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Promise of Combining a Bcl-2 Family Inhibitor with Bortezomib or SAHA for Adult T-cell Leukemia/Lymphoma

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Abstract. *Background:* Adult T-cell leukemia/lymphoma (ATL) is an aggressive malignancy of peripheral T-lymphocytes and its prognosis still remains very poor. *Materials and Methods:* The potential of combining the Bcl-2 homology 3 mimetic ABT-737, which blocks Bcl-2, Bcl-X_L, and Bcl-w, with either the proteasome inhibitor bortezomib or histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) to inhibit the growth of human T-lymphotropic virus type-I (HTLV-1) infected T-cell lines and its mechanism was further evaluated. *Results:* ABT-737 synergistically induced apoptosis when combined with either bortezomib or SAHA in HTLV-1 infected T-cell lines and fresh ATL cells. Bortezomib increased the expression of Noxa, which subsequently enhanced the formation of Mcl-1-Noxa complexes, resulting in the functional neutralization of Mcl-1, an inducer of resistance to ABT-737. On the other hand, SAHA reduced the expression of survivin, an anti-apoptotic molecule that confers drug resistance on ATL cells. *Conclusion:* The combination of ABT-737 with bortezomib or SAHA is promising for the treatment of ATL.

Adult T-cell leukemia/lymphoma (ATL) is an aggressive malignancy of peripheral T-lymphocytes associated with human T-cell lymphotropic virus type I (HTLV-1) (1, 2). The clinical subtypes of ATL have been divided into acute, lymphoma, chronic and smoldering (1), and a recent retrospective study showed that the median survival times of patients with these subtypes were 8.3, 10.6, 30.2 and 36.7 months and overall survival rates at 4 years were 11.4%, 16.2%, 35.0% and 43.2%, respectively (3). Novel therapeutic options such as allogeneic stem cell transplantation and anti-

CCR4 monoclonal antibody have been recently introduced; however, the outcome of patients with ATL is still very poor and novel therapeutic approaches are urgently required.

Members of the Bcl-2 family proteins are critical regulators of apoptosis and interactions between anti-apoptotic and pro-apoptotic members are the major determinants of cell death and survival. ABT-737 is a small molecule Bcl-2 homology 3 (BH3) mimetic that binds to surface hydrophobic grooves of anti-apoptotic Bcl-2 family members and has been shown to strongly and selectively inhibit Bcl-2, Bcl-X_L and Bcl-w, but not Mcl-1 or A1. In preclinical studies, ABT-737 exhibited single-agent activity and also increased the sensitivities of malignant lymphoma and small cell lung carcinoma cell lines to chemotherapeutics (4, 5). ABT-263 (Navitoclax), an analogue of ABT-737, was also reported to be effective on various cancer-derived cell lines *in vitro*, *in vivo* animal model, and in several clinical trials (6-11). We previously demonstrated that Bcl-2 and Bcl-X_L, as well as the Mcl-1 protein, were highly expressed in ATL cells and HTLV-1 infected T-cell lines. Fresh tumor cells derived from ATL patients were less sensitive to ABT-737 than those derived from chronic lymphocytic leukemia (CLL) patients *in vitro*, however, ABT-737 synergistically enhanced apoptosis induced by current key conventional chemotherapeutics in HTLV-1 infected T-cell lines. Furthermore, the inhibition of Mcl-1 expression significantly enhanced the induction of apoptosis by ABT-737 (12). In addition, the nuclear factor-kappa-light-chain-enhancer of activated B (NF-κB) pathway, which induces anti-apoptotic and survival signals, was also shown to be constitutively activated in ATL cells (13).

The clinical activity of proteasome inhibitors against multiple myeloma has already been established. Proteasome inhibition is known to affect various intracellular signaling pathways, including NF-κB, cell-cycle regulation, modulation of Bcl-2 family members and accumulation of p53 (14). Previous studies have demonstrated that the proteasome inhibitor bortezomib induced cell death in HTLV-1 infected T-cell lines and ATL cells, and its potential activity has also been reported in a xenograft murine model

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Key Words: Adult T-cell leukemia/lymphoma, Bcl-2 family protein, ABT-737, bortezomib, suberoylanilide hydroxamic acid.

(15-18). We are currently conducting a multi-center phase II clinical trial of bortezomib in relapsed and refractory ATL patients (clinical trial registry number; UMIN000004061).

Histone deacetylase inhibitors (HDACi) are novel agents that inhibit the enzymatic activity of HDAC. HDACi can induce the acetylation of histone and non-histone proteins, which have been shown to affect various physiological and pathological processes controlling apoptosis, autophagy, cell cycle, immune response, inflammation and metabolism through its downstream molecular targets (19). HDACi were shown to influence the expression and activity of apoptotic proteins favoring a pro-apoptotic response and lowered the cellular apoptotic threshold (20). HDACi, such as suberoylanilide hydroxamic acid (SAHA), romidepsin and panobinostat, have shown promise in pre-clinical and/or clinical studies against T-cell malignancies including ATL (21, 22). However, the overexpression of anti-apoptotic Bcl-2 or Bcl-X_L has been previously shown to reduce the efficacy of HDACi (23, 24). ABT-737 has been reported to sensitize Bcl-2 overexpressing tumors to HDACi-mediated apoptosis (25). Therefore, we postulated that ABT-737, combined with SAHA, may synergistically induce apoptosis in HTLV-1-infected T-cells.

In the present study, we examined the synergistic effects of the combination of ABT-737 with bortezomib or SAHA and further clarified the molecular sequences of HTLV-1 infected T-cell lines.

Materials and Methods

Cells. Two HTLV-1 infected T-cell lines MT-1 and MT-2 (kindly provided by Dr. Miyoshi I. (Kochi University, Nangoku, Japan)) were used in this study. MT-1 cells were established from the peripheral blood (PB) tumor cells of ATL patients (26), while MT-2 cells were established from cord blood T-cells by the co-cultivation of normal human cord lymphocytes and PB tumor cells of an ATL patient (27). Fresh PB ATL cells were separated by density sedimentation using LymphoPrep (PROGEN Biotechnik, Heidelberg Germany) from heparinized PB obtained from two acute-type ATL patients whose number of ATL cells comprised more than 85% of mononuclear cells, after obtaining informed consent. Cells were cultured at 37°C in Roswell Park Memorial Institute (RPMI) 1640 containing 15% fetal bovine serum (Sigma, St Louis, MO, USA), 2 µM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Grand Island, NY, USA).

Reagents. An inhibitor of Bcl-2 family proteins, ABT-737 was provided by Abbott Laboratories (Abbott Park, IL, USA). The proteasome inhibitor bortezomib, HDACi SAHA and survivin inhibitor YM-155 were obtained from Toronto Research Chemicals Inc. (Ontario, Canada), Cayman Chemical (Michigan, IL, USA) and Selleck Chemicals (Houston, TX, USA), respectively. The pan-caspase inhibitor z-VAD-fmk (Z-VAD) was obtained from Bachem (Bubendorf, Switzerland). The Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) was used to assess cellular proliferation by a colorimetric assay.

Detection of apoptosis. APO 2.7 staining (Immunotech, Marseille, France) was used to determine apoptosis and was evaluated using an EPICS XL flow cytometer (Beckman Coulter, Hialeah, FL, USA).

Western blotting. Western blotting was performed as previously described (28) with the following antibodies: anti-caspase 3, -caspase 9, -PARP, -Bcl-2, -Bcl-X_L, -Bcl-w and -survivin (Cell Signaling, Danvers, MA, USA), and -Noxa and -Mcl-1 (Santa Cruz Biotechnology city, CA, USA). Immunoblotting with anti-α-Tubulin (Cell Signaling) confirmed equivalent protein loading.

Immunoprecipitation. Cells cultured under the indicated conditions were harvested, washed twice with ice-cold PBS, lysed in radioimmunoprecipitation assay (RIPA) buffer (Wako Pure Chemical Industries, Osaka, Japan), incubated with 2 µg anti-Mcl-1 antibody and 15 µl protein G-Sepharose was added. The immunocomplexes were collected and detected by Western blotting.

Analysis of drug synergy. The effects of combining ABT-737 with either bortezomib or SAHA were evaluated using the Chou-Talalay method to determine the combination index using the CalcuSyn software (Biosoft, Ferguson, MO, USA). Each affected fraction (Fa) was calculated by comparing the absorbance values of drug-treated wells measured by a colorimetric assay, to the absorbance of control wells. The drug concentration that induced Fa=0.25 signified a 75% decrease in absorbance and growth (*i.e.*, IC₂₅ concentration). Background absorbance was set at Fa=1. Based on this approach, combination index (CI) values of <0.9 were considered synergistic, >1.1 were antagonistic and values of 0.9 to 1.1 were additive (29, 30).

Results

Either bortezomib or SAHA synergistically enhanced the effects of ABT-737. We first examined the single-agent activity of ABT-737, bortezomib or SAHA on HTLV-1 infected T-cell lines by the colorimetric assay and showed that they inhibited the growth of MT-1 and MT-2 cells (Figure 1A). We then investigated the effects of combining ABT-737 with either bortezomib or SAHA. Dose-effect and Fa-CI plots revealing the effects of fixed drug ratio combinations are shown in Figure 1B. The combination of ABT-737 with bortezomib or SAHA displayed a strong synergism (CI<0.9) for inhibiting the growth of MT-1 and MT-2 cells.

To clarify the *in vitro* anti-tumor effects achieved by these combinations, we examined the induction of apoptosis in MT-1, MT-2 cells and fresh ATL cells, treated by ABT-737 with or without bortezomib or SAHA. Apoptosis induced by ABT-737 was significantly enhanced by either bortezomib or SAHA. Of note, it was accompanied by the cleavage of caspase 3, caspase 9 and PARP, and was blocked by the pan-caspase inhibitor Z-VAD in MT-1 and MT-2 cells (Figure 2A and B).

