Hypomethylation of the Treg-specific demethylated region in FOXP3 is a hallmark of the regulatory T-cell subtype in adult T-cell leukemia

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Abstract

Adult T-cell leukemia (ATL) is an aggressive T-cell malignancy caused by human T-cell leukemia virus type 1. Because of its immunosuppressive property and resistance to treatment, ATL patients have poor prognoses. ATL cells possess the regulatory T cell (Treg) phenotype such as CD4, CD25, and usually express forkhead box P3 (FOXP3). However, the mechanisms of FOXP3 expression and its association with Treg-like characteristics in ATL remain unclear. Selective demethylation of the Treg-specific demethylated region (TSDR) in the FOXP3 gene leads to stable FOXP3 expression and defines natural Tregs. Here, we focus on the functional and clinical relationship between the epigenetic pattern of the TSDR and ATL. Analysis of DNA methylation in specimens from 26 ATL patients showed that 15 patients (58%) hypomethylated the TSDR. The FOXP3⁺ cells were mainly observed in the TSDR-hypomethylated cases. The TSDR-hypomethylated ATL cells exerted more suppressive function than the TSDR-methylated ATL cells. Thus, the epigenetic analysis of the FOXP3 gene identified a distinct subtype with Treg properties in heterogenous ATL. Furthermore, we observed that the hypomethylation of TSDR was associated with poor outcomes in ATL. These results suggest that the DNA methylation status of the TSDR is an important hallmark to define this heterogeneous disease and to predict ATL-patient prognosis.
Introduction

Adult T-cell leukemia (ATL) is an aggressive T-cell malignancy caused by human T-cell leukemia virus type 1 (HTLV-1) (1, 2). HTLV-1 is endemic in southwest Japan, Sub-Saharan Africa, the Caribbean Basin, and South America (3). Approximately 5% of HTLV-1-infected individuals develop ATL after a latency period of 40- to 70-years from the initial HTLV-1 infection (3, 4). The prognosis of ATL is usually poor with a median survival period of approximately 1 year (5). The major obstacles in the treatment of ATL patients include resistance to a variety of cytotoxic agents and susceptibility to opportunistic infections because of its immunosuppressed property (3,6,7).

In HTLV-1-infected individuals, HTLV-1 proviral DNA has been detected mainly in CD4+ T cells (8). However, human CD4+ T cells are heterogeneous, and the subset of initial HTLV-1 infection in ATL patients remains to be clarified. Most ATL cells express CD3, CD4, and CD25 (9). This phenotype resembles regulatory T cells (T\textsubscript{regs}), and several previous studies have shown that 60% to 70% of ATL cases expressed forkhead box P3 (FOXP3) (10, 11), which is a master regulator of T\textsubscript{regs} (12).

T\textsubscript{regs} play an important role in immune tolerance, and they prevent the development of autoimmune and inflammatory diseases by suppressing autoreactive T
cells (13). FOXP3, a key transcription factor, controls both the development of T_{reg}s and their suppressive function (12–14). However, a substantial amount of evidence indicates that FOXP3 expression itself is not sufficient to establish the full phenotype and function of Treg cells (15). In humans, FOXP3^{+} cells are heterogeneous and are not always suppressive (16). T_{reg}s are divided into two main subpopulations: natural or induced T_{reg}s. The former is a genuine T_{reg}, developing in the thymus at the stage of CD4 single-positive thymocytes (17,18), whereas the latter is induced by differentiation in the periphery after antigenic stimulation (19,20) and cannot fully exert suppressive activity (21, 22). Selective DNA hypomethylation of conserved CpG residues within the FOXP3 locus, termed the T_{reg}-specific demethylated region (TSDR), is important for distinguishing natural T_{reg}s from peripherally induced T_{reg}s and for stabilizing FOXP3 expression in both humans and mice (23, 24).

