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

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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). The prognosis of aggressive types of ATL is poor. 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. 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 and the aberrant activation of nuclear factor-kappa B (NF-kB) contribute to ATL leukemogenesis. 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. Constitutive HH activation leads to the overproliferation or survival of several cancer cells, such as basal cell carcinoma or B-cell lymphomas. There are some HH inhibitors under clinical trial as drug candidates against those cancers.

In the present study, using ATL patient samples and some ATL models, we found two specific gene overproductions in ATL, Ellis Van Creveld syndrome I (EVC1) and EVC2, which belong to the EVC family of genes that are implicated in HH regulation. We demonstrated that epigenetically upregulated EVC was associated with cellular HH activity. 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.

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
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. 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. 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). Briefly, 7X GLI binding site (GA-ACACCCA)-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. 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 immunoprecipitatio-
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 ± 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 μM of anacardic acid for 48 h and the EVC mRNA level was then analyzed (n = 3, mean ± SD). *P < 0.05. (f) Epigenetic changes in primary ATL samples. Three independent clinical samples were compared with normal PBMC (n = 3, mean ± SD).
tion (ChIP) assay was conducted as described previously. 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-μm 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. Briefly, replication-defective, self-inactivating lentivirus vectors were used. shRNA were cloned into a CS-H1-EVBsd. High-titer viral solutions prepared using a centrifugation-based concentration were transduced into ATL cell lines using the spinoculation method. The transduced 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.

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![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 ± 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 ± SD). *P < 0.05. **P < 0.01. (c) EVC and PTCH1 levels in Jurkat cells expressing Tax (n = 3, mean ± 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).](image-url)
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. 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 data-set from CADM1 versus CD7 plot subpopulation samples. 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, we analyzed the epigenetic status of the EVC locus to clarify the possible
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, failed to induce EVC transcription 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 expression.

**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-κB activation-defected Tax mutant, M22, showed similar EVC1 induction, suggesting that EVC1 transcriptional activation was independent from NF-κB activation. Indeed, the pharmacological inhibition of NF-κB 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-κB-dependent manner (Fig. 3b). The experimental condition was validated by the evaluation of PTHrP, which has been known to be a Tax and NF-κB-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, 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 (78.75%; 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. 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 ± SD). *P < 0.05. (d–e) GANT61 reduced ATL cell viability (n = 3, mean ± SD). The cells were treated with the indicated concentrations of GANT61 for 96 h (d) or with 5 μM 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 ± SD). **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 PTC1H1 and GLI1 were well-established HH activity markers.\(^{120}\) The EVC depletion resulted in reduction of PTC1H1 and GLI1 mRNA levels (Fig. 5a). Next we established a luciferase reporter containing 7 × sequential GLI-binding sites (Fig. 5b), which strongly responded against GLI2ΔN, a constitutive active form of GLI2.\(^{12}\) 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 GLI1-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 the EVC locus was coordinated by epigenetic alteration. In particular, the lymphoma-associated H3Ac and H3K4me3 accumulations appeared to dominate EVC epigenetic state. 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 mechanism. Nuclear localization-deficient Tax mutant was unable to induce EVC expression, implying that Tax may participate directly in the regulation 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, SUV39H1, and SMYD3. 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. 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 HH-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, 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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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. HTLV-1 HBZ does not affect EVC expression.

Fig. S2. EVC knockdown reduces ATL cell proliferation.

Methods S1. Including: details of clinical samples; and primer sequences used in the present study.