Bortezomib induced the expression of Noxa. To explore the mechanism of the synergistic effects of ABT-737 and

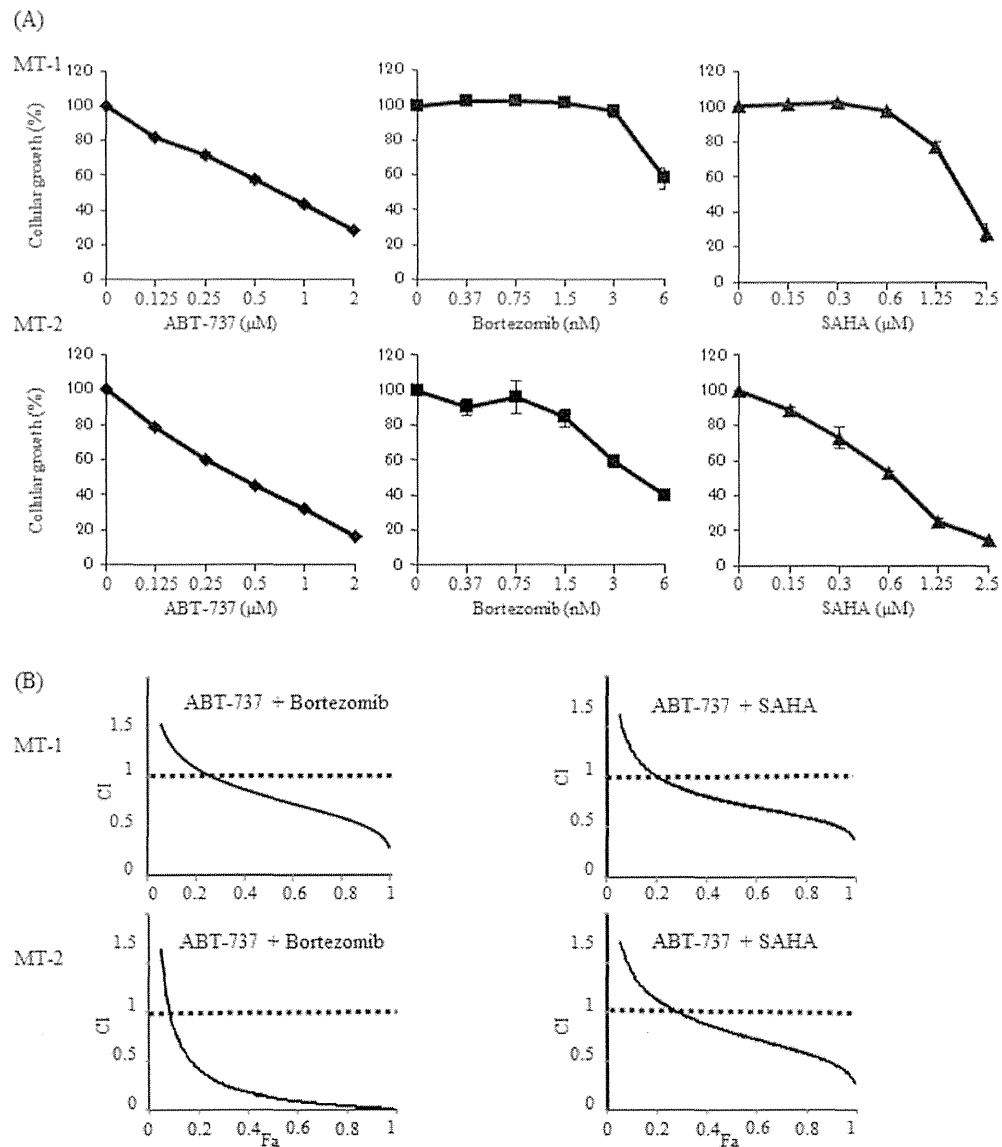


Figure 1. Combination of ABT-737 with bortezomib or SAHA synergistically inhibited the growth of HTLV-1-infected T-cell lines. (A) The growth inhibition of MT-1 and MT-2 cells by ABT-737, bortezomib, and SAHA assessed by a colorimetric assay after a 72-h culture. Data represent means \pm SD (standard deviation) of 3 independent experiments. (B) MT-1 and MT-2 cells were treated with ABT-737 in combination with either bortezomib or SAHA for 72 h and the effects of the combined treatments were evaluated using the CalcuSyn software. Dose-effect and Fa-CI plots, illustrating the effects of fixed drug ratio combinations, are depicted. CI values <0.9 were considered synergistic, >1.1 were antagonistic and values of 0.9 to 1.1 were additive.

bortezomib, we examined the modulation of Bcl-2 family proteins in MT-1 and MT-2 cells. Bortezomib induced the expression of Noxa and Mcl-1 without modulating the expression of Bcl-2, Bcl-w or Bcl-X_L (Figure 3A). Immunoprecipitation using the anti-Mcl-1 antibody revealed the enhanced formation of the Mcl-1-Noxa complex in both cell lines treated with bortezomib-alone and ABT-737 combined with bortezomib (Figure 3B).

SAHA reduced the expression of survivin. We next elucidated the mechanism of synergy between ABT-737 and SAHA. No significant change was observed in the expression of Bcl-2, Bcl-w or Bcl-X_L. SAHA alone and ABT-737 combined with SAHA slightly decreased the expression of Mcl-1. The expression of survivin was reduced by SAHA and was significantly reduced by combining SAHA with ABT-737 (Figure 4). To confirm the

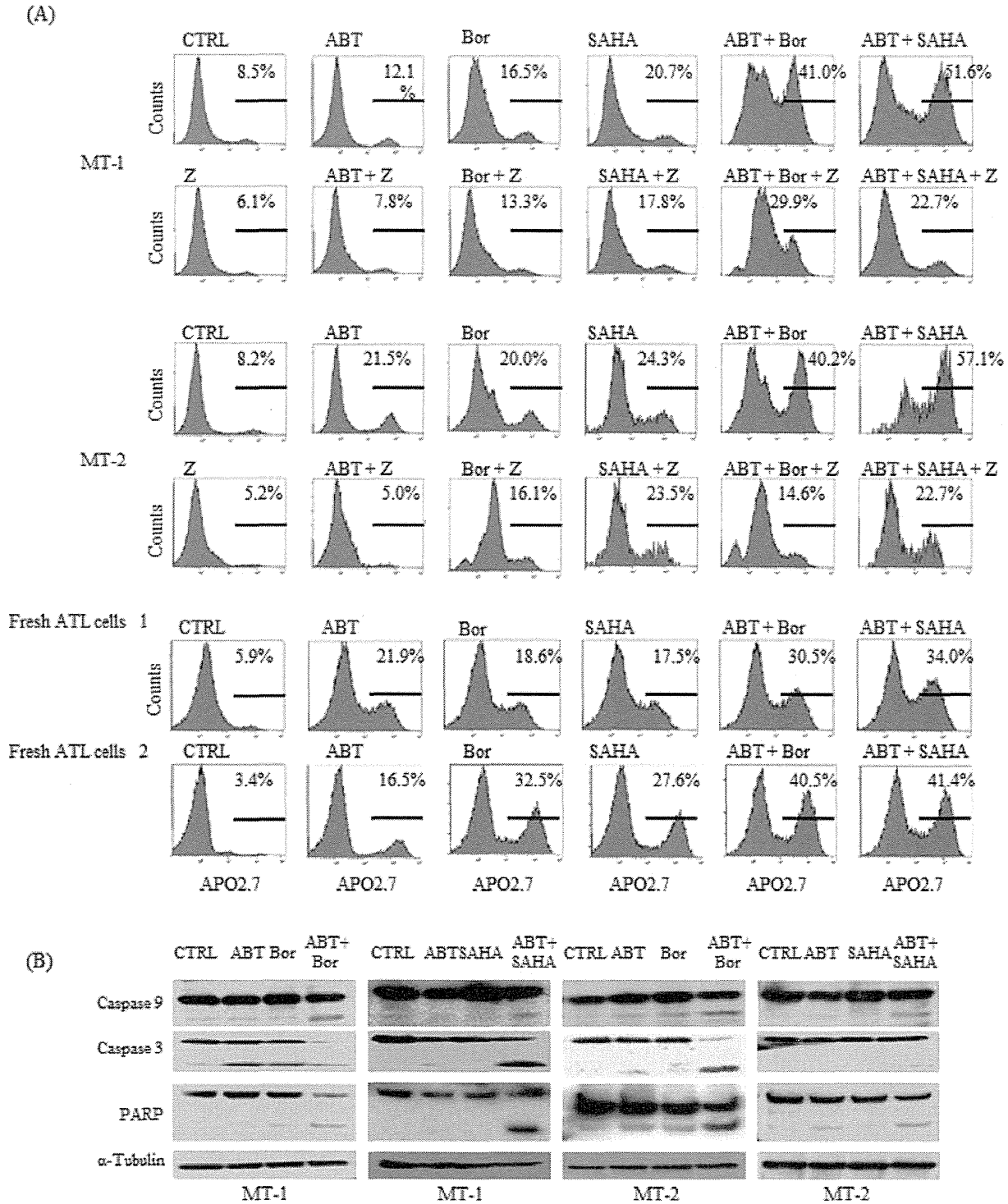


Figure 2. Combination of ABT-737 and bortezomib or SAHA synergistically induced apoptosis in HTLV-1-infected T-cell lines and fresh ATL cells. (A) MT-1 and MT-2 cells were treated by ABT-737 (1 μ M), bortezomib (4 nM), SAHA (2 μ M) or by their combination in the absence or presence of Z-VAD (25 μ M) for 72 h. Fresh ATL cells were treated by ABT-737 (1 μ M), bortezomib (4 nM), SAHA (1 μ M) or by their combination for 72 h. The induction of apoptosis was assessed by using the APO2.7 assay. The percentage of APO2.7-positive cells is shown. Data are representative of two independent experiments. CTRL, Control; ABT, ABT-737; Bor, bortezomib; Z, Z-VAD. (B) Cells were treated with ABT (1 μ M), Bor (4 nM), SAHA (2 μ M) or their combination for 24 h. Whole-cell lysates were subjected to western blotting to assess the cleavage of caspase 9, caspase 3 and PARP. CTRL, Control; ABT, ABT-737; Bor, bortezomib.

