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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 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.

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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. Satijn 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, Fiegl 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, Hirama 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, Chinniyan 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.

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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-CTCGGTCACCATGTGGTCCTCAGATGA-TAMRA-3'	
RYBP (NM_012234)	F	5'-CTGACATTCTGAAAGATCCTCC-3'	143
	R	5'-AGTTACTGCCAACTGCTGTG-3'	

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	P	5'-FAM-TGCAAATGCTACAACAAAGACCAGCGA-TAMRA-3'	
RBBP4 (NM_05610)	F	5'-ATGCCCCAGAACCCTTGT-3'	132
	R	5'-ATGTCCACGGAGACGCAA-3'	
	P	5'-FAM-CTCCTTCCAGTGATGTTCTTGTCTTTGACT-TAMRA-3'	
EED (NM_152991)	F	5'-GAATATCCAGACGGACACTC-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-ACGGCTCCTCTAACCATGTTTACAACTATCA-TAMRA-3'	
PBGD (NM_000190)	F	5'-AACCAGCTCCCTGCGAAGA-3'	134
	R	5'-CCAGGATGATGGCACTGAACT-3'	
	P	5'-FAM-ACTCCTGAACTCCAGATGCGGGAACT-TAMRA-3'	

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

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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

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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

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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 ×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 μM but showed no effect on normal $CD4^+$ 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*

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transcript in ST1, SO4, and KK1 but increased it in KOB (B), which results were confirmed at protein level (C). (D, E) Effects of PS (LBH589) on *EZH2* transcript (D) or EZH2 protein expression (E) were examined. PS (LBH589) was added at final concentrations of 50 nM and 100 nM for (D) and 20 nM and 100 nM for (E). One hundred nM of PS (LBH589) decreased the expression of EZH2 at both transcript (D) and protein levels (E) after 24 hours of culture. (F) Effects of combined treatment with DZNep and PS (LBH589) on LM-Y1 and KOB cells were analyzed. Cells were treated with DZNep (0.3-5.0 μM) and PS (LBH589) (3-50 nM) for 48 hours. After evaluation of cell proliferation status by a MTS assay (upper panel), the combination index (CI) for each drug combination was obtained using commercially available software Calcusyn (lower panel). CI < 1 indicates synergism.

