

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 3A, 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 functionally active EZH2, and as a result, their H3K27 are trimethylated, and that ATL cell lines LMY1 and KOB preserve such character 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 (Figure 4 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 (Figure 4 and *data not shown*).

***Down-regulation of miR-101 and miR-128a may be responsible for increased EZH2 expression***

So far, more than 200 miRNAs have been identified in human, 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.<sup>34,35</sup> miR-128a is known to be a negative regulator of *BMI1* and has been reported to be involved in glioma cell proliferation.<sup>36</sup> 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 5A,C). Notably, there were significant inverse correlations between *EZH2* expression and miR-101 expression or *EZH2* expression and miR-128a expression (Figure 5D, 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,<sup>34</sup> 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 2*). The expression of miR-26a was, in contrast, not different between ATL cells and cells from HTLV-1 carriers (Figure 5B). Unexpectedly, there was no significant correlation between *BMI1* expression and miR-128a expression (Figure 5F).

### ***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.<sup>22,23</sup> DZNep inhibited the proliferation of these cell lines, at concentrations above 0.5  $\mu$ M (Figure 6A). In contrast, CD4<sup>+</sup> T cells from healthy adults as a normal control were resistant to DZNep even at 5 $\mu$ M. Notably, although DZNep decreased *EZH2* expression in ST1, SO4, and KK1, it did not decrease but rather increased the expression in KOB, which results were confirmed by Western blot (Figure 6B, C). PS (LBH589) is also known to decrease the level of EZH2 in several types of leukemia cells.<sup>24</sup> 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 (Fig. 6D, E). We next examined whether these agents show a synergistic effect or just an additive effect. As shown in Figure 6F (upper panel), the cell viabilities of LM-Y1 treated with 25 nM PS (LBH589) or 2.5  $\mu$ M 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  $\mu$ M DZNep, or a combination of these agents were 86%, 93%, and 48%, respectively. By calculating CI according to the method of Chou and Talalay,<sup>29</sup> we found a

strong synergistic antiproliferative effect in both cell lines (Figure 6F, lower panel).

## Discussion

EZH2 is a critical component of PRC2, which mediates epigenetic gene silencing through trimethylation of H3K27.<sup>37,38</sup> EED and SUZ12 are also required for the exhibition of methyltransferase activity and for the localization of this complex to target genes.<sup>39</sup> 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.<sup>40</sup> Ectopic expression of EZH2 is capable of providing a proliferative advantage to primary cells, and its gene locus is amplified in primary tumors.<sup>41</sup> Indeed, increased EZH2 expression has been reported in several types of cancer cells, and its clinical significance is extensively studied in prostate cancer.<sup>42</sup> 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 hematological malignancies.

In the present study, we showed for the first time that EZH2 was overexpressed 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 overexpressed 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.<sup>43</sup> 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.<sup>44,45</sup> 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 hematological 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.<sup>34</sup> 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.<sup>31,36</sup> ATL cells showed a decreased level of miR-101 expression compared with the cells from HTLV-1 carriers, which is not caused by genomic loss of the *miR-101* gene, different from the case of prostate cancer.<sup>34</sup> 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*.<sup>46</sup> 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 (Supplementary Figure 3). 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.<sup>14,15,19,20</sup> *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.<sup>41</sup> 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*.<sup>47</sup> Since somatic mutations altering *EZH2* (Tyr641) have recently been reported in follicular and diffuse large B-cell lymphomas of germinal-center origin,<sup>48</sup> we performed a similar analysis in 10 primary ATL samples. There were however no such mutations (Supplementary Figure 4).

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.<sup>49</sup> 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.<sup>21</sup> DZNep has drawn attention recently 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.<sup>22,23</sup> 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<sup>+</sup> 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.<sup>50</sup> 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.<sup>51</sup> 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 bring breakthrough in treatment of aggressive ATL.