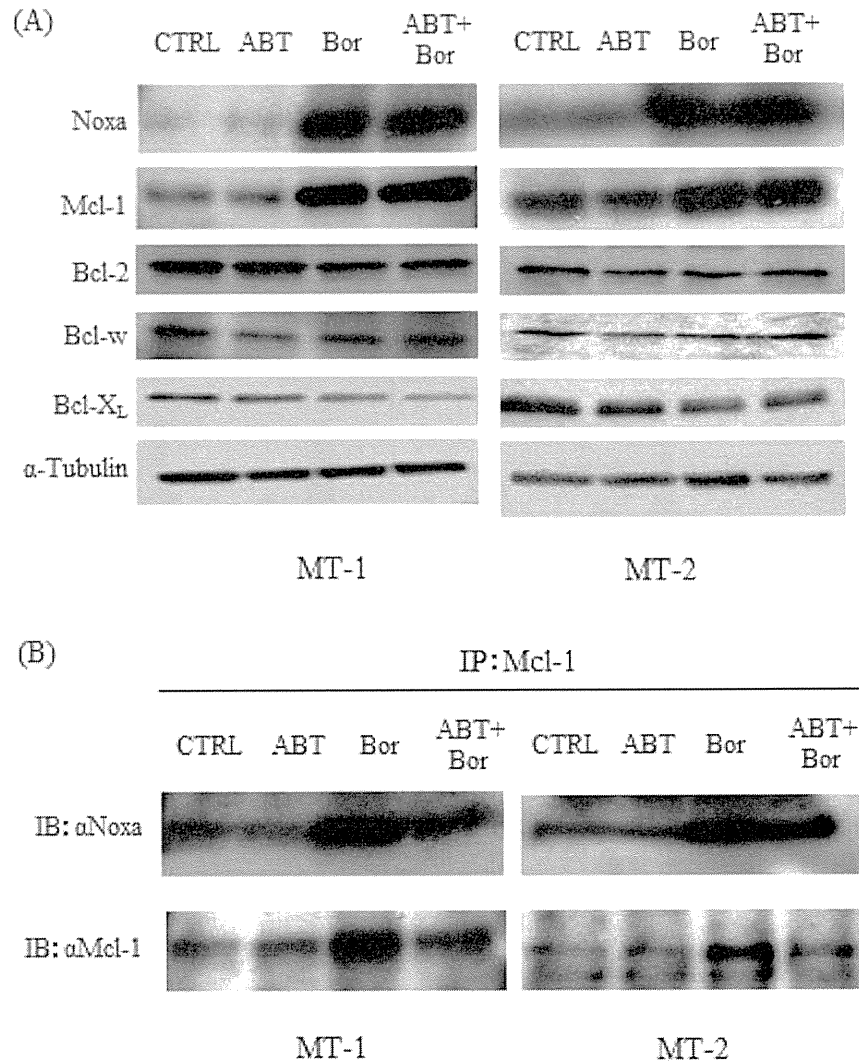


Figure 3. The effects of the combination treatment with ABT-737 and bortezomib on NOXA and Mcl-1 protein expression. (A) Cells were treated with ABT (1 μ M), Bor (4 nM) or combination of both for 24 h. Whole-cell lysates were subjected to western blotting to assess Noxa, Mcl-1, Bcl-2, Bcl-w and Bcl-X_L. CTRL, control; ABT, ABT-737; Bor, bortezomib. (B) Cells were treated with ABT (1 μ M), Bor (4 nM) or combination of both for 24 h. Whole-cell lysates were immunoprecipitated with the anti-Mcl-1 antibody. Immunoprecipitates were then detected by western blotting using the anti-Noxa or anti-Mcl-1 antibody. CTRL, control; ABT, ABT-737; Bor, bortezomib.

biological relevance of survivin in ABT-737-induced apoptosis, we demonstrated that the small-molecule inhibitor of survivin YM-155 significantly enhanced apoptosis induced by ABT-737 (Figure 5).

Discussion

In the present study, we investigated the synergistic anti-ATL effects by combining ABT-737 and a proteasome inhibitor, bortezomib, or an HDACi, SAHA, in HTLV-1 infected T-cell lines and elucidated their mechanism of action.

ABT-737 in combination with bortezomib induced a synergistic apoptotic response in HTLV-1 infected T-cell lines and fresh ATL cells. Mcl-1 cleavage by bortezomib has been reported in multiple myeloma cells (31). The cleavage of Mcl-1 was not observed in our study; however, bortezomib up-regulated the expression of Noxa, resulting in the enhanced formation of Mcl-1-Noxa complexes. Because the BH3 domain of Noxa has been shown to antagonize Mcl-1, the up-regulation of Noxa resulted in the functional repression of Mcl-1 (32). Thus, the mechanism that enhances the activity of ABT-737 by bortezomib is suggested to cancel the anti-apoptotic effect of Mcl-1.

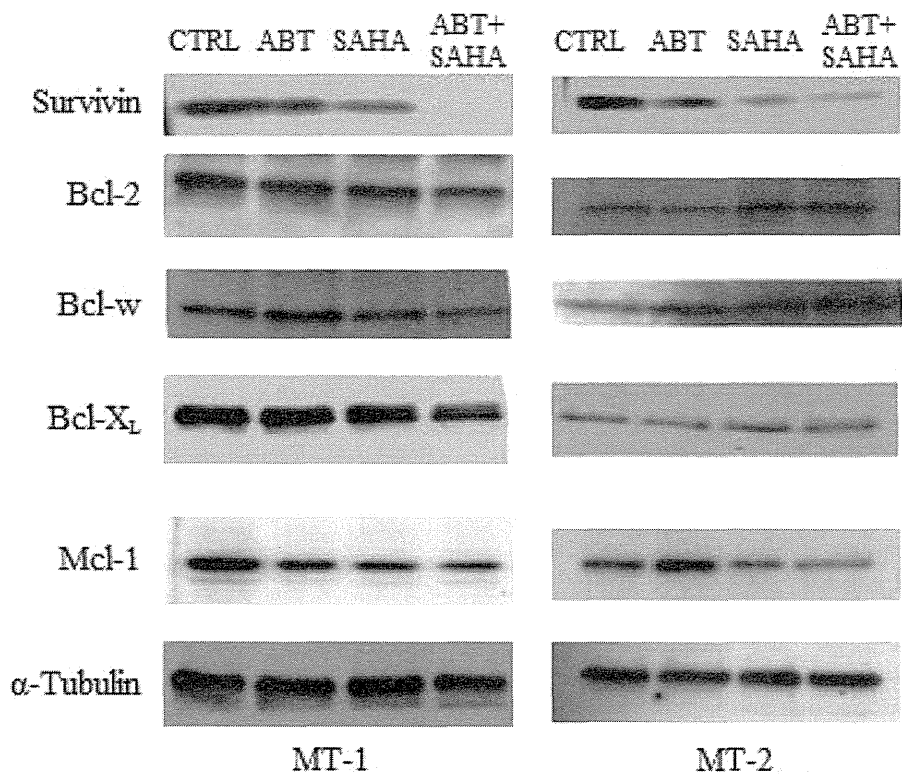


Figure 4. The effect of combination of ABT-737 and SAHA on survivin levels. Cells were treated with ABT (1 μ M), SAHA (2 μ M) or their combination for 24 h. Whole-cell lysates were subjected to Western blotting to assess survivin, Bcl-2, Bcl-w, Bcl-X_L and Mcl-1. CTRL, control; ABT, ABT-737= Bor, bortezomib.

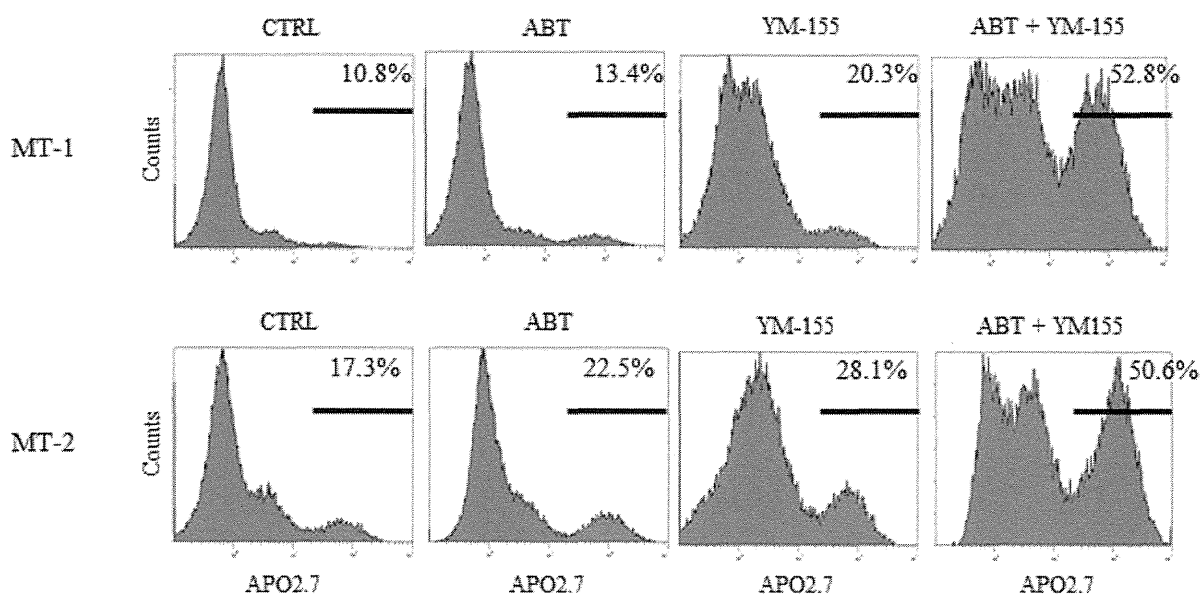


Figure 5. Inhibition of survivin sensitizes HTLV-1-infected T-cell lines to ABT-737. Cells were treated with ABT (1 μ M), YM-155 (10 nM) or their combination for 72 h and induction of apoptosis was assessed using an APO2.7 assay. The percentage of APO2.7-positive cells is shown. CTRL, Control; ABT, ABT-737.

The combination of ABT-737 and SAHA also synergistically enhanced the induction of apoptosis in HTLV-1-infected T-cells by down-regulating the expression of survivin. Caspases are regulated by several intrinsic inhibitors of apoptosis proteins, in which survivin is a major one. Previous studies have shown that survivin induced resistance to ABT-737 (33, 34). In ATL, survivin has been suggested to be an important anti-apoptotic molecule that confers drug resistance, and high mRNA expression of survivin was shown to be a risk factor for the prognosis (35, 36). We also demonstrated that the inhibition of survivin by YM-155 sensitized MT-1 and MT-2 cells for induction of apoptosis by ABT-737. Our results suggest that SAHA increases sensitivity to ABT-737 by down-regulating the expression of survivin. The down-regulation of Mcl-1 was less significant than that of survivin, but may also contribute to the induction of apoptosis. Consequently, the combination of ABT-737 and SAHA is a promising treatment that targets multiple anti-apoptotic molecules such as Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and survivin in HTLV-1 infected T-cells.