Several studies have shown that FOXP3 expression varies among ATL patients (10, 25), and that FOXP3-expressing ATL cells are not always suppressive (26). Therefore, we investigated the DNA methylation pattern of the TSDR and its relevance to T_{reg} properties and clinical outcomes in ATL.
Materials and methods

Primary ATL cells and control cells

Primary ATL cells were obtained from 40 patients hospitalized at Kyoto University Hospital or at affiliated hospitals during the period from 2001 to 2014. The diagnosis of ATL was based on the Shimoyama classification (7). For CpG methylation analysis, we included only male patients, because one allele of the TSDR on the \textit{FOXP3} locus of the X-chromosome was methylated in females (24). The characteristics of 26 male patients are summarized in Table 1 and Supplementary Table S1. Peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Paque (Pharmacia Biotech, Little Chalfont, UK) density-gradient centrifugation. If the proportion of abnormal cells in the PBMCs was less than 60%, we purified the ATL cells by sorting (Supplementary Fig. S1). Control peripheral blood samples were obtained from eight healthy volunteers.

This study was performed in compliance with the Declaration of Helsinki after approval by the Ethics Committee of Kyoto University, Graduate School of Medicine. Written informed consent was obtained from all patients and healthy volunteers.

Cell lines

We used HTLV-1-infected cell lines, ATL-43T (27), MT-1 (28), MJ (29), MT-2
(30), MT-4 (30), and Hut102 (31). ATL43T were kindly provided by Dr M.Maeda (Virus Institute, Kyoto University, Japan) in 1991. MT-1, MT-2 and MT-4 were obtained from Japanese Collection of Research Bioresources (JCRB) Cell Bank and Hut102 and MJ were obtained from American Type Culture Collection (ATCC) between 2000 and 2014. All cell lines were authenticated using HTLV-1 clonal integration and tested for mycoplasma contamination in 2014. These cell lines were cultured in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 2 mM penicillin, streptomycin, and glutamine (Gibco BRL, Grand Island, NY) at 37°C in a humidified incubator with 5% CO₂ in air. For the ATL-43T cell line, which is interleukin (IL)2-dependent, the medium was supplemented with 0.5 nmol/L recombinant IL2 (Shionogi, Osaka, Japan). All cell lines were not cultured for longer than 1 month.

**Bisulfite sequencing**

A CpG methylation analysis was performed as described with slight modifications (23). Briefly, genomic DNA was isolated from cells and bisulfite treatment was performed using the MethylEasy Xceed Kit (Human Genetic Signatures, North Ryde, NSW, Australia). Modified DNA was amplified by PCR and cloned into a
pGEM-T Easy Vector Systems (Promega, Madison, WI). The bisulfite sequencing-specific primers are listed in Supplementary Table S2. The independent colonies (16 colonies/region) were amplified with the Illustra TempliPhi Amplification Kit (GE Healthcare, Buckinghamshire, UK) and sequenced using a 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA). The percentage of methylation was calculated by dividing the number of demethylated colonies at the CpG site by 16.

Quantitative reverse transcription-(qRT)-PCR analysis

Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and converted into cDNA using a SuperScript Reverse Transcriptase (Invitrogen, Carlsbad, CA) and Random Hexamers (Applied Biosystems). A qRT-PCR was performed using the TaqMan gene expression kit (Applied Biosystems). The primers and probes for tax and HTLV-I bZIP factor (HBZ) were as described (32, 33). Those for human FOXP3, CTLA4, HELIOS, EOS, β-ACTIN and 18S rRNA were purchased from Applied Biosystems. We calculated the relative mRNA expression based on the levels of expression in the MJ cell line. All standards and samples were analyzed in duplicate, and the average value was used for the calculations.
Flow cytometric analysis

A phenotypic analysis was performed on the purified PBMCs. The data were acquired using a FACS LSR II instrument (BD Bioscience, San Diego, CA) or FACS Aria instrument (BD Bioscience) and analyzed with FlowJo software (ver. 9.7.5; Tree Star, Inc., San Carlos). For the sorting of primary ATL cells, cells were stained with anti-CD3 (UCHT1, eBioscience, San Diego, CA), anti-CD4 (RPA-T4, eBioscience), anti-CD5 (UCH-T2, Biolegend, San Diego, CA), anti-CD7 (CD7-6B7, Biolegend), anti-CD25 (M-A251, BD Bioscience) and LIVE/DEAD Fixable Dead Cell Stain Kits (Invitrogen). Cells were sorted with FACS Aria (BD Bioscience) and used for further experiments (Supplementary Fig. S1).