#### **Authorship and Disclosures**

DS and YI contributed equally to this work. DS, YI, HH, AO, YLC, TH, and YuM performed research and interpreted data; YI, KT, and HM designed the microarray study, collected samples, and analyzed data; VEM provided DZNep, interpreted data, and wrote the paper; KY, YaM, and SK provided administrative support; YY conceived of and designed the study, interpreted data, and wrote the paper.

The authors reported no potential conflicts of interest.



## References

1. Sparmann A, van Lohuizen M. Polycomb silencers control cell fate, development and cancer. *Nat Rev Cancer*. 2006;6(11):846-56.
2. Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, Kumar RM, et al. Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell*. 2006;125(2):301-13.
3. Kamminga LM, Bystrykh LV, de Boer A, Houwer S Douma J, Weersing E, et al. The Polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. *Blood*. 2006;107(5):2170-9.
4. van Lohuizen M, Tijms M, Voncken JW, Schumacher A, Magnuson T, Wientjens E. Interaction of mouse polycomb-group (Pc-G) proteins Enx1 and Enx2 with Eed: Indication for separate Pc-G complexes. *Mol Cell Biol*. 1998;18(6):3572-9.
5. Satiijn DP, Hamer KM, den Blaauwen J, Otte AP. The Polycomb group protein EED interacts with YY1, and both proteins induce neural tissue in *Xenopus* embryos. *Mol Cell Biol*. 2001;21(4):1360-9.
6. van der Vlag J, Otte AP. Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. *Nat Genet*. 1999;23(4):474-8.
7. Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, et al. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature*. 2006;439(7078):871-4.
8. Cao R, Zhang Y. The functions of E(Z)EZH2-mediated methylation of lysine

- 27 in histoneH3. *Curr Opin Genet Dev.* 2004;14(2):155-64.
9. Widschwendter M, Fiegler H, Egle D, Mueller-Holzner E, Spizzo G, Marth C, et al. Epigenetic stem cell signature in cancer. *Nat Genet.* 2007;39(2):157-8.
10. Simon JA, Lange CA. Roles of the EZH2 histone methyltransferase in cancer epigenetics. *Mutat Res.* 2008;647(1-2):21-9.
11. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood.* 1977;50(3):481-92.
12. Yoshida M, Seiki M, Yamaguchi K, Takatsuki K. Monoclonal integration of human T-cell leukemia provirus in all primary tumors of adult T-cell leukemia suggests causative role of human T-cell leukemia virus in the disease. *Proc Natl Acad Sci USA.* 1984;81(8):2534-7.
13. Shimoyama M and members of the Lymphoma Study Group (1984-1987): Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984-1987). *Br J Haematol.* 1991;79(3):428-37.
14. Hatta Y, Hiramata T, Miller CW, Yamada Y, Tomonaga M, Koeffler HP. Homozygous deletions of p15 (MTS2) and p16 (CDKN2/MTS1) genes in adult T-cell leukemia. *Blood.* 1995;85(10):2699-704.
15. Yamada Y, Hatta Y, Murata K, Sugawara K, Ikeda S, Mine M, et al. Deletions of p15 and/or p16 genes as a poor-prognosis factor in adult T-cell leukemia. *J Clin Oncol.* 1997;15(5):1778-85.
16. Nagai H, Kinoshita T, Imamura J, Murakami Y, Hayashi K, Mukai K, et al. Genetic alteration of p53 in some patients with adult T-cell leukemia. *Jpn J Cancer Res.* 1991;82(12):1421-7.