ABT-737 is a known inhibitor of some anti-apoptotic proteins. Bortezomib targets the proteasome/ubiquitin pathway, which is active on apoptosis pathway regulators, while SAHA targets histone and non-histone deacetylases and indirectly modulates apoptosis. Numerous regulators are known to be involved in apoptotic pathways; therefore, inhibiting a single pathway is not sufficient. ABT-737, bortezomib and SAHA by themselves exhibited limited activity against HTLV-1 infected T-cell lines. However, ABT-737 combined with bortezomib or SAHA synergistically enhanced the induction of apoptosis because they could compensate for the functional deficits of each other. Taken together, these novel strategies targeting multiple apoptotic pathways appear promising for ATL but should be validated by *in vivo* animal model and clinical studies.

Conflicts of Interest

The Authors declare no conflicts of interest.

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Epigenetic deregulation of *Ellis Van Creveld* confers robust Hedgehog signaling in adult T-cell leukemia

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Key words

ATL, epigenetics, EVC, Hedgehog, HTLV-1

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One of the hallmarks of cancer, global gene expression alteration, is closely associated with the development and malignant characteristics associated with adult T-cell leukemia (ATL) as well as other cancers. Here, we show that aberrant overexpression of the *Ellis Van Creveld* (EVC) family is responsible for cellular Hedgehog (HH) activation, which provides the pro-survival ability of ATL cells. Using microarray, quantitative RT-PCR and immunohistochemistry we have demonstrated that EVC is significantly upregulated in ATL and human T-cell leukemia virus type I (HTLV-1)-infected cells. Epigenetic marks, including histone H3 acetylation and Lys4 trimethylation, are specifically accumulated at the *EVC* locus in ATL samples. The HTLV-1 Tax participates in the coordination of EVC expression in an epigenetic fashion. The treatment of shRNA targeting EVC, as well as the transcription factors for HH signaling, diminishes the HH activation and leads to apoptotic death in ATL cell lines. We also showed that a HH signaling inhibitor, GANT61, induces strong apoptosis in the established ATL cell lines and patient-derived primary ATL cells. Therefore, our data indicate that HH activation is involved in the regulation of leukemic cell survival. The epigenetically deregulated EVC appears to play an important role for HH activation. The possible use of EVC as a specific cell marker and a novel drug target for HTLV-1-infected T-cells is implicated by these findings. The HH inhibitors are suggested as drug candidates for ATL therapy. Our findings also suggest chromatin rearrangement associated with active histone markers in ATL.

Adult T-cell leukemia (ATL) is a malignant T-cell disorder caused by infection with a human retrovirus, human T-cell leukemia virus type I (HTLV-1).^(1–3) The prognosis of aggressive types of ATL is poor.⁽⁴⁾ At present, ATL is an intractable disease in human beings. To prevent the development of ATL and the poor prognosis that is associated with it, the development of effective therapies based on the molecular characteristics is needed.

To explore effective drugs, precise understanding of the molecular mechanism of ATL pathogenesis is essential. We have previously reported that genetic and epigenetic imbalances and following aberrant gene expressions are the main framework for ATL tumor cells.^(5,6) In addition, the involvement of systemic downregulation of cellular microRNA has been implicated in the leukemogenesis of ATL. So far, several host cellular signaling abnormalities induced by HTLV-1 Tax in the early phase of infection^(6–8) and the aberrant activation of nuclear factor-kappa B (NF-κB) contribute to ATL leukemogenesis.^(9,10) Although other several molecular deregulations have been suggested in ATL, we have not completely covered the landscape of signaling networks in ATL.

Recently, Hedgehog (HH) signaling has been reported as an oncogenic pathway in many types of cancers.^(11,12) Constitutive HH activation leads to the overproliferation or survival of

several cancer cells, such as basal cell carcinoma or B-cell lymphomas.^(13–15) There are some HH inhibitors under clinical trial as drug candidates against those cancers.⁽¹⁶⁾

In the present study, using ATL patient samples and some ATL models, we found two specific gene overproductions in ATL, *Ellis Van Creveld syndrome 1* (*EVC1*) and *EVC2*, which belong to the EVC family of genes that are implicated in HH regulation.^(17–19) We demonstrated that epigenetically upregulated EVC was associated with cellular HH activity. EVC and other regulatory factors for HH signaling were responsible for the survival of ATL cell lines and also primary ATL samples. Direct evidence from the ATL samples revealed that universal epigenetic marks associated with actively transcribed genes were rearranged in the leukemic cells. These findings may shed light on the abnormal gene expression signature and leukemic cell traits observed in ATL.

Materials and Methods

Patient samples. The primary peripheral blood mononuclear cells (PBMC) from ATL patients and healthy volunteers were a part of those collected with informed consent as a collaborative project of the Joint Study on Prognostic Factors of ATL Development (JSPFAD). The project was approved by the University

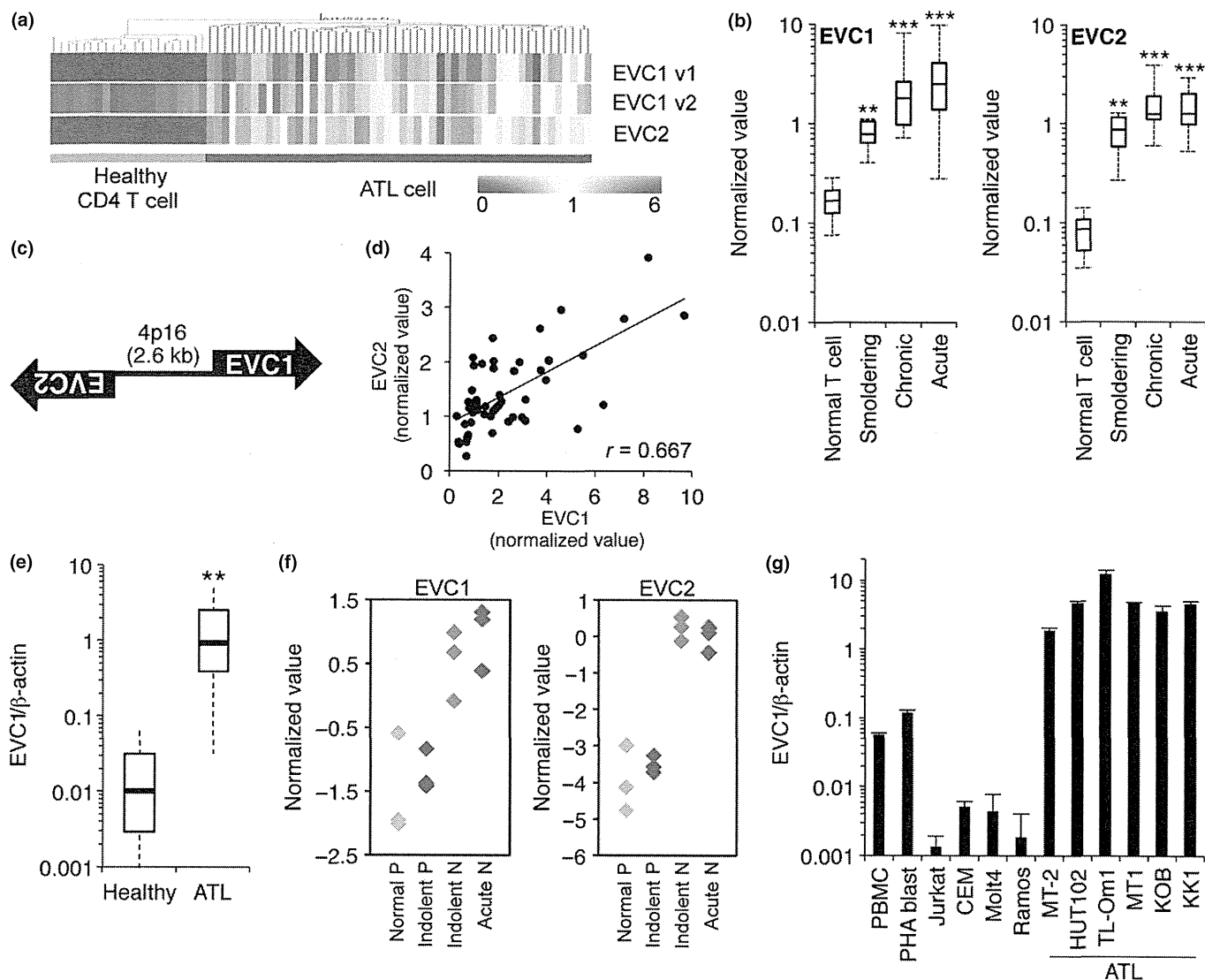


Fig. 1. *EVC* overexpression in ATL. (a, b) Microarray heatmap (a) and box plot (b) of *EVC*. $**P < 0.01$. $***P < 0.001$. (c) Schematic illustration of locus encoding *EVC1/2*. (d) Individual expression values ($n = 52$) between *EVC1* and *EVC2*. (e) *EVC1* mRNA level in ATL patient PBMC (total, $n = 11$; acute, $n = 7$; chronic, $n = 4$) and in CD4⁺ T cells from healthy donors ($n = 6$) evaluated using quantitative RT-PCR (qRT-PCR). $**P < 0.01$. (f) *EVC1* and *EVC2* levels in CADM1 versus CD7 plot subpopulations. Normal P, CD4⁺/CADM1⁻/CD7⁺ T cells from healthy donors; Indolent P, CD4⁺/CADM1⁻/CD7⁺ from indolent ATL patients; Indolent N, CD4⁺/CADM1⁺/CD7⁻ from indolent ATL patients; Acute N, CD4⁺/CADM1⁺/CD7⁻ from acute ATL patients. The gene expression microarray dataset is available in Kobayashi et al.⁽²⁵⁾ (g) *EVC1* levels in various cell lines examined using qRT-PCR ($n = 3$, mean \pm SD).

of Tokyo and Showa University research ethics committees. The PBMC were isolated using Ficoll separation and maintained in RPMI1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 1% of self-serum and antibiotics (Invitrogen). Clinical information is shown in the Supporting Information Methods.