Functional assay to evaluate the suppressive activity of ATL cells in vitro

The suppressive function of ATL cells was assayed using a modified protocol (16). Briefly, CD4⁺CD25⁻CD45RA⁺ T cells from a healthy donor were used as responder cells and stained with the Cell Trace Violet Cell Proliferation Kit (Molecular Probes, Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. CD4⁻CD8⁻ cells were used as antigen-presenting cells (APCs) after irradiation (18.5 Gy). Responder cells were cocultured with the same numbers of ATL cells with 10-fold
APCs, and stimulated with 0.5 μg/mL soluble anti-CD3 (eBioscience) and 0.1 μg/mL soluble anti-CD28 (eBioscience) in supplemented RPMI medium. The number of proliferated violet-labeled cells was assessed by flow cytometry after culturing for 84-90 h.

**Cytokine staining**

Cytokine staining was performed as described (16). Briefly, purified T cells were stimulated by phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and ionomycin (Sigma-Aldrich) for 4 h in the presence of a Golgi transport inhibitor, monensin (GolgiStop; BD Pharmingen, San Diego, CA). After staining with antibodies to surface moieties, the cells were fixed and permeabilized by Cytofix/Cytoperm and PermWash (BD Pharmingen) according to the manufacturer’s instructions. Cells were then stained with anti-cytokine or isotype-matched antibodies and analyzed by flow cytometry.

**Statistical analysis**

Statistical analyses were performed using Graph Pad Prism 6.0 (Graph Pad Software, San Diego, CA). Survival curves of the ATL patients were calculated by the
Kaplan-Meier method using the SPSS Statistics software program, version 22.0 (IBM, Armonk, NY). Differences between two groups were evaluated by unpaired t-tests. For the comparison of multiple groups, we used by Tukey’s method. Comparisons of overall survival (OS) were carried out between two groups with two-sided log rank tests. 

$P$ values < .05 were considered significant.

Results

Methylation status of $FOXP3$, $CTLA4$, and $HELIOS$ genes in ATL cells

We first investigated the DNA methylation status of CpG residues in the $FOXP3$ gene locus from amplicon (amp) 1 to 11 by using the bisulfite sequencing method, based on a previous report (24). The MJ cell line and ATL cells from Case 2 showed hypomethylation states of $FOXP3$ that were similar to those of $T_{regs}$, whereas the MT-1 cell line and ATL cells from Case 17 showed methylation states similar to those of CD4$^+$CD25$^-$ conventional T cells (Fig. 1A). Subsequently, we analyzed the DNA methylation status of amp 5 of $FOXP3$ by the same method, because this region, referred to as the TSDR, is important in the development and function of $T_{regs}$ (24). We calculated the mean methylation percentage in the TSDR. A rate lower than 50%, was considered hypomethylated, and greater than 50%, was considered methylated. ATL
cells from 15 (58%) out of the 26 patients analyzed have hypomethylation states similar to normal Tregs (Fig. 1B, left panel). The median methylation rate of the TSDR-hypomethylated cases and that of the TSDR-methylated cases were 15% (range, 0%–40%) and 85% (range, 73%–96%), respectively.

The CTLA4 and HELIOS regions are also differentially methylated in Tregs and CD4⁺CD25⁻ conventional T cells (23). We confirmed that the CTLA4 exon 2 and HELIOS intron 5 regions were hypomethylated in normal Tregs, but highly methylated in CD4⁺CD25⁻ conventional T cells (Supplementary Fig. S2). Therefore, we examined the DNA methylation of both of these areas in primary ATL cells and found that they were both predominantly hypomethylated, except for four cases in CTLA4 exon 2 and for one case in HELIOS intron 5. All cases with CTLA4 exon 2-methylation or HELIOS intron 5-methylation were included in the TSDR-methylated cases.