17. Sakashita A, Hattori T, Miller CW, Suzushima H, Asou N, Takatsuki K, et al. Mutations of the p53 gene in adult T-cell leukemia. *Blood*. 1992;79(2):477-80.
18. Tawara M, Hogerzeil SJ, Yamada Y, Takasaki Y, Soda H, Hasegawa H, et al. Impact of p53 aberration on the progression of adult T-cell leukemia/lymphoma. *Cancer Lett*. 2006;234(2):249-55.
19. Kohno T, Yamada Y, Tawara M, Takasaki Y, Kamihira S, Tomonaga M, et al. Inactivation of p14ARF as a key event for the progression of adult T-cell leukemia/lymphoma. *Leuk Res*. 2007;31(12):1625-32.
20. Nosaka K, Maeda M, Tamiya S, Sakai T, Mitsuya H, Matsuoka M. Increasing methylation of the CDKN2A gene is associated with the progression of adult T-cell leukemia. *Cancer Res*. 2000;60(4):1043-8.
21. Glazer RI, Hartman KD, Knode MC, Richard MM, Chiang PK, Tseng CK, et al. 3-Deazaneplanocin: a new and potent inhibitor of S-adenosylhomocysteine hydrolase and its effects on human promyelocytic leukemia cell line HL-60. *Biochem Biophys Res Commun*. 1986;135(2):688-94.
22. Miranda TB, Cortez CC, Yoo CB, Liang G, Abe M, Kelly TK, et al. DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation. *Mol Cancer Ther*. 2009;8(6):1579-88.
23. Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, et al. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. *Genes Dev*. 2007;21(9):1050-63.
24. Fiskus W, Pranpat M, Balasis M, Herger B, Rao R, Chinniyar A, et al.

- Histone deacetylase inhibitors deplete EZH2 and associated Polycomb Repressive Complex 2 proteins with attenuation of HOXA9 and MEIS1 and loss of survival of human acute leukemia cells. *Mol Cancer Ther.* 2006;5(12):3096-104.
25. Choi YL, Tsukasaki K, O'Neill MC, Yamada Y, Onimaru Y, Matsumoto K, et al. A genomic analysis of adult T-cell leukemia. *Oncogene.* 2007;26(8):1245-55.
26. Yamada Y, Ohmoto Y, Hata T, Yamamura M, Murata K, Tsukasaki K, et al. Features of the cytokines secreted by adult T cell leukemia (ATL) cells. *Leuk Lymphoma.* 1996;21(5-6):443-7.
27. Choi YL, Makishima H, Ohashi J, Yamashita Y, Ohki R, Koinuma K, et al. DNA microarray analysis of natural killer cell-type lymphoproliferative disease of granular lymphocytes with purified CD3(-) CD56(+) fractions. *Leukemia.* 2004;18(3):556-65.
28. Hasegawa H, Yamada Y, Komiyama K, Hayashi M, Ishibashi M, Sunazuka T, et al. A novel natural compound, a cycloanthranilylproline derivative (Fuligocandin B), sensitizes leukemia cells to apoptosis induced by tumor necrosis factor related apoptosis-inducing ligand (TRAIL) through 15-deoxy-Delta 12, 14 prostaglandin J2 production. *Blood.* 2007;110(5):1664-74.
29. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul.* 1984;22:27-55.
30. Yasunaga J, Taniguchi Y, Nosaka K, Yoshida M, Satou Y, Sakai T, et al. Identification of aberrantly methylated genes in association with adult T-cell

- leukemia. *Cancer Res.* 2004;64(17):6002-9.
31. Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene *bmi-1* regulates cell proliferation and senescence through the *ink4a* locus. *Nature.* 1999;397(6715):164-8.
32. Scott CL, Gil J, Hernando E, Teruya-Feldstein J, Narita M, Martinez D, et al. Role of the chromobox protein CBX7 in lymphomagenesis. *Proc Natl Acad Sci USA.* 2007;104(13):5389-94.
33. Cha TL, Zhou BP, Xia W, Wu Y, Yang CC, Chen CT, et al. Akt-mediated phosphorylation of EZH2 suppresses methylation of Lysine 27 in histone H3. *Science.* 2005;310(5746):306-10.
34. Varambally S, Cao Q, Mani RS, Shankar S, Wang X, Ateeq B, et al. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. *Science.* 2008;322(5908):1695-6.
35. Sander S, Bullinger L, Klapproth K, Fiedler K, Kestler HA, Barth TF, et al. MYC stimulates EZH2 expression by repression of its negative regulator miR-26a. *Blood.* 2008;112(10):4202-12.
36. Godlewski J, Nowicki MO, Bronisz A, Williams S, Otsuki A, Nuovo G, et al. Targeting of the *Bmi-1* oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal. *Cancer Res.* 2008;68(22):9125-30.
37. Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science.* 2002;298(5595):1039-43.
38. Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, Pirrotta V. *Drosophila* enhancer of Zeste/ESC complexes have a histone H3 methyltransferase

