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, patients with ATL have poor prognoses. ATL cells possess the regulatory T cell (Treg) phenotype, such as CD4 and 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 patients with ATL 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 heterogeneous 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. Cancer Immunol Res; 4(2); 1–10. ©2015 AACR

Introduction

Adult T-cell leukemia (ATL) is an aggressive T-cell malignancy caused by human T-cell leukemia virus type 1 (HTLV-1; refs. 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 patients with ATL 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 that of regulatory T cells (Tregs), and several previous studies have shown that 60% to 70% of ATL cases expressed forkhead box P3 (FOXP3; refs. 10, 11), which is a master regulator of Tregs (12).

Tregs 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 Tregs 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 Tregs (15). In humans, FOXP3+ cells are heterogeneous and are not always suppressive (16). Tregs are divided into two main subpopulations: natural or induced Tregs. The former is a genuine Treg, 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 cytosine-phosphate-guanine (CpG) residues within the FOXP3 locus, termed the Treg-specific
demethylated region (TSDR), is important for distinguishing natural Tregs from peripherally induced Tregs 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 Treg 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 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 (PBMC) were isolated with Ficoll-Paque (Pharmacia Biotech) density-gradient centrifugation. If the proportion of nuclear cells (PBMC) were isolated with Ficoll-Paque (Pharmacia

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). ATL-43T 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 (ICRB) Cell Bank, and Hut102 and MJ were obtained from the American Type Culture Collection between 2000 and 2014. All cell lines were not cultured for more than 1 month. The data were acquired using a FACS LSR II instrument (BD Bioscience) and sequenced using a 3130xl Genetic Analyzer (Applied Biosystems). The percentages of methylation were calculated by dividing the number of demethylated colonies at the CpG site by 16.

qRT-PCR analysis

Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen) and converted into cDNA using a SuperScript Reverse Transcriptase (Invitrogen) and Random Hexamers (Applied Biosystems). qRT-PCR was performed using the TaqMan gene expression Kit (Applied Biosystems). The primers and probes for tax and HTLV-1 bZIP factor (HBZ) were 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) or FACS Aria instrument (BD Bioscience) and analyzed with FlowJo software (ver. 9.7.5; Tree Star, Inc.). The characteristics of 26 male patients are summarized in Table 1 and Supplementary Table S1. Peripheral blood mononuclear cells (PBMC) were isolated with Ficoll-Paque (Pharmacia Biotech) density-gradient centrifugation. If the proportion of nuclear cells (PBMC) were isolated with Ficoll-Paque (Pharmacia

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Table 1. Patients’ characteristics

<table>
<thead>
<tr>
<th>Age (year), median (range)</th>
<th>Hypomethylated patients</th>
<th>Methylated patients</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td>57 (33–87)</td>
<td>54 (29–80)</td>
<td></td>
</tr>
<tr>
<td>Lymphoma</td>
<td>10 (66.7%)</td>
<td>7 (63.6%)</td>
<td></td>
</tr>
<tr>
<td>Chronic</td>
<td>3 (20.0%)</td>
<td>2 (18.1%)</td>
<td></td>
</tr>
<tr>
<td>Smoldering</td>
<td>1 (6.7%)</td>
<td>1 (9.0%)</td>
<td></td>
</tr>
<tr>
<td>Alb (g/dL), median (range)</td>
<td>3.9 (2.3–4.4)</td>
<td>3.6 (2.6–4.5)</td>
<td>0.63</td>
</tr>
<tr>
<td>LDH (U/L), median (range)</td>
<td>1.037 (323–6,451)</td>
<td>485 (195–3,092)</td>
<td>0.46</td>
</tr>
<tr>
<td>Ca (mg/dL), median (range)</td>
<td>9.7 (8.8–16.9)</td>
<td>11.2 (9.1–16.9)</td>
<td>0.38</td>
</tr>
<tr>
<td>IgG (mg/dL), median (range)</td>
<td>1.037 (323–6,451)</td>
<td>990 (624–1,460)</td>
<td>0.80</td>
</tr>
<tr>
<td>sIL2R (U/mL), median (range)</td>
<td>30,071 (758–104,000)</td>
<td>18,670 (1,220–232,000)</td>
<td>0.55</td>
</tr>
<tr>
<td>OS (days), median (range)</td>
<td>283 (16–3,099)</td>
<td>627 (116–3,923)</td>
<td>0.1</td>
</tr>
<tr>
<td>Cause of death</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATL</td>
<td>8 (66.6%)</td>
<td>5 (71.4%)</td>
<td></td>
</tr>
<tr>
<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>