**DNA methylation of the TSDR, FOXP3 expression and Treg signatures**

We analyzed the expression of Treg-related genes and various cytokines. With the use of flow cytometric analysis, FOXP3 expression was observed in almost all of the patients identified with a hypomethylated state. The percentage of FOXP3⁺ cells was significantly higher in the TSDR-hypomethylated cases than in the methylated cases.
The mean fluorescence intensity (MFI) of FOXP3 tended to be higher in the hypomethylated cases, but the difference was statistically marginal. Transcriptional analysis by qRT-PCR presented a trend of more FOXP3 mRNA in the TSDR-hypomethylated cases (Fig. 2B and Supplementary Fig. S3B). We could not exclude the possibility that contamination of the normal T_{reg} population might lead to the elevation of FOXP3 expression in whole PBMC samples, so we isolated the ATL cells by sorting. Purified ATL cells from the TSDR-hypomethylated cases had significantly more FOXP3 mRNA compared to the methylated cases ($P = 0.01$) (Fig. 2B and Supplementary Fig. S3C).

In order to further characterize the ATL cells, we subdivided T_{reg} into three fractions (fraction I, II, and III) according to the expressions of CD45RA and FOXP3, as described (16). We analyzed 8 TSDR-hypomethylated cases and 8 methylated cases. ATL cells from the TSDR-hypomethylated cases were mainly classified into fraction II or fraction III, whereas those from the TSDR-methylated cases belonged to various subsets (Fig. 3). In most of the TSDR-hypomethylated cases, CD45RA FOXP3^{high}--activated T_{reg} constituted a proportion of the ATL cells. ATL cells from case 8, which had about 40% methylation of the TSDR, also were primarily in fraction II.
The expression of other T<sub>reg</sub>-related proteins besides FOXP3, such as CD25, CTLA4, GITR, CCR4, and HELIOS, was analyzed in 14 ATL patients, including 8 TSDR-hypomethylated cases (Supplementary Fig. S4). Most patients had expression of CD25, CTLA4, GITR, CCR4, and HELIOS, consistent with the phenotype of T<sub>reg</sub>s. However, the TSDR-hypomethylated and methylated cases were not significantly different (Supplementary Fig. S4A–E). In addition, expression of proliferation marker Ki67 (16) was not significantly different between the groups (Supplementary Fig. S4F).

We analyzed cytokine secretion from the ATL cells of 14 patients whose cells were stimulated with PMA and ionomycin (Supplementary Fig. S5). The cells secreted low amounts of IFNγ, IL4, IL17, IL10, IL2, and TNFα with no differences between the TSDR-hypomethylated and methylated cases. Expression of mRNA from CTLA4, HELIOS, and EOS, which forms a complex with FOXP3 (34), in the cells of 25 ATL patients varied among the ATL cases, with no significant differences in methylation status of TSDR (Supplementary Fig. S6). Together, these results indicated that the common characteristics of T<sub>reg</sub> (<i>e.g.</i>, FOXP3 expression, cell surface phenotype, and cytokine secretion) were indicated by hypomethylation of the TSDR in ATL cells. However, only FOXP3 expression could discriminate between the TSDR-hypomethylated and methylated cases.
Functional analysis of suppressive activity of primary ATL cells in vitro

To clarify the functional differences between the TSDR-hypomethylated and the methylated cases, we measured suppressive activity, a major characteristic of Tregs. We analyzed 10 ATL cells (5 TSDR-hypomethylated cases and 5 methylated cases). When we cocultured healthy CD4^+CD45RA^+ naïve T cells as responders with the TSDR-hypomethylated ATL cells as effectors, the proliferative activity and number of responder T cells were suppressed (Fig. 4). In contrast, the TSDR-methylated ATL cells could not suppress. Similar results were seen in the HTLV-1-infected cell lines (Supplementary Fig. S7). Thus, the methylation status of the TSDR was associated with suppressive activity.

HTLV-1 encoded tax and HBZ not related to the hypomethylation of TSDR

It was reported that the HTLV-1–associated molecules tax and HBZ affected the expression of FOXP3 (35, 36). To clarify the relationship between FOXP3 expression and these molecules, we analyzed mRNA expression. Consistent with previous reports (37), ATL cells contained little or no tax mRNA. The methylation status of the TSDR was not related to the amount of tax mRNA (Supplementary Fig.
HBZ mRNA was also independent of the methylation status of the TSDR in ATL patients (Supplementary Fig. S8B). Taken together, these results show that there is no obvious relation between HTLV-1 viral molecules and the methylation pattern of the TSDR in ATL.