- activity that marks chromosomal Polycomb sites. *Cell*. 2002;111(2):185-96.
39. Cao R, Zhang YI. SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. *Mol Cell*. 2004;15(1):57-67.
40. Yu J, Yu J, Rhodes DR, Tomlins SA, Cao X, Chen G, et al. A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. *Cancer Res*. 2007;67(22):10657-63.
41. Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, Helin K. EZH2 is down stream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. *EMBO J*. 2003;22(20):5323-35.
42. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature*. 2002;419(6907):624-9.
43. Schlesinger Y, Straussman R, Keshet I, Farkash S, Hecht M, Zimmerman J, et al. Polycomb-mediated methylation of Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nat Genet*. 2007;39(2):232-6.
44. Wei Y, Xia W, Zhang Z, Liu J, Wang H, Adsay NV, et al. Loss of trimethylation at lysine 27 of histone H3 is a predictor of poor outcome in breast, ovarian, and pancreatic cancers. *Mol Carcinog*. 2008;47(9):701-6.
45. Sun F, Chan E, Wu Z, Yang X, Marquez VE, Yu Q. Combinatorial pharmacologic approaches target EZH2-mediated gene repression in breast cancer cells. *Mol Cancer Ther*. 2009;8(12):3191-202.
46. Cao P, Deng Z, Wan M, Huang W, Cramer SD, Xu J, et al. MicroRNA-101 negatively regulates Ezh2 and its expression is modulated by androgen receptor and HIF-1alpha/HIF-1beta. *Mol Cancer*. 2010;9:108.

47. Bracken AP, Kleine-Kohlbrecher D, Dietrich N, Pasini D, Gargiulo G, Beekman C, et al. The polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. *Genes Dev.* 2007;21(5):525-30.
48. Morin RD, Johnson NA, Severson TM, Mungall AJ, An J, Goya R, et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. *Nat Genet.* 2010;42(2):181-5.
49. Yamada Y, Tomonaga M, Fukuda H, Hanada S, Utsunomiya A, Tara M, et al. A new G-CSF-supported combination chemotherapy, LSG15, for adult T-cell leukaemia-lymphoma: Japan Clinical Oncology Group Study 9303. *Br J Haematol.* 2001;113(2):375-82.
50. Ellis L, Pan Y, Smyth GK, George DJ, McCormack C, Williams-Truax R, et al. Histone deacetylase inhibitor panobinostat induces clinical responses with associated alterations in gene expression profiles in cutaneous T-cell lymphoma. *Clin Cancer Res.* 2008;14(14):4500-10.
51. Fiskus W, Wang Y, Sreekumar A, Buckley KM, Shi H, Jillella A, et al. Combined epigenetic therapy with the histone methyltransferase EZH2 inhibitor 3-deazaneplanocin A and the histone deacetylase inhibitor panobinostat against human AML cells. *Blood.* 2009;114(13):2733-43.

Table 1. PCR primers and probes for PcG protein genes.

Gene name (Accession no.)	Primer and probe sequence	Product size (bp)
BMI1 (NM_005180)	F 5'-GCCTACATTTATTCCTGGAGAAG-3'	135
	R 5'-CCCAGAGTCACTTTCCAGTT-3'	
	P 5'-FAM-TTGTCAGTCCATCTCTCTGGTGACTGATCT-TAMRA-3'	
YY1 (NM_003403)	F 5'-CAACAAGAAGTGGGAGCAG-3'	143
	R 5'-GAGGTGAGTTCTCTCCAATGAT-3'	
	P 5'-FAM-CTCGGTCACCATGTGGTCTCAGATGA-TAMRA-3'	
RYBP (NM_012234)	F 5'-CTGACATTCTGAAAGATCCTCC-3'	143
	R 5'-AGTTACTGCCAACTGCTGTG-3'	