Microarray analysis. Gene expression profiling of ATL patient samples and normal CD4⁺ T cells has been performed previously.⁽⁵⁾ The coordinate has been deposited in the Gene Expression Omnibus database (GSE33615).

Cell culture. The HTLV-1-infected cell lines MT-2 and HUT102, ATL-derived cells MT-1 and TL-Om1, and other leukemic cell lines were cultured in RPMI1640 with 10% FCS. ATL-derived KOB and KK1 were cultured in RPMI1640 with 10% FCS and 10 ng/mL recombinant human IL-2 (R&D Systems, Minneapolis, MN, USA). The 293T cell was cultured in DMEM with 10% FCS. All cell lines were cultured at 37°C, with 5% CO₂.

Plasmids and HH activity analysis. Tax-encoding plasmids have been described previously.⁽²⁰⁾ *EVC1* cDNA was amplified as two fragments from the human cDNA library. Cellular HH activity was evaluated using a dual-luciferase assay (Promega, Madison, WI, USA).⁽²¹⁾ Briefly, 7 × GLI binding site (GAACACCCA)-luciferase plasmid and control RSV-Renilla plasmid were co-transfected into target cells using Lipofectamine2000 (Invitrogen). At 24 h post-transfection, the cells were collected and analyzed using a dual-luciferase assay.

Quantitative RT-PCR. Procedures for RNA isolation and RT-PCR have been described previously.⁽⁵⁾ Primer sets for quantitative RT-PCR (qRT-PCR) are provided in the Supporting Information Methods.

Epigenetic analyses. Bisulfite treatment was conducted using a MethylEasy Xceed Rapid DNA Bisulphite Modification kit (Human Genetic Signatures, NSW, Australia). For evaluating histone covalent modifications, a chromatin immunoprecipita-

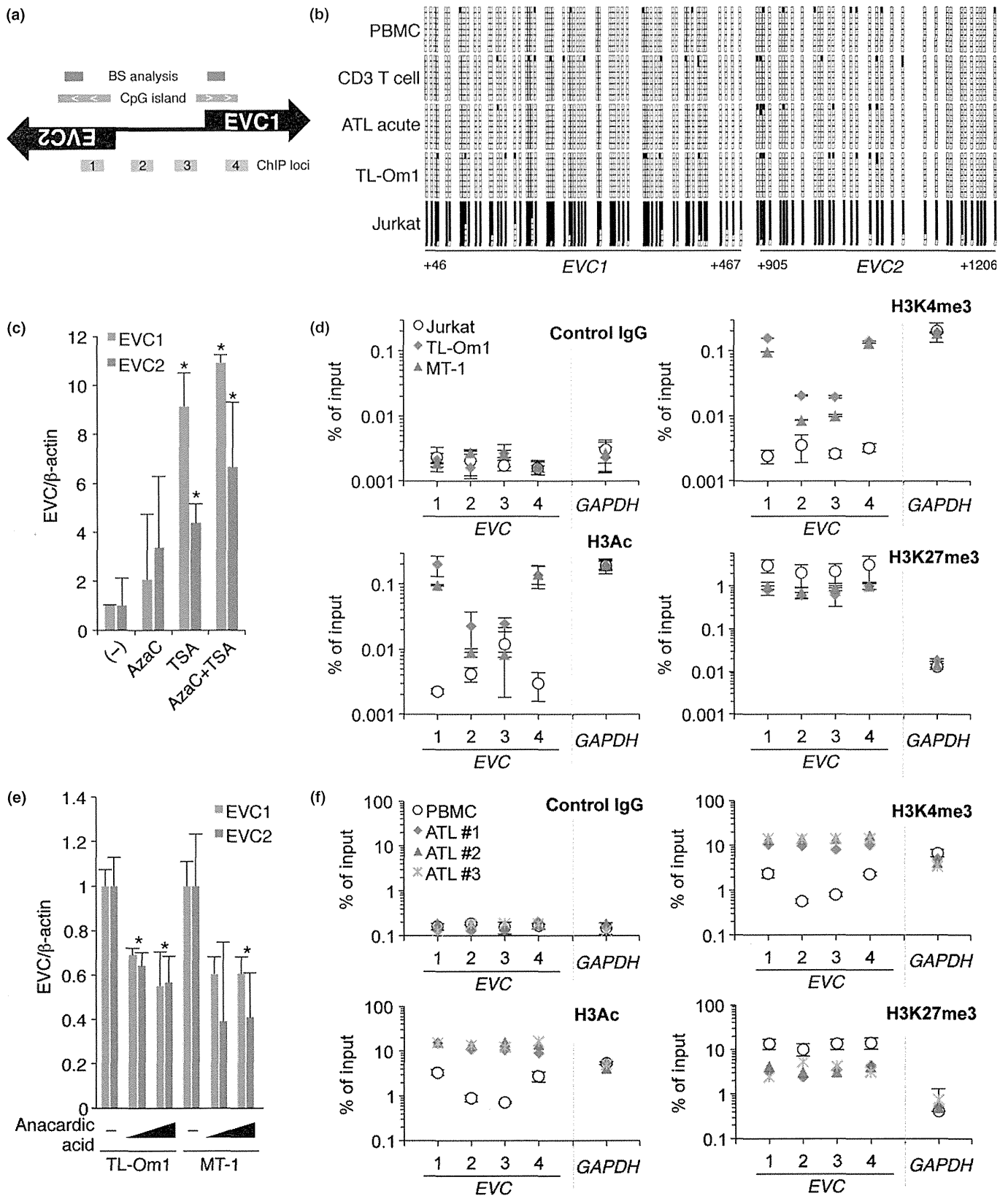


Fig. 2. Epigenetic reprogramming in the *EVC* locus. (a) Schematic of CpG islands and chromatin immunoprecipitation (ChIP) loci. (b) Results of bisulfite sequencing (+46 to +466 from *EVC1* transcription start site [TSS]; +905 to +1206 from *EVC2* TSS). The black and empty boxes represent methylated and unmethylated CpG, respectively. (c) *EVC* RNA levels in Jurkat cells in the presence or absence of epigenetic drugs ($n = 3$, mean \pm SD). * $P < 0.05$. (d) Histone covalent modifications at *EVC* and *GAPDH* loci in three cell lines were analyzed using PCR-based ChIP assay with specific antibodies. Positions of primer sets for the real-time PCR are indicated in (a). Enrichment values relative to input samples are plotted. (e) TL-Om1 and MT-1 cells were treated with 1 or 5 μ M of anacardic acid for 48 h and the *EVC* mRNA level was then analyzed ($n = 3$, mean \pm SD). * $P < 0.05$. (f) Epigenetic changes in primary ATL samples. Three independent clinical samples were compared with normal PBMC ($n = 3$, mean \pm SD).

tion (ChIP) assay was conducted as described previously.^(5,22) Anti-H3K4me3 (#9751S; Cell Signaling, Danvers, MA, USA), anti-AcH3 (#06-599; Millipore, Billerica, MA, USA), anti-H3K27me3 (#39155; Active Motif, Carlsbad, CA, USA) and control IgG (I5381; SIGMA, St. Louis, MO, USA) were used for ChIP. Primers for the qPCR are provided in the Supporting Information Methods.

Immunohistochemistry. For preparation of the paraffin block of 293T cells, the cells were fixed in 20% of formalin/PBS for 24 h. After removing the formalin, alcohol dehydration and paraffin permeation were done using Tissue-Tek VIP5Jr (Sakura, Alphen aan den Rijn, The Netherlands). Paraffin blocks were sectioned at 3- μ m thickness. The sections were then transferred to coating slide glasses (Muto pure chemicals, Bunkyo-ku, Tokyo, Japan). After paraffin removal, the paraffin sections of the 293T and ATL

cells were treated with 3% H₂O₂. Antigen-retrieval treatment was done using Histofine antigen retrieval solution pH9 (Nichirei, Chuo-Ku, Tokyo, Japan) for 20 min under microwave radiation. After reaction with the first antibody, anti-EVC antibody (HPA008703, 1:400; SIGMA), and the second antibody (K5027, ENVISION Kit/HRP [DAB]; Dako, Bunkyo-ku, Tokyo, Japan), the sections were colored using ENVISION Kit/HRP [DAB] DAB+ (K3468; DAKO). Finally, the sections were stained with hematoxylin.

Lentivirus construction and production. Detailed procedures for lentivirus production have been described previously.⁽⁵⁾ Briefly, replication-defective, self-inactivating lentivirus vectors were used.^(23,24) shRNA were cloned into a CS-H1-EVBs. High-titer viral solutions prepared using a centrifugation-based concentration were transduced into ATL cell lines using the spinoculation method. The transduced cells were