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Figure 1

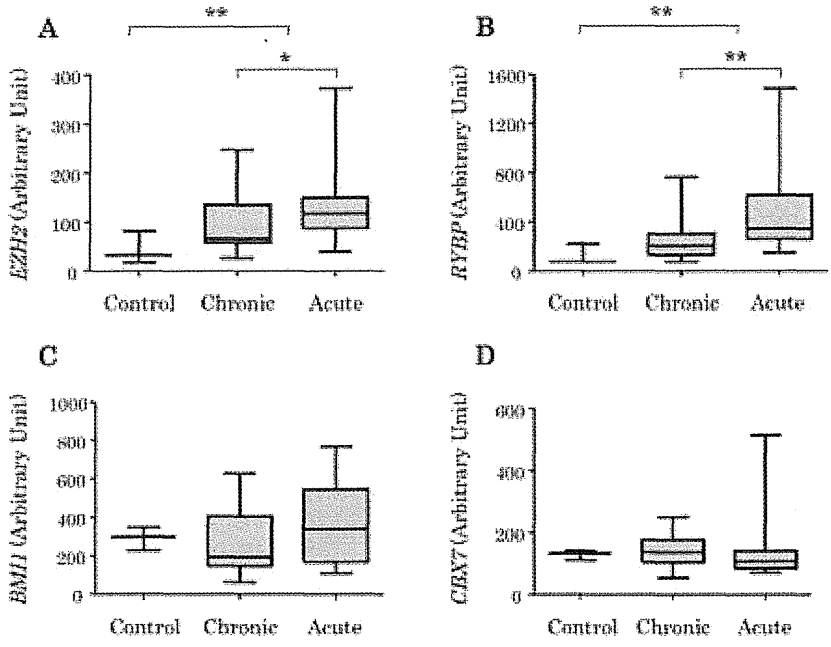

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Figure 1

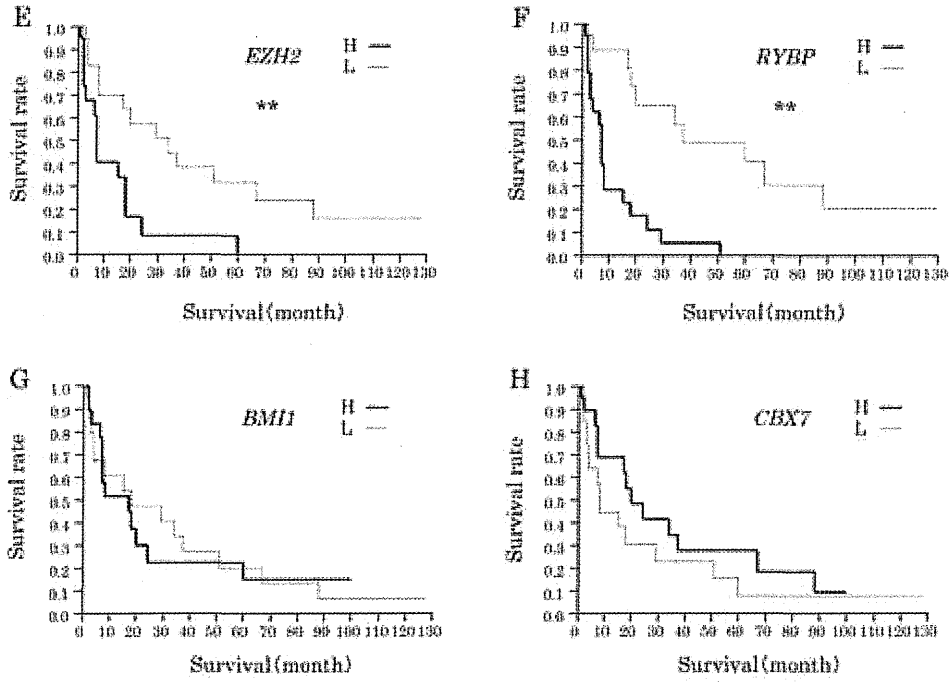

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Figure 2

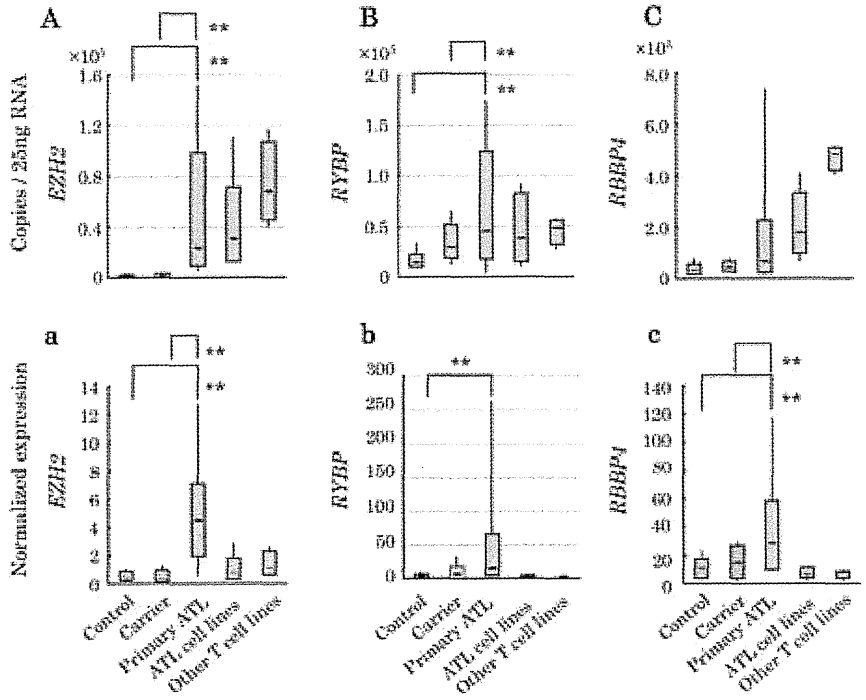