	P	5'-FAM-TGCAAATGCTACAACAAAGACCAGCGA-TAMRA-3'	
RBBP4 (NM_05610)	F	5'-ATGCCCCAGAACCCTGT-3'	132
	R	5'-ATGTCCACGGAGACGCAA-3'	
	P	5'-FAM-CTCCTTCCAGTGATGTTCTTGTCTTTGACT-TAMRA-3'	
EED (NM_152991)	F	5'-GAATATCCAGACGGACTC-3'	126
	R	5'-AGAGAATGATCCATACCACAG-3'	
	P	5'-FAM-ATAATCAGCACTTAGAACTTCATCTCTGTGCC-TAMRA-3'	
EZH2 (NM_152998)	F	5'-GATGTGGATACTCCTCCAAG-3'	149
	R	5'-GAACTGTCACAAGGCTGC-3'	
	P	5'-FAM-ACGGCTCCTCTAACCATGTTTACAACATCA-TAMRA-3'	
PBGD (NM_000190)	F	5'-AACCAGCTCCCTGCGAAGA-3'	134
	R	5'-CCAGGATGATGGCCTGAAC-3'	
	P	5'-FAM-ACTCCTGAACTCCAGATGCGGGAAC-TAMRA-3'	

F: forward primer, R: reverse primer, P: TaqMan probe

### Figure legends

#### 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). \* $p < 0.05$ , \*\* $p < 0.01$

#### Figure 2. Quantitative real-time RT-PCR for PcG genes. (A-F, a-f)

Expressions of PcG protein genes *EZH2* (A, a), *RYBP* (B, b), *RBBP4* (C, c), *BMI1* (D, d), *YY1* (E, e), and *EED* (F, f) were compared among healthy adults (Control), HTLV-1 carriers (Carrier), ATL patients (Primary ATL), ATL cell lines, and non-ATL T-cell lines. Capital letters (A-F) indicate absolute copy number per 25 ng of total RNA, and small letters (a-f) indicate normalized expression. ATL cells showed significantly higher levels of *EZH2* and *RYBP* transcripts than

the cells from healthy adults and HTLV-1 carriers, in terms of both absolute copy number and normalized expression (A, a, B, b, Mann-Whitney's U test). *RBBP4* transcript was significantly increased in ATL cells only in terms of normalized expression (C, c, Mann-Whitney's U test). There was no difference in *BMI1*, *YY1*, and *EED* expression levels among these groups (D, d, E, e, F, f).  
**\*\* $p < 0.01$**

**Figure 3. 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.

**Figure 4. Immunostaining for EZH2 and H3K27me3 in lymph nodes.** Lymph nodes from patients with lymphoma-type ATL and follicular lymphoma (FL) were stained for EZH2 and H3K27me3. Representative results of 3 ATL lymph nodes and 1 FL lymph node are shown. ATL lymph nodes were all strongly positive for both EZH2 and H3K27me3 without exception in their cell

nuclear staining (brown color). In contrast, FL lymph nodes were sparsely positive for EZH2 and mostly negative for H3K27me3. HE: hematoxylin-eosin stain. EZH2 and H3K27me3: immunostaining, Nikon Eclipse 80i, magnification  $\times 200$ .

**Figure 5. 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). \* $p < 0.05$ , \*\* $p < 0.01$

**Figure 6. Sensitivities of cell lines to DZNep and PS (LBH589).** (A) Sensitivities of cell lines to DZNep were examined after 72 hours of culture. DZNep suppressed the proliferation of all cell lines examined at concentrations above 0.5  $\mu\text{M}$  but showed no effect on normal CD4<sup>+</sup> T cells (control 1-4, dotted lines). (B, C) Effects of DZNep on *EZH2* transcript (B) or *EZH2* protein expression (C) were examined in ATL and HTLV-1-infected cell lines. DZNep was added at final concentrations of 0.5 and 5 nM. DZNep decreased *EZH2*