Hypomethylation of TSDR as a predictive factor for survival in ATL

To assess the clinical significance of the epigenetic status of FOXP3, we divided 25 patients into two groups according to the TSDR methylation status. One patient (Case 5) was excluded in this analysis because of missing data. Clinical characteristics including the median age, clinical type, history of stem cell transplantation (SCT), and putative prognostic factors such as concentrations of serum albumin, lactate dehydrogenase (LDH), calcium, IgG, and soluble IL2 receptor not significantly different between the TSDR-hypomethylated group and the TSDR-methylated group (Table 1 and Supplementary Table S1). The median OS for the entire group was 315 days (range, 16–3923 days) and 19 of the 25 patients (76%) died. The median OS of the TSDR-hypomethylated group and that of the TSDR-methylated group were 283 days (range, 16–3099 days) and 627 days (range, 116–3923 days), respectively (Supplementary Fig. S9A). As the patients with chronic and smoldering types of ATL
had better prognoses than those with the acute or lymphoma types, we excluded both of these clinical types and reanalyzed OS. The TSDR-hypomethylated group had significantly inferior OS compared to the TSDR-methylated group:

TSDR-hypomethylated, median OS of 186 days (range, 16–691 days),

TSDR-methylated, median of 627 days (range, 116–3923 days), $P = .02$ (Fig. 5). Of the ten deceased patients in the TSDR-hypomethylated group, eight patients succumbed to ATL, one patient to infection, and one to treatment-related mortality. Of the six deceased patients in the TSDR-methylated group, four patients succumbed to ATL, and two to infection (Supplementary Table S1). These results suggest that the methylation pattern of TSDR could be associated with survival for ATL patients.

Next, we analyzed the relationship between the expression of FOXP3 and survival. Although the high frequency of CD45RA$^-$FOXP3$^{high}$ T cells had a tendency to reduce survival, this association was not statistically significant ($P = 0.14$) (Supplementary Fig. S9B). Even when we include 14 female patients for the analysis, the difference was not apparent ($P = 0.16$) (Supplementary Fig. S9C, 10 and Supplementary Table S3).

Thus, the methylation pattern of the TSDR may provide more precise prognostic stratification than expression of FOXP3.
Discussion

In this study, we found that ATL cells contain a unique cell subtype with a T\textsubscript{reg}-like hypomethylation status in the TSDR. The TSDR-hypomethylated ATL cells not only had higher expression of FOXP3 and more suppressive activity, but also poorer clinical outcomes.

FOXP3 expression in ATL cell lines and primary ATL cells has been investigated by many researchers at the mRNA and protein levels, and it varies widely among cell lines and individual cases (10, 11, 25, 26, 38, 39). Our data also showed a wide range of FOXP3 expression among primary ATL cells. Among these heterogeneous clusters, we could differentially identify one specific subtype, which shows the TSDR-hypomethylated status similar to that of natural T\textsubscript{regs}. These TSDR-hypomethylated ATL cells resemble natural T\textsubscript{regs} not only in their expression of FOXP3, but also other T\textsubscript{reg}-specific markers such as CD25, CTLA4, HELIOS, and GITR. However, the TSDR-methylated ATL cells also expressed T\textsubscript{reg}-specific markers other than FOXP3. In conventional T cells, the expression of CD25 and CTLA4 is readily induced by T cell activation, irrespective of their DNA methylation status (23). This may explain the expression of such molecules in ATL cells with a methylated TSDR.
The suppressive function of ATL cells has also been a subject of considerable discussion. Some studies show suppressive activity of ATL cells (39, 40), but others did not (26). We demonstrated that the TSDR-hypomethylated ATL cells, which showed higher FOXP3 expression, had suppressive function, whereas the TSDR-methylated ATL cells, which had less FOXP3 expression, did not. In natural Tregs, the DNA hypomethylation status of the TSDR is essential for stable FOXP3 expression and suppressive function (24, 41, 42). Similarly, in ATL cells, we found that the DNA hypomethylation status of the TSDR was closely associated with FOXP3 expression and function.