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Figure 2

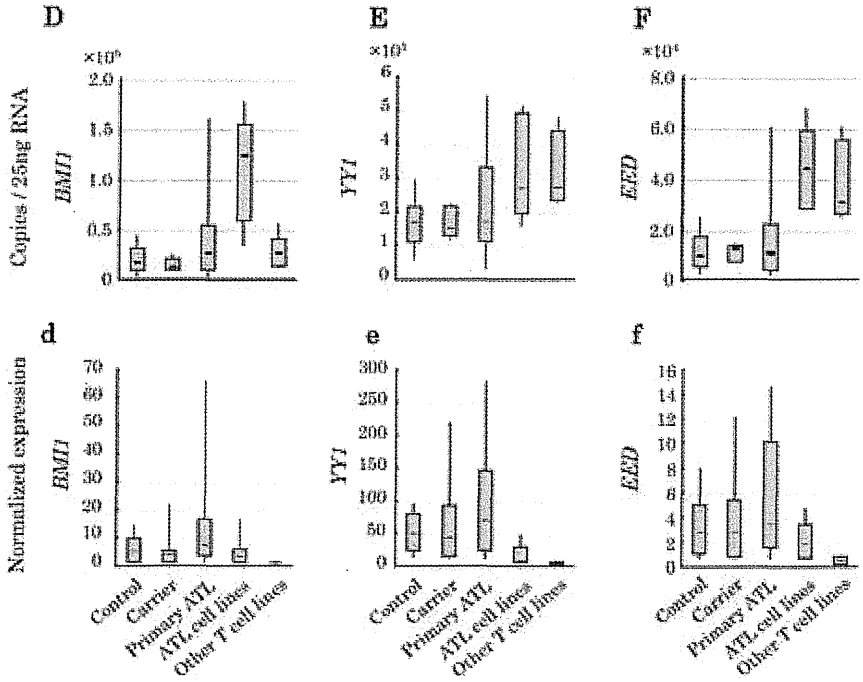

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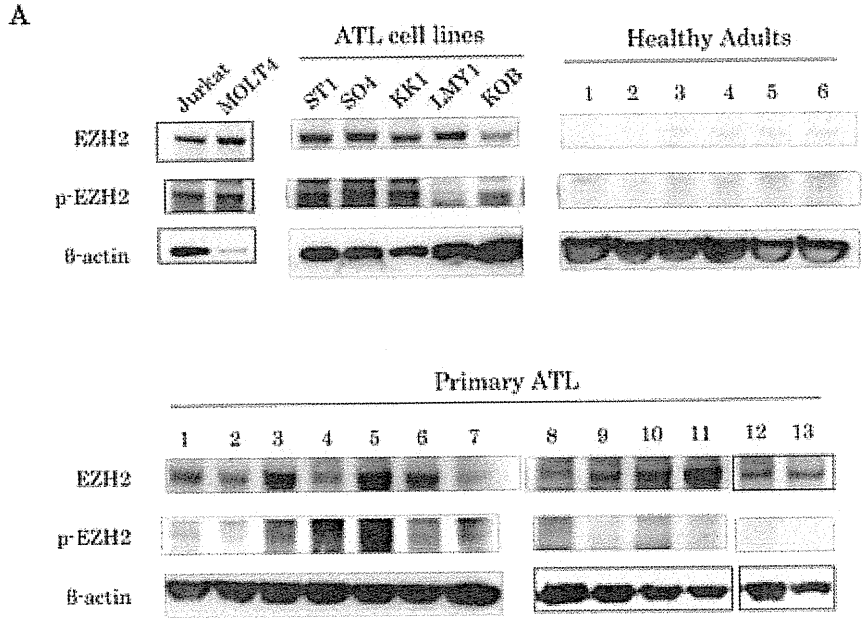
Figure 3



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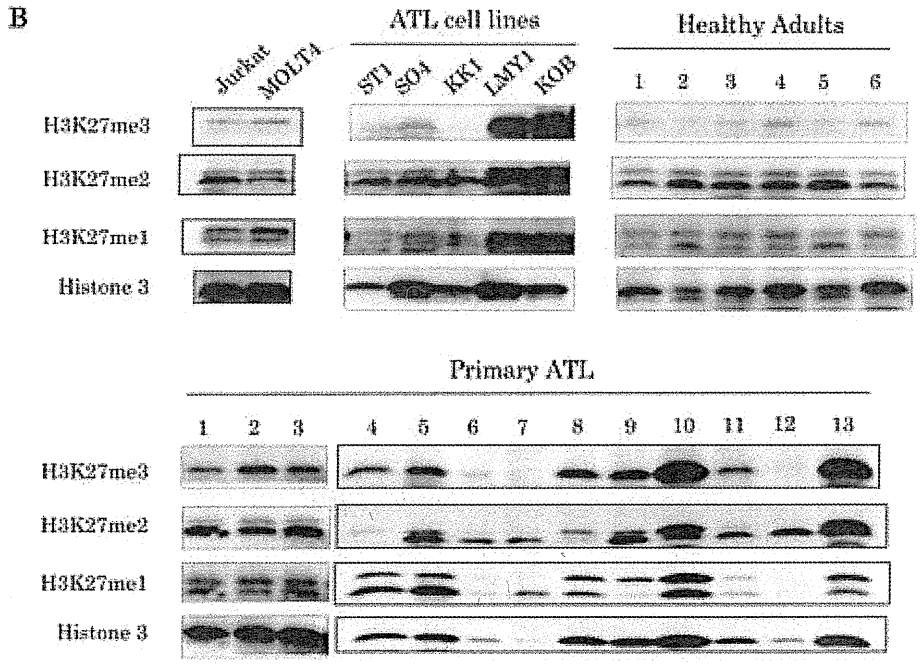
Figure 3