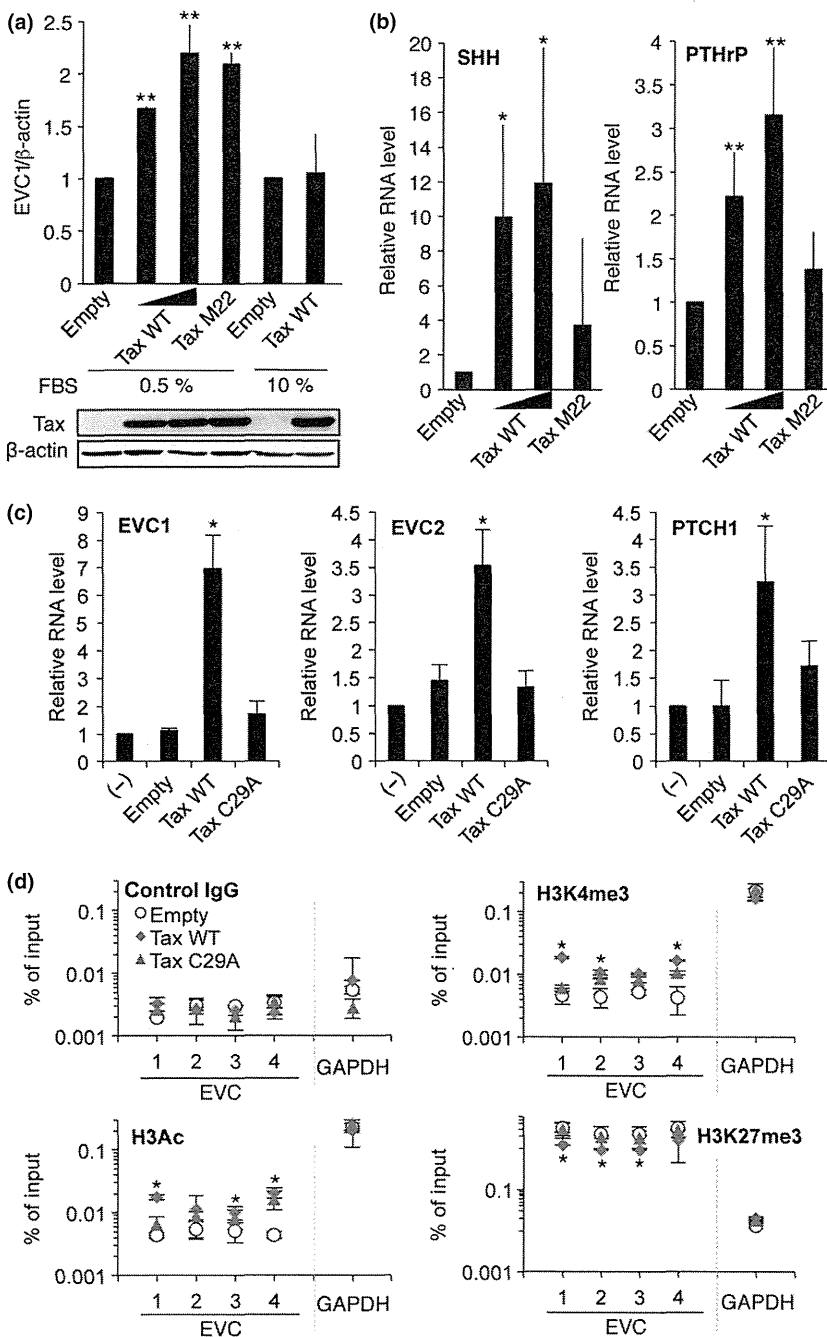


Fig. 3. Role of Tax in EVC regulation. (a) *EVC1* RNA levels are affected by Tax. The 293T cells in different FBS condition were transfected with the indicated plasmids. Relative *EVC1* levels were evaluated using quantitative RT-PCR (qRT-PCR) (top panel, $n = 3$, mean \pm SD). ** $P < 0.01$. Tax expression was confirmed using western blotting with an anti-Tax antibody (Lt-4) (bottom panel). (b) Levels of *SHH* and *PTHrP* in the presence or absence of Tax ($n = 3$, mean \pm SD). * $P < 0.05$. ** $P < 0.01$. (c) *EVC* and *PTCH1* levels in Jurkat cells expressing Tax ($n = 3$, mean \pm SD). * $P < 0.05$. (d) Tax-mediated epigenetic changes. Histone modifications at *EVC* and *GAPDH* loci in Tax-expressing Jurkat cells were analyzed using a ChIP assay. * $P < 0.05$ (Tax WT vs Empty). Primer positions are shown in Figure 2(a).

further selected by blastcidin and used within 14 days. shRNA sequences are described in the Supporting Information Methods.

Cell viability and apoptosis analyses. For the cell proliferation assay, 5000 cells were plated in a 96-well flat bottom plate with RPMI1640 medium supplemented with 1% FCS. After 1–3 days culture, cell numbers were evaluated using Cell Counting kit-8 (Dojindo, Kumamoto, Japan). The apoptosis cell was determined using PE Annexin V/7-AAD stainings (BD Pharmingen, San Jose, CA, USA). Detection of apoptotic cells was performed using FACSCalibur (Becton, Dickinson, Franklin Lakes, NJ, USA). Primary ATL cells were defined using sequential gating based on a Forward scatter/Side Scatter (FSC/SSC) pattern and a CD4-positive population (anti-CD4-FITC; BD Pharmingen). Collected data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Results

Epigenetic abnormalities in *EVC* regulation in ATL. We have determined the gene expression signature of ATL tumor cells by conducting massive microarrays.⁽⁵⁾ The gene expression profiles from 52 ATL patients and 21 healthy donors identified a

large number of specific gene upregulations in ATL cells. Among these, the genes encoding *EVC1* and *EVC2* were strikingly overexpressed in ATL patient samples, which had a relationship to disease progression (Fig. 1a,b). These genes are located in an identical chromosome *4p16*, under a bi-directional promoter (Fig. 1c), and their expressions have shown a strong positive correlation (Fig. 1d). The qRT-PCR revealed that the median of the *EVC1* mRNA level in ATL was 90.9-fold higher than that of normal CD4+ T-cells (Fig. 1e). Specificity of tumor-associated *EVC* expression was confirmed using the dataset from *CADM1* versus *CD7* plot subpopulation samples.⁽²⁵⁾ *CADM1* expression and *CD7* loss have recently been identified as highly sensitive molecular markers of HTLV-1-infected cells. *EVC1* and *EVC2* were significantly expressed in the *CADM1*+/*CD7*- tumorous population (Fig. 1f). The HTLV-1-infected and ATL-derived cells showed higher levels of *EVC1* mRNA compared with those in other leukemia and lymphoma cell lines and those of healthy PBMC (Fig. 1g). The MT-2 and HUT102 cells, which highly express HTLV-1 genes, showed high *EVC1* mRNA levels similar to those in ATL-derived cells.

Looking at the tumor-associated epigenetic reprogramming that was frequently observed in ATL,^(5,6) we analyzed the epigenetic status of the *EVC* locus to clarify the possible

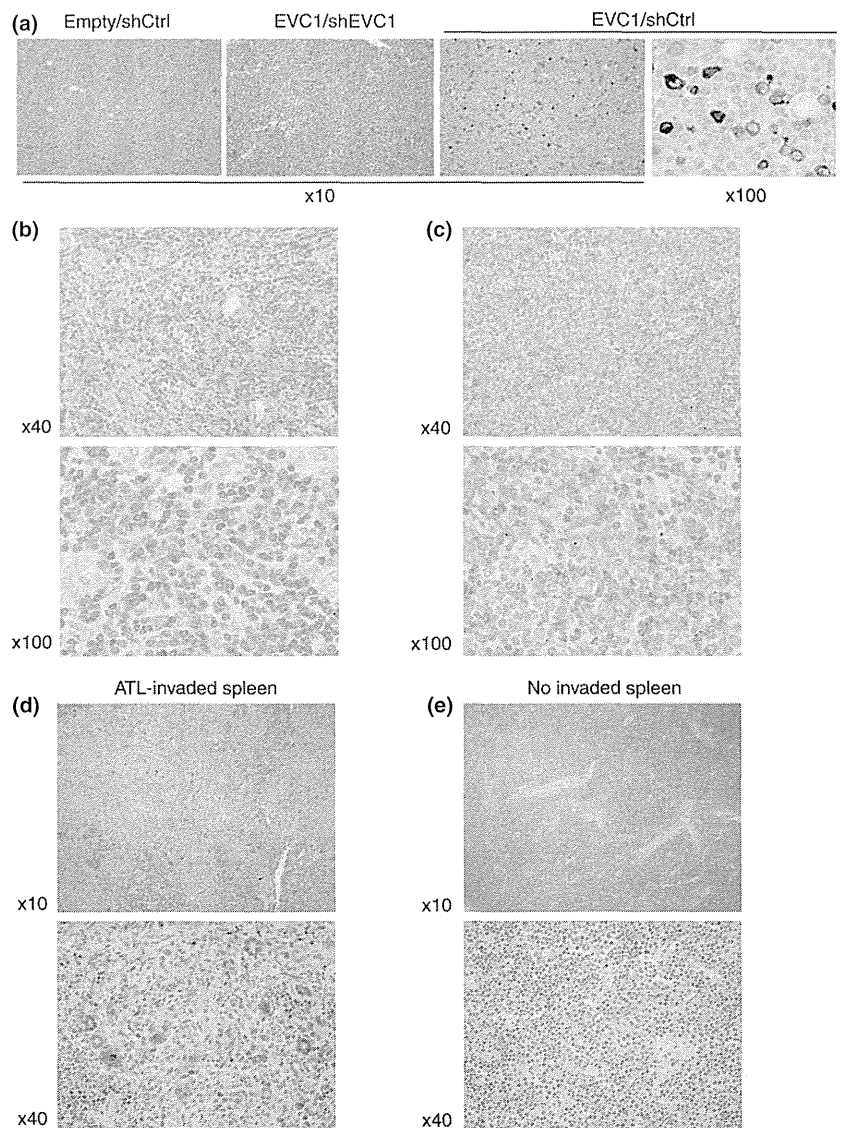


Fig. 4. *EVC1* expression in ATL cells. (a–e) Immunohistochemistry-based *EVC1* protein detection in paraffin-embedded samples: 293T transfected with the indicated plasmids (a), primary ATL lymph node (b, c, representative data are shown) and spleen from mice engrafted with primary ATL cells (d, tumor invasive, $n = 3$; e, non-invasive). These samples were stained with anti-*EVC1* antibody and hematoxylin.

involvement of epigenetic variation in *EVC* deregulation. There were two typical CpG islands in the *EVC* locus whose transcription may be tightly regulated by the gain of CpG methylation (Fig. 2a). However, bisulfite sequencing revealed that DNA methylation was not acquired in normal lymphocytes, as well as in the primary ATL sample (Fig. 2b). CpG hypermethylation within the *EVC* locus was found only in Jurkat cells where the *EVC* expression was nearly undetectable (Fig. 1g). Instead, treatment with epigenetic drugs, particularly a histone deacetylase (HDAC) inhibitor tricostatin A (TSA), reactivated the *EVC* transcription in Jurkat cells, suggesting that histone modifications such as acetylation were involved in *EVC* regulation (Fig. 2c). To further address the epigenetic implication, we performed ChIP assays to assess the possible contribution of histone modification in *EVC* upregulation. The ATL cell lines showed significant accumulation (log-scale) of histone H3 acetylation (H3Ac) and H3K4 trimethylation (H3K4me3), which have been recognized generally as positive transcription marks around the transcription start site region of both *EVC1* and *EVC2* (Fig. 2d). Treatment with a pan-histone acetylase inhibitor, anacardic acid, reduced *EVC* transcription in ATL cell lines (Fig. 2e). Interestingly, H3K27me3, which has been implicated as a poor prognostic marker in ATL,^(5,26) was decreased at the *EVC* locus in ATL cells. We confirmed directly the epigenetic reprogramming at the *EVC* locus in primary ATL samples (Fig. 2f). In summary, it appeared that the acquisition of active histone modifications and the reciprocal disappearance of H3K27me3 contributed to aberrant *EVC* transcription.

