicity to normal $CD4^+$ T cells as a normal control. Acute T-lymphoblastic leukemia cell lines showed similar sensitivities to DZNep, which may indicate that DZNep exerts general toxicity to leukemia and lymphoma cells not necessarily associated with histone modification. Indeed, although DZNep rather increased EZH2 expression in KOB cells, this cell line was equally sensitive as other cell lines to DZNep. HDAC inhibitor PS (LBH589) is an effective agent for cutaneous T-cell lymphoma and induced complete remission in 2 of 9 patients involved in a phase I clinical trial.[50] More interestingly, it has been reported recently that combined use of DZNep and PS (LBH589) yielded more depletion of EZH2 and induced more apoptosis of leukemia cells, but not normal CD34 (+) bone marrow progenitor cells.[51] In the present study, we showed that the combination of DZNep and PS (LBH589) exhibited a synergistic effect in killing ATL cells. Thus, epigenetic therapy by the combined use of these agents that inhibit histone methylation could lead to a breakthrough in the treatment of aggressive ATL.

Authorship and Disclosures

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