The HTLV-1-encoded genes, tax and HBZ, can modulate the expression of FOXP3. Transfection of tax DNA to CD4⁺CD25⁺ T cells resulted in a reduction of FOXP3 mRNA and suppressive function (35). In HBZ transgenic mice, the number of FOXP3⁺CD4⁺ T cells increased and these HBZ-induced Tregs had unstable FOXP3 expression, impaired function, and methylated TSDRs (36). However, primary ATL cells were not assessed in these experiments. In the present study, no correlation was detected between HTLV-1-associated molecules and the expression of FOXP3 or the methylation status of the TSDR.

Several studies have tried to identify the origin of ATL cells (11, 43), but the
answer as to whether the origin of ATL cells is natural T\textsubscript{reg} or conventional T cells has remained controversial. The DNA demethylation of the \textit{FOXP3} gene is thought to be a specific phenomenon of natural T\textsubscript{reg}. In addition, the HTLV-1 gene itself could change FOXP3 expression through the TGF\textbeta signal pathway (44), but such FOXP3\textsuperscript{+} T cells are induced T\textsubscript{reg}, not natural T\textsubscript{reg}. We therefore speculate that the demethylation of the TSDR in ATL cells is not a secondary event after HTLV-1 infection, and that the ATL cells with the hypomethylated TSDR may be derived from natural T\textsubscript{reg}. However, we cannot exclude the possibilities that (i) the HTLV-1 virus may have infected CD4\textsuperscript{+}Foxp3\textsuperscript{−} non-Treg cells, and (ii) epigenetic change had occurred during the development of ATL. Therefore, we cannot make any conclusions about the origin of ATL cells from the findings of the present study. More detailed characterizations of the TSDR-hypomethylated ATL cells at different time points during ATL progression would provide useful information about the etiology of ATL cells.

ATL is divided into four clinical variants: acute, lymphoma, chronic, and smoldering. The former two variants have a more aggressive clinical course and shorter OS than the latter two. The ATL-prognostic index was recently proposed to identify the clinical risk among acute and lymphoma type patients (45). Until now, the biological phenotype could not be used to distinguish the prognosis (46).
With respect to the FOXP3 expression, some studies have evaluated ATL cells by immunohistochemical staining (25, 47) or qRT-PCR (48), but they did not show a correlation with OS. Similarly, we couldn’t show a significant association between the FOXP3 expression and the OS. But the results of the present study demonstrated that the methylation pattern of the TSDR is correlated with the OS, regardless of the treatment with SCT (data not shown), and it was maintained when we omitted TSDR-methylated but FOXP3+ cases (data not shown). The hypomethylation of the TSDR in ATL cells led to ¥ FOXP3 expression, but even the TSDR-methylated cases often expressed FOXP3 (Figs. 2 and 3). The hypomethylation of the TSDR in ATL cells is closely associated with their suppressive function, like natural Tregs. In contrast, the expression of FOXP3 is not specific to Tregs, and the FOXP3-expressing ATL cells with the methylated TSDR may be classified with non-Tregs that do not possess suppressive function. Such functional deference according to the methylation status might influence the clinical course of ATL. However, the intrinsic immunosuppression in the TSDR-hypomethylated patients seems to be unrelated to the cause of death in the present small cohort, and we could not elucidate the reason for their poor survival. The possibility of other causes, such as drug resistance, will be a subject for future investigation.
The limitation of our study was that we could only analyze 26 patient cases of ATL regarding the methylation pattern of the TSDR, and the treatment strategies differed among patients. It is necessary to confirm our results by conducting a large-scale prospective clinical study. In addition, we could not evaluate the methylation patterns of the TSDR in women. We analyzed other T<sub>reg</sub>-specific demethylation regions, e.g., CTLA4 exon 2 and HELIOS intron 5, but the hypomethylation of such regions was not specific to ATL patients with the hypomethylated TSDR. Therefore, to analyze female cases, other assessment strategies are needed. In the male transplant setting, incidence of ATL in Japan is generally higher and its prognosis is worse than in females (49). We do not know whether our results could be applied to cases involving women. To date, the definition of hypomethylation is not standardized. We tentatively defined the cut-off values for FOXP3 DNA hypomethylation as 50%. According to this definition, we can classify the hypomethylated cases with high FOXP3 expression in this small cohort, but the relevance of this threshold should be reevaluated with a more large-scale study. However, we believe that it is important to recognize the unique ATL subtype defined according to the hypomethylation status of the TSDR. In the future, different treatment strategies might be needed for the treatment of this poor-prognosis ATL subtype.
In conclusion, we propose that a distinct subtype of the heterogeneous ATL disease can be defined according to the hypomethylated status of the TSDR. This T_{reg}-subtype is related to the biological phenotype and clinical course of ATL. The hypomethylated status of the TSDR in ATL cells might be a promising tool for risk classification.