Role of HTLV-1 Tax in *EVC* transcription. Next we addressed whether Tax could participate in the deregulated *EVC1* transcription. Although Tax expression did not influence the *EVC1* mRNA levels in 293T cells at complete growth conditions, Tax activated *EVC1* transcription in a dose-dependent manner in serum-starved conditions (Fig. 3a). A NF- κ B activation-defected Tax mutant, M22,⁽²⁷⁾ showed similar *EVC1* induction,

suggesting that *EVC1* transcriptional activation was independent from NF- κ B activation. Indeed, the pharmacological inhibition of NF- κ B activity failed to prevent *EVC* transcription in ATL cell lines (data not shown). Meanwhile, Tax induced transcription of *Sonic hedgehog* (*Shh*), which encodes the HH activation ligand, in a NF- κ B-dependent manner (Fig. 3b). The experimental condition was validated by the evaluation of *PTHrP*, which has been known to be a Tax and NF- κ B-targeted gene. HTLV-1 *HBZ* did not affect *EVC* transcription (Supporting Information Fig. S1).

We examined the possible relationship between Tax and histone modifications. For this purpose, we established lentiviral vectors inducing stable Tax expression in Jurkat cells. More than 80% of transduction efficiencies were achieved in all tested cells. Tax induced transcription of *EVC1* and *EVC2*, as well as the HH target gene *PTCH1* (Fig. 3c). Interestingly, the Tax C29A mutant, which was unable to localize in the nucleus,⁽²⁸⁾ failed to induce *EVC*, suggesting that *EVC* induction was directly caused by the nuclear-localized Tax. A ChIP assay revealed that the Tax wild type, but not the C29A mutant, directly accumulated H3K4me3 and H3Ac in the *EVC* locus (Fig. 3d). Thus, Tax appeared to, at least partially, induce *EVC* expression through epigenetic reprogramming.

***EVC1* expression in primary ATL cell.** We performed immunohistochemistry (IHC) with a commercially available antibody that recognized *EVC1*. First, we stained paraffin-embedded 293T cells transduced with the *EVC1*-expressing plasmid to test the antibody specificity. Strong positivity was detected in the plasmid-transduced sample but not in samples with untreated or concomitantly treated with shRNA targeting *EVC1* (Fig. 4a). Using this antibody we investigated *EVC1* expression in several aggressive ATL cases. Most ATL cases showed stable *EVC1* positivity (7/8, 87.5%; two representatives in Fig. 4b,c). We noted that all *EVC1*-positive cells were dysplastic. Furthermore, *EVC1* expression was clearly detected in a mouse ATL model that was established using xenotrans-

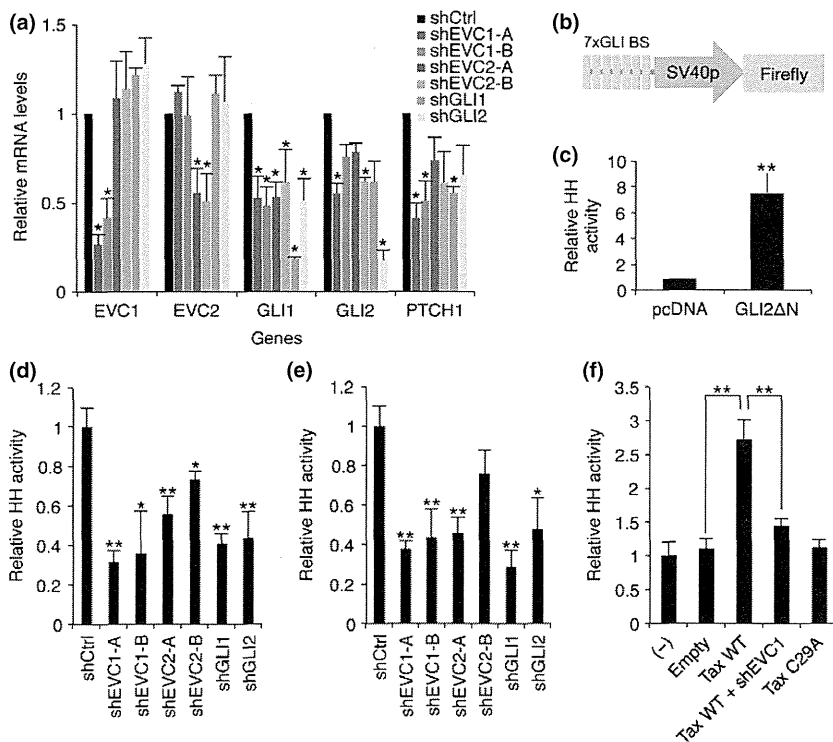


Fig. 5. *EVC* supports Hedgehog (HH) activity. (a) Relative RNA levels in shRNA-expressing TL-Om1 cells ($n = 3$, mean \pm SD). * $P < 0.05$. (b) Luciferase reporter plasmid containing 7 \times sequential GLI binding sites. (c) *GLI2* Δ N activated HH activity ($n = 3$, mean \pm SD). ** $P < 0.01$. (d–f) Hedgehog activity in various shRNA-expressing TL-Om1 (d), MT-2 (e) and Tax and shEVC1-expressing Jurkat (f) ($n = 3–4$, mean \pm SD). * $P < 0.05$. ** $P < 0.01$.

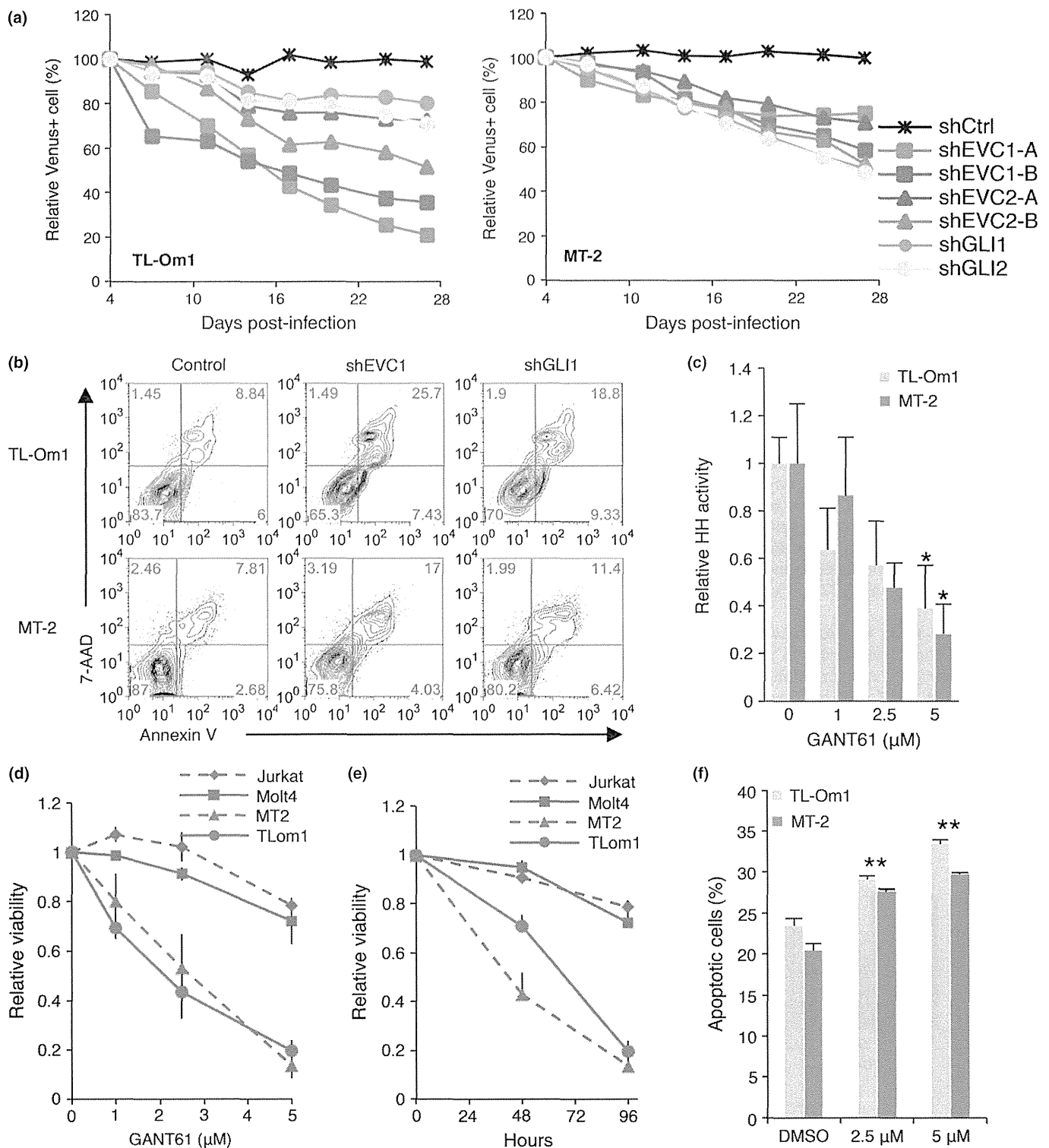


Fig. 6. Hedgehog (HH)-dependent ATL cell survival. (a) Time course of the abundance of Venus+ TL-Om1 (left) and MT-2 (right) infected with lentiviral vector expressing control shRNA (shCtrl), either of two shRNA targeting EVC1 and EVC2, or shRNA targeting GLI1 and GLI2, then cultured for 27 days together with uninfected cells. Data are representative of three independent experiments. Results are presented relative to those of cells at 4 days post-infection. (b) shRNA-mediated apoptosis induction. shRNA-expressing cells were cultured in 1% FBS for 72 h. The apoptotic pattern was defined by gating with Venus+ and Annexin V/7-AAD ($n = 3$, representative data). (c) GANT61 inhibited HH activity in ATL cells ($n = 3$, mean \pm SD). * $P < 0.05$. (d-e) GANT61 reduced ATL cell viability ($n = 3$, mean \pm SD). The cells were treated with the indicated concentrations of GANT61 for 96 h (d) or with 5 μ M of GANT61 for the indicated time periods (e). Cells were maintained in 1% FCS. (f) GANT61-dependent apoptosis analyzed using Annexin V/7-AAD staining ($n = 3$, mean \pm SD). ** $P < 0.01$.