Abbreviation: sIL2R, soluble IL2 receptor.
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) according to the manufacturer’s instructions. CD4+ CD8+ cells were used as antigen-presenting cells (APC) after irradiation (18.5 Gy). Responder cells were cocultured with the same numbers of ATL cells with 10-fold APCs and stimulated with irradiation (18.5 Gy). Responder cells were cocultured with the CD25

Results

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 hours in the presence of a Golgi transport inhibitor, monensin (GolgiStop; BD Pharmingen). 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). Survival curves of the ATL patients were calculated by the Kaplan–Meier method using the SSPI Statistics software program, version 22.0 (IBM). Differences between two groups were evaluated by unpaired t tests. For the comparison of multiple groups, we used the Tukey method. Comparisons of overall survival (OS) were carried out between two groups with two-sided log-rank tests. P values < 0.05 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 amplicrons (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 Tregs, 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 Tregs (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%) of the 26 patients analyzed have hypomethylation states similar to normal Tregs (Fig. 1B, left). 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 4 cases in CTLA4 exon 2 and 1 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 (Fig. 2A and Supplementary Fig. S3A). 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 Treg 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 with the methylated cases (P = 0.01; Fig. 2B and Supplementary Fig. S3C).

In order to further characterize the ATL cells, we subdivided Tregs into three fractions (fractions I, II, and III) according to the expression 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+ FOXP3hi activated Tregs 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 Treg-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 Tregs. However, the TSDR-hypomethylated and -methylated cases were not significantly different (Supplementary Fig. S4A–S4E). 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 Tregs (e.g., 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 TSDR-methylated cases). When cocultured healthy CD4+CD45RA- naive 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. S8A). 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, are 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, 16–3,923 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, 16–3,099 days) and 627 days (range, 116–3,923 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 with the TSDR-methylated group: TSDR-hypomethylated, median OS of 186 days (range, 16–691 days); TSDR-methylated, median of 627 days (range, 116–3,923 days), P = 0.02 (Fig. 5). Of the 10 deceased patients in the TSDR-hypomethylated group, 8 patients succumbed to ATL, 1 patient to infection, and 1 to treatment-related mortality. Of the 6 deceased
patients in the TSDR-methylated group, 4 patients succumbed to ATL and 2 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<sup>+</sup> FOXP3<sup>high</sup> T cells had a tendency to reduce survival, this association was not statistically significant ($P = 0.14$; Supplementary
FOXP3 expression among primary ATL cells. Among these heterogenous clusters, we could differentially identify one specific subtype, which shows the TSDR-hypomethylated status is similar to that of natural Tregs. These TSDR-hypomethylated ATL cells resemble natural Tregs not only in their expression of FOXP3, but also other Treg-specific markers, such as CD25, CTLA4, HELIOS, and GITR. However, the TSDR-methylated ATL cells also expressed Treg-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 showed 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 Tregs or conventional T cells has remained controversial. The DNA demethylation of the FOXP3 gene is thought to be a specific phenomenon of natural Tregs. In addition, the HTLV-1 gene itself could change FOXP3 expression through the TGFβ signal pathway (44), but such FOXP3+ T cells are induced Tregs, not natural Tregs. 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 Tregs. However, we cannot exclude the possibilities that (i) the HTLV-1 virus may have infected CD4+ FOXP3+ non-Treg cells and (ii) epigenetic change had occurred during the development of ATL. Therefore, we cannot draw 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 biologic phenotype could not be used to distinguish the clinical risk among acute and lymphoma-type patients (45). Until now, the biologic phenotype could not be used to distinguish the clinical risk among acute and lymphoma-type patients (45). Until now, the biologic phenotype could not be used to distinguish the clinical risk among acute and lymphoma-type patients (45). Until now, the biologic phenotype could not be used to distinguish the clinical risk among acute and lymphoma-type patients (45).
the TSDR in women. We analyzed other Treg-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, 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 cutoff 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 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 Treg subtype is related to the biologic phenotype and clinical course of ATL. The hypomethylated status of the TSDR in ATL cells might be a promising tool for risk classification.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Shimazu, Y. Shimazu, M. Hishizawa, M. Hamaguchi, N. Kadowaki, A. Takaori-Kondo
Development of methodology: Y. Shimazu, Y. Shimazu, S. Sakaguchi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Shimazu, Y. Shimazu, M. Hishizawa, Y. Nagai, N. Sugino, S. Fujii
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Shimazu, Y. Shimazu, M. Hishizawa, M. Kawahara, H. Nishikawa, A. Takaori-Kondo
Writing, review, and/or revision of the manuscript: Y. Shimazu, Y. Shimazu, M. Hishizawa, M. Hamaguchi, M. Kawahara, A. Takaori-Kondo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Shimazu, M. Kawahara, H. Nishikawa, A. Takaori-Kondo
Study supervision: M. Hishizawa, N. Kadowaki, S. Sakaguchi, A. Takaori-Kondo

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References


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