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References


42 Morikawa H, Sakaguchi S. Genetic and epigenetic basis of Treg cell development and function: from a FoxP3-centered view to an epigenome-defined view of natural Treg cells. *Immunol Rev* 2014; 259: 192–205.


Table 1. Patients’ characteristics

<table>
<thead>
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<th></th>
<th>Hypomethylated patients</th>
<th>Methylated patients</th>
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<tr>
<td>Age (year), median (range)</td>
<td>57 (33-87)</td>
<td>54 (29-80)</td>
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<tr>
<td>Clinical type</td>
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<tr>
<td>Acute</td>
<td>10 (66.7%)</td>
<td>7 (63.6%)</td>
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<td>Lymphoma</td>
<td>1 (6.7%)</td>
<td>2 (18.1%)</td>
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<tr>
<td>Chronic</td>
<td>3 (20.0%)</td>
<td>1 (9.0%)</td>
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<td>Smoldering</td>
<td>1 (6.7%)</td>
<td>1 (9.0%)</td>
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<tr>
<td>Alb (g/dl), median (range)</td>
<td>3.9 (2.3-4.4)</td>
<td>3.6 (2.6-4.5)</td>
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<td>LDH (IU/L), median (range)</td>
<td>1036.5 (323-6451)</td>
<td>485 (195-3092)</td>
<td>.46</td>
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<td>Ca (mg/dl), median (range)</td>
<td>9.7 (8.8-16.9)</td>
<td>11.2 (9.1-16.9)</td>
<td>.38</td>
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<tr>
<td>IgG (mg/dl), median (range)</td>
<td>1093 (236-1395)</td>
<td>990 (624-1460)</td>
<td>.80</td>
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<td>sIL-2R (U/ml), median (range)</td>
<td>30071 (758-104000)</td>
<td>18670 (1220-232000)</td>
<td>.55</td>
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<td>OS (days), median (range)</td>
<td>283 (16-3099)</td>
<td>627 (116-3923)</td>
<td>.1</td>
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<td>Cause of death</td>
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<td>ATL</td>
<td>8 (66.6%)</td>
<td>5 (71.4%)</td>
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<td>Infection</td>
<td>2 (16.7%)</td>
<td>2 (28.6%)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>2 (16.7%)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

LDH indicates lactate dehydrogenase; sIL-2R, soluble interleukin-2 receptor; and OS, overall survival.
Figure Legends

Figure 1. The \( T_{\text{reg}} \)-specific demethylated region (TSDR) of ATL cells exhibits regulatory T cell-like hypomethylated status or CD4\(^+\) conventional T cell-like methylated status.

Bisulfite sequencing was performed using genomic DNA from each sample. The frequency of the demethylation of CpG is shown. Yellow = hypomethylated and blue = methylated in each CpG. (A) The DNA methylation status in the TSDR of the HTLV-1-infected cell lines and primary ATL cells was analyzed, and all TSDR, from amplicon (amp) 1 to 11, are illustrated. MJ cell line was used as the TSDR-hypomethylated representative, and MT-1 cell line was used as the TSDR-methylated representative. For primary ATL cells, Case 2 was used as the TSDR-hypomethylated representative, and Case 17 was used as the TSDR-methylated representative. (B) The DNA methylation status of the TSDR of a healthy donor and primary ATL cells. Regulatory T cells from a healthy donor were used as the TSDR-hypomethylated control, and CD4\(^+\)CD25\(^-\) conventional T cells from a healthy donor were used as the TSDR-methylated control. The number of primary ATL cells corresponds to each case number shown in Supplementary Table S1. The data shown are the sum of three independent experiments.
Figure 2. The hypomethylation of the TSDR correlates with FOXP3 expression.