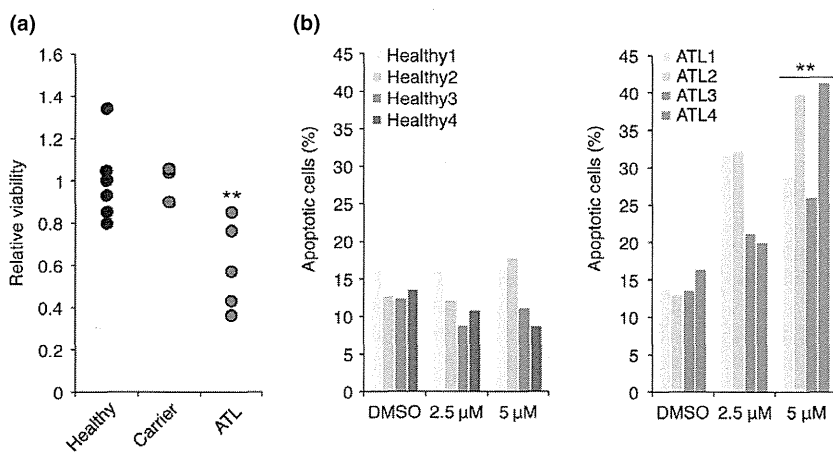


Fig. 7. GANT61 treatment reduced cell viabilities of primary ATL samples. (a) Effect of GANT61 in primary PBMC samples. The PBMC from healthy donors ($n = 7$), asymptomatic carriers ($n = 3$) and ATL patients ($n = 5$) were exposed in $5 \mu\text{M}$ of GANT61 for 72 h. Cells were maintained in media with 1% self-serum. $**P < 0.01$. (b) GANT61-dependent apoptosis in ATL samples. The PBMC from healthy donors ($n = 4$) and ATL patients ($n = 4$) were treated with $5 \mu\text{M}$ of GANT61 for 72 h. Graphs show percentiles of apoptotic population in CD4^+ cells $**P < 0.01$.

plantation of primary tumor cells derived from an ATL patient. The lymphoma cells specifically expressed EVC1 (Fig. 4d,e). Taken together, EVC1 protein was definitely expressed in ATL cells.

EVC in HH activation. The EVC family has been implicated in HH signaling.^(18,19) We performed the knockdown of EVC in TL-Om1 and MT-2 cells, which all highly expressed EVC (Fig. 1g). Specific knockdown was accomplished using lentivirus harboring specific and previously validated shRNA against EVC1, EVC2 or GLI transcription factors in the HH cascade. The qRT-PCR revealed the knockdown efficiency and also the HH activity as the RNA levels of *PTCH1* and *GLII* were well-established HH activity markers.⁽²⁹⁾ The EVC depletion resulted in reduction of *PTCH1* and *GLII* mRNA levels (Fig. 5a). Next we established a luciferase reporter containing $7 \times$ sequential GLI-binding sites (Fig. 5b), which strongly responded against *GLI2ΔN*, a constitutive active form of *GLI2*⁽¹²⁾ (Fig. 5c). As expected, the knockdown of EVC1 and EVC2 represented diminished HH activity in TL-Om1 and MT-2 cells (Fig. 5d,e). In addition, Tax activated the HH signal in Jurkat cells (Fig. 5f). Knockdown of EVC1 cancelled Tax-directed HH activation, suggesting that Tax affects HH signaling through, at least partially, EVC induction epigenetically.

EVC-dependent cell survival in ATL. Aberrant activation of HH provides cell survival ability in myeloma and lymphoma.^(14,15) We found that different shRNA targeting EVC and GLI caused a progressive depletion of Venus+ cells (Fig. 6a). Knockdown of EVC1 or EVC2 attenuated ATL cell proliferation (Fig. S2). The growth defect was associated with a substantial decrease in the expression of targeted genes (Fig. 5). We then measured the apoptotic status by staining Annexin V/7-AAD. Specific analyses within the knocked down cells were achieved by gating with Venus fluorescence. At complete growth condition, slight but steady apoptosis was induced by EVC1 knockdown in MT-2 and TL-Om1 cells (data not shown). Furthermore, strong apoptosis was observed in EVC1-depleted cells at low FCS condition (Fig. 6b). This cell death appeared to be due to HH inactivation because *GLII*-knocked down cells showed similar results.

Specific killing of ATL cell by GANT61. GANT61 is a cell-permeable hexahydropyrimidine compound, which has been shown to be a well-established inhibitor of GLI-mediated gene transactivation.⁽²⁹⁾ GANT61 treatment successfully reduced GLI binding to the target sequence (Fig. 6c). In that condition, MT-2 and TL-Om1 cells showed remarkable reduction of cell

viability by GANT61 treatment in dose- and time-dependent manners (Fig. 6d,e), which may be caused by apoptosis (Fig. 6f).

Finally, we evaluated the pharmacological activity of GANT61 on primary ATL samples. Although GANT61 did not show a clear effect on PBMC derived from healthy donors and HTLV-1 carriers, its treatment specifically reduced the viability of ATL samples significantly (Fig. 7a). Flow cytometry demonstrated that GANT61 specifically killed CD4^+ leukemic cells from ATL patients via apoptosis induction (Fig. 7b).

Discussion

A large number of efforts have collectively concluded that aberrant gene expression patterns contribute to the malignant characteristics in ATL and other neoplastic cells. In the present study, based on the careful analyses of patient samples, we have demonstrated that *EVC* is drastically overexpressed in mRNA and its protein can be specifically detected in ATL cells in contrast to normal CD4^+ T-cells. To the best of our knowledge, this is the first report regarding *EVC* expression and function in lymphocytes. The results of the microarray indicate that *EVC* expression appears to be induced in accordance with disease progression. *EVC1* protein expression is observed in dysplastic ATL cells derived from patients and a xenotransplantation model. Because the EVC family may be membrane-associated proteins (Fig. 4),⁽³⁰⁾ the present study provides us with the possibility that *EVC* expressions might be useful cell markers of HTLV-1-infected T-cells for future clinical purposes.

The EVC family has been identified initially as the responsible genes for one morphogenic disorder, Ellis van Creveld syndrome; it is also believed to play a role in the determination of body-axis or morphogenesis by usually bearing one step of the HH signaling pathway.^(12,17–19) Knockout studies have demonstrated that EVC1 and EVC2 cooperatively act as positive modulators of the HH pathway in mouse fibroblasts and chondrocytes. However, abnormal EVC upregulation has not been reported in any cancers; whether the HH pathway is sensitive to cellular dynamism of EVC has not been elucidated as yet. Herein, we demonstrated that overexpression of EVC can be linked to HH activity in T cells for the first time. In addition, several experimental results, including the knockdown assay and GANT61 treatment, suggest totally that the HH pathway was activated in ATL, which in turn contributed to ATL cell survival. Further study will be required for mechanistic insights on how EVC activates HH in T cells.

Further investigation uncovered that transcription from the *EVC* locus was coordinated by epigenetic alteration. In particular, the lymphoma-associated H3Ac and H3K4me3 accumulations appeared to dominate *EVC* upregulation. Direct evidence from patient samples supported the epigenetic reprogramming, including previously unappreciated H3K4me3 rearrangements, conferring robust *EVC* expression. Interestingly, repressive histone mark H3K27me3 was mutually reduced at the *EVC* locus in the ATL samples, suggesting that cooperative regulation in this bivalent domain may define the *EVC* expression and possibly HH activity.

HTLV-1 Tax was involved in the regulation of *EVC* via epigenetic regulation. Nuclear localization-deficient Tax mutant was unable to induce *EVC* expression, implying that Tax may participate directly in determination of chromatin architecture. Indeed, lentiviral expression of Tax partially increased active histone modifications, which in turn activated HH signaling. Previously, we and others have reported that Tax physically binds with histone modifying factors, including HDAC,⁽³¹⁾ SUV39H1⁽³²⁾ and SMYD3.⁽²⁰⁾ Interplay between Tax and epigenetic rearrangement may be closely involved in the progression of HTLV-1-infected cells to leukemic cells. Meanwhile, other ATL-specific epigenetic events including significant modifications on histone acetylation, H3K4me3 and H3K27me3 clearly dominate stable *EVC* expression. The alteration of the epigenetic landscape by Tax and other molecular mechanisms such as expression changes of epigenetic modifiers will be elucidated by comprehensive analysis such as a genome-wide ChIP analysis.

In the context of molecular targeting, a new possibility for the HH inhibitor was suggested. Recently, aberrant HH activation and its contribution to cell survival and the cell cycle have been reported in various cancer cells.^(11,12) In agreement with other tumors where HH is active, we found that ATL was sensitive against GANT61. This compound can inhibit HH signaling

by preventing DNA binding of the GLI family and has few impacts on the viability of healthy CD4+ T cells. We note that we could not confirm the *EVC*-directed upregulation of common HH target genes such as *Cyclin D1* and *Bcl-2* in ATL models (data not shown). Given that the HH pathway regulates transcription of many genes important for cell fate and many inhibitors against HH cascade have been developed,⁽¹⁶⁾ our findings suggest that pharmacological drugs that can inhibit the HH pathway may be feasible for ATL treatment. Identification of ATL-specific HH target genes will help understanding of the HH roles in survival capability.

In summary, we have identified *EVC* overexpression as a specific character of ATL and HTLV-1-infected T cells. We have demonstrated the molecular mechanism that overexpressed *EVC1* contributes to ATL cell survival. Considering aberrant gene expression associated with cancers, the emerging relationship between epigenetic regulation and the HH pathway provides us with conceptual advance in understanding the broad-acting oncogenic signaling.

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Disclosure Statement

The authors have no conflict of interest.

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