(A) Primary ATL cells were stained with FOXP3 antibody, and the expression level of FOXP3 was assessed by flow cytometry. Representative histogram data of FOXP3 expression are shown. Case 13 and Case 22 are representatives of the hypomethylated TSDR (Hypomethylation) and the methylated TSDR (Methylation), respectively. The frequency of FOXP3-positive cells and mean fluorescence intensity (MFI) of FOXP3 were calculated and plotted. Each dot represents individual ATL cells from each patient, and bar indicates the median. (B) The FOXP3 mRNA levels of primary ATL cells were assessed by quantitative RT-PCR method. The FOXP3 mRNA level was divided by the β-actin mRNA level. The relative mRNA levels were calculated based on the expression of mRNA level in the MJ cell line. Bars indicate the median. Bold circles and squares indicate sorted samples. Solid circles and squares indicate unsorted samples. Statistical analysis was performed and P-value was shown in the figure.

Figure 3. Subpopulation of FOXP3-positive ATL cells.

The expressions of CD45RA and FOXP3 of primary ATL cells are shown. CD45RA: y-axis, FOXP3: x-axis. We analyzed total 16 cases. 8 TSDR-hypomethylated cases and
8 methylated cases). The representative data of a healthy individual is also shown. As mentioned in a previous study (16), Tregs can be divided into three groups according to their function: fractions I, II and III. Each square indicates fraction I (Fr I), fraction II (Fr II) and fraction III (Fr III), respectively. Numbers indicate the frequency of CD4$^+$ T cells in each fraction.

**Figure 4.** Primary ATL cells with the hypomethylated TSDR showed suppressive function resembling regulatory T cells.

CD4$^+$CD45RA$^+$ naïve T cells from a healthy donor were used as responder cells, cultured with irradiated APC with or without primary ATL cells from patients. Responder cells and primary ATL cells were added at a 1:1 ratio. ATL cells from Cases 5 and 17 were used as representative TSDR-hypomethylated cases (Hypomethylation) and TSDR-methylated cases (Methylation), respectively. The proliferation of responder cells was assessed by the dilution of labeled dye. Representative data of responder cells alone, ATL cells from the hypomethylated TSDR added, and ATL cells from the methylated TSDR added (top panel). The numbers indicate the frequency of proliferating responder cells. The percentage of proliferating responder cells (middle panel) and the numbers of proliferating cells (bottom panel) were plotted as mean ±
standard deviation. Representative data were shown, as the experiments were
independently performed three times using different ATL cells with similar results. *P*

values were indicated in each figure; NS, not statistically significant.

**Figure 5. Overall survival according to the methylation status of the TSDR.**

Survival curves of the hypomethylated TSDR (solid line) and the methylated TSDR
dotted line) were calculated by the Kaplan-Meier method. *P* value was shown in the
figure.
Healthy donor

Fr I: 3.1%
Fr II: 4.3%
Fr III: 4.1%

Figure 3

FOXP3
CD45RA

Hypomethylation

Methylation

Case 4
0% 84%
7% 1%

Case 5
0% 70%
20% 8%

Case 8
0% 34%
63% 6%

Case 10
5% 51%
28% 5%

Case 11
1.0% 36%
53% 1%

Case 12
0% 82%
16% 0%

Case 13
0.4% 4%
93% 16%

Case 14
0.8% 55%
35% 1%

Case 16
0% 25%
0.4% 53%

Case 17
0% 87%
0.3% 16%

Case 18
0% 64%
31% 1%

Case 19
0.2% 5%
7.5% 2.8%

Case 20
0% 25%
0.4% 0%

Case 21
0% 72%
0.4% 0%

Case 22
0% 93%
4% 0.3%

Case 23
1% 0%
0.5% 0%

Case 24
3% 40%
6% 72%

Case 25
0% 79%
0.3% 0%

Case 26
0.2% 14%
3% 3%

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Figure 5

Overall survival

Methylation

Hypomethylation

Days after diagnosis (days)

Overall survival

Days after diagnosis (days)

P = .02
Hypomethylation of the Treg-specific demethylated region in FOXP3 is a hallmark of the regulatory T-cell subtype in adult T-cell leukemia

Yayoi Shimazu, Yutaka Shimazu, Masakatsu Hishizawa